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Characterisation of dietary fibre, bioactive compounds and prebiotic effect of plant food by-products for their valorisation as high added-value ingredients

Caracterización de la fibra dietética, compuestos bioactivos y efecto prebiótico de subproductos vegetales para su valorización como ingredientes de alto valor añadido

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## ABBREVIATIONS

| | |
|---|---|
| **AAPH** | 2,2'-azobis(2-amidinopropane)dihydrochloride |
| **ANOVA** | One-way analysis of variance |
| **AOA** | Association of Official Agricultural Chemists |
| **DAD** | Diode array |
| **DBS** | Freeze-dried broccoli stalk |
| **DF** | Dietary fibre |
| **DW** | Dry weight |
| **EFSA** | European Food Safety Authority |
| **EPP** | Extractable (poly)phenols |
| **ESI** | Electrospray ionisation |
| **EU** | European Union |
| **FAC** | Fat absorption capacity |
| **FAO** | Food and Agricultural Organization of the United Nations |
| **FDB** | Freeze-dried berries |
| **FOS** | Fructo-oligosaccharides |
| **FRAP** | Ferric reducing antioxidant power |
| **FW** | Fresh weight |
| **GAE** | Gallic acid equivalents |
| **GBS** | Glucobrassicin |
| **GDRI** | Glucose diffusion retardation index |
| **GIB** | Glucoiberin |
| **GLC-FID** | Gas liquid chromatography coupled to flame ionisation detector |
| **GLS** | Glucosinolates |
| **GOS** | Galacto-oligosaccharides |
| **GRA** | Glucoraphanin |
| **HGB** | Hydroxyglucobrassicin |
| **HPLC-DAD** | High-performance liquid chromatography–diode array detection |
| **HPLC-DAD-ESI-MSn** | High-performance liquid chromatography coupled to electrospray ionisation mass spectrometric detection |
| **HRMS** | High-resolution mass spectrometry |
| **IDF** | Insoluble dietary fibre |
| **IFB** | Insoluble dietary fibre fraction from broccoli stalk |
| **IFF** | Insoluble fibre fraction |
| **LC** | Liquid chromatography |
| **LDL** | Low-density lipoprotein |
| **MAPA** | Ministry of Agriculture, Fisheries and Food |
| **MAU** | Mili absorbance units |
| **MGB** | Methoxyglucobrassicin |
| **MSIMI** | Metabolite standards initiative metabolite identification level |
| **ND** | Not detected |
| **NEPP** | Non extractable (poly)phenols |
| **NGB** | Neoglucobrassicin |
| **NIDF** | Normal-weight insoluble dietary fibre |
| **NPEX** | Normal-weight (poly)phenol extract |

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|---|---|
| **NRAS** | Normal-weight raspberry |
| **NSP** | Non starch polysaccharides |
| **NTDF** | Normal-weight total dietary fibre |
| **NW** | Normal-weight |
| **OIDF** | Overweight insoluble dietary fibre |
| **OP** | Orange peel |
| **OPEX** | Overweight (poly)phenol extract |
| **ORAC** | Oxygen radical absorbance capacity |
| **ORAS** | Overweight raspberry |
| **OTDF** | Overweight total dietary fibre |
| **OW** | Overweight |
| **PCA** | Principal component analysis |
| **PEX** | Extract rich in (poly)phenols from raspberry |
| **PRE** | (Poly)phenol rich extract from berries |
| **QE** | Quercetin equivalents |
| **RAS** | Freeze-dried raspberry |
| **RS** | Resistant starch |
| **SCFA** | Short chain fatty acids |
| **SD** | Standard deviation |
| **SFB** | Soluble dietary fibre fraction from broccoli stalk |
| **SFF** | Soluble fibre fraction |
| **SWC** | Swelling capacity |
| **TDF** | Total dietary fibre |
| **TE** | Trolox equivalents |
| **TF** | Total flavonoids |
| **TFB** | Total dietary fibre fraction from broccoli stalk |
| **TGD** | Total glucose diffused |
| **TPC** | Total phenolic content |
| **UHPL-HRMS** | Ultra-high performance liquid chromatography high-resolution mass spectrometry |
| **USDA** | United States Department of Agriculture |
| **UV** | Ultraviolet |
| **WBC** | Water binding capacity |
| **WRC** | Water retention capacity |
| **WSE** | Water-soluble extract |
| **TR** | Retention time |

## ABSTRACT

The activity of the agri-food industry generates the production of a large quantity of by-products, whose management cause a negative impact both at economical and at environmental level. Since the agri-food by-products remain similar nutritional composition (macronutrients and micronutrients) to the initial plant food products, their valorisation by obtaining added-value food ingredients can be considered as a strategy for their reintroduction into the food chain, implementing the circular economy strategies. Particularly, these by-products are rich in dietary fibre and in bioactive compounds, such as (poly)phenols, carotenoids and glucosinolates. All these compounds are recognised to have beneficial effects for human health, so according to their properties, the by-products can be used to obtain ingredients that could be included in the formulations of functional foods, developing healthy foods.

Based on the importance of the agro-industrial sector in the Spanish economy, berries, broccoli and orange by-products have been selected for the development of the experiments carried out in the present PhD Thesis. Firstly, because all of them are a source of dietary fibre and due to their chemical composition contains different bioactive compounds, a wide variety of (poly)phenols, and in addition broccoli contains glucosinolates and orange contains carotenoids. Secondly, they represent some of the main cultivars in Spain and in the Region of Murcia. In this sense, the production of berries has increased in the last years in Spain, becoming one of the main European producers, generating by-products mainly as whole fruits, which do not meet the quality requirements, and pomaces obtained after berries transformation to derived products such as juices and jams. In addition, the Region of Murcia is the first producer of broccoli in Europe, being stalks and leaves the main broccoli by-products, like as the third producer of oranges in Spain, being the orange peel, pulp and seeds the major by-product from the juice processing industry.

Therefore, the general aim of this PhD Thesis has been to valorise different by-products generated in the agri-food industry, derived from berries, broccoli and orange to obtain ingredients rich in dietary fibre and with a high content of bioactive compounds ((poly)phenols, carotenoids and glucosinolates), which can be used as functional ingredients in the food industry to design and develop functional foods.

In order to achieve the general objective, four different experiments were carried out according to the by-product used as samples (raspberries, mixed of berries, broccoli stalks and orange peels). First, the different by-products were subjected to the extraction

processes to separate the fibre-rich fraction. The use of enzymatic and water-ethanol extraction allowed to obtain different fractions from the by-products selected. The fractions obtained were nutritionally characterised to know its proximate composition by analysing the moisture content, protein, fat, ash, and carbohydrates using the AOAC Official methods. Dietary fibre was also analysed to determine both fractions of insoluble dietary fibre (IDF) and soluble dietary fibre (SDF), by ethanol precipitation. Moreover, the dietary fibre was characterised measuring the proportion of neutral sugars using gas-liquid chromatography and uronic acids by spectrophotometry. Furthermore, based on the results of the neutral sugars and uronic acids, the pectin, cellulose and hemicellulose content and the structure characteristics of pectin were theoretically calculated. Water retention capacity (WRC), swelling capacity (SWC), fat absorption capacity (FAC), glucose diffusion retardation index (GDRI), and osmotic pressure were analysed as physicochemical properties to evaluate the potential physiological and technological functions of the different fractions obtained from the by-products. On the other hand, the content of extractable (poly)phenols (EPP), extracted using organic solvents, and non-extractable (poly)phenols (NEPP), obtained by hydrolysis with sulphuric acid, were analysed by liquid chromatography, and in these EPP and NEPP fractions total phenolic content was determined by the Folin-Ciocalteu method and the total flavonoids, which were analysed by spectrophotometry. Glucosinolates present in broccoli stalks and carotenoids in the orange peel samples were also determined by liquid chromatography analyses. Moreover, the antioxidant capacity was also evaluated by using two spectrophotometric assays, the ferric reducing antioxidant power (FRAP) and the oxygen radical absorbance capacity (ORAC).

To determine the prebiotic effect of the different fractions obtained from the by-products an *in vitro* digestion following an oral, gastric and intestinal phases was performed. After that, the samples digested were incubated *in vitro* with human faeces during 48 h. Aliquots of faecal slurries were taken at different points to analyse the production of short chain fatty acids (SCFAs) (acetate, propionate, butyrate, isobutyrate, isovalerate, valerate, isocaproate, caproate and heptanoate) by gas-liquid chromatography and ammonium production as a measure of the microbial activity. Finally, the production of phenolic catabolites produced by the microbiota were also analysed by liquid chromatography, analysing urolithins from the catabolism of ellagitannins and ellagic acid; and phenolic acids, including phenylpropionic, phenylacetic and benzoic acid derivatives, from the flavanones and hydroxycinnamic acids catabolism.

In order to achieve the general objective, four different experiments were carried out. In **Experiment 1**, frozen commercial raspberries were used, representing the surplus of the fruits that can be considered as by-products. Three dietary fibre fractions were obtained by an enzymatic process: 1) total dietary fibre (TDF), 2) insoluble dietary fibre (IDF), and 3) soluble dietary fibre (SDF), as well as freeze-dried raspberry sample (RAS). The SDF fraction showed the highest pectin content (67%) related to the uronic acid content, compared to TDF and IDF which were mainly composed of cellulose (41% and 50%) and hemicellulose (31% and 36%). Moreover, SDF was the fraction with the highest hydration properties. In terms of (poly)phenols content, RAS had the highest content of EPP, represented by anthocyanins, ellagitannins, ellagic acid derivatives, flavonols and caffeic acid, with 42 mg/g of dry weight (d.w.), while the fibre fractions had the highest content of NEPP, represented by ellagic acid derivatives (8 mg/g of d.w.), being TDF the fraction with the highest content, indicating that these compounds may be bound to the components of dietary fibre. The *in vitro* fermentation in this experiment were carried out using faecal samples from six female volunteers, three normal-weight and three overweight, both groups were selected because obesity is known to cause dysbiosis of the microbiota and we wanted to evaluate the potential effect on (poly)phenol metabolism. The fermentations were carried out individually with each inoculum from each volunteer during 48 h with the four samples (RAS, TDF, IDF, SDF) and with an extract rich in (poly)phenols (PEX) obtained from RAS to test the prebiotic-like effect previously associated to these compounds. In terms of SCFAs production as a prebiotic effect indicator, no differences were observed between normal-weight and overweight inoculums due to the high individual variability. However, in both inoculums, there was higher SCFAs production in RAS and PEX compared to the fibre fractions. Although there were no significant differences in the production of urolithins from ellagic acid catabolism, a trend was observed. In this regard, inoculums from normal-weight volunteers produced more urolithins from the TDF and IDF fractions, in which ellagic acid was mainly non-extractable. However, overweight inoculums produced more urolithins from RAS and PEX, where ellagic acid was mostly extractable.

In **Experiment 2**, as no surplus or by-products were available as in experiment 1, a commercial mixture of frozen berries was used, and three different samples were obtained by water-ethanol extraction method. However, the enzymatic extraction was not carried out to reduce overall costs and simplify the process compared to experiment 1. The berries were freeze-dried to obtain the freeze-dried berries sample (FDB) and two fibre-rich fractions, the insoluble fibre-rich fraction (IFF) and the soluble fibre-rich fraction (SFF), which were extracted by the water-ethanol extraction procedure. The IFF fraction

showed the highest fibre content with 65%, mainly represented by pectin and hemicellulose (52% and 42%, respectively), with a similar profile to FDB, while SFF had lower content of TDF (47%) than IFF, which was mainly composed by pectin (93%). For this reason, SFF was the fraction with the highest hydration properties and the highest linearity of pectin. As for the (poly)phenols profile, the highest amount of EPP was obtained in FDB and IFF with 403 and 342 mg/100 g respectively, represented by anthocyanins, ellagic acid and ellagitannins. However, as observed in the previous experiment, the highest amount of NEPP was obtained for the fibre fractions, IFF (496 mg/100 g) followed by SFF (414 mg/100 g), represented by hydroxycinnamic acids and ellagic acid. Because of the high individual variability observed in experiment 1 due to the differences in microbiota composition, not only due to the body mass index, but also because other factors that influence the bacterial populations. The *in vitro* fermentation in this experiment was carried out using a pooled faecal sample from nine healthy females to increase the microbial diversity and thus reducing the individual variability. The three fibre-rich fractions obtained (FDB, IFF, SFF), as well as an extract rich in (poly)phenols (PRE) were *in vitro* incubated, to test its potential prebiotic effect. No differences were observed in the production of SCFAs between the different samples tested. On the other hand, for the urolithin production, IFF showed the highest production due to the highest amount of (poly)phenols in this fraction, followed by FDB, showing PRE and SDF the lowest production.

In **Experiment 3**, the effect of seasonal variation on bioactive compounds in broccoli florets in spring and autumn was determined in order to select raw materials and obtain broccoli stalks as industrial by-products. Fresh broccoli stalks were freeze-dried to obtain freeze-dried broccoli stalk (DBS) and two fibre-rich fractions were obtained from the raw samples: total fibre ($TF_B$) and insoluble fibre ($IF_B$). In the seasonal study, for the content of (poly)phenols no differences were observed between the two seasons, but for glucosinolates and carotenoids the content was 2.5 and 3.3-fold higher in autumn than in spring. Therefore, broccoli stalks from the autumn trial were selected to obtain the fibre-rich samples. The fibre fractions ($TF_B$ and $IF_B$) had the highest content of TDF (69% and 61%, respectively), being mainly insoluble, representing 79% and 89% from the TDF respectively. In terms of composition, pectin was the major component in all samples due to the high content of uronic acids, with cellulose being the minor component. $IF_B$ was the fraction with the highest hydration properties, due to the highest content of soluble hemicellulose in this fraction. The glucosinolate content was highest in DBS (140 mg/100 g), followed by $IF_B$ and $TF_B$ (100 and 69 mg/100 g, respectively), being glucoraphanin the major glucosinolate found. Moreover, EPP was highest in DBS with a

mean value of 11 mg/100 g and NEPP was highest in $IF_B$, with a mean value of 73 mg/100 g, being represented for both EPP and NEPP for chlorogenic and sinapic acid derivatives. Pooled of faeces were also used in this experiment based on the advantages observed in experiment 2, incubating only DBS and $IF_B$ during 48 h. The production of SCFAs was higher in $IF_B$ than in DBS, for its higher content of fibre and (poly)phenols.

In **Experiment 4**, orange peels were used as an industrial by-product. Three different samples were obtained, the first one after drying the whole peel (OP) and two fractions obtained from a green method: the fraction rich in insoluble dietary fibre (IFF) and the fraction of water-soluble extract (WSE). The IFF fraction showed the highest fibre content (76%), being mostly insoluble for OP and IFF, representing 80% and 85% from the TDF, while the WSE fraction, although showing the lowest TDF content, was the sample with the highest proportion of SDF, representing 30% from the TDF. In addition, WSE was the fraction with the highest carbohydrates content represented by soluble sugars. The fibre of the fractions was mainly composed by pectin ranging between 69%-85%, being cellulose the minority component, ranging between 4%-5%. In this sense, IFF was the fraction with the highest water retention capacity due the highest pectin content and the highest linearity of pectin. On the other hand, WSE was the fraction with the highest swelling capacity due to the highest content of soluble hemicellulose, with the highest mannose contribution. Regarding the composition of (poly)phenols, the WSE fraction showed a higher amount of EPP (4589 mg/100 g) and NEPP (1146 mg/100 g), than the other two samples, represented by flavanones and hydroxycinnamic acid derivatives. On the other hand, the carotenoid content was highest in OP and IFF with a mean value around 12 mg/100 g for both samples. As in the two previous experiments a pooled of faeces were used from eight healthy female volunteers, incubating OP, IFF, WSE and a negative control (C-) without any sample and a positive control (C+) with glucose to compare with WSE for its highest content of carbohydrates, during 48 h. Regarding SCFAs, a highest production was observed in IFF while WSE showed the lowest production, because this fraction showed the lowest content of TDF. The degradation of the parent compounds in the orange peel samples coincided with the concomitant production of catabolites from these compounds. In this sense, the metabolisation of flavanones and hydroxycinnamic acid derivatives present in the different fractions showed a higher production of catabolites in the WSE fraction, compared to the other samples, producing mainly phenylpropionic, phenylacetic and benzoic acid derivatives, derived from the higher presence of flavanones in this fraction, compared to the other samples.

The use of enzymatic and water-ethanol extraction methods allowed the extraction of fibre-rich fractions, whose characteristics depend on the initial by-product and the method used for the extraction. All fibre fractions obtained showed antioxidant capacity related with the content of (poly)phenols. The content of EPP and NEPP was also related with the extraction method used, because the fractions of soluble fibre which were extracted with ethanol showed lower content of EPP than the other fractions, because part of the EPP were removed during the extraction. Moreover, glucosinolates were also found in the broccoli stalk fractions and carotenoids in the orange peel fractions. All fibre fractions obtained showed a prebiotic effect, measured by the production of SCFAs, producing mainly acetate, followed by propionate and butyrate. The (poly)phenols extracts tested in the two first experiments also showed prebiotic-like effect by increasing the production of SCFAs. Finally, the fermentation of the microbiota of fibre-rich fractions with (poly)phenols led to the production of catabolites, which vary depending on the parent compound. These catabolites are more bioavailable compounds than the parent compounds, which allows their absorption in the colon and their systemic distribution and may have beneficial effects on consumer health. In this sense, the fractions obtained can be used as potential ingredients for the development of functional foods with enhanced nutritional properties.

## RESUMEN

La actividad de la industria agroalimentaria genera la producción de una gran cantidad de subproductos, cuya gestión provoca un impacto negativo tanto a nivel económico como medioambiental. Dado que los subproductos agroalimentarios mantienen una composición nutricional similar (macronutrientes y micronutrientes) a la de los productos vegetales de partida, su valorización mediante la obtención de ingredientes alimentarios con valor añadido puede considerarse como una estrategia para su reintroducción en la cadena alimentaria, implementando las estrategias de economía circular. En particular, estos subproductos son ricos en fibra dietética y en compuestos bioactivos, como (poli)fenoles, carotenoides y glucosinolatos. Todos estos compuestos son reconocidos por sus beneficios para la salud humana, por lo que, de acuerdo a sus propiedades, los subproductos pueden ser utilizados para obtener ingredientes que puedan ser empleados para el desarrollo de formulaciones de alimentos funcionales, permitiendo la obtención de alimentos más saludables.

En base a la importancia del sector agroindustrial en la economía española, se han seleccionado los subproductos de frutos rojos, brócoli y naranja para el desarrollo de los experimentos realizados en la presente Tesis Doctoral. En primer lugar, se han seleccionado porque todos ellos son fuente de fibra dietética y por su composición química contienen diferentes compuestos bioactivos, una amplia variedad de (poli)fenoles, y además el brócoli contiene glucosinolatos y la naranja contiene carotenoides. En segundo lugar, se han seleccionado estos subproductos ya que representan algunos de los principales cultivos en España y en la Región de Murcia. En este sentido, la producción de frutos rojos se ha incrementado en los últimos años en España, convirtiéndose en uno de los principales productores europeos, generando principalmente frutos enteros como subproductos, debido a que no cumplen los requisitos de calidad, y orujos obtenidos tras la transformación de los frutos rojos a productos derivados como zumos y mermeladas. Además, la Región de Murcia es la primera productora de brócoli de Europa, siendo los troncos y las hojas los principales subproductos del brócoli, al igual que es la tercera productora de naranjas de España, siendo la piel, la pulpa y las semillas de la naranja el principal subproducto de la industria de transformación de zumos.

Por tanto, el objetivo general de esta Tesis Doctoral ha sido valorizar diferentes subproductos generados en la industria agroalimentaria, derivados de los frutos rojos, del brócoli y de la naranja para obtener ingredientes ricos en fibra dietética y con un alto

contenido en compuestos bioactivos ((poli)fenoles, carotenoides y glucosinolatos), que puedan ser utilizados como ingredientes funcionales en la industria alimentaria para diseñar y desarrollar alimentos funcionales.

Para alcanzar el objetivo general, se realizaron cuatro experimentos diferentes según el subproducto utilizado (frambuesas, mezcla de frutos rojos, troncos de brócoli y piel de naranja). En primer lugar, los diferentes subproductos se sometieron a procesos de extracción para separar la fracción rica en fibra. El uso de la extracción enzimática y de métodos que emplean agua y etanol, permitió obtener diferentes fracciones a partir de los subproductos seleccionados. Las fracciones obtenidas fueron caracterizadas nutricionalmente para conocer su composición proximal mediante el análisis del contenido de humedad, proteínas, grasas, cenizas y carbohidratos utilizando los métodos oficiales de la AOAC. También se analizó la fibra dietética para determinar las dos fracciones, fibra dietética insoluble (IDF) y fibra dietética soluble (SDF), mediante precipitación con etanol. Además, la fibra dietética se caracterizó midiendo la proporción de azúcares neutros mediante cromatografía gas-líquido y los ácidos urónicos mediante espectrofotometría. Adicionalmente, basándonos en los resultados de los azúcares neutros y los ácidos urónicos, se calculó teóricamente el contenido de pectina, celulosa y hemicelulosa y las características de la estructura de la pectina. Se analizaron la capacidad de retención de agua (WRC), la capacidad de hinchamiento (SWC), la capacidad de absorción de grasa (FAC), el índice de retardo de difusión de la glucosa (GDRI) y la presión osmótica como propiedades fisicoquímicas para evaluar las potenciales funciones fisiológicas y tecnológicas de las diferentes fracciones obtenidas de los subproductos. Por otro lado, se analizó por cromatografía líquida el contenido de (poli)fenoles extraíbles (EPP), extraídos mediante disolventes orgánicos, y de (poli)fenoles no extraíbles (NEPP), obtenidos por hidrólisis con ácido sulfúrico, y en estas fracciones (EPP y NEPP) se determinó el contenido fenólico total por el método de Folin-Ciocalteu y los flavonoides totales, que se analizaron por espectrofotometría. Los glucosinolatos presentes en el tronco de brócoli y los carotenoides en las muestras de naranja también se determinaron mediante análisis de cromatografía líquida. Además, también se evaluó la capacidad antioxidante mediante dos ensayos espectrofotométricos, el poder antioxidante para reducir el hierro (FRAP) y la capacidad de absorción de radicales de oxígeno (ORAC).

Para determinar el efecto prebiótico de las diferentes fracciones obtenidas de los subproductos se realizó una digestión *in vitro* siguiendo una fase oral, gástrica e intestinal. Posteriormente, las muestras digeridas se incubaron *in vitro* con heces

humanas durante 48 h. Se tomaron alícuotas en diferentes puntos para analizar la producción de ácidos grasos volátiles de cadena corta (AGVCC) (acetato, propionato, butirato, isobutirato, isovalerato, valerato, isocaproato, caproato y heptanoato) mediante cromatografía gas-líquido y la producción de amonio como medida de la actividad microbiana. Por último, también se analizó la producción de catabolitos fenólicos producidos por la microbiota mediante cromatografía líquida, analizando las urolitinas procedentes del catabolismo de los elagitaninos y del ácido elágico; y los ácidos fenólicos, incluidos los derivados del ácido fenilpropiónico, fenilacético y benzoico, procedentes del catabolismo de las flavanonas y de los ácidos hidroxicinámicos.

Para lograr el objetivo general, se llevaron a cabo cuatro experimentos diferentes. En el **experimento 1**, se utilizaron frambuesas comerciales congeladas, que representan el excedente de las frutas que pueden considerarse como subproductos. Se obtuvieron tres fracciones de fibra dietética mediante un proceso enzimático 1) fibra dietética total (TDF), 2) fibra dietética insoluble (IDF) y 3) fibra dietética soluble (SDF), así como una muestra de frambuesa liofilizada (RAS). La fracción SDF mostró el mayor contenido de pectina (67%) relacionado con el contenido de ácido urónico, en comparación con la TDF y la IDF, que estaban compuestas principalmente por celulosa (41% y 50%) y hemicelulosa (31% y 36%). Además, la SDF fue la fracción con mayores propiedades de hidratación. En cuanto al contenido de (poli)fenoles, la RAS tuvo el mayor contenido de EPP, representado por antocianinas, elagitaninos, derivados del ácido elágico, flavonoles y ácido cafeico, con 42 mg/g de peso seco (p.s.), mientras que las fracciones de fibra tuvieron el mayor contenido de NEPP, representado por los derivados del ácido elágico (8 mg/g de p.s.), siendo la TDF la fracción con mayor contenido indicando que estos compuestos pueden estar unidos a los componentes de la fibra dietética. Las fermentaciones *in vitro* en este experimento se llevaron a cabo utilizando muestras fecales de seis mujeres voluntarias, tres con normo-peso y tres con sobrepeso, ambos grupos fueron seleccionados porque se sabe que la obesidad causa disbiosis de la microbiota y queríamos evaluar el efecto potencial sobre el metabolismo de los (poli)fenoles. Las fermentaciones se realizaron individualmente con cada inóculo de cada voluntaria durante 48 h con las cuatro muestras (RAS, TDF, IDF, SDF) y con un extracto rico en (poli)fenoles (PEX) obtenido a partir de RAS para comprobar el efecto prebiótico previamente asociado a estos compuestos. En cuanto a la producción de AGVCC como indicador del efecto prebiótico, no se observaron diferencias entre los inóculos procedentes de voluntarias con normo-peso y los de sobrepeso debido a la alta variabilidad individual. Sin embargo, en ambos inóculos, hubo una mayor producción de AGVCC en RAS y PEX en comparación con las fracciones de fibra. Aunque no hubo

diferencias significativas en la producción de urolitinas procedentes del catabolismo del ácido elágico, se observó una tendencia. En este sentido, los inóculos de voluntarios con normo-peso produjeron más urolitinas a partir de las fracciones TDF e IDF, en las que el ácido elágico era principalmente no extraíble. Sin embargo, los inóculos de personas con sobrepeso produjeron más urolitinas de las fracciones RAS y PEX, en las que el ácido elágico era principalmente extraíble.

En el **experimento 2**, al no disponer de excedentes o subproductos como en el experimento 1, se utilizó una mezcla comercial de frutos rojos congelados y se obtuvieron tres muestras diferentes empleando un método de extracción con agua y etanol. No se realizó la extracción enzimática para reducir los costes totales y simplificar el proceso en comparación con el experimento 1. Los frutos rojos se liofilizaron para obtener la muestra de frutos rojos liofilizados (FDB) y dos fracciones ricas en fibra, la fracción rica en fibra insoluble (IFF) y la fracción rica en fibra soluble (SFF), que se extrajeron mediante un procedimiento de extracción con agua y etanol. La fracción IFF mostró el mayor contenido de fibra, con un 65%, representado principalmente por pectina y hemicelulosa (52% y 42%, respectivamente), con un perfil similar al de la FDB, mientras que la SFF tuvo un menor contenido de TDF (47%) que la IFF, que estaba compuesta principalmente por pectina (93%). Por esta razón, la SFF fue la fracción con mayores propiedades de hidratación y mayor linealidad de pectina. En cuanto al perfil de (poli)fenoles, la mayor cantidad de EPP se obtuvo en FDB e IFF con 403 y 342 mg/100 g respectivamente, representados por antocianinas, ácido elágico y elagitaninos. Sin embargo, como se observó en el experimento anterior, la mayor cantidad de NEPP se obtuvo en las fracciones de fibra, IFF (496 mg/100 g), seguida de SFF (414 mg/100 g), representadas por los ácidos hidroxicinámicos y el ácido elágico. La elevada variabilidad individual observada en el experimento 1 se debe a las diferencias en la composición de la microbiota, no sólo por el índice de masa corporal, sino también por otros factores que influyen en las poblaciones bacterianas. La fermentación *in vitro* en este experimento se llevó a cabo utilizando una muestra fecal conjunta de nueve mujeres sanas para aumentar la diversidad microbiana y reducir así la variabilidad individual. Se incubaron *in vitro* las tres fracciones ricas en fibra obtenidas (FDB, IFF, SFF), así como un extracto rico en (poli)fenoles (PRE), para comprobar su potencial efecto prebiótico. No se observaron diferencias en la producción de AGVCC entre las diferentes muestras probadas. Por otro lado, en cuanto a la producción de urolitina, IFF mostró la mayor producción debido a la mayor cantidad de (poli)fenoles en esta fracción, seguido de FDB, mostrando PRE y SDF la menor producción.

En el **experimento 3**, se determinó el efecto de la variación estacional en los compuestos bioactivos de los floretes de brócoli en primavera y otoño para seleccionar las materias primas y obtener troncos de brócoli como subproductos industriales. Los troncos de brócoli frescos se liofilizaron para obtener troncos de brócoli liofilizados (DBS) y de las muestras crudas se obtuvieron dos fracciones ricas en fibra: fibra total ($TF_B$) y fibra insoluble ($IF_B$). En el estudio estacional, para el contenido de (poli)fenoles no se observaron diferencias entre las dos estaciones, pero para los glucosinolatos y los carotenoides el contenido fue 2,5 y 3,3 veces mayor en otoño que en primavera. Por lo tanto, se seleccionaron los troncos de brócoli del ensayo de otoño para obtener las muestras ricas en fibra. Las fracciones de fibra ($TF_B$ e $IF_B$) tuvieron el mayor contenido de TDF (69% y 61%, respectivamente), siendo principalmente insolubles, representando el 79% y el 89% de la TDF respectivamente. En cuanto a la composición, la pectina fue el componente mayoritario en todas las muestras debido al alto contenido en ácidos urónicos, siendo la celulosa el componente minoritario. La $IF_B$ fue la fracción con mayores propiedades de hidratación, debido al mayor contenido de hemicelulosa soluble en esta fracción. El contenido de glucosinolatos fue mayor en DBS (140 mg/100 g), seguido de $IF_B$ y $TF_B$ (100 y 69 mg/100 g, respectivamente), siendo la glucorafanina el principal glucosinolato encontrado. Además, el EPP fue más alto en la DBS con un valor medio de 11 mg/100 g y el NEPP fue más alto en la $IF_B$, con un valor medio de 73 mg/100 g, estando representados tanto el EPP como el NEPP por los derivados del ácido clorogénico y sinápico. En este experimento también se utilizó una mezcla de heces en base a las ventajas observadas en el experimento 2, incubando sólo DBS e $IF_B$ durante 48 h. La producción de SCFAs fue mayor en $IF_B$ que en DBS, por su mayor contenido en fibra y (poli)fenoles.

En el **experimento 4** se utilizaron pieles de naranja como subproducto industrial. Se obtuvieron tres muestras diferentes, la primera tras el secado de la piel entera (OP) y dos fracciones obtenidas a partir de un método limpio: una fracción rica en fibra dietética insoluble (IFF) y la fracción hidrosoluble (WSE). La fracción IFF mostró el mayor contenido en fibra (76%), siendo mayoritariamente insoluble para la OP y la IFF, representando el 80% y el 85% de la TDF, mientras que la fracción WSE, aunque mostró el menor contenido en TDF, fue la muestra con mayor proporción de SDF, representando el 30% de la TDF. Además, la WSE fue la fracción con mayor contenido en carbohidratos representados por azúcares solubles. La fibra de las fracciones estaba compuesta principalmente por pectina que oscilaba entre el 69%-85%, siendo la celulosa el componente minoritario, que oscilaba entre el 4%-5%. En este sentido, IFF fue la fracción con mayor capacidad de retención de agua debido al mayor contenido de

pectina y a la mayor linealidad de la misma. Por otro lado, WSE fue la fracción con mayor capacidad de hinchamiento debido al mayor contenido de hemicelulosa soluble, con mayor contribución de manosa. En cuanto a la composición de (poli)fenoles, la fracción WSE mostró una mayor cantidad de EPP (4589 mg/100 g) y NEPP (1146 mg/100 g), que las otras dos muestras, representadas por flavanonas y derivados del ácido hidroxicinámico. Por otra parte, el contenido en carotenoides fue mayor en OP e IFF, con un valor medio en torno a 12 mg/100 g para ambas muestras. Al igual que en los dos experimentos anteriores, se utilizó una mezcla de heces de ocho mujeres voluntarias sanas, incubando OP, IFF, WSE y un control negativo (C-) sin ninguna muestra y un control positivo (C+) con glucosa para comparar con WSE por su mayor contenido en carbohidratos, durante 48 h. En cuanto a los AGVCC, se observó una mayor producción en IFF mientras que WSE mostró la menor producción, ya que esta fracción mostró el menor contenido en TDF. La degradación de los (poli)fenoles iniciales en las muestras de piel de naranja coincidió con la producción concomitante de catabolitos a partir de estos compuestos. En este sentido, la metabolización de flavanonas y derivados de ácidos hidroxicinámicos presentes en las diferentes fracciones mostró una mayor producción de catabolitos en la fracción WSE, produciéndose principalmente derivados de los ácidos fenilpropiónico, fenilacético y benzoico, derivados de la mayor presencia de flavanonas en esta fracción, en comparación con las otras muestras.

El uso de métodos de extracción enzimática y métodos que emplean agua y etanol permite la extracción de fracciones ricas en fibra, cuyas características dependen del subproducto inicial y del método utilizado para la extracción. Todas las fracciones de fibra obtenidas mostraron capacidad antioxidante relacionada con el contenido de (poli)fenoles. El contenido de EPP y NEPP también se relacionó con el método de extracción utilizado, ya que las fracciones de fibra soluble que fueron extraídas con etanol mostraron un menor contenido de EPP, debido que parte de los EPP fueron eliminados durante la extracción. Además, también se encontraron glucosinolatos en las fracciones de troncos de brócoli y carotenoides en las fracciones de piel de naranja. Todas las fracciones de fibra obtenidas mostraron un efecto prebiótico, medido por la producción de AGVCC, produciendo principalmente acetato, seguido de propionato y butirato. Los extractos de (poli)fenoles incubados en los dos primeros experimentos también mostraron un efecto similar al de los prebióticos al aumentar la producción de AGVCC. Por último, la fermentación por parte de la microbiota de las fracciones ricas en fibra en presencia de (poli)fenoles da lugar a la producción de catabolitos, que varían en función del compuesto inicial. Estos catabolitos son compuestos más biodisponibles

que los compuestos iniciales, lo que permite su absorción en el colon y su distribución sistémica, lo cual puede tener efectos beneficiosos para la salud del consumidor. En este sentido, las fracciones obtenidas pueden ser utilizadas como potenciales ingredientes para el desarrollo de alimentos funcionales con propiedades nutricionales mejoradas.

# General Index

# Chapter 1. General introduction

# 1. Importance of by-products from agri-food industry

## 1.1. By-products definition and classification

According to Directive 2018/851 of the European Union (EU), a by-product is a substance or object, resulting from a production process, whose primary purpose is not the production of that substance or object, and is not considered as a waste, for which the following conditions must be fulfilled: (a) there is certainty that the substance or object will be used subsequently; (b) the substance or object can be used directly without any further processing other than normal industrial practice; (c) the substance or object is produced as part of a production process; and (d) further use is lawful, e.g. the substance or object fulfils all relevant product, environmental and health protection requirements for the specific use and will not lead to overall adverse environmental or human health impacts (EUR-Lex, 2018).

Related to the activity of the plant food industries, several by-products are generated, which could be consider as raw material for other industrial practices. The most practical classification is made in terms of the moment in which they are generated within the agri-food chain as is shown in Figure 1 (Morales-Moreno., 2015; Sadh *et al*., 2018). In this sense, **field by-products** are those that remain after harvesting, including leaves, stems, roots, or seeds, or even though the edible part of the plant, that are considered super plus, which are not harvested. After that, plant foods are subjected to sorting, grading and/or cleaning processes, according to specific quality criteria, leading to the generation of **process by-products,** including husks, straw, leaves, stubble, and shells among others, which are separated during processing. In this regard, there are whole fruits and vegetables, which are removed because their quality standards are not met, and which are usually produced as field or process by-products, depending on if they are discarded prior to processing or during processing. Finally, **industrial by-products** are those generated from the different industrial transformation processes and are by-products of different physical and chemical nature being peels, pulp, pomaces and cakes, among others.

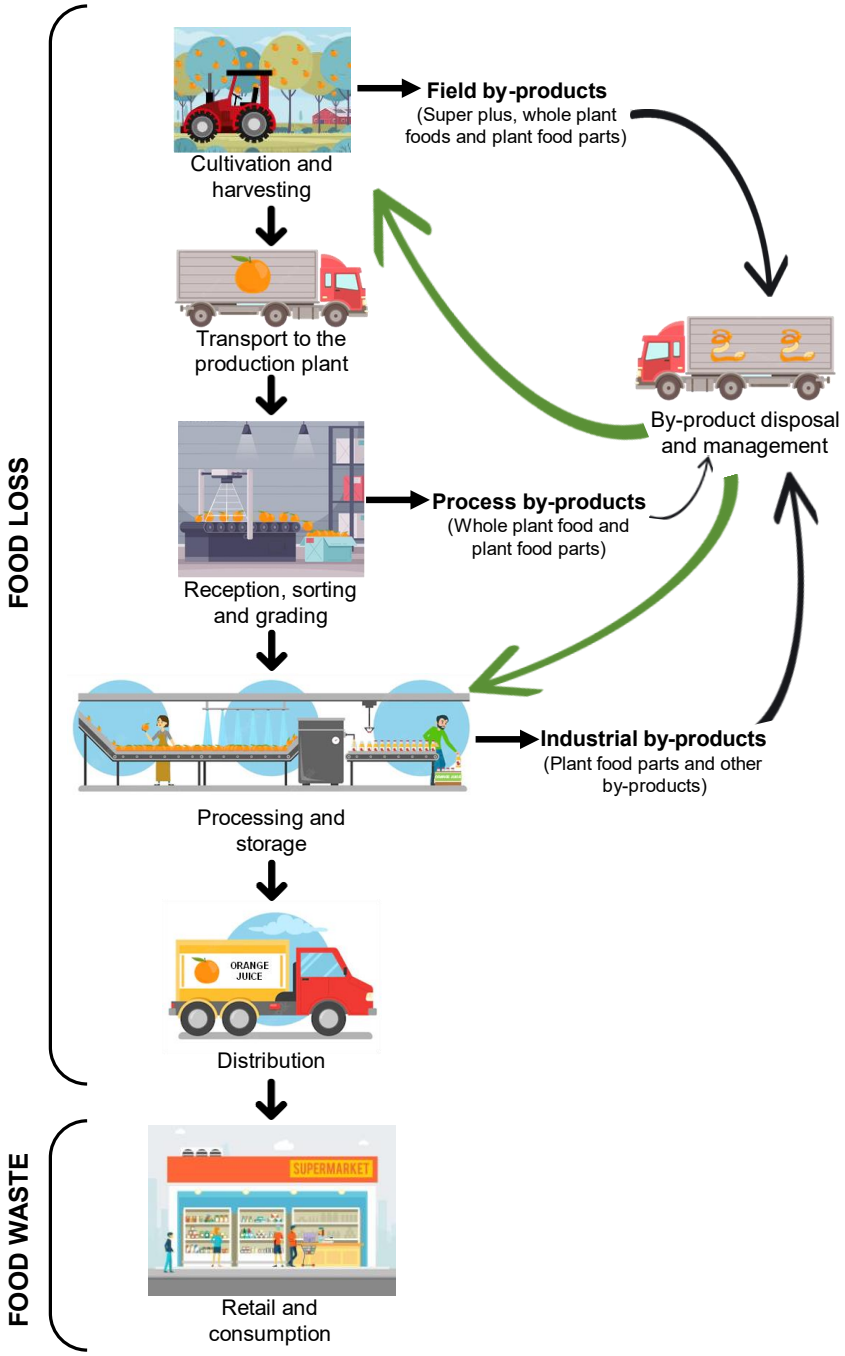



**Figure 1.** General diagram of by-product generation and management along the food chain (Gustavsson *et al.*, 2011; Sadh *et al.*, 2018).

### 1.2. By-products environmental issues

The Food and Agricultural Organization of the United Nations (FAO) has described the generation of by-products as food losses, referring to the decrease in edible food mass throughout the part of the supply chain that specifically leads to edible food for human consumption. Food losses take place at production, post-harvest and processing

stages in the food supply chain. On the other hand, the food losses that occur at the end of the food chain (retail and consumption) are considered as food waste (Figure 1) (Gustavsson *et al.*, 2011).

According to FAO, the food loss and waste in developed countries are higher than in developing ones. In Europe, North and South America and Oceania the food losses *per capita* were 550 kg/year. In contrast, in Sub-Saharian Africa and South and Southeast Asia the food losses *per capita* were 275 kg/year. At global level, 75% from the total, correspond to food loss occurring during production and postharvest, being the total *per capita* value for both food loss and waste 1,532 kg/year (Gustavsson *et al.*, 2011). Regarding the food loss and waste database between 2016-2017, the greatest losses around the world for fruit and vegetable group occurred in the pre-harvest and distribution steps, which highlights the importance of taking advantage of the by-products generated at these stages (FAO, 2022).

In the EU, 88 million tonnes of food are wasted every year, which are associated with costs of 143 billion euros, of which 35% is lost between primary production and trade (European Comission, 2016). In Spain, according to Ministry for Ecological Transition and the Demographic Challenge, around 7.7 million tonnes of food are wasted every year, about 9% of the EU total, 39% corresponding to food loss at manufacturing level (ECODES, 2018). In the plant food industrial processing the main by-products produced are peels (3-40% of the total fruit and vegetable fresh weight), seeds (15-40% of the total fruit fresh weight), pomaces and cores among others (Del Rio *et al*., 2021). In particular, within the fruit and vegetable subsector, according to Ministry of Agriculture, Fisheries and Food (MAPA, 2021), the fruit juice industry generates most of the by-products, specifically the orange juice industry (with approximately 163,000 tonnes/year), followed by the fresh fruit and vegetable industry (with approximately 76,000 tonnes/year), the vegetable canning industry, the frozen plant food industry and the olive industry (MAPA, 2021).

The generation and management of by-products cause social, environmental and economic problems for companies and for the whole society. It can have social impact associated with ethics and morals, as according to FAO in 2019 there were 688 million of people undernourished in the world. So, a good management of by-products that allows their reintroduction into the food chain could be a way to manage nutritional problems at a global level (Torres-León *et al.*, 2018). Regarding environmental problems, global food loss and waste are estimated to generate a total carbon footprint of 3.6 Gigatons of $CO_2$ eq. The carbon footprint of a food product is the total amount of

greenhouse gas emissions emitted throughout its life cycle; expressed in kilograms of $CO_2$ equivalents, from the production phase through to processing, transport, food preparation and waste disposal. Taking into account the Sustainable Development Goals, the valorisation of the by-products is related to the sustainable development goal 12 “responsible consumption and production”, since this goal could be achieve by reducing food loss and waste to decrease the impact on climate change (FAO, 2019). Moreover, in most cases, by-products are made up of large amounts of biodegradable compounds that can be used by bacteria and are consequently susceptible to content pathogen bacteria, that can cause communicable diseases (Socas-Rodríguez *et al.*, 2021). Therefore, and taking into account the current globalisation level, they may become real problems for society, as has been the case of the current COVID-19 pandemic (Socas-Rodríguez *et al.*, 2021).

From an economic perspective, a reduction in food loss and waste and the valorisation due to their reintroduction into the food chain lead to a significant reduction in by-products management costs, at the same time that the industries may have a profit from the products that have been obtained from these by-products (Socas-Rodríguez *et al.*, 2021). In other words, increased profitability along the food chain is achieved through the efficient use of materials, which consists of using materials that would otherwise have been discarded and doing so in an efficient way (e.g., by reusing by-products as raw material, recovering solvents or reusing water in different processes). In this way, the costs of purchasing new raw materials are saved and the valorisation of a by-product is gained, to which, through an industrial process, value has been added by taking advantage of its properties (Otles *et al.*, 2015).

In this regard, two new concepts have been introduced in recent decades, such as the circular economy and the bioeconomy (Sillanpää and Ncibi, 2019). In the literature, there are several definitions that have been proposed to define them. The most accurate definition for circular economy was described by Murray *et al*. (2017), which includes economic, environmental and human aspects. The definition suggested by these authors reads: “Circular economy is an economic model wherein planning, resourcing, procurement, production, and reprocessing are designed and managed, as both process and output, to maximise ecosystem functioning and human well-being*”.*

The term bioeconomy is directly related to the circular economy, even though some authors have even gone so far, speaking of a new concept “the circular bioeconomy” (Sheridan, 2016). According to the European Commission bioeconomy – which encompasses the sustainable production of renewable resources from land, fisheries

and aquaculture environments and their conversion into food, feed, fibre bio-based products and bioenergy as well as the related public goods – is an important element of the European response to the future challenges. The bioeconomy includes primary production, such as agriculture, forestry, fisheries and aquaculture, and industries using/processing biological resources, such as the food, pulp and paper industries, and parts of the chemical, biotechnological and energy industries (European Comission, 2012). The Spanish bioeconomy strategy defines the bioeconomy as the whole of the economic activities that provide goods and services, and thus generate economic value, through the use, as fundamental elements, of resources of biological origin in an efficient and sustainable manner (Laínez and Periago, 2019). In this sense, considering that the agri-food sector has an important weight in the Spanish economy, accounting for 8.9% of the Gross Domestic Product in 2018 (MAPA, 2018). It highlights the importance of the engagement of the Spanish agri-food industry, in leading its value chain towards a circular production based in bioeconomy, developing strategies aimed at efficiency in the use of resources and energy, and the reuse and recovery of waste and by-products. A recent study, based on the economic impact of the bioeconomy in Spain, showed that although it is still far from being an economic system based on this concept, strategies should be developed mainly within the sectors of food, biomass and agriculture, as they have the greatest impact due to the type of products that they develop (Ferreira *et al.*, 2021).

### 1.3. Composition of the agro-industrial by-products and industrial applications

The strategy for the utilisation of by-products from the plant food industry should take into consideration their composition. In this sense, these by-products have important contents of proteins, dietary fibre (DF), starch, micronutrients (Torres-León *et al.*, 2018), as well as lipids, being seeds the by-product with the highest content of fat. For example more than 20% for pepper and melon seeds are constituted by fat (Zou *et al*., 2015; Mallek-Ayadi *et al*., 2018). However, the content of DF stands out, as have been described in the scientific literature for some by-products, and as is shown in Table 1. So, the content of total dietary fibre (TDF) in apple, redcurrant, rowanberry and tomato pomace are 51.1%, 58.1%, 67.2% and 64.1%, respectively (Table 1) (Reißner *et al.*, 2019; Azabou *et al.*, 2020); whereas in citrus, mango, plantain and tomato peels the mean contents of TDF are 67.4%, 69.9%, 64.3% and 86.2% (Table 1), respectively (Navarro-González *et al.*, 2011; Jayalaxmi *et al.*, 2018; Pacheco *et al.*, 2019). On the other hand, the content of TDF in seeds is lower compared with the pomace and peels, ranging from 2.9-26.3% (Table 1).

**Table 1.** Dietary fibre (%) and bioactive compounds of fruit and vegetable by-products based on data reported by Cassani and Gomez-Zavaglia (2022).

| Fruit/vegetable | TDF* | IDF | SDF | (Poly)phenols | Carotenoids |
|---|---|---|---|---|---|
| **Pomace** | | | | | |
| Apple | 51.1 | 36.5 | 14.6 | 10.2 mg GAE/g | - |
| Redcurrant | 58.1 | 51.1 | 7.1 | 20.0 mAU min/g | - |
| Rowanberry | 67.2 | 59.5 | 7.7 | 37.0 mAU min/g | - |
| Tomato | 64.1 | 58.5 | 5.6 | 55.1 mg GAE/g | - |
| **Peel** | | | | | |
| Citrus | 67.4 | 62.5 | 4.9 | 145.5 mg GAE/100 g | - |
| Mango | 69.9 | 44.2 | 24.6 | 4.5 mg GAE/100 g | 5.6 μg β-carotene/g |
| Plantain | 64.3 | 56.9 | 7.5 | 15.2 mg QE/g | - |
| Tomato | 86.2 | 71.8 | 14.3 | 158.1 mg GAE/100 g | 3-4 mg lycopene/100 g |
| **Seed** | | | | | |
| Avocado | 3-26 | - | - | 0.3-0.5% | - |
| Grapes | 8.2 | - | - | 0.5 g/100 g | - |
| Papaya | 8-9 | 2.5-3.4 | 5.2-5.4 | 34-92 mg GAE/g | - |

*Total dietary fibre (TDF); insoluble dietary fibre (IDF); soluble dietary fibre (SDF); gallic acid equivalents (GAE); mili-absorbance unit (mAU); quercetin equivalents (QE).

Of the TDF, insoluble dietary fibre (IDF) has been described as the major fraction in these by-products, being represented by cellulose, hemicellulose and lignin (Cassani and Gomez-Zavaglia, 2022). Moreover, the amounts of soluble dietary fibre (SDF) range from 7 to 35%, represented mainly by pectin content, with a high percentage of TDF in apple pomace and mango peel (Cassani and Gomez-Zavaglia, 2022). In addition, plant food by-products are rich in antioxidant bioactive compounds ((poly)phenols and carotenoids), showing different composition according to the raw material. In Table 1 are shown the total content of (poly)phenols in different samples of pomace and peels, and the content of carotenoids in mango and tomato peels (Cassani and Gomez-Zavaglia, 2022). Other authors have reported that grape, tomato pomace and red corn cob contain important concentrations of gallic acid, rutin, epicatechin and apigenin (Abbasi-Parizad *et al.*, 2021). Also relevant contents of carotenoids have been described in orange,

mango, carrot and tomato by-products (Navarro-González *et al*., 2011; Sharma *et al*., 2021).

In the agro-industrial sector, around the 50% of the whole mass of the vegetables and fruit products are discarded during the course of the industrial process (Ayala-Zavala *et al.*, 2011). Traditionally landfilling or incinerating has been used for by-products disposal, but these practices lead to the problem of polluting the environment (air, water and soil). Although they are now in disuse, they are still actively used in some countries (Socas-Rodríguez *et al.*, 2021). As an alternative, these by-products have been also used as fertilisers in the agronomical practices and/or has been used as animal feed, since they still maintaining the chemical composition of the raw material and provide different amounts of protein, cellulose and hemicellulose (Otles *et al.*, 2015). However, the utilisation of by-products in the animal nutrition practices has certain problems. One of them is the potential presence of toxic or chemical compounds which could be danger for the livestock, affecting the performance and the food production. In addition, the moisture content, which allow the growth of pathogenic microorganisms and may endanger the quality of life of the livestock, at the same time that difficult their transportation and storage. Moreover, the composition of the by-products varies seasonally, so that reformulations have to be made constantly to prepare the feed and sometimes in order to ensure their use, the by-products often have to be subjected to industrial treatment, which leads to an increase of the price of the feed, making the process more difficult and unprofitable (San Martin *et al.*, 2016).

Despite of the application of plant foods by-products has been used from many years ago, nowadays, new trends in by-product utilisation have focused on the recovery and reuse of valuable components. This aspect has attracted increased interest from companies, mainly due to the application of the circular economy and bioeconomy approaches. In these sense, by-products can be used to obtain compost, biofuel, bio-adsorbent materials, biopolymers, new textile materials or bioactive compounds to be used in food or pharmaceutic industries (Jõgi and Bhat, 2020).

Composting is a technique that has been widely used for the transformation of fruit and vegetable by-products due to its composition (Ghinea and Leahu, 2020). It is an old and cheap technique, consisting of an exothermic biodegradation process of biochemical reactions in which facultative aerobic microorganisms use the plant substrates for the development of their metabolic needs. In general terms, the microorganisms act on carbonaceous substrates to hydrolyse and oxidise them, giving rise to carbon dioxide, heat, and compost as the final products, which is used as soil improver (Chang *et al.*,

2006). There are two main types of composting, normal composting, where microorganisms such as bacteria, fungi and protozoa are involved, and vermicomposting, where earthworms are involved, generating an humus-like output as the end product (Otles *et al.*, 2015).

The use of agri-food waste to generate biofuels has emerged as an important valorisation strategy due to the depletion of fossil resources (Nayak and Bhushan, 2019). Aerobic and anaerobic digestion and microbial fermentation have been the main technologies employed. In this sense, several studies have been carried out that showed the potential of different matrixes rich in carbohydrates and sugars to generate biofuels, such as wheat and rice straw or sugar cane bagasse, among others (Sharma *et al*., 2020). Some biofuels produced are biomethane and biohydrogen, which are classified as gas biofuels, as well as bioethanol and biodiesel, which are considered liquid biofuels. According to Stephen and Periyasamy (2018) liquid biofuels, in particular biodiesel, are gaining more prominence due to greater efficiency in their production and high economic performance.

Another application for which agro-industrial by-products have been used, especially lignocellulosic by-products, is for the development of bio-adsorbent materials for wastewater treatments, as adsorption is one of the cheapest and cleanest technologies (Solangi *et al.*, 2021). Commercial activated carbon is commonly used, but due to its high cost and the difficulty in the subsequent management of the waste, obtaining activated carbon from industrial by-products, has become one of the most sustainable and advantageous alternatives (Nayak and Bhushan, 2019). Among some of the by-products used for the development of bio-adsorbents are banana bunch, pomelo peel and apple seed husks (Allwar *et al.*, 2018; Abatan *et al.*, 2019; Wang *et al.*, 2020).

In the last few decades, there has been a growing awareness to reduce the consumption of petroleum-based plastics (Ibrahim *et al.*, 2019), at the same time that the production of biopolymers have gained popularity. Biopolymers are polymers derived from renewable organic substrates that have several advantages over traditional polymers (Nayak and Bhushan, 2019). Firstly, they increase the organic content of the soil and reduce the presence of chemicals. They also have environmental advantages, as they are produced from renewable sources, using less energy in their production and also producing less greenhouse gases (Ibrahim *et al.*, 2019). For their production, agro-industrial by-products such as citrus peel and pulp, seed residues and sugar bagasse, among others, are used (Ranganathan *et al.*, 2020). The carbohydrates, fibres, proteins,

organic acids, and oils present in these by-products are extracted and subsequently processed using fermentative and enzymatic processes that produce biopolymers as final products. Some of the most common biopolymers are poly-lactic acid, poly-butylene succinate and polyethylene, which are used in packaging, agriculture, biomedical and cosmetic industries (Ibrahim et al., 2019; Nayak and Bhushan, 2019).

Regarding the textile industry, in recent years the development of new materials from industrial by-products has been implemented, such as rice, corn and wheat straws. These by-products are used to produce bio-resins, which combined with other vegetable fibres such as linen are used to produce vegetable leather which is used for the manufacture of footwear (Faria-Silva *et al.*, 2020). The development of new yarns from oranges has also been implemented, as well as the development of fabrics from coffee by-products, which also have the capacity to adsorb odours (Faria-Silva *et al.*, 2020).

Other recent applications are the extraction of different bioactive compounds for food industry, nutraceutical and pharmacological application (Domínguez-Perles *et al.*, 2018; Socas-Rodríguez *et al.*, 2021), since by-products from fruits and vegetables are rich in phytochemicals, which are considered natural active substances with significant health-promoting effects (Kumar, 2020).

In the scientific literature, several techniques are reported that have been used for the extraction of the different bioactive compounds, which will be use later as ingredient in different industries, including their use as nutraceutical products. The most common are the atmospheric liquid extraction methods, including microwave and ultrasound-assisted extractions, which have been used to extract (poly)phenols, hydrophobic and hydrophilic pigments (Alirezalu *et al.*, 2020; Cassani and Gomez-Zavaglia, 2022). Other method is the pressurised liquid extraction, which have been defined as “accelerated solvent extractions”, due to high pressure helps to accelerate the processes. This method is used to extract a large variety of phytochemicals depending on the chemical characteristics of the compounds and the solvent used (Manzoor *et al.*, 2021). The technique based in the enzyme-assisted extraction, uses hydrolytic enzymes alone or combined with other extraction techniques, allowing the unbounding of the phytochemical compounds from the cell wall (Manzoor *et al.*, 2021). The supercritical fluid extraction, is the technique used to extract thermolabile compounds, such as pigments and (poly)phenols (Ahangari *et al.*, 2021). The pulsed electric field assisted extraction, is applied by using a high voltage pulses, facilitating extraction of phytosterols, (poly)phenols and carotenoids (Kumar, 2020; Cassani and Gomez-Zavaglia, 2022).

Once extracted, the phytochemicals or the bioactive compounds can be used as nutraceuticals, as in the case of extracts from berry pomace (Dienaitė *et al.*, 2021). In addition, others have shown pharmacological properties, as is the case of orange peel components, which showed potential use in the prevention of membrane oxidative stress (Castro-Vázquez *et al.*, 2021) and berry pomace with potential use in antimicrobial therapies (Silvan and Martinez-Rodriguez, 2018). Also orange juice by-products have shown neuroprotective properties (Sanchez-Martinez *et al.*, 2021) and flavonoids from citrus peel have shown potential cancer prevention properties (Koolaji *et al.*, 2020).

Furthermore, new uses have also been reported within the cosmetics industry. In this sense, grape by-products could be used to treat skin wrinkling and pigmentations disorders (Leal *et al.*, 2020). Furthermore, other *in vitro* and *in vivo* studies have linked (poly)phenols to the prevention of skin damage, rosacea and psoriasis, among others (Faria-Silva *et al.*, 2020). Other compounds such as β-carotene in tomato skin as a by-product are used in the development of sunscreen formulations (Faria-Silva *et al.*, 2020).

Nowadays, due to the chemical properties and the compounds present in fruit and vegetables by-products, similar to those present in the raw materials, they can be reused in the food industry after further processing. Currently, the reintroduction of these by-products into the food chain is one of the most important ways of recovery, as they can be reused in the food chain following the circular economy model. For example, carotenoids and (poly)phenols obtained from plant by-products have been used as natural food colourants and preservatives (Oreopoulou and Tzia, 2007). Betalains have also been used as natural colourants (Amaya-Cruz *et al.*, 2019). The natural colourants extracted from industrial by-products can be considered new ingredients and allow the development of innovative foods with natural or sometimes fortified ingredients, in which the artificial colourant has been replaced by one with a higher nutritional value and which is often even cheaper (Majerska *et al.*, 2019). Other important use of the plant food by-products is the extraction of TDF, IDF and SDF, that can be used as ingredients to the design and development of fibre-rich products. So, the DF can be added to cereal flour for the development of bakery products, improving the nutritional value and the health-promoting properties (Ma and Han, 2019). The industrial applications of the fibre are based on its physicochemical properties, which are described in section 2.2.

## 2. Dietary fibre

Dietary fibre has been extensively studied because of its physicochemical properties which are directly related to its physiological effects. In this sense, there is still no consensus on the definition of DF as it has been changing over time according to different authors. The concept of fibre was first mentioned in 1953, when Hipsley associated the presence of fibre in the diet with the potential inhibition of preeclampsia. Later, Southgate in 1969 used the term “unavailable carbohydrates” to define DF, which is related to a previous definition given by Mccance and Lawrence in 1929 that differentiated between "available" and "unavailable" carbohydrates, the latter being those that cannot be hydrolysed by human digestive enzymes and are thus not absorbed. The term of DF was implemented a few years later by Trowell, in 1972. This researcher offered the first nutritional definition of this compound, differentiating between crude fibre and DF, being the crude fibre “the part of carbohydrates of food that resists extraction by boiling, first with sulphuric acid and then with sodium hydroxide, being mainly formed by cellulose and lignin”. On the other hand, the concept of DF, was defined “as the skeletal remains of plant cells that are resistant to digestion by human enzymes”. Furthermore, this author pointed out that it was DF and not crude fibre that could help to prevent diseases. Because, in the South Africa population, which had high consumption of fibre, both coronary heart disease and diverticular disease were very rare, hypothesising that consumption of fibre-rich carbohydrates may protect against atherosclerosis and reduce hypercholesterolaemia (Trowell, 1972).

The DF concept has been modified over the years according to the analytical methods of determination and quantification (Cruz-Requena *et al.*, 2019). In 2009 Codex Alimentarius Commission defined DF “as carbohydrate polymers with 10 or more monomeric units, which are not hydrolysed by the endogenous enzymes in the small intestine of humans and belong to the following categories: edible carbohydrate polymers naturally occurring in the food as consumed; carbohydrate polymers, which have been obtained from food raw material by physical, enzymatic or chemical methods, and which have a physiological effect or health benefit as demonstrated by generally accepted scientific evidence, as well as synthetic carbohydrate polymers, which have a beneficial physiological effect or benefit to health as demonstrated by generally accepted scientific evidence” (Lupton *et al.*, 2009).

Other authorities have offered other definitions of DF, such as European Food Safety Authority (EFSA), which defines DF “as non-digestible carbohydrates plus lignin

and hence includes: non-starch polysaccharides (NSP) (cellulose, hemicelluloses, pectins and hydrocolloids, e.g., gums, mucilages, β-glucans), resistant oligosaccharides (fructo-oligosaccharides, FOS, and galacto-oligosaccharides, GOS), other resistant oligosaccharides, resistant starch (RS) (consisting of physically enclosed starch, some types of raw starch granules, retrograded amylose, chemically and/or physically modified starches), and lignin associated with the DF polysaccharides" (EFSA, 2016).

Additionally, the Food and Drug Administration has provided a definition of fibre "as non-digestible soluble and insoluble carbohydrates (with 3 or more monomeric units), and lignin that are intrinsic and intact in plants; as well as isolated or synthetic non-digestible carbohydrates (with 3 or more monomeric units), which have physiological effects that are beneficial to human health" (Food and Drug Administration, 2018).

Around these definitions, debate has arisen related to the inclusion of oligosaccharides within the definition, so that different authorities and organisations have been modifying the DF definition. In this sense, a scientific consensus has emerged within the scientific community to include oligosaccharides in the definition to facilitate international trade and the comparison of consumption between different regions, as well as to simplify the interpretation in physiological effect studies (De Menezes *et al.*, 2013).

**Table 2.** Classification of DF based on its different characteristics.

| Fibre type | Examples |
|---|---|
| Dietary fibre* | Lignin, cellulose, pectin, gums and starch |
| Insoluble fibre | Cellulose lignin and some pectin and hemicellulose |
| Soluble fibre | Pectin, gum, β-glucan, psyllium and inulin |
| Fermentable fibre | Pectin, guar gum, β-glucan and inulin |
| Non-fermentable fibre | Lignin |
| Viscous fibre | Pectin, β-glucan, guar gum and psyllium |
| Non-viscous fibre | Polydextrose and inulin |
| Functional fibre | Psyllium, resistant dextrin, RS and FOS |

*Based on data reported by Slavin (2013).

Moreover, DF can be classified not only according to the chemical compounds but also according to its properties as is shown in Table 2. Each type of fibre shows different properties of solubility, fermentability and viscosity, which determine the beneficial effect for human health (Slavin, 2013).

In addition, there are five different types of DF according to their solubility in water and the capacity to be fermented by the gut microbiota (Abreu *et al*., 2021). In this sense, DF can be classified in:

- **Soluble and highly fermentable short-chain fibre:** it consists of oligosaccharides, such as FOS and GOS, which stimulate *Bifidobacteria* growing. It has a weak laxative effect and does not affect intestinal transit time, although it causes flatulence.
- **Soluble and highly fermentable long-chain fibre:** it stimulates bacterial growth. Has weak laxative effect, does not affect intestinal transit time and causes moderate flatulence.
- **Partially soluble and moderately fermentable fibre:** it has a good laxative effect, accelerates intestinal transit, stimulates general bacterial growth and causes moderate flatulence.
- **Insoluble and slow-fermentable fibre:** it has a good laxative effect, accelerates intestinal transit, stimulates bacterial growth, and causes moderate flatulence.
- **Insoluble and non-fermentable fibre:** it has a good laxative effect, accelerates intestinal transit, and only stimulates the growth of specific bacteria that degrade it, such as *Xylanibacter* and *Prevotella*.

## 2.1. Dietary fibre components

The main compounds of DF are the polysaccharides of the cell wall, being mainly composed by cellulose, hemicellulose, pectin and other components (Figure 2), such as gums, starch, oligosaccharides and lignin, which are described below (Rezende *et al.*, 2021).

### *2.1.1. Cellulose*

Cellulose is an homopolysaccharide consisting of monomeric units of D-anhydroglucose ($C_6H_{11}O_5$) linked together by β-(1→4)-glycosidic bonds (Abdul Khalil *et al.*, 2017). Each monomer consists of three hydroxyl groups, which form bonds that are

essential for the crystalline packing on which the physical properties of cellulose depend (Brinchi *et al.*, 2013). It is the most abundant biopolymer in nature, as it is the main component in the structure of plant cell walls. In nature, cellulose is not found as independent units but in the form of fibres, whose structure depends on the degree of polymerisation or number of glucose units, which varies over a wide range (Brinchi *et al.*, 2013). By-products are an important source of DF, being cellulose found in high amounts in stems, straw and peels, with 42% in banana stem, 51% in maize straw and 41% and 39% respectively in onion and oat peel (Ahmad Khorairi *et al.*, 2021), it have been also found in high proportions in citrus peel and grapefruit wastes with 34% and 27%, respectively (Rivas-Cantu *et al.*, 2013). Cellulose is insoluble in water and it is not digested by human enzymes, but when reaches the colon, around 50% is degraded by some microbial communities with cellulose degrading capacity, being *Ruminococcus* sp. and some *Enterococcus* sp. the most described groups with this capacity (Chassard *et al*., 2010; Mudgil and Barak, 2013). Cellulose provides important beneficial effects for human health such as increasing faecal bulk and promoting large intestine movements.

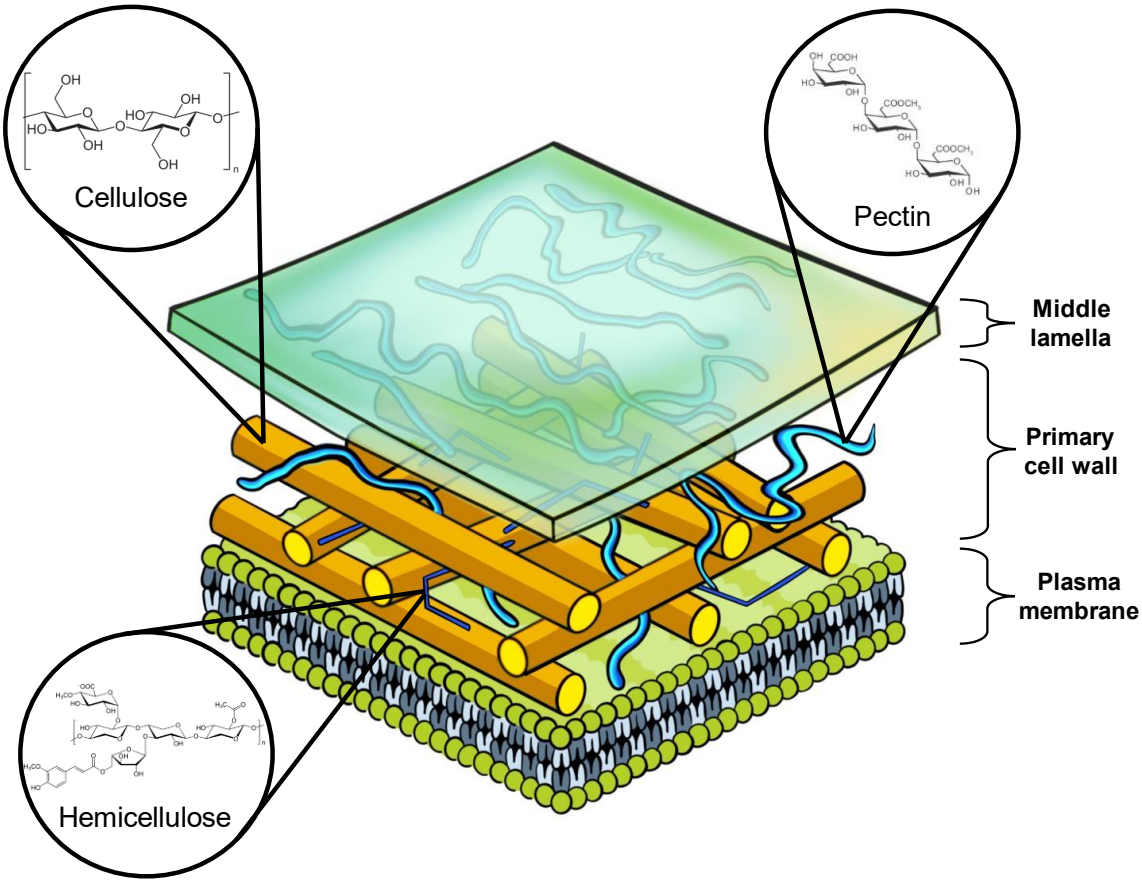



**Figure 2.** Structure and major components of plant cell wall. Adapted from Loix *et al.* (2017).

### *2.1.2. Hemicellulose*

Unlike cellulose, hemicellulose is an heteropolysaccharide consisting of monomers other than glucose, including pentose units (xylose and arabinose) and hexose units (glucose, galactose, mannose, rhamnose, glucuronic and galacturonic acids). Both linear and branched molecules are included in its structure (Hu and Yu, 2013; Mudgil and Barak, 2013). It is the second most abundant component of lignocellulosic biomass after cellulose, and is also found in plant cell walls (Farhat *et al.*, 2017; Cruz-Requena *et al.*, 2019). Hemicellulose have been extracted from agro-industrial by-products, being found in blackcurrant pomace in 25%, 34% in chokeberry, 11% in cherry, 10% in citrus peel and 6% in grapefruit waste (Nawirska and Kwaśniewska, 2005; Rivas-Cantu *et al.*, 2013). Hemicellulose is soluble in alkali solutions and its water solubility depends on its structure, being hemicellulose with high mannose contribution more soluble in water than that with high xylose contribution (Dhingra *et al.*, 2012; Peng *et al.*, 2019). Moreover, they promote regular bowel movements by increasing the hydration of the stool, as well as binding to cholesterol in the intestine, preventing its absorption (Mudgil and Barak, 2013).

### *2.1.3. Pectin*

Pectin is a family of heteropolysaccharides consisting of galacturonic acid and other neutral sugars like rhamnose, arabinose and galactose monomers bound by α–(1→4) linkages, which is partly esterified as methyl esters (Flutto, 2003; Mudgil and Barak, 2013). They also have a structural role in plant cell walls, as well as acting as intercellular binders (Dhingra *et al.*, 2012). It should be noted that pectins are mainly found in fruit and vegetable peels, being found in orange peel ranging between 8%-53%, in lemon peel ranging from 1%-17% whereas in grapes may vary between 2%-16%, according to the different analytical and extraction methods (Venkatanagaraju *et al.*, 2019). Pectins are water-soluble in hot water and have the ability to form gel when cooled, so their consumption is of special interest since gelling traps cholesterol molecules in their structure, preventing their absorption and facilitating their elimination, as well as their hypoglycemic properties, by interfering the absorption of glucose in the large intestine (Mudgil and Barak, 2013; Zaitseva *et al*., 2020). The degree of esterification of pectins is an important parameter defining their applications. The degree of esterification is understood as the percentage of carboxyl groups esterified with methanol present in the molecule. On this basis, pectins are divided into high methoxylation pectins with a degree of esterification above 50% and low methoxylation

pectins below 50%. In terms of their characteristics, the high methoxylation pectins can form gels when heated in solutions at low pH with high concentration of sugars, whereas the low methoxylation pectins can form gels in a wide pH range with or without sugars and in the presence of divalent ions such as calcium (Hosseini *et al.*, 2016). Pectin molecules can be linear or branched, the side chains being linked to the galacturonic acid backbone by rhamnose units, all these structural characteristics and the length of the side chains determine the physicochemical properties of the pectin molecule (Naqash *et al.*, 2017).

### *2.1.4. Gums and seaweed polysaccharides*

This group is comprised by a type of fibres that do not constitute part of the cell wall structures but are excreted by specialised cells (Dhingra *et al.*, 2012). Within this group, there are vegetable exudates, gum arabic and tragacanth, those obtained from seeds, such as guar gum and locust bean gum, and finally those obtained from algae, which include agar, carrageenan, and alginate. These are highly branched polysaccharides that have gel forming properties and are therefore widely used in industry as gelling agents, thickeners, stabilisers and emulsifiers in certain food products (Dhingra *et al*., 2012; Mudgil and Barak, 2013). Agar have been found between 10%-18% in *Gracilaria sp.,* carragenan have been found between 22%-36% in *Kappaphycus alvarezii* and 21%-34% in E*ucheuma spinosum* using different extraction methods, and alginate in 32%, 34% and 27% in *Laminaria japonica, Ecklonia cava* and *Sargassum duplicatum,* respectively (Istinii *et al.*, 1994). They have several health benefits due to their ability to form gels. They slow down gastric emptying, thereby increasing the feeling of satiety and helping to reduce body weight (Butt *et al.*, 2007). There is also a reduction in free cholesterol, because there is an increase in the excretion of bile acids in the faeces and a decrease in intrahepatic bile acids, increasing the production of bile acids from cholesterol (Rideout *et al.*, 2008). It also has hypotriacylglycerolaemic effects, as lipid absorption is decreased and fatty acid synthesis in the liver is reduced (Mudgil *et al.*, 2014).

### *2.1.5. Resistant starch*

RS is the fraction of starch present in a food product that is not digested by human digestive enzymes, hence reach the colon, where may be fermented by the microbiota. Starch is synthesised in the plastids and stored in the amyloplasts as granules (Mudgil and Barak, 2013). The structure of these granules depends on their botanical origin, and they are mainly composed of amylose and amylopectin biopolymers. Amylose is a glucan

linked by α(1→4) bonds and amylopectin has chains with α(1→4) linkages and also has multiple branches α(1→6) (Gidley *et al*., 2010; Mitchell and Hill, 2021).

Starch is classified into five sub-fractions, encapsulated starch (RS I), which is inaccessible to enzyme digestion due to the cell walls and protein barriers formed; resistant granules (RS II), is resistant to digestion due to its crystalline structure, when reaches the intestine it may allow the growth of beneficial bacteria (*Bifidobacterium* and *Bacteroidetes*); retrograded amylose (RS III), this type of starch is formed when starch containing food are cooked and then cooled, forming double helices in amylopectin chains that cannot be hydrolysed by digestive enzymes, but it may increase the short-chain volatile fatty acids (SCFAs) production and increase the growth of beneficial bacteria in the intestine (mainly *Bifidobacterium*); chemically modified starch (RS IV), which may change the profile of intestine bacterial communities and increase the SCFAs production and decrease the pH; and amylose-lipid complex (RS V), that due to its structure is resistant to both amylolytic and enzymatic hydrolysis and may help to control glycemic and insulinemic, helping also to prevent colon cancer by stimulation of SCFAs production (Hasjim *et al*., 2013; Lockyer and Nugent, 2017; Rezende *et al*., 2021). The content of total starch have been reported in rice in 81% with 1.5% of RS, 60% of total starch with 3.5% of RS in pea and 86% of total starch with 1.8% of RS in potato (Yadav and Yadav, 2010). It is also noted other nutritional properties for RS, such us potential reduction of glucose and insulin response, as well as a reduction in transit time and increasing stool frequency (Zhao *et al.*, 2018).

### *2.1.6. Oligosaccharides*

Oligosaccharides are carbohydrates consisting of 3-10 monomeric units linked by α- and/or β-glycosidic bonds (Laurentin and Edwards, 2012; Cruz-Requena *et al*., 2019). The main components of monosaccharides include D-glucose, D-galactose, D-mannose, L-glucose, N-acetyl-D-glucosamine and others. Oligosaccharides occur naturally in plants but can also be synthesised from monosaccharides and disaccharides by chemical or enzymatic processes and from polysaccharides by hydrolysis. The main oligosaccharides include FOSs, GOSs and lactulose among others (Cruz-Requena *et al.*, 2019; Navarro *et al.*, 2019). Although the content of this compounds is low, FOSs have been found in broccoli and kale around 1% of fresh weight and in berries around 0.5% of fresh weight (Jovanovic-Malinovska *et al.*, 2014), and GOSs have been found around 3% of fresh weight in legumes (Njoumi *et al.*, 2019). The intake of oligo-saccharides has a beneficial effect on health, as they are indigestible and are able to reach the colon where they can be metabolised by the microbiota, triggering a prebiotic

effect by increasing the number of beneficial bacteria and decreasing the number of pathogens (Laurentin and Edwards, 2012; Mudgil and Barak, 2013).

#### *2.1.7. Lignin*

Lignin is not considered a polysaccharide, it is a polymer of phenylpropane units through chemical linkages of alkyl-alkyl, alkyl-aryl and aryl-aryl groups. It consists of precursors, some of which are *p*-coumaryl alcohol, coniferyl alcohol and sinapyl alcohol. Due to strong intramolecular binding with C-C bonds, which makes it very inert and more resistant than any other natural polymer (Dhingra *et al.*, 2012; Huang *et al.*, 2019). It has been also described in fruit and vegetable by-products, being found mainly in onion and pineapple peel with 39% and 29% respectively and in 24% in sugarcane bagasse (Ahmad Khorairi *et al.*, 2021). As for their health benefits, their potential applications in the treatment of obesity, diabetes, as well as viral infections and cancer stand out, due to their potent antioxidant capacity, which may be related to the presence in their structure of monomers derived from hydroxycinnamic acids and guaiacyl units (Vinardell and Mitjans, 2017).

### 2.2. Physicochemical properties

#### *2.2.1. Solubility*

As already mentioned, fibre is classified as soluble and insoluble depending on whether they form a solution when mixed with water or not (Mudgil and Barak, 2019). Solubility refers to the ability to hydrate easily in water, which is affected both by the structure of the molecules and by the conditions of the medium, such as temperature and ionic strength (Elleuch *et al*., 2011; Mudgil and Barak, 2019). It has been shown that modifications in the structure of the molecules increases the hydration capacity, as is the case of gum arabic with a high number of branches, pectin methoxylation with presence of ionic groups, and the different types of bonds that occur in β-glucans β(1→3) β-(1→4) (Dhingra *et al.*, 2012). In this sense, soluble fibres are characterised by their ability to increase viscosity, which is why they can be used at an industrial level to make gels or emulsions, and insoluble fibres, which are characterised by their porosity and their low density (Elleuch *et al*., 2011; Mudgil and Barak, 2013).

#### *2.2.2. Viscosity*

Viscosity is the resistance offered by a fluid to its flow. This characteristic is mainly associated with soluble fibre, which, after mixing with water, leads to viscous solutions

due to its water retention capacity (Mudgil and Barak, 2019). For this reason, they are used at industrial level for gel and emulsion production, which can be affected positively by the concentration of the fibres. The shear rate also affects the viscosity inversely, as the polysaccharides suffer an opening of their chains due to shearing, decreasing the viscosity. Another important factor at industrial level is the temperature, which also affects the viscosity inversely (Elleuch *et al*., 2011; Mudgil and Barak, 2013; Mudgil and Barak, 2019). The structure of the molecules is also a factor to be considered in gel formulation, since long-chain polymers with high molecular weight give rise to more viscous gels, whereas if the polymers are highly branched or have short chains, the viscosity will be low (Dhingra *et al.*, 2012).

### *2.2.3. Hydration properties*

Water binding capacity (WBC), water retention capacity (WRC), and swelling capacity (SWC) reflect the hydration properties of fibres and are useful for the design of fibre-supplemented foods (Elleuch *et al.*, 2011; Dhingra *et al.*, 2012). WBC is associated with the amount of water retained in the structure of a gel after exposure to external force or stress. For industrial purposes, it is a very interesting characteristic because many processes apply external forces, such as kneading, centrifugation or extrusion, being related to the porosity of the sample (Dhingra *et al*., 2012; Mudgil and Barak, 2019). WRC is defined as the amount of water retained per 1 g of dry fibre under specified conditions of time, temperature and centrifugation speed. Regarding SWC, this refers to the amount of water absorbed that makes fibres swell (Elleuch *et al.*, 2011). These properties can be affected by several factors such as the source of fibre, which determines its chemical composition, and other factors as microstructure, particle size and structure, as well as processing and environmental conditions, pH, temperature, presence of other ingredients and mechanical processing (Elleuch *et al*., 2011; Dhingra *et al*., 2012; Mudgil and Barak, 2019).

### *2.2.4. Fat absorption capacity (FAC)*

Fibres also could retain oil, which is of great interest for the design of new products and for the industrial processes. This capacity refers to the amount of oil that can be retained by the fibres after mixing with a defined amount of oil or fat. Important factors determining this capacity are the structure and chemical composition of the fibre components and the porosity of the fibres, as well as the nature of the oil involved. In some industrial processes, the fibre samples are pre-mixed with water to prevent the pores from being occupied by the oil after mixing with it, thus reducing the fat content of

the product, and making it healthier. In addition, this capability is also used to develop healthier meat emulsions with better texture (Elleuch *et al*., 2011; Mudgil and Barak, 2019).

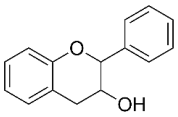
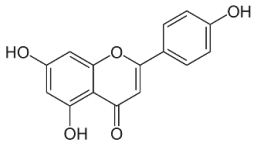
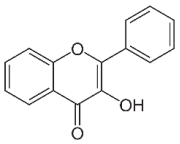
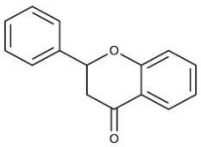
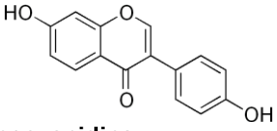
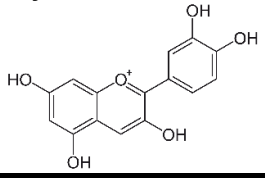
## 3. (Poly)phenols

(Poly)phenols are compounds derived from the secondary metabolism of plants. They play important roles in the plant kingdom, such as reproduction and growth, as well as protection from adverse environmental factors and against pathogens and herbivores (Waterman, 1992). They are also responsible for some of the organoleptic properties of plants, such as colour, taste or astringency, and its consumption has preventive properties against diseases such as obesity, diabetes, cardiovascular and neurodegenerative diseases, and some types of cancer (Scalbert *et al.*, 2005). These beneficial properties for human health derive mainly from its antioxidant capacity (Del Rio *et al.*, 2013; Marquardt and Watson, 2013).

(Poly)phenols have as a common structure one or more aromatic or benzyl rings to which one or more hydroxyl groups are attached. Generally, (poly)phenols are not found free, but are linked to sugars by β-glycosidic bonds to an hydroxyl group (O-glycosides) or to a carbon atom of the aromatic ring (C-glycosides). (Poly)phenol content and composition may vary depending on physiological, genetic and agronomic factors such as the cultivar, the soil composition, agronomic treatments, climatic conditions and pre- and post-harvest treatments (Tiwari and Cummins, 2013). More than 8,000 compounds have been identified in nature (Harborne and Williams, 2000), which are classified according to their origin, structure and biological function into two main groups being flavonoids and non-flavonoids (Table 3 and 4) (Tsao, 2010; Del Rio *et al.*, 2013).

In addition to the chemical classification, (poly)phenols can be also classified according to their binding to food matrix molecules in extractable and non-extractable compounds. In this sense, extractable (poly)phenols (EPP) are low molecular weight (poly)phenols that are easily released from the food matrix, which are extracted by using organic solvents (Hümmer and Schreier, 2008). This group includes proanthocyanidins (dimers and trimers) as extractable proanthocyanidins and hydrolysable tannins of low molecular weight (Durazzo, 2018; Plumb *et al.*, 2020).

**Table 3.** Flavanoids structure, main compounds and food sources.

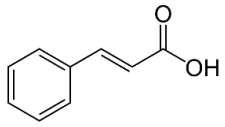
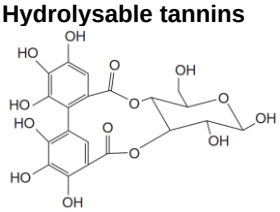
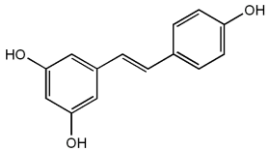
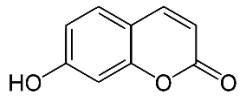
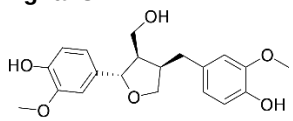
| Structure | Main compounds | Food sources |
|---|---|---|
| **Flavan-3-ols***  | Catechin<br>Epicatechin<br>Epicatechin gallate<br>Gallocatechin | Grape<br>Cocoa<br>Berries<br>Tea<br>Apple |
| **Flavones**  | Luteolin<br>Apigenin<br>Chrysin | Artichoke<br>Pepper<br>Citrus fruit<br>Cereals<br>Lettuce |
| **Flavonols**  | Quercetin<br>Kaempferol<br>Myricetin<br>Isoharmentin | Onion<br>Berries<br>Mango<br>Broccoli<br>Kale |
| **Flavanones**  | Naringenin<br>Hesperetin | Orange<br>Grapefruit<br>Lemon<br>Lime |
| **Isoflavones**  | Daidzein<br>Genistenin<br>Glycitein | Soybean<br>Lentil<br>Pea<br>Broad bean |
| **Anthocyanidins**  | Cyanidin<br>Delphinidin<br>Pelargonidin<br>Peonidin | Berries<br>Grape<br>Cherries<br>Eggplant<br>Onion<br>Pistachio nut |

*Based on data reported by González-Sarrías *et al.* (2020).

On the other hand, the non-extractable (poly)phenols (NEPP), represent the fraction which cannot be extracted easily with organic solvents, because they are bounded to the food matrix and mainly to the cell walls (Pérez-Jiménez *et al.*, 2013). Within this group there are two subgroups, hydrolysable (poly)phenols, which are low molecular weight (poly)phenols bound to the food matrix, generally to the fibre fraction (Pérez-Jiménez and Saura-Calixto, 2018). Furthermore, there are the non-extractable proanthocyanidins, which are high molecular weight proanthocyanidins with long chains, which, due to their structure, limit their release from the food matrix (Pérez-Jiménez and Torres, 2011; Martínez-Meza *et al.,* 2021). It is remarkable that some vegetables such as broccoli, carrot, orange and tomato have more than half of their (poly)phenols as NEPP (Martínez-Meza *et al.,* 2021).

Moreover vegetable by-products, which are a source of DF, are also a promising source of NEPP compounds (Pérez-Jiménez and Saura-Calixto, 2018). These compounds, due to their distribution in the food matrix, have important beneficial effects on human health due to its antioxidant effect, microbiota modulation and biological activities of metabolites produced during NEPP catabolism (Martínez-Meza *et al*., 2021). More information about NEPP is described behind in section 7.5.

**Table 4.** Non-flavanoid structure, main compounds and food sources.

| Structure | Main compounds | Food sources |
|---|---|---|
| **Phenolic acids***  | Caffeic acid<br>Ferulic acid<br>Sinapic acid<br>Ellagic acid<br>Gallic acid | Coffee<br>Broccoli<br>Raspberry<br>Blackcurrant<br>Blueberry |
| **Hydrolysable tannins**  | Punicallagin<br>Pedunculagin<br>Galloyl-hexoside<br>Digalloyl-hexoside | Berries<br>Pomegranate<br>Nuts<br>Mango<br>Red sword bean<br>Green and black tea |
| **Stilbenes**  | Resveratrol | Grapes<br>Red wine |
| **Coumarins**  | Umbelliferona<br>Esculetina<br>Scoparone | Citrus<br>Parsley<br>Celery |
| **Lignans**  | Secoisolariciresinol<br>Matairesinol<br>Pinoresinol<br>Lariciresinol | Seeds |

*Based on data reported by González-Sarrías *et al.* (2020).

### 3.1. Flavonoids

Flavonoids are the largest and most studied group of (poly)phenols, with more than 4,000 types of compounds identified. Their structure is formed by two aromatic rings joined by three carbon atoms forming an oxygenated heterocycle (Pandey and Rizvi, 2009). Depending on the degree of hydroxylation, oxidation and saturation of the central pyran ring, flavonoids are divided into several subgroups, flavan-3-ols, flavones,

flavonols, flavanones, isoflavones and anthocyanidins (Table 3). They occur naturally as glycosides rather than aglycones, mainly bound to glucose, xylose, rhamnose or galactose (Del Rio *et al.*, 2013; González-Sarrías *et al.*, 2020).

### *3.1.1. Flavan-3-ols*

Favan-3-ols have an hydroxyl group on the heterocyclic ring and are characterised not as glycosides, but as aglycones. They have different molecular weight structures ranging from monomers (catechin and epicathechin) to oligomers and polymeric proanthocyanidins, also known as condensed tannins. The most common proanthocyanidins in nature are called procyanidins which contained only catechin and epicathechin units. The proanthocyanidins formed by gallocatechin and epigallocatechin units are known as prodelphinidins while those formed with afzelechin and epiafzelechin units are called as propelargonidins (Tsao, 2010; Favari *et al.*, 2020; González-Sarrías *et al.*, 2020). In terms of health benefits, a beneficial effect on the reduction of the risk of cardiovascular diseases has been reported after the intake of flavan-3-ols (Kuhnle, 2018). They are mainly found in tea, berries, cocoa and nuts (Vadivel *et al*., 2012; Schulz and Chim, 2019; Kiyama, 2020; Martin and Ramos, 2021).

### *3.1.2. Flavones*

The structure of flavones consists of a double bond and an oxygen atom in the heterocyclic ring (González-Sarrías *et al.*, 2020). They are mainly found in the form of glycosides and the most important in this group are luteolin and apigenin (Dos Santos *et al.*, 2020). This group reacts by hydroxylation, acylation and glycosylation to form C- and O-glucosides (Del Rio *et al.*, 2013). In terms of its effects on human health, apigenin could be used in the prevention and treatment of cancer, cardiovascular and degenerative diseases (Kashyap *et al.*, 2018). Luteolin also has a preventive effect against certain types of cancer (Imran et al., 2019). The main food sources include parsley, red peppers, celery, chamomile and mint, and some citrus fruits such as oranges and grapefruit (Shukla and Gupta, 2010; Panche et al., 2016).

### *3.1.3. Flavonols*

The structure of flavonols is similar to that of flavones, hence the name 3-hydroxyflavones, due to the presence of an hydroxyl group on carbon 3 of the flavone nucleus. In nature they are usually found in glycoside form (González-Sarrías *et al.*, 2020). Among the most common in this group are quercetin, kaempferol and myricetin

(Del Rio *et al.*, 2013). In terms of their beneficial effects on health, they have antioxidant, anti-inflammatory, anti-angiogenic and anti-metastatic activity, with beneficial effects against cancer and cardiovascular diseases (Wang *et al.*, 2016; Kashyap *et al.*, 2017). They are found in leafy vegetables, apples, broccoli, berries, spices, orange and other citrus fruits (Imran *et al*., 2019; Dos Santos *et al*., 2020).

#### *3.1.4. Flavanones*

The structure of flavanones is similar to the structure of flavones and flavonols, with the exception that a reducing reaction on the double bond of the heterocyclic ring takes place (Del Rio *et al.*, 2013). In nature they are mainly found in glycosylated form (González-Sarrías *et al.*, 2020; Pereira-Caro *et al.*, 2020). This group of compounds is especially abundant in oranges and other citrus fruits, such as limes, lemons and grapefruit among others, being hesperidin and naringenin the main compounds (Khan *et al*., 2014). Regrading to the beneficial effect for human health, they have antimicrobial, antioxidant and anti-inflammatory effects (Frattaruolo *et al.*, 2019), and also against the risk of suffering non communicable diseases, such as diabetes, cancer, cardiovascular and neurodegenerative diseases (Joshi *et al*., 2018; Pandey and Khan, 2021).

#### *3.1.5. Isoflavones*

The structure of the isoflavones differs from the other flavonoids because the B-ring is attached to the heterocyclic ring at the C3 position instead of the C2 position (Del Rio *et al.*, 2013). It is mainly found in β-glycoside form and its acetyl and carbonyl derivatives, but due to their structure they show great structural variation (González-Sarrías *et al.*, 2020). Among the most important compounds are daidzein and genistein, which are mainly found in legumes, with soya being one of the main food sources in diets (González-Sarrías *et al.*, 2020). In terms of the beneficial effects derived from their consumption, the treatment of inflammation, cancer, metabolic syndrome, osteoporosis and the regulation of the microbiota stand out. In addition, it is currently being used to treat menopausal symptoms (Hsiao *et al.*, 2020).

#### *3.1.6. Anthocyanidins*

The structure of anthocyanidins is characterised by the presence of a positive charge (flavinium cation) on the oxygen atom in the heterocyclic ring. They are commonly found in the glycoside form, linked to several sugars, known as anthocyanins (González-Sarrías *et al.*, 2020). The main compounds in this group are cyanidin, delphinidin,

pelargonidin, malvidin and petunidin. They are responsible for the purple, blue and red pigmentation of most fruits and vegetables (Tsao, 2010; Del Rio *et al.*, 2013), being mainly found in berries, grapes, cherries, pomegranate and eggplant among others (Cassidy, 2018; González-Sarrías *et al.*, 2020). They have anti-inflammatory and antioxidant properties, and prevent against some diseases such as diabetes, cardiovascular diseases and cancer (Amini *et al.*, 2017; Rupasinghe *et al.*, 2018).

### 3.2. Non-flavonoids

Non-flavonoids are the second largest group of (poly)phenols, they have a simpler chemical structure than flavonoids and include phenolic acids, hydrolysable tannins, stilbenes, coumarins and lignans (Table 4) (Del Rio *et al.*, 2013).

#### *3.2.1. Phenolic acids*

Phenolic acids are classified as hydroxybenzoics (C1-C6 chains) and hydroxycinnamics (C3-C6 chains) derivatives (Kumar and Goel, 2019). Benzoic acid derivatives include: gallic acid, *p*-hydroxybenzoic acid, salicylic acid, ellagic acid, gentisic acid, protocatechuic acid, syringic acid and vanillic acid. These are mainly found in vegetables as glycosides (Rashmi and Negi, 2020). These compounds are found in cauliflower, berries, nuts, spinach and onions, among others (Bunea *et al.*, 2008; Lin *et al.*, 2020; Picchi *et al.*, 2020; Lo Scalzo *et al.*, 2021). The main hydroxycinnamic acid derivatives are: *p*-coumaric acid, cinnamic acid, caffeic acid, ferulic acid, sinapic acid, isoferulic acid and *p*-hydroxycinnamic acid. These compounds can be found in plants as esters and are more abundant than hydroxybenzoic acid derivatives. More specifically, chlorogenic acid is one of the most common and is made from the combination of caffeic acid and quinic acid (Rashmi and Negi, 2020). These are found mainly in broccoli, tomato, aubergine, artichoke and cocoa among others (Nagraj *et al*., 2020; Gerschenson *et al*., 2020; Mauro *et al*., 2020; Martin and Ramos, 2021; Cárdenas-Castro *et al*., 2021).

#### *3.2.2. Hydrolysable tannins*

The structure of hydrolysable tannins consists of a sugar that can be partially or totally esterified, glucose being the most common, which binds to gallic acid, giving rise to gallotannins, or to ellagic acid, giving rise to ellagitannins, which can release gallic acid and ellagic acid units among others when is subjected to acid/basic hydrolysis (Das *et al.*, 2020; González-Sarrías *et al.*, 2020). The most common hydrolysable tannins are sanguiin H-6, lambertianin C, punicalagin and galloylhexoside, which are found in

berries, pomegranate and nuts (Mullen *et al.*, 2003; González-Sarrías *et al.*, 2020). Related to their beneficial health effects for human health, different authors have reported that they have anti-inflammatory and prebiotic effect (Kiss and Piwowarski, 2016).

#### *3.2.3. Stilbenes*

Stilbenes are phytoalexins with a C6-C2-C6 structure. They can be found as monomers and oligomers by oxidative coupling between monomeric stilbenes. They are minority components, resveratrol being the most representative, found in *cis* and *trans* forms and in glycosylated form (astringin and piceid), as well as oligomers containing two (ε-viniferin) or three resveratrol units (α-viniferins) (González-Barrio *et al.*, 2006). They are mainly found in red wines, although in much lower concentrations than other (poly)phenols (Del Rio *et al.*, 2013; González-Sarrías *et al.*, 2020). These compounds have been related with the prevention of oxidative stress and inflammation (Reinisalo *et al.*, 2015).

#### *3.2.4. Coumarins*

Coumarins are a family of benzopyrenes derived from the lactonisation of hydroxycinnamic acids. The most common are isocoumarins, furanocoumarins and benzocoumarins. They are highly bioactive compounds and are mainly found in oranges, celery and parsley (González-Sarrías *et al.*, 2020). These compounds are commonly used in the treatment of some types of cancer (Akkol *et al.*, 2020).

#### *3.2.5. Lignans*

Lignans are phytoestrogens whose structure is derived from the oxidative dimerisation of two phenyl-propanoid units linked at the central carbon. They are generally found in free form. Lignans are metabolised by the gut microbiota to the enterolignans (enterodiol and enterolactone) which contain a structure with only two phenolic hydroxyl groups. These compounds are mainly found in seeds (González-Sarrías *et al.*, 2020).

## 4. Glucosinolates

Glucosinolates are a large group of bioactive compounds, with more than 120 different chemical compounds identified. Their structure is based on an *S*-β-D-glucopyrano unit anomerically connected to an *O*-sulfated (*Z*)-thiohydroximate function. The rest of the molecule is known as an aglycone and constitutes the side-chain ("R-group") which has highly variable structures, depending on the precursor amino acid (Blažević *et al.*, 2020). Glucosinolates are hydrolysed by a plant enzyme called myrosinase (EC: 3.2.1.147), which hydrolyses the thioglycoside bond, to yield metabolites such as isothiocyanates, thiocyanates, nitriles, epithionitriles and oxazolidine-2-thione, whose production depend on the content, the kind of the glucosinolate and the environmental conditions (Castro-Torres *et al.*, 2019). The content and composition of glucosinolates vary depending on several factors such as genotype variability, development stage at harvest, environmental and seasonal variations, agricultural practices (irrigation, ferlisation and elicitation) and other post-harvest factors (storage, processing and packaging) (Ilahy *et al.*, 2020).

Glucosinolates can be classified according to several criteria, but one of the most common classifications is according to their precursor. Aliphatic glucosinolates, include methionine, isoleucine, leucine, alanine and valine derivatives, being the most common the glucoraphanin. Indolic glucosinolates, include tryptophan derivatives, with glucobrassicin as the main one; whereas aromatic glucosinolates are derivatives of phenylalanine and tyrosine (Di Gioia *et al.*, 2019).

Glucosinolate are abundant in plant foods of the *Brassicaceae* family, which includes vegetables such as broccoli, brussels sprouts, cabbage, mustard, radish, watercress and wasabi, among others. In addition to the *Brassicaceae* family, other edible dicotyledons contain glucosinolates, such as capers, papaya and moringa (Possenti *et al.*, 2016; Tacer-Caba, 2019). Broccoli is one of the most widely consumed vegetables and its consumption has increased in recent years due to the health benefits associated with its consumption (Hassini *et al.*, 2019). In broccoli the predominant glucosinolate is glucoraphanin followed by progroitin and glucoiberin within the aliphatic ones. As for the indole content, the content of glucobrassicin stands out, although the profile changes mainly due to the variation of environmental conditions caused by seasonality (Vallejo *et al.*, 2002; Possenti *et al.*, 2016).

Although glucosinolates can act in plants as a reserve of sulphur and nitrogen, their main role is as a defense mechanism against pests, mainly against herbivores. In this

sense, when tissue rupture occurs due to pest damage, myrosinase, which is stored in the idioblasts, is released. Then, it hydrolyses the glucosinolates, producing toxic products such as isothiocyanates, acting against pests (Andréasson and Jørgensen, 2003; Shakour *et al*., 2021). However, it should be noted that glusinolates not only respond to biotic stress, but also to environmental stress, since when atmospheric or soil conditions are unfavourable (low temperatures and water deficit) there is an increase in these compounds (Vallejo *et al.*, 2003; Martínez-Ballesta *et al.*, 2013).

Glucosinolates and their metabolites have many beneficial effects on human health derived from their consumption. In this regard, their anticarcinogenic activity stands out, which derives from different mechanisms. Through detoxification, as isothiocyanates may alter the metabolic pathways, reducing the activation of procarcinogens and improving their excretion. Other mechanisms include cell apoptosis, ROS-mediated oxidative stress, inhibition of cell cycle progression, angiogenesis, histone deacetylase or by altering estrogen metabolism (Akram *et al.*, 2021). Furthermore, glucosinolates also exhibit an antioxidant effect, but not directly, because they do not scavenge free radicals. However, they are able to remove the free radicals by modulating the enzymatic activities involved in the metabolism of xenobiotics, thus resulting in long-lasting antioxidant activity (Vig *et al.*, 2009). In addition, isothiocyanates also have immunomodulatory and anti-inflammatory activity, which are directly related to several chronic diseases (Björkman *et al.*, 2011). The consumption of foods rich in glucosinolates has also been linked to a reduction in endogenous cholesterol synthesis, thus helping to prevent coronary heart disease (Bahadoran *et al.*, 2012; Akram *et al.*, 2021). Moreover, their properties have also shown a beneficial effects against neurological diseases and diabetes (Bahadoran *et al.*, 2011; Giacoppo *et al.*, 2015; López-Chillón *et al.*, 2018).

## 5. Carotenoids

Carotenoids are $C_{40}$ tetraterpenoid pigments widely distributed in plants, algae, fungi and bacteria. They consist of eight isoprenoid units inverted in the centre of the molecule by a double molecule system. They can be found either in free form or esterified with fatty acids, this type of bonding allows the storage of carotenoids (Saini *et al.*, 2015; Langi *et al.*, 2018;).

They are located in the different parts of plants, such as leaves, roots, fruits and seeds, mainly in fatty tissues, as they are fat-soluble compounds. As for their location in cells, they are located in photosynthetic tissues, known as thylakoids, together with

chlorophylls, some of them helping in photosynthesis by capturing light energy (Delgado-Vargas *et al.*, 2000).

Carotenoids are classified into two subgroups according to the functional group. In this sense, xanthophylls are oxygenated derivatives, as they have oxygen in their structure, including lutein, zeaxanthin and cryptoxanthin. The other group is known as carotenes, which are hydrocarbon derivatives and do not have any group attached to their structure, within this group are α-carotene, β-carotene and lycopene (Saini *et al.*, 2015; Rodriguez-Amaya, 2018).

Carotenoids cannot be synthesised by the human body, so human have to obtain them from the diet. Generally, the beneficial effects attributed to these type of compound are due to its activity as provitamin A, for which it must have an unsubstituted ring structure with an 11-carbon polyene chain (Rodriguez-Amaya, 2018; Meléndez-Martínez *et al.*, 2022). Carotenoids are strong antioxidant compounds and they are involved in reducing the effects of ageing, which is related to the progressive loss of cellular functions (Rivera-Madrid *et al.*, 2020). Specifically, effects have been demonstrated against skin, eye and vascular ageing, mainly due to the protection against cellular oxidation, due to their ability to scavenge free radicals (Grether-Beck *et al.*, 2017; Rivera-Madrid *et al.*, 2020; Arunkumar *et al.*, 2021). In addition, they also have beneficial effects in neurodegenerative diseases such as Alzheimer, improving the cognitive functions (Lindbergh *et al.*, 2018; Polidori *et al.*, 2021). They also exert a preventive effect against other diseases related to oxidative stress, such as osteoporosis (Rivera-Madrid *et al.*, 2020). Furthermore, they prevent some types of cancer such as breast, prostate, liver and lung cancer, among others. This anticarcinogenic activity is related to their different mechanisms, since they can act as antioxidant and prooxidant (Rodriguez-Amaya, 2018; Rivera-Madrid *et al.*, 2020). It has also been reported to have a beneficial effect on cardiovascular diseases through its protective effect against oxidation of low-density lipoprotein (LDL) cholesterol and even by reducing blood cholesterol by inhibiting the enzymatic activity of 3-hydroxy-3-methylglutaryl-CoA reductase, which is involved in cholesterol biosynthesis pathway (Navarro-González *et al.*, 2014). Other related effects, are the hepatoprotective effect due to their ability to reduce oxidative stress and regulate hepatocyte lipid metabolism, reducing the risk of some diseases such as non-alcoholic fatty liver disease (Elvira-Torales *et al.*, 2019).

Regarding the content of carotenoids in foods, these can be tentatively identified by the colour of the food. As for yellow and orange, they are related to β-carotene, which is an orange pigment, and α-carotene which is yellow, the former is present in carrots,

sweet potatoes, tomatoes and in green leafy vegetables, some fruits such as orange, watermelon and apricot also present high contents. While α-carotene is mainly found in carrots (Saini *et al.*, 2015). Zeaxanthins are found as yellow-orange pigments, with lutein being mostly yellow and zeaxanthin and cryptoxanthin being yellow orange. They are found mainly in green leafy vegetables and yellow fruits, including spinach, broccoli, green beans, corn, peppers, persimmon and papaya, being orange one of the main dietary sources of β-cryptoxanthin (Saini *et al.*, 2015). Red pigments include δ-carotene, which is present in red-orange, but lycopene and astaxanthin are the main pigments in this group. Lycopene is mainly found in tomatoes, persimmon, pink grapefruit and watermelon (Khoo *et al.*, 2011; Saini *et al.*, 2015; Langi *et al.*, 2018). It should be noted that the carotenoid content and composition, as in the case of the other bioactive compounds, can be affected by several factors such as the stage of ripening, variety, growing conditions, time of harvest, post-harvest treatments and storage conditions (Maiani *et al.*, 2009; Zakynthinos and Varzakas, 2016).

## 6. Dietary fibre extraction methods

Dietary fibre can be extracted from plant foods by-products and fibre-rich residues. In the scientific literature different extraction methods have been reported to obtain DF, but the selection of a specific method should be based on the complexity and composition of DF. Moreover, the different methods determine the characteristics of the fibre-rich extract obtained, which should be take into account based on the extraction purpose (Maphosa and Jideani, 2016; Tejada-Ortigoza *et al.*, 2016; Cruz-Requena *et al.*, 2019). A brief description of the different methods is given below.

### 6.1. Physical extraction

Physical methods, such us extrusion, preserve the intact structure of the fibres without damaging their polymeric chains, and the fibres obtained may have a high cation exchange capacity due to the unaltered side chains (Cruz-Requena *et al.*, 2019). The most widely used is thermal extraction, which uses hot water. In this method it is important to manage the pH and time conditions to improve the yields, although these will be lower than in the chemical and enzymatic methods (Tejada-Ortigoza *et al.*, 2016; Garcia-Amezquita *et al.*, 2020).

Another method is the dry milling method, which consists of milling the samples to form particles of different size and density (Cruz-Requena *et al.*, 2019). Afterwards, air

classification in several phases is used to separate the different types of particles, obtaining a light and fine powder containing mainly starch and fibre, and a heavier powder fraction containing proteins and lipids. This method allows to reduce the use of water and energy and is most effective when the fibre has a high starch content (Maphosa and Jideani, 2016).

### 6.2. Chemical extraction

Chemical methods can be alkaline, acidic or can use other chemical reagents such as ethanol, EDTA or CDTA, among others. However, these methods affect the structure and properties of the fibres obtained, as the time, reagents and temperature conditions are sometimes aggressive (Tejada-Ortigoza *et al.*, 2016).

Wet extraction methods use water and different chemical reagents, which vary depending on the procedure employed. In the conventional wet extraction method, samples are ground and subsequently treated usually with an alkaline solution to extract the proteins, which are removed by acid precipitation or ultrafiltration. $SO_2$ is used for the separation of the components. The wet extraction requires a lot of time and energy and is not environmentally friendly because it uses different chemical reagents (Maphosa and Jideani, 2016). To obtain fibre for food grade, this method is modified and the fibre separation is carried out by the different swelling conditions between starch and fibre, instead of using $SO_2$ (Maphosa and Jideani, 2016; Cruz-Requena *et al*., 2019).

Alkali wet milling is used as an alternative to the conventional process, in which $SO_2$ is replaced by NaOH at 85 °C. After that, samples are immersed in NaOH at 45 °C and finally ground and sieved (Cruz-Requena *et al.*, 2019).

### 6.3. Enzymatic extraction

The enzymatic extraction method consists of simulating the digestive process that occurs in the human body. This method has advantages over the chemical method as it is more environmentally friendly, but it still requires long times and sometimes high temperatures which make the extraction process difficult. Moreover, the use of digestive enzymes makes the processes more expensive (Tejada-Ortigoza *et al.*, 2016; Spotti and Campanella, 2020).

The enzymes used are pancreatin, pepsin, trypsin, lipase, protease, amylase and amyloglucosidase, among others, the last three being the most commonly used. In this sense, protease hydrolyses the protein matrix; amylase gelatinises, hydrolyses and

depolymerises starch; and amyloglucosidase breaks down starch fragments into glucose, allowing NSP or fibre recovery. All these methods can be used individually or in combination to optimise conditions in order to improve yields (Spotti and Campanella, 2020; Tejada-Ortigoza *et al.*, 2016).

### 6.4. Emerging technologies assisted methods

All previous methods described before are not considered environmentally friendly either because they require to use a large amount of resources (water and energy) or because they consume significant amounts of solvents or toxic substances. Emerging technologies may be used to extract DFs or for use in combination with the methods described before (Maphosa and Jideani, 2016; Tejada-Ortigoza *et al*., 2016; Cruz-Requena *et al*., 2019).

In this sense, microwave radiation is used, which due to the rapid increase in temperature it reduces the viscosity and breaks the outer films, helping to implement the extractions and improving the yield (Tejada-Ortigoza *et al.*, 2016).

Ultrasound has also been used, which creates a continuous cycle of increasing and decreasing pressure, causing the cavitation effect, which is produced after the explosion of small bubbles generated in the pressure cycles. This process causes a structural alteration that aids the permeation of the solvents and the release of the cellular components (Tejada-Ortigoza *et al.*, 2016).

High pressures can also be used for fibre extraction. Although not much work has been done using this technology, it has been observed that the localised use of pressure, saves time in extractions and also causes structural changes and consequently causes changes in the functional properties of the fibres obtained, so its use may be interesting for implementing new applications of DF (Tejada-Ortigoza *et al.*, 2016).

## 7. Digestion and functionality of dietary fibre

### 7.1. Gut motility

It has been shown that DF has beneficial health effects that are exceptionally related to its soluble and insoluble properties as previously thought, but more to its physicochemical properties (Gidley and Yakubov, 2019). Among them, one of the best known is the prevention of constipation and the promotion of laxation. For this, DF must

meet two requirements. Firstly, it must be resistant to fermentation along the entire gastrointestinal tract to reach the colon practically intact and mixes with the faeces. The second is related to its hydration properties, which is mainly associated with the soluble compounds. So, the capacity to retain water lead to an increase in the water content of the faeces and consequently decreases their consistency, which increases the bowel movements and stool frequency (Fuller *et al*., 2016; McRorie and McKeown, 2017; Barber *et al*., 2020). In contrast, a laxative effect of insoluble fibres has been described, which is not related to their water retention capacity. This effect is related to the presence of insoluble particles, which have a mechanically irritating effect on the large intestine mucosa, stimulating the secretion of water and mucus as a defense mechanism to protect against abrasion, resulting in a decrease in stool consistency and therefore a greater laxative effect (McRorie and McKeown, 2017).

### 7.2. Postprandial glucose and insulin responses

The beneficial effect of DF on postprandial blood glucose and insulin response has been demonstrated, being related to its viscosity and gel-forming ability. Increased viscosity causes a slowing down of the breakdown of complex nutrients, which slows the absorption of glucose and other nutrients in the small intestine (McRorie and McKeown, 2017). This results in a knock-on effect by stimulating mucosal L-cells, which release glucagon-like peptide-1 into the bloodstream, decreasing appetite and increasing the growth of pancreatic beta-cells (insulin-producing). Leading to an increase in insulin production and sensitivity and reducing glucagon secretion in the liver (Dailey and Moran, 2013). This effect is also related with the effect of DF on the body weight regulation, as it produces a decrease in the gastric emptying and reduces transit in the small bowel, which reduces hunger and consequently food intake (Hasbay, 2019). Moreover, DF also acts as a substrate for the intestinal microbiota, which also modulates energy expenditure and may help to control the body weight (Fuller *et al.*, 2016; Hasbay, 2019; Barber *et al.*, 2020).

### 7.3. Cholesterol lowering effects

One of the most remarkable and documented effects of DF on the body is its cholesterol-lowering effect, being related to highly viscous soluble fibres, such as β-glucan, raw guar gum and psyllium (McRorie and McKeown, 2017). The mechanism of action is similar as it has been described before to the reduction of postprandial glucose. Since at the same time that DF may interfere in the digestion and absorption process of

fat and cholesterol due to the gel formation in the large intestine by soluble compounds, DF may also trap bile within its structure. This effect limits its reabsorption, since this takes place only in the terminal ileum, unlike the absorption of nutrients, which can be absorbed along the whole ileum. In this sense, bile does not return to the enterohepatic circulation and is excreted in faeces, this decrease in bile acids is compensated by stimulating LDL receptor expression/increasing LDL cholesterol clearance to synthesise more bile acids, decreasing the LDL and total cholesterol serum levels, affecting in a low extent the high-density lipoprotein cholesterol concentration (Fuller *et al*., 2016; McRorie and McKeown, 2017). According to its cholesterol lowering effect, the consumption of DF has been considered beneficial on the risk reduction of cardiovascular diseases, mainly for the prevention of those pathologies associated with a high plasmatic levels of cholesterol (Elizabeth and Evans, 2019; Barber *et al*., 2020).

### 7.4. Prebiotic effect

As previously mentioned, DF is resistant to the digestive process in the human body and reaches the colon almost intact, where it can be fermented by the microbiota, being considered as a prebiotic compound. The fermentability of DFs is one of their most remarkable properties at nutritional and physiological level since they can act as a substrate for colonic bacteria (Slavin, 2013).

Prebiotics were defined for the first time in 1995 by Gibson and Roberfroid as “non-digestible food ingredients that beneficially affect the host by selectively stimulating the growth and/or activity of one or a limited number of bacteria already resident in the colon”. This term was modified by Gibson *et al*., in 2004 to be defined as “selectively fermented ingredients that allow specific changes, both in the composition and/or activity in the gastrointestinal microflora that confers benefits upon host well-being and health”. Later, in 2008, the Food and Agricultural Organization of the United Nations (FAO) organised a consensus panel to establish the definition of prebiotics, which has been defined as “non-viable food components that confer a health benefit on the host associated with modulation of the microbiota” (Pineiro *et al.*, 2008). Finally, two more definitions for the term prebiotic have been given, in 2015 by Bindels *et al*., as “a non-digestible compound that, through its metabolisation by microorganisms in the gut, modulates the composition and/or activity of the gut microbiota, thus, conferring a beneficial physiological effect on the host” which have been modified for the International Scientific Asociation for Probiotics and Prebiotics being finally defined as “a substrate that is selectively utilised by host micro-organisms conferring a health benefit” (Gibson *et al.*, 2017). In this sense,

GOS, FOS, DF and other polysaccharides such as pectin, inulin, fructans and RS, are included in the term prebiotic (Hutkins *et al.*, 2016).

The prebiotic effect of DF varies according to the composition of fibre, being classified as highly fermentable, slowly fermentable and non-fermentable. Within the first group are those present in fruits and vegetables and are generally represented by a high proportion of soluble compounds. Slow fermentable fibres are those found in cereals and are mainly insoluble compounds, whereas the non-digestible group is represented by synthetic fibres, such as methyl cellulose (Mudgil and Barak, 2019; Gill *et al*., 2021).

In humans, one of the main functions of the gut microbiota is the breakdown of the dietary components that scape digestion by host enzymes. Non-digestible polysaccharides include resistant starch, cellulose, pectin, hemicellulose and inulin, as well as the lignin which are in the plant cells, representing the main substrate source for intestinal bacteria (Blaut, 2018). The first step in the utilisation of non-digestible polymeric carbohydrates by the microbiota requires their breakdown leading to the formation of oligomeric and monomeric carbohydrates. In a second step, microbiota employ them for the fermentation process, producing SCFAs as the end products (Blaut, 2018).

The fermentation process of DF by the microbiota involves the conversion of complex polysaccharides into monosaccharides through several metabolic pathways. These processes are mediated by the enzymatic activity of the microorganism, which varies depending on the type of microorganism involved. As it is shown in Figure 3, the end products of this fermentation are SCFAs, the main ones being acetate, propionate, butyrate and other minor SCFAs (isobutyrate, isovalerate, valerate, isocaproate, caproate and heptanoate), gases are also produced, mainly $H_2$ and $CO_2$. The highest concentration of SCFAs is found in the proximal colon, decreasing along the distal colon, which are absorbed or used by the microbiota, with a small percentage found in the peripheral circulation (5%) (Holscher, 2017). After their absorption, SCFAs enter into the enterohepatic circulation, being acetate and butyrate reported to provide energy to the host after oxidation. In this respect, butyrate provides 70% of the energy required by colonic epithelial cells. In addition, acetate acts as a lipogenic substrate in the liver and propionate as a substrate for gluconeogenesis (Blaut, 2018).

Due to the fermentation that occurs in the colon, a decrease in pH is caused by the production of SCFAs, low pH has been reported to reduce the amount of pathogenic species by increasing other species such as *Bifidobacerium* and *Lactobacillus* (Yegin *et al.*, 2020). Reductions in colon pH have been linked to a low incidence of cancer, by enhancing apoptosis and preventing cancer cells proliferation (Sánchez-Alcoholado *et*

*al.*, 2020). Specifically, the reduction in pH causes a decrease in the activity of the enzyme 7-α-dehydroxylase and prevents the transformation of primary bile acids into secondary bile acids and their metabolites. Because of this decrease, there is also a reduction in the absorption of calcium, increasing its concentration in the intestine, which has been linked to a lower incidence of colon cancer (Periago *et al.,* 1993). Another mechanism of colon cancer prevention is the stimulation of blood flow in the colon cell walls by SCFAs (Periago *et al.,* 1993). In this sense, altering the composition and metabolic activity of the microbiota has been linked to the prevention of certain types of cancer, in particular colorectal cancer. For this reason, the prevention of some types of cancer is directly related to the production of SCFAs, with butyrate playing an important role in reducing the risk of colon cancer as it has been found that it stimulates the growth of colon epithelial cells and reduces the growth of cancer cells by promoting DNA repair (Periago *et al.,* 1993; Gill *et al*., 2018).

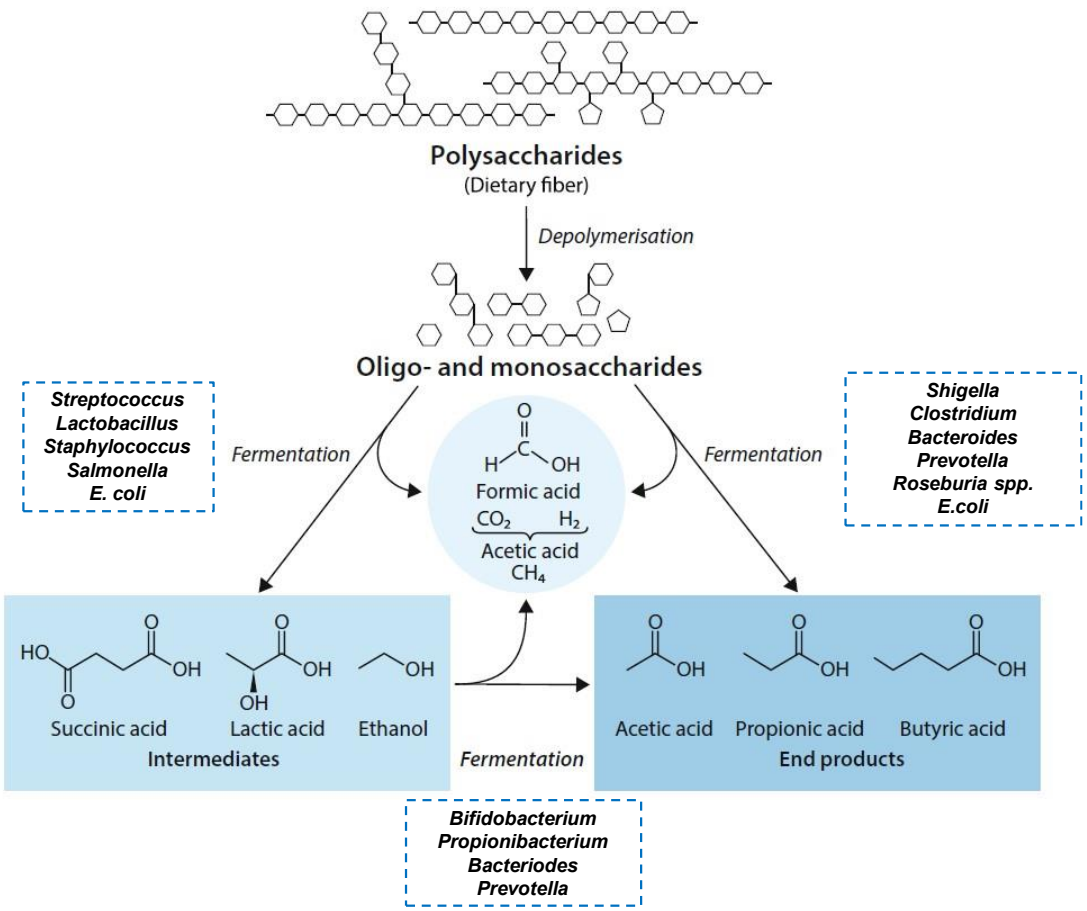



**Figure 3.** Complex polysaccharides breakdown by the colonic microbiota and species involved. Adapted from Blaut (2018) and Nyangale *et al*. (2012).

But dietary fibre not only has a preventive effect against cancer due to the production of SCFAs, but also due to the formation of gel structures, that traps cancer-causing molecules in its structure, reducing contact with mucous membranes and thus reducing the production of harmful compounds, as is the case of bile acids, preventing their conversion by bacteria into cocarcinogenic factors (Periago *et al.,* 1993; Barber *et al.*, 2020; Yegin *et al.*, 2020). Acetate, propionate and butyrate have other common health benefits such as anti-inflammatory activity by modulating the release of reactive oxygen species, immune cell chemotaxis and cytokine release (Tan *et al.*, 2014). They have also been linked to the maintenance of mucosal homeostasis, whose disruption is associated with diseases such as celiac disease and inflammatory bowel disease (Tan *et al.*, 2014; Blaak *et al.*, 2020). In addition, they may also have an effect on metabolism by contributing to obesity control and appetite regulation, glucose homeostasis and blood pressure regulation (Gill *et al.*, 2018).

In general, it should be noted that to have a healthy and diverse microflora is very important to maintain a healthy physiology, immune development, metabolic pathways and emotional and mental development. Gut dysbiosis, on the other hand, is linked to the development of chronic diseases, so maintaining a healthy microbiota is crucial to the maintenance of good health status (Barber *et al.*, 2020).

### 7.5. Antioxidant dietary fibre

Moreover, as previously highlighted, the presence of bioactive compounds bound to the components of fibre should be taken into account from a chemical and nutritional point of view. Since these bioactive compounds provide antioxidant activity to the fibre and therefore also determine their beneficial effects on health (Welti-Chanes *et al.*, 2020).

In 1998, Saura-Calixto (1998) defined the concept of antioxidant DF, which refers to plant food material that contains at least a 50% of total DF on a dry matter basis with the following requirements according to its antioxidant capacity: one gram of the material must have the capacity to inhibit lipid oxidation equivalent to 200 mg of vitamin E and to eliminate free radicals equivalent to 50 mg of vitamin E. These capacities must be intrinsic and not derived from the addition of antioxidants. Hence, the properties of the antioxidant DF are related to the bioactive compounds that are attached to the cell walls, and the intrinsic properties depend on the chemical characteristics of these compounds. So, antioxidant DF is a component that contains both high proportion of DF and

significant amounts of natural antioxidants associated with the fibre matrix (Saura-Calixto, 1998).

The main antioxidant compounds of the DF are (poly)phenols, previously described as NEPP. The aromatic rings of these compounds have hydrophilic groups that can bind to polysaccharides or cell wall proteins; the bindings occur between the (poly)phenol hydroxyl group and the oxygen atom from the glycosidic bond of the polysaccharide (Renard *et al.*, 2017). This interaction is determined by some factors such as the porosity of the microstructure, as the pore size restrict the entry of certain (poly)phenol molecules (Saura-Calixto, 2011; Eskicioglu *et al.*, 2015).

The (poly)phenols composition of the DF is expected to be related to the main (poly)phenols present in the plant foods. In this sense, phenolic acids, including ferulic acid, sinapic and *p*-coumaric acid among others, are mainly found in cereal fibres (Vitaglione *et al.*, 2008; Fărcaș *et al.*, 2021). On the other hand, flavonoids have been described to be the major (poly)phenols in citrus fibre (Russo *et al.*, 2014; Buljeta *et al.*, 2021); and hydrolysable tannins have been found mainly in pomegranate and nut fibres (Vikas Dadwal *et al*., 2017; Cardullo *et al.*, 2021).

Nevertheless, it should be noted that he antioxidant capacity of DF is not only due to the presence of (poly)phenols, but also, although in smaller quantities, rich fibre fraction may have other compounds that provide also this properties, such as carotenoids and melanoidins or Maillard reaction products (Saura-Calixto, 2011; Eskicioglu *et al.*, 2015).

## 8. Bioavailability of bioactive compounds

### 8.1. (Poly)phenols bioavailability

From a nutritional point of view the bioavailability of bioactive compounds is defined as the fraction of a given compound that can be utilised by the body, depending on various processes such as release from the food matrix, absorption, distribution, metabolism and elimination (Rein *et al.*, 2013).

The term bioavailability is related to other concepts such as bioaccessibility, absorption, metabolism, and bioactivity, all of which lead to the concept of bioefficiency. A compound is bioefficient if it provides, on the one hand, energetic, biochemical, and mechanical support for body functions and, on the other hand, contributes to the maintenance of human health (Dima *et al.*, 2020). To study the bioavailability of bioactive

compounds present in foods, several factors must be taken into account, including the matrix, the food composition and processing, since the intermolecular bonds that may occur, as well as the molecular structure and size may affect bioavailability (Palafox-Carlos *et al.*, 2011; Dima *et al.*, 2020). It has been reported that this factors affect phenolic acid bioavailability (Bento-Silva *et al.*, 2020). Other host related factors are gender, age, pathologies, genetics, physiological condition, enzyme activity and microbiota status among others (D'Archivio *et al.*, 2010). Moreover, (poly)phenol structure also affect the bioavailability and it has been determined that according to the class its bioavailability is ranked as follows: phenolic acids > isoflavones > flavonols > catechins > flavanones, proanthocyanidins > anthocyanins (Williamson and Manach, 2005; Di Lorenzo *et al.*, 2021).

According to the classification of the (poly)phenols in EEP and NEPP, as is shown in Figure 4, the EPP may be absorbed partially in the intestinal enterocytes of the upper part of the small intestine, because they are not bound to other food components. The EPP that are not absorbed in phase I metabolism, pass into the colon where they undergo conversion by microbiota. After microbiota biotrasformation into catabolites, some of them may exert gut benefits or may be absorbed in the colon and transported through the portal vein to the liver, where the phase II metabolism takes place (Figure 4). Phase II metabolites enter in the bloodstream and finally are excreted in urine, while those that are not absorbed are excreted in faeces (Martínez-Meza *et al.,* 2021).

On the other hand, NEPP needs to be released from molecular interactions with food components such as fibres, proteins or lipids, before to be absorbed (Figure 4). The digestion starts with a mechanical separation by mastication and oral grinding, where saliva also helps to release (poly)phenols, as it has enzymes with β-glucosidase activity. This release continues in the stomach and small intestine, through the release of gastric acid and stomach and intestinal enzymes that hydrolyse the macrostructures of the food components (Gioxari *et al.*, 2015; Neilson *et al.*, 2017). Finally, (poly)phenols that have been released during the digestion process and are soluble, can diffuse for absorption in enterocytes. They may reach the bloodstream after several metabolic processes, but it should be noted that the solubilisation and consequent absorption of NEPP in this digestion stage is minimal (Martínez-Meza *et al.,* 2021). Most NEPP can reach the lower gastrointestinal tract almost intact, due to their binding to other food components (Ludwig *et al.*, 2018), mainly DF. When NEPP reach the colon, and according to the extension that DF is fermented, they can be released from the food matrix and then used by the intestinal microbiota and transformed into catabolite products (Figure 4). These

catabolites followed the same process as those produced by the EPP after microbial metabolisation (phase II metabolism) (Martínez-Meza *et al.,* 2021).

Health benefits derived from the (poly)phenol consumption are mainly related with its antioxidant capacity. As already mentioned in the previous sections, this effect is related mainly to the free radical scavenging capacity, reduction of iron (III) ion and inhibition of lipid peroxidation (Shubina *et al.*, 2021). In this regard, most of the current studies only take into account the antioxidant capacity of EPP, which are extracted with organic solvents from the food matrix, while several studies have shown that NEPP significantly increase the antioxidant capacity of the foods in which they are present (Matsumura *et al.*, 2016;Liu *et al.*, 2018). But in addition, due to the interactions with DF, (poly)phenols have other beneficial effects, which are described below.

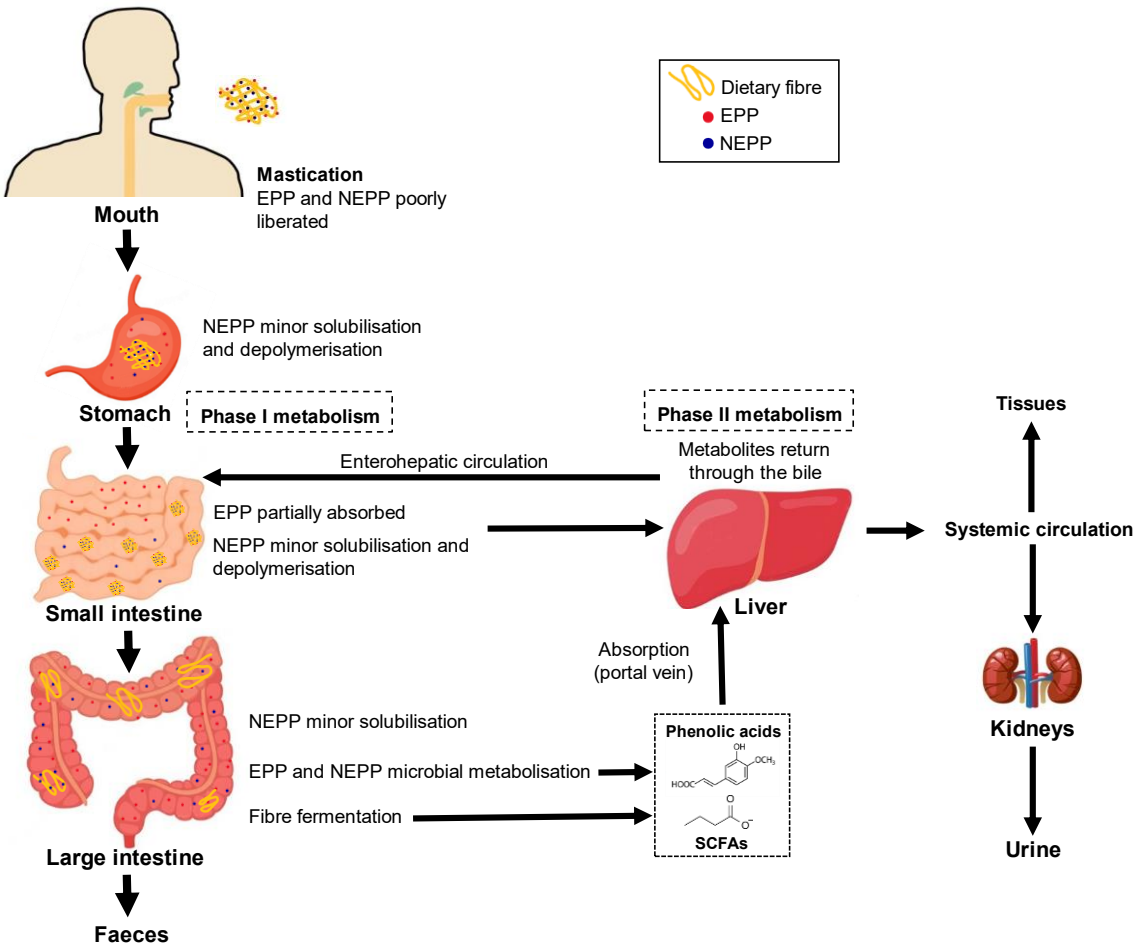



**Figure 4.** Metabolic fate of extractable (poly)phenols (EPP) and non-extractable (poly)phenols (NEPP). Adapted from Martínez-Meza *et al.* (2021).

The microbiota can use the (poly)phenols by dihydroxylation, ester cleavage, decarboxylation and ring cleavage among other metabolic processes (Tabernero and Gómez de Cedrón, 2017; Dingeo *et al*., 2020). Through these processes it can metabolise the (poly)phenols increasing the number and profile of microorganisms with several beneficial effects for the host health, showing prebiotic-like effect. In this sense, they promote the growing of *Lactobacillus* and *Bifidobacterium* (Jaquet *et al.*, 2009; Dueñas *et al.*, 2015), having also effect on the positive modulation of *Akkermansia muciniphila* and *Faecalibacterium prausnitzii*, which have been described as the anti-obesity bacteria (González-Sarrías *et al*., 2017; Martínez-Meza *et al.,* 2021), they also may increase the *Firmicutes*/*Bacteriodetes* ratio, related with beneficial host health effects (Tomás-Barberán *et al.*, 2016). Furthermore, these compounds are able to reduce the number of pathogenic species, which also benefits the growth of the beneficial microbiota (Tabernero and Gómez de Cedrón, 2017; Dingeo *et al*., 2020). It should be noted that (poly)phenols have other mechanisms of modulation of the microbiota, such as antimicrobial effect, inhibition of microbial enzymes and alterations in mucus viscosity (Havlik and Edwards, 2018).

Catabolites derived from the metabolism of EPP and NEPP are the same, but NEPP derivatives are absorbed with some delay compared to the ones derived from EPP, which may cause prolongation of their biological activity over time (Martínez-Meza *et al.,* 2021). Some of the reported catabolites from (poly)phenol metabolism are urolithins which are derived from ellagitannins and ellagic acid metabolism (González-Barrio *et al.*, 2012), phenolic acids (phenylpropionic, phenylacetic acid and benzoic acid derivatives) from flavanones, flavonols and anthocyanins metabolism (Del Rio *et al.*, 2010; Nurmi *et al.*, 2009) and *y*-valerolactones and phenylvaleric acid from flavan-3-ols metabolism (Curti *et al.*, 2015; Di Pede *et al.*, 2022). These catabolites exhibited some benefits for host health (anticancer, anti-inflammatory, antiglycative, neuroprotective effect, antiatherogenic, and also inhibition of lipid synthesis or insulin modulating activities) (Mele *et al*., 2016; Pereira-Caro *et al*., 2016; Martínez-Meza *et al*., 2021).

A potential synergy between (poly)phenols and DF has been observed in several studies, with increased SCFAs production observed in the presence of (poly)phenols (Grabber *et al.*, 2012; Parkar *et al.*, 2013). However, it should be noted that this synergy and its mechanisms are not clear, as the opposite effect has been observed in other studies (Bazzocco *et al.*, 2008; Jalil *et al.*, 2019), so it is important to carry out further *in vitro* and *in vivo* studies to elucidate the simultaneous effects of both substrates and the

effects on microbiota, taking into account the preference of gut bacteria in the transformation of DF or (poly)phenols (Martínez-Meza *et al*., 2021).

### 8.2. Bioavailability of other bioactive compounds

Carotenoids are mainly found in plant foods and their bioavailability depends on several factors such as the food composition and the food matrix. Moreover, the processing and the presence of oil in the food matrix are important factors, due to the fat-soluble properties of carotenoids. The bioavailability of β-carotene ranged between 3.5% and 90% (Desmarchelier and Borel, 2017). The cooking process helps the release and absorption of carotenoids, and the presence of oil favours this process due to its fat-soluble character, but on the other hand it should be noted that the application of high temperatures may cause the degradation, reducing their bioavailability and isomerisation of carotenoids, which depending on the carotenoid, may increase or decrease its bioavailability (Colle *et al.*, 2016; Bohn, 2018; Elvira-Torales *et al.*, 2019). However, the presence of other components such as protein and fibre can hinder their release and subsequent absorption (Schweikert, 2017; Walayat, 2018; Elvira-Torales *et al.*, 2019).

Therefore, the first step prior to digestion, by mechanical processes carried out during mastication, is critical, as it releases the carotenoids from the cell structures, since they must be released from the plant cell walls and then from the chromoplast. After the release of the carotenoids, they must diffuse into a lipid emulsion and then be solubilised by pancreatic lipases and bile salts, leading to the production of mixed micelles, which finally allow absorption in the small intestine and entry into the systemic circulation. The micelle assembly is critical for the bioavailability of carotenoids, and thus the factors affecting its formation (Saini *et al.*, 2015).

When carotenoids are not included in the micelles, they are able to reach the colon, although micelles can also reach the colon but to a lesser extent. To our knowledge, not much information has been elucidated on how the microbiota can metabolise these compounds, and the potential metabolites that are produced. But it has been observed that they accumulate in the colon as they cannot be absorbed, which is important for the potential beneficial effects that may have on the microbial communities and colonic cells (Periago *et al.*, 2016; Bohn, 2018). In this sense, apo-carotenoids have been described as potential metabolites that have been attributed the ability to react with different transcription factors (e.g., Nrf2, RARs, RXRs, PPARs), and regulate key pathways of lipid metabolism and may also stimulate the expression of phase II enzymes with antioxidant, cryoprotective or immunomodulatory activities (Bohn, 2018; Harrison and

Quadro, 2018; Saini *et al.*, 2020; Böhm *et al.*, 2021). The capacity of several sporulated bacteria belonging to the *Bacillus* genus to synthesise apo-carotenoids *de novo* has been observed (Perez-Fons *et al.*, 2011; Steiger *et al.*, 2012). In another study, it was observed that supplementation with *B. indicus* spores, improved metabolic syndrome symptoms in rats, although there were no changes in the microbiota, suggesting that the benefits could be mediated by bacterial metabolites (Crescenzo *et al.*, 2017). Furthermore, in an *in vitro* study it has been reported that fucoxanthin inhibits the growth of *Escherichia coli* and promotes the growth of *Lactobacilli*, via metabolism that may involve bacterial metabolisation of fucoxanthin to fucoxanthinol (Liu *et al*., 2019).

Glucosinolates may also be present in DF by-products obtained from *Brasicaceae* due to their occurrence in the different plant foods. The bioavailability of glucosinolates depends on several factors, including the concentration of glucosinolates and its hydrolytic products in the food; the concentration of myrosinase and its stability; the effect on processing and storage; particular physico-chemical characteristics of glucosinolates, the digestive process and host characteristics (Prieto *et al.*, 2019). The bioavailability of different glucosinolates and isothiocyanates ranged from 0.7 to 80% (Baenas *et al.*, 2017). After ingestion, a small proportion are absorbed directly in the stomach, most of them reaching the small intestine, where plant myrosinase, when is present, can hydrolyse them, being isothiocyanates the hydrolysis products, which can be absorbed. Due to the fact that plant materials containing glucosinolates are mostly consumed cooked, the myrosinase enzyme is inactivated, which may determine that reach the colon intact (Barba *et al.*, 2016). Myrosinase activity has been described in several microbial strains; consequently, in the studies carried out, the metabolism of glucosinolates is different depending on the composition of the microbiota. In this sense, it has been described the production of erucin, erucin nitrile, iberverin and iberverin nitrile from the degradation of glucoerucin, glucoiberin and glucoraphanin by a selection of human gut bacteria(Luang-In *et al.*, 2014). These glucosinolate derivatives may have an important impact on human health, such as anti-cancer and anti-inflammatory effects (Sikorska-Zimny and Beneduce, 2021).

## 9. Dietary fibre-rich functional foods and claims

The production of fibre-rich ingredients may allow their use in the food industry for the development of functional foods from by-products (Eskicioglu *et al.*, 2015). In this sense, functional foods have been defined as “natural or processed foods that contain biologically-active compounds; which, in defined, effective, non-toxic amounts, provide

a clinically proven and documented health benefit utilising specific biomarkers, to promote optimal health and reduce the risk of chronic/viral diseases and manage their symptoms" (Martirosyan *et al.*, 2022). For the development of this type of food, fortification is used as a technique, which consists of adding ingredients with functional properties to different foods to improve their biological activity (Arroyo *et al*., 2018; Mohanty and Singhal, 2018). In this sense, this technique has been widely used in industry for the development of bakery products. Fibre-rich plant extracts and bioactive compounds are the main ingredients used for this purpose. Other foods, such as beverages are enriched with fibre, with the aim of replacing the fibre content that has been removed during industrial processing and thus reduce the sugar content. Preferably soluble fibres are usually used for their dispersion capacity, although sometimes other types of fibres are used in products that have a cloud or more viscous fibres to stabilise beverages (Viscione, 2013). They are also used in jam production to reduce the caloric content with good sensory properties (Belović *et al.*, 2017). When by-products with functional properties are used as ingredients for the development or fortification of other foods, some nutrition and health claims may be included in their labelling as indicated below.

Two nutrition claims have been approved by the European Commission in the Regulation No 1924/2006 related to the DF content in foods:

- **Source of fibre:** a food is considered a source of fibre if contains at least 3 g of fibre per 100 g or at least 1,5 g of fibre per 100 kcal.
- **High fibre:** a food is high in fibre if contains at least 6 g of fibre per 100 g or at least 3 g of fibre per 100 kcal.

Related to the DF health claims, EFSA has approved different claims which are based on the functionality of the fibres, and the requirements for their use are set out in the Commission Regulation (EU) No 432/2012 (EUR.Lex, 2012). The approved health claims for fibre and its components are described below, but only are available to wheat bran, some kinds of fibres from cereal grains, different soluble and viscose fibres and methylcellulose.

- Consumption of β-glucans from oats or barley, arabinoxylan, hydroxypropyl methylcellulose, pectin and RS as part of a meal contributes to the reduction of the blood glucose rise after that meal.
- Barley grain fibre, oat grain fibre and wheat bran fibre contribute to an increase in faecal bulk.

- β-glucans, glucomannan, guar gum, hydroxypropyl methylcellulose and pectin contribute to the maintenance of normal blood cholesterol level.
- Glucomannan in the context of an energy restricted diet contributes to weight loss.
- Rye fibre contributes to normal bowel function.
- Wheat bran fibre contributes to an acceleration of intestinal transit.

These declarations may be used on the labelling of products provided that they comply with the requirements of the regulation. In this way they are used as marketing techniques to make health foods more attractive to consumers. However, it deserves to be mentioned that there are no health claims for fibre from fruits and vegetable, probably because they are more complex material and their characterisation is more difficult and depends on the method used for their extraction.

In this regard, when a new by-product ingredient has been obtained with a potential functional effect, an application for authorisation as a novel food must be made in accordance with EU Regulation (2015/2283). There are several definitions of novel food according to categories, including vegetables, which consider a novel food as “a food consisting of, isolated from or produced from plants or their parts, except when the food has a history of safe food use within the Union and is consisting of, isolated from or produced from a plant or a variety of the same species obtained by traditional or non-traditional propagating practices”. In terms of the application process, the novel food should be properly identified, describing the manufacturing process, composition details, proposed uses and levels, and intended intake. In addition, information on the history of use and/or its origin, absorption, distribution, metabolism, excretion, nutritional information, toxicological information and allergenicity should also be included (EFSA, 2021). Finally, after obtaining a positive report for its use, it can be employed for the development of functional foods as described before.

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# Chapter 2. Objectives

According to the previously described, the agri-food industry is one of the most important industrial sectors worldwide. As a result of its intense processing activity, large amounts of by-products are produced throughout the world, which a priori, have no value for the companies that produce them. However, its management causes a negative impact both at an economical and environmental level.

The valorisation of agri-food industry by-products, turning them into materials of interest for their use in the same or other industrial processes, has currently become one of the main objectives of the European Union in support to Sustainable Development Goals.

In addition, their valorisation as natural ingredients with beneficial health properties in the development of new food products, or in the reformulation of existing ones, responds to the growing demand of consumers, who are increasingly looking for new functional products that preserve their organoleptic and nutritional characteristics.

For all these reasons, the general objective of this PhD Thesis has been to valorise different by-products generated in the agri-food industry to obtain dietary fibre-rich fractions with high content of bioactive compounds ((poly)phenols, carotenoids and glucosinolates), which could be used as functional ingredients in the food industry to design and develop functional foods.

## Specific objectives

In order to achieve the general objective, the following specific objectives have been proposed:

1) To apply different extraction methods to obtain fractions rich in dietary fibre and bioactive compounds from berries, broccoli stalks and orange peel by-products.

2) To characterise the fibre-rich samples and determine their content of soluble and insoluble dietary fibre, the composition in neutral sugars and uronic acids of the non-starch polysaccharides (cellulose, hemicellulose and pectin) and to evaluate their physicochemical properties.

3) To determine the antioxidant capacity and (poly)phenols content in the fibre-rich fractions and quantify the extractable and non-extractable compounds attached to dietary fibre components.

4) To quantify other bioactive compounds (glucosinolates and carotenoids) in the different fractions obtained from broccoli stalk and orange peel by-products.

5) To evaluate the prebiotic effect of the fibre-rich fractions obtained from the different by-products, by carrying out *in vitro* fermentation tests with human faeces, measuring the metabolic activity of the colonic microbiota by the production of SCFAs and ammonium.

6) To evaluate the impact of *in vitro* colonic fermentation on the (poly)phenols present in the fibre-rich fractions obtained and to determine how the presence of fibre can influence the metabolism of these bioactive compounds.

# Chapter 3. Experimental design

## Experiment 1

For the development of experiment 1 (Figure 5), a purée from commercial frozen raspberries was used and nutritionally characterised. Three dietary fibre (DF) fractions were obtained using an enzymatic process: total dietary fibre (TDF), insoluble dietary fibre (IDF) and soluble dietary fibre (SDF); as well as a liquid extract (PEX) and the raspberry as a freeze-dried ingredient (RAS) were used as samples. The neutral sugars and uronic acids of the three fibre fractions and their physicochemical properties were analysed. Moreover, the content of pectin, cellulose and hemicellulose was calculated. Then, the content of (poly)phenols, their profile and antioxidant capacity, of fibre fractions and raspberry were determined. Finally, an *in vitro* fermentation study was carried out using the faecal samples from six female volunteers, three of them with normal-weight and three with overweight. The fermentation was carried out during 48 h, individually with each inoculum from each volunteer, with the five samples obtained. Aliquots were collected at baseline (0 h) and at 6, 24 and 48 h of fermentation to analyse SCFAs production by gas chromatography (GLC-FID) and urolithin production by high-performance liquid chromatography–diode array detection (HPLC-DAD).

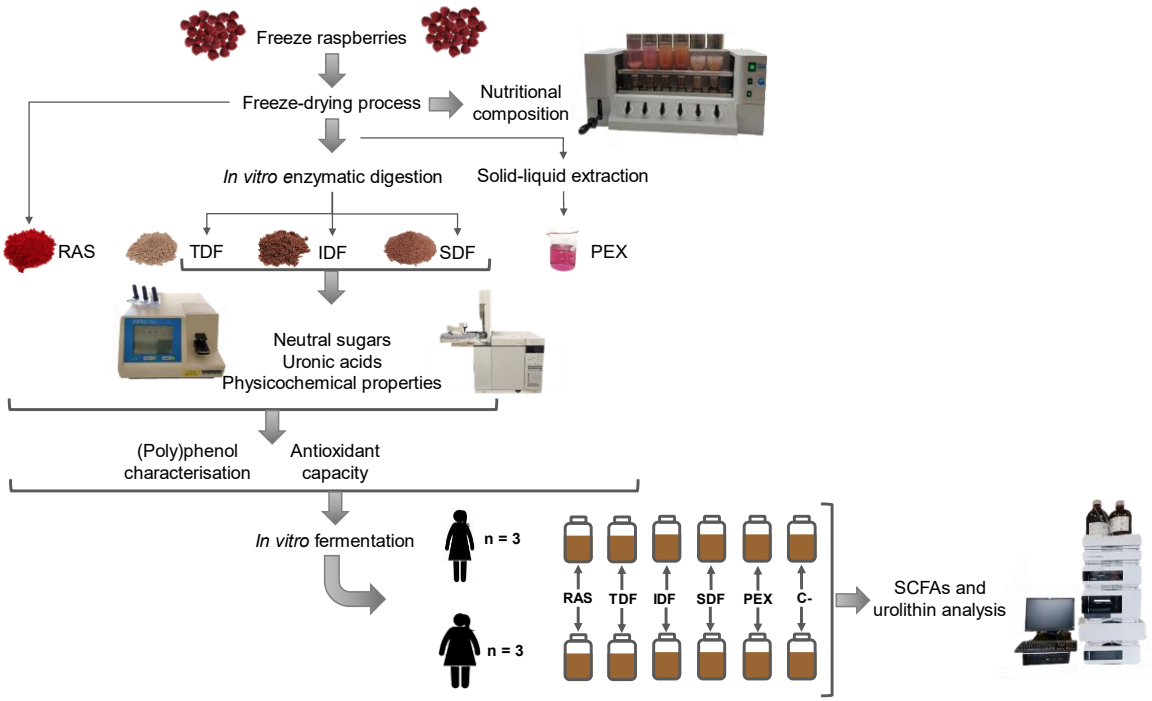


**Figure 5.** Experimental design for experiment 1.

## Experiment 2

For the development of experiment 2 (Figure 6), a purée of commercial frozen berry mixture was used. Four different samples were obtained, the first one after the freeze-drying of the berry mixture (FDB); an aqueous extract rich in (poly)phenols from the FDB (PRE); and two fibre-rich fractions, insoluble fibre-rich fraction (IFF) and soluble fibre-rich fraction (SFF). The proximate composition of the three solid samples were analysed, as well as the composition of DF according to the analysis of neutral sugar and uronic acid profile. Moreover, the cellulose, hemicellulose and pectin content were calculated, as well as the pectin structure characteristics, and the physicochemical properties were analysed. The total phenolic content (TPC), and the antioxidant capacity were analysed using spectrophotometric methods and (poly)phenol characterisation was also performed by HPLC-DAD. Finally, the samples were subjected to an *in vitro* digestion, and then an *in vitro* faecal fermentation was performed using a pooled faecal sample from nine healthy female volunteers to avoid individual variability observed in experiment 1 and increase microbial diversity. The samples (FDB, IFF, SFF and PRE) were incubated to test its prebiotic effect compared with a negative control (C) without any sample incubated. The fermentation experiment was carried out during 48 h, collecting aliquots at 0, 4, 8, 24 and 48 h to analyse the SCFAs and ammonium production as a prebiotic effect indicator of the samples, as well as the production of urolithin by HPLC-DAD.

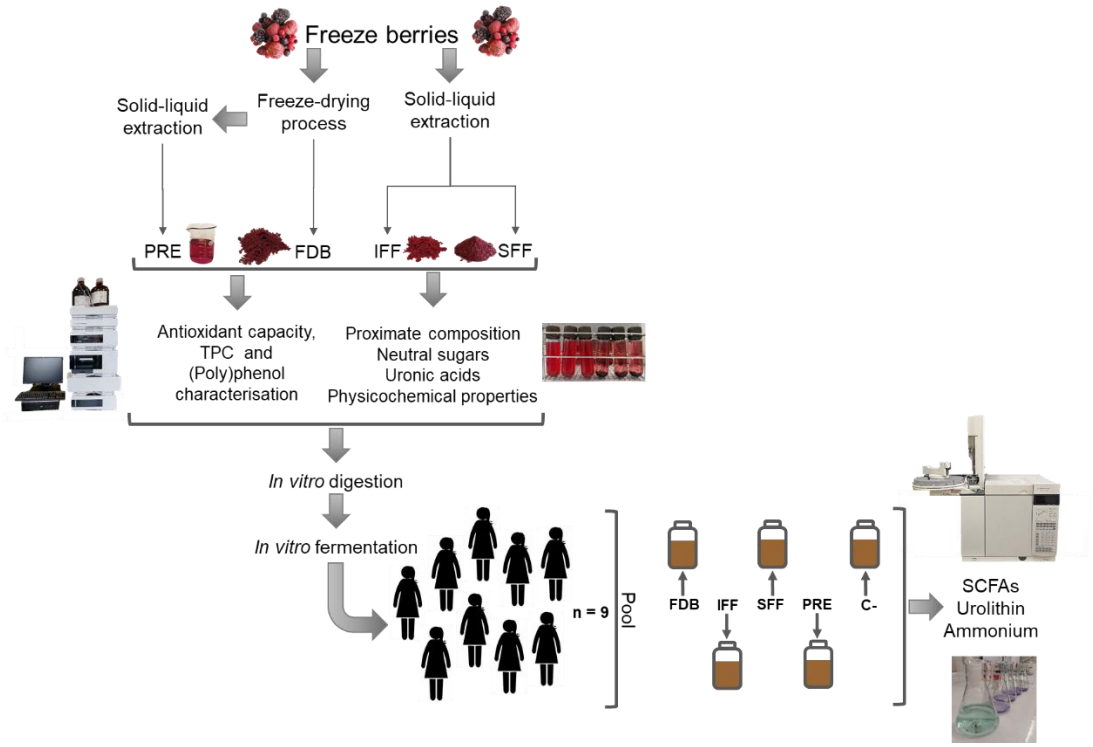


**Figure 6.** Experimental design for experiment 2.

## Experiment 3

Firstly, the seasonal variation effect on bioactive compounds in broccoli florets was determined in spring and autumn, to select the raw materials for obtaining broccoli stalks as by-products. In experiment 3, broccoli stalks selected from the seasonal assay were used as an industrial by-product (Figure 7). Fresh broccoli stalk was freeze-dried (DBS) and from raw samples two different fibre-rich fractions were obtained: total dietary fibre ($TF_B$) and insoluble dietary fibre ($IF_B$). Samples were analysed to determine their nutritional composition, physicochemical properties, as well as neutral sugars and uronic acids were analysed to estimate the main polysaccharides profile (proportion of pectin, hemicellulose, cellulose) of the DF. The total phenolic content (TPC), and the antioxidant capacity were analysed using spectrophotometric methods, and the glucosinolate and (poly)phenol characterisation was performed by high-performance liquid chromatography coupled to electrospray ionisation mass spectrometric detection (HPLC-DAD-ESI-$MS_n$). Finally, DBS and $IF_B$, previously digested, were subjected to an *in vitro* faecal fermentation, to evaluate the prebiotic effect. For that, pooled faecal sample from nine healthy female volunteers was used and the fermentation experiment was carried out during 48 h, collecting aliquots at 0, 4, 8, 24 and 48 h to analyse the SCFAs by gas chromatography (GLC-FID) and ammonium production by the microbiota activity.

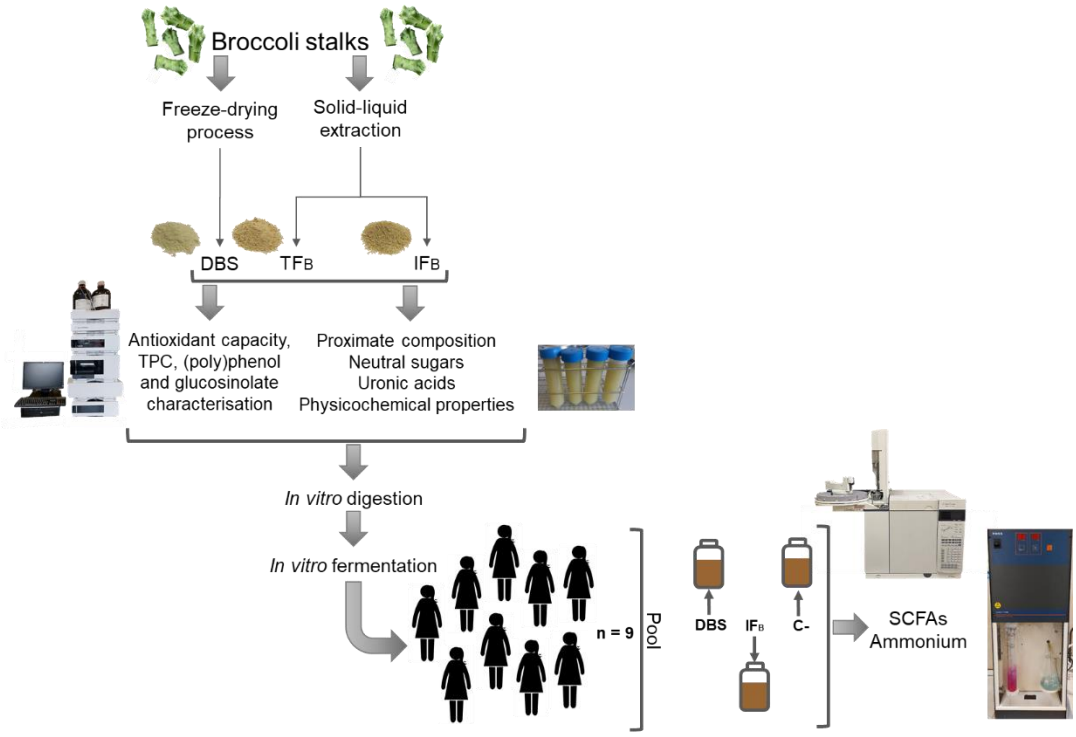


**Figure 7.** Experimental design for experiment 3.

### Experiment 4

In experiment 4, orange peels were used as an industrial by-product (Figure 8). Three different fractions were obtained, the first one after drying of the whole peel (OP) and two fractions obtained from a clean method performed with water: insoluble dietary fibre fraction (IFF) and water-soluble extract (WSE). To characterise the samples, the proximate composition, the DF composition and profile, as function of the neutral sugar and uronic acids, and physicochemical properties were analysed. Moreover, the cellulose, hemicellulose and pectin content were calculated, as well as the pectin structure characteristics. The total phenolic content (TPC), total flavonoids (TF) and the antioxidant capacity were analysed using spectrophotometric methods and (poly)phenol and carotenoid characterisation was also performed by HPLC-DAD. Finally, samples were subjected to an *in vitro* digestion, followed by an *in vitro* faecal fermentation to evaluate the prebiotic effect of the orange peel samples (OP, IFF and WSE). The *in vitro* fermentation was carried out with a pooled faecal sample from eight healthy female volunteers during 48 h, collecting aliquots at 0, 4, 8, 24 and 48 h to analyse the SCFAs and ammonium production as well as the catabolites production from (poly)phenols, formed by the microbiota, which were analysed by ultra-high-performance liquid chromatography-high-resolution mass spectrometry (UHPLC-HRMS).

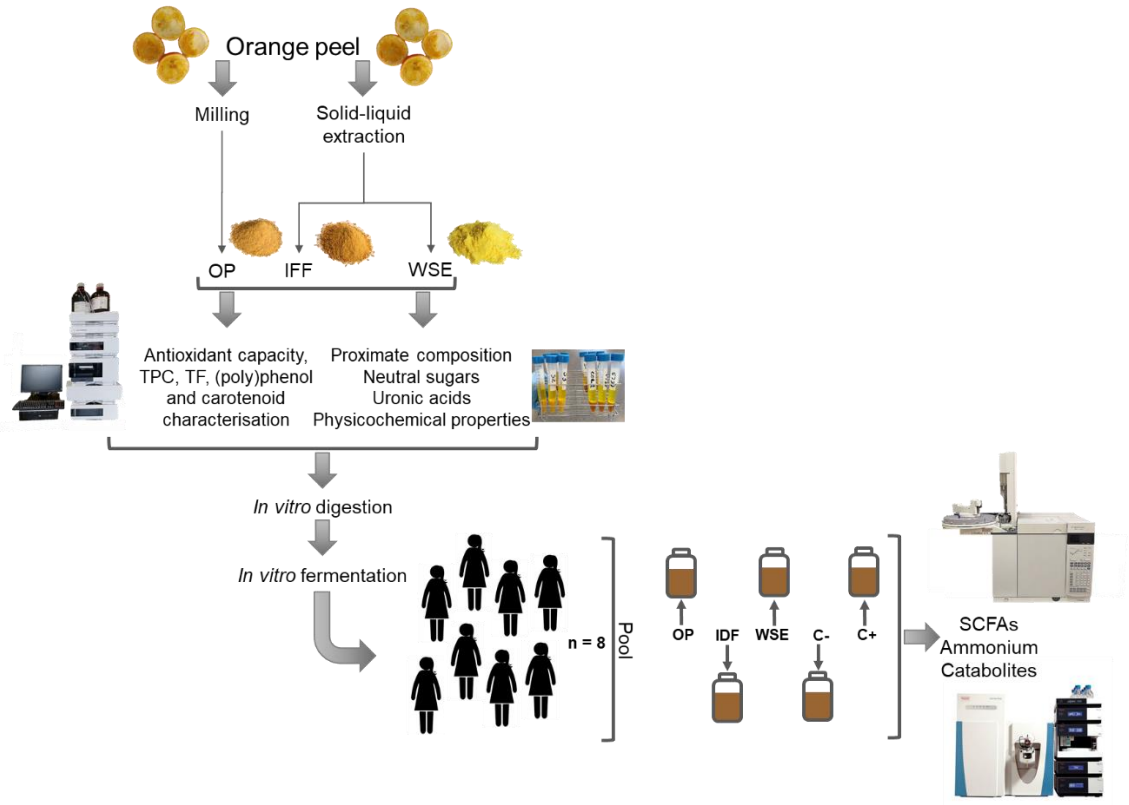


**Figure 8.** Experimental design for experiment 4.

# Chapter 4. By-products from raspberry

## 1. Introduction

Raspberries (*Rubus ideaus*), which are usually cultivated in temperate regions, are mainly harvested in Europe and Northern Asia (Hidalgo and Almajano, 2017). Both consumption and production have increased in recent years due to the growing adherence to healthier diets (Vázquez-González *et al.*, 2020). Increasing demand has led to an increase in the generation of by-products. This biomass could be used to obtain ingredients with high added value to be used in the development of new products, and thus implementing production processes that minimise wastes and maximise reuse and recycling (Laínez and Periago, 2019). This is because this fruit is a source of vitamins, mainly C, E and K and also of minerals, including Ca, Mg and P (Simmonds and Preedy, 2015). In addition, its composition is characterised by its dietary fibre (DF) content and bioactive compounds, particularly (poly)phenols, including mainly anthocyanins and ellagitannins, which have effects on some diseases due to their antioxidant, anti-inflammatory and antimicrobial properties (Szajdek and Borowska, 2008; Ludwig *et al.*, 2015).

DF intake may influence several metabolic processes, such as nutrient absorption, as well as glucose and cholesterol metabolism (Fuller *et al.*, 2016). This substance is of particular importance due to its prebiotic effect, since, being non-digestible compounds, it may reach the colon and be used by the microbiota. This effect is associated with the prevention of cardiovascular diseases, diabetes, obesity and colon cancer (Fuller *et al.*, 2016; Hijová *et al.*, 2019). The term prebiotic has been defined as a substrate that is selectively utilised by host micro-organism conferring a health benefit to the host health (Gibson *et al.*, 2017). In this sense, this definition not only includes carbohydrates, but also other molecules such as (poly)phenols (Espín *et al.*, 2017; Holscher, 2017). This is because in many cases the (poly)phenols are bound to the cell walls that constitute the fibre molecules, which allow them to reach the large intestine and act together with fibre.

After reaching the colon, fermentation of the fibre through the action of the gut microbiota takes place, leading to short-chain volatile fatty acids (SCFAs) as end products, the main ones being acetate, propionate and butyrate (Wang *et al.*, 2019). These compounds, after absorption by colonic epithelial cells, have several functions, including the maintenance of the intestinal barrier function and the promotion of the intestinal immune system (Hijová *et al.*, 2019; Wang *et al.*, 2019). In addition, when ellagitannins and ellagic acid reach the intestine, they may also be metabolised by the microbiota, in this case to form urolithins. These compounds, after absorption have some

biological activities, such as anti-inflammatory, anti-microbial, anti-cancer and antiglycative (Kujawska and Jodynis-Liebert, 2020; Jiang *et al.*, 2021).

However, the status and composition of the gut microbiota is very important in the performance of its essential functions in the host: fermentation of indigestible food components, regulation of immune system, competition with pathogens and removal of toxic compounds (Heintz-Buschart and Wilmes, 2018; Cunningham *et al.*, 2021). Consequently, it is important to consider many diseases that may cause alterations in the microbiota, such as obesity, which results in dysbiosis, leading to changes in the metabolism of substrates and consequent production of catabolites (Liu *et al.*, 2017). In this sense, it has been described a decrease in genetic richness and microbial diversity in obese subjects, although its prevalence depends on several factors such as the occurrence of other diseases (hypertension and diabetes) and dietary habits (Debédat *et al.*, 2019). Several studies have associated obesity with a decrease in the ratio of *Bacteroidetes*/*Firmicutes* (Turnbaugh *et al.*, 2008; Debédat *et al.*, 2019), although it cannot be used as a biomarker of obesity as other authors have not observed this effect or have observed the opposite (Duncan *et al.*, 2008; Schwiertz *et al.*, 2010; Liu *et al.,* 2017). In addition to these phylogenetic changes, it has been observed that the microbiota of obese subjects is able to obtain more energy during fermentation than the microbiota of normal-weight subjects (Debédat *et al.*, 2019).

Given the above, the aim of this chapter was to evaluate the chemical and physicochemical properties of raspberry and raspberry fibre fractions (TDF, IDF and SDF), through the characterisation of the chemical composition of DF, physicochemical properties, the profile of extractable (poly)phenols (EPP) and non-extractable (poly)phenols (NEPP) and antioxidant capacities. In addition, the prebiotic *in vitro* effect of the raspberry and its fractions was determined using faecal inoculum from normal-weight and overweight subjects, evaluating the production of SCFAs and (poly)phenol catabolites.

## 2. Material and methods

### 2.1. Samples

Frozen raspberries (*Rubus idaeus*) were purchased from a local supermarket in Murcia (Spain) and were taken directly to the laboratory. This fruit were purchased because we did not dispose of surplus production in the Region of Murcia and because

the companies that use these products use them as concentrates, for this reason by-products were also not available. Some samples were kept frozen for the evaluation of the proximate composition and others were freeze-dried during 72 h before being ground using a Thermomix TM-31. The raspberry powder was stored at -20 °C prior to extractions and analyses.

### 2.2. Proximate composition of raspberry

Frozen raspberry samples were analysed to determine the proximate composition using the Association of Official Agricultural Chemists (AOAC) Official methods (AOAC, 2016): moisture (method 964.22), protein (method 955.03), fat (method 920.39C) and ash (method 923.03).

The crude protein content was determined by the Kjeldahl method, the samples were subjected to an acid digestion for 1.5 hours at 425 °C, in which $H_2SO_4$ and a catalyst were used. The ammonium sulphate generated in the digestion was taken to a distiller, in which, after the addition of sodium hydroxide, it was transformed into ammonia and collected in 4% boric acid. Finally, it was titrated with 0.1N HCl, recording the volume used to determine the protein content.

The crude fat was analysed using a Soxhlet extractor. Petroleum ether was used to perform the solid-liquid extraction of the samples, which was finally evaporated to obtain the fat content by gravimetry. The ash content was determined by incineration of the samples at 525 °C and total carbohydrates were calculated by difference with the other components.

Total dietary fibre (TDF), insoluble dietary fibre (IDF) and soluble dietary fibre (SDF) were determined following the enzymatic and gravimetric method described by Prosky *et al*., (1988), using a Fibertec E 1023 system (Hogänas, Sweden). Briefly, an enzymatic digestion was performed with α-amylase incubated at 95 °C for 30 minutes, followed by a digestion with α-amyloglucosidase at 60 °C for 30 minutes and protease for 35 min at 60 °C. Finally, to obtain TDF, 95% ethanol was added and allowed to precipitate for one hour. In the case of IDF, it was filtered prior to ethanol precipitation and then 95% ethanol was added to the filtered liquid, which was filtered after one hour of precipitation to obtain the SDF, the fibre content results were obtained by gravimetry. The quantification of soluble sugars was estimated by the difference between carbohydrates and TDF.

### 2.3. Extraction of raspberry dietary fibre fractions

Three different fibre fractions (TDF, SDF and IDF) were extracted from the freeze-dried raspberries, following the procedure based on enzymatic extraction as is described in Figure 9.

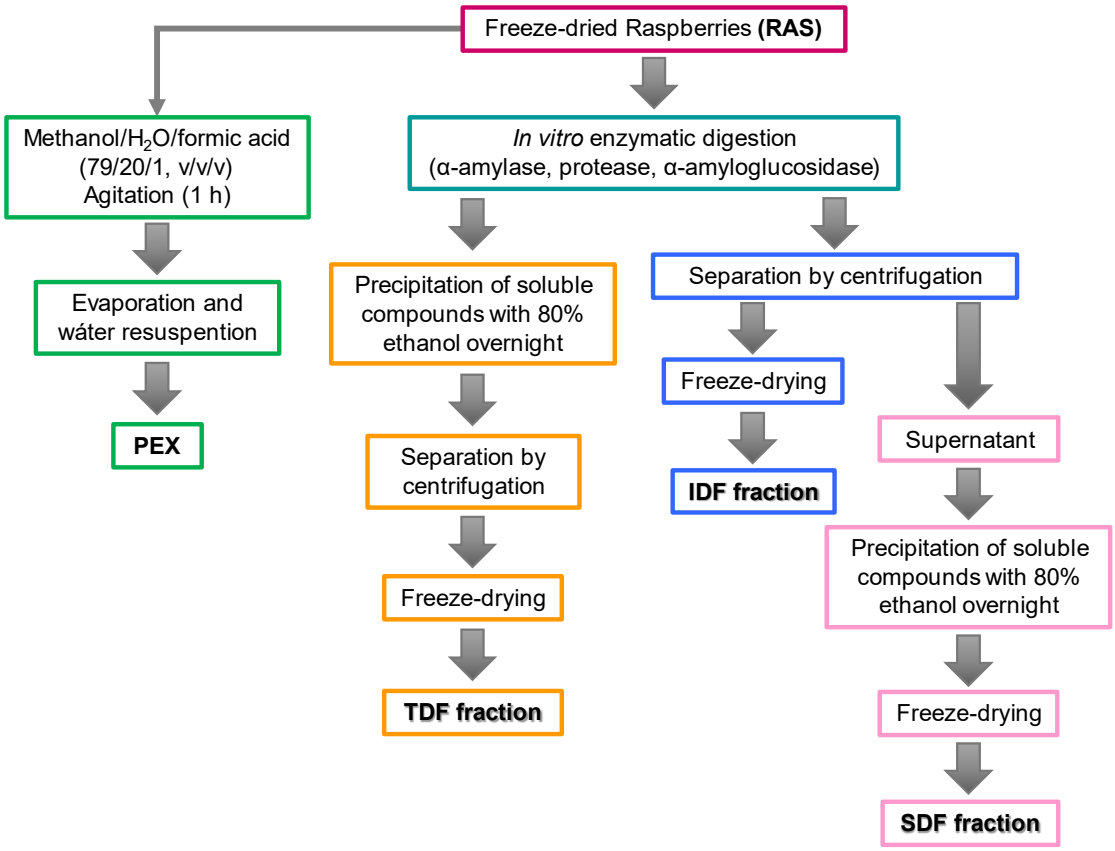


**Figure 9.** Flow diagram of the procedure used to obtain the different samples from raspberry (RAS), (poly)phenol extract (PEX), total dietary fibre (TDF), insoluble dietary fibre (IDF) and soluble dietary fibre (SDF).

Firstly, raspberry powder samples were dissolved in phosphate buffer solution, and immediately, samples were digested following an *in vitro* intestinal digestion with α-amylase heat-stable (A3306 Sigma, St. Louis, USA) at 95 °C for 30 min, with protease from *Bacillus licheniformis* (P3910 Sigma, St. Louis, USA) at 60 °C for 35 min, and with α-amyloglucosidase from *Aspergillus niger* (A9913 Sigma, St. Louis, USA) at 60°C for 35 min. After completing the digestion, samples containing the DF residues were treated in different ways to obtain the TDF and their fractions (soluble and insoluble fibre). For TDF isolation, 80% ethanol were added maintaining overnight to allow precipitation of soluble components. After that, samples were centrifuged at 8820*g*, 20 °C, for 30 min in

a Centrifuge Beckman J2-21 (Indianapolis, USA), supernatants were discarded and the precipitates, containing TDF, were stored at -20 °C and then freeze-dried. For soluble and insoluble fibre isolation (SDF and IDF), digested samples were centrifuged at 25920*g* for 30 min. The precipitates were taken as IDF. The supernatants were collected and were mixed with 80% ethanol to allow soluble fibre precipitation during all night. Samples were centrifuged and the precipitates were taken as SDF. Both fractions insoluble and soluble were stored at -20° C and freeze-dried.

### 2.4. Extraction of (poly)phenol extract

A (poly)phenol aqueous extract (PEX) from RAS was obtained, for which 0.2 g of the freeze-dried material was added to a solution of methanol/water/formic acid (79/20/1, v/v/v) and left in rotational agitation and darkness for 1 h, after which it was evaporated, resuspended in water and stored at -20 °C. The PEX was extracted to be used only for *in vitro* fermentation, to ascertain the prebiotic-like effect related to the (poly)phenols.

### 2.5. Neutral sugars and uronic acids in raspberry fibre fractions

For the characterisation of neutral sugars, the gas–liquid chromatography method described by Englyst *et al*. (1992) was followed. Briefly, the polysaccharides in the samples were firstly hydrolysed with 12 M $H_2SO_4$ during 30 min at 35 °C and then, during 1 h at 100 °C. Finally, the alditol acetate derivatives were obtained from the hydrolysates and were analysed by gas chromatography in an Agilent 7890B (Mechelen, Germany) with a flame ionisation detector.

A capillary column DB-225 (Supelco, USA) was used to identify and quantify the neutral sugars in the sample. The temperature conditions were as follows: injector at 280 °C, the oven was set at 210 °C for 5 minutes, increasing by 5 °C/min until 240 °C for 9 minutes, and the detector was set at 280 °C. A neutral sugars mix solution (rhamnose, fucose, arabinose, xylose, mannose, galactose and glucose) was used as standards and β-D-allose (2595-97-3, Thermo Scientific, Madrid, Spain) as internal standard (Figure 10). The content of individual neutral sugars was expressed as percentage (%). The pectin, hemicellulose and cellulose content were calculated based on the calculations proposed by Houben *et al*. (2011) and Umaña *et al*. (2016).

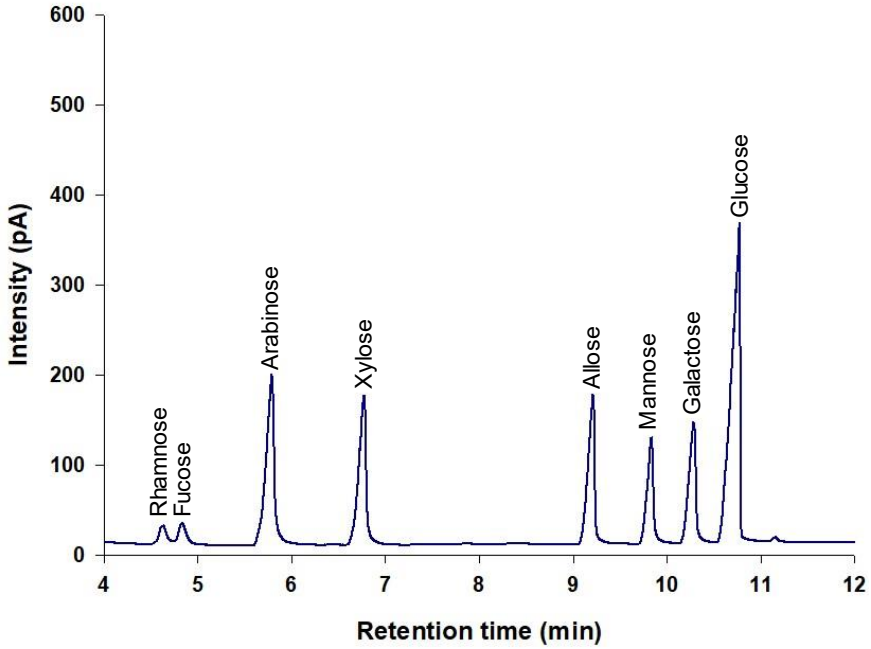


**Figure 10.** Chromatogram of neutral sugars standard mixture and allose as internal standard.

The determination of uronic acids was carried out by the colorimetric method described by Scott (1979). Briefly, the hydrolysed samples were mixed with $H_2SO_4$ and left during 40 min at 70 °C in the presence of 2% NaCl and 3% $H_3BO_3$. From this reaction, 5-formyl-2-furancarboxylic acid is generated and acts selectively with the added 3-5-dimethylphenol. The absorbance is measured at 400 and 450 nm, using galacturonic acid as standard (Figure 11). The results were expressed as g/100 g of dry weight (d.w.) of sample.

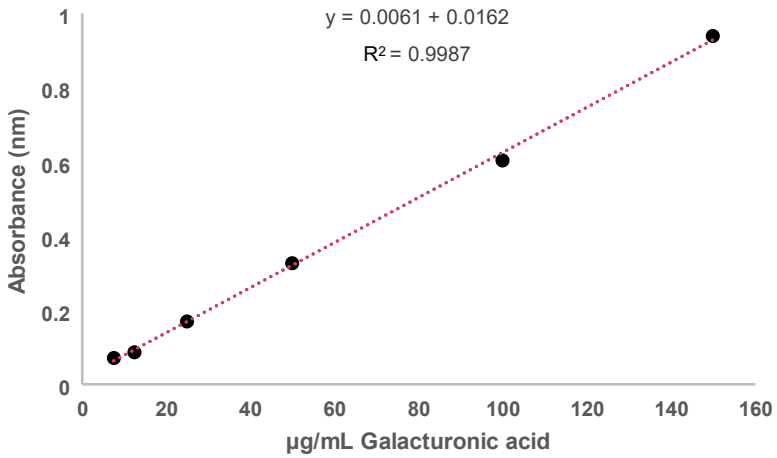


**Figure 11.** Standard calibration curve of galacturonic acid used for the quantification of uronic acids.

### 2.6. Physicochemical properties of raspberries fibre fractions

The water retention capacity (WRC), swelling capacity (SWC), fat absorption capacity (FAC), glucose diffusion retardation index (GDRI) and osmotic pressure were analysed as physicochemical properties of the fibre fractions (TDF, IDF and SDF), following the methodology previously described by Navarro-González *et al*. (2011).

In brief, to obtain the WRC 30 mL of distilled water were added to 1 g of sample and hydrated during 18 h at room temperature (25 °C). The sample was centrifuged at 3000*g* for 20 min, removing the supernatant. The pellet was weighed (fresh weight) and then dried at 100 °C until constant weight, weighing the final residue (dry weight). The results were calculated as the amount of water retained by the sample and expressed as g of water/g of d.w.

For the SWC, 1 g of sample was placed in a graduated cylinder to which 10 mL of distilled water with 0.02% sodium azide was added and vigorously mixed. It was left during 18 h at room temperature (25 °C), and finally the volume occupied by the sample was measured and expressed as mL of water/g of d.w.

For FAC, 24 mL of sunflower oil were added to 4 g of sample and mixed on a rotational stirrer during 30 min. The mixture was centrifuged at 1600*g* for 25 min, and the supernatant removed, the pellet was weighed, and the results were calculated as the amount of sunflower oil retained by the sample and expressed as g of oil/g of d.w.

For the GDRI, the samples were previously hydrated during 14 h. Then, the dialysis bags (5–25/32, Medicell International, London) were perfused in sodium azide solution (1 g/L). The bags were filled with 6 mL of the previous sodium azide solution and 0.2 g of each sample, 36 mg of glucose were added as control in one of the bags. Each bag was placed on shaking with 100 mL of the previous solution at 37 °C for 1 h. At 30 and 60 min, 10 µL of the dialysate was analysed to determine the total glucose diffused (TGD) from the bags using a glucose assay kit (Chronolab System, S.L, Spain). The rate of diffusion of glucose was calculated using Equation 1.

$$GDRI = \frac{TGD\ containing\ fibre\ x\ 100}{TGD\ from\ control}$$

**Equation 1.** Where TGD containing fibre was the total glucose diffused from each sample bags; TGD from control was the total glucose diffused from the control bag.

The osmotic pressure was determined in 2% (w/v) solution of the sample in distilled water and in 0.15 M NaCl using a Knauer osmometer (Berlin, Germany).

### 2.7. Analysis of (poly)phenols by HPLC-DAD

Two different extractions were performed on each sample (RAS, TDF, IDF and SDF) to determine the EPP and NEPP (Figure 12). For the extraction process the method described by Arranz *et al*., (2009) was followed with some modifications.

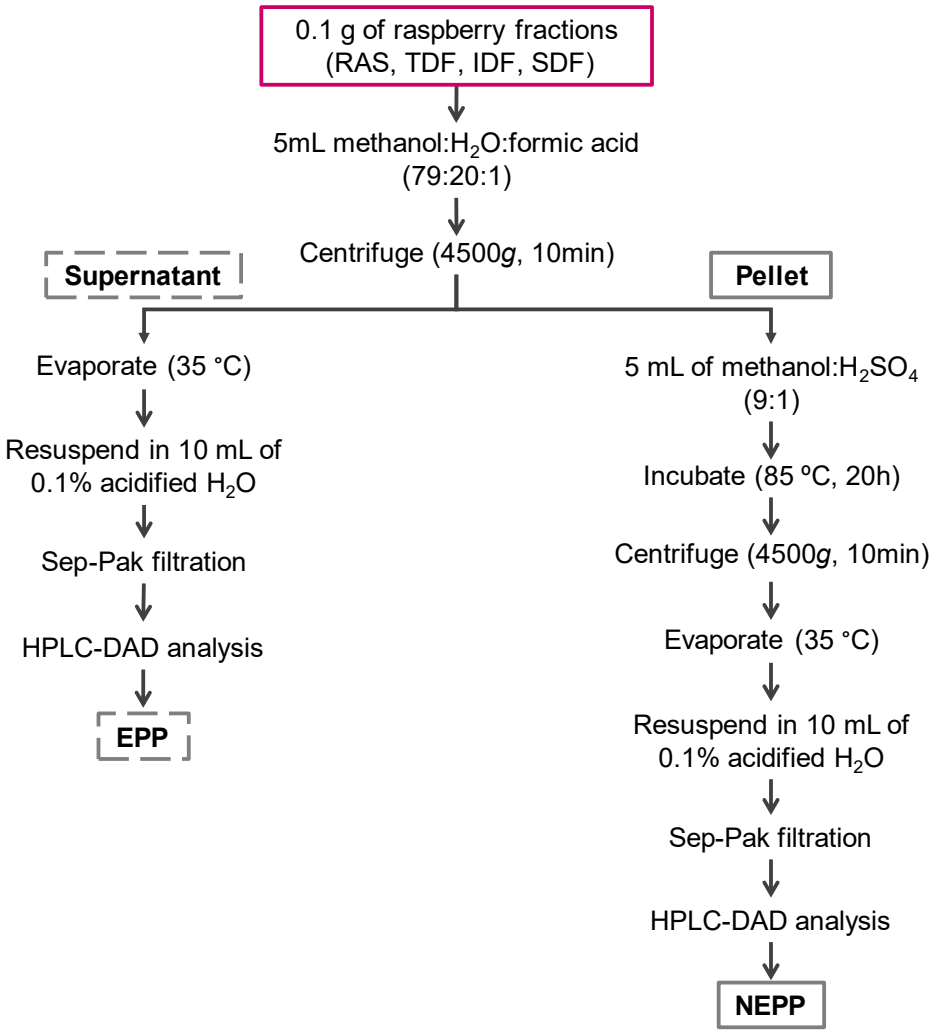


**Figure 12.** General flow diagram of the extractable (EPP) and non-extractable (poly)phenol (NEPP) extraction process. Raspberry (RAS); TDF (total dietary fibre); IDF (insoluble dietary fibre); SDF (soluble dietary fibre).

Briefly, for the EPP, 5 mL of methanol/$H_2O$/formic acid (79/20/1, v/v/v) were added to 0.1 g of sample. Then, samples were shacked and centrifuged at 4500*g* for 10 min.

The supernatant obtained from the extraction was dried under vacuum at 35 °C in a Laborota-4002 rotatory evaporator (Heidolph, Schwabach, Germany). Afterthought, the residue was re-dissolved in 10 mL of mili-Q water and 10 µl of 98% formic acid were added, then, samples were loaded into pre-conditioned Sep-Pak C18 cartridge (Waters Corporation, Milford, Massachusetts, USA), which was washed with 10 mL of mili-Q water before elution. Finally, the compounds were recovered in 1 mL of methanol. For the NEPP the pellet from this extraction was treated with methanol/$H_2SO_4$ (9/1, v/v) and incubated in a shaking incubator (VorTemp 1550, LabNet Biotécnica, Spain) during 20 h at 85 °C. Then, the supernatant was treated following the same method described before, recovering the compounds in 1 mL of methanol.

(Poly)phenols in the samples were quantified following the method described previously by González-Barrio *et al*. (2018). Briefly, an HPLC 1200 series with diode array detector (DAD) (Agilent Technologies, Waldbronn, Germany) was used, scanning from 200 to 600 nm. Separation of the different (poly)phenols was performed using a LiChroCART RP-18 column (250 × 4.6 mm, i.d. 5 µm), with a pre-column (4 × 4 mm) of the same material (Merck, Darmstadt, Germany). The mobile phases used were 1% aqueous formic acid (solvent A) and acetonitrile (solvent B), at a flow rate of 1 mL/min. Elution began with a linear gradient from 2 to 40% B in 50 min, followed by washing and then return to the initial conditions. Chromatograms were recorded at 280, 305, 320, 360, and 520 nm. Anthocyanins were quantified by comparison with the standard cyanidin-3-O-glucoside at 520 nm, flavonols as quercetin-3-O-rutinoside at 360 nm, hydroxycinnamic acid derivatives at 320 nm as chlorogenic acid, ellagic acid derivatives at 360 nm as ellagic acid and ellagitanins at 280 nm, using punicalagin as standard.

### 2.8. Antioxidant capacity of raspberry and its fibre fractions

For the antioxidant capacity analyses, 1 g of RAS and 0.2 g of TDF, SDF and IDF were mixed with 80% methanol sonicated during 10 min at room temperature using an ultrasonic bath (Branson Digital model 250 (Danbury, USA) and centrifuged at 4500*g* during 10 min at room temperature. The supernatants were evaporated in a vacuum concentrator (Eppendorf model 5301, Hamburg, Germany) and then resuspended in methanol. The antioxidant capacity of the samples was evaluated by two different antioxidant assay methods: the ferric reducing antioxidant power (FRAP) and the oxygen radical absorbance capacity (ORAC).

FRAP assay was carried out as described by Benzie and Strain, (1996), adapted to a microplate spectrophotometer (BioTek Instruments, Winooski, USA). This method

is based on the reduction of the ferric ion ($Fe^{+3}$) present in the FRAP reagent to its ferrous ion form ($Fe^{+2}$) caused by the presence of antioxidants, producing a colour change that determines its spectrophotometric absorbance. After the extraction of the samples, FRAP reagent, which have been previously prepared with acetate buffer/2,4,6-Tris(2-pyridyl)-s-triazine/$FeCl_3 \cdot 6H2O$ (10/1/1, v/v/v), was added and the absorbance was measured at 593 nm after 4 min. The results were expressed as µmol of Trolox equivalents (TE)/g of d.w.

The ORAC method was performed according to Ou *et al*. (2001), in a microplate spectrophotometer (BioTek Instruments, Winooski, USA). The ORAC assay is based on the inhibition of the peroxyl radical generated from thermal decomposition of the 2,2'-azobis(2-amidinopropane) dihydrochloride (AAPH) (Sigma-Aldrich, USA) by the antioxidants in the sample. Fluorescein (Sigma-Aldrich, USA) was used as fluorescence probe, whose signal intensity is attenuated by the oxidation caused by the AAPH radical. After sample extraction, the samples were placed in a microplate, where the spectrophotometer will add the fluorescein and the AAPH reading the absorbance at an excitation wavelength of 485 nm and an emission wavelength of 520 nm during 90 min. Trolox was used as the standard, and the results were expressed as µmol of TE/g of d.w.

### 2.9. *In vitro* faecal fermentation of raspberry and its fractions

The prebiotic effect of RAS and its extracted fractions (TDF, IDF, SDF and (poly)phenol extract (PEX)) (Figure 13) was evaluated by performing *in vitro* fermentations according to the method described by González-Barrio *et al*. (2011a), using human faecal samples.


**Figure 13.** Samples incubated in the *in vitro* fermentation. RAS (freeze-dried raspberry), TDF (total dietary fibre), IDF (insoluble dietary fibre), SDF (soluble dietary fibre) and PEX ((poly)phenol extract).

**Human faecal samples.** Faecal samples were collected from three healthy normal-weight woman (NW) and three healthy overweight women (OW), aged 35 to 60 years old. The number of volunteers has been selected based on previous *in vitro* studies (Jaganath *et al.*, 2009; Ludwig *et al.*, 2013). The inclusion criteria were to be non-smoker, with stable food habits, who did not present any symptoms of gastrointestinal disease, had not taken antibiotics for at least 4-6 months before the study, not to follow any dietary restrictions, not take any food supplements, prebiotics or probiotics, and were free of any gastrointestinal disease. Moreover, the volunteers had to follow a pre-established diet for two days prior to the study, mainly this diet removed the main sources of fibre and (poly)phenols. This study was approved by the Research of Ethics Commission (CEI code number: 2664/2019) and by the Experimentation Biosafety Committee (CBE code number: 282/2019) of the University of Murcia (Annex I). Written informed consent was obtained from each subject. Fresh faeces were collected in a flask containing an AnaeroGen™ Sacket (AN35, Oxoid®, UK), to produce anaerobic conditions and avoid microbial modifications, and were processed in the following 2 h.

**Fermentation Medium.** The fermentation medium was prepared following the protocol described by Jaganath *et al*. (2009). In short, 2 g of tryptone were dissolved in 400 mL of distilled water and 100 µL of micromineral solution (consisting of 13.2 g of $CaCl_2 \cdot 2H_2O$, 10 g of $MnCl_2 \cdot 4H_2O$, 1 g of $CoCl_2 \cdot 6H_2O$, 8 g of $FeCl_3 \cdot 6H_2O$, and distilled water up to 100 mL), 200 mL of buffer (2 g of $NH_4HCO_3$, 17.5 g of $NaHCO_3$, and distilled water up to 500 mL), 200 mL of macromineral solution (2.85 g of $Na_2HPO_4$, 3.1 g of $KH_2PO_4$, 0.3 g of $MgSO_4 \cdot 7H_2O$, and distilled water up to 500 mL), and 1 mL of 1% of resazurin solution (w/v), which was used as a redox indicator, were added. Then, the medium was adjusted to pH 7 using 6 M HCl and then sterilised by boiling for a few minutes before allowed to cool under oxygen free nitrogen for 30 min, the medium changed from blue to pink when all the oxygen was removed. Reducing solution (312 mg of cysteine hydrochloride, 2 mL of 1 M NaOH, 312 mg of $Na_2S \cdot 9H_2O$, and distilled water up to 50 mL) was added in a proportion of 1 mL of reducing solution per 20 mL of medium, after which the solution was purged with nitrogen until anaerobic conditions.

***In vitro* incubations.** Samples of fresh faeces were homogenised with phosphate buffer to obtain 32% faecal suspensions. Five mL of faecal suspension was added to 44 mL of fermentation medium at pH 7 and placed in a 100 mL McCartney bottle. Freeze-dried *in vitro* digested raspberries (200 mg) and the corresponding fractions of TDF (94 mg), IDF (67 mg), SDF (28 mg) and (poly)phenols (1.5 mL) were dissolved in 1 mL of water and added to each fermentation bottle. After this, the fermentation bottles were

purged with nitrogen and then incubated for 48 h at 37 °C in a shaking bath, simulating colonic lumen conditions. Aliquots of the fermented faecal samples were collected at baseline (0 h) and after 6, 24 and 48 h to analyse microbial catabolites. Samples were immediately stored at -80 °C prior to analysis.

### 2.10. Analysis of SCFAs by gas liquid chromatography with flame ionisation detector (GLC-FID)

The prebiotic activity of the substrates was measured by evaluation of the main SCFAs produced (acetate, propionate and butyrate) and the minor ones (isobutyrate, isovalerate, valerate, isocaproate, caproate and heptanoate), as catabolites produced by the gut microbiota. The SCFAs were analysed in faecal fermentation aliquots from 0, 6, 24 and 48 h, following the protocol described by Anson *et al*., (2011) with some modifications.

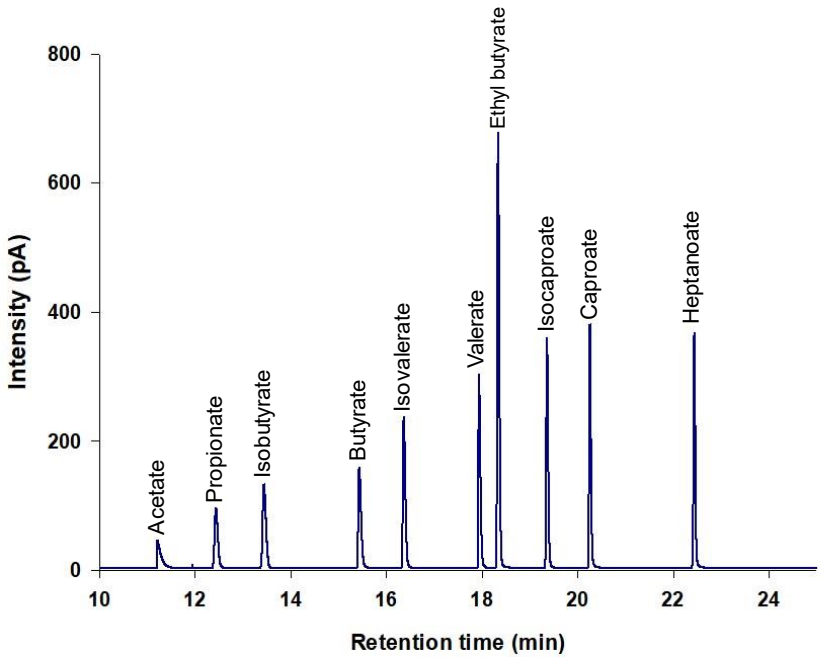


**Figure 14.** Chromatogram of SCFAs mixture and 2-ethyl butyrate as internal standard.

Briefly, the fermented faecal samples were mixed during 5 min with a solution of 20% formic acid/methanol/2-ethyl butyric acid (internal standard, 2 mg/mL in methanol) (1/4.5/1, v/v/v). After that, samples were centrifuged at 16110*g* for 15 min at room temperature and the supernatant obtained was filtered (Ø 13 mm, pore size 0.22 µm, PTFE, VWR International, USA) and analysed by GC-FID. Chromatographic analysis was carried out using an Agilent 7890A GC system equipped with a flame ionisation

detector (FID) and a 7683B automatic injector (Agilent Technologies, USA). A fused-silica capillary column (Nukol TM, Supelco, USA) of 30 m × 0.25 mm, i.d. 0.25 µm coated, was used to separate the SCFAs. Helium was supplied as the carrier gas at a flow rate of 25 mL/min. The initial oven temperature was 80 °C and it was kept constant for 5 min and then raised to 185 °C at a rate of 5 °C/min. Samples (2 µL) were injected in splitless mode, with an injection port temperature of 220 °C. The flow rates of hydrogen, and air as makeup gas were 30 and 400 mL/min, respectively. The temperature of the FID was 220 °C and the running time for each analysis was 26 min.

SCFAs were identified by comparison with the retention times of authentic standards (Figure 14) (Supelco, USA). Quantification was based on calibration curves constructed for a set of SCFAs standards (acetate, propionate, isobutyrate, butyrate, isovalerate, valerate, isocaproate, caproate and heptanoate). The concentration was expressed as mM and was calculated using linear regression equation ($R^2$ 0.99) from the corresponding standards curve. All chemicals were at least GC grade quality.

### 2.11. Analysis of microbial (poly)phenol catabolites by liquid chromatography (HPLC-DAD)

Fermented faecal samples (1 mL) were mixed with water acidified with 0.1% formic acid and passed through a pre-conditioned Sep-Pak C18-SPE column (Waters Corporation, Milford, Massachusetts, USA), and the compounds of interest were eluted in 1 mL of methanol. The methanolic extract was analysed by HPLC–DAD (Agilent Technologies, Waldbronn, Germany), using the same method as for the analysis of (poly)phenols in section 2.7. (González-Barrio *et al*., 2018).

Urolithins and ellagic acid derivatives were identified according to their absorbance spectra, based on data previously reported and by comparison with authentic standards (González-Barrio *et al*., 2011b). Urolithins were quantified using urolithin B at 305 nm as standard and ellagic acid using the pure standard at 360 nm. The results were expressed in µg/mL.

### 2.12. Statistical analysis

The statistical analysis was carried out using R studio, version 3.4.3. (R Foundation for Statistical Computing, Vienna, Austria). Normality was determined by the Shapiro-Wilk test. The homogeneity of variances was analysed using the Bartlett test. One-way analysis of variance (ANOVA) was performed to determine significant differences among

different samples for the parameters analysed. A two-way analysis of variance (two-way ANOVA) was performed, considering the effect of the different samples and the body weight condition of the subjects. A correlation analysis was also performed between the physicochemical properties and fibre composition parameters. Tukey´s test was used as a post-hoc test. Differences were considered significant at a *p*-value<0.05. A principal component analysis (PCA) was also performed, to correlate the metabolic activity of the microbiota with the health status of the subjects. The ellagic acid degradation and the production of urolithins and SCFAs were expressed as Δ from the baseline.

## 3. Results and discussion

### 3.1. Nutritional composition of raspberry

The data of the proximate composition of the raspberries are shown in Table 5. These berries showed a higher moisture content (87%) and lower contents of total protein and fat (0.05% and 0.01%, respectively) compared to the values presented by the United States Department of Agriculture (USDA) database (USDA, 2018) and other authors (Probst, 2012; De Souza *et al.*, 2014). With regard to the ash content, our samples had a higher mean value (0.8%) when we compared with the scientific literature (De Souza *et al.*, 2014).

**Table 5.** Raspberries chemical composition (g/100 g of f.w.).

| Parameters | Content |
|---|---|
| **Moisture** | 87.28 ± 0.60* |
| **Protein** | 0.05 ± 0.01 |
| **Fat** | 0.02 ± 0.01 |
| **Ash** | 0.80 ± 0.10 |
| **Carbohydrates** | 11.85 ± 0.28 |
| **Soluble sugars** | 5.75 ± 0.11 |
| **Total dietary fibre (TDF)** | 6.10 ± 0.57 |
| **Insoluble dietary fibre (IDF)** | 4.26 ± 0.16 |
| **Soluble dietary fibre (SDF)** | 1.90 ± 0.09 |

*Values are expressed as mean ± standard deviation (SD) (n = 3). Fresh weight (f.w.).

The carbohydrate content (11.9 g/100 g of fresh weight (f.w.)) was similar to that in other reports (USDA, 2018) and corresponded to the sum of the soluble sugars (5.8 g/100 g of f.w.), mainly glucose, fructose and sucrose (Dincheva *et al.*, 2013), and TDF. According to these results, raspberries are low-energy fruits composed mainly of carbohydrates and DF, principal components of a healthy and balanced diet (Lunn and Buttriss, 2007). During the last decade, a wide variety of genotypes of raspberry have been developed to obtain commercial cultivars with increased productivity, valuable nutrients and bioactive contents (Bobinaite *et al.*, 2015). However, different cultivars, environmental factors, processing and storage time may influence its composition (Zhang *et al.*, 2019).

The raspberries were found to have a high content of TDF (6.1 g/100 g of f.w.), higher than in other berries - such as strawberry (1.3 g/100 g of f.w.), blueberry (1.9 g/100 g of f.w.) and blackberry (4.5 g/100 g of f.w.) - and cherry (2.1 g/100 g of f.w.) (De Souza *et al.*, 2014), and fruits such as kiwi (3.0 g/100 g of f.w.), oranges (4.5 g/100 g of f.w.), apples (2.4 g/100 g of f.w.) and pears (3.1 g/100 g of f.w.) (USDA, 2018).

Regarding the raspberry fibre fractions, the IDF represented 70% of the TDF (Table 5) with a mean content of 4.3 g/100 g of f.w. The IDF fraction was mainly represented by cellulose, insoluble hemicellulose and lignin, which facilitate the gastrointestinal tract passage (Lattimer and Haub, 2010). The SDF (30% of the TDF, with a mean value of 1.9 g/100 g of f.w.) was composed of soluble hemicellulose and pectin, these being indigestible compounds fermented by the microbiota in the colon (Laroze *et al.*, 2010). The IDF/SDF ratio was 2.3, generally acceptable for the use of fibre fractions as ingredients in the food industry. This value was lower than in guava, tomato peel, grapefruit, lemon, orange and apple (Figuerola *et al.*, 2005; Navarro-González *et al.*, 2011), due to raspberry is a fruit with an important content of SDF, lower IDF/SDF ratios have been associated with more metabolic, appetite and energy intake control (Burton-Freeman *et al.*, 2017). Moreover, papaya, mango and passion fruit have been reported to have similar ratios than that found in our raspberry samples, because these fruits have also a significant amount of SDF (Martínez *et al.*, 2012; Nieto Calvache *et al.*, 2016).

Taking into consideration the high content of DF of raspberries, these fruits, as well as their by-products from the processing industry, such as seeds and pomace, may be used as ingredients for the development of innovative fibre containing foods, such as beverages and dairy or bakery products (Cho and Samuel, 2009; Shahidi *et al.,* 2019), obtaining a valorisation of the by-products, being necessary to characterise its physicochemical properties and chemical composition of the new ingredients.

### 3.2. Neutral sugars and uronic acids of dietary fibre fractions

To know the composition of the DF fractions (TDF, IDF, SDF) isolated from raspberries, the individual neutral sugars profile, the uronic acids content and pectin, hemicellulose and cellulose profile were determined (Table 6). Fibre is constituted by cellulose, uronic acids (composing pectins) and hemicellulose with different solubility properties. Hemicellulose is a heteropolysaccharide constituted by different sugars, which could be soluble or insoluble in water depending on its chemical composition. Besides glucose, sugar monomers in hemicellulose can include the five-carbon sugars, xylose and arabinose, the six-carbon sugars, mannose and galactose, and the six-carbon deoxy sugar, rhamnose (Căpriţă *et al*., 2010). The TDF and IDF showed similar profiles of neutral sugars, being glucose the main compound in the samples (48% and 54%, respectively), followed by xylose, arabinose, galactose and mannose. Other authors have reported the same order but lower proportions, except for glucose, in a deoiled red raspberry pomace with 83%, 6%, 3% and 2% respectively for glucose, xylose, arabinose and galactose. On the other hand, the same order and similar proportion except for glucose, have been found in TDF from deoiled red raspberry pomace, with 19%, 32%, 16% and 13%, respectively (Li *et al.*, 2022). The SDF fraction had a contrasting profile of neutral sugars, being glucose (28%) and arabinose (34%), the most abundant monomers, followed by galactose, mannose and xylose. In contrast, the results found in the soluble fraction of a blackcurrant pomace showed that the most abundant monomers were arabinose and galactose, followed by glucose with 9%, 8% and 4%, respectively (Alba *et al.*, 2018).

Hence, these results suggest that hemicellulose in the soluble fraction was mainly constituted by mannose, whereas the hemicellulose in the insoluble fraction was comprised mostly by xylose, which was also found at higher concentrations in the TDF (3.3 and 2.7-fold higher than in the SDF), as the predominant component of the insoluble hemicellulose (Mudgil, 2017). The results showed that the hemicellulose in SDF was mostly soluble, compared to that in TDF and IDF, which was mostly insoluble due to the higher amount of xylose (Peng *et al.*, 2019). The content of hemicellulose was highest in IDF (36%), followed by SDF (33%) and TDF (31%) although without significant differences. Moreover, the xylose/arabinose ratio in the present study may indicate the successful separation of hemicelluloses during the extraction of the TDF, IDF and SDF, being 1.8, 4.7 and 0.3, respectively. A low value in this ratio indicated the extraction of hemicellulose with more side chains and complex structures. Suggesting this results that IDF hemicellulose have a longer and linear backbones, instead the ones in SDF with

more branches (Peng *et al*., 2019). Other component of the insoluble fraction of DF is the cellulose, an insoluble complex carbohydrate constituted by glucose monomers joined by β-(1→4) linkages (Abdul Khalil *et al.*, 2017). In these samples, the contents of cellulose in TDF and IDF were 41% and 50% (Table 6), respectively, showing higher values than that reported in a deoiled red raspberry pomace and its TDF being 11% and 18% respectively (Li *et al.*, 2022).

**Table 6.** Percentage (%) of neutral sugars, uronic acids, hemicellulose, cellulose and pectin in raspberry dietary fibre fractions.

| Sugars/Polysaccharides | TDF | IDF | SDF |
|---|---|---|---|
| **Arabinose** | 13.5 ± 4.8$^{b}$* | 6.2 ± 3.1$^{b}$ | 34.0 ± 3.7$^{a}$ |
| **Xylose** | 24.2 ± 5.8$^{a}$ | 29.4 ± 4.9$^{a}$ | 8.9 ± 2.7$^{b}$ |
| **Mannose** | 5.7 ± 0.9$^{b}$ | 4.2 ± 0.4$^{b}$ | 10.2 ± 0.5$^{a}$ |
| **Galactose** | 8.8 ± 0.6$^{b}$ | 5.5 ± 0.2$^{b}$ | 19.3 ± 1.3$^{a}$ |
| **Glucose** | 47.8 ± 0.5$^{a}$ | 53.7 ± 1.2$^{a}$ | 27.5 ± 2.4$^{b}$ |
| **Uronic acids** | 11.0 ± 2.8$^{b}$ | 3.4 ± 0.3$^{c}$ | 35.4 ± 2.0$^{a}$ |
| **Hemicellulose$^{A}$** | 30.9 ± 3.3 | 35.7 ± 4.3 | 33.2 ± 1.6 |
| **Cellulose$^{B}$** | 41.3 ± 0.6$^{b}$ | 49.6 ± 3.5$^{a}$ | – |
| **Pectin$^{C}$** | 27.8 ± 3.0$^{b}$ | 14.8 ± 1.6$^{c}$ | 66.8 ± 1.6$^{a}$ |

*Values are expressed as mean ± standard deviation (SD) (n = 3). Different letters (a-c) within the same row indicate significant differences among samples ($p$<0.05). Total dietary fibre (TDF); insoluble dietary fibre (IDF); soluble dietary fibre (SDF); – (not calculated). $^{A}$ Hemicellulose: (Fucose + Xylose + Mannose + (Glucose x 0.1); $^{B}$ Cellulose: Glucose x 0.9; $^{C}$ Pectin: (Rhamnose + Arabinose + Galactose + Uronic acids).

Regarding the uronic acids, higher values were found in the SDF compared to the IDF and TDF (10- and 3-fold, respectively), representing the content of pectin. In a blackcurrant pomace the content of uronic acids was 3.5-fold higher in the soluble fraction compared with the insoluble fraction (Alba *et al.*, 2018), being lower than that reported in our results. Pectins are heteropolysaccharides constituted by neutral sugars and uronic acids. Most of pectins have neutral sugars covalently linked to uronic acids as side chains, mainly arabinose and galactose, and to a lesser extent, xylose, rhamnose, and glucose (Căpriţă *et al.*, 2010). For this reason, the pectin content was highest in SDF, followed by TDF and IDF with 67%, 28% and 15%, respectively. Moreover, SDF fraction presented a higher proportion of arabinose and galactose than

TDF and IDF fractions. Similar values of neutral sugars and uronic acids were found by Ross *et al*. (2015) after analysing crude polysaccharides isolated from raspberry. In addition, the uronic acids present in the IDF may be derived from the insoluble hemicelluloses (Huffman, 2003).

The different compositions of the TDF, SDF and IDF may determine their beneficial physiological effects. Thus, a high amount of uronic acids in SDF, representing the pectin content which is soluble in water, indicates that this fraction was likely to be fermented by the microbiota, which exert several beneficial effects for human health such as lipid-lowering effect which helps to reduce serum cholesterol levels and glucose-lowering effect (Surampudi *et al.*, 2016). On the contrary the IDF fractions did not form gels and its fermentation will be severely limited (Lattimer and Haub, 2010). The content of insoluble hemicellulose in the IDF has been associated with a lower postprandial glucose response in humans, which could be related to a faster bowel transit and reduced glucose absorption that would improve insulin resistance and reduce the risk of developing diabetes mellitus type II (Mudgil, 2017; Weickert, 2018).

### 3.3. Physicochemical properties of dietary fibre fractions

In addition to the chemical composition, the physicochemical properties of the fibre fractions were evaluated to ascertain their potential physiological functions related to homeostatic and therapeutic effects in the gastrointestinal system. These properties should be taken into consideration when selecting a fibre source as ingredient, due to its possible individual responses to processing conditions. In this chapter, the functionality of DFs was determined by the study of its physical and hydration properties, such as the WRC, SWC, FAC, GDRI and osmotic pressure (Table 7).

The hydration properties of DFs, evaluated as WRC and SWC, described the ability of the fibre matrix to retain water, which may affect the pattern of nutrient absorption, postprandial satiety, intestinal motility in the upper intestine, and increase the stool weight (Navarro-González *et al.*, 2011; Tan *et al.*, 2017). The TDF fraction showed a WRC of 5.3 g of water/g, this parameter showed the highest value in the SDF fraction (10.4 g of water/g), due to its highest content of pectin and soluble hemicellulose, showing IDF fraction the lowest value (0.6 g of water/g). These values agree with the SWC, which was also greatest in the SDF fraction (2.0 mL of water/g), whereas the low value of SWC observed in the TDF fraction (0.8 mL of water/g) might be related to the proportion of SDF in the raspberry (30%) (Table 5).

The soluble polysaccharides (mainly pectin and soluble hemicellulose) are the structures capable of absorbing water (swelling) (Lattimer and Haub, 2010), being the first step of polysaccharide solubilisation, which may decrease the postprandial glucose and insulin response being directly related with a slowing of gastric emptying and an increase in satiety (Tan *et al.*, 2016; McRorie and McKeown, 2017). However, in our study only significantly positive correlations between hydration properties (WRC and SWC) and pectin content were observed. In accordance with our results, other plant materials containing around 70% of IDF showed similar hydration properties, such as tomato peel (Navarro-González *et al.*, 2011) or seaweeds (Gómez-Ordóñez *et al.*, 2010). Despite the importance of the hydrophilic properties of the fractions, the water affinity of fibre is affected by other physical characteristics, such as structure, viscosity and particle size, as well as by the extraction method (Chau *et al.*, 2007).

**Table 7.** Physicochemical properties of raspberry dietary fibre fractions.

| Physicochemical properties | TDF | IDF | SDF |
|---|---|---|---|
| **Water retention capacity** (g water/g) | 5.3 ± 0.3$^{b}$* | 0.6 ± 0.0$^{c}$ | 10.4 ± 0.4$^{a}$ |
| **Swelling capacity** (mL water/g) | 0.8 ± 0.0$^{b}$ | 0.5 ± 0.0$^{c}$ | 2.0 ± 0.0$^{a}$ |
| **Fat absorption capacity** (g oil/g) | 3.9 ± 0.5$^{a}$ | 2.1 ± 0.1$^{b}$ | 1.6 ± 0.1$^{b}$ |
| **GDRI 30 min** (%) | 6.1 ± 0.5$^{b}$ | 0.0 ± 0.0 | 8.9 ± 0.7$^{a}$ |
| **GDRI 60 min** (%) | 7.0 ± 0.6$^{a}$ | 3.1 ± 0.9$^{b}$ | 3.0 ± 0.5$^{b}$ |
| **Osmotic pressure** (mmol/kg) | 302.0 ± 14.8$^{b}$ | 321.3 ± 1.5$^{ab}$ | 342.7 ± 2.9$^{a}$ |

*Values are expressed as mean ± standard deviation (SD) (n = 3). Different letters (a-c) within the same row indicate significant differences among samples ($p$<0.05). Total dietary fibre (TDF); insoluble dietary fibre (IDF); soluble dietary fibre (SDF); glucose diffusion retardation index (GDRI).

Regarding FAC, the ability of fibre to adsorb fat or oil, the TDF showed a significantly higher value (3.9 g of oil/g of sample) compared to the SDF and IDF fractions (1.6 and 2.0 g of oil/g of sample, respectively). This physicochemical property is an important characteristic of ingredients to be used in the food industry, to avoid fat loss from foods during cooking, stabilise emulsions and improve shelf-life (Elleuch *et al.*, 2011). The raspberry TDF and its fractions showed FAC values higher than those of other vegetable materials, such as tomato peels, grapefruits, lemon and apple fibre concentrates (Figuerola *et al.*, 2005; Navarro-González *et al.*, 2011), but similar to those

reported in orange peels (De Moraes Crizel *et al.*, 2013) and pomegranate peel (Hasnaoui *et al.*, 2014). Thus, the TDF from raspberry could be useful in the formulation of fibre-enriched foods that require emulsifying properties or even as fat replacers in low-calorie products, as have been described for other fibres (De Moraes Crizel *et al.*, 2013). These differences are due to the diverse chemical composition of the dietary vegetable fibres, with regard to the surface properties, particle size, overall charge and grade of lipophilicity of the components (Karaman *et al.*, 2017).

The GDRI of DF fractions predicts the ability of fibre components to retain glucose and, therefore, to delay glucose absorption by the intestine. The results showed that after 30 min of dialysis the SDF had the highest GDRI value (8.9%), while the IDF did not retain glucose, allowing the dialysis of glucose through the membrane. In contrast, the two fibre fractions showed similar glucose retention values (around 3%) at 60 min. This behaviour can be explained because the SDF had stronger properties of hydration, which reduced the absorption of glucose more quickly, whereas the IDF required a longer hydration time to reduce the glucose dialysis. For the TDF, similar behaviour along the study was observed, with a dialysis trend almost constant in time (Table 7). We can assert that the soluble and insoluble fractions acted at different times due to their differences in composition. However, the TDF showed a constant capacity to retard glucose diffusion at different times, which could be explained by the mix of the fibre components with different solubilities. This property is important to determine the functional effect of fibres in relation to the glycaemic index of foods. At 60 min, the GDRI of cocoa TDF was lower (4%) than TDF and similar to IDF and SDF (Lecumberri *et al.*, 2007), while the value obtained with tomato peel DF at 60 min was higher (39%) (Navarro-González *et al.*, 2011).

In addition, the osmotic pressure of the raspberry fibre fractions was also evaluated and compared to the control (300 ± 2 mmol/kg NaCl in the physiological state) (data not shown). The results showed that the IDF and SDF may slightly increase the intestinal osmotic pressure; however, these values were not expected to induce diarrhoea after consumption.

### 3.4. (Poly)phenols and antioxidant capacity of raspberry and its fibre fractions

(Poly)phenols are the predominant bioactive compounds in raspberry, among them, anthocyanins and ellagitannins are the main groups present in this fruit (González-Barrio *et al*., 2010; Bobinaite *et al*., 2015). Generally, the evaluation of these compounds reported in the literature refers to the EPP analysed by aqueous-organic solvents

extraction. Nevertheless, a significant non-extractable amount of these bioactive compounds, the NEPP, remains in the sample and should be extracted by acidic hydrolysis of the extracted residues (Arranz *et al.*, 2009). These dietary NEPP are not significantly released from the food matrix by digestion; therefore, they reach the colon where they would be subjected to the activity of the gut microbiota and may be bio-accessible, thus, allowing the absorption of derived catabolites and providing beneficial effects on gastrointestinal health (Pérez-Jiménez *et al.*, 2008).

**Table 8.** Extractable (EPP) and non-extractable (NEPP) (poly)phenols (mg/g of d.w.) by HPLC-DAD and antioxidant capacity (mg TE/g of d.w.) of raspberry and its dietary fibre fractions.

| (Poly)phenols | RAS | TDF | IDF | SDF |
|---|---|---|---|---|
| ***Extractable (poly)phenols (EPP)*** | | | | |
| Anthocyanins | 38.0 ± 4.4$^{a}$* | 0.1 ± 0.0$^{b}$ | 0.3 ± 0.0$^{b}$ | 0.1 ± 0.0$^{b}$ |
| Ellagitannins | 4.1 ± 0.3$^{a}$ | 0.1 ± 0.0$^{c}$ | 0.4 ± 0.1$^{b}$ | 0.1 ± 0.0$^{c}$ |
| Ellagic acid derivatives | 0.2 ± 0.0$^{b}$ | 0.3 ± 0.0$^{b}$ | 1.0 ± 0.2$^{a}$ | 0.1 ± 0.0$^{c}$ |
| Flavonols | 0.1 ± 0.0 | nd | nd | nd |
| Caffeic acid | 0.01 ± 0.00 | nd | nd | nd |
| ***Non-extractable (poly)phenols (NEPP)*** | | | | |
| Ellagic acid derivatives | 0.1 ± 0.0$^{d}$ | 7.5 ± 0.2$^{a}$ | 6.0 ± 0.9$^{b}$ | 1.8 ± 0.1$^{c}$ |
| ***Antioxidant capacity*** | | | | |
| FRAP | 32.6 ± 0.2$^{a}$ | 3.9 ± 0.1$^{c}$ | 9.4 ± 0.2$^{b}$ | 0.6 ± 0.0$^{d}$ |
| ORAC | 69.1 ± 5.3$^{a}$ | 10.7 ± 0.7$^{c}$ | 20.2 ± 0.7$^{b}$ | 7.8 ± 0.8$^{d}$ |

*Values are expressed as mean ± standard deviation (SD) (n = 3). Different letters (a-c) within the same row indicate significant differences among samples ($p$<0.05). Raspberry (RAS); total dietary fibre (TDF); insoluble dietary fibre (IDF); soluble dietary fibre (SDF); not detected (nd); dry weight (d.w.).

Moreover, the (poly)phenols from the EPP and NEPP fractions of RAS and DF fractions (TDF, SDF, IDF) were analysed by HPLC-DAD. The total content of (poly)phenols in the samples were calculated as the sum of the individual compounds. Among the EPP, anthocyanins and ellagitannins were found as the main groups of (poly)phenols in RAS, being 38 mg/g of d.w. and 4.6 mg/g of d.w., respectively, followed by ellagic acid derivatives (0.2 mg/g of d.w.), flavonols (0.1 mg/g of d.w.) and caffeic acid (0.01 mg/g of d.w.) (Table 8). As it is shown in Figure 15, RAS was the sample with the

highest content of EPP, followed by IDF, TDF and SDF. Differences in the contents of (poly)phenols reported in raspberries are quite remarkable maybe because the existing differences among analytical methods, cultivars, conventional and organic farming, harvested time, processing and manipulation (Hidalgo and Almajano, 2017; Ponder and Hallmann, 2019; Stamenković *et al.*, 2019). However, total contents of (poly)phenols reported in this study were slightly higher compared to other research works for raspberry (11 – 37 mg/g of d.w.) (Pantelidis *et al.*, 2007; Bobinaite *et al.*, 2015).

Compared to the TDF, IDF and SDF fractions, RAS showed the highest content of anthocyanins and ellagitannins (Table 8). These results suggest that these (poly)phenols are mostly solubilised in the organic extraction solvents after disruption of the cells during the isolation of the fibres, since their potential to be attached to the polysaccharides of the plant cell wall is low (Padayachee *et al.*, 2012).

Regarding the NEPP in RAS, ellagic acid derivatives were also found (0.1 mg/g of d.w.), being these compounds released from the ellagitannins associated with the cell wall polysaccharides after hydrolysis (Ross *et al*., 2007; Arranz *et al*., 2010). Thus, in addition to the ellagic acid from EPP fraction reported before, RAS contained a total amount of 0.3 mg/g of d.w. of ellagic acid derivatives. This value was within the range of the data found in the literature (0.01 – 5 mg/g of d.w.). This variation may have been due to the difficult extraction of ellagic acid from the fruit, which requires an acid hydrolysis of the ellagitannins present in the samples (Mullen *et al.*, 2002; Rothwell *et al.*, 2013).

On the contrary, DF fractions (TDF, IDF, SDF) contained higher contents of (poly)phenols in the NEPP fraction compared to the EPP content, representing 94%, 78% and 86% of the total content for the TDF, SDF and IDF, respectively (Figure 15). These NEPP were represented by ellagic acid derivatives, as it was found in RAS. These findings indicate the presence of ellagitannins attached to the polysaccharides of the raspberry fibre fractions, which after resisting the intestinal digestion, reach the colon, being also relevant to the health benefits related to fibre consumption (Quirós-Sauceda *et al.*, 2014; Macagnan *et al.*, 2016). Ellagic acid derivatives are of great interest due to their health effects related to gastric mucosa protection through anti-inflammatory and antioxidant activities (Sangiovanni *et al.*, 2013). These compounds have also been reported in other fruit by-products containing fibre, such as pomegranate husk, tea production residues, oak acorns and orange peels (Sepúlveda *et al.*, 2020). These results point out the relevance of the extraction, not only for the EPP using aqueous-organic solvents, but also for the NEPP fraction.

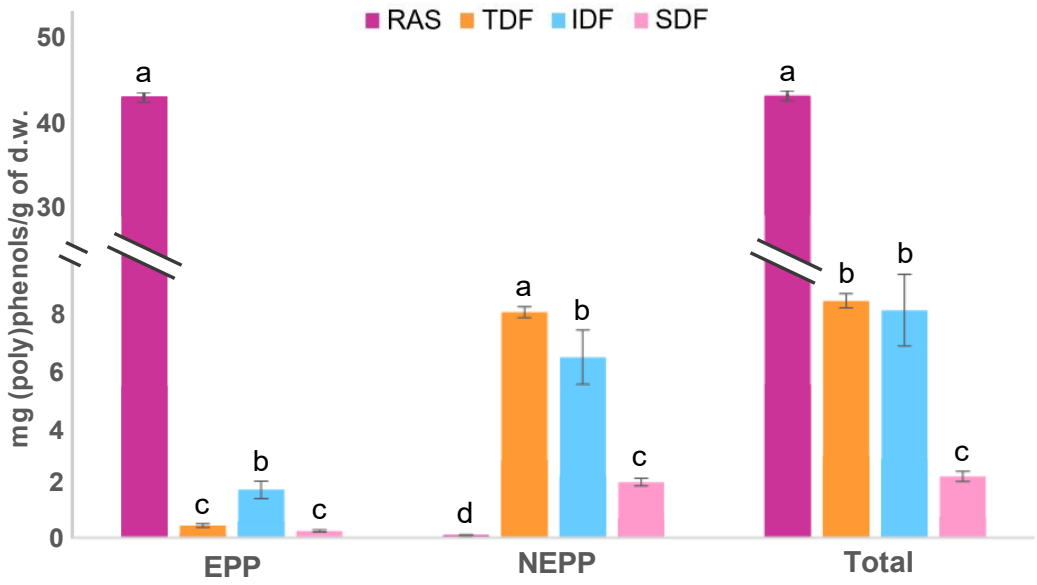


**Figure 15.** Contents of extractable (poly)phenols (EPP), non-extractable (poly)phenols (NEPP) and total (poly)phenols as the sum of EPP and NEPP (mg/g of d.w.) analysed by HPLC-DAD for raspberry (■RAS), total dietary fibre (■TDF), insoluble dietary fibre (■IDF) and soluble dietary fibre (■SDF). Values are expressed as mean ± SD (n = 3). Different letters (a–d) indicate significant differences ($p$<0.05) among the different samples.

Related to the differences in the (poly)phenols profile among the three fibre fractions, TDF and IDF showed a similar content of (poly)phenols, being the NEPP significantly higher in TDF (7.5 mg/g of d.w.) than in IDF (6.0 mg/g of d.w.) (Table 8). By contrast, the contents of EPP (anthocyanins, ellagitannins and ellagic acid derivatives) were higher in the IDF than in the TDF, may be due to the different isolation procedure of these fibre fractions, being ethanol not added for the IDF extraction (Figure 9), avoiding the solubilisation of a considerable part of the EPP. Curiously, the highest content of extractable ellagic acid derivatives were found in the IDF (1 mg/g of d.w.) (Table 8), maybe due to the release of a portion of these compounds contained in the insoluble fibre with the hydromethanolic extraction of EPP. On the other hand, the SDF fraction showed the lowest content of (poly)phenols, suggesting that these compounds were mainly solubilised by ethanol during the extraction procedure of this soluble fraction. In addition, the SDF did not contain cellulose, where the (poly)phenols are bound to the DF (Căpriţă *et al.*, 2010). Regarding to the total content of (poly)phenols as the sum of EPP and NEPP, RAS was the fraction with the highest content due to the high content of EPP with a mean value of 42.5 mg/g of d.w. Followed by TDF and IDF (8 and 7.7 mg/g of d.w.) without significant differences among them and being SDF the one with the lowest content (2.1 mg/g of d.w.) (Figure 15).

With respect to the *in vitro* antioxidant capacity of the samples, it was evaluated by two different methods the FRAP assay and the ORAC assay. Both assays are used to obtained more reliable assessment of the antioxidant profile of the samples, showing also a high correlation with total (poly)phenols (Du and Xu, 2014). RAS showed the highest antioxidant capacity (33 and 69 mg TE/g of d.w., for FRAP and ORAC, respectively) (Table 8), which was highly related to the high content of extractable anthocyanins and ellagitannins, in agreement with the scientific literature (Pantelidis *et al.*, 2007). In spite of the similar content of (poly)phenols in the TDF and IDF, higher antioxidant capacity was found in the IDF, suggesting that EPP might be largely responsible for the antioxidant capacity in the fibre, as the extraction procedure in the antioxidant capacity assays did not break down the (poly)phenols attached to the cell walls (corresponding to > 80% of the (poly)phenols in the fibre fractions). The FRAP antioxidant capacity of the IDF from raspberry (33 mg TE/g of d.w.) was higher than that of other fibre extracts, such as papaya fibre (2.5-6 mg TE/g of d.w.) (Nieto Calvache *et al.*, 2016) or mango, passion fruit, pineapple and guava ranging from 0.7-5 mg TE/g of d.w. (Martínez *et al.*, 2012). Therefore, the fibre fractions of raspberry had important contents of EPP and NEPP with their potential free radical scavenging activity and antioxidant capacity *in vivo*, being the EPP partially absorbed in the small intestine, most of them reaching the colon and increasing the antioxidant nature of the environment (Martínez-Meza *et al*., 2021).

### 3.5. SCFAs production during *in vitro* fermentation

The potential prebiotic effect of the different samples was measured by the evaluation of the SCFAs produced by the gut microbiota, following an *in vitro* fermentation method with faecal slurries from normal-weight (NW) and overweight (OW) women. Both types of inoculums were used to evaluate a potential difference between them, because as other authors have previously reported, the dysbiosis observed in overweigh subjects may lead to alterations in the microbiota metabolic activity (Liu *et al.*, 2017).

As it is shown in Table 9, acetate was the major SCFAs produced, for both NW and OW groups, since acetate is recognised as the main SCFA produced by the intestinal bacteria (Karimi *et al*., 2020; Nogacka *et al*., 2020). RAS produced the highest amounts of acetate and propionate, followed by the PEX fraction. These results reveal that not only DF from raspberry exhibited a prebiotic effect (Hijová *et al.*, 2019) but also its (poly)phenols (Table 9 and Figure 16) may be considered as potential prebiotics,

showing a synergistic effect (Alves-Santos *et al.*, 2020). The butyrate content showed the smallest differences when comparing TDF, IDF and SDF with RAS and PEX, since butyrate is mainly produced from non-digestible polysaccharides, and therefore, in the fibre fractions the production was equalised to the other two fractions (Bas-Bellver *et al.*, 2020).

For the fibre-rich fraction, SDF produced the highest amount of acetate, propionate and butyrate of all fibre fractions but without significant differences, being IDF the one with the lowest production. SDF also produced the highest amount of other minor SCFAs (valerate and isovalerate) of all the fractions, although there were also no significant differences. The prebiotic effect of fibre depends on its solubility, in this sense, SDF was the fraction with the highest values of soluble fibre based on its pectin content, followed by TDF (Table 6), whereas IDF fraction contains the non-fermentable carbohydrates (mostly cellulose and insoluble hemicellulose). For this reason, the fermentation of SDF fraction led to a higher formation of SCFAs than TDF and IDF fractions.

The major SCFAs produced for all samples in both inoculums was acetate. In contrast, there was a different trend at 48 h for propionate, butyrate and other minor SCFAs, as they were the majority in that order for RAS and PEX, but the opposite was found for the fibre fractions (TDF, IDF and SDF), which may be due to high content of non-digestible carbohydrates increasing the butyrate content as they are mainly produced from these degradation pathways (Szentirmai *et al.*, 2019; Widaningrum *et al.*, 2020). In addition, as there was a greater challenge in the degradation of the fibres, there may be an increase in the digestion of the amino acids found in the fermentation medium, leading to an increase in isobutyrate and isovalerate as the majority SCFAs in the minor group, which have been described as derivatives from the protein and amino acid degradation pathways (Shortt *et al.*, 2018).

It is known that overweight leads to a higher amount of SCFAs in faeces because the intestinal microbiota from OW people showed a better ability to produce SCFAs (Nogacka *et al.*, 2020). However, we did not observe a clear effect, and the amounts of SCFAs were mainly related to the fermentable substrate or sample. The propionate production from RAS was highest in the NW at 6 h. On the other hand, the production of acetate and propionate from the PEX fraction was highest in the OW at 6 h, and acetate production from the IDF fraction was highest in OW at 48 h. This could be due to the high production of urolithins in the OW explained in the next section, since urolithins have antimicrobial activity (Singh *et al.*, 2020) and may cause a decrease in the gut microbial activity leading to a reduction in SCFAs production.

**Table 9.** Increase (Δ) in short-chain fatty acids (SCFAs) (mM) (acetate, propionate, butyrate and other minor SCFAs (isobutyrate, isovalerate, valerate, isocaproate, caproate and heptanoate)) produced by human faecal microbiota from normal-weight and overweight volunteers after 6, 24 and 48 h of *in vitro* fermentation with different substrates: RAS, TDF, IDF, SDF and PEX.

| | RAS | | | TDF | | | IDF | | | SDF | | | PEX | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | 6 h | 24 h | 48 h | 6 h | 24 h | 48 h | 6 h | 24 h | 48 h | 6 h | 24 h | 48 h | 6 h | 24 h | 48 h |
| **Normal-weight** | | | | | | | | | | | | | | | |
| *Acetate* | 12.5±1.6$^{a*}$ | 15.9±5.3 | 14.5±5.5$^{a}$ | 4.6±0.9$^{b}$ | 9.4±2.6 | 9.3±4.4$^{ab}$ | 4.1±1.1$^{b}$ | 6.4±0.6 | 6.8±0.9$^{*b}$ | 4.1±1.7$^{b}$ | 6.2±1.7 | 10.0±4.2$^{ab}$ | 6.4±1.3$^{*b}$ | 9.0±6.3 | 13.3±2.4$^{ab}$ |
| *Propionate* | 3.1±0.7$^{*a}$ | 3.7±0.7$^{a}$ | 3.6±0.5 $^{a}$ | 1.0±0.4$^{b}$ | 1.8±1.2 $^{ab}$ | 1.9±1.1$^{b}$ | 1.0±0.4$^{b}$ | 1.4±0.5$^{b}$ | 1.6±0.7$^{b}$ | 0.9±0.5$^{b}$ | 1.9±1.2$^{ab}$ | 2.3±.1.0$^{b}$ | 1.3±0.1$^{*b}$ | 1.9±1.0$^{ab}$ | 2.7±0.6 $^{ab}$ |
| *Butyrate* | 1.7±0.2 | 2.5±0.4 | 2.6±1.0 | 1.5±0.6 | 1.9±0.5 | 2.1±0.6 | 1.4±0.1 | 1.7±0.5 | 1.9±0.6 | 1.3±0.3 | 1.6±0.7 | 2.3±0.2 | 1.4±0.5 | 2.1±1.1 | 2.8±0.2 |
| *Minor* | 0.3±0.1$^{*ab}$ | 2.3±0.7 | 2.1±.1.5 | 0.3±0.1$^{*ab}$ | 1.6±0.6 | 2.5±0.5 | 0.3±0.0$^{ab}$ | 1.9±1.1 | 2.4±1.0 | 0.9±0.4$^{a}$ | 2.1±0.5 | 3.0±2.2 | 0.2±0.0$^{*b}$ | 1.5±0.9 | 2.5±0.8 |
| **Overweight** | | | | | | | | | | | | | | | |
| *Acetate* | 9.9±5.5$^{ab}$ | 22.2±5.9$^{a}$ | 22.1±9.1$^{a}$ | 5.0±0.4$^{b}$ | 9.1±2.1$^{b}$ | 9.3±1.4$^{b}$ | 4.1±2.0$^{b}$ | 8.3±2.7 $^{b}$ | 9.0±0.1$^{*b}$ | 5.2±1.8$^{b}$ | 9.7±3.9$^{b}$ | 10.9±5.6$^{ab}$ | 15.0±2.6$^{*a}$ | 21.1±7.6$^{a}$ | 20.4±10.6$^{ab}$ |
| *Propionate* | 0.9±0.9* | 3.6±1.2 | 3.9±2.2 | 0.7±0.2 | 1.5±0.5 | 1.6±0.4 | 0.7±0.4 | 1.2±0.5 | 1.6±0.6 | 1.0±0.4 | 1.9±1.1 | 2.3±1.1 | 1.7±0.3* | 3.5±1.5 | 3.6±2.4 |
| *Butyrate* | 0.9±1.0$^{b}$ | 2.4±0.8 | 2.5±0.8 | 1.5±0.2$^{ab}$ | 1.8±0.3 | 1.9±0.4 | 1.0±0.4$^{ab}$ | 1.9±0.3 | 1.9±0.5 | 1.3±0.5$^{ab}$ | 1.9±0.5 | 2.3±1.4 | 2.3±0.2$^{a}$ | 3.0±0.7 | 2.9±0.6 |
| *Minor* | 0.8±0.3* | 1.8±0.3 | 1.9±0.3 | 1.1±0.2* | 1.9±0.2 | 2.4±0.7 | 0.8±0.4 | 1.5±0.4 | 2.5±1.1 | 0.9±0.4 | 2.1±0.5 | 3.0±2.2 | 1.0±0.5* | 2.1±0.5 | 2.6±0.8 |

*Values are expressed as mean ± SD (n =3). Different letters (a–c) indicate significant differences ($p$<0.05) among the samples at the same fermentation time. *Indicates significant differences ($p$<0.05) between normal-weight and overweight volunteers for the same SCFA at the same fermentation time. Raspberry (RAS); total dietary fibre (TDF); insoluble dietary fibre (IDF); soluble dietary fibre (SDF); (poly)phenol extract (PEX).

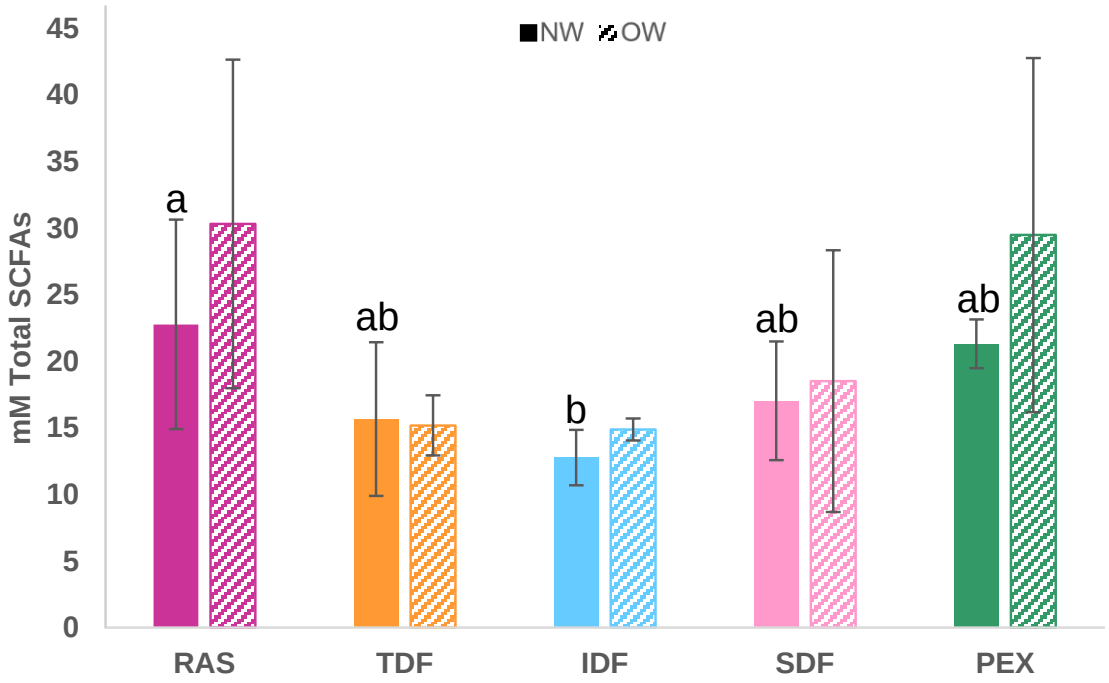


**Figure 16.** Total SCFAs production (mM) at 48 h of *in vitro* fermentation of raspberry (■RAS), total dietary fibre (■TDF), insoluble dietary fibre (■IDF), soluble dietary fibre (■SDF) and (poly)phenols extract (■PEX) for normal-weight subjects (■NW) and overweight subjects (■OW). Values are expressed as mean ± SD (n =3). Different letters (a–b) indicate significant differences ($p$<0.05) among the different samples for each NW and OW condition.

Regarding total SCFAs produced at 48 h (Figure 16), significant differences were only found between the different fractions for the *in vitro* fermentation carried out with faeces of NW volunteers, showing the lowest production the IDF fraction. On the contrary, the total SCFAs produced at 48 h after the fermentation of the samples with faeces from OW donors, did not show significant differences ought to the high variability (Figure 16). In general, it was observed that for both NW and OW volunteers, there was a higher production of SCFAs from RAS and PEX, being lower for the fibre fractions (TDF, IDF and SDF), which indicates a lower prebiotic effect compared with RAS and PEX fraction (Figure 16). It should be noted that there were no significant differences between NW and OW for the same fraction.

Finally, the highest proportion of acetate at 48 h (Figure 17) was found in RAS for both NW and OW subjects, being the lowest acetate proportion found in IDF and SDF for NW and OW, respectively. Moreover, the propionate was lowest in TDF and IDF for NW and OW. For butyrate and other minor SCFAs the same pattern was observed, showing IDF the highest values and RAS the lowest, for both types of inoculums.

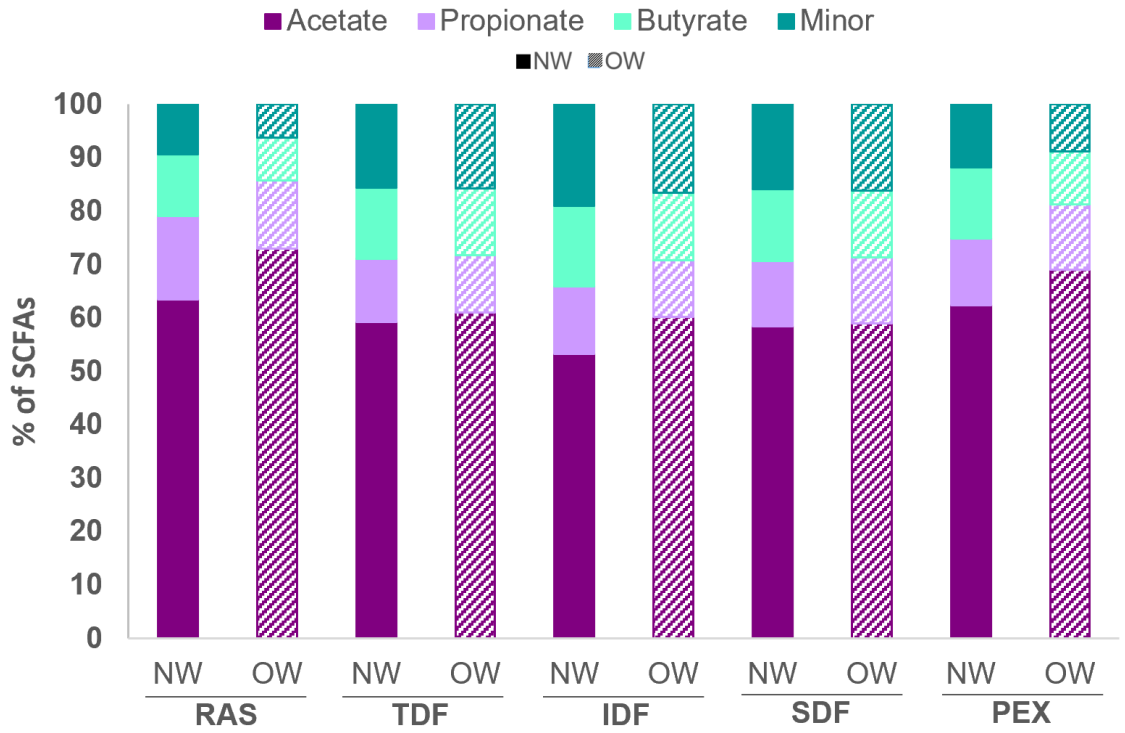


**Figure 17.** Proportion (%) of acetate (■), propionate (■), butyrate (■) and other minor SCFAs (■) (isobutyrate, isovalerate, valerate, isocaproate, caproate and heptanoate) after 48 h of *in vitro* fermentation of raspberry (RAS), total dietary fibre (TDF), insoluble dietary fibre (IDF), soluble dietary fibre (SDF), (poly)phenol extract (PEX) for normal-weight (■NW) and overweight (■OW). Values are expressed as mean (n =3).

### 3.6. Microbial (poly)phenol catabolites

As it has been reported previously (González-Barrio *et al.*, 2010; Kujawska and Jodynis-Liebert, 2020; Hao *et al.*, 2021), ellagic acid reaches the large intestine, where it can be degraded by the microbiota to urolithins. Our results showed that after 24 h of *in vitro* fermentation, the bulk of the ellagic acid had been degraded by the gut microbiota to urolithin A (3,8-Dihydroxydibenzo[b,d]pyran-6-one) (Figure 18). The urolithin production rate being highest from 0 to 24 h, reaching the highest accumulation after 48 h (Figure 18.b.). However, no significant differences were found in urolithin A production, neither among the different raspberry samples (RAS, TDF, IDF and PEX), within the same group, nor between the study groups NW and OW.

It should be noted that no urolithin production was observed for SDF as no ellagic acid was found in the fermentations, this may be due to this fraction was the one with the lowest amount of extractable ellagic acid, although there was ellagic in the non-extractable fraction, it is possible that due to the physicochemical properties of the SDF

it was not bioaccessible to the microbiota. However, the results showed a clear tendency (Figure 18), since the faecal incubations with RAS and PEX showed the highest ellagic acid degradation (Figure 18.a.), for both groups, which resulted in higher production of urolithin A in comparison with the fermentation of DF fractions (TDF and IDF). This behaviour could be related to the differences in the content of (poly)phenols of the substrates. So, RAS had the highest amounts of ellagic acid and ellagitannins, which were hydrolysed to ellagic acid that was then converted into urolithins, whereas TDF fraction showed the lowest ellagic acid degradation rate, and consequently the lowest urolithin production due to the lowest content of extractable ellagitannins and ellagic acid. Moreover, this fraction had the highest amount of NEPP, whose metabolisation by the microbiota may have been hampered since they are bound to cell walls. So, the high production of urolithins from RAS and PEX fractions appeared to be associated to the bioaccessibility of their precursors, because the EPP can be rapidly metabolised by the microbiota. It was also notable that the urolithin production (Figure 18.b.) was similar for RAS and PEX during the first 6 h, but after 24 h differences were apparent, might be due to the distinct compositions of the two fractions. PEX fraction only contained extractable ellagic acid, while ellagic acid in RAS was also present attached to the matrix and, therefore, it might be released from the cell walls and promote urolithin production over a longer period.

As far as we know, only Inada *et al*. (2019) analysed *in vivo* the production of urolithins after ingestion of a berry powder in NW and OW subjects, reporting similar results that are in agreement with our findings, without significant differences between the weight condition. However, a tendency was also observed related to the body weight condition, showing overweight volunteers a higher urolithin production than normal-weight volunteers. It is important to highlight that the faecal inoculum of NW produced more urolithins from the fibre fractions (TDF and IDF) than those of OW. By contrast, the fermentation with the faecal inoculum of OW produced more urolithins from the RAS and PEX fractions, probably due to their amount of EPP (Figure 18.b.). Hence, our results showed that when the bulk of the (poly)phenols were extractable, the microbiota of OW was able to produce a greater quantity of urolithins than the microbiota from NW. However, for the fractions with more NEPP (TDF and IDF), the microbiota from OW was less able to bio-transform them into urolithins.

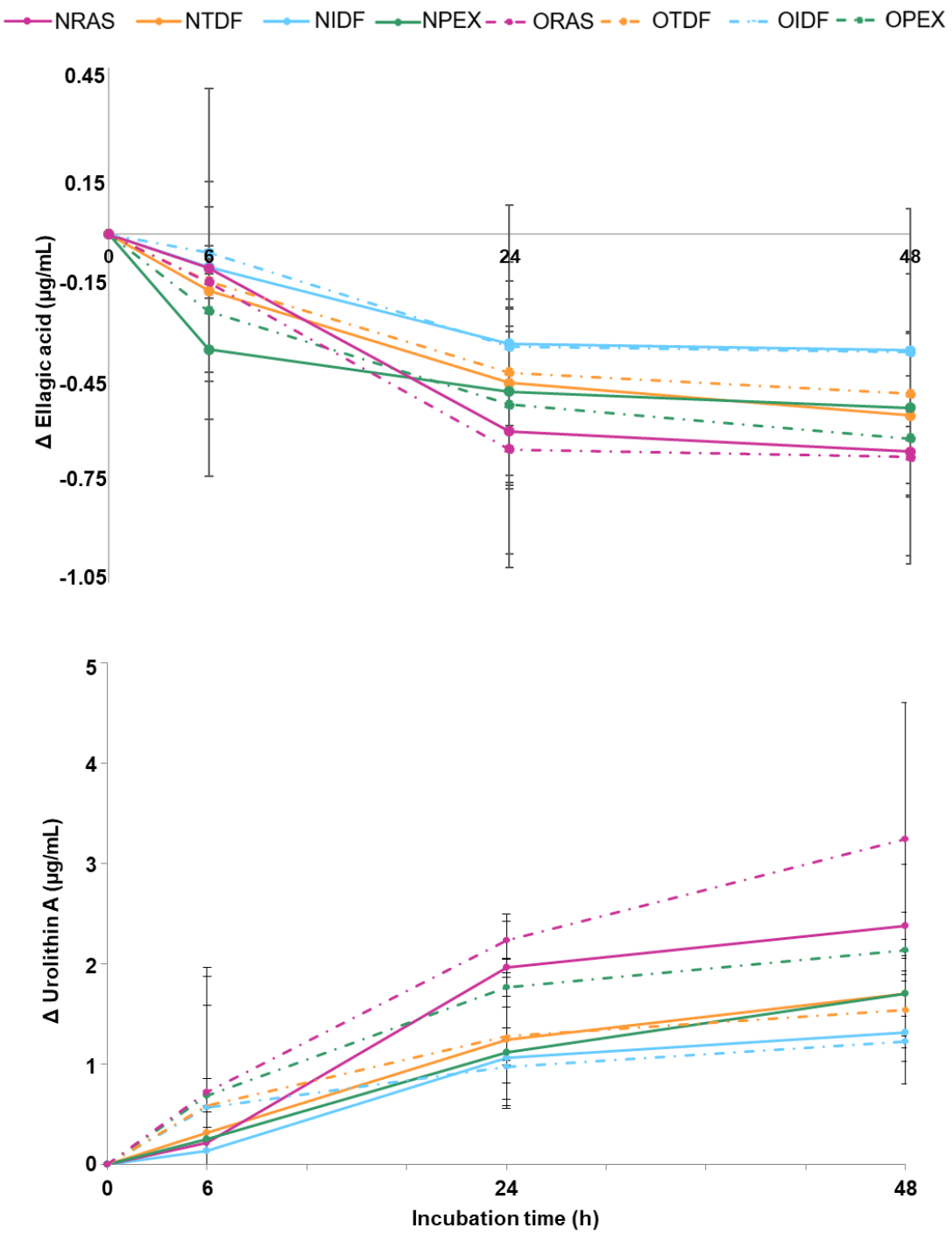


**Figure 18.** Decrease (Δ) in the content of ellagic acid and increase (Δ) in urolithin production (µg/mL) produced by the faecal microbiota of humans (normal-weight and overweight volunteers) after 6, 24 and 48 h of *in vitro* fermentation with different substrates: NRAS ( normal-weight, raspberry),NTDF (normal-weight, total dietary fibre), NIDF (normal-weight, insoluble dietary fibre), NPEX (normal-weight, (poly)phenol extract), ORAS (overweight, raspberry), OTDF (overweight, total dietary fibre), OIDF (overweight, insoluble dietary fibre), OPEX (overweight, (poly)phenol extract). Values are expressed as mean ± SD (n =3).

### 3.7. Prebiotic effect and microbial catabolites relationship

Multivariate statistical analysis was used to determine the relationship between the substrates (RAS, TDF, IDF and PEX) and the catabolites produced during fermentation, with the aim to ascertain the differences in the prebiotic effect according to the chemical composition of each fraction. SDF was not included in this analysis due to it showed no presence of ellagic acid and no production of urolithins. In this sense, a PCA was performed separately for NW and OW (Figure 19).

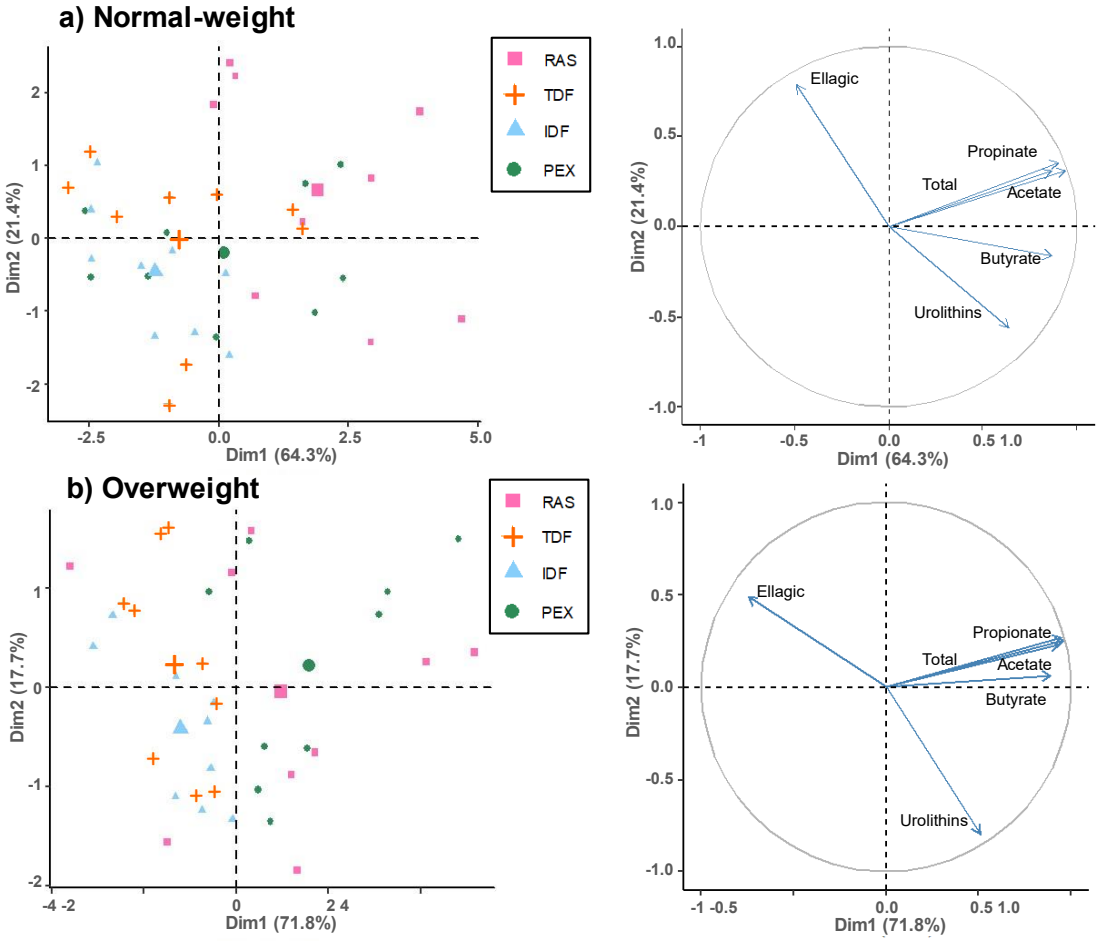


**Figure 19.** Principal component analysis (PCA) showing individual distribution (on the left) and variables distribution (on the right) for selected variables (Δ ellagic acid, urolithin A, acetate, propionate, butyrate and total SCFAs) in normal-weight volunteers (a) and overweight volunteers (b) after 48 h of *in vitro* fermentation. Different colours show different samples: raspberry (■RAS), total dietary fibre (+TDF), insoluble dietary fibre (▲IDF) and (poly)phenol extract (●PEX).

The representation of the samples is shown on the left side of the figure, and that of the variables on the right. The analysis identified six dimensions, the first two (Dim 1

and Dim 2) explained 85.7% of the total variance for NW and 89.5% for OW. The contents of individual acetate, propionate, butyrate, total SCFAs and urolithins were positively correlated with Dim 1, in both groups, representing this dimension the fermentation process, whereas ellagic acid was negatively correlated in this dimension. Dim 2 represented the formation of urolithins, being urolithins negatively correlated in this dimension for both groups and positively for ellagic acid. It is noteworthy that the raspberry samples were clearly separated but in a different way depending on the body weight condition, indicating the large symbol within each figure the centroid of the data groups. Figure 19.a. shows that RAS was the substrate that gave rise to the greatest formation of beneficial catabolites from the activity of the NW microbiota, followed by PEX with a clear difference among both samples. Contrastingly, in OW volunteers, PEX was the fraction that led to the greatest formation of these compounds, followed by RAS with a smaller difference between them compared to NW (Figure 19.b.). In addition, it is noteworthy that in the NW volunteers there was small difference between the fibre fractions and the PEX, showing that the prebiotic effect was almost equally from these fractions. In contrast, in the case of the OW there was a greater difference, with a higher prebiotic-like effect from (poly)phenols, which showed a lower ability of the microbiota from these volunteers to ferment the non-digestible fractions compared to PEX. These results indicate that body weight and the related condition of the microbiota could determine the beneficial effects of prebiotic functional food and functional ingredients.

To sum up, raspberry is a rich source of fibre, with a high proportion of SDF and high content of EPP compounds. The by-products of raspberries could be used to obtain a rich dietary fibre ingredient which have great physicochemical properties (WRC, FAC and GDRI) and a high amount of (poly)phenols link to the components of cell walls, which predominate over the extractable ones. It should be noted that the content of these bioactive compounds in the different fibre fractions depended on the type of fibre, since the insoluble fraction contained a high amount of (poly)phenols, giving it a high antioxidant capacity. The raspberry exhibits a prebiotic effect which is mainly due to its (poly)phenol content and not so much to its fibre content. Moreover, the (poly)phenol in the DF fractions also contribute to the activity of the gut microbiota. These results are interesting for the development of prebiotic functional ingredients, but the individual characteristics of the target groups must be considered, since the beneficial effect could be different depending on the body weight condition and thus on the catabolites formed in the colon. Further studies of the changes in the individual groups comprising the microbiota are required.

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# Chapter 5. By-products from berries

## 1. Introduction

The consumption of fruit and vegetables is part of the current models of a balanced diet for its vitamin, mineral and bioactive compounds content, for which it is important to take into account both the quantity and the variety ingested (Arroyo *et al*., 2018; Harvard Chan, 2022). An adequate consumption of these foods is associated with a healthier lifestyle and might exert beneficial effect to human health by reducing the risk of some diseases such as cancer, diabetes, neurodegenerative and cardiovascular diseases (Schulz and Chim, 2019). In this sense, berries, including strawberries, blackberries, raspberries and blueberries, which are the most widely consumed worldwide, are of particular importance due to their nutritional and beneficial properties for human health. They are rich in dietary fibre (DF), vitamins (A, C, E and B) and minerals (Ca, Na, P, Fe and K) (Giampieri *et al.*, 2018; Bilawal *et al.*, 2021), and also non-nutritive compounds with important health-promoting properties. These are (poly)phenols, including tannins, phenolic acids, stilbenes and other (poly)phenols. Several studies have attributed cardiovascular, antimicrobial, antioxidant, anti-inflammatory, anticancer, anti-ageing and neuroprotective properties to these compounds (De Pascual-Teresa *et al.*, 2010; Inada *et al.*, 2021; Mithul Aravind *et al.*, 2021).

According to their composition, not only due to the DF content but also due to the amounts and profile of (poly)phenol compounds, berries have an important prebiotic effect. The prebiotic effect refers to the specific changes in the composition and/or activity of the gastrointestinal microbiota, thus conferring health benefits to the host, caused by the fermentation of different compounds or ingredients (Gibson *et al.*, 2017). The DF from berries, with a high proportion of soluble compounds, reaches the colon, where is fermented by the intestinal microbiota. As result, short-chain fatty acids (SCFAs) are produced as bacterial metabolism end products, mainly acetate, propionate and butyrate. These compounds in turn act as a source of energy for bacteria, and may inhibit the development of pathogenic organisms, modulating intestinal immunity and reducing inflammatory responses (Akhtar *et al.*, 2021; Ma *et al.*, 2021).

In addition, modulation of the microbiota by (poly)phenols has been described in several studies (Lee *et al.*, 2006; Tzounis *et al.*, 2011), highlighting its prebiotic-like effect which has been summarised in several recent review reports (Alves-Santos *et al.*, 2020; Rodríguez-Daza *et al.*, 2021). In this sense, (poly)phenols have a double action, on the one hand, they have an antimicrobial effect by suppressing the growth of pathogens, and on the other hand they stimulate the growth of the microbiota, which has a positive effect

on host health through the reduction of proinflammatory response and metabolic syndrome among others, derived also from the production of (poly)phenol catabolites (Alves-Santos *et al.*, 2020; Mithul Aravind *et al.*, 2021; Rodríguez-Daza *et al.*, 2021). However, the mechanism of action of the microbiota on each individual (poly)phenol is quite specific. Urolithins, which are derived from the metabolism of ellagic acid and ellagitannins by the microbiota, are of particular importance. These compounds improve cellular health and increase mitochondrial function and mitophagy by reducing inflammation, and also protect against age-related diseases (D'Amico *et al.*, 2021).

Taking this into account, the by-products derived from berries have an interesting composition from a nutritional point of view, therefore their valorisation as potential ingredients for the food industry is interesting, as they are rich in fibre and also have bioactive compounds, which may allow the development of new formulations with health benefits for consumers.

Based on this background and considering the high content of DF and (poly)phenols in berries, the aim of this chapter was to extract different fibre-rich dietary fractions from a mix of different berries and characterise their chemical composition, including the bioactive compounds, as well as to evaluate their *in vitro* prebiotic effect.

## 2. Material and methods

### 2.1. Sample

As in the case of raspberries and because in the Region of Murcia surplus or by-products from berries were not available, a commercial mix of frozen berries, containing 25% of strawberries (*Fragaria ananassa*), 20% of blackberries (*Rubus ulmifolius*), 20% of blackcurrants (*Ribes nigrum*), 20% of redcurrants (*Ribes rubum*), 10% of raspberries (*Rubus idaeus*) and 5% of wild blueberries (*Vaccinium myrtillus*), was purchased in a local supermarket and was stored at -20 °C. Before defrosting, an amount of the berries mix was transfer to -80 °C charm and after 24 h was put into a freeze-dryer to obtain freeze-dried berries (FDB). Freeze-dried berries were crushed and stored at -20 °C until the analysis. The rest of the berries mix was defrosted in refrigerated charm at 5 °C to proceed with the DF extraction process (Figure 20).

### 2.2. Extraction process of dietary fibre-rich fractions

Two fractions, with a high content in DF, were obtained from the defrosted berries (Figure 20).

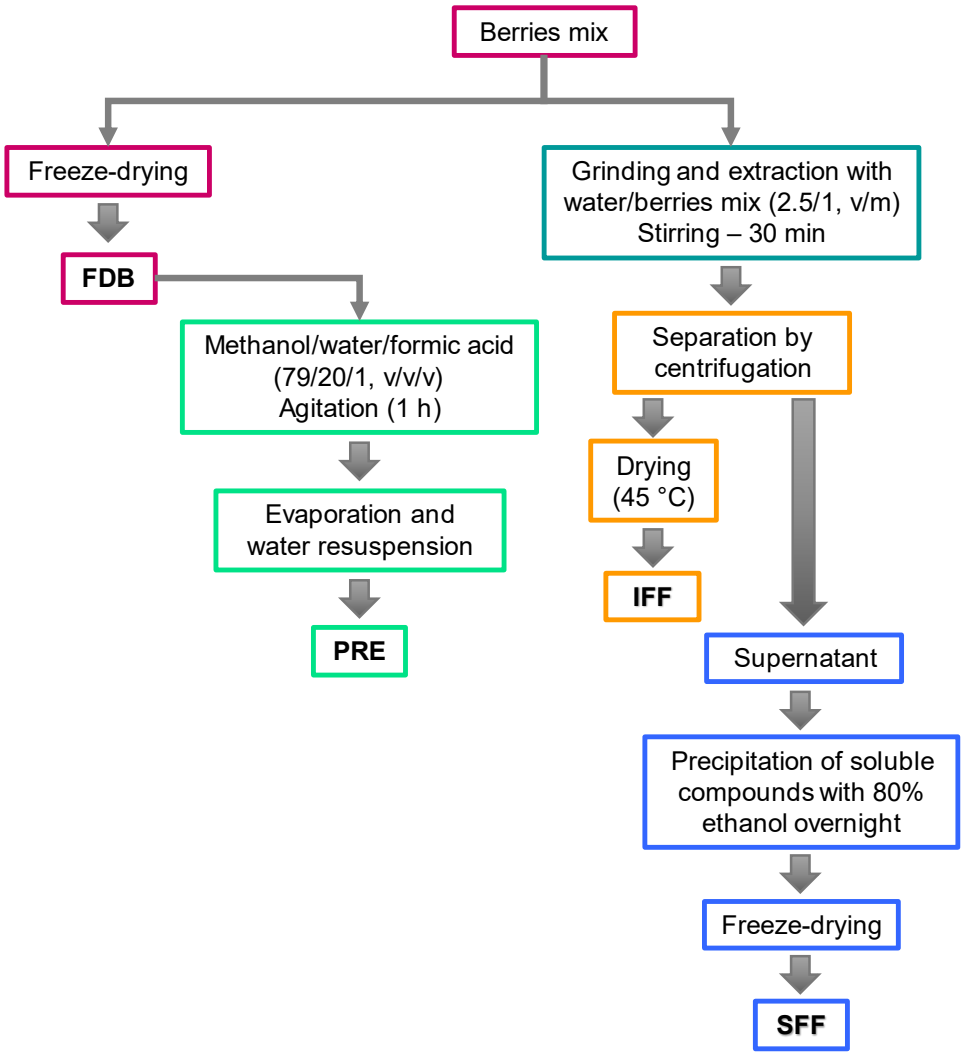


**Figure 20.** Flow diagram of the procedure used to obtain the different samples, freeze-dried berries (FDB), (poly)phenol extract (PRE), insoluble fibre-rich fraction (IFF) and soluble fibre-rich fraction (SFF) from the berry mixture.

First, the mix was grinded in a Thermomix TM-31 to homogenise the sample. Then, the mixture was dissolved in water, with a water/sample ratio of 2.5/1 (v/m) and stirred for 30 minutes. After that was centrifuged at 4500*g* for 5 minutes, to separate the insoluble water compounds, and the pellet was placed on a stove at 45 °C to constant weight, corresponding to the insoluble fibre-rich fraction (IFF). The supernatant obtained in the last extraction, containing soluble water compounds, was collected and mixed with

absolute ethanol (with a ratio of 5/1, ethanol/supernatant, v/v) and left overnight at room temperature for precipitation of the soluble fibre. The mixture was separated by decantation and the solid phase was freeze-dried, corresponding to soluble fibre-rich fraction (SFF). When the fibre samples reached constant weight, they were crushed separately and stored at -20 °C, until the different analysis were performed.

### 2.3. Extraction process of the (poly)phenol extract

An aqueous extract rich in (poly)phenols (PRE) was obtained from FDB, for which 0.5 g of the freeze-dried material was added to a solution of methanol/water/formic acid (79/20/1, v/v/v) and left in rotational agitation and darkness for 1 h, after which it was evaporated, resuspended in water and stored at -20 °C. The PRE was extracted to be used only for *in vitro* fermentation, to ascertain the prebiotic-like effect related to the (poly)phenols.

### 2.4. Physicochemical characterisation of the fibre fractions

The different fibre samples (FDB, IFF and SFF) were analysed for the proximate composition (moisture, protein, ash, total dietary fibre (TDF), insoluble dietary fibre (IDF) and soluble dietary fibre (SDF)); chemical composition of the dietary fibre in each fraction (neutral sugars and uronic acids); physicochemical properties (water retention capacity (WRC), swelling capacity (SWC), fat absorption capacity (FAC) and osmotic pressure). Moreover, the antioxidant capacity by FRAP and ORAC and the extractable (poly)phenols (EPP) and non-extractable (poly)phenols (NEPP) were analysed according to the methodology previously described in Chapter 4 (pages 73-80).

### 2.5. Total phenolic content

The extracts obtained from the EPP and the NEPP extractions described in Chapter 4 (pages 78-79) were used to determine the total phenolic content (TPC).

Total phenolic content were analysed using Folin-Ciocalteu's colorimetric assay as described by Singleton and Rossi (1965) with some modifications. For the colorimetric assay a microplate spectrophotometer (BioTek Instruments, Winooski, USA) was used. This method is based on the interaction of (poly)phenols at basic pH with the Folin-Ciocalteu reagent. The phosphomolybdic-tungstic acid present in the reagent with yellowish colouration is reduced by the phenolic groups giving rise to blue colour, which is measured at 765 nm. Gallic acid (Riedel-de Haën, Hannover, Germany) was used as

the standard, and the TPC in the samples was expressed as mg of gallic acid equivalents (GAE)/g of dry weight (d.w.).

### 2.6. *In vitro* gastrointestinal digestion and *in vitro* faecal fermentation of samples

Simulated gastrointestinal digestion was carried out following the *in vitro* method from INFOGEST protocol (Brodkorb *et al*., 2019). To start the process with the oral phase, 0.5 g of each FDB, IFF and SFF and 5 mL of PRE were mixed in individual containers with simulated salivary fluid, which includes α-amylase. The sample was stirred for 2 min at 37 °C, to simulate mastication. The gastric phase was then carried out using simulated gastric fluid, which includes pepsin, the sample was adjusted to pH 3 and was placed in a water bath with stirring for 2 h at 37 °C. The last step was the intestinal phase, in this case simulated intestinal fluid, which includes bile salts and pancreatin was used, the mixture was adjusted to pH 7 and then placed in a water bath with stirring for 2 h at 37 °C. Finally, to slow down the enzymatic activity, the samples were stored at -80 °C and then they were freeze-dried and kept on cold until the *in vitro* fermentation process was carried out (Brodkorb *et al.*, 2019).

Moreover, after the *in vitro* digestion, the prebiotic effect was evaluated by performing *in vitro* fermentations with the four samples extracted from berries (FDB, IFF, SFF and PRE) (Figure 21) in a proportion of 1% (0.5 g). In addition, a negative control (C) with no sample added were also analysed to test the prebiotic effect of the berry samples. The method used was the same as described in Chapter 4 (pages 81-82), with the exception that in this experiment a homogeneous group of 10 healthy normal-weight woman was selected (20-28 years old), to reduce individual variability, but only 9 women provided a sample on the day of the experiment. The inclusion criteria were to be non-smoker, with stable food habits, who did not present any symptoms of gastrointestinal disease, had not taken antibiotics for at least 4-6 months before the study, not to follow any dietary restrictions and not take any food supplements, prebiotics or probiotics. Moreover, the volunteers had to follow a pre-established diet for two days prior to the study, mainly this diet removed the main sources of fibre, (poly)phenols and glucosinolates that could influence the study. Written informed consent was obtained from the volunteers. The present study was conducted according to guidelines and procedures approved by Research of Ethics Commission (CEI code number: 2664/2019) and by the Experimentation Biosafety Committee (CBE code number: 282/2019) of the University of Murcia (Annex I).


**Figure 21.** Samples incubated in the *in vitro* fermentation. Freeze-dried berries (FDB); insoluble fibre-rich fraction (IFF); soluble fibre-rich fraction (SFF); (poly)phenol extract (PRE).

In this study pooled faecal samples were used because the high homogeneity allows for a better validation of the prebiotic effect of the ingredients (Aguirre *et al.*, 2014; Pérez-Burillo *et al.,* 2021). Previously to the preparation of faecal inoculum 12 g of fresh faeces from each volunteer were pooled and homogenised. As indicated before, the rest of the process was the same as described in Chapter 4 (pages 81-82). During fermentation process aliquots were collected at baseline (0 h), and after 4, 8, 24 and 48 h. Finally, SCFAs and microbial (poly)phenol catabolites were analysed by gas chromatography and liquid chromatography respectively, following the methods described in Chapter 4 (pages 82-84).

### 2.7. Ammonium analysis

The ammonium analysis was adapted from de determination of total volatile base nitrogen method (Sewwandi *et al.*, 2016). To determine the ammonium content generated during *in vitro* fermentation, 0.9 mL of samples were placed in a Kjeldahl tube and dissolved with 20 mL of distilled water and 1 mL of 20% NaOH solution obtaining basic conditions. The tube was placed in a Kjeltec 2100 distiller (Foss, USA) for 4 min and the ammonium was collected as ammonia in a flask containing 25 mL of 3% boric acid solution. Finally, the nitrogen content was quantified by neutralisation titration with 0.01 N HCl. The total content was calculated using Equation 2 and expressed as mg of ammonium/mL of fermented medium.

$$\boldsymbol{mg\ ammonium/mL = \left(\frac{V1\ x\ 0.01\ x\ 1.4}{V2}\right) x\ 1.2878}$$

**Equation 2.** Where, V1 was the volume (mL) of HCl used for the titration; 0.01 corresponded to HCl normality; V2 to the volume of the fermented medium (mL); 1.2878 to the nitrogen-ammonium conversion factor.

### 2.8. Statistical analysis

The statistical analysis was carried out using R studio, version 4.0.5 (R Foundation for Statistical Computing, Vienna, Austria). Normality was determined by the Shapiro-Wilk test. The homogeneity of variances was analysed using the Bartlett test. One-way analysis of variance (ANOVA) was performed to determine significant differences among different samples for the parameters analysed. A correlation analysis was also performed between physicochemical properties and fibre composition parameters, and among the TPC and antioxidant capacity, and between SCFAs and composition parameters. Tukey´s test was used as a post-hoc test. Differences were considered significant at a $p$-value<0.05. A principal component analysis (PCA) was also performed, to correlate the metabolic activity of the microbiota with the different samples inoculated.

## 3. Results and discussion

### 3.1. Nutritional composition

The nutritional composition of the three samples is shown in Table 10. As the samples were stored in powder form, the moisture content was very low being in all samples lower than 1%. For protein content, although there were no significant differences, the highest protein content was obtained for IFF. The FDB showed a lower content of protein than that obtained for different strawberry genotypes or that obtained by USDA database for strawberries, blackberries and blueberries, ranging from 7.4 to 11.8 g/100 g of d.w. (Hossain *et al*., 2016; USDA, 2022). Regarding carbohydrates content, representing the soluble fractions, FDB exhibited the high concentration as expected, since in the freeze-dried sample all the sugars found in the berries mix were contained, while for the fibre fractions most of these sugars were lost during the extraction process. It is noteworthy that in FDB the major component were carbohydrates, in contrast to the fibre fractions (IFF and SFF), where TDF was the major component.

For ash content, SFF showed the highest values compared with the other two samples (around 12% vs. 3%). FDB showed a similar content of ashes than those reported by USDA for the individual berries included in the mix (USDA, 2022). Whereas the high mineral content detected in SFF might be due to the fact that soluble polysaccharides, like pectin, can join mineral to their structure because of their cation

binding capacity, which depends on several factors such as fibre structure and others including pH or cation type (Celus *et al.*, 2018).

Regarding the fibre composition, the content for FDB was similar to that reported by USDA database for the individual compounds, and also similar to that reported for strawberries (Hossain *et al*., 2016; USDA, 2022). It is remarkable that the composition of TDF of the FDB showed approximately an 84% of IDF and a 16% of SDF (22.9% and 4.5%, respectively). Similar proportions have been found by other authors, reporting 22% of SDF for Goji berries and blackberries (Ilić *et al.*, 2020; Moraes *et al.*, 2020), and also the results showed for raspberry in Chapter 4, with 30% of SDF. However, in the dietary fibre-rich fractions the contents of TDF were significantly higher than in FDB, reaching a mean values of 65.1% and 47.4% in the IFF and SFF, obtaining two fractions with an increased content of TDF (1.7 and 2.4-fold than the FDB, respectively). According to the extraction process, both samples showed a different composition, with a high percentage of IDF (56.4%) in IFF, in comparison with SFF, which showed a high amount of SDF (44.8%). The amount of fibre and the IDF/SDF proportion found in IFF (6.5) were similar to those reported by other authors for different berries by-products, being 7.5, 4.4 and 10 for bilberry press cake, blueberry puree and cranberry fibre, respectively (Alba *et al.*, 2019). Hence, the percentage of IDF and SDF in both fractions were 87% vs. 13% for IFF and 5% vs. 95% for SFF.

**Table 10.** Nutritional composition of berry samples expressed in percentage or g/100 g of d.w.

| Parameters | FDB | IFF | SFF |
|---|---|---|---|
| **Moisture** | $0.3 \pm 0.0^{a*}$ | $0.2 \pm 0.0^{b}$ | $0.2 \pm 0.0^{b}$ |
| **Protein** | $1.7 \pm 0.4$ | $4.3 \pm 1.3$ | $1.6 \pm 0.8$ |
| **Carbohydrates**** | $67.5 \pm 0.4^{a}$ | $28.0 \pm 3.1^{c}$ | $39.2 \pm 1.2^{b}$ |
| **Ash** | $3.1 \pm 0.1^{b}$ | $2.6 \pm 0.1^{b}$ | $11.7 \pm 0.3^{a}$ |
| **Total dietary fibre (TDF)** | $27.4 \pm 0.2^{c}$ | $65.1 \pm 1.4^{a}$ | $47.4 \pm 2.3^{b}$ |
| **Insoluble dietary fibre (IDF)** | $22.9 \pm 0.5^{b}$ | $56.4 \pm 0.5^{a}$ | $2.6 \pm 0.6^{c}$ |
| **Soluble dietary fibre (SDF)** | $4.5 \pm 0.7^{b}$ | $8.7 \pm 0.9^{b}$ | $44.8 \pm 2.9^{a}$ |

*Values are expressed as mean ± SD (n =3). Different letters (a–c) indicate significant differences ($p$<0.05) among the samples. Freeze-dried berries (FDB); insoluble fibre-rich fraction (IFF); soluble fibre-rich fraction (SFF); d.w. (dry weight). **Carbohydrates were calculated by difference between the other components.

Taking into account the content of DF in the samples, the extraction procedure reported before was useful to separate soluble and insoluble fractions, in order to obtain two new fractions. However, due to the composition of fibre in the mix of berries (FDB) the performance was higher for the extraction IFF than SFF, with mean values of 67% and 11%, respectively.

### 3.2. Neutral sugars and uronic acids

In order to know the DF composition of the samples, DF was isolated and hydrolysed with $H_2SO_4$ to determine the content of neutral sugars and uronic acids, by GLC-FID and colorimetric method, respectively. The profile of neutral sugars and uronic acids is expressed as percentage, and the results are shown in Table 11. In a general view, significant differences were observed in the content of all neutral sugars (with the exception of galactose) and uronic acids, among the three samples. It is remarkable that fucose was not found in any of the samples, because it is commonly found in leafy vegetables and *Brassica* family group (Bala *et al.*, 2019). Glucose was the most abundant sugar in FDB, whereas the proportion of arabinose, xylose and mannose were significantly higher in IFF compared with the other two samples. However, SFF had the highest proportion of rhamnose and uronic acids, while FDB and IFF showed a greater similarity in their composition, without significant differences in the proportion of uronic acids.

**Table 11.** Proportion of neutral sugars and uronic acid in berry samples expressed in percentage (%).

| Sugars | FDB | IFF | SFF |
|---|---|---|---|
| **Rhamnose** | $0.9 \pm 0.1^{c*}$ | $1.2 \pm 0.2^{b}$ | $2.3 \pm 0.1^{a}$ |
| **Arabinose** | $7.3 \pm 0.5^{b}$ | $8.7 \pm 0.6^{a}$ | $5.8 \pm 0.2^{c}$ |
| **Xylose** | $17.4 \pm 0.7^{b}$ | $22.1 \pm 2.2^{a}$ | nd |
| **Mannose** | $13.0 \pm 2.6^{b}$ | $18.5 \pm 1.9^{a}$ | $1.3 \pm 0.2^{c}$ |
| **Galactose** | $7.3 \pm 2.7$ | $7.1 \pm 0.3$ | $5.3 \pm 0.2$ |
| **Glucose** | $16.1 \pm 0.7^{a}$ | $7.9 \pm 1.9^{b}$ | $6.0 \pm 0.6^{b}$ |
| **Uronic acid** | $38.1 \pm 2.4^{b}$ | $34.5 \pm 3.5^{b}$ | $79.4 \pm 0.4^{a}$ |

*Values are expressed as mean ± SD (n =4). Different letters (a–c) indicate significant differences ($p$<0.05) among the samples. Freeze-dried berries (FDB); insoluble fibre-rich fraction (IFF); soluble fibre-rich fraction (SFF); nd (not detected).

The SFF was the sample that exhibited more differences in its composition mainly due to the proportion of uronic acid content, which indicated that in this fraction more pectins were isolated during the extraction process. Our findings are in agreement with those reported by Ahmadi *et al*. (2021), who described a higher content of uronic acids with 47 and 65% for pectin extracted from Goji berry and raspberry, respectively. But in contrast to our results, the other two major sugars were rhamnose with 19% and 12% and galactose with 17% and 9%, respectively for Goji berry and raspberry pectin, these differences regarding our SFF may be due to the extraction process used to obtain the pectin fraction and the type of berries analysed. The results showed in Chapter 4 for SDF fraction from raspberry, although in higher amounts, showed the same profile than that described for SFF with uronic acids, glucose, arabinose and galactose as the main components. Moreover, the IDF fraction from raspberry showed higher content of xylose as it was also found in this chapter for IFF.

According to the neutral sugars and uronic acids, the proportion of cellulose, hemicellulose and pectin were calculated based on the method described by Umaña *et al*. (2016), as it is shown in Table 12.

**Table 12.** Proportion of cellulose, hemicellulose and pectin in berry samples expressed in percentage (%).

| Polysaccharides | FDB | IFF | SFF |
|---|---|---|---|
| **Cellulose[A]** | 14.5 ± 0.6[a]* | 7.1 ± 1.7[b] | - |
| **Hemicellulose[B]** | 32.0 ± 3.1[b] | 41.5 ± 1.5[a] | 7.4 ± 0.5[c] |
| **Pectin[C]** | 53.6 ± 3.6[b] | 51.5 ± 3.1[b] | 92.7 ± 0.5[a] |

*Values are expressed as mean ± SD (n =4). Different letters (a–c) indicate significant differences ($p$<0.05) among the samples. Freeze-dried berries (FDB); insoluble fibre-rich fraction (IFF); soluble fibre-rich fraction (SFF); - (not calculated). [A] Cellulose: Glucose x 0.9; [B] Hemicellulose: (Fucose + Xylose + Mannose + (Glucose x 0.1); [C] Pectin: (Rhamnose + Arabinose + Galactose + Uronic acids).

Cellulose and hemicellulose have been described as two of the main insoluble fibre components, as already mentioned in Chapter 4 (Dhingra *et al.*, 2012; Holscher, 2017). The highest content of cellulose (represented by glucose) was obtained for FDB with a mean value of 14%, while in the SFF sample, the glucose found was that which is part of soluble polysaccharides such as hemicellulose and pectin. Hemicellulose (represented by xylose, mannose and glucose) was found in the three samples, with the lowest proportion in SFF (7.4%) and the highest proportion in IFF (41.5%). It should be

noted that the sample with the highest content as the sum of cellulose and hemicellulose was the IFF, but not much different from FDB. These results are in concordance with those reported before, which showed that FDB and IFF have a similar proportion of insoluble fibre. The contribution of mannose to the hemicellulose structure was calculated based on Houben *et al*. (2011) (Table 13), to obtain a general view about the solubility properties of the hemicellulose. The mannose contribution of FDB and IFF did not show significant differences. Furthermore, this ratio could not be calculated for SFF, because no xylose was detected in this sample, which indicates that the hemicelluloses present in this fraction are mostly soluble due to their predominant mannose content as have been previously reported (Peng *et al*., 2019).

On the other hand, the highest pectin content (determined from uronic acids, rhamnose, arabinose and galactose) was observed for SFF, being 1.7-fold compared to the other two samples, which did not show significant differences between them. These results could be explained based on the different extraction method used, in which the soluble polysaccharides were precipitated with ethanol, allowing the isolation of the pectin content of berries. As has been mentioned above the SFF contained a 95% of soluble compounds of DF, mainly represented by uronic acids and pectins.

Finally, using also the results from neutral sugar analysis, pectin structure ratios were calculated based on the previous work published by Houben *et al*. (2011), in order to know the pectin structure (Table 13).

**Table 13.** Sugar ratios for pectin and hemicellulose characterisation from berry samples.

| Ratio | FDB | IFF | SFF |
|---|---|---|---|
| ***Man* contribution[A]** | 0.75 ± 0.14* | 0.85 ± 0.16 | - |
| **Linearity[B]** | 1.17 ± 0.15$^{b}$ | 0.89 ± 0.14$^{c}$ | 6.00 ± 0.17$^{a}$ |
| ***Rha-Uro* contribution[C]** | 0.02 ± 0.00 | 0.04 ± 0.01 | 0.03 ± 0.00 |
| **RG-I branching[D]** | 16.83 ± 3.45$^{a}$ | 13.00 ± 2.00$^{b}$ | 4.86 ± 0.10$^{c}$ |

*Values are expressed as mean ± SD (n =4). Different letters (a–c) indicate significant differences ($p$<0.05) among the samples. Freeze-dried berries (FDB); insoluble fibre-rich fraction (IFF); soluble fibre-rich fraction (SFF); - (not calculated). [A] contribution of mannans to hemicelluloses: Mannose/Xylose; [B] Linearity of pectins: Uronic acids/(Fucose + Rhamnose + Arabinose + Galactose + Xylose); [C] Contribution of rhamnose and uronic acids to pectins: Rhamnose/Uronic acids; [D] Branching of RG-I: (Arabinose + Galactose)/Rhamnose.

Rhamnose contribution to the uronic acids gives a general idea about the ramification and lateral chains of the pectin. Regarding the rhamnose contribution no significant differences were observed between samples. Because although the extraction process was different, the ramification structure of the original pectin was not altered and therefore neither the rhamnose-uronic acid ratio (Mohnen, 2008). Moreover, the results showed that SFF pectin molecules seemed to be the linear and longer ones, as they have the lowest degree of branching and the highest content of uronic acids, which could provide some interesting functional and physicochemical properties. Because, due to their flexible structure they interact less with each other resulting in less rigid structures that allow for example a better stabilisation of emulsions (Shafie *et al.*, 2020; Mendez *et al.*, 2021). Other parameter to characterise pectin structure in the isolated samples was the length of the side chains, mainly formed by arabinose and galactose units. The length of the side chains was highest in FDB, followed by IFF and SFF, indicating that the pectins present in FDB may have stronger molecular interactions, leading to more consistent structures, being this characteristic interesting at an industrial level (Shafie *et al.*, 2020).

### 3.3. Physicochemical properties

Table 14 shows the mean values for the WRC, FAC, SWC and osmotic pressure for the three samples. Physicochemical properties are important, not only because they provide information on potential physiological effects, but also because they provide information on how the samples will behave technologically when they are used as ingredient in the food industry (Song *et al*., 2019; Liu *et al*., 2021). Both WRC and SWC refer to the hydration properties and the capacity of the samples to hold water within its fibrous structure (Song *et al.*, 2019). The SFF showed a highest WRC than the other two samples, with a mean value of 4.8 g of water/g, and it also exhibited a highest SWC, being 1.5 and 1.8-fold higher than FDB and IFF respectively. The data of WRC were similar to that reported by pectin extracted from strawberry, blackberry and strawberry with mean values of 2.5, 4.2 and 2.4 g of water/g (Muñoz-Almagro *et al.*, 2021). Moreover, SWC results were similar to those obtained by other authors for coconut cake DF (Zheng and Li, 2018), ranging from 8.5-13.3 mL of water/g, for apple fibre powder with mean values of 7.1 mL of water/g (Lian *et al.*, 2015) and different berry pomace powders ranging between 5.5 and 7.1 mL of water/g (Reißner *et al.*, 2019).

As other authors have previously shown, a high amount of SDF and pectin increases the capacity to retain water within the fibrous structure (Zheng and Li, 2018; Li

*et al*., 2022), being these both parameters positively correlated with the hydration properties (WRC and SWC) in our study. The results for FDB and IFF were similar for both parameters, since the percentage of SDF and pectin in both samples did not show differences. These results showed that SFF fraction will have a greater capacity to increase stool volume and reduce the transit time of faeces in the large intestine, decreasing the risk of intestinal diseases (McRorie and McKeown, 2017). In addition, in the technological processes it will reduce water loss and dehydration processes, as well as improving the texture and viscosity of the products (Song *et al*., 2019; Liu *et al*., 2021).

**Table 14.** Physicochemical properties of berry samples.

| Physicochemical properties | FDB | IFF | SFF |
|---|---|---|---|
| **Water retention capacity** (g of water/g) | $1.7 \pm 0.0^{b*}$ | $1.5 \pm 0.1^{b}$ | $4.8 \pm 0.0^{a}$ |
| **Swelling capacity** (mL of water/g) | $8.9 \pm 0.9^{b}$ | $7.2 \pm 0.5^{c}$ | $12.9 \pm 1.0^{a}$ |
| **Fat absorption capacity** (g of oil/g) | $1.7 \pm 0.3$ | $1.8 \pm 0.1$ | $2.0 \pm 0.1$ |
| **Osmotic pressure** (mmol/kg) | $195.3 \pm 5.9^{a}$ | $156.3 \pm 4.0^{b}$ | $164.3 \pm 0.6^{b}$ |

*Values are expressed as mean ± SD (n =3). Different letters (a–c) indicate significant differences ($p$<0.05) among the samples. Freeze-dried berries (FDB); insoluble fibre-rich fraction (IFF); soluble fibre-rich fraction (SFF).

In terms of FAC, no differences were observed between the different samples, showing a mean value around 2 g oil/g of sample. These results were similar to that reported for blackcurrant, redcurrant, gooseberry, rowanberry and chokeberry pomaces with values ranging between 1.9 and 2.3 g of oil/g of d.w. (Reißner *et al.*, 2019). Moreover, these values were also similar to that reported for mango, orange, pomegranate and lemon DF powder with values of 1.6, 1.6, 1.4 and 1.3 g of oil/g, respectively.

Regarding the osmotic pressure, although there were differences between the samples, being the osmotic pressure in FDB sample higher than the other two samples with a value of 195.3 mmol/kg, it is noteworthy that any of them are expected to induce diarrhoea after consumption, because they are under the physiological values (290 mmol/kg) (Lawrence and Joseph., 2015).

### 3.4. (Poly)phenols and antioxidant capacity

The (poly)phenol content in FDB and in both fibre fractions (IFF and SFF) was identified and quantified by HPLC-DAD. The identification was performed in both extractable (EPP) and non-extractable (NEPP) fractions and the results are showed in Figure 22. It is known that berry pomaces are rich in (poly)phenols, but the total amount and profile depend on the type of berry. Different authors have reported the following mean values: 0.25 g/100 g of d.w. in saskatoon berry (De Souza *et al.*, 2019), 10.5 g/100 g of d.w. for chokeberry, 1 g/100 g of d.w. for black chokeberry and 4 g/100 g of d.w. for strawberry (Struck *et al.*, 2016). Taking this into account, when we obtain a fibre-rich fractions from the pomace, part of the (poly)phenols remain in the extracted fraction, because they are bounded to the fibre components.

In EPP, solubilised in 80% acidified methanol, the major compounds were anthocyanins, ranging from 148 mg/100 g in SFF to 403 mg/100 g in FDB (Figure 22.a.), being higher than that reported for berries press residues by Tumbas Šaponjac *et al.* (2014), who reported a mean value ranging from 52 to 182 mg/100 g of d.w. The second largest were ellagic acid and ellagitannins. The IFF showed the highest content of extractable ellagic acid (27 mg/100 g), and the SFF showed the lowest amount of the three compounds analysed (Figure 22.a.). FDB showed the highest content of EPP since it is constituted by the whole fruits, and during DF extraction process, (poly)phenols were partially removed, mainly in the isolation of SFF after precipitation with 80% ethanol. For the NEPP, only ellagic acid and hydroxycinnamic acids were identified, the first one being the majority for all samples (Figure 22.b.). In addition, IFF was the one with the highest content of both compounds, as well as the total, followed by SFF and showing FDB the lowest content. This is because these two fibre-rich fractions have (poly)phenols bound to the fibre molecules, which makes these samples more interesting regarding its composition and its health related properties, as other authors have previously reported (Peng *et al*., 2021).

The compounds in the FDB sample were mostly EPP, representing around 67% of the total content, being anthocyanins the majority group. In contrast, in the fibre-rich fractions the NEPP, represented 57% and 73% from the total, for IFF and SFF respectively, being ellagic acid the predominant between the identified compounds. Tomas *et al*. (2020) showed that adding pectin, as a soluble fibre, to a blackberry puree significantly increased the content of NEPP, and consequently reduced the extractable ones, because these compounds were complexed with the fibre molecules, showing this fibre the same effect as was observed in our study. In this sense, it should be noted that

the total content, as the sum of EPP and NEPP, was higher in IFF being 1.4 and 1.5-fold compared to FDB and SFF, respectively. These results agree with those obtained by Reynoso-Camacho *et al*. (2021) for a by-product of strawberry and blueberry decoction process, which showed a higher content of non-extractable compounds, whereas for raspberries and blackberries by-products the majority were extractables, this could be due to the content of fibre present in these by-products, which was not reported in the publication.

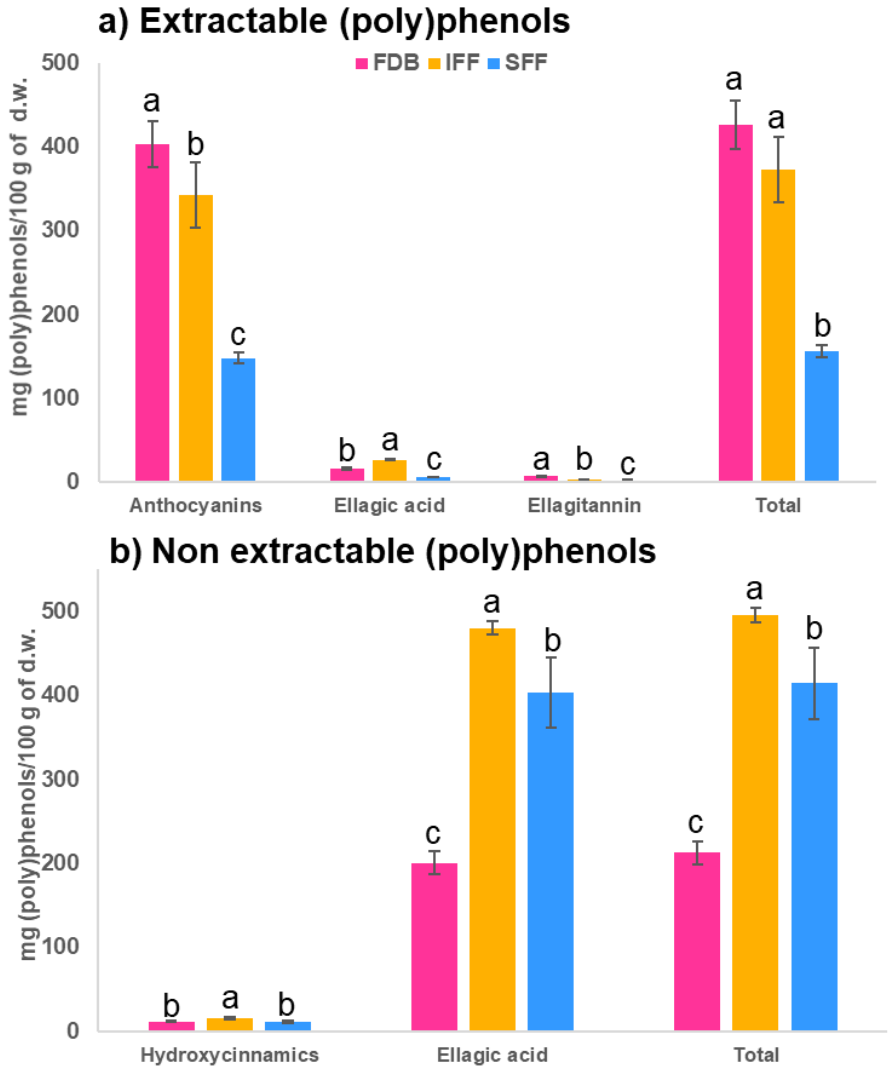


**Figure 22.** Contents of individual (poly)phenols (anthocyanins, ellagic acid, ellagitannins and hydroxycinnamics) and the total contents of (poly)phenols determined as the sum of the individual compounds (mg/100 g of d.w.), analysed by HPLC-DAD in berry samples for freeze-dried berries (■FDB), insoluble fibre-rich fraction (■IFF) and soluble fibre-rich fraction (■SFF). Values are expressed as mean ± SD (n = 3). Different letters (a–c) indicate significant differences ($p$<0.05) among the samples for the individual and total (poly)phenols.

Table 15 shows the total phenolic content (TPC, Folin-Ciocalteu method) and antioxidant capacity (FRAP and ORAC) for the different extracted fractions (EPP and NEPP), as well as the total values calculated as the sum of both fractions.

**Table 15.** Total phenolic content (TPC) (mg GAE/g of d.w.), and antioxidant capacity by FRAP and ORAC (µmol TE/g of d.w.) of berry samples from extractable (EPP) and non-extractable (NEPP) fraction.

| | FDB | IFF | SFF |
|---|---|---|---|
| ***Extractable fraction (EPP)*** | | | |
| TPC | 12.2 ± 1.6$^{a}$* | 11.1 ± 0.6$^{a}$ | 4.3 ± 0.2$^{b}$ |
| FRAP | 19.3 ± 2.7$^{b}$ | 25.3 ± 1.9$^{a}$ | 12.3 ± 5.3$^{b}$ |
| ORAC | 128.0 ± 3.0$^{a}$ | 128.4 ± 2.9$^{a}$ | 60.7 ± 1.3$^{b}$ |
| ***Non-extractable fraction (NEPP)*** | | | |
| TPC | 8.5 ± 0.4$^{b}$ | 13.7 ± 1.5$^{a}$ | 9.4 ± 1.2$^{b}$ |
| FRAP | 16.5 ± 1.9$^{b}$ | 28.4 ± 7.1$^{a}$ | 15.2 ± 2.9$^{b}$ |
| ORAC | 107.6 ± 2.3$^{b}$ | 121.4 ± 6.7$^{a}$ | 100.5 ± 11.6$^{b}$ |
| ***Total*** | | | |
| TPC | 20.7 ± 1.3$^{b}$ | 24.7 ± 1.1$^{a}$ | 13.7 ± 1.2$^{c}$ |
| FRAP | 35.8 ± 0.8$^{b}$ | 53.6 ± 7.7$^{a}$ | 27.6 ± 7.8$^{b}$ |
| ORAC | 235.6 ± 3.7$^{b}$ | 249.8 ± 6.2$^{a}$ | 161.1 ± 11.0$^{c}$ |

*Values are expressed as mean ± SD (n =3). Different letters (a–c) indicate significant differences ($p$<0.05) among the samples. Ferric reducing antioxidant power (FRAP); oxygen radical absorbance capacity (ORAC); freeze-dried berries (FDB); insoluble fibre-rich fraction (IFF); soluble fibre-rich fraction (SFF).

Although the Folin-Ciocalteu method overestimates the content of (poly)phenols in comparison with those obtained by HPLC. It should be noted that these results are consistent with those obtained by HPLC-DAD, since a higher content of EPP was observed in the samples FDB and IFF, while for NEPP the IFF fraction showed the highest content. Even though, the content of NEPP for SFF was higher than for FDB there was no significant differences, in contrast to the HPLC analysis. Furthermore, the TPC was higher in IFF than in the other samples, showing SFF fraction the lowest content. The results obtained for EPP in FDB were lower than those obtained by Schulz *et al*. (2019) for blackberry samples (19.2 mg GAE/g of d.w.), and those obtained for blackberry puree (24.5 mg GAE/g of d.w.) by Tomas *et al*. (2020). On the other hand, for

NEPP in FDB we obtained higher values than that have been reported by Huang *et al*. (2022) for strawberry samples (3 mg GAE/g of d.w.). Moreover, Basanta *et al*. (2014) reported a total content of (poly)phenols ranging from 7.2 to 10 mg/g for cherry fibres, representing the bounded (poly)phenols more than 60% of the total. In our study the mean contents of EPP and NEPP were 11.1 and 4.3 mg/g and 13.7 and 9.4 for IFF and SFF, respectively. Representing NEPP 56% and 69% from the total content for IFF and SFF, respectively.

Related to the antioxidant capacity, the values showed that the EPP and NEPP fractions of IFF exhibited the highest FRAP and ORAC activities. However, the EPP from FDB showed the same scavenging activity (128 µmol TE/g) than IFF. Hence, IFF showed a higher antioxidant capacity compared to SFF, similar to those reported by Xu *et al*. (2020) for insoluble fibre of lychee pulp, due to a high concentration of bound (poly)phenols. In addition, the FRAP activity obtained for IFF were similar to the data reported by Azman *et al*. (2022) for blackcurrant pomace (58 µmol TE/g). The results were compared with those obtained by other authors for fruit by-products showing similar values, being 38 µmol TE/g for apple pomace, 52 µmol TE/g for orange peel and 29 µmol TE/g for blueberry peel (Hernández-Carranza *et al.*, 2016; Struck *et al.*, 2016). Furthermore, correlation analysis was performed to evaluate the relationship between the antioxidant capacity results with the TPC. The total content, calculated as the sum of EPP and NEPP, showed a positive correlation between both antioxidant capacity methods with a correlation coefficient for FRAP of r=0.81 ($p$<0.01) and for ORAC of r=0.97 ($p$<0.001). These results suggest that (poly)phenols are one of the main compounds that confer antioxidant capacity to the samples.

### 3.5. Microbial (poly)phenol catabolites

The changes in the amount of anthocyanins and ellagic acids and the production of (poly)phenol catabolites during *in vitro* fermentation, analysed by HPLC-DAD is shown in Figure 23. As mentioned above the *in vitro* fermentation was carried out with the three samples, FDB, IFF and SFF fractions, and also with a (poly)phenol rich extract (PRE), containing the EPP. Hence, the mean content of (poly)phenols in PRE fraction were: 40.4 mg of anthocyanins /100 mL, 2 mg of ellagic acid /100 mL and 1 mg of ellagitannins /100 mL. The PRE was included in the *in vitro* fermentation assay to compare the activity of the microbiota on (poly)phenol extract, without the presence of non-starch polysaccharides from DF.

During the *in vitro* fermentation, the content of anthocyanins and ellagic acid decreased in all samples due to the metabolism of the microbita whereas the catabolites were generated. However, only urolithin A was detected as a compound derived from ellagic acid metabolism, being both identified and quantified by its spectral characteristics previously reported by González-Barrio, *et al*. (2011) (Figure 23).

Regarding anthocyanins degradation (Figure 23), it is noteworthy that at the beginning of the fermentation, the amount found in the FDB and PRE fractions was approximately two times higher than that found in IFF and SFF, due to the content of EPP. In terms of degradation, it should be noted that the anthocyanins disappeared completely after 24 h of fermentation in all samples, which indicates that they may have been metabolised by the microbiota. Although the anthocyanins catabolites have not been measured in this study, the metabolisation of these compounds by the microbiota have been associated with the production of different phenolic acids due to the etherocyclic breakage of the C ring that constitutes the structure of its molecules (González-Barrio *et al.*, 2011; Tian *et al.*, 2019; Bresciani *et al.*, 2021). In this regard, it should be noted that the degradation curves follow more or less the same trend for all samples, except for SFF where a slower degradation was observed, probably due to a lowest content of these compounds in this fraction.

According to the chemical analysis, ellagic was observed in highest quantities in fermentation sample of IFF and FDB (Figure 23). As for the degradation of ellagic acid, it should be noted that in both FDB and PRE there was complete degradation after 8 and 4 h of incubation, respectively. In contrast, the content of the ellagic acid from IFF and SFF fractions was not completely degraded, with 95% degradation at the end of the *in vitro* fermentation for IFF and 53% for SFF. This effect is ought to the differences in the (poly)phenol composition of the samples, which could explain that the final content of ellagic acid that reaches the human intestine depends on the presence of other components in the food matrix, and depend on how the ellagic acid is bound to them, allowing in more or less extension its fermentation by microbiota (Jakobek and Matić, 2019; Liu *et al*., 2020).

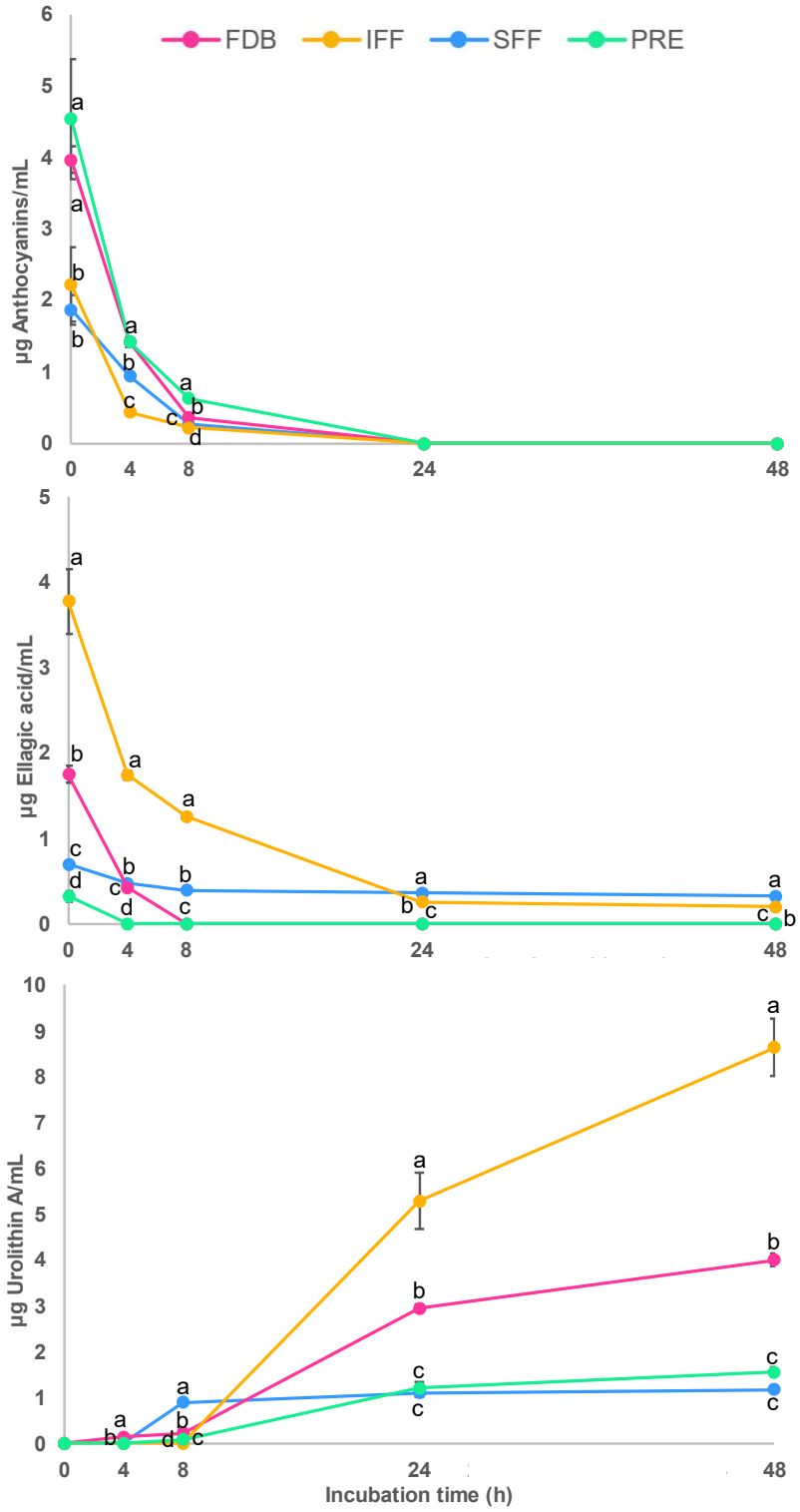


**Figure 23.** Decrease in the content of anthocyanins and ellagic acid and production of urolithin (µg/mL) by the faecal microbiota of volunteers at 0, 4, 8, 24 and 48 h of *in vitro* fermentation with different samples from de berries mix, freeze-dried berries fraction (●FDB), insoluble fibre-rich fraction (●IFF), soluble fibre-rich fraction (●SFF) and (poly)phenol extract (●PRE). Values are expressed as mean ± SD (n =3). Different letters (a–d) indicate significant differences ($p$<0.05) among the samples at the same incubation time.

At the beginning of the fermentation of PRE fraction, containg only EPP, which were extracted from 0.5 g of FDB, the ellagic acid should be at a similar concentration that in the whole berries sample. However, the content was relatively low compared to FDB. This differences could be explained because these compounds could have been lost during the previous gastrointestinal *in vitro* digestion process, as other authors have previously reported for an extrac rich in (poly)phenols (Dacrema *et al.*, 2020). In addition, due to the content of non-extractable ellagic acid in DBS, it may be that a release effect occurs during the *in vitro* digestion process, and therefore a higher amount of (poly)phenols were found in FDB at the beginning of fermentation compared to PRE.

Regarding to the production of urolithin A (Figure 23), the highest content was observed after the fermentation of the IFF during 48 h, followed by FDB, showing significant differences. However, the SFF and PRE did not show significant differences among them. Both IFF and FDB showed a high content of ellagic acid, and hence was used by the microbiota leading to a high formation of its catabolite. Although, the (poly)phenol profile of SFF showed a significant amount of ellagic acid, it was degraded towards urolithin A in a significantly lower percentage than the other samples. This could be explained because the fibre compounds might interfer the bioaccessibility of the microbiota to the ellagic acid (Palafox-Carlos *et al.*, 2011). This finding can be support by the fact that the fermentation of PRE, containing lower amount of ellagic acid alone in absence of fibre, led to a similar formation of urolithins than SFF, because in PRE ellagic acid were more bioaccessible since this fraction was only represented by EPP. Other authors also showed that after the fermentation of cyanidin-3-O-glucoside, alone and in presence of other soluble fibres, protocatechuic acid was produced in lowest amounts when fibres are present (Yang *et al.*, 2021). Although, the production of this catabolites depend on the type of fibre, being in agreement with the results reported in our study.

It have been recently reported that there are three phenotypes according to the capacity of the microbiota to produce urolithins. In this study, although we used a pooled faecal samples from female volunteers, the phenotype A was predominant, since resulted only in the production of urolithin A. As have been reported by Tomás-Barberán *et al*. (2014), the phenotype A is present in a wide range of the volunteers (25-80%) and depends mainly on the composition of the gut microbiota, however the profile of microbiota has not been investigated in our study, and further study will carry out with these samples to ascertain the changes in the microbiota.

### 3.6. Evaluation of the potential prebiotic effect and ammonium production

Finally, to evaluate the prebiotic effect of FBD, IFF, SFF and PRE samples, the SCFAs were analysed at different times along the fermentation assay. In addition, a negative control was also performed, where no sample was added, showing more or less a constant behaviour during the *in vitro* fermentation, except for the content of other minor SCFAs.

Figures 5 and 6 show that as has been described in the scientific literature, acetate was the most abundant SCFA (51-71%), followed by propionate (15-27%) and butyrate (8-21%). The proportions found for the main SCFAs were similar to those obtained by Cui *et al*. (2020) after *in vitro* fermentation of orange pectin, that reported a ratio of 67%-77% for acetate, 17%-26% for propionate and 6%-11% for butyrate. Moreover, the content of other minor SCFAs was also analysed, and the concentration of isobutyrate and isovalerate stood out, although valerate, isocaproate, caproate and heptanoate were also found but in smaller concentrations. The total content of other minor SCFAs, is depicted as the sum of all of them.

It is noteworthy that SFF was the one that showed the highest production of acetate (Figure 24), ranging from 8.2 to 84.7 mM, which was mainly due to the content of soluble fibres and mainly pectin (Table 10 and 12), being both pectin content and acetate production positively correlated ($r=0.84$, $p<0.01$). Other authors have also reported that the production of acetate is mainly due to the presence of galacturonic acid and consequently to the pectin content (Tian *et al.*, 2016; Zhao *et al.*, 2021). Moreover, PRE and IFF fractions were the second ones with the highest production, ranging from 8.9 mM to 74.4 mM, and from 6.4 mM from 70.4 mM, respectively. It is remarkable that the PRE, which is a source of EPP, increased the production of acetate as have been previously reported in an *in vitro* fermentation of (poly)phenol extract from Chilean currants (Burgos-Edwards *et al.*, 2020). Finally, FDB was the sample with the less production of acetate ranging from 9.8 to 61 mM.

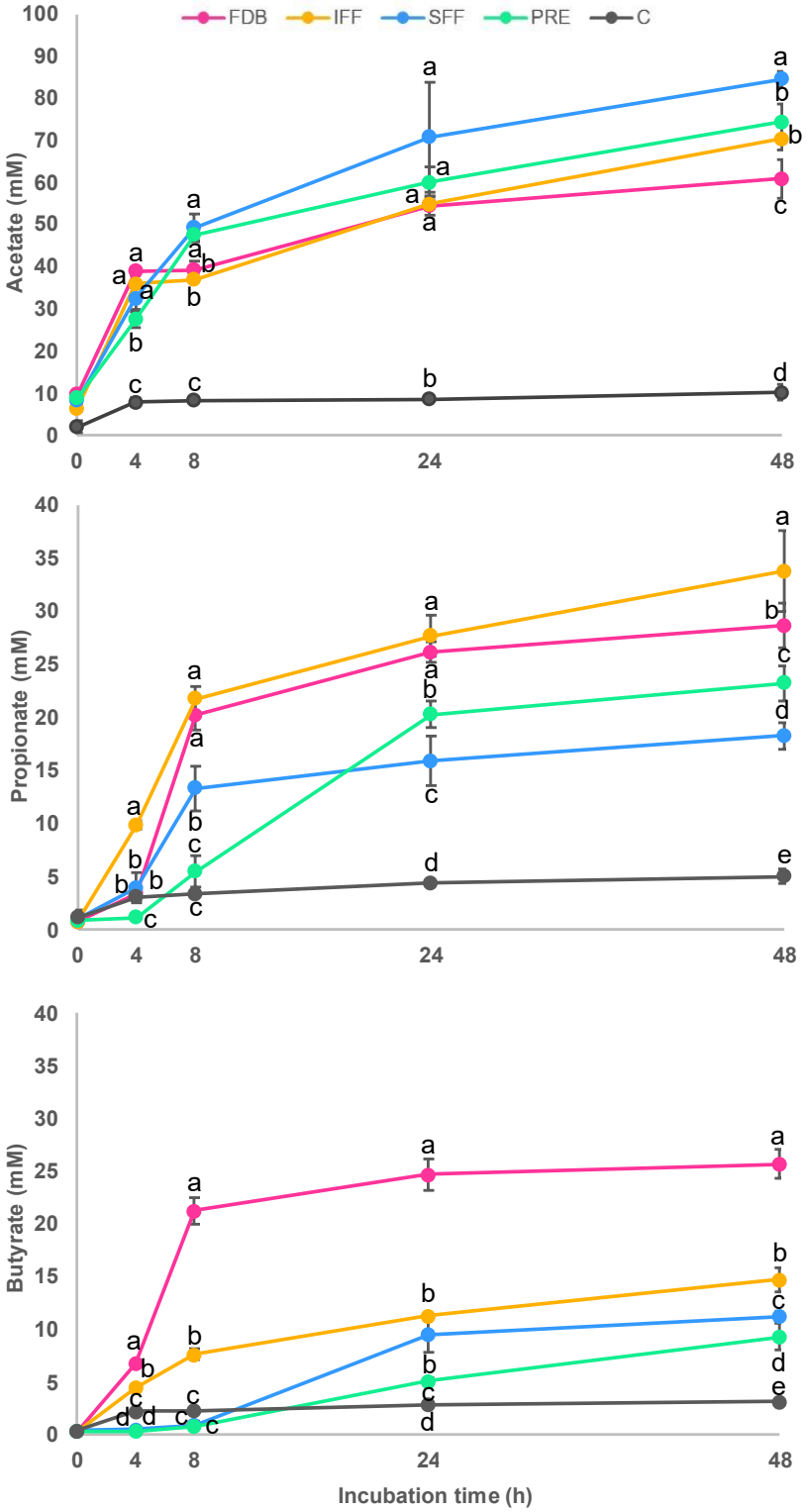


**Figure 24.** SCFAs production (acetate, propionate and butyrate) (mM) after 0, 4, 8, 24 and 48 h of *in vitro* fermentation of freeze-dried berries (●FDB), insoluble fibre-rich fraction (●IFF), soluble fibre-rich fraction (●SFF), (poly)phenol extract (●PRE) and control (●C). Values are expressed as mean ± SD (n =3). Different letters (a–e) indicate significant differences ($p$<0.05) among the samples at the same incubation time.

Propionate was mainly produced in greater quantities after the fermentation of the IFF and FDB substrates (Figure 24), in quantities ranging from 0.7 to 33.8 mM for IFF and from 0.8 to 28.6 mM for FDB. Zhao *et al*. (2021) has reported that after *in vitro* fermentation of pectin and homogalacturonan, propionate production is positively correlated with the content of rhamnose, arabinose and xylose. These neutral sugars are related with cellulose and hemicellulose content, which showed a higher concentration in IFF and FDB samples when we compared with SFF (Table 12). In terms of propionate production from the PRE fraction, it is noted that it was higher than that produced from SFF, being EPP and propionate production positively correlated (r=0.83, $p$<0.01). This effect highlights the ability of the microbiota to metabolise (poly)phenols from an extract, compared to a sample with a higher amount of SDF and bound (poly)phenols.

Butyrate production (Figure 24) was highest for FDB, ranging from 0.3 to 25.7 mM. These results could be related to the high content of carbohydrates (67.5%) in this sample, showing a positive correlation (r=0.86, $p$<0.01) between both parameters. This effect could be explained because one of the metabolic pathway that originates butyrate, has monosaccharides as starting compounds, leading to a higher production of butyrate, compared with the other fractions (Liu *et al*., 2018). The second fraction with the highest butyrate production was IFF, followed by SFF and PRE, with values ranging from 0.3-14.7 mM for IFF, 0.3-11.2 for SFF and 0.3-9.3 mM for PRE.

Finally, other minor SCFAs (isobutyrate, isovalerate, valerate, isocaproate, caproate and heptanoate) (Figure 25) were highest in IFF and PRE, followed by the negative control. This results suggest that in the control, where no sample was incubated, the microbiota may use tryptone as nitrogen base of the medium, leading to higher production of isobutyrate and isovalerate as end products from the amino acids metabolisation, than in the other samples (Rios-Covian *et al.*, 2020). Finally, FDB and SFF were the ones with the lowest production of other minor SCFAs. Although the data obtained are not shown individually, it should be noted that the production of both isovalerate and isobutyrate as major compounds in this group at 48 h of fermentation, being highest for PRE with 2.9 and 4.6 mM and lowest for SFF with 1 and 2 mM, respectively.

As for the total SCFAs content (Figure 25), it should be noted that there were no significant differences between the different samples, although there were significant differences with the control incubation in which no sample was added. It is remarkable that PRE showed similar production for total SCFAs that the fibre-rich fractions, highlighting the prebiotic-like effect of (poly)phenols, which have been previously

reported by other authors (Anhê *et al.*, 2015; Roopchand *et al.*, 2015; Moreno-Indias *et al.*, 2016). Moreover, our results are consistent with those reported by Wu *et al*. (2020), who showed an increase in the production of acetate, propionate and butyrate after *in vitro* incubation of blueberry extract when compared with a control where sample were no added. These authors compared the prebiotic effect of the samples with other substrates showing similar proportions of SCFAs production than gelatin, soy protein isolate, maltodextrin and Arabic gum.

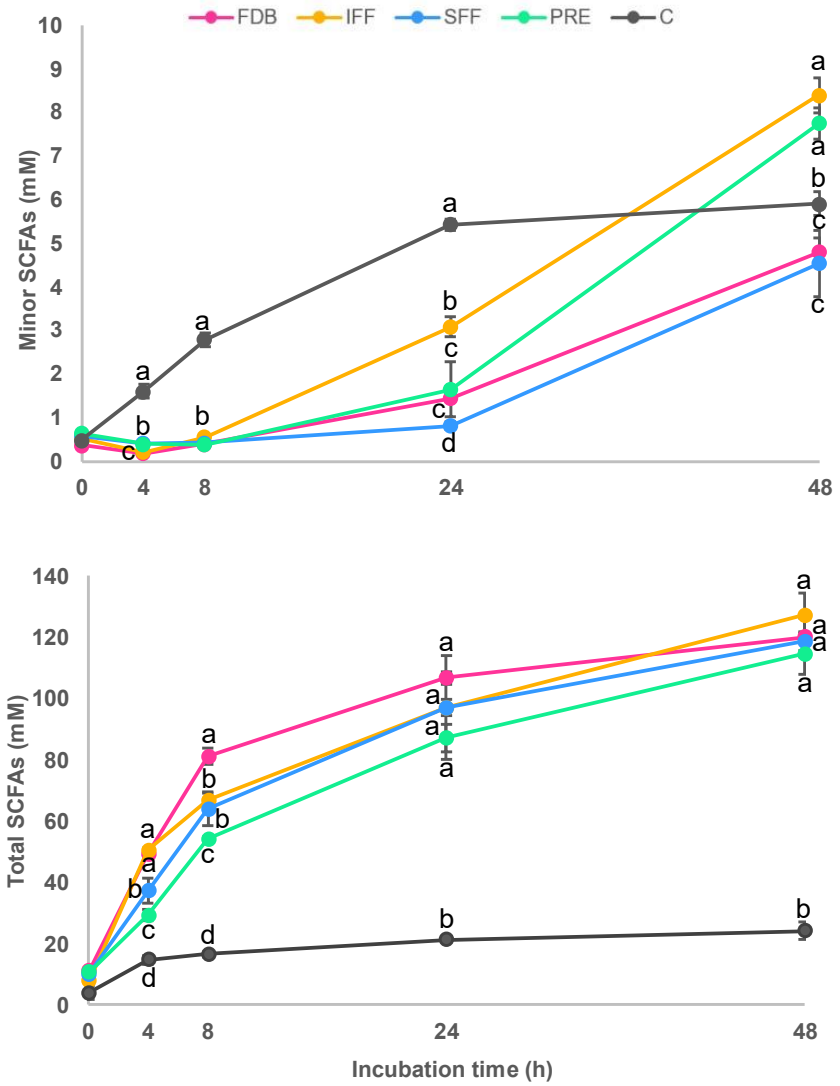


**Figure 25.** SCFAs production (other minor SCFAs (isobutyrate, isovalerate, valerate, isocaproate, caproate and heptanoate) and total SCFAs) (mM) after 0, 4, 8, 24 and 48 h of *in vitro* fermentation of freeze-dried berries (●FDB), insoluble fibre-rich fraction (●IFF), soluble fibre-rich fraction (●SFF), (poly)phenol extract (●PRE) and control (●C). Values are expressed as mean ± SD (n =3). Different letters (a–d) indicate significant differences ($p$<0.05) among the samples at the same incubation time.

The production of ammonium during *in vitro* ferementation of the different substrates might result from the consumption by the bacteria of tryptone, the culture medium used, which is composed mainly by nitrogen. But it should be noted that although there were differences between the different samples assayed (Figure 26), the values obtained were not likely to be harmful. The lowest values were found in the negative control (C), and could be due to after the first 4 h of *in vitro* fermentation the microbial activity was notably reduced, reflected in a lower production of ammonium and also of SCFAs as have been mentioned before.

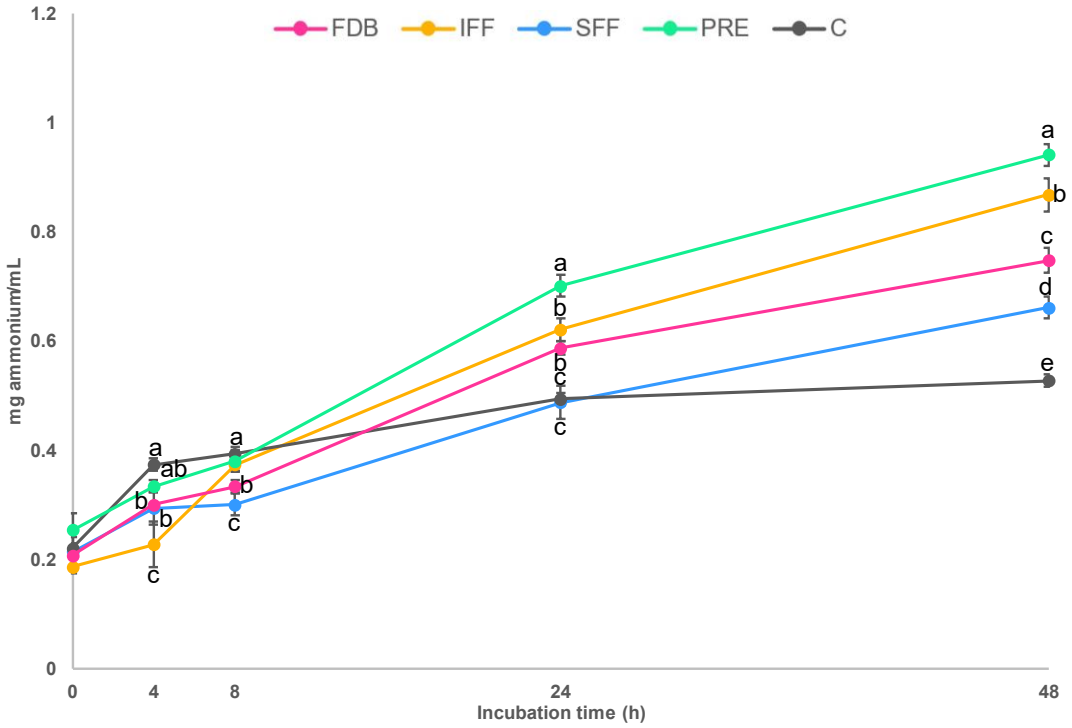


**Figure 26.** Ammonium production (mg ammonium/mL faecal slurry) during *in vitro* fermentation for berries mix samples. Freeze-dried berries (●FDB), insoluble fibre-rich fraction (●IFF), soluble fibre-rich fraction (●SFF), (poly)phenol extract (●PRE) and control (●C). Values are expressed as mean ± SD (n =3). Different letters (a–e) indicate significant differences ($p$<0.05) among the samples at the same incubation time.

On the contrary, PRE was the sample that showed the highest ammonium production which could be explaind based on PRE composition, which was composed by EPP, and had no non-starch polysaccharides or other carbohydrates as carbon source for bacteria, which consequently consumes tryptone as a source of nitrogen leading to increased ammonium production. This effect was related to that previously described for other minor SCFAs, with PRE being the fraction with the highest production

with IFF. The increased production of isobutyrate, isovalerate and valerate was related to the production of ammonium, as these are also end products from amino acid metabolism (Shortt *et al.*, 2018). On the other hand, the samples with the lowest production of ammonium were FDB and SFF, being also related with the production of other minor SCFAs. Our findings are in agreement with that reported by Zhao *et al*. (2021), who showed that bacteria may have a certain preference for the consumption of carbohydrates when pectins are present, and therefore stop consuming the tryptone from the medium.

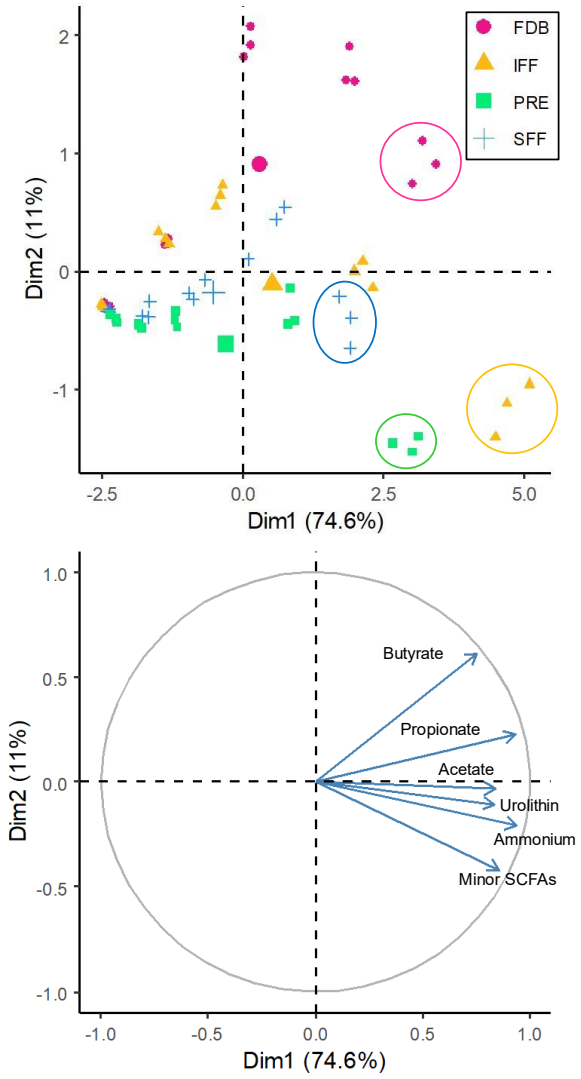


**Figure 27.** Principal component analysis (PCA) showing individual distribution (on top) and variables distribution (on bottom) for selected variables (urolithin, acetate, propionate, butyrate, minor SCFAs and ammonium). Different colours show different samples: freeze-dried berries (●FDB), insoluble fibre-rich fraction (▲IFF), soluble fibre-rich fraction (+SFF), (poly)phenol extract (■PRE). Circles represent samples at 48 h of fermentation.

A multivariate statistical analysis was carried out to determine the relationship between the different samples and the overall catabolite production of the fermentation (Figure 27). The data have been represented in two dimensions, which explain 85.6% of the total variance, the large symbol represents the centroid of the data groups. All variables were positively correlated in Dim 1, which represents the fermentation process. Whereas only butyrate was positively correlated in Dim 2. The PCA shows on top the representation of the samples and on bottom the representation of the variables. This graph shows that the presence of mostly insoluble fibre, in combination with soluble fibre and a high amount of (poly)phenols as shown for FDB and IFF, leads to a higher catabolite production compared to the other samples. On the other hand, the only presence of (poly)phenols, as in the case of PRE, or soluble fibre with a low amount of (poly)phenols, the case of SFF, showed that the microbiota of the volunteers was less able to produce catabolites at a general level from these samples. The circles in the figure indicate the distribution of the samples at 48 h, the time at which the greatest differences between the different samples were observed. At this fermentation time, IFF showed a greater production of catabolites and therefore a greater prebiotic effect, with fewer differences between the other three samples and SFF being the one that showed the lowest prebiotic effect.

Summing up our findings, the four samples exhibit a significant prebiotic effect, but the one with the most interesting profile, based on the composition and the catabolites production was IFF, which led to a great amount of urolithin production. This together with its content of mainly non-extractable (poly)phenols and fibre, make it an interesting fraction with potential nutritional properties. In addition, taking into account the procedure used to isolate the different fractions, both soluble and insoluble fibre-rich fractions can be extracted using a green procedure. So, the by-products and super-plus from berries can be valorised by the extraction of new ingredients with nutritional and technological properties. Although in this study the analysis of microbiota has not been conducted, a further microbiota analysis would help to elucidate new correlations that would allow us to assess catabolite production with specific microbial groups, and to give a better understanding of the beneficial effects for human health of the different isolated fibre-rich fractions from berries.

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# Chapter 6. By-products from broccoli

## 1. Introduction

*Brassica oleracea* vegetables are one of the ten most important vegetable groups in world agriculture, which includes broccoli, cabbage, cauliflower and kale among others (Francisco *et al*., 2017). The consumption of vegetables of this group provides benefits for human health due to their content in glucosinolates, (poly)phenols, carotenoids, vitamins and minerals (Liu *et al*., 2018), showing a protective effect against chronic diseases such as inflammatory disorders, cardiovascular diseases and some types of cancer (Raiola *et al*., 2018). However, the content of these bioactive compounds varies depending on the agronomic and environmental conditions, with significant seasonal variations (Shiva and Jung-Ho, 2014).

The broccoli production and consumption have increased in the last decade due to changes in the population lifestyles, leading to a high adherence to healthy diets (Shi *et al*., 2019). Murcia region (southeastern Spain) is the major producer area in Europe (FAO, 2019; MAPA, 2019), and hence the waste from broccoli industry (leaves and stalks) has become an environmental problem for the agri-food industries. In this regard, only a 15% of total plant biomass is constituted by the florets, which represents the edible part, and a large number of by-products are generated after harvesting. The other aerial parts of the plants are stalks and leaves, which represent approximately 21% and 47%, respectively, whereas the roots represent 17% (Liu *et al*., 2018). Despite of these by-products are sometimes used for animal feeding; in many cases they are left in the field being an environmental problem. Nowadays, there is an increased interest to reduce the by-products disposal, implementing a transition to circular economy process, in which new products with added value are designed to give a second useful life to by-products, reducing also the energy and raw material consumption (Laínez and Periago, 2019). Therefore, the by-products from agri-food industry can be processed for the extraction of ingredients such as bioactive compounds, dietary fibre (DF) and enzymes, with the aim to valorise, and create new production chains.

The broccoli stalks are rich in DF, containing also significant amounts of some minerals, and bioactives compounds (glucosinolates, (poly)phenols, and carotenoids) and some vitamins, but in smaller quantities than in florets and leaves (Liu *et al*., 2018). Taking into consideration their composition, stalks represent a source of DF which can be extracted from the by-products. DF can be classified according to its solubility in water into soluble and insoluble dietary fibre (IDF). Soluble dietary fibre (SDF) includes pectin, soluble hemicellulose, gums and mucilages, which are more fermentable by the

microbiota than insoluble fibre, which includes cellulose, insoluble hemicelluloses and lignin (Soliman, 2019). DF has many health benefits, because can be considered a prebiotic ingredient, and after fermentation by gut microbiota end products, such as short chain fatty acids (SCFAs), are produced (Holscher, 2017; Rezende *et al*., 2021). Main SCFAs (acetate, propionate and butyrate) are produced from the fibre fermentation, and after their absorption in the large intestine, they are distributed through the blood stream modulating several functions, such as immunity response, appetite regulation, glucose homeostasis and lipid metabolism, as well as regulating the body weight and reducing risk of some types of cancer (Morrison and Preston, 2016; Yegin *et al*., 2020).

(Poly)phenols can be found in free form (extractable (poly)phenols (EPP)) and can therefore be extracted with aqueous-organic solvents; but they can also be found bound to fibre molecules to form non-extractable (poly)phenols (NEPP), so that these bonds need to be broken for their extraction. It is important to take into account the NEPP, as they make up more than 50% of the total (poly)phenols and in most of the research works they are not considered (Hümmer and Schreier, 2008; Martínez-Meza *et al.,* 2021). In this regard, the methods used for fibre extraction are very important. Extraction methods using solvents or enzymes have been commonly employed, which leads to more expensive processes, as well as toxic and environmental problems due to the toxicity of the solvents used. As an alternative, extraction techniques using microwaves, ultrasounds or supercritical fluids have emerged, but in this case, they require a large amount of energy as well as specific equipment (Dong *et al*., 2020; Li *et al*., 2020). Therefore, the development of new extraction techniques, which are industrially simple and environmentally friendly, is very important for the implementation of the circular economy system (Pagano *et al*., 2021).

Against this background, the aim of this work was to determine the seasonal effect on the content of bioactive compounds in broccoli florets, in order to select the raw materials for obtaining broccoli stalks as by-products. In addition, to determine the nutritional composition of the broccoli stalk and to extract different DF fractions with the objective to valorise this by-product obtaining new potential ingredients. Furthermore, the different samples obtained have been analysed to characterise their chemical composition, including the bioactive compounds, physicochemical properties as well as their *in vitro* prebiotic effect.

## 2. Material and methods

### 2.1. Seasonal floret assay

Broccoli plants (*Brassica oleracea* L. var. *italica* cv. Parthenon) (Sakata seeds, Uchaud, France) were cultivated by the company "Agrícola San Luis" (La Hoya, Murcia, Spain) under real field conditions. The farm is located in Caniles, in Mediterranean South-Eastern Spain (Granada, lat. 37˚26'03"N, long. 2˚43'28"W). 'Parthenon' is a 90-days from sowing to harvest broccoli variety, is very well adapted to the growing area and is one of the best-quality varieties for international trade.

Two different assays were carried out in the same field and with the same agronomic conditions, using broccoli plants, which were seeded and harvested in different seasons, from two consecutive sowings: the first set of plants was grown in spring (March to early June 2018) and the second in autumn (mid-September to mid-December 2018). Finally, broccoli was taken to the laboratory where the florets were analysed to determine differences between both seasons.

### 2.2. Extraction process of broccoli stalk fibre samples

Broccoli stalks were provided by the company "Agrícola San Luis" (La Hoya, Murcia, Spain) as an industrial by-product from broccoli cultivars (October 2019). The stalks correspond to the season selected in the seasonal assay described before. The samples were directly processed in the laboratory and stored at -20 °C.

Different samples were obtained from the fresh broccoli stalks following the processes described in Figure 28, which were used as samples in this experimental work. Firstly, the stalks were freeze-dried in a Lyoquest freeze-dryer (Telstar, USA), to obtain the freeze-dried broccoli stalks sample (DBS). After removing the total water content, the sample was milled to obtain a fine powder. The other three samples were the fibre-rich fractions, extracted from fresh broccoli stalks, which were classified according to the extraction process in: 1) total dietary fibre fraction ($TF_B$), 2) insoluble dietary fibre fraction ($IF_B$) and 3) soluble dietary fibre fraction ($SF_B$) (Figure 28).

For $TF_B$ the broccoli stalks were ground in a Thermomix TM-31 and mixed with 80% ethanol, with a ratio of 1/2.5 (m/v). 80% ethanol was previously heated to 70 °C to facilitate the precipitation of the soluble and insoluble components and to inactivate myrosinase. Samples were stirred for 30 minutes and after that were centrifuged at 4500*g* for 5 minutes and the pellet was placed on a stove at 45 °C to constant weight.

To obtain the other two fractions ($IF_B$ and $SF_B$) broccoli stalks were mixed with water, previously heated to 70 °C, in order to inactivate myrosinase, with a ratio of 1/2.5 (m/v) and then shacked for 30 minutes. Samples were centrifuged at 4500*g* for 5 minutes and the pellet was placed on a stove at 45 °C to constant weight, obtaining the $IF_B$. The supernatant, containing the SDF, was collected in a flask and then 80% ethanol was added and left overnight at room temperature to allow the precipitation of soluble complex polysaccharides. The samples were centrifuged at 4500*g* for 5 minutes and the pellet was placed on a stove at 45 °C to constant weight, corresponding to $SF_B$. When the samples reached constant weight, they were crushed separately and stored at -20 °C, until the different analyses were performed. The yield was calculated for the three different fibre-rich fractions, and due to low proportion of $SF_B$, this fraction was discarded.

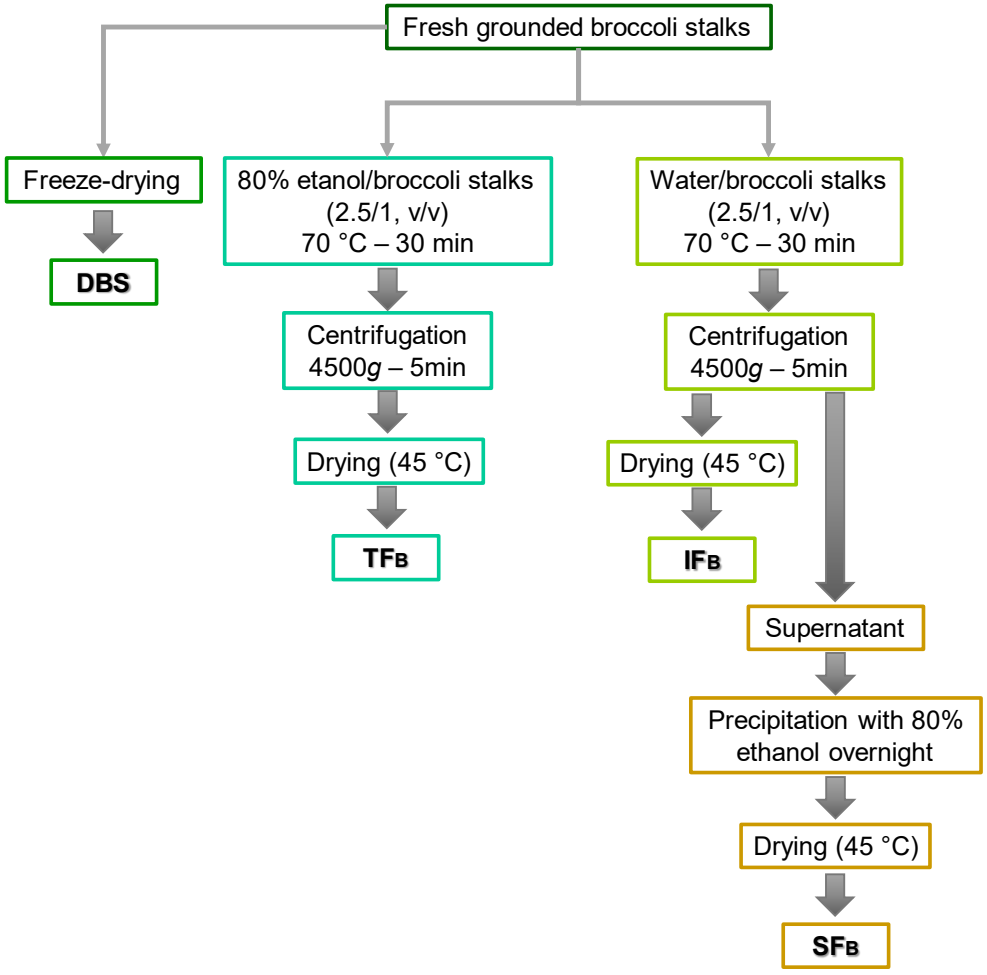


**Figure 28.** Flow diagram of the procedure used to obtain the freeze-dried broccoli stalks sample (DBS) and the three fibre-rich fractions, total dietary fibre fraction ($TF_B$), insoluble dietary fibre fraction ($IF_B$) and soluble dietary fibre fraction ($SF_B$) from broccoli stalks.

### 2.3. Physicochemical properties and prebiotic effect

The proximate composition of the samples (moisture, protein, ash, total dietary fibre (TDF), IDF and SDF), the chemical composition of the fibre, determining the neutral sugars and uronic acids, the physicochemical properties, the total phenolic content (TPC) and the antioxidant capacity were determined in all the samples obtained (Chapter 4, pages 73-80; Chapter 5, page 114). Moreover, an *in vitro* gastrointestinal digestion and the *in vitro* faecal fermentation were also performed with DBS and $IF_B$ fraction (Figure 29), determining the prebiotic effect by the evaluation of the SCFAs production, and also the ammonium production was measured. These analyses were carried out following the methods previously described in Chapter 5 (pages 115-116).


**Figure 29.** Samples inoculated in the *in vitro* fermentation. Freeze-dried broccoli stalks sample (DBS) and insoluble dietary fibre fraction ($IF_B$).

Furthermore, the mineral composition (calcium, magnesium, sodium, potassium, iron, and zinc) was analysed by inductively coupled plasma optical emission spectroscopy, using an ICAP 6500 Duo (Thermo Fisher Scientific, Waltham, Massachusetts, USA) after microwave-assisted digestion (UltraCLAVE, Milestone) with $H_2O_2/HNO_3$ (1/4, v/v).

### 2.4. Analysis of carotenoids by HPLC-DAD

The analysis of carotenoids in broccoli florets was performed using the method described by González-Barrio *et al*. (2018), with some modifications. For the sample extraction, 0.1 g of freeze-dried broccoli florets were extracted with 5 mL of tetrahydrofuran/methanol (50/50, v/v) containing 0.1% butylated hydroxytoluene, in an ultrasonic bath for 5 min at room temperature. The extraction process was repeated, after which the residual tissue was colourless. The supernatants obtained from both extractions were mixed and dried under vacuum at 30 °C in a Laborota-4002 rotatory evaporator (Heidolph, Schwabach, Germany). The residue was re-dissolved in 2 mL of methyl tert-butyl ether/methanol (50/50, v/v) and finally was centrifuged at 20817*g* for 5

min. This extract was analysed by HPLC-DAD in an Agilent 1100 (Agilent Technologies, Germany). A C30 column (250×4.6 mm, 5 µm i.d.) (Trentec, Gerlingen, Germany) was used to perform the chromatographic separation at 17 °C. The mobile phases used were methyl tert-butyl ether (A) and methanol (B), at a flow rate of 1 mL/min. The gradient started with 2% of A, reaching 35% at 35 min, 60% at 45 min until 55 min, followed by washing. Chromatograms were recorded at 472 and 450 nm.

Carotenoids were identified by comparison of their wavelength of maximum absorption in the UV spectra and their retention times with those of the authentic standards, when available, and with published results when a standard was not available (Strati *et al*., 2012; Becerra-Moreno *et al*., 2014; Villarreal-Garciá *et al*., 2015). Quantification was based on calibration curves constructed using 5 to 100 µg/mL of lutein and β-carotene (Sigma-Aldrich, St Louis, MO, USA). The contents of total and individual carotenoids were expressed as mg/kg of fresh weight (f.w.).

### 2.5. Analysis of (poly)phenols and glucosinolates in the extractable and non-extractable fractions by HPLC-DAD

To analyse EPP and NEPP, two different extractions procedures were followed according to Arranz *et al*. (2009) with some modifications. For the EPP 0.35 g of each sample were mixed with methanol/water/formic acid (79/19/1, v/v/v), vortexed for 1 min and centrifuged at 4500*g* during 10 min at room temperature. The supernatants were evaporated with a Laborota-4002 rotatory evaporator (Heidolph, Schwabach, Germany), and resuspended in 10 mL of distilled water, after that the samples were passed through a C18-SPE cartridge (Waters Corporation, Milford, Mas-sachusetts, USA) previously activated (10 mL methanol, 10 mL $H_2O$ and 10 mL air flow). After passing the sample through the cartridge, the column was washed with 10 mL of milli-Q water and the compounds of interest were recovered in 1 mL of methanol, which was evaporated in vacuum concentrator (Eppendorf model 5301, Hamburg, Germany) and redissolved in 0.25 mL of methanol/formic acid/acetonitrile (49.5/0.5/50, v/v/v).

For NEPP, present in the pellet obtained from the extraction of EPP, the residues were resuspended in 5 mL of methanol/$H_2SO_4$ (9/1, v/v) and incubated at 85 °C during 20 h. Then the samples were centrifuged at 4500*g* during 10 min at room temperature and were evaporated with a Laborota-4002 rotatory evaporator (Heidolph, Schwabach, Germany). After resuspending in 10 mL of distilled water, the samples were passed through a pre-conditioned C18-SPE cartridge as described before (Waters Corporation, Milford, Mas-sachusetts, USA), and the compounds were recovered in 1 mL of methanol,

which was evaporated in vacuum concentrator (Eppendorf model 5301, Hamburg, Germany) and redissolved in 0.25 mL of methanol/formic acid/acetonitrile (49.5/0.5/50, v/v/v).

For glucosinolates extraction, the method described by Baenas *et al*. (2012) was followed with slight modifications. Briefly, 0.1 g of sample were extracted with 1.5 mL of methanol/water (70/30, v/v) and heated at 70 °C for 30 min, shaking every 5 min, then centrifuged at 12000*g* for 10 min. The supernatant was dried under vacuum in a Laborota-4002 rotatory evaporator (Heidolph, Schwabach, Germany), re-dissolved in 1 mL of water and filtered through a 0.22 µm polyvinylidene fluoride membrane (PVDF) filter.

The LC method described by Francisco *et al*. (2009) was followed with slight modifications to perform analysis to identify glucosinolates and (poly)phenols in the samples extracted. Both compounds were identified in an HPLC 1200 (Agilent Technologies, Germany), equipped with a mass detector in series (HPLC-DAD-ESI-$MS_n$) following their MS and $MS^2$ $[M-H]^-$ fragmentation ions, UV-visible spectra, and elution order. The HPLC system consisted of a binary capillary pump (model G1376A), an autosampler (model G1377A), a degasser (model G1379B), a sample cooler (model G1330B), and a photodiode array detector (model G1315D), and was controlled by ChemStation software (v.B.0103-SR2). The column used for the separation of the compounds was a Luna C18 (250 mm x 4.6 mm, 5 µm particle size; Phenomenex, Macclesfield, UK). The mobile phases used were 0.1% trifluoroacetic acid (A) and acetonitrile/trifluoroacetic acid (99.9/0.1, v/v) (B). The injection volume was 20 µL and the flow rate was 1 mL/min with linear gradient, starting with 100% of phase A from 0-5 min, reaching 83% of A from 15-17 min, 75% at 22 min, 65% at 30 min, 50% at 35 min, 1% at 50 min and 100% at 55-65 min. The mass detector was a Bruker, model UltraHCT (Bremen, Germany), ion trap spectrometer equipped with an electrospray ionisation interface (ESI) and controlled by Bruker Daltonic Esquire software (v.6.1). The ionisation conditions were adjusted at 4 kV and 350 °C for voltage and capillary temperature, respectively. The flow rate of nitrogen was 11 mL/min and the nebuliser pressure was 65 psi. The full scan mass covered the from *m/z* 100 up to *m/z* 1500 range. Collision-induced fragmentation experiments were performed in the ion trap using helium as the collision gas, with voltage ramping cycles from 0.3 up to 2 V. Mass spectrometry data were acquired in the negative ionisation mode. $MS_n$ was carried out in the automatic mode on the more abundant fracragment ion in $MS(n^{-1})$.

The quantifications of both glucosinolates and (poly)phenols were carried out by using another HPLC-DAD Agilent 1260 Infinity (Agilent Technologies, Waldbronn, Germany), using the conditions described before. The quantification was carried out according to their UV spectra and order of elution already described for similar acquisition conditions (Baenas *et al*., 2012). The HPLC-DAD was equipped with a binary pump (model G 1312 B), a degasser (model G 1379 B), an autosampler (model G 1377 A), and a diode array detector, DAD (model G 4212 B) that is controlled by the Agilent software B. 02. 02. Chromatograms were recorded at 330 nm for (poly)phenols, using chlorogenic acid (Sigma-Aldrich Chemie GmbH, Steinheim, Germany) and sinapic acid (Sigma, St. Louis, MO, USA) respectively, and 227 nm for glucosinolates, using sinigrin and glucobrassicin (Phytoplan, Germany) as standard of aliphatic and indole glucosinolates, respectively. Results were expressed as mg/kg of f.w. for the broccoli florets and mg/100 g of dry weight (d.w.) for the samples obtained from the broccoli stalks.

### 2.6. Statistical analysis

The statistical analysis was carried out using R studio, version 4.0.5 (R Foundation for Statistical Computing, Vienna, Austria). Normality was determined by the Shapiro-Wilk test. The homogeneity of variances was analysed using the Bartlett test. One-way analysis of variance (ANOVA) was performed to determine significant differences among different seasons and different samples for the parameters analysed. Tukey´s test was used as a post-hoc test. Moreover, two-way analysis of variance (two-way ANOVA) was performed to determine the prebiotic effect of the samples, considering the effect of the different samples and the time of fermentation in the parameters measured (SCFAs and ammonium) during the *in vitro* fermentation analysis. A correlation analysis was also performed between physicochemical properties and fibre composition parameters, and among the parameters of the composition of the DF of the samples (DBS and $IF_B$) and the SCFAs produced during fermentation. Differences were considered significant at a *p*-value< 0.05.

# 3. Results and discussion

## 3.1. Seasonal variation of broccoli florets composition

The content of (poly)phenols, glucosinolates and carotenoids was analysed in broccoli florets in two different seasons, spring and autumn. The characterisation is included in Table 16.

Regarding the (poly)phenol composition, the results were expressed as individual groups of flavonols, chlorogenic acid derivatives and sinapic acid derivatives, and the total content as the sum of the three groups. There was a significant effect of the season on the individual groups, being flavonols and sinapic derivatives higher in spring assay, whereas the chlorogenic derivatives were higher in autumn assay, and no differences were observed for the total (poly)phenol content. As other authors have previously reported, flavonols and sinapic acid derivatives were positively correlated with sunlight, UV-B and temperature exposure (Neugart *et al*., 2014), which may explain the trend observed in our results. Moreover, water deficit have been related with a reduction in flavonols (Martínez-Lüscher *et al*., 2014), supporting also our data because in the spring assay the total precipitation was 361 mm compared with 185 mm in the autumn assay.

The glucosinolate content was expressed per family of compounds, including glucoiberin (GIB) and glucoraphanin (GRA) as aliphatics, and 4-hydroxyglucobrassicin (HGB), glucobrassicin (GBS), 4-methoxyglucobrassicin (MGB) and neoglucobrassicin (NGB) as indoles and the total content of glucosinolates was expressed as the sum of both groups. Regarding the individual glucosinolates, the main compound in the samples of the florets cultivated in spring was GIB, followed by GRA. By contrast, in the florets cultivated in autumn, the order was the opposite, GRA being the main one, followed by GIB. Related to our results, Mølmann *et al*. (2015) reported that, under controlled conditions, at 18 °C the GIB content was higher than that of GRA, while the opposite occurred at 12 °C. This explains our results because the mean temperature in the spring assay was higher (15 °C) than that registered in the autumns assay (14 °C).

Considering the two glucosinolates classes present in broccoli, the aliphatics were predominant in our samples, representing 79% and 72% of the total, respectively for the spring and autumn assay. Chiu *et al*. (2019) reported that aliphatic glucosinolates represented around 66% of the total content in ‘Green Magic’ broccoli. Comparing both seasons, the content for aliphatic, indole and total glucosinolates was 2.3, 3.3 and 2.5-fold higher, respectively, in autumn than in spring assay. Previously, contrary to our

results, it has been reported that the total content of glucosinolates was higher in spring than in autumn, in two different cultivars (Charron *et al*., 2005), since the sunlight exposure and temperature were higher in srping season. However, other authors have stated that the synthesis of glucosinolates was not only determined by light and temperature exposure, since other agronomic factors, such as water stress, could increase the content (Vallejo *et al.*, 2003; Ku *et al*., 2013). Our results are in agreement with these findings, because in the spring assay the total precipitation was higher than in the autumn assay. So, the water deficit in autumn could have contributed to the increase in the total glucosinolates content.

**Table 16.** Bioactive characterisation of (poly)phenols, glucosinolates and carotenoids (mg/kg of f.w.) of broccoli florets in the seasonal assay (spring and autumn).

| Bioactive compounds | Spring assay | Autumn assay |
|---|---|---|
| **Flavonols** | 49.4 ± 1.4$^{a}$* | 42.2 ± 1.5$^{b}$ |
| **Chlorogenic derivatives** | 14.1 ± 1.3$^{b}$ | 30.9 ± 1.6$^{a}$ |
| **Sinapic derivatives** | 38.9 ± 1.1$^{a}$ | 32.6 ± 0.8$^{b}$ |
| **Total (poly)phenols** | 102.4 ± 2.7 | 105.8 ± 1.7 |
| **Aliphatics** | 245.3 ± 15.7$^{b}$ | 553.6 ± 63.5$^{a}$ |
| **Indoles** | 65.1 ± 2.4$^{b}$ | 212.3 ± 15.9$^{a}$ |
| **Total glucosinolates** | 310.4 ± 14.5$^{b}$ | 765.9 ± 76.6$^{a}$ |
| **B-carotene** | 6.1 ± 0.9$^{b}$ | 25.4 ± 1.0$^{a}$ |
| **Lutein** | 1.4 ± 0.1 | 1.7 ± 0.4 |
| **Neoxanthin** | 0.8 ± 0.0$^{b}$ | 1.3 ± 0.0$^{a}$ |
| **Violaxanthin** | 0.8 ± 0.0$^{b}$ | 1.5 ± 0.1$^{a}$ |
| **Total carotenoids** | 9.1 ± 1.1$^{b}$ | 29.9 ± 1.0$^{a}$ |

*Values are expressed as mean ± SD (n =3). Different letters (a-b) indicate significant differences ($p$<0.05) between both seasonal assays.

For the carotenoid composition, it can be seen that β-carotene was the main carotenoid of broccoli, representing 67% and 85% of the total carotenoids in the spring and autumn assay respectively, being similar to the 51% previously reported for broccoli samples (Dos Reis *et al*., 2015). Moreover, β-carotene was followed by lutein, which was

found in 15.4% and 5.7%, neoxanthin in 8.8% and 4.3% and violaxanthin in 8.8 and 5%, respectively in the spring and autumn assays. In general, the content of carotenoids was higher in broccoli cultivated in autumn than in spring-grown florets, the season showing a significant effect on the contents of individual and total carotenoids, except for lutein, where differences were not observed. The content of total carotenoids in broccoli cultivated in autumn represented an increase of 3.3-fold compared to broccoli florets grown in spring (9 mg/kg). Although the effect of sunlight on the biosynthesis of *Brassica* carotenoids is not clear (Neugart *et al*., 2018), in florets of the broccoli variety ´Green´, the control group showed higher levels when compared with samples subjected to light at different wavelengths (Samuolienė *et al*., 2019; Thoma *et al*., 2020). Moreover, other authors have reported that temperatures >15 °C could lead to a decrease in carotenoids (Schonhof *et al*., 2007). Among other agronomic factors, the high temperature and the greater sunlight exposure during spring, compared to autumn, may have affected the samples of broccoli florets, which showed lower contents of total and individual carotenoids.

To summarise, the content of carotenoids and glucosinolates were higher in the autumn assay, with no differences for (poly)phenols between the two season experiments. Based on these results, and despite broccoli stalks were not analysed in this previous study, the by-products used for the next experiment were from the autumn harvest, since they exhibited the highest content of total bioactive compounds and hence is expected to be also higher in the stalks.

### 3.2. Performance of the samples obtained from broccoli stalk

The aim of this research has been to extract different DF fractions from broccoli stalk selected from the autumn harvest, obtaining different samples with a high content of complex polysaccharides. In plant food products, the complex polysaccharides are mainly represented by starch and non-starch polysaccharides (NSP), which are constituted by the compounds of the cell wall (cellulose, hemicellulose and pectin), representing the DF.

The polysaccharides that are part of the DF are generally either water-soluble or non-water soluble, and can be extracted using hot water, alcohol precipitation, ultrasonic-assistant extraction, acid-assisted extraction, high-pressure hot water extraction, alkali water extraction, ultrasonic-assisted enzyme extraction, microwave assisted extraction, subcritical water extraction and ultrahigh pressure extraction (Huo *et al*., 2022).

The methods used in this research for the extraction of the DF fractions consisted in: 1) the precipitation of the soluble and insoluble compounds by ethanol precipitation to extract the $TF_B$; 2) the extraction with hot water to separate the insoluble NSP, obtaining the $IF_B$; and 3) the precipitation of soluble water compounds, previously isolated, using ethanol precipitation, obtaining $SF_B$ (Figure 28). After the extraction, the samples were dried at low temperature (45 °C) until constant weight, obtaining a yield of 67% for $TF_B$, 70% for $IF_B$ and 1.5% for $SF_B$ (data not shown).

Despite of the methods for the extraction of DF include the purification of crude polysaccharides removing protein, fats and pigments, in this study we have analysed the DF rich fractions as have been obtained, with the aim to maintain other beneficial bioactive compounds and to stablish a clean extraction procedure, reducing the use of organic solvents.

According to the yield, the broccoli stalk is not a source of soluble polysaccharides ($SF_B$) because only a 1.5% of yield was obtained. However, broccoli stalk can be considered a plant material for the extraction of total and insoluble DF rich fractions with an average value of 70% ($TF_B$ and $IF_B$). Taking into in account these results, $SF_B$ was discarded and not analysed in this study.

### 3.3. Nutritional composition of samples obtained from broccoli stalk

The proximate composition of the three samples DBS, $TF_B$ and $IF_B$ is shown in Table 17. The main component of the samples was the DF in comparison with other constituents. The DBS showed a similar proximate composition when compared with USDA database results for broccoli stalk (USDA, 2019). Regarding to the protein content, it ranged from 5.6 in DBS to 3.8 in $IF_B$, with a significantly lower content in the fractions compare to the DBS. These results suggest that some of the proteins were removed from the fibre-rich fractions during the extraction process, mainly due to the fact that a proportion of the proteins are soluble in ethanol or water and therefore are removed during the isolation process. So, the fibre-rich fractions had a lower content compared to DBS, leading to a decrease of 29-32% of the total proteins. The results were similar to that obtained by Campas-Baypoli *et al*., (2009) for broccoli stalk flour, with a notably lower content than the protein content reported for broccoli florets (Campas-Baypoli *et al*., 2009; Li *et al*., 2022). Fat content was not analysed, as the proportion reported in the literature was very low (<5%) and consequently not relevant for the present study (Nagraj *et al*., 2020; Li *et al*., 2022).The total carbohydrates were estimated by difference ranging from 19% in $TF_B$ to 44% in the DBS. In DBS the total carbohydrates were significantly

higher than in the other two samples because the whole sample of broccoli stalk also has starch as complex polysaccharides.

**Table 17.** Nutritional composition of broccoli stalk samples expressed as percentage or g/100 g d.w., and mineral composition.

| Parameters | DBS | $TF_B$ | $IF_B$ |
|---|---|---|---|
| **Moisture** | 0.3 ± 0.0[a]* | 0.2 ± 0.0[b] | 0.2 ± 0.0[b] |
| **Protein** | 5.6 ± 0.2[a] | 4.0 ± 0.8[b] | 3.8 ± 0.0[b] |
| **Carbohydrates**** | 43.9 ± 0.4[a] | 19.3 ± 1.2[c] | 24.4 ± 0.7[b] |
| **Total dietary fibre (TDF)** | 38.0 ± 0.4[c] | 68.9 ± 0.4[a] | 60.8 ± 0.4[b] |
| **Insoluble dietary fibre (IDF)** | 34.9 ± 0.1 [b] | 54.3 ± 0.4[a] | 54.0 ± 0.1[a] |
| **Soluble dietary fibre (SDF)** | 3.2 ± 0.5[c] | 14.7 ± 0.1[a] | 6.8 ± 1.0[b] |
| **Ash** | 12.2 ± 0.2[a] | 7.5 ± 0.0[c] | 10.8 ± 0.2[b] |
| **K** (g/kg) | 47.4 ± 0.4[a] | 14.5 ± 0.3[c] | 31.7 ± 1.6[b] |
| **Ca** (g/kg) | 4.9 ± 0.1[b] | 6.5 ± 0.3[a] | 6.2 ± 0.3[a] |
| **P** (g/kg) | 4.1 ± 0.0[a] | 4.1 ± 0.2[a] | 2.7 ± 0.2[b] |
| **Na** (g/kg) | 2.9 ± 0.0[b] | 0.9 ± 0.1[c] | 8.2 ± 0.5[a] |
| **Mg** (g/kg) | 2.6 ± 0.0[a] | 1.3 ± 0.1[b] | 2.6 ± 0.2[a] |
| **Mn** (mg/kg) | 39.6 ± 0.6[a] | 24.4 ± 1.2[b] | 39.9 ± 1.4[a] |
| **Zn** (mg/kg) | 23.1 ± 0.8[b] | 14.6 ± 0.7[c] | 27.9 ± 2.9[a] |
| **Fe** (mg/kg) | 15.7 ± 0.8[c] | 25.0 ± 1.8[a] | 18.9 ± 1.4[b] |

*Values are expressed as mean ± SD (n =3). Different letters (a–c) indicate significant differences ($p$<0.05) among the samples. Freeze-dried broccoli stalk (DBS), total fibre fraction ($TF_B$), insoluble fibre fraction ($IF_B$).
**Carbohydrates were calculated by difference between the other components.

Related to the content of TDF, DBS sample showed a mean concentration of 38%, but in the fibre-rich fractions the content of TDF was 68.9% for $TF_B$ and 60.8% for $IF_B$, showing significant differences. Related to the classification of the different fractions of DF, the content of IDF ranged from 35% to 54%, whereas the SDF content showed mean values of 14.7% for $TF_B$, 6.8% for $IF_B$ and 3.2% for DBS. The content of IDF and SDF in DBS found in this study, was similar to that described by Schäfer *et al*. (2017), who reported a mean content of 32% and 3% of IDF and SDF, respectively, for fresh stalk. $TF_B$ was the fraction with the highest content of TDF (68.9%) compared with $IF_B$, since the extraction procedure allowed the extraction of the insoluble NSP as well of the soluble NSP, which were precipitated with ethanol. However, for the $IF_B$, the extraction

procedure allowed the isolation mainly of the insoluble NSP, since the soluble compounds are solubilised in the hot water.

The Figure 30 shows the proportion of the IDF and SDF in DBS and in the fibre-rich fractions. The proportion of SDF in broccoli stalks was low, under 10%, for this reason the yield of the extraction of SDF was very low. In $TF_B$ the percentage of SDF and IDF was 21.3% and 78.7%, because the soluble compounds were precipitated with ethanol. Although, $IF_B$ was only extracted with hot water, showing a small proportion of SDF. The procedure used for $IF_B$ led to a green extraction process compared with the extraction process of $TF_B$, allowing the extraction of a rich DF fraction.

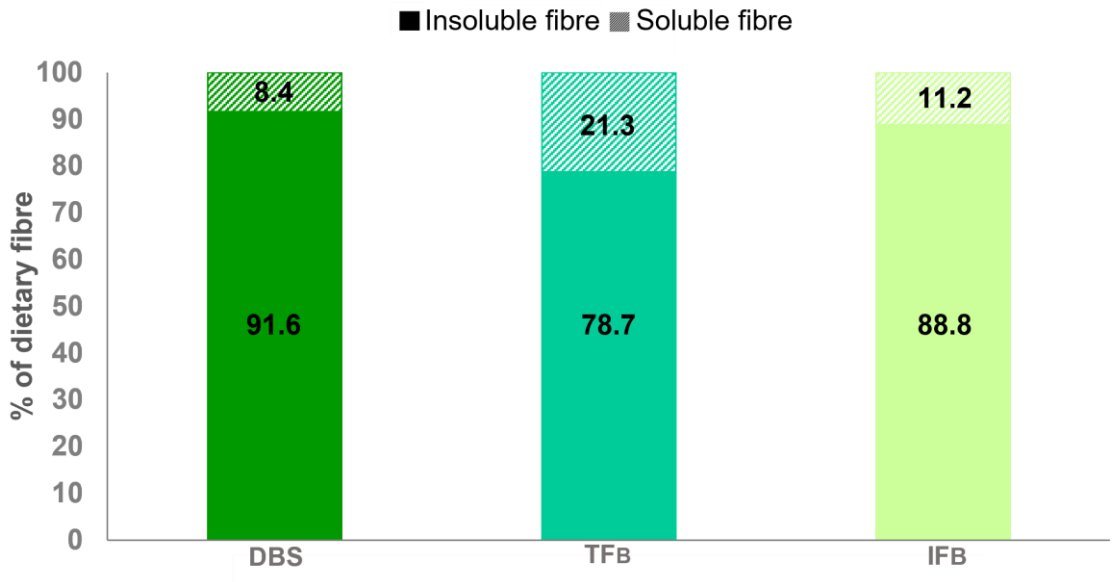


**Figure 30.** Proportion (%) of insoluble fibre (■) and soluble fibre (■) for the freeze-dried broccoli stalk (■DBS) and the fibre-rich fractions, total dietary fibre fraction (■$TF_B$) and insoluble dietary fibre fraction (■$IF_B$).

The content of ashes was highest in DBS, with a mean value of 12.2%, when compared with the other two samples, being these results also higher than those reported in the scientific literature, which reported a mean value around 9-10% (Campas-Baypoli *et al*., 2009; USDA, 2019).

On the other hand, regarding to the mineral composition of the samples, potassium and calcium showed the highest concentrations, while trace elements, zinc and iron, showed the lowest content (Table 17). These values are in agreement with the content of minerals in broccoli stalks as have been reported by USDA (2019) and Liu *et al*. (2018). In addition, the two fibre-rich fractions obtained from broccoli stalks showed an

important content of minerals. The binding properties of minerals to fibre have been reported in several studies. The binding is affected by several factors such as the type and amount of fibre, the pH and the ionic strength of the fibre. In this sense, it has been reported that iron has a binding affinity with pectin, and the highest amount of iron was observed in $TF_B$ which had also the highest proportion of SDF. In addition, it has also been reported that zinc has a high binding affinity to insoluble fibres, an effect that has been also reflected in our study, where $IF_B$ showed the highest content of zinc at the same time that had the highest proportion of IDF (Miyada *et al*., 2011; Baye *et al*., 2017).

### 3.4. Composition of neutral sugars and uronic acids in the samples obtained from broccoli stalk

To assess the composition of DF present in the samples obtained from broccoli stalk, the neutral sugar profile and uronic acids were analysed (Table 18). Moreover, cellulose, pectin and hemicellulose content were calculated as function of the neutral sugar and uronic acid content, according to Umaña *et al*. (2016) (Table 19). In addition, the structure of the pectin was also calculated based on the composition of DF (Table 20) (Houben *et al*., 2011).

**Table 18.** Proportion of neutral sugars and uronic acid in broccoli stalk samples expressed as percentage (%).

| Sugars | DBS | $TF_B$ | $IF_B$ |
|---|---|---|---|
| **Rhamnose** | $1.8 \pm 0.1^{b*}$ | $1.7 \pm 0.1^{b}$ | $2.3 \pm 0.4^{a}$ |
| **Fucose** | $0.8 \pm 0.0^{b}$ | $0.7 \pm 0.1^{b}$ | $1.1 \pm 0.1^{a}$ |
| **Arabinose** | $15.9 \pm 0.6^{b}$ | $16.7 \pm 0.8^{b}$ | $18.5 \pm 0.5^{a}$ |
| **Xylose** | $16.4 \pm 0.5^{b}$ | $14.3 \pm 0.8^{c}$ | $20.2 \pm 0.6^{a}$ |
| **Mannose** | $4.5 \pm 0.1^{a}$ | $2.4 \pm 0.2^{c}$ | $2.7 \pm 0.1^{b}$ |
| **Galactose** | $10.0 \pm 0.3^{b}$ | $11.4 \pm 0.2^{a}$ | $10.7 \pm 0.7^{ab}$ |
| **Glucose** | $20.6 \pm 2.3^{a}$ | $3.6 \pm 0.3^{b}$ | $4.0 \pm 0.3^{b}$ |
| **Uronic acid** | $30.1 \pm 2.8^{c}$ | $49.3 \pm 1.6^{a}$ | $40.6 \pm 1.0^{b}$ |

*Values are expressed as mean ± SD (n =4). Different letters (a–c) indicate significant differences ($p$<0.05) among the samples. Freeze-dried broccoli stalk (DBS); total fibre fraction ($TF_B$); insoluble fibre fraction ($IF_B$).

The DF is constituted by NSP, polymers made up of more than 10 monosaccharides, being cellulose, hemicellulose and pectin the main components of the cell wall (Rezende *et al*., 2021). Table 18 shows the composition of different monosaccharides (pentose and hexoses) and uronic acids in the DBS and both fibre fractions ($TF_B$ and $IF_B$). The major component in the three samples was the uronic acid, showing significant differences among the samples. For the concentration of the neutral sugars, glucose was the main monosaccharide in DBS, whereas arabinose and xylose were the main monosaccharides in the $TF_B$ and $IF_B$, respectively. The main differences found in the composition of the three samples have been: DBS had more contribution of glucose and mannose, and less for uronic acids; $TF_B$ had more contribution of galactose and uronic acid and less of xylose and mannose, whereas $IF_B$ had highest content of rhamnose, fucose, arabinose and xylose. It was also remarkable that the sugar proportions were similar for DBS when compared to those obtained by other authors (Femenia *et al*., 2000; Schäfer *et al*., 2017), being glucose and uronic acids the main components, and the minority ones rhamnose and fucose. Regarding the uronic acid content, other authors found a proportion of 51% and 36% for alcohol-insoluble residue in broccoli stalks and broccoli florets respectively, due to the high fibre concentration in stalks (Houben *et al*., 2011). These results showed that the uronic acids are the main compounds of the DF in broccoli stalks and florets, as we have observed in our samples, ranging from 30% for DBS to 49.3% for $TF_B$ (Table 18). On the contrary, Schäfer *et al*. (2017), found arabinose as the main component for insoluble fibre obtained from broccoli stalk by methanolysis and glucose for insoluble fibre obtained from broccoli stalk obtained by acid hydrolysis, which showed that the extraction method used determines the composition of the fibre fractions obtained.

According to the composition of neutrals sugars and uronic acids, the content of cellulose, hemicellulose and pectin was estimated (Table 19). DBS was the samples with the highest proportion of cellulose (18.5%), being this component found in significantly lower proportion in $TF_B$ and $IF_B$ (around 3.5%) (Table 19).

Hemicellulose heteropolymer is divided into four major classes, xylans, mannans, xyloglucans and mixed linkage β-glucans, which are constituted by units of pentose sugars, such as arabinose and xylose, or hexose sugars, such as mannose, glucose and galactose (Holscher, 2017). Hemicellulose is insoluble in water solutions but soluble in alkaline ones (Dhingra *et al*., 2012). As show in Table 18, $IF_B$ showed the highest proportions of arabinose, xylose and galactose, without significant differences for galactose with $TF_B$. Regarding mannose and glucose proportions, the highest ones were

found for DBS. Consequently, the highest proportion of hemicellulose was found in $IF_B$ and DBS, but not significant differences were observed among them (Table 19). This fact is in concordance with the higher proportion of IDF observed in DBS and $IF_B$ (Figure 30), whereas $TF_B$ showed the higher content of pectin, being the fraction with the highest proportion of SDF (Figure 30). The contribution of mannose to the hemicellulose heteropolymer was calculated using a ratio previously described by Houben *et al*. (2011), and is shown in Table 20. The results showed that the mannose contribution was highest for DBS when compared with both fibre fractions. This effect could explain that DF in DBS were mainly composed by soluble hemicellulose due to the highest mannose contribution (Peng *et al*., 2019). In contrast, the highest xylose content, and therefore a lower value of this ratio, would indicate that the hemicellulose in $IF_B$ and $TF_B$ was mostly insoluble compared with DBS.

**Table 19.** Proportion of cellulose, pectin and hemicellulose of samples obtained from broccoli stalk expressed as percentage (%).

| Polysaccharydes | DBS | $TF_B$ | $IF_B$ |
|---|---|---|---|
| **Cellulose** [A] | 18.5 ± 2.1[a]* | 3.3 ± 0.2[b] | 3.6 ± 0.3[b] |
| **Pectin** [B] | 57.8 ± 2.8[c] | 79.1 ± 1.2[a] | 72.1 ± 0.7[b] |
| **Hemicellulose** [C] | 24.1 ± 0.3[a] | 17.6 ± 1.2[b] | 24.4 ± 0.6[a] |

*Values are expressed as mean ± SD (n =4). Different letters (a–c) indicate significant differences ($p$<0.05) among the samples. Freeze-dried broccoli stalk (DBS); total fibre fraction ($TF_B$); insoluble fibre fraction ($IF_B$). [A] Cellulose: Glucose x 0.9; [B] Pectin: (Rhamnose + Arabinose + Galactose + Uronic acids); [C] Hemicellulose: (Fucose + Xylose + Mannose + (Glucose x 0.1).

Pectin is a family of heteropolysaccharides consisting of a backbone of α–(1→4) galacturonic acid and other neutral sugars like rhamnose, arabinose and galactose. Regarding uronic acid the highest proportion was found in $TF_B$, being 1.2 and 1.6-fold higher than in $IF_B$ and DBS, respectively. In this sense, $TF_B$ also showed the highest proportion of pectin, followed by $IF_B$ and DBS. Three ratios were calculated to obtain information about the pectin structure (Table 20) (Houben *et al*., 2011). The results showed that pectin molecules in $TF_B$ seemed to be more linear in comparison to pectin molecules from $IF_B$ and DBS, being longer due to the highest content of SDF. The linearity of the pectin chains could provide some interesting physicochemical properties such as the emulsifying ability, which due to a high flexibility of the molecules has better

interfacial properties to stabilise emulsions (Houben *et al*., 2011; Mendez *et al*., 2021). Moreover, the rhamnose contribution was highest in DBS and $IF_B$, indicating that the pectin in these samples showed highest ramification with lateral chains of galactose and arabinose. However, the lateral chains were longer in $TF_B$, determined by the highest content of arabinose and galactose. Hence the different extraction procedures used in the isolation of $TF_B$ and $IF_B$ led to slightly changes in the molecular structure of pectin compared with the original pectins from DBS. As other authors have previously reported, the different conditions used in the extraction process, temperature, solvents or pH among others, may led to changes in the molecular structure of pectin (Belkheiri *et al*., 2021).

**Table 20.** Sugar ratios for pectin and hemicellulose characterisation from broccoli stalk samples.

| Ratio | DBS | $TF_B$ | $IF_B$ |
|---|---|---|---|
| **Man contribution[A]** | $0.3 \pm 0.0^{a*}$ | $0.2 \pm 0.0^{b}$ | $0.1 \pm 0.0^{c}$ |
| **Linearity[B]** | $0.7 \pm 0.1^{c}$ | $1.1 \pm 0.1^{a}$ | $0.8 \pm 0.0^{b}$ |
| **Rha-Uro contribution[C]** | $0.1 \pm 0.0^{a}$ | $0.03 \pm 0.00^{b}$ | $0.1 \pm 0.0^{a}$ |
| **RG-I branching[D]** | $14.7 \pm 1.0^{ab}$ | $16.7 \pm 1.3^{a}$ | $12.9 \pm 1.9^{b}$ |

*Values are expressed as mean ± SD (n =3). Different letters (a–c) indicate significant differences ($p$<0.05) among the samples. Freeze-dried broccoli stalk (DBS); total fibre fraction ($TF_B$); insoluble fibre fraction ($IF_B$). [A] contribution of mannans to hemicelluloses: Mannose/Xylose; [B] Linearity of pectins: Uronic acids/(Fucose + Rhamnose + Arabinose + Galactose + Xylose); [C] Contribution of rhamnose and uronic acids to pectins: Rhamnose/Uronic acids; [D] Branching of RG-I: (Arabinose + Galactose)/Rhamnose.

### 3.5. Physicochemical properties of dietary fibre present in the samples obtained from broccoli stalk

The physicochemical properties of fibre provide information about the technological properties and physiological effects in the gastrointestinal system (Dhingra *et al*., 2012; Baenas *et al*., 2020). Table 21 shows the results for water retention capacity (WRC), swelling capacity (SWC), fat absorption capacity (FAC) and osmotic pressure. The hydration properties were analysed according to the WRC and SWC, which indicate the capacity of the fibre to retain water in its structure. Highest values of WRC means an increase in faecal volume and a reduction of rectum pressure which help to prevent intestinal diseases, furthermore, when SWC increases also the satiety feelings increases (Liu *et al*., 2021).

$IF_B$ was the fraction with the highest values of hydration properties, and $TF_B$ showed the lowest values, with significant differences among the samples. According to Belkheiri *et al*. (2021), who reported that branched chains may negatively affect the gelation properties, it was observed in the present study that $TF_B$ which showed the highest branching ratio of pectin was the one that showed the lowest WRC and SWC, affecting this properties to the gelling capacity related with both parameters. Hydration properties of fibres are related to the content of pectin and hemicellulose. We have observed that these physicochemical properties were highest in $IF_B$, being positively correlated with hemicellulose content but not with the content of pectin. However, the higher WRC and SWC observed in DBS, when compared with $TF_B$, could be related to the retention of water for other components, such as the starch, due to the highest content of carbohydrates in this sample (Table 17). As other authors have shown, physicochemical properties are not only related to soluble or insoluble fibre content, but also to the method used for the extraction, that determines the physical structure, the particle size, porosity, hydrophobicity, which have not been measured in this work (Ahmed *et al*., 2013; Meng *et al*., 2019).

**Table 21.** Physicochemical properties of samples obtained from broccoli stalk.

| Physicochemical properties | DBS | $TF_B$ | $IF_B$ |
|---|---|---|---|
| **Water retention capacity** (g of water/g) | $6.4 \pm 0.9^{b*}$ | $3.9 \pm 0.3^{c}$ | $8.2 \pm 0.8^{a}$ |
| **Swelling capacity** (mL of water/g) | $17.1 \pm 0.8^{b}$ | $10.2 \pm 0.8^{c}$ | $20.3 \pm 0.8^{a}$ |
| **Fat absorption capacity** (g of oil/g) | $4.0 \pm 0.0^{a}$ | $3.7 \pm 0.0^{b}$ | $2.6 \pm 0.1^{c}$ |
| **Osmotic pressure** (mmol/kg) | $225.0 \pm 1.0^{a}$ | $157.0 \pm 1.0^{c}$ | $187.3 \pm 3.8^{b}$ |

*Values are expressed as mean ± SD (n =3). Different letters (a–c) indicate significant differences ($p$<0.05) among the samples. Freeze-dried broccoli stalk (DBS); total fibre fraction ($TF_B$); insoluble fibre fraction ($IF_B$).

The WRC values obtained in the samples from broccoli stalks were a bit higher than those reported for some fruit by-products, such as apple and grapefruit, which ranged between 1.6 and 2.3 g of water/g respectively, and were similar compared to that obtained for tomato peel fibre (6.7 g of water /g) (Figuerola *et al*., 2005; Navarro-González *et al*., 2011). The results obtained for $IF_B$ were similar to that reported for a mixture of broccoli stalks and leaves being 8.8 g of water/g (Shi *et al*., 2019). Regarding

SWC, the results were similar to that obtained by Wang *et al*. (2015) for five different types of citrus fibre ranging from 15 to 24 mL of water/g. However, they were higher than those obtained in the Chapter 4 for raspberry fractions.

The FAC was the capacity to retain fat or oil, which is used in the industry to stabilise fatty products and it depends on the chemical and physical structure of the fibres. The highest value was obtained for DBS (4g of oil/g), being higher to those obtained by other authors for TDF and IDF from pomelo (1.2 and 1.1 g of oil/g, respectively) and similar to other citrus fruits (3.6-8.2 g of oil/g) (Wang *et al*., 2015; Liu *et al*., 2021).

Regarding the osmotic pressure, the lowest value was obtained for $TF_B$ (157 mmol/kg) and the highest for DBS (225 mmol/kg) but any of them were expected to induce diarrhoea after consumption, as they were below the physiological values previously reported (290 mmol/kg) (Lawrence and Joseph, 2015).

### 3.6. Glucosinolate content of samples obtained from broccoli stalk

Glucosinolates content was identified by HPLC-DAD-ESI-MSn and quantified by HPLC-DAD. The identification was performed in both extractable and non-extractable fractions, but no glucosinolates were identified in the second fraction, showing that these bioactive compounds can be extracted from plant food and are not attached to cell walls. The content of the identified glucosinolates is shown in Figure 31, being GRA the only aliphatic glucosinolate, while the rest of the compounds belong to the indolic group. These results are in agreement with those reported by other authors, who have described that the major glusosinolate is the GRA, being in some cases up to 70% of the total (Alvarez-Jubete *et al*., 2014; Liu *et al*., 2018; Thomas *et al*., 2018). The GRA, MGB and NGB were found in the three samples, whereas GBS was only found in DBS and $IF_B$, and HGB was only quantified in DBS.

Moreover, the content of NGB, GBS and HGB were higher in DBS sample than in the fibre fractions ($TF_B$ and $IF_B$), except for the content of MGB, which was significantly higher in both DBS and $IF_B$ compared to $TF_B$. The content of GRA and consequently the aliphatic glucosinolates did not show significant differences between the three samples. On the other hand, the indolic group was significantly higher in DBS and lower in $TF_B$ ranging from 92 to 22.4 mg/100 g, respectively. The total contents of glucosinolates were approximately within the range reported by other authors for broccoli stalk samples,

values ranging between 69 and 140 mg/100 g of d.w. (Domínguez-Perles *et al*., 2010; Thomas *et al*., 2018).

The content of glucosinolates in DBS samples can be considered relatively high, since it is remarkable that the content of these bioactive compounds in stalks is lower 3 or 5-fold than the content in the florets (Alvarez-Jubete *et al*., 2014; Liu *et al*., 2018). The differences in the total content of glucosinolates in the samples could be due to the extraction process. While DBS was the freeze-dried stalk, and hence contains all the glucosinolates of the fresh sample, the $TF_B$ and $IF_B$ were extracted after removing some alcohol and water-soluble compounds. Glucosinolates are soluble in alcohol and water and for this reason the samples of fibre showed a significant lowest content, being discarded in a higher proportion during the extraction of $TF_B$ than during isolation of $IF_B$, due to the use of ethanol.

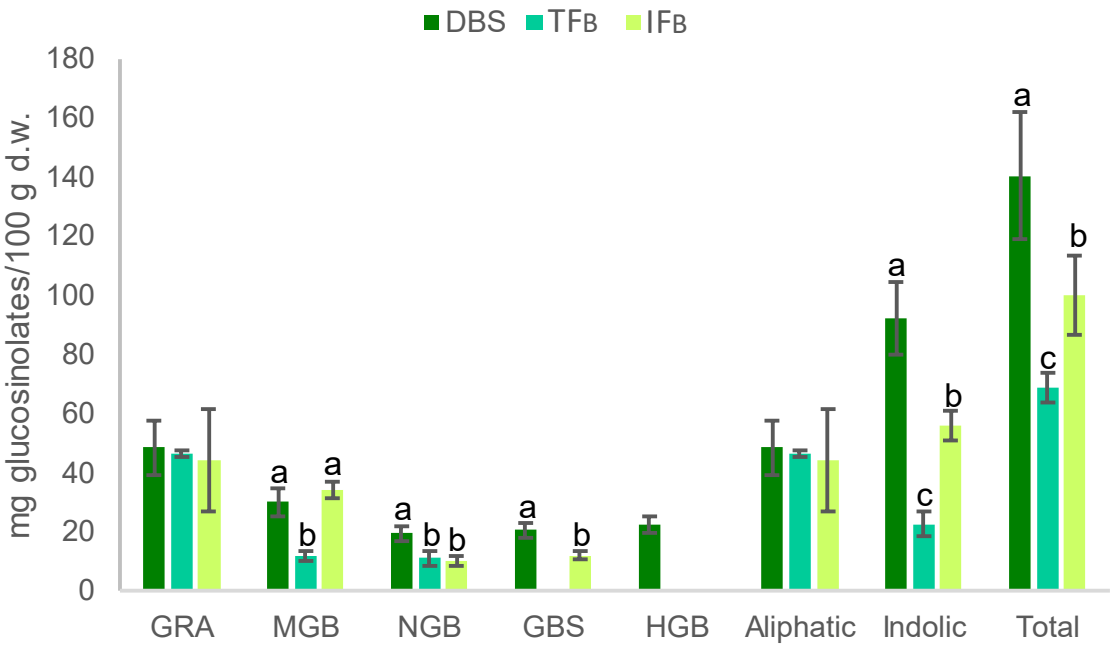


**Figure 31.** Contents of glucosinolates (mg/100 g of d.w.) analysed by HPLC-DAD of freeze-dried broccoli stalk (■DBS), total fibre fraction (■$TF_B$) and insoluble fibre fraction (■$IF_B$). Glucosinolates are presented as individual compounds (GRA: glucoraphanin (4-Methylsulphinybutyl-gls), MGB: 4-Methoxyglucobrassicin (4-Methoxy-3-indolylmethyl-gls), NGB: neoglucobrassicin (1-Methoxy-3-indolylmethyl-gls), GBS: glucobrassicin (3-Indolylmethyl-gls), HGB: 4-Hydroxyglucobrassicin (4-hydroxy-3-indolylmethyl)). Values are expressed as mean ± SD (n = 3). Different letters (a–c) indicate significant differences ($p$<0.05) among the samples for each individual and total glucosinolates.

### 3.7. Total (poly)phenols and antioxidant capacity in the samples obtained from broccoli stalk

(Poly)phenols were identified by HPLC-DAD-ESI-MSn and quantified by HPLC-DAD, in both EPP and NEPP fractions and the results are showed in Figure 32. The majority of the (poly)phenols quantified in the samples were NEPP, representing a 98%, 97% and 79% from the total (poly)phenols, for $TF_B$, $IF_B$ and DBS, respectively. On the other hand, the EPP represented a fraction around 21% in DBS and a very small fraction in the fibre-rich samples.

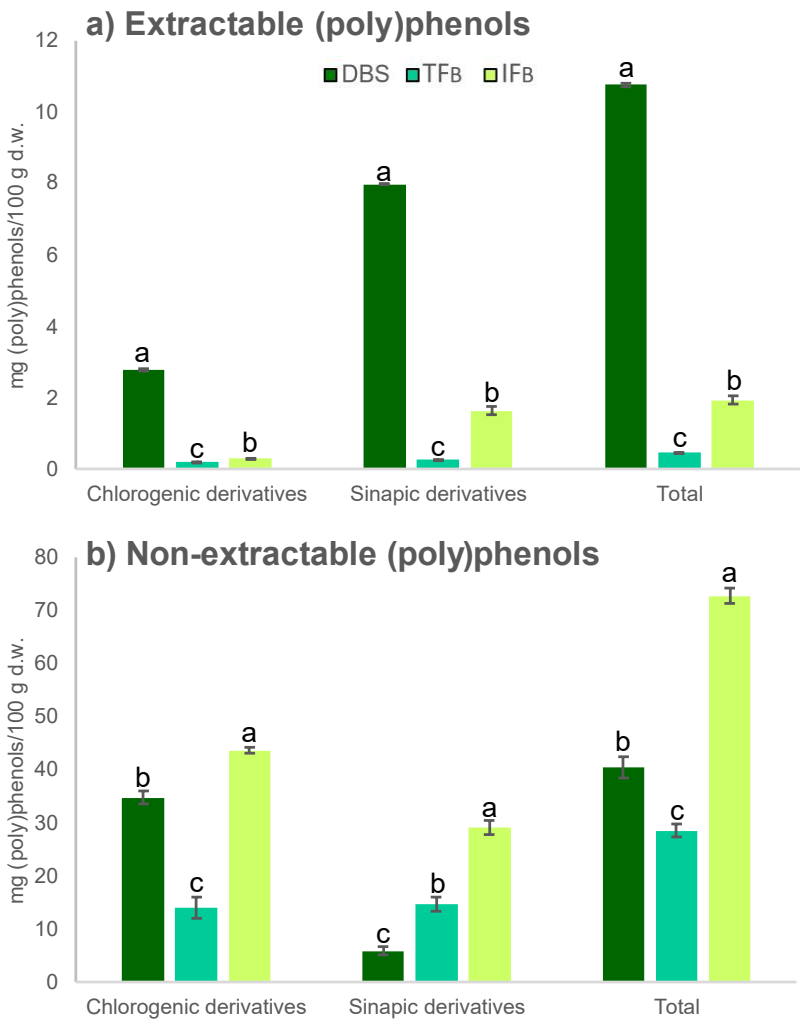


**Figure 32.** Contents of individual (poly)phenols (chlorogenic acid derivatives and sinapic acid derivatives) and the total contents of (poly)phenols determined as the sum of the individual compounds (mg/100 g d.w.), being a) extractable (poly)phenols (EPP) and b) non-extractable (poly)phenols (NEPP) analysed by HPLC-DAD for freeze-dried broccoli stalk (■DBS), total fibre fraction (■$TF_B$) and insoluble fibre fraction (■$IF_B$). Values are expressed as mean ± SD. Different letters (a–c) indicate significant differences among the samples ($p$<0.05) per each individual group and total (poly)phenols.

The total EPP was significantly higher in DBS, than in $IF_B$ and $TF_B$, with mean values of 10.8, 2.0 and 0.5 mg/100 g of d.w., respectively. The identified (poly)phenols in the EPP were the sinapic derivatives and chlorogenic derivatives groups. On the other hand, the content of NEPP was significantly highest in $IF_B$ (72.7 mg/100 g d.w.), followed by DBS (40.5 mg/100 g of d.w.) and $TF_B$ (28.5 mg/100 of g d.w.). Chlorogenic and sinapic derivatives were also quantified but chlorogenic derivatives were the main (poly)phenols for $IF_B$ and DBS. These findings showed that sinapic derivatives and chlorogenic derivatives were mainly bound to the fibre compounds, as part of the cell wall.

Although studies on the analysis of individual (poly)phenols in broccoli stalks are very scarce, Domínguez-Perles *et al*. (2010) and Thomas *et al*. (2018), showed that the main (poly)phenols in broccoli stalks of different cultivars are the hydroxycinnamic acids, being chlorogenic acid derivatives the main group. In contrast to our study, flavonoids have been found by Domínguez-Perles *et al*. (2010), although it is noteworthy that only in two cultivars of the three varieties analysed (Nubia and Viola). In our study, Parthenon cultivar was used and did not show flavonoids neither in the DBS nor in the fibre-rich fractions.

The differences observed in the content of EPP and NEPP in the three samples will be explained by the extraction procedure applied for each sample. While in DBS, whole fresh stalks, the content of (poly)phenols was not modified, in $TF_B$ and $IF_B$ the extraction procedure removed part of the EPP compounds, discarding the (poly)phenols solubilised in 80% ethanol and in water, respectively. For this reason, the total content of EPP was 22-fold and 5.5-fold higher in the DBS when compared with $TF_B$ and $IF_B$. However, in the NEPP, the chlorogenic and sinapic derivatives linked to DF compounds, were in $IF_B$ 2.6-fold and 1.8-fold higher than DBS and $TF_B$, respectively. The total content of (poly)phenols was lower in this study when the results are compared with that reported by Domínguez-Perles *et al*. (2010), with values ranging from 8 to 12 mg/g for broccoli stalk from three different cultivars.

Results for TPC measured by spectrophotometric method are shown in Table 22. This method provided a measure of the EPP, and hence DBS showed the highest values (154.7 mg/100 g of d.w.), followed by $IF_B$ (139 mg/100 of g d.w.) and $TF_B$ (39.3 mg/100 g of d.w.), obtaining the same order than in the HPLC-DAD analysis. When we compare the results of our samples with the results obtained by other authors for other parts of the plant (Liu *et al*., 2018), we can see that the content in leaves (414 mg/100 g) and florets (251 mg/100 g) was also highest, as previously demonstrated, since the presence of (poly)phenols in the stalk is lower than in other parts of the plant (Domínguez-Perles

*et al*., 2010; Thomas *et al*., 2018). It is remarkable that other authors have obtained higher TPC for stalks when compared with our results, these differences as mentioned above may be due to the different varieties or growing conditions (Domínguez-Perles *et al*., 2010). On the contrary, Liu *et al*. (2018) found similar TPC content in broccoli stalks (1.3 mg GAE/g) to that reported in our study for DBS and $IF_B$.

**Table 22.** Total phenolic content (TPC) (mg GAE/100 g of d.w.), ferric reducing antioxidant power (FRAP) (µmol TE/100 g of d.w.) and oxygen radical absorbance capacity (ORAC) (µmol TE/100 g of d.w.) in the samples obtain from broccoli stalk.

| | **DBS** | **$TF_B$** | **$IF_B$** |
|---|---|---|---|
| **TPC** | 154.7 ± 8.3$^{a}$* | 39.3 ± 2.4$^{c}$ | 139.0 ± 8.7$^{b}$ |
| **FRAP** | 264.0 ± 9.6$^{a}$ | 102.7 ± 2.1$^{c}$ | 229.6 ± 10.4$^{b}$ |
| **ORAC** | 2821.7 ± 96.5$^{a}$ | 1666.9 ± 40.8$^{c}$ | 1856.4 ± 19.0$^{b}$ |

*Values are expressed as mean ± SD (n=3). Different letters (a–c) in the same row indicate significant differences among the samples ($p<0.05$). Freeze-dried broccoli stalk (DBS); total fibre fraction ($TF_B$); insoluble fibre fraction ($IF_B$).

The antioxidant capacity measured by FRAP and ORAC methods (Table 22) were analysed to test ferric reducing capacity and scavenging capacity. It is remarkable that in both cases DBS showed the highest antioxidant capacity, followed by $IF_B$ and $TF_B$. These results are correlated with the content of glucosinolates and (poly)phenols in the samples, which both have antioxidant capacity (Biegańska-Marecik *et al*., 2017), showing a correlation coefficient of r=0.86 and r=0.88 ($p<0.05$), respectively. Both groups of bioactive compounds were significantly higher in DBS samples than in $TF_B$ and $IF_B$, and hence this sample showed the highest antioxidant capacity. It is remarkable that in both analytical procedures the antioxidant capacity only provides activities of the extractable compounds. When we compared our results with those obtained by Shi *et al*. (2019), we noticed that these authors found an antioxidant capacity of 203.7 µmol TE/g for pomace from broccoli stalks and leaves, which was higher than that reported in our data because, as previously indicated, other parts of the plant such as florets and leaves have a higher content of compounds and therefore a higher antioxidant capacity. In contrast, these authors also analysed the content for a washed pomace, which was obtained by a similar process to the one used to obtain $IF_B$ in our study. In this case, an antioxidant capacity of 7.3 µmol TE/g was observed in ORAC assay, which was lower than our results, indicating that during the extraction process of that fraction part of the

original compounds are lost and therefore the antioxidant capacity is reduced (Shi *et al*., 2019).

### 3.8. Evaluation of the potential prebiotic effect and ammonium production

To evaluate the potential prebiotic effect of samples obtained from broccoli stalk, only DBS and $IF_B$ samples were subjected to an *in vitro* fermentation using human faecal samples. DBS was used because it represents the whole broccoli stalk, where $IF_B$ was chosen, instead of $TF_B$, because showed a similar content of IDF but a highest content in bioactive compounds (glucosinolates and (poly)phenols). Although $TF_B$ contains more SDF, the extraction procedure of $IF_B$, using only hot water, was cleaner, more environmentally friendly and less contaminant, avoiding the application of organic solvents.

To determine the prebiotic effect 1% (0.5 g) of digested DBS and $IF_B$ were inoculated into the *in vitro* fermentation batch, using as substrate for the microbiota from the faecal slurry. Moreover, a negative control with the fecal slurry but without sample was also tested. The content and the evolution on the formation of main SCFAs (acetate, propionate and butyrate) during the *in vitro* fermentation is represented in Figure 33, other minor SCFAs and the total SCFAs are represented in Figure 34. The two-way ANOVA (data not shown), showed that both factors (sample and time of fermentation), significantly determined the content of all SCFAs content, as well as the interaction sample *x* time ($p<0.05$).

As was previously reported, the main SCFAs was acetate followed by propionate and butyrate, with an approximate molar ratio of 60:23:17, respectively (Blaut, 2018). Other minor SCFAs were represented by the content of isobutyrate, valerate, isovalerate, caproate and isocaproate, being isobutyrate and isovalerate the most abundant (Tejada-Ortigoza *et al*., 2019; Karimi *et al*., 2020).

As shown in Figure 33 and Figure 34, the fermentation of $IF_B$ led to a highest formation of acetate, other minor SCFAs and total SCFAs at 48 h. On the contrary, the fermentation of DBS allowed to obtain a higher content of butyrate at 48 h than $IF_B$. The negative control, without sample inoculated, increased slightly the content of SCFAs in the first 4 hours of fermentation, reaching after that a constant value being 4.1 and 4.6-fold lower than for DBS and $IF_B$, respectively. Only, for other minor SCFAs at 24 h of fermentation, the control showed a value until 4-fold higher compared with the fermentations carried out with the addition of the samples, indicating that the bacteria

were consuming tryptone and leading to a highest production of isobutyrate, valerate and isovalerate as end products from this amino acid degradation pathway (Rios-Covian *et al*., 2020).

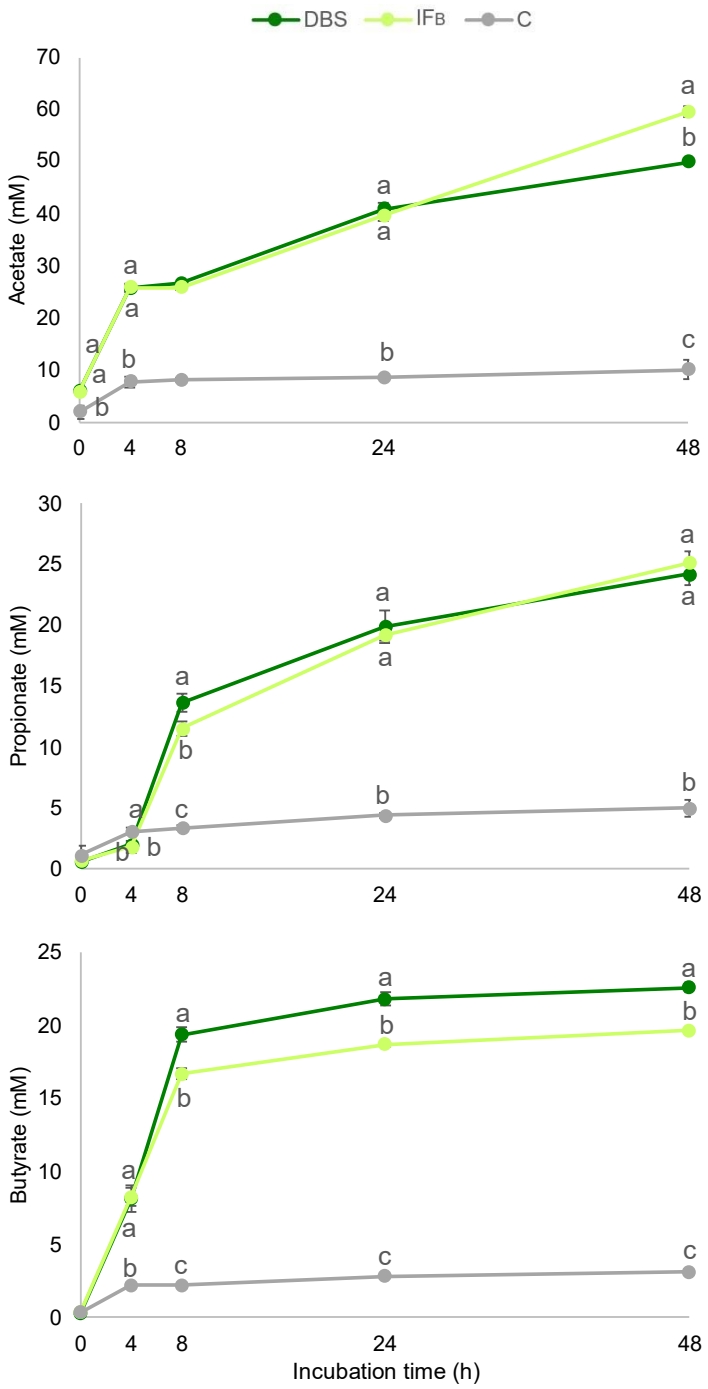


**Figure 33.** SCFAs production (acetate, propionate, butyrate) (mM) during *in vitro* fermentation of samples obtained from broccoli stalk. Freeze-dried broccoli stalk (●DBS); insoluble fibre fraction (●$IF_B$) and control (●C). Values are expressed as mean ± SD (n =3). Different letters (a–c) indicate significant differences ($p$<0.05) among the samples at the same incubation time.

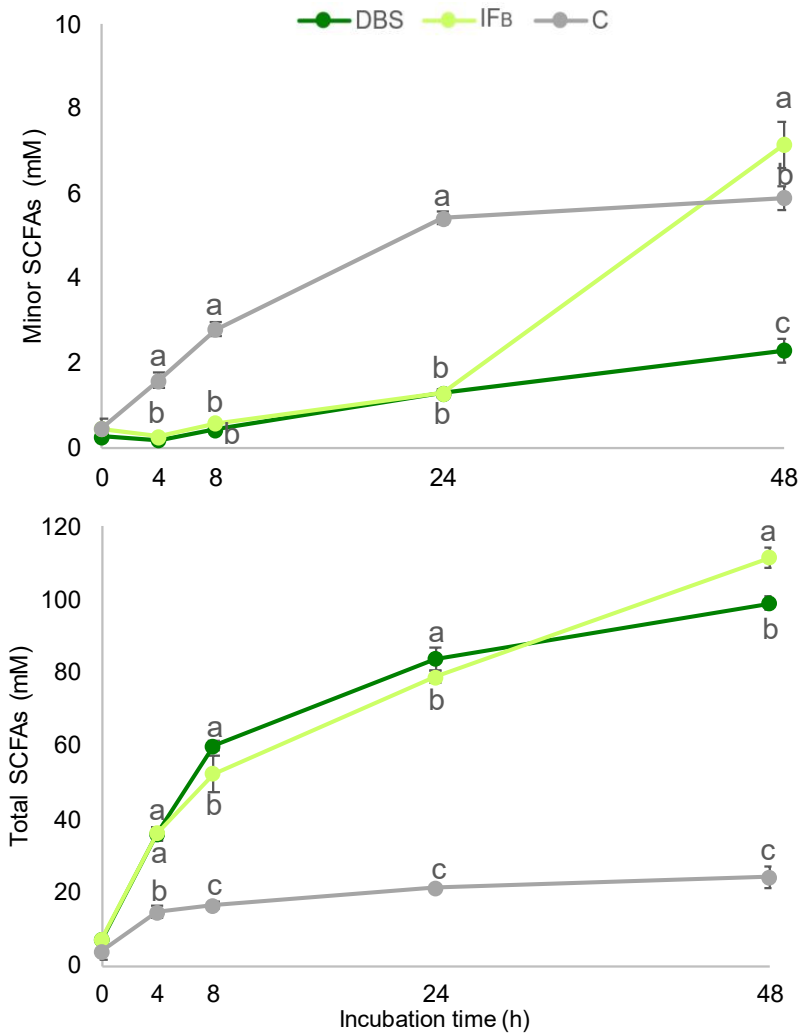


**Figure 34.** Other minor SCFAs (isobutyrate, isovalerate, valerate, isocaproate, caproate and heptanoate) and total SCFAs production (mM) during *in vitro* fermentation of samples obtained from broccoli stalk. Freeze-dried broccoli stalk (●DBS); insoluble fibre fraction (●$IF_B$) and control (●C). Values are expressed as mean ± SD (n =3). Different letters (a–c) indicate significant differences ($p$<0.05) among the samples at the same incubation time.

A highest butyrate content after fermentation of DBS could be explained because the sample of whole stalks showed a highest content of carbohydrates, including starch polysaccharides. It is assumed that starch is mainly absorbed in the small intestine, but some fraction of starch can be resistant to the digestion, reaching the colon where it can be fermented by microbiota leading to the formation of butyrate (DeMartino and Cockburn, 2020). Despite of the content of resistant starch was not analysed, a small fraction could be in the sample after the manipulation and freeze-drying process. Moreover, the whole stalk sample showed a highest content of carbohydrates, being these components around 44% of the total (Table 17).

So, with the exception of the formation of propionate, where differences were not observed. In general, the production of acetate, butyrate and other minor SCFAs determined the prebiotic effect of the samples. $IF_B$ exhibits a higher prebiotic effect than DBS, leading to a higher content of total SCFAs. It is known that the molar distribution and the total content of SCFAs depend on the type and amount of the DF, and in an *in vitro* fermentation assay, is determined by the composition of the substrate. Moreover, not only the chemical composition of fibre is important to determine the prebiotic effect of plant foods, but also the physicochemical properties such as the viscosity and hydration properties influence the fermentability, being pectin the fraction the most fermentable by the microbiota (Prandi *et al*., 2018; Gallotti *et al*., 2020; Widaningrum *et al*., 2020), but also cellulose and hemicellulose could be used and converted in to SCFAs. According to other authors (Widaningrum *et al*., 2020), the content of uronic acids and cellulose in apple and celery stalk led to a higher production of total SCFAs during *in vitro* fermentation, compared with banana samples, which showed no uronic acids and a low content of cellulose. In addition, the *in vitro* fermentation of citrus pectin increased significantly the production of acetate and butyrate (Bang *et al*., 2018).

Due to the higher content of pectin in $IF_B$ than in DBS (Table 19), the formation of total SCFAs was significantly higher in this fibre-rich fraction. Moreover, the prebiotic effect of $IF_B$ could be attributed to a synergistic effect between fibre and the bioactive compounds, since this fraction showed highest content in fibre and also contains significant amounts of (poly)phenols and glucosinolates, which have previously been attributed a prebiotic-like effect leading to the modulation of the microbiota (Kaczmarek *et al*., 2019; Alves-Santos *et al*., 2020; Shock *et al*., 2021).

As for the SCFAs proportion after 48 h of fermentation (Figure 35), as indicated above, the main part was represented by acetate. It should be noted that for DBS and $IF_B$, the second most important was propionate and other minor SCFAs being the ones found in the lowest proportion. On the other hand, in the control, the other minor SCFAs were the second most important, as have been previously described, due to no sample was inoculated. In addition, the minor one was butyrate, as this SCFA is derived from the degradation of non-digestible carbohydrates (Shortt *et al*., 2018; Szentirmai *et al*., 2019).

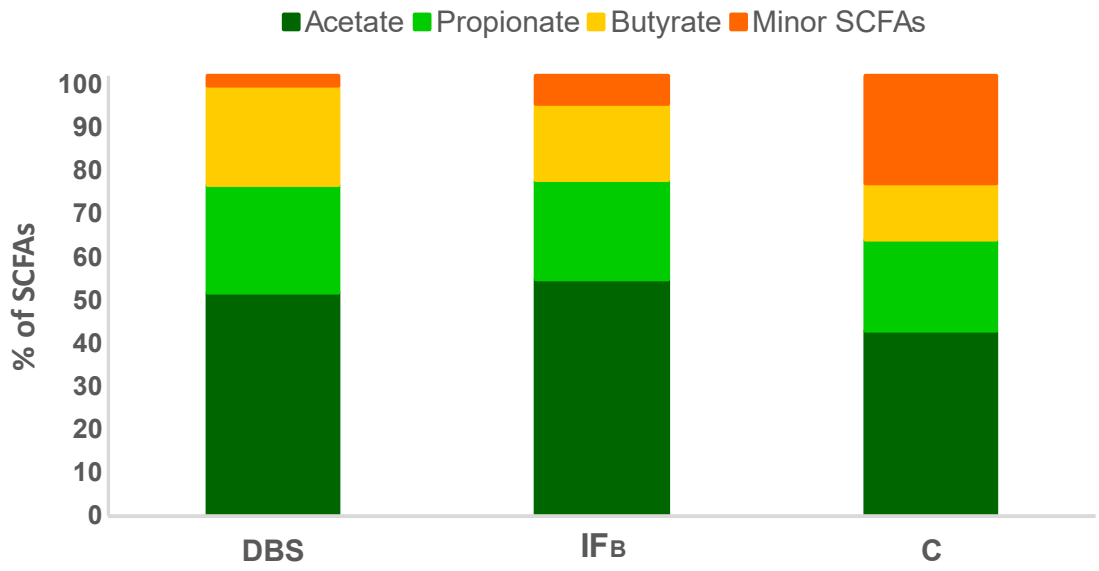


**Figure 35.** Acetate (■), propionate (■), butyrate (■) and other minor SCFAs (■) proportion (%) at 48 h of *in vitro* fermentation of freeze-dried broccoli stalk (DBS); insoluble fibre fraction ($IF_B$) and control (C). Values are expressed as mean (n =3).

Ammonium is a fermentation by-product from protein substrates, and it is formed when the content of carbohydrate is limited in the intestinal lumen (Blaut, 2018). Protein degradation in the colon occurs in several steps and involves different bacteria leading to formation of metabolites (amines, ammonia, sulfur compounds and mercaptans) that due to its toxic effects may affect host health. In our study, ammonium production comes mainly from the consumption of tryptone by bacteria, which is used as fermentation medium and had nitrogen as its main component. Although there were significant differences between the different samples tested (Figure 36), it should be noted that the levels of ammonium production are very low and therefore not likely to cause toxic effects. It is remarkable that the lowest content was obtained in the control, because after 4 hours of fermentation, the microbial activity was notably reduced due to the lack of substrate. After 24 h and 48 h of fermentation the ammonium production was highest in $IF_B$ than in DBS. This effect could be due to the highest content of pectin in $IF_B$, because as other authors have previously reported, pectin increases the microbial activity and metabolism leading to a highest production of SCFAs as previously described, and using proteins from the medium with the subsequent increase in ammonium production due to the highest metabolic activity (Barry *et al*., 2010; Blanco-Pérez *et al*., 2021).

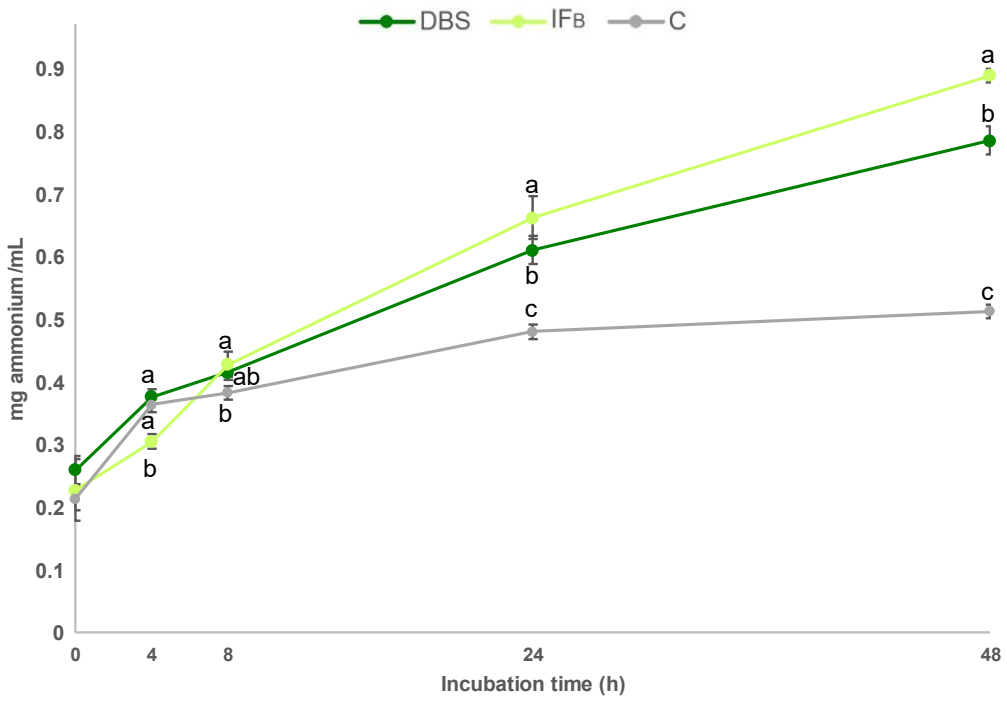


**Figure 36.** Ammonium production (mg ammonium/mL faecal slurry) during *in vitro* fermentation of samples obtained from broccoli stalk. Freeze-dried broccoli stalk (●DBS); insoluble fibre fraction (●$IF_B$) and control (●C). Values are expressed as mean ± SD (n =3). Different letters (a–c) indicate significant differences ($p$<0.05) among the samples at the same incubation time.

The Pearson´s correlation (Figure 37) has shown that the content of pectin and NEPP contents were positively correlated significantly with the production of acetate (r = 0.9 and r = 1), other minor SCFAs (r = 0.9 and r = 1), total SCFAs (r = 0.9 and r = 0.9), and ammonium (r = 0.9 and r = 1), during the *in vitro* fermentation. However, the production of butyrate was negative correlated with pectin and NEPP content in the samples (r = -1 and r = -1). On the other hand, cellulose and EPP was negatively correlated with the production of acetate, minor and total SCFAs, and ammonium. Being no significant in the case of propionate and being positively correlated with the production of butyrate. It was also noteworthy that glucosinolates content was positively correlated with butyrate production (r = 0.9), and negatively with acetate (r = -0.8), not being significant the correlations with propionate, minor and total SCFAs. This behaviour may be explained because the sample of DBS led to the highest formation of butyrate, probably ought to the non-digestible carbohydrates content, and was the sample with more NEEP and glucosinolates. The other components of DF, such as hemicellulose, did not show significant correlation with the microbiota metabolites (SCFA and

ammonium), as well as any correlation was observed between DF components and the production of propionate.

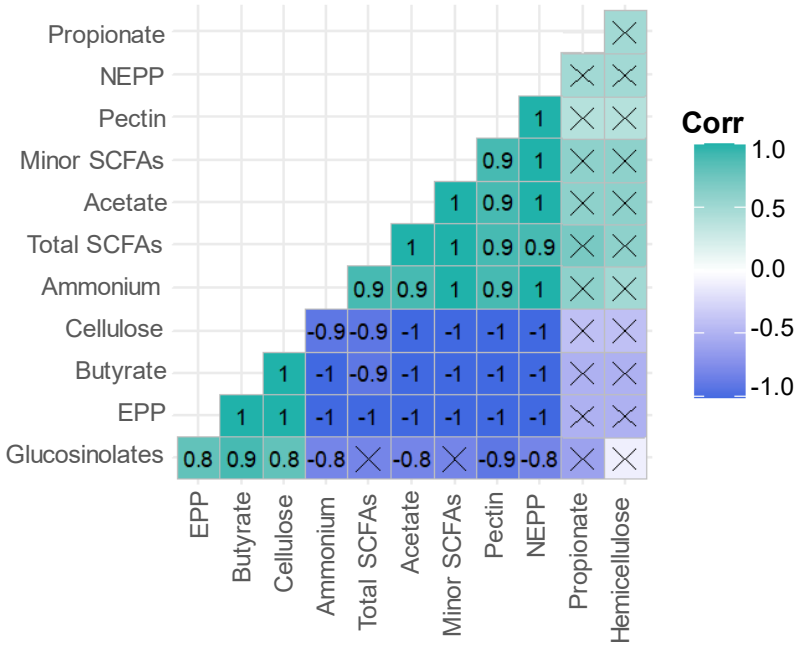


**Figure 37.** Correlation analysis between cellulose, pectin, hemicellulose content, short chain fatty acids (SCFAs) (acetate, propionate, butyrate, minor SCFAs and total SCFAs), ammonium production, extractable (poly)phenols (EPP) and non-extractable (poly)phenols (NEPP) at 48 h of fermentation of freeze-dried broccoli stalk (DBS) and insoluble fibre fraction ($IF_B$). The cross (X) means non-significant differences for the labelled correlations. Significant differences were set at $p<0.05$.

To sum up, in both fibre-rich fractions, the presence of fibre, (poly)phenols and glucosinolates could be considered interesting from a nutritional point of view. Regarding the proportion of glucosinolates in the fibre-rich fractions, GRA represented the highest proportion, followed by MGB, being the indolic glucosinolates the major group. In addition, $IF_B$ showed a good prebiotic effect resulting in the production of SCFAs, this together with its content of bioactive compounds and fibre, make it an interesting fraction both at nutritional and technological level. Being this a way of reintroducing an industrial by-product as in a new ingredient format which has been extracted with water, using a clean and environmentally friendly method.

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# Chapter 7. By-products from orange

## 1. Introduction

Citrus fruits, originally from the tropical and subtropical regions of Asia and Oceania, are nowadays one of the most widespread and well-known fruits worldwide, representing oranges more than half of the citrus fruit production. Brazil and China are the largest orange producers in the world, whereas in Europe, Spain accounts for more than half of the European production, being Murcia Region the third largest producer after Andalucía and Valencia (FAO, 2020; FAO, 2021; MAPA, 2021).

One-third of the production of oranges is used for industrial processing (FAO, 2021), and the consumption of oranges as fresh fruit has been replaced over the years by the consumption of juice. Orange juice is the most widely consumed fruit juice in the world due to its organoleptic and nutritional properties, as well as its simple preparation (Naz, 2018; Sahar *et al.*, 2019). So, fresh oranges and its fresh juice are one of the main sources of vitamin C in the diet, and also provide (poly)phenols, carotenoids, terpenoids and folic acid with important antioxidant and anti-inflammatory health properties (Sahar *et al.*, 2019).

From the industrial production of orange juice, a large volume of by-products is generated, such as peel, pulp, membranes and seeds, which account for approximately 70% of the weight of fresh fruit. These by-products have been either used for animal feed or landfilled, as they are often a problem of management for the producers (Siles *et al.*, 2016; Terzioğlu *et al.*, 2021). Some juice industries recover some of their by-products for reintroduction into the food chain, although it should be noted that the large amount of by-products produced is not recovered and that many industries lack of the resources to carry out these procedures. In this sense, some companies make products from the mixture of the by-products obtained, such as comminuted, which is used to give colour and flavour to soft drinks (Berk, 2016). Nowadays, new methods have been developed for the valorisation of these by-products, among them the production of biopolymers, biofuels and the extraction of compounds of interest (Liu *et al.*, 2021; Terzioğlu *et al.*, 2021). The orange peel is constituted by flavedo, which is the external part with an orange colour, and albedo which is the internal part with a whitish colour. Despite of the orange peel is not extensively used in human nutrition, it has been considered a source of several bioactive compounds, and hence this by-product can be valorised after using different extraction methods. Firstly, orange peel is a source of soluble fibre, such as pectin, and insoluble fibre such as cellulose, hemicellulose and lignin (Tütem *et al.*, 2020). In addition to fibre, orange peel also includes other nutritional compounds, such

as vitamin C, flavanones and carotenoids, which are mainly found in flavedo, whereas in the albedo there are mainly flavanones (Escobedo-Avellaneda *et al.*, 2014). These bioactive compounds have multiple functions, including antioxidant, antimicrobial, anti-inflammatory and anti-cancer effects, among others (Luiza Koop *et al.*, 2022).

Dietary fibre, as prebiotic compound, can be totally or partially fermented in the large intestine leading to the modulation of microbiota. From this fermentation short-chain fatty acids (SCFAs) are derived that have important effects on consumers health. Fibre consumption has therefore been linked to the prevention or amelioration of diseases such as obesity, diabetes, some types of cancer and intestinal diseases (He *et al.*, 2022). At meantime, the (poly)phenols present in the orange peel by-products can also reach the colon. As has been shown in studies with ileostomist, ~70% of ingested flavanones reach the colon intact, where they can be metabolised by the colonic microbiota (Borges *et al.*, 2013), which through enzymatic transformation gives rise to less complex compounds, such as phenolic and aromatic acid catabolites, which are absorbed into the circulatory system, and may play a key role in the beneficial health effects that exert the consumption of fruit and vegetables (Corrêa *et al.*, 2019; Martínez-Meza *et al*., 2021). The production of SCFAs and phenolic catabolites could be modified by the presence of fibre, which may enhance the health benefits (Mansoorian *et al.*, 2019; Havlik *et al.*, 2020).

Based on this background, the aim of this chapter was to extract different dietary fibre-rich fractions from orange peel to valorise this by-product. The fibre-rich ingredients were characterised to know the physicochemical properties and the chemical composition of dietary fibre, including their content in bioactive compounds ((poly)phenols and carotenoids). Moreover, to study the functionality and the prebiotic effect, an *in vitro* faecal fermentation assays were carried out measuring the SCFAs and phenolic acid catabolites production.

## 2. Material and methods

### 2.1. Extraction process of orange peel by-products

Three samples were extracted from orange peel by-products of Navelate variety (Cieza, Spain) (Figure 38). The extraction procedure is described in Figure 39. The orange peel was milled in a Thermomix TM-31. After milling the whole peel was dried at 42 °C until constant weight to obtain the orange peel extract (OP). On the other hand,

the whole peel was mixed with water in proportion 2.5/1 (v/m) and stirred for 15 min. After that the mixture was centrifuged at 4500 *g* during 5 min at room temperature, and then the supernatant (completely gelled) was separated from the pellet. Supposing that the supernatant fraction contains the soluble dietary fibre, was freeze dried until constant weight obtaining the water-soluble extract (WSE), whereas the pellet, containing the insoluble fibre, was dried until constant weight at 42 °C to obtain the insoluble fibre fraction (IFF).


**Figure 38.** Fractions incubated in the *in vitro* fermentations. Orange peel (OP); insoluble fibre fraction (IFF); water-soluble extract (WSE).

To characterise the different fractions, the proximate and nutritional composition (protein, ash, TDF, IDF, SDF and mineral composition), proportion of neutral sugars and uronic acids in the dietary fibre, as well as its physicochemical properties were determined according to the methods described in Chapter 4 (pages 75-78). Moreover, total phenolic content (TPC) (Chapter 5, page 114), antioxidant capacity, and the qualitative and quantitative profile of (poly)phenols (Chapter 4, pages 78-80) were determined.

Due to the high content of carotenoids as esterified compounds present in orange, its analysis was carried out after saponification according to the method described by Nan *et al.* (2012). Briefly, 0.1 g of sample was mixed with $NaHCO_3$ and ethyl acetate/methanol/petroleum ether (1/1/1, v/v/v) with 0.1% butylated hydroxytoluene, the samples were mixed and put in ultrasonic bath during 5 min. The samples were centrifuged, recovering the supernatant, and repeating this process twice. Finally, the supernatants were washed with 5% NaCl, evaporating in a rotary evaporator at 35 °C and resuspended in diethyl ether and mixed with 30% KOH, then stirred during 8 h in darkness. After that, the samples were washed with 5% NaCl until neutral pH, evaporating the supernatant in a rotary evaporator at 35 °C and resuspended in methyl tert-butyl ether/methanol (1/1, v/v), following the same process described in Chapter 6 (pages 149-150). To evaluate the prebiotic effect, the samples obtained following the

flow diagram indicated in Figure 39, were *in vitro* digested to simulate gastrointestinal conditions. After that, the three samples were *in vitro* fermented using a pooled faecal inoculum. In addition, a negative control (C-) without any sample added and a positive control (C+) with glucose to compare with WSE due to its high content of carbohydrates were also incubated. To prepare the pooled of faeces a group of 10 healthy normal-weight women volunteers were selected (aged between 25-35 years old), but the day of the experiment only 8 volunteers provide the faecal sample. Volunteers had to follow nutritional restrictions two days prior to the study, excluding all the citrus fruits and its derived products in the diet, and meet the requirements described in Chapter 5 (pages 115-116). *In vitro* fermentations were carried out during 48 h and samples were taken at 0, 4, 8, 24 and 48 h, in which SCFAs and ammonium were measured following the methods described in Chapter 5 (page 116). In addition, in this experiment the analysis of the phenolic acids catabolites produced by the microbiota was carried out by UHPLC-HRMS, to identify and quantify the degradation of the orange (poly)phenols as well as the production of catabolites over 48 h of faecal fermentation with different fractions of orange peel (Figure 38).

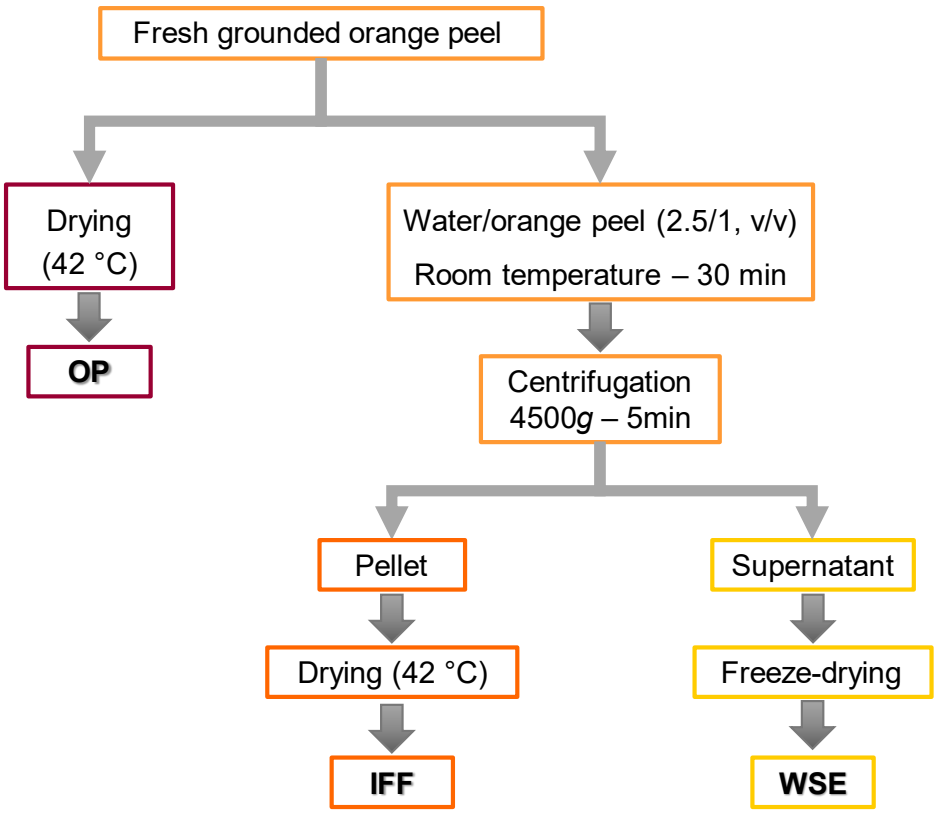


**Figure 39.** Flow diagram of the procedure used to obtain the three orange peel fractions, orange peel extract (OP), insoluble fibre fraction (IFF) and water-soluble extract (WSE).

### 2.2. UHPLC-HRMS (poly)phenol catabolite analysis

Faecal slurry samples collected from the *in vitro* fermentation at 0, 4, 8, 24 and 48 h, were weighted (0.5 g) and mixed with 2.5 mL of methanol/water/formic acid (79/20/1, v/v/v), stirred for 30 seconds and put in an ultrasonic bath during 5 min. Then, the samples were centrifuged at 4500*g* during 5 min at 4 °C and placed in a vacuum concentrator (Eppendorf model 5301, Hamburg, Germany). Finally, the dry residue was redissolved in 0.5 mL of methanol/water/formic acid (79/20/1, v/v/v).

The extracted samples were analysed following the method described by Ordoñez-Díaz *et al*. (2020). Briefly, the analysis was carried out in an Ultimate 3000 RS UHPLC system (Dionex, San José, CA, USA), comprising of an UHPLC pump and an autosampler operating at 4 °C (Dionex Ultimate 3000 RS, Thermo Corporation). The (poly)phenol separation was performed on a Zorbax SB-C18 RRHD column (100 × 2.1 mm i.d., 1.8 µm (Agilent,Santa Clara, CA, USA)) with a precolumn of the same stationary phase, both maintained at 40 °C. The mobile phases used were 0.1% acidified water with formic acid (A) and acetonitrile/formic acid (99.9/0.1, v/v) (B). The gradient started with 3% of B during 2 min, rising to 65% in 18 min, increasing until 80% in 1 min and maintained 6 min more in a 26 min gradient, followed by a stabilisation phase with the starting conditions during 10 min. The flow rate was 0.2 mL/min. The Exactive Orbitrap mass spectrometer, fitted with a heated electrospray ionisation probe (ThermoFisher Scientific, San José, CA, USA), operated in negative ionisation mode (scanning from 100 to 1000 m/z). The capillary temperature was set to 300 °C and the heater temperature were 150 °C. The sheath gas and the auxiliary gas flow rate were both 20 units, the sweep gas was 3 units, and the spray voltage was 4.00 kV. Data acquisition and processing were carried out using Xcalibur 3.0 software.

Orange (poly)phenols and their catabolites produced during *in vitro* fermentation were identified in the faecal slurry samples by comparison of their exact mass and retention time with authentic commercial standards. When the standards were not available, the compounds were tentatively identified by comparing the theoretical exact mass of the molecular ion with its measured accurate mass, and referred to databases or libraries containing HRMS spectral information such as Metlin (https://metlin.scripps.edu/landing_page.php?pgcontent=mainPage) and Phytohub (https://phytohub.eu/) databases. Identifications were categorised according to the annotation previously described using the metabolite standards initiative metabolite identification level (MSIMI) (Sumner *et al.*, 2007). The compounds were quantified by selecting the theoretical exact mass of the molecular ion using standard curves (0.01-

100 µg/mL). When standards were not available (poly)phenols, and their catabolites were quantified by using calibration curve of structurally similar compounds.

### 2.3. Statistical analysis

The statistical analysis was carried out using R studio, version 4.0.5 (R Foundation for Statistical Computing, Vienna, Austria). Normality was determined by the Shapiro-Wilk test. The homogeneity of variances was analysed using the Bartlett test. One-way analysis of variance (ANOVA) was performed to determine significant differences among different samples for the parameters analysed. Tukey´s test was used as a post-hoc test to determine the differences among means. A correlation analysis was also performed between the physicochemical properties and fibre compositions and betweenTPC, TF and antioxidant capacity. Differences were considered significant at a *p*-value< 0.05.

## 3. Results and discussion

### 3.1. Proximate composition

The proximate composition of OP, IFF and WSE is shown in Table 23. The major component in OP and IFF was the dietary fibre, while in WSE was the carbohydrates. This is due to the extraction process used for this samples, in which alcohol was not used to remove the soluble sugars, as have been mentioned before, WSE fraction comprised the water phase extraction fraction, and therefore includes soluble sugars of the orange peels, that have been reported to be around 17% of dry weight (d.w.) for orange peel (Rivas *et al.*, 2008).

The moisture content of all samples was less than 0.5% and for this reason these values have not been included in Table 23. Garcia-Amezquita *et al*. (2019) and USDA (2022) have reported a content of protein in the orange peels that range from 4.9 to 5.5 g/100 g of d.w. In this study the OP showed the lowest content of protein with a mean value of 4.9 g/100 g, showing IFF and WSE a mean value of 5.3 and 5.2 g/100 g, respectively, being similar to that previously reported. The fat content was not analysed in this study because the values reported in the literature for dry peel were very low (<2%), and therefore no major changes are likely to occur between the different fractions obtained (Ahmed *et al.*, 2016; Garcia-Amezquita *et al.*, 2019). The carbohydrates content was calculated by differences with the other components. OP showed a mean value of 45.6 g/100 g, similar to the data reported for dry orange peel by other authors (41 g/100

g) (Garau *et al.*, 2007; Garcia-Amezquita *et al.*, 2019). The carbohydrate content was significantly different ($p$<0.05) among the three samples, showing the highest value in the WSE fraction and the lowest in the IFF. These differences are due to the extraction method, since WSE represents the water-soluble compounds, and hence this fraction contains the carbohydrates, soluble sugars and soluble polysaccharides.

**Table *23*.** Nutritional composition expressed as percentage, and minerals content of orange peel samples.

| Parameters | OP | IFF | WSE |
|---|---|---|---|
| **Protein** | 4.9 ± 0.0$^{b}$* | 5.3 ± 0.1$^{a}$ | 5.2 ± 0.2$^{a}$ |
| **Carbohydrates**** | 45.6 ± 2.9$^{b}$ | 15.6 ± 0.8$^{c}$ | 68.2 ± 2.7$^{a}$ |
| **Ash** | 3.0 ± 0.1 | 3.4 ± 0.2 | 3.1 ± 0.2 |
| **Total dietary fibre (TDF)** | 46.5 ± 4.1$^{b}$ | 75.8 ± 0.8$^{a}$ | 23.5 ± 4.1$^{c}$ |
| **Insoluble dietary fibre (IDF)** | 37.3 ± 2.2$^{b}$ | 64.6 ± 2.5$^{a}$ | 16.5 ± 3.1$^{c}$ |
| **Soluble dietary fibre (SDF)** | 9.2 ± 1.9 | 11.2 ± 1.7 | 7.0 ± 1.0 |
| **Ca** (g/kg) | 9.2 ± 0.0$^{b}$ | 13.5 ± 0.9$^{a}$ | 6.2 ± 0.1$^{c}$ |
| **K** (g/kg) | 9.0 ± 0.0$^{b}$ | 4.1 ± 0.2$^{c}$ | 1.1 ± 0.1$^{a}$ |
| **Mg** (g/kg) | 1.1 ± 0.0$^{c}$ | 2.0 ± 0.1$^{a}$ | 1.5 ± 0.0$^{b}$ |
| **Na** (g/kg) | 0.2 ± 0.0$^{b}$ | 1.1 ± 0.1$^{a}$ | 1.1 ± 0.0$^{a}$ |
| **P** (mg/kg) | 803.9 ± 7.2$^{b}$ | 449.9 ± 25.0$^{c}$ | 992.6 ± 4.8$^{a}$ |
| **Mn** (mg/kg) | 12.3 ± 0.1$^{c}$ | 19.8 ± 0.8$^{a}$ | 13.4 ± 0.2$^{b}$ |
| **Fe** (mg/kg) | 12.3 ± 0.3$^{b}$ | 15.0 ± 1.2$^{a}$ | 14.4 ± 0.6$^{a}$ |
| **Zn** (mg/kg) | 6.7 ± 0.2$^{b}$ | 8.7 ± 0.7$^{a}$ | 8.4 ± 0.3$^{a}$ |

*Values are expressed as mean ± SD (n =3). Different letters (a–c) indicate significant differences ($p$<0.05) among the samples. Orange peel (OP); insoluble fibre fraction (IFF); water-soluble extract (WSE). **Carbohydrates were calculated by difference between the other components.

The content of TDF and IDF showed significant differences ($p$<0.05) among the three samples, ranging the TDF content from 23.5% and 75.8% and the IDF content from 16.5% to 64.6%, for WSE and IFF, respectively. On the other hand, the content of SDF in the three samples did not show significant differences containing 7% and 11.2% for the WSE and IFF fractions, respectively. Different authors have reported that the content of TDF in orange peels is around 63% expressed in dry weight (de Moraes Crizel *et al*., 2013; Wang *et al*., 2015). We have found a lower content of TDF in OP, but our results for the fibre fractions (IFF and WSE) agree with those obtained by the mentioned

authors, since the major fraction of TDF is represented by IDF. The IFF was the fraction with the highest content of TDF, because the insoluble water compounds of the orange peels were extracted, with 64.6% of IDF and 11.2% of SDF. As for the WSE fraction, which comprised the supernatant collected during centrifugation, the content of dietary fibre was very low because the soluble carbohydrates from orange peel were solubilised in this fraction.

Figure 40 shows the proportion of both IDF and SDF for the three samples, being highest the IDF for all samples, showing the IFF fraction the highest percentage (85.2%). On the contrary, WSE showed the highest proportion of SDF (29.8%), having highest amount of soluble non-starch polysaccharides. Although, this fraction also showed a 70.2% of IDF which could be due to the fact that the insoluble compounds of fibre were retained during the extraction due to the gelation of the pectin. Regarding the fibre proportions for OP, it was similar to the IFF, and also to that reported by other authors, IDF ranging from 76-88% (De Moraes Crizel *et al.*, 2013; Wang *et al.*, 2015; Garcia-Amezquita *et al.*, 2019).

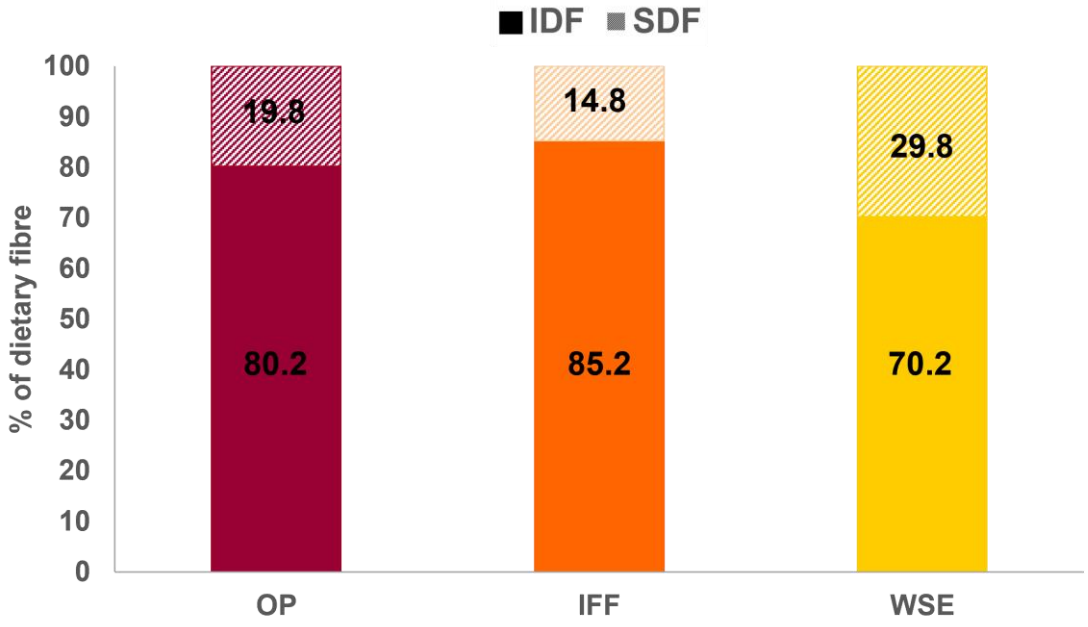


**Figure 40.** Proportion (%) of insoluble dietary fibre (IDF) and soluble dietary fibre (SDF) in the orange peel samples. Orange peel (■OP); insoluble fibre fraction (■IFF); water-soluble extract (■WSE).

The three samples had a similar content of ashes being for all samples around 3% without significant differences among them. These results were in agreement with those

obtained by other authors ranging between 2.5% and 3.0% for orange peel waste and peel fibre, respectively (De Moraes Crizel *et al.*, 2013; Lei *et al.*, 2021). The mineral composition of the samples is also shown in Table 23. The minor ones were the trace elements zinc, manganese, and iron. On the contrary, the majority ones were calcium and potassium; similar profiles of mineral for orange peels were reported by Czech *et al*. (2020). As previously reported, minerals have the ability to bind to the fibre and therefore be extracted with it, although these bindings depend on several factors, such as the pH or the ionic strength (Baye *et al.*, 2017). In our samples, IFF showed the highest content of all minerals (divalent cations) except for potassium and phosphorus, this effect is due to the highest content of fibre in this sample, having the other two samples a lower amount of minerals due to the lower fibre content. It deserves to mention, that the IFF fraction showed a high content of Ca, so at the same time, this fraction is a source of dietary fibre and this mineral.

### 3.2. Composition of dietary fibre, neutral sugars and uronic acids

The composition of dietary fibre, based on the percentage of neutral sugars and uronic acids, in the three samples is described in Table 24. The dietary fibre of the samples was constituted mainly for arabinose, followed by uronic acids and galactose, being IFF the sample with the highest proportion of the three compounds. On the contrary, the lowest proportion of the neutral sugars was observed for xylose in OP and WSE, and for mannose in IFF.

**Table 24.** Proportion (%) of neutral sugars and uronic acid in the orange peel samples obtained from orange by-products.

| Sugars | OP | IFF | WSE |
|---|---|---|---|
| **Rhamnose** | $5.1 \pm 0.5^{b*}$ | $4.1 \pm 0.3^{c}$ | $9.4 \pm 0.4^{a}$ |
| **Arabinose** | $28.0 \pm 1.6^{b}$ | $31.5 \pm 1.5^{a}$ | $21.0 \pm 0.5^{c}$ |
| **Xylose** | $4.8 \pm 0.2^{b}$ | $5.6 \pm 0.3^{a}$ | $4.8 \pm 0.2^{b}$ |
| **Mannose** | $5.3 \pm 0.5^{b}$ | $3.6 \pm 0.1^{c}$ | $8.8 \pm 0.1^{a}$ |
| **Galactose** | 19.0 ± 1.0 | 18.8 ± 1.3 | 17.8 ± 0.8 |
| **Glucose** | $10.5 \pm 0.7^{b}$ | $5.9 \pm 0.8^{c}$ | $17.9 \pm 2.2^{a}$ |
| **Uronic acid** | $27.3 \pm 1.2^{b}$ | $30.5 \pm 1.9^{a}$ | $20.3 \pm 1.6^{c}$ |

*Values are expressed as mean ± SD (n =4). Different letters (a–c) indicate significant differences ($p$<0.05) among the samples. Orange peel (OP); insoluble fibre fraction (IFF); water-soluble extract (WSE).

Related to the proportion of the neutral sugars and uronic acids significant differences were observed among the three samples, except for galactose, which showed a mean proportion around 18.5%. Moreover, fucose was not detected in any sample. The main differences between the samples obtained from orange peel was that IFF had more contribution of arabinose, xylose and uronic acids, while WSE had more of rhamnose, mannose and glucose. Although OP did not have any outstanding compound over the other fractions, it had a composition that for all its constituents was between the two other fractions, which indicates that the extraction carried out allowed to obtain two clearly differentiated ingredients.

Other authors have described that uronic acids, arabinose and galactose, were the majority components of orange peel, ranging from 38% to 20% for uronic acids, from 16.4% to18% for arabinose, and from 13.6% to 8.4% for galactose (Garau *et al.*, 2007; Wang *et al.*, 2015). On the contrary, Garau *et al*. (2007) have reported that rhamnose, xylose and mannose were the components found in the lowest proportion. Peels from other citrus fruits, such as grapefruit and lemon peels showed arabinose as the main component followed by uronic acids, so the results were similar to those obtained in our study for OP and its fractions (Wang *et al.*, 2015). It is noteworthy, that as have been previously reported, the extraction procedure can influence the composition of dietary fibre, since an increase in the drying temperature of the dietary fibre extracts lead to a significant decrease in the uronic acids content and then change the overall profile, as have been shown for orange peel (Garau *et al*., 2007).

Based on the proportion of neutral sugars and uronic acids, the non-starch polysaccharides proportion was estimated as proposed by Umaña *et al*. (2016), providing the percentage of cellulose, hemicellulose and pectin in the samples (Table 25).

Cellulose, which is an unbranched homopolymer insoluble in water (Ali and Arafat, 2021), was the minority component present in all fractions as other authors have previously reported (Rivas *et al.*, 2008). This compound was only quantified for OP and IFF without significant differences, since in the case of WSE the glucose found is that which is part of the soluble polysaccharides.

Hemicelluloses are heteropolysaccharides that can be soluble or insoluble in water solutions. Hemicellulose was the second most abundant component of fibre in the orange peel fractions, being significantly higher in WSE, showing IFF the lowest content with 11.3%. The contribution of mannose to hemicellulose structure was calculated based on Houben *et al*. (2011), the results are shown in Table 26. The fraction that

showed the highest contribution of mannose was WSE and the lowest was IFF, showing significant differences among the three samples. Other authors have previously reported for hemicellulose from maize stems that the water-soluble hemicelluloses showed highest content of mannose and less of xylose (Peng *et al.*, 2019). This could explain that hemicellulose of WSE, with highest contribution of mannose, was composed mainly of water-soluble hemicellulose. On the other hand, IFF which showed the lowest contribution of mannose and therefore had the highest xylose content, was composed mostly of non-water-soluble hemicelluloses, which is consistent with the results obtained for the analysis of the fibre fractions where this fraction showed a highest proportion of IDF, while WSE showed the highest SDF content (Figure 40).

**Table 25.** Proportions of cellulose, pectin and hemicellulose in the orange peel samples obtained from orange by-products expressed as percentage (%).

| Polysaccharides | OP | IFF | WSE |
|---|---|---|---|
| **Cellulose** [A] | 5.4 ± 0.9* | 3.8 ± 0.8 | - |
| **Hemicellulose** [B] | 15.3 ± 0.4$^{b}$ | 11.3 ± 0.4$^{c}$ | 31.5 ± 1.5$^{a}$ |
| **Pectin** [C] | 79.3 ± 1.3$^{b}$ | 84.9 ± 1.1$^{a}$ | 68.5 ± 2.2$^{c}$ |

*Values are expressed as mean ± SD (n =4). Different letters (a–c) indicate significant differences ($p$<0.05) among the samples. Orange peel (OP); insoluble fibre fraction (IFF); water-soluble extract (WSE). [A] Cellulose: Glucose x 0.9; [B] Hemicellulose: (Fucose + Xylose + Mannose + (Glucose x 0.1); [C] Pectin: (Rhamnose + Arabinose + Galactose + Uronic acids).

Pectin is one of the most abundant heteropolysaccharides in the plant cell walls, which are abundant in fruits particularly in citrus and apples (Zdunek *et al.*, 2021). This component was the main one in our samples, with significant differences among them ranging from 68.5% to 84.9%. In this sense, IFF was the one with the highest content, results that are in accordance with the proportion of uronic acid content (Table 24). Moreover, using the ratios proposed by Houben *et al*. (2011), information related to the structure of pectin was obtained (Table 26).

Results showed that pectin molecules from IFF and OP were more linear than that obtained from WSE, having the linearity important contribution to the technological properties of the sample. The ability to form emulsions is enhanced when the linearity is highest because the hydration properties of the pectin are increased (Mendez *et al.*, 2021). Moreover, the rhamnose-uronic acid ratio was significantly higher for WSE compared to IFF and OP, which means that there were more domains of RG-I or that

they were longer. On the other hand, regarding RG-I branching, which shows the degree of branching of the pectin molecules, IFF showed the highest values, while WSE had the lowest value for this parameter. IFF pectin showed a highest number of branches, being both more linear and branched molecules compared to the pectin of the other two samples (Van Audenhove *et al.*, 2021). As we can see in Table 26, only there were differences between OP and IFF for the branching ratio of pectin, showing WSE more differences regarding the other two samples. This behaviour might be related to the procedure used for the preparation and extraction of the samples, since the OP and IFF were dried at 45 °C, whereas the WSE fraction, was freeze-dried. These results are supported by other authors, who determined that differences in the extraction process may directly affect the structure of the pectin obtained (Belkheiri *et al.*, 2021).

**Table 26.** Sugar ratios for pectin and hemicellulose characterisation from orange peel samples obtained from orange by-products.

| Ratio | OP | IFF | WSE |
|---|---|---|---|
| **Man contribution [a]** | 1.1 ± 0.1[b]* | 0.6 ± 0.0[c] | 1.8 ± 0.0[a] |
| **Linearity [b]** | 0.5 ± 0.0[a] | 0.5 ± 0.0[a] | 0.4 ± 0.0[b] |
| **Rha-Uro contribution [c]** | 0.2 ± 0.0[b] | 0.1 ± 0.0[b] | 0.5 ± 0.0[a] |
| **RG-I branching [d]** | 9.3 ± 1.1[b] | 12.3 ± 0.7[a] | 4.2 ± 0.1[c] |

*Values are expressed as mean ± SD (n =4). Different letters (a–c) indicate significant differences ($p$<0.05) among the samples. Orange peel (OP); insoluble fibre fraction (IFF); water-soluble extract (WSE). [A] contribution of mannans to hemicelluloses: Mannose/Xylose; [B] Linearity of pectins: Uronic acids/(Fucose + Rhamnose + Arabinose + Galactose + Xylose); [C] Contribution of rhamnose and uronic acids to pectins: Rhamnose/Uronic acids; [D] Branching of RG-I: (Arabinose + Galactose)/Rhamnose.

### 3.3. Physicochemical properties of fibre

Table 27 shows the mean values for the water retention capacity (WRC), swelling capacity (SWC), fat absorption capacity (FAC), and osmotic pressure of the three samples. The hydration properties, WRC and SWC, indicate the ability of the fibre matrix to retain water inside of the structure. The WRC showed significant differences among the samples, and the highest value of WRC was observed in the IFF fraction (8.8 g water/g of sample). The results obtained for WRC were similar to that reported by other authors being 9.6 g water/g for orange peel fibre and 9.7 g water/g for orange peel dried with hot air and milled at room temperature, respectively (De Moraes Crizel *et al.*, 2013; Sankalpa *et al.*, 2016). Regarding the SWC, which shows the ability to form gel structures

and therefore the ability to modify the texture after addition into food formulations, ranged from 6.6 to 20.9 mL of water/g, showing the WSE fraction the highest values and the OP the lowest. Similar values have been reported in the literature for orange peels ranging from 12-21 mL of water/g (Garau *et al.*, 2007; Wang *et al.*, 2015; Sankalpa *et al.*, 2016). Despite of the highest branching of pectin molecules reduces the ability to form gels, because the molecules become more rigid (Belkheiri *et al.*, 2021); the WSE fraction, which was the sample with the lowest branched pectin structure, had the highest SWC. Although, the ability to form gels is also positively correlated to the presence of pectin and hemicellulose, pectin was not present in a high proportion in this sample, indicating the positive correlation of SWC and hemicellulose, since hemicellulose with high mannose contribution could exert water binding capacity. On the other hand, samples with high branched pectin and hemicelluloses with low mannose contribution are less soluble, hence the SWC is reduced and for this reason the IFF and OP showed a significantly lower SWC than WSE. The hydration properties contribute to stomach distension and increased satiety due to the slowdown emptying, reduce the absorption of nutrients and increase stool volume reducing constipation (McRorie and McKeown, 2017; Tan *et al.*, 2017).

**Table 27.** Physicochemical properties of orange peel samples obtained from orange by-products.

| Physicochemical properties | OP | IFF | WSE |
|---|---|---|---|
| **Water retention capacity** (g of water/g) | $4.0 \pm 0.2^{c*}$ | $8.8 \pm 1.2^{a}$ | $6.2 \pm 1.3^{b}$ |
| **Swelling capacity** (mL of water/g) | $6.6 \pm 0.1^{c}$ | $14.6 \pm 0.3^{b}$ | $20.9 \pm 3.8^{a}$ |
| **Fat absorption capacity** (g of oil/g) | $1.6 \pm 0.1^{b}$ | $1.8 \pm 0.2^{b}$ | $2.8 \pm 0.0^{a}$ |
| **Osmotic pressure** (mmol/kg) | $173.0 \pm 0.0^{b}$ | $146.3 \pm 2.1^{c}$ | $196.3 \pm 0.6^{a}$ |

*Values are expressed as mean ± SD (n =3). Different letters (a–c) indicate significant differences ($p$<0.05) among the samples. Orange peel (OP); insoluble fibre fraction (IFF); water-soluble extract (WSE).

The FAC helps to prevent fat loss during cooking helping also to the flavour retention (Wang *et al.*, 2015). Significant differences were observed for the FAC between the samples with values that ranged from 1.6 to 2.8 g of oil/g, similar to those reported by other authors for orange peel samples (1.6 and 3.6 g of oil/g) (Garau *et al.*, 2007; De Moraes Crizel *et al.*, 2013; Wang *et al.*, 2015; Sankalpa *et al.*, 2016).

As for the osmotic pressure (146.3-196.3 mmol/kg), all the samples were found to be below physiological values (290 mmol/kg) (Lawrence and Joseph., 2015), which indicates that there was no risk of these fibres causing osmotic diarrhoea, as in the case of xylitol, which after high intakes can cause it because its osmotic pressure is higher than physiological values (Yu *et al.*, 2014; Mäkinen, 2016).

### 3.4. (Poly)phenols and antioxidant capacity

TPC measured by Folin method, and antioxidant capacity measured by FRAP and ORAC methods, in both EPP and NEPP fractions, is shown in Table 28. The TPC was higher in the EPP fraction than in NEPP fraction, representing EPP a 68% for both OP and IFF and 76% for WSE. Significant differences were observed for the TPC (in EPP, NEPP and total fractions) among the three samples, showing WSE fraction the highest amount and IFF fraction the lowest content, with an average value of 61 and 40.6 mg GAE/g, respectively. Lagha-Benamrouche and Madani (2013) have reported a content of TPC from 9.6 to 31.6 mg GAE/g of d.w. for orange peels, but only considering EPP fraction. Moreover, also information for both TPC of flavedo and albedo have been reported by other authors (Escobedo-Avellaneda *et al.*, 2014; Wang *et al.*, 2022), that showed higher proportion of EPP fraction according to our results.

TF followed a similar trend to that of TPC, being mostly present in the EPP rather than in NEPP (Table 28). Regarding to the proportion of TF in the EPP with respect to the total, as the sum of EPP and NEPP, a 77, 80 and 94% were observed for OP, IFF and WSE, respectively. In the case of EPP, WSE showed the highest content, while IFF showed the lowest, with a mean value of 8 mg CE/g and 1.6 mg CE/g, respectively. In the case of NEPP, OP showed the highest content, with a mean value of 0.7 mg CE/g and IFF showed the lowest, with a mean value of 0.4 mg CE/g. The results reported in our study were a bit higher than that reported by other authors for some citrus peels, being 0.5, 0.4, 0.4 and 0.5 mg of catechol/g for orange, lemon, tangerine and grapefruit peels, respectively (Shehata *et al.*, 2021). Wang *et al*. (2022) have reported similar values to that obtained for WSE, with 9.6 mg rutin equivalents/g of d.w. for orange flavedo and 10.5 mg rutin equivalents/g of d.w. for orange albedo.

The antioxidant capacity for the three samples is shown in table 28. In this regard, the antioxidant capacity (FRAP and ORAC) was related to the TPC and TF content, showing a positive correlation with both values, being r = 0.99 for TPC ($p$<0.05) and r = 0.79 for TF ($p$<0.05). Thus, the antioxidant capacity was significantly higher for the EPP, NEPP and total extracts obtained from WSE, being 1.3 and 2.6-fold higher in FRAP

assay, and 1.1 and 1.5-fold higher in ORAC assay than the values obtained for OP and IFF, respectively. Our results were lower in the FRAP assay than that reported by other authors for orange flavedo (101 µmol TE/g) and albedo (142 µmol TE/g) (Escobedo-Avellaneda *et al.*, 2014), and also for other orange peels ranging between 75 and 155 µmol TE/g (Castro-Vázquez *et al.*, 2021).

**Table *28*.** Total phenolic content (TPC) (mg GAE/g of d.w.), total flavonoids (TF) (mg CE/g of d.w.) and antioxidant capacity (µmol TE/g of d.w.) by FRAP and ORAC of orange peel samples obtained from orange by-products, from extractable (EPP) and non-extractable fractions (NEPP).

| | OP | IFF | WSE |
|---|---|---|---|
| ***Extractable fraction (EPP)*** | | | |
| TPC | 38.2 ± 0.5$^{b}$* | 27.9 ± 0.8$^{c}$ | 46.4 ± 0.8$^{a}$ |
| TF | 2.3 ± 0.3$^{b}$ | 1.6 ± 0.4$^{c}$ | 8.0 ± 0.1$^{a}$ |
| FRAP | 51.7 ± 3.1$^{b}$ | 25.4 ± 1.5$^{c}$ | 82.2 ± 2.8$^{a}$ |
| ORAC | 454.0 ± 17.7$^{b}$ | 301.6 ± 11.1$^{c}$ | 502.6 ± 11.7$^{a}$ |
| ***Non-extractable fraction (NEPP)*** | | | |
| TPC | 17.6 ± 0.3$^{a}$ | 12.7 ± 0.8$^{c}$ | 14.6 ± 0.3$^{b}$ |
| TF | 0.7 ± 0.0$^{a}$ | 0.4 ± 0.1$^{c}$ | 0.5 ± 0.0$^{b}$ |
| FRAP | 29.9 ± 1.0$^{a}$ | 15.4 ± 2.1$^{c}$ | 21.4 ± 0.7$^{b}$ |
| ORAC | 193.9 ± 4.1$^{a}$ | 153.0 ± 10.7$^{c}$ | 182.3 ± 6.9$^{b}$ |
| ***Total*** | | | |
| TPC | 55.7 ± 0.8$^{b}$ | 40.6 ± 1.6$^{c}$ | 61.0 ± 1.1$^{a}$ |
| TF | 3.0 ± 0.3$^{b}$ | 2.0 ± 0.4$^{c}$ | 8.5 ± 0.1$^{a}$ |
| FRAP | 81.6 ± 5.9$^{b}$ | 40.8 ± 2.8$^{c}$ | 104.6 ± 4.9$^{a}$ |
| ORAC | 647.9 ± 21.7$^{b}$ | 454.6 ± 21.8$^{c}$ | 684.9 ± 18.5$^{a}$ |

*Values are expressed as mean ± SD (n =3). Different letters (a–c) indicate significant differences ($p$<0.05) among the samples. Orange peel (OP); insoluble fibre fraction (IFF); water-soluble extract (WSE). Ferric reducing antioxidant power (FRAP); oxygen radical absorbance capacity (ORAC).

In addition to the spectrophotometric method, the different samples were also analysed by HPLC-DAD to identify and quantify both EPP and NEPP. The results shown in Figure 41 highlight that most of the compounds in these samples were extractable as other authors have described before for orange, mandarin and grapefruit by-products (Reynoso-Camacho *et al.*, 2021), representing EPP 57, 58 and 78% from the total for

OP, IFF and WSE, respectively. On the contrary, the NEPP fraction represented almost half of the total compounds for OP and IFF, but only 22% in WSE, being the fraction with the lowest proportion of these compounds and the lowest content of TDF.

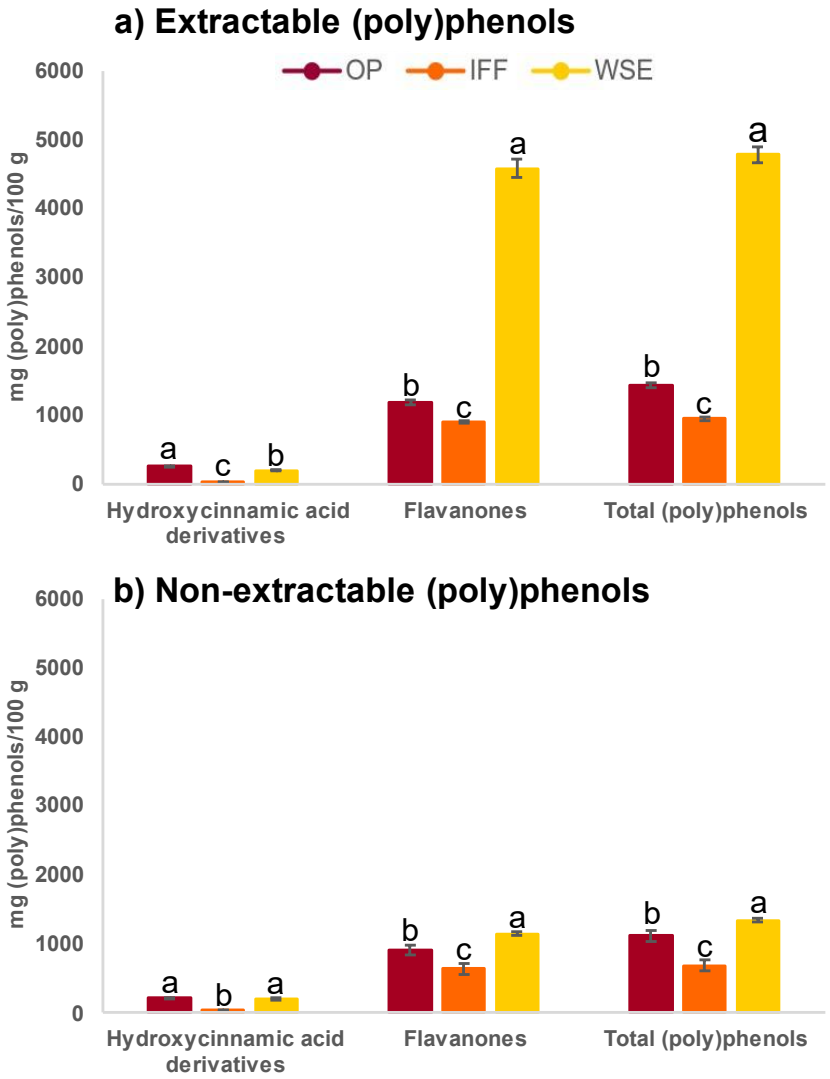


**Figure 41.** Contents of individual (poly)phenols (hydroxycinnamic acid derivatives and flavanones) and the total (poly)phenols determined as the sum of the individual compounds (mg/100 g of d.w.), being a) extractable (poly)phenols and b) non-extractable (poly)phenols analysed by HPLC-DAD for orange peel (■OP) and the fibre fractions, insoluble fibre fraction (■IFF) and water-soluble extract (■WSE). Values are expressed as mean ± SD (n = 3). Different letters (a–c) indicate significant differences ($p$<0.05) among the samples for the individual and total (poly)phenols.

As it has been described before for TPC, the individual (poly)phenols showed significant differences ($p$<0.05) among the three samples, for both EPP and NEPP. Total

EPP (Figure 41.a.), which was mainly represented by flavanones, showed a mean value of 4589 mg/100 g of d.w. for WSE, followed by OP and IFF with 1185 and 909 mg/100 g of d.w., respectively. Hydroxycinnamic acids were also found but in smaller concentrations than flavanones, varying between 41 and 259 mg/100 g of d.w, being significantly higher also in OP sample.

Regarding the NEPP, the same profile as in EPP was observed. The WSE showed the highest concentration, being mainly represented by flavanones due to its water-soluble character (Vallejo *et al.*, 2010), with 1146 mg/100 g of d.w., followed by OP and IFF with 903 and 640 mg/100 g of d.w., respectively. The hydroxycinnamic acids were found in smaller concentrations than flavanones, showing similar amount than in the EPP fraction. In this sense, were OP and WSE the samples with the highest amount, 210 and 196 mg/100 g of d.w., being IFF the one with the lowest value, 42 mg/100 g of d.w.

It is know that orange peel is rich in flavanones (Barrales *et al.*, 2018; Angoy *et al.*, 2020; Shehata *et al.*, 2021) being hesperidin the main flavanone (Wang *et al.*, 2022). In addition, as was observed in our study, where around 48% of the hydroxycinnamic acids were found in the NEPP fraction, other authors have shown that hydroxycinnamic acids are attached in large quantities to the fibre molecules (Wang *et al.*, 2022).

### 3.5. Carotenoid content

Carotenoid profile was identified after saponification by HPLC-DAD according to their spectrum and their elution times, comparing with authentic standards, and according to their spectral characteristics previously reported in the literature (Meléndez-Martínez *et al.*, 2005; Murador *et al.*, 2019). The quantification was also carried out on an HPLC-DAD using calibration curves from authentic carotenoid standards. The individual carotenoids identification and their contents in the three samples are included in Table 29.

Twenty different peaks were found in the different fractions (OP, IFF, WSE), and ten of them were tentatively identified (Table 29), being leuteoxanthin ($t_R$=5.7); sintaxanthin ($t_R$=6.3), which was an apocarotenoid; violaxanthin ($t_R$=7.9); lutein ($t_R$=9.1); lutein isomer I ($t_R$=9.4); lutein isomer II ($t_R$=10.2); α-carotene ($t_R$=19.4); β-cryptoxanthin ($t_R$=21.1); antheraxanthin isomer ($t_R$=22.5), and β-carotene ($t_R$=31.5). All the individual carotenoids were identified in all samples, except for peak 7 which was only identified in OP and IFF, while peak 8 only was identified in OP. Furthermore, other carotenoid-like compounds were also detected with similar spectral characteristics (Table 29).

**Table 29.** Spectral characteristic and content (mg/100 g of d.w.) of the carotenoids detected by HPLC-DAD in the orange peel samples obtained from orange by-products.

| Peak | Carotenoid | $t_R$ (min)[a] | λmax (nm)[b] | *Content (mg/100 g d.w.)* | | |
|---|---|---|---|---|---|---|
| | | | | OP | IFF | WSE |
| 1 | Luteoxanthin | 5.7 | 400, 422, 447 | 0.8 ± 0.1* | 0.8 ± 0.1 | 0.5 ± 0.2 |
| 2 | Sintaxanthin | 6.3 | 419, 438, 466 | 1.0 ± 0.1$^{a}$ | 0.8 ± 0.1$^{a}$ | 0.7 ± 0.1$^{b}$ |
| 3 | Carotenoid-like 1 | 6.9 | 380, 402, 424, 434 | 1.0 ± 0.1$^{a}$ | 0.9 ± 0.1$^{ab}$ | 0.7 ± 0.1$^{b}$ |
| 4 | Carotenoid-like 2 | 7.5 | 426, 442, 460 | 1.3 ± 0.1$^{a}$ | 1.1 ± 0.0$^{b}$ | 1.0 ± 0.0$^{c}$ |
| 5 | Violaxanthin | 7.9 | 418, 438, 465 | 0.7 ± 0.1$^{b}$ | 1.3 ± 0.2$^{a}$ | 1.5 ± 0.2$^{a}$ |
| 6 | Carotenoid-like 3 | 8.1 | 398, 418, 442 | 0.6 ± 0.1$^{b}$ | 0.7 ± 0.0$^{a}$ | 0.3 ± 0.0$^{c}$ |
| 7 | Carotenoid-like 4 | 8.3 | 400, 418, 444 | 0.2 ± 0.3$^{b}$ | 0.9 ± 0.1$^{a}$ | nd |
| 8 | Carotenoid-like 5 | 8.7 | 380, 428, 446 | 0.4 ± 0.0 | nd | nd |
| 9 | Lutein | 9.1 | 423, 444, 468 | 0.5 ± 0.1 | 0.6 ± 0.1 | 0.4 ± 0.1 |
| 10 | Lutein isomer I | 9.4 | 418, 444, 466 | 0.3 ± 0.0$^{b}$ | 0.5 ± 0.1$^{a}$ | 0.4 ± 0.0$^{ab}$ |
| 11 | Lutein isomer II | 10.2 | 418, 442, 468 | 0.3 ± 0.0 | 0.3 ± 0.0 | 0.2 ± 0.1 |
| 12 | Carotenoid-like 6 | 11.4 | 404, 426, 450 | 0.6 ± 0.1 | 0.6 ± .0.1 | 0.5 ± 0.0 |
| 13 | Carotenoid-like 7 | 11.8 | 398, 424, 450 | 1.0 ± 0.1 | 1.1 ± 0.1 | 0.8 ± 0.1 |
| 14 | Carotenoid-like 8 | 15.7 | 412, 444, 470 | 0.2 ± 0.0 | 0.1 ± 0.0 | 0.1 ± 0.0 |
| 15 | Carotenoid-like 9 | 17.9 | 406, 432, 450 | 0.3 ± 0.0$^{a}$ | 0.3 ± 0.0$^{a}$ | 0.2 ± 0.0$^{b}$ |
| 16 | α-carotene | 19.4 | 414, 444, 470 | 0.2 ± 0.0$^{b}$ | 0.3 ± 0.0$^{a}$ | 0.2 ± 0.0$^{b}$ |
| 17 | β-cryptoxanthin | 21.1 | 424, 450, 476 | 1.2 ± 0.0$^{a}$ | 1.1 ± 0.0$^{b}$ | 0.9 ± 0.1$^{c}$ |
| 18 | Antheraxanthin isomer | 22.5 | 414, 436, 468 | 0.1 ± 0.0$^{b}$ | 0.2 ± 0.0$^{a}$ | 0.1 ± 0.0$^{b}$ |
| 19 | Carotenoid-like 10 | 29.9 | 414, 430, 450 | 0.1 ± 0.0$^{c}$ | 0.2 ± 0.0$^{a}$ | 0.2 ± 0.0$^{b}$ |
| 20 | β-carotene | 31.5 | 436, 452, 476 | 0.2 ± 0.0 | 0.2 ± 0.0 | 0.2 ± 0.0 |

*Values are expressed as mean ± SD (n =3). Different letters (a–c) indicate significant differences ($p$<0.05) among the samples. [a] Retention time on the $C_{30}$ column; [b] λmax, maximum absorption wavelength (nm); nd, not detected; orange peel (OP); insoluble fibre fraction (IFF); water-soluble extract (WSE).

Sintaxanthin was significantly higher in OP and IFF than in WSE, while violaxanthin and lutein isomer I were significantly higher in WSE and IFF than in OP. On the other hand, IFF showed the highest content of α-carotene and antheraxanthin isomer, while OP showed the highest content of β-cryptoxanthin. The differences were therefore greater on a quantitative than on a qualitative level, as all fractions were obtained from the same starting material.

Regarding the carotenoid profile reported by other authors it was similar to the one reported in our study, with some differences. For OP sample, β-cryptoxanthin was the highest carotenoid identified (11.1%), followed by sintaxanthin (9.3%) and luteoxanthin (7.4%). On the contrary, for IFF and WSE violaxanthin was the highest carotenoids with 11% and 16.8%, respectively. In addition, β-cryptoxanthin with 9.2% and 9.6%, and sintaxanthin with 6.8 and 7.3% were also found, respectively for IFF and WSE. In agreement with the scientific literature, violaxanthin and its isomers have been described as the major carotenoids from the total content in orange flavedo (Rodrigo and Zacarias, 2007; Escobedo-Avellaneda *et al.*, 2014; Ma *et al.*, 2015). By contrast, β-cryptoxanthin, which was the major carotenoid found in OP samples, has been described as the main carotenoid in orange juice (Escobedo-Avellaneda *et al.*, 2014). In our study, we only identified one violaxanthin, but this was the majority in proportion, although it should be noted that other authors have obtained a high proportion of violaxanthin and its isomers (60%) (Escobedo-Avellaneda *et al.*, 2014). On the other hand, for the rest of the compounds there was more similarity in the proportions compared with that obtained for orange flavedo and albedo by other authors (Escobedo-Avellaneda *et al.*, 2014), ranging luteoxanthin in our study from 5.7 to 7.4%, lutein from 4.5-4.7% and β-cryptoxanthin from 9.2 to 11%. Finally, β -carotene ranged from 1.8 to 2.3%.

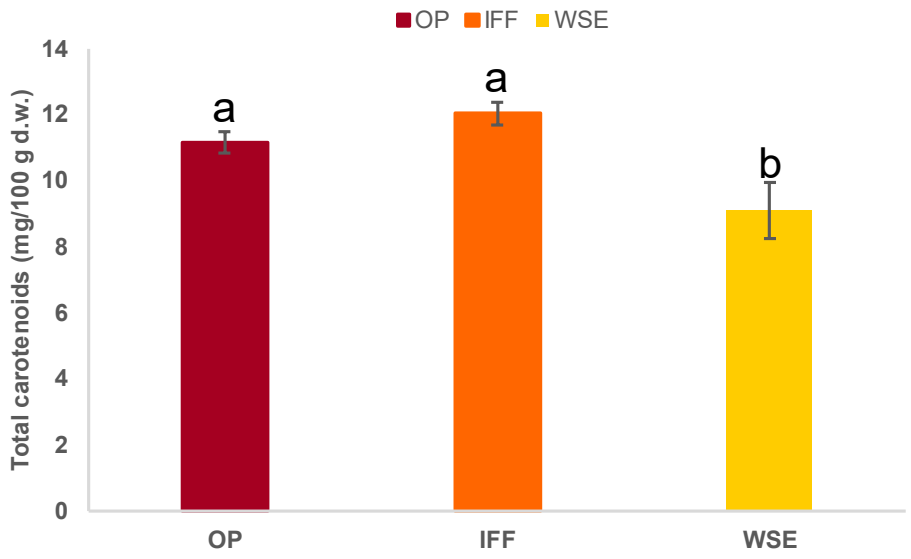


**Figure 42.** Content of total carotenoids (mg/100 g of d.w.) analysed by HPLC-DAD for orange peel (■OP) and the fibre fractions, insoluble fibre fraction (■IFF) and water-soluble extract (■WSE). Values are expressed as mean ± SD (n = 3). Different letters (a–b) indicate significant differences ($p$<0.05) among the samples.

Regarding the total carotenoid content as the sum of all individual carotenoids (Figure 42), IFF and OP showed higher content (~12 mg/100 g of d.w), than WSE fraction, with a significant lowest content (9.1 mg/100 g of d.w.). The OP and IFF, represented the dried peel and the insoluble compounds of orange peel, respectively, and hence they contain the carotenoids that provides orange colour. Although soluble compounds of orange peel are in the WSE fraction, carotenoids were also included in this fraction providing a light orange colour. These results were within the range to that reported for total carotenoids in orange flavedo by other authors, ranging from 6 to 24 mg/100 g of d.w. (calculated based on 73% moisture content ((USDA), 2022)) (Rodrigo and Zacarias, 2007; Escobedo-Avellaneda *et al.*, 2014; Ma *et al.*, 2015; Murador *et al.*, 2019).

### 3.6. Evaluation of the potential prebiotic effect and ammonium production

The prebiotic effect of the samples was evaluated by the analysis of SCFAs produced after the *in vitro* fermentation carried out with 1% of the three samples (OP, IFF and WSE) as has been described in Figure 8. The results of the SCFAs production and evolution during *in vitro* fermentation are shown in (Figure 43 and 44).

To check the *in vitro* fermentation a negative and positive controls were assayed together with the samples. In the positive control, fermentation was carried out with 1% of glucose and in the negative control no substrate was added to the faecal slurry. As for the negative control (C-), a low production of SCFAs were observed, followed by a rapid stabilisation after 4-8 hours, due to the lack of a fermentable substrate. On the contrary in the positive control (C+), a high production of SCFAs was observed during the first 4 hours, due to the presence of free glucose available in the medium, which can be easily and rapidly used by the microbiota. However, from 8 h onwards the production becomes stable due to depletion of fermentable nutrients (Figures 43 and 44).

As is shown in Figure 43, after the *in vitro* fermentation, the IFF sample led to the highest amount of acetate and propionate, as well as total SCFAs (Figure 44), followed by OP and WSE, showing significant differences ($p$<0.05) between them at 48 h. This was due to the IFF fraction contains the highest TDF content, being 1.6 and 3.2-fold higher than OP and WSE, respectively (Table 23). Furthermore, IFF fraction had also the highest content of uronic acids and therefore higher pectin content, compounds that have been positively linked to acetate production (Gómez *et al.*, 2014; Widaningrum *et al.*, 2020), which was the major SCFA produced in this study. Moreover, also IFF fraction showed the highest propionate production, which is mainly produced when gut

microbiota ferment IDF (Widaningrum *et al.*, 2020), being IFF the fraction with the highest amounts of IDF (Table 23). On the other hand, the fermentation of WSE fraction, which only had a 23.5% of TDF, with 7% of SDF and 16.5% of IDF (Table 23), led to the lowest production of acetate, propionate and total SCFAs.

Regarding the butyrate production, it was noteworthy that the production in the C+ was significantly higher than in the rest of the samples. This was due to one of the metabolic pathways of the microbial production of butyrate starts directly from glucose as a starting compound, which can be directly degraded to butyrate or follow another route in which acetate acts as an intermediate compound (Esquivel-Elizondo *et al.*, 2017). For the incubated samples, the production of butyrate was significantly highest during fermentation of WSE fraction within the first 24 h. This tendency might be explained because this fraction contains a high amount of soluble carbohydrates, including glucose, that are used by faecal bacteria to produce butyrate, as is shown in the positive control. In addition, WSE fraction also exhibited the highest contents of both EPP and NEPP, which also are considered prebiotic-like compounds, and have been related to highest production of butyrate (Alves-Santos *et al.*, 2020; Shock *et al.*, 2021).

The other minor SCFAs production is represented as the sum of the individual minor compounds (isobutyrate, valerate, isovalerate, caproate, iso-caproate and heptanoate) (Figure 44), being mainly represented by caproate (data not shown). During *in vitro* fermentation the highest amount of these SCFAs were produced in the C+. This behaviour could be explained because after the first 4 h of fermentation, the bacteria started to use the tryptone in the medium leading to a production of caproate, which is produced when the microbiota use the protein or amino acids (Rios-Covian *et al.*, 2020). This effect was slightly observed also in C-, but to a lesser extent, because the microbiota of C+ had high microbial activity due to the presence of glucose at the beginning of fermentation.

The SCFAs proportion at 48 h of fermentation (data not shown) showed in a general view that the proportion of SCFAs after the *in vitro* fermentation, was similar to the values reported in the literature (Blaut, 2018; Cui *et al.*, 2020), showing a high proportion of acetate > propionate > butyrate, and followed by the group of minor SCFAs. So, for all samples, acetate was the predominant, ranging between 62.5%-69.9%, followed by propionate (12.3% to 21.4%) and butyrate (8.7% to 19.5%), whereas other minor SCFAs represented a proportion lower than 4%. The inoculated fibre samples showed few differences in the percentage of produced SCFAs, but it is remarkable that

WSE fraction showed a higher proportion of butyrate and a lower proportion of propionate, in comparison with the other two samples.

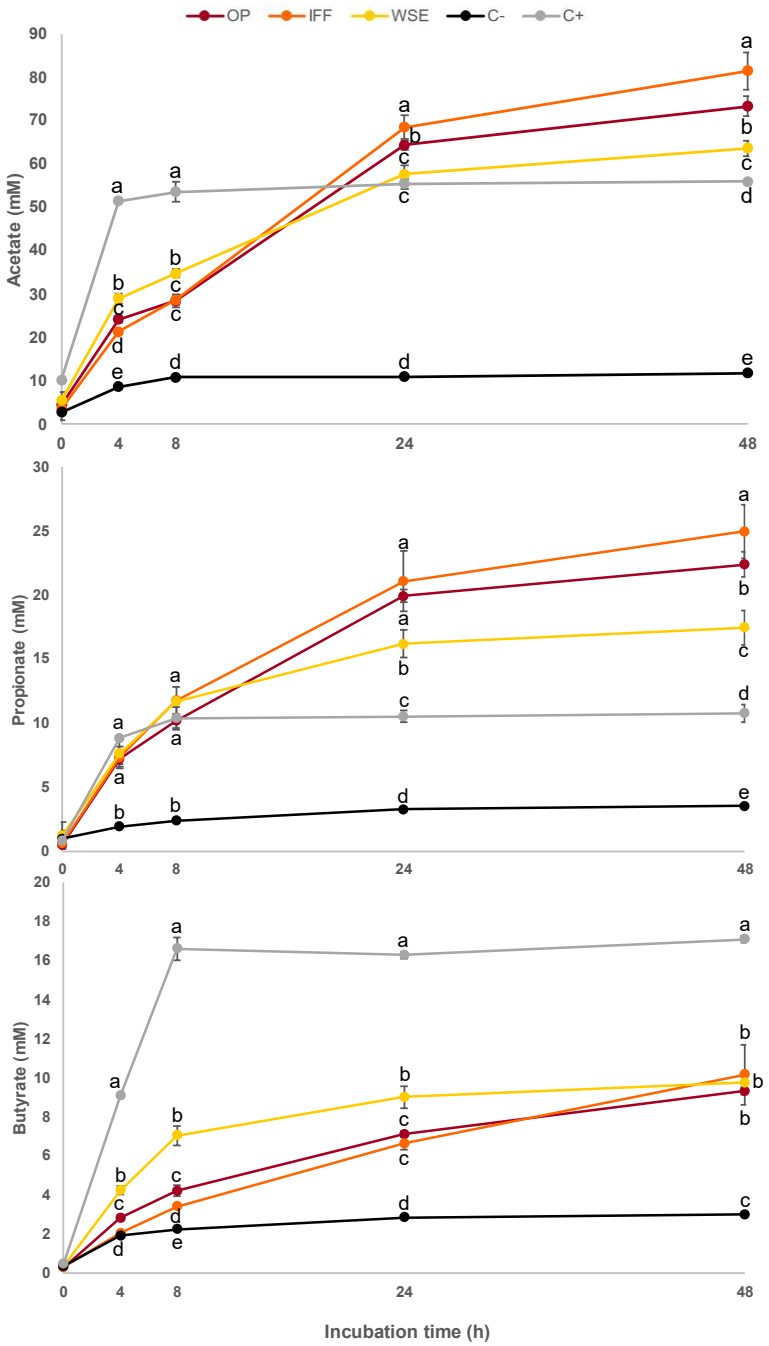


**Figure 43.** SCFAs production (acetate, propionate, butyrate) (mM) during *in vitro* fermentation for orange peel samples at 0, 4, 8, 24 and 48 h. Orange peel (●OP) and the fibre fractions, insoluble fibre fraction (●IFF) and water-soluble extract (●WSE), and the negative control (●C-) and positive control (●C+). Values are expressed as mean ± SD (n =3). Different letters (a–e) indicate significant differences ($p$<0.05) among the samples.

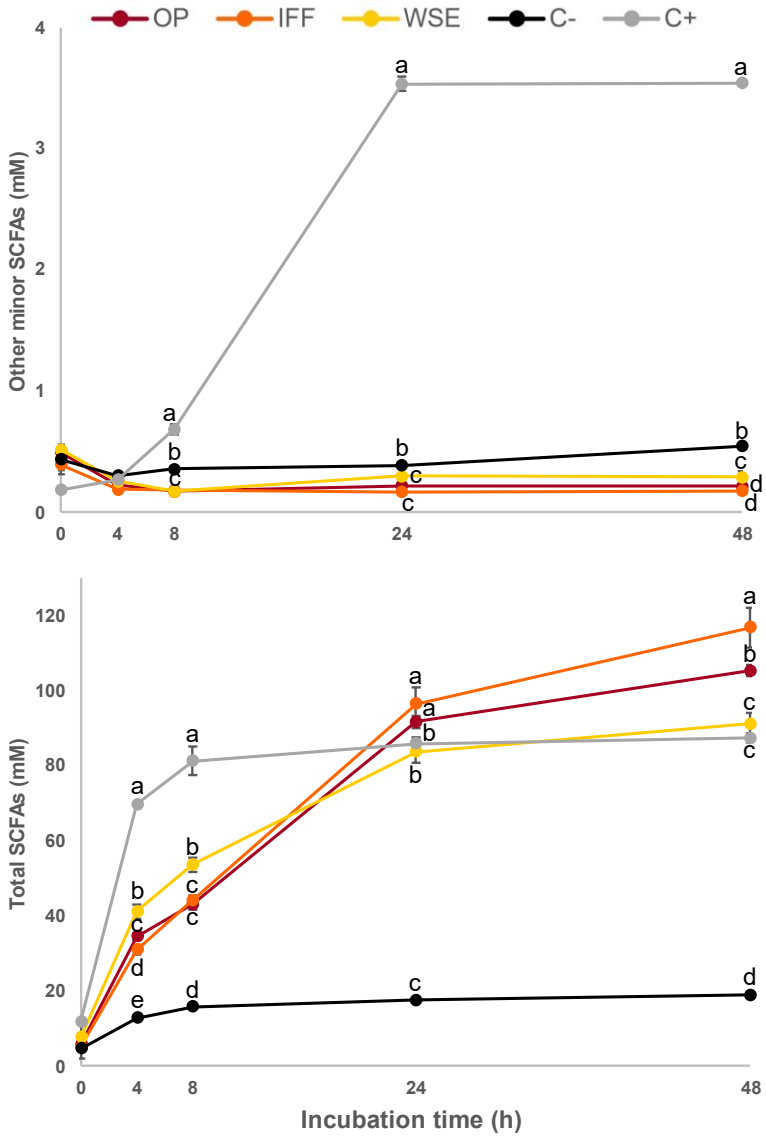


**Figure 44.** Other minor SCFAs and total SCFAs production (mM) during *in vitro* fermentation of orange peel samples at 0, 4, 8, 24 and 48 h. Orange peel (●OP) and the fibre fractions, insoluble fibre fraction (●IFF) and water-soluble extract (●WSE), and the negative control (●C-) and positive control (●C+). Values are expressed as mean ± SD (n =3). Different letters (a–d) indicate significant differences ($p$<0.05) among the samples.

In general, the three samples, OP and the extracted fractions IFF and WSE, exhibited a prebiotic effect *in vitro*, since led to a significant increase of the total SCFAs from the beginning to 48 h. Moreover, at the end of fermentation, it was observed that the fibre fractions followed a slow fermentation pattern. This may be beneficial, as slow-fermenting prebiotics prevent the onset of flatulence and are able to reach the last parts of the gastrointestinal tract promoting further benefits for the microbiota (Cui *et al.*, 2020; Hou *et al.*, 2022). The potential used of these samples as ingredients may allow the

development of healthy food, since the SCFAs produced after their fermentation in the colon, provides health benefits such as prevention of some types of cancer, inflammatory activity, and metabolism regulation, contributing to obesity control, appetite regulation, glucose homeostasis and blood pressure regulation (Tan *et al.*, 2014; Gill *et al.*, 2018; Sánchez-Alcoholado *et al.*, 2020).

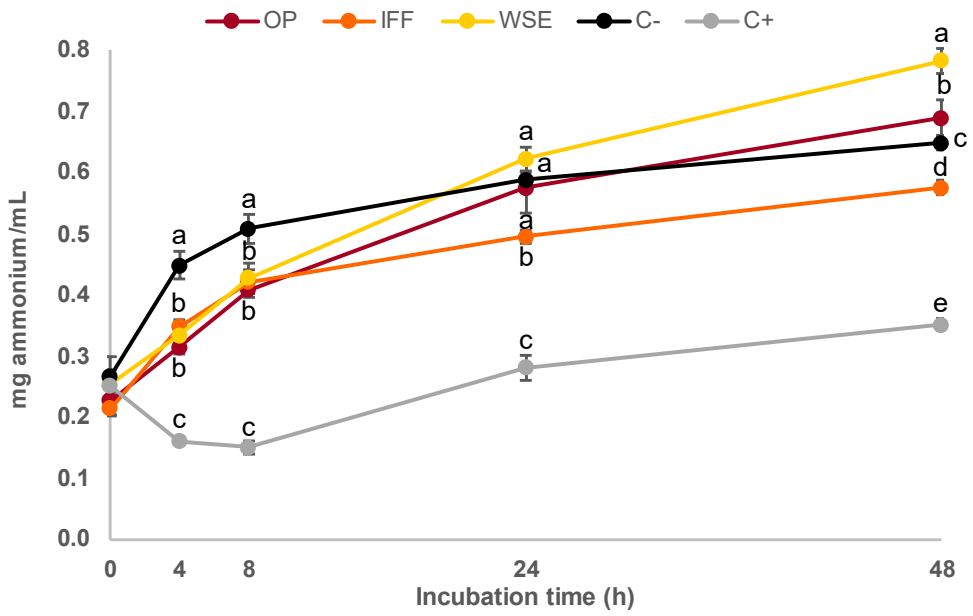


**Figure 45.** Ammonium production (mg ammonium/mL of faecal slurry) during *in vitro* fermentation for orange peel samples at 0, 4, 8, 24, and 48 h. Orange peel (●OP) and the fibre fractions, insoluble fibre fraction (●IFF) and water-soluble extract (●WSE), and the negative control (●C-) and positive control (●C+). Values are expressed as mean ± SD (n =3). Different letters (a–e) indicate significant differences ($p$<0.05) among the samples.

The ammonium produced during the *in vitro* fermentation process is shown in Figure 45. The ammonium is derived from the metabolisation of proteins and peptides present in the medium and in the samples, by the bacteria. It is noteworthy that in all samples there was an increase from the beginning of the fermentation, except in the case of C+, due to the content of glucose that is available for bacteria to produce mainly SCFAs. But, when glucose was depleted, the bacteria started to consume tryptone, from 8 h onwards, leading to an increase in ammonium production. Related to the production of ammonium in the *in vitro* fermentation of the samples significant differences were observed at 48 h. The WSE fraction showed the statistically highest production of ammonium, followed by OP and IFF. This behaviour was negatively corelated (r=-0.97, $p$<0.001) with the content of dietary fibre, which is used during fermentation to produce

SCFAs. So, in IFF fraction, as there was a highest TDF content, the bacteria mainly consumed it, digesting tryptone to a lesser extent, whereas in WSE fraction the TDF content was lower, so there was a greater consumption of tryptone simultaneously with the fibre, which led to a greater production of ammonium. Moreover, during the fermentation of OP the ammonium content showed an intermedium value in comparison with both extracted fractions, due to its TDF content was also between both fibre fractions.

### 3.7. Colonic *in vitro* biotransformation of orange peel (poly)phenols

The evaluation of the catabolites produced over 48 h of *in vitro* fermentation of the three samples obtained from orange peel (OP, IFF and WSE), was based on the degradation pathways proposed by Pereira-Caro *et al* (2016), in which the catabolites found in the different samples are indicated with orange labels (Figure 46 and 47).

UHPLC-HRMS analysis of the faecal slurries revealed degradation by the colonic microbiota over the time of incubation, of the flavanones and hydroxycinnamic acids derivatives, present in the samples of orange peel (Table Annex II). Seven parent flavanones were identified, including hesperitin-7-*O*-rutinoside, hesperetin hexoside, hesperetin, naringenin-7-*O*-rutinoside, naringenin-7-*O*-glucoside, naringenin, and isosakuranetin-7-*O*-rutinoside. Moreover, seven hydroxycinnamic acid derivatives were identified and quantified. These compounds were identified and quantified by comparison with authentic standards, except for hesperetin, hesperetin hexoside and ferulic acid hexoside which were tentatively identified by their exact mass and elution order and quantified using the most analogous standard. In addition, different phenolic acids catabolites derived from microbiota-mediated ring fission of flavanones and hydroxycinnamates were also detected in the faecal slurry samples. Eighteen phenolic catabolites have been identified and quantified as derived from from phenylpropionic acid, phenylacetic acid, benzoic acid and benzenetriol using authentic standards. When the standard was not available, which was the case of 3-(3′-hydroxy-4′-methoxyphenyl)hydracrylic acid and dihydroisoferulic acid, the most analogous standard was used.

Degradation of the parent flavanones were monitored over a 48 h incubation period, and the data obtained are included in Table 30. Hesperetin and naringenin-rutinosides, as well as their respective glucosides and aglycones, in addition to isosakuranetin-7-*O*-rutinoside have been found as the main flavanones at baseline of the fermentation, being hesperetin-7-*O*-rutinoside the major one. These compounds

have been previously described as the major components of orange peel by other authors (Zhu *et al.*, 2020; Wang *et al.*, 2022).

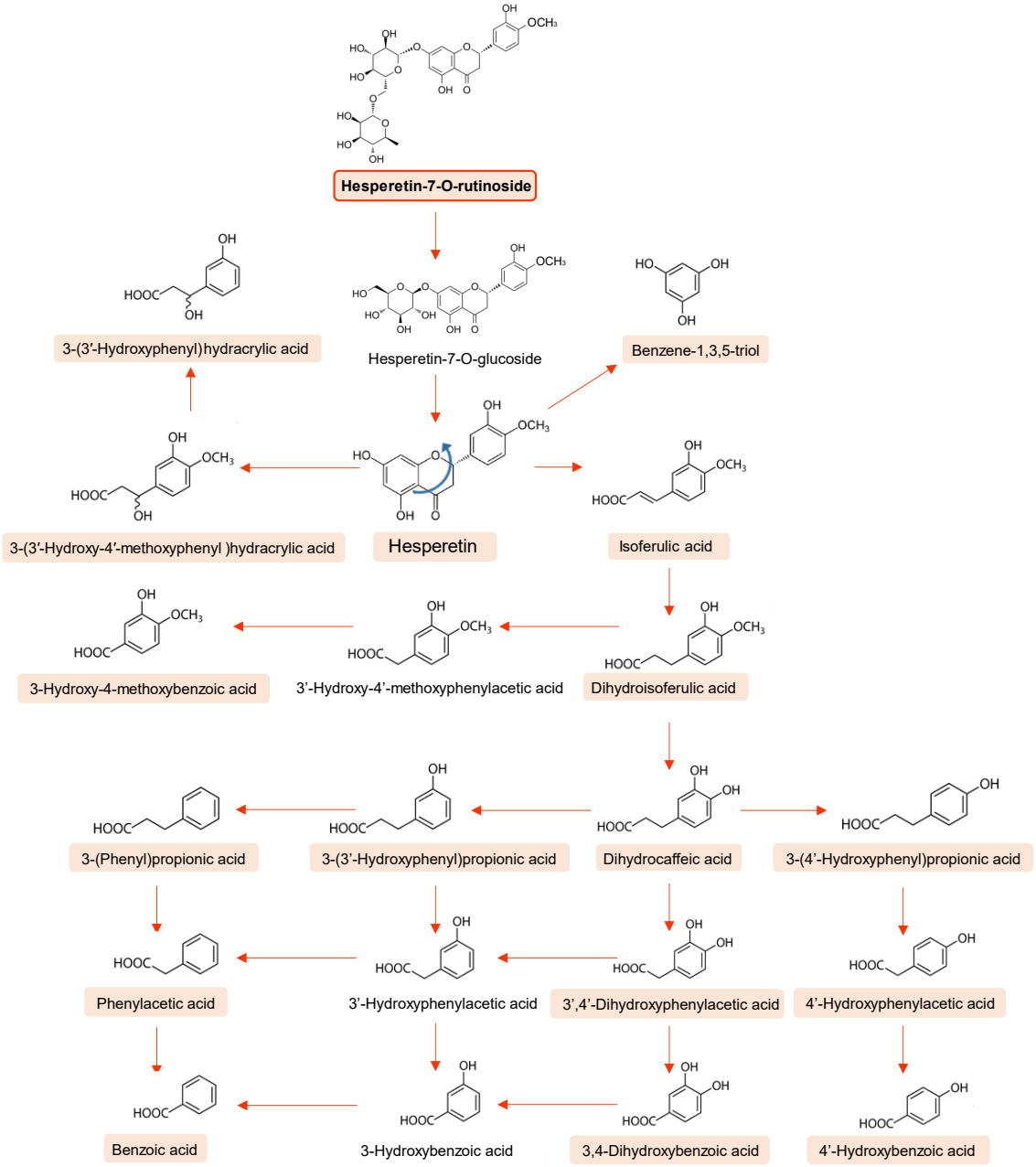


**Figure 46.** Proposed degradation pathway from hesperetin-7-*O*-rutinoside based on Pereira-Caro *et al*. (2016). Orange labels indicate compounds found in the present study.

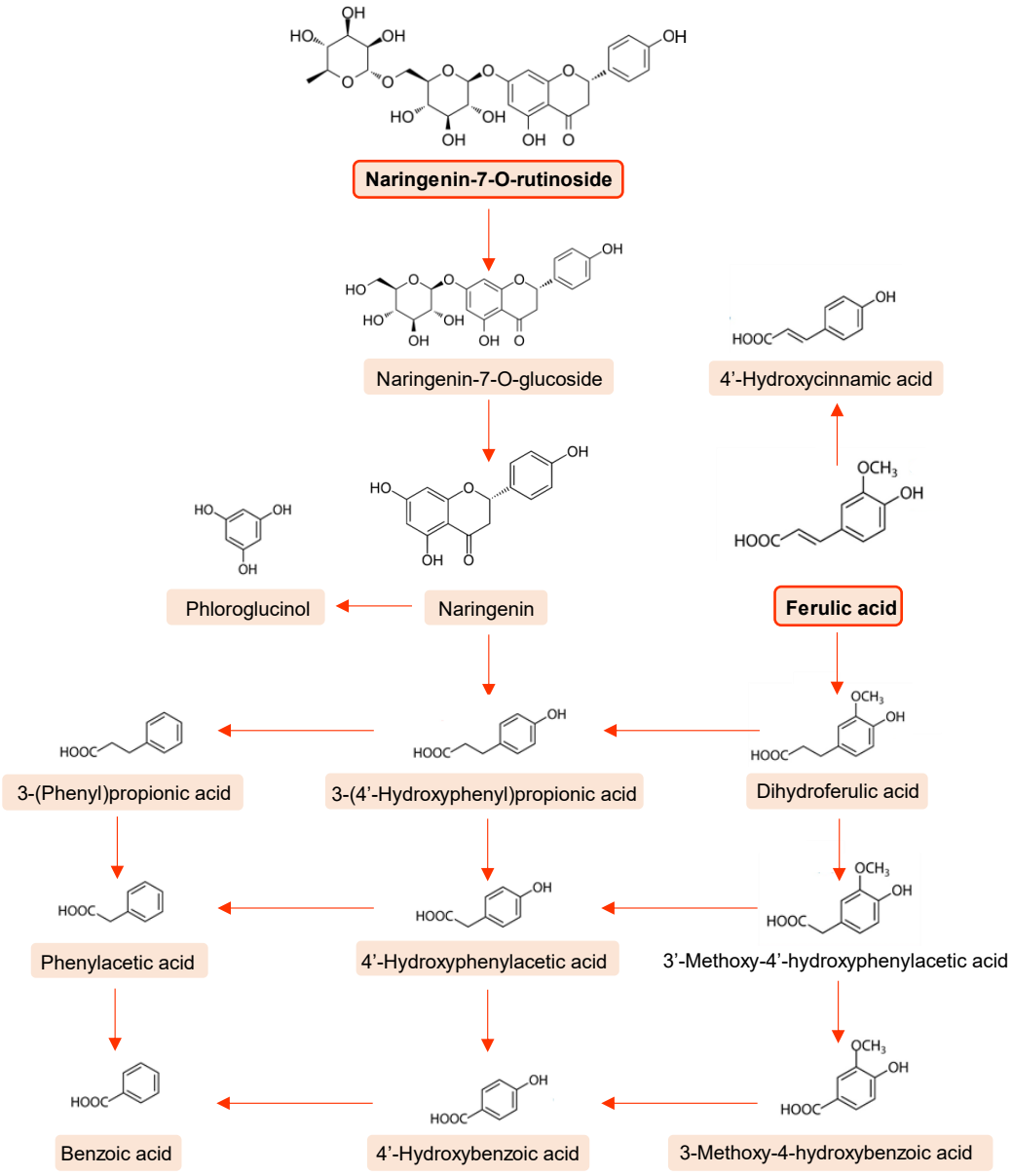


**Figure 47.** Proposed degradation pathway from naringenin-7-*O*-rutinoside based on Pereira-Caro *et al*. (2016). Orange labels indicate compounds found in the present study.

As for the evolution of flavanones during the fermentation time, the aglycone naringenin was significantly reduced at 4 h, showing a decrease of 67, 60 and 76% respectively for OP, IFF and WSE. Pereira-Caro *et al*. (2015) have shown also a significant decrease in naringenin content after 4 h of its faecal fermentation, and after 6 h it was metabolised completely, faster than other flavanones. For most of the flavanones it was necessary to reach more than 24 h of incubation to complete their degradation. This effect being observed mainly in the fibre-rich samples (IFF and OP), which indicates that these compounds must be released from their fibre bonds by the action of the

bacteria prior to be metabolised. The effect observed for the aglycone naringenin was not detected for the aglycone hesperetin, which after 4 h of incubation reached significantly higher amount compared to the beginning of the incubation period, which indicated its hydrolysis from heperetin-7-*O*-rutinoside and from hesperetin hexoside by the action of α-rhamnosidases and β-glucosidases of the colonic microbiota (Pereira-Caro *et al*., 2018).

**Table 30.** Degradation of flavanones (µg/g of faecal slurry) in faecal slurries after 0, 4, 8, 24 and 48 h of *in vitro* fermentation with different samples of orange peel.

| Compound | Sample | Fermentation time (h) | | | | |
|---|---|---|---|---|---|---|
| | | **0** | **4** | **8** | **24** | **48** |
| **Hesperetin-7-*O*-rutinoside** | *OP* | 63.6±5.5* | 70.7±0.3$^{a}$ | 56.4±18.3 | 17.8±6.7 | *tr* |
| | *IFF* | 51.9±9.3 | 73.7±10.3$^{a}$ | 47.5±19.3 | 19.1±11.0 | *tr* |
| | *WSE* | 52.7±2.7 | 52.8±5.0$^{b}$ | 58.2±6.3 | *tr* | *tr* |
| **Hesperetin hexoside** | *OP* | 0.9±0.0 | 0.2±0.0 | 0.4±0.1 | 0.2±0.1$^{a}$ | *tr* |
| | *IFF* | 0.7±0.1 | 0.3±0.1 | 0.3±0.2 | 0.2±0.1$^{a}$ | *tr* |
| | *WSE* | 0.8±0.1 | 0.2±0.0 | 0.3±0.1 | 0.02±0.0$^{b}$ | 0.01±0.0 |
| **Hesperetin** | *OP* | 5.6±0.5$^{ab}$ | 25.9±2.9 | 13.4±2.4 | *tr* | *tr* |
| | *IFF* | 7.6±1.3$^{a}$ | 15.5±7.3 | 12.8±4.5 | *tr* | *tr* |
| | *WSE* | 4.1±0.9$^{b}$ | 20.2±0.8 | 11.5±4.3 | *tr* | *tr* |
| **Naringenin-7-*O*-rutinoside** | *OP* | 4.2±0.6$^{b}$ | 4.1±0.3 | 2.7±1.4 | 0.8±0.4 | *tr* |
| | *IFF* | 2.5±0.8$^{c}$ | 4.0±1.1 | 2.4±1.7 | 0.7±0.4 | *tr* |
| | *WSE* | 6.4±0.9$^{a}$ | 5.3±0.2 | 4.3±0.3 | *tr* | *tr* |
| **Naringenin-7-*O*-glucoside** | *OP* | 0.3±0.0$^{b}$ | *tr* | *tr* | *tr* | *tr* |
| | *IFF* | 0.1±0.0$^{c}$ | *tr* | *tr* | *tr* | *tr* |
| | *WSE* | 0.6±0.1$^{a}$ | 0.01±0 | *tr* | *tr* | *tr* |
| **Naringenin** | *OP* | 1.8±0.1$^{b}$ | 0.6±0.0 | 0.3±0.1 | 0.1±0.0 | *tr* |
| | *IFF* | 1.0±0.1$^{c}$ | 0.4±0.2 | 0.3±0.1 | *tr* | *tr* |
| | *WSE* | 2.5±0.1$^{a}$ | 0.6±0.1 | 0.4±0.1 | *tr* | *tr* |
| **Isosakuranetin-7-*O*-rutinoside** | *OP* | 2.8±0.5 | 3.1±0.2 | 1.6±0.7 | 0.5±0.3 | *tr* |
| | *IFF* | 1.9±0.7 | 2.9±0.7 | 1.5±1.1 | 0.3±0.2 | *tr* |
| | *WSE* | 2.6±0.1 | 3.1±0.2 | 2.7±0.3 | *tr* | *tr* |

*Values are expressed as mean ± SD (n =3). Different letters (a–c) indicate significant differences ($p$<0.05) among the fractions at the same fermentation time. Orange peel (OP); insoluble fibre fraction (IFF); water-soluble extract (WSE); traces (tr).

Regarding the total content of flavanones shown in Figure 48.a., a difference was observed between OP and IFF compared to WSE, although there were no significant differences between the samples for the same fermentation times, but a trend was noted. In the *in vitro* fermentation performed with OP and IFF a clear increase was observed after 4 h of incubation time, as previously indicated, this increase was not so notable for WSE. In this fraction the content remained practically unchanged until 8 h of fermentation, disappearing almost completely at 24 h, while for the other two fractions, a gradual decrease was observed after 4 h, disappearing almost completely at 48 h. This may be due to the gradual release of compounds from the cell walls, which makes the metabolism of the compounds slower than when the amount of fibre was lower, as in the case of WSE, and therefore the compounds were released to a lesser extent. This decrease showed that the compounds have been metabolised by the microbiota, as previously mentioned Pereira-Caro *et al*. (2015), who demonstrated that after 6 hours of fermentation, the flavanones content was significantly reduced and appeared in very low concentration at the end of the fermentation process.

In the case of hydroxycinnamic acid derivatives, at the beginning of the *in vitro* fermentation, sinapic, isoferulic and ferulic acid were detected as the major ones (Table 31). Wang *et al*. (2022) showed that in orange peel the major components within this group were sinapic, ferulic and *p*-coumaric acid, the latter not being found in significant quantities in our samples, which may be due to the different orange varieties used.

As for the evolution of these compounds, sinapic acid increased in IFF at 8 and 24 h, which showed a similar effect to that observed for flavanones, which may be releasing from the cell walls due to the bacterial activity. On the other hand, in WSE, where it was found in higher amounts than in the other fractions, a clear decrease was observed from the beginning of fermentation. Ferulic acid and caffeic acid and the rest of the minorities decreased throughout the fermentation process, with ferulic disappearing after 24 h of incubation without differences among fractions. Pereira-Caro *et al*. (2015) showed that, in the *in vitro* fermentation of ferulic acid, it was completely metabolised at 6 h of fermentation, being faster compared to our study due to this *in vitro* fermentation was performed using the standard in contrast with our study where ferulic acid prior to be metabolised has to be release from the cell walls, being its degradation slower. As for isoferulic acid, it showed slight ups and downs throughout the fermentation process, appearing in similar concentrations at the beginning and end of the process, indicating that although this compound was being degraded, it was also being produced as a

catabolite from the degradation of hesperetin, as shown in Figure 46 of the degradation pathway based on Pereira-Caro *et al*. (2016).

**Table 31.** Hydroxycinnamic acid derivatives (µg/g of faecal slurry) detected in fermentation slurries after 0, 4, 8, 24 and 48 h of *in vitro* fermentation with different samples of orange peel.

| Compound | Sample | Fermentation time (h) | | | | |
|---|---|---|---|---|---|---|
| | | 0 | 4 | 8 | 24 | 48 |
| **Sinapic acid** | *OP* | 10.0±1.0$^{b}$* | 6.9±0.3$^{b}$ | 8.7±1.4$^{b}$ | 7.8±1.4$^{b}$ | 3.6±0.1$^{b}$ |
| | *IFF* | 2.7±0.1$^{c}$ | 2.9±0.1$^{c}$ | 4.2±0.0$^{c}$ | 7.8±0.2$^{b}$ | 3.5±0.2$^{b}$ |
| | *WSE* | 26.2±1.0$^{a}$ | 13.6±0.6$^{a}$ | 13.8±1.2$^{a}$ | 13.1±1.1$^{a}$ | 4.6±0.5$^{a}$ |
| **Caffeic acid** | *OP* | 0.5±0.1$^{b}$ | 0.1±0.0$^{b}$ | 0.1±0.0$^{b}$ | 0.1±0.0$^{b}$ | 0.1±0.0 |
| | *IFF* | 0.1±0.0$^{c}$ | 0.02±0.0$^{c}$ | 0.03±0.0$^{c}$ | 0.1±0.0$^{a}$ | 0.03±0.0 |
| | *WSE* | 1.2±0.0$^{a}$ | 0.1±0.0$^{a}$ | 0.1±0.0$^{a}$ | 0.1±0.0$^{a}$ | 0.04±0.0 |
| **Ferulic acid** | *OP* | 3.4±0.0$^{b}$ | 2.8±0.1$^{b}$ | 2.4±0.0$^{a}$ | nd | nd |
| | *IFF* | 2.2±0.04$^{c}$ | 2.1±0.0$^{c}$ | 2.1±0.1$^{b}$ | *tr* | nd |
| | *WSE* | 4.6±0.2$^{a}$ | 3.2±0.0$^{a}$ | 2.4±0.1$^{a}$ | nd | nd |
| **Isoferulic acid** | *OP* | 9.4±0.5$^{c}$ | 11.5±0.7$^{a}$ | 12.4±0.6 | 13.0±0.94$^{a}$ | 8.9±1.2$^{b}$ |
| | *IFF* | 10.3±0.4$^{b}$ | 9.9±0.4$^{b}$ | 11.6±0.6 | 8.9±0.9$^{b}$ | 11.5±0.7$^{a}$ |
| | *WSE* | 15.1±0.4$^{a}$ | 10.6±0.3$^{b}$ | 12.0±0.5 | 8.3±0.3$^{b}$ | 8.4±0.8$^{b}$ |
| **Ferulic acid hexoside** | *OP* | 0.21±0.0$^{b}$ | 0.20±0.0$^{b}$ | 0.19±0.0 | 0.16±0.0 | 0.15±0.0 |
| | *IFF* | 0.18±0.0$^{b}$ | 0.19±0.0$^{b}$ | 0.18±0.0 | 0.16±0.0 | 0.15±0.0 |
| | *WSE* | 0.28±0.0$^{a}$ | 0.24±0.0$^{a}$ | 0.20±0.0 | 0.18±0.0 | 0.15±0.0 |
| **3'-hydroxycinnamic acid** | *OP* | 0.4±0.0$^{a}$ | 0.3±0.0$^{b}$ | 0.3±0.0 | 0.3±0.0 | 0.2±0.0$^{b}$ |
| | *IFF* | 0.4±0.1$^{ab}$ | 0.4±0.0$^{a}$ | 0.3±0.0 | 0.6±0.0 | 0.3±0.0$^{a}$ |
| | *WSE* | 0.3±0.04$^{b}$ | 0.3±0.0$^{b}$ | nd | nd | nd |
| **4'-hydroxycinnamic acid** | *OP* | 0.4±0.0$^{b}$ | 0.2±0.0 | nd | nd | nd |
| | *IFF* | 0.2±0.0$^{c}$ | nd | nd | nd | nd |
| | *WSE* | 0.7±0.0$^{a}$ | 0.3±0.1 | 0.2±0.1 | nd | nd |

*Values are expressed as mean ± SD (n =3). Different letters (a–c) indicate significant differences ($p$<0.05) among the fractions at the same fermentation time. Orange peel (OP); insoluble fibre fraction (IFF); water-soluble extract (WSE); traces (tr); not detected (nd).

As for the total content of hydroxycinnamics (Figure 48.b.) observed at the beginning of the incubation, this was lower than the content of flavanones, agreeing with the data obtained for the characterisation of the three fractions. In this sense, it should be noted that a clear difference was observed between OP and IFF regarding WSE. Since in the first two fractions, the content remains constant with a slight decrease at 48

h. On the other hand, in WSE, a higher content was observed respect to the other two fractions in the beginning of fermentation, which was statistically significant at 0, 4 and 8 h, being higher at the beginning and decreasing significantly after 4 h (48%) of incubations, which indicated a greater metabolisation of the compounds present in this fraction.

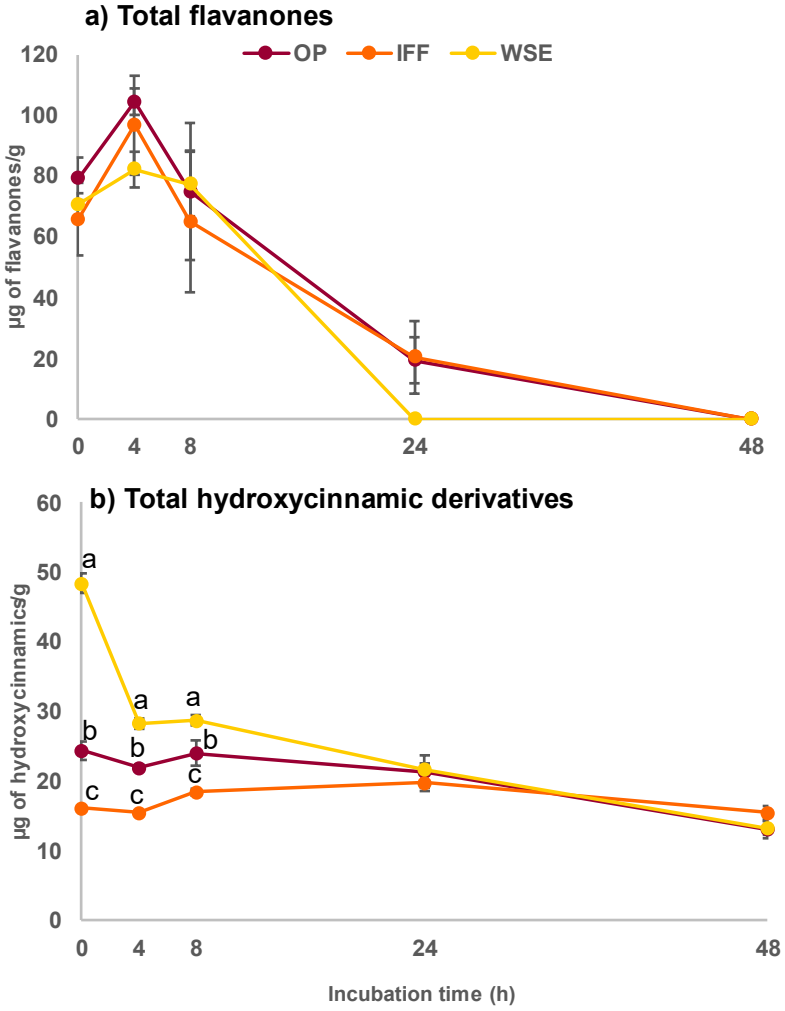


**Figure 48.** Total flavanones (a) and total hydroxycinnamic acid derivatives (b) found in fermentation slurries after 0, 4, 8, 24 and 48 h of *in vitro* fermentation with different samples of orange peel. Orange peel (●OP) and the fibre fractions, insoluble fibre fraction (●IFF) and water-soluble extract (●WSE). Values are expressed as mean ± SD (n =3). Different letters (a–c) indicate significant differences ($p$<0.05) among the fractions at the same fermentation time.

The breakdown products produced over 48 h of *in vitro* faecal fermentation with different fractions obtained from orange peel, were also investigated as an integral part of the overall bioavailability of orange (poly)phenols. Most of these products were derived

from the microbiota action on the orange (poly)phenols as a result of the ring fission as well as demethylation and dehydroxylation steps, and also shortening of the side chain which generated mainly derived of phenylpropionic acid, phenylacetic acid, benzoic acid and benzenetriol.

Figure 49 shows the phenylpropionic acid derivatives catabolites produced at 0, 4, 8, 24 and 48 h of faecal fermentation with the three fractions, being dihydroferulic acid, dihydroisoferulic acid, 3-(4′-hydroxyphenyl)propionic acid and 3-(3′-hydroxyphenyl)propionic acid the major ones. This group have been described in our samples as the major group of phenolic catabolites produced during fermentation for all the samples tested.

The results obtained shown that the major transformation products of hesperetin were dihydroisoferulic and 3-(3′-hydroxyphenyl)propionic acid, while 3-(4′-hydroxyphenyl)propionic acid was the main catabolite that came from naringenin degradation and dihydroferulic from ferulic acid degradation (Figure 46 and 47) in agreement with other authors (Pereira-Caro, *et al*., 2015). The amounts of these catabolites increased along the incubation time with the concomitant decrease of hesperetin, naringenin, and their rutinoside and glucoside derivatives, as well as the hydroxicinnamic acid derivatives. These catabolites started to appear mainly after 4 h of incubations, reaching the highest accumulation at 8, 24 and 48 h for dihydroferulic acid, dihydroisoferulic, and 3-(4′-hydroxyphenyl)propionic acid and 3-(3′-hydroxyphenyl)propionic acid, respectively.

As for the differences between the fractions, at 4 and 8 h, dihydroferulic was highest in WSE, while dihydroisoferulic was highest in OP, this was because even though at the beginning of fermentation process both ferulic and isoferulic were highest in WSE, after 4 h of fermentation the isoferulic content was highest in OP, which explains why after its conversion there was also more production of dihydroisoferulic in this fraction. 3-(4′-hydroxyphenyl)propionic acid and 3-(3′-hydroxyphenyl)propionic acid which come from the dehydroxylation of the two previous compounds, presented a significant increase in their production after 8 h, reaching their maximum at 48 h, due to their precursors (dihydroferulic and dihydroisoferulic) decreased significantly at this time. Both compounds were found in highest amounts in WSE along the fermentation process, being IFF the fraction with the lowest production. Although it was found in small quantities, it was worth noting the presence of 3-(3′-hydroxy-4′-methoxyphenyl)hydracrylic acid, whose concentration increased between 8 and 24 h and disappeared after 48 h of fermentation, being a compound that has been described

as a biomarker of orange juice intake (Pereira-Caro *et al.*, 2014; Pereira-Caro, *et al.*, 2015).

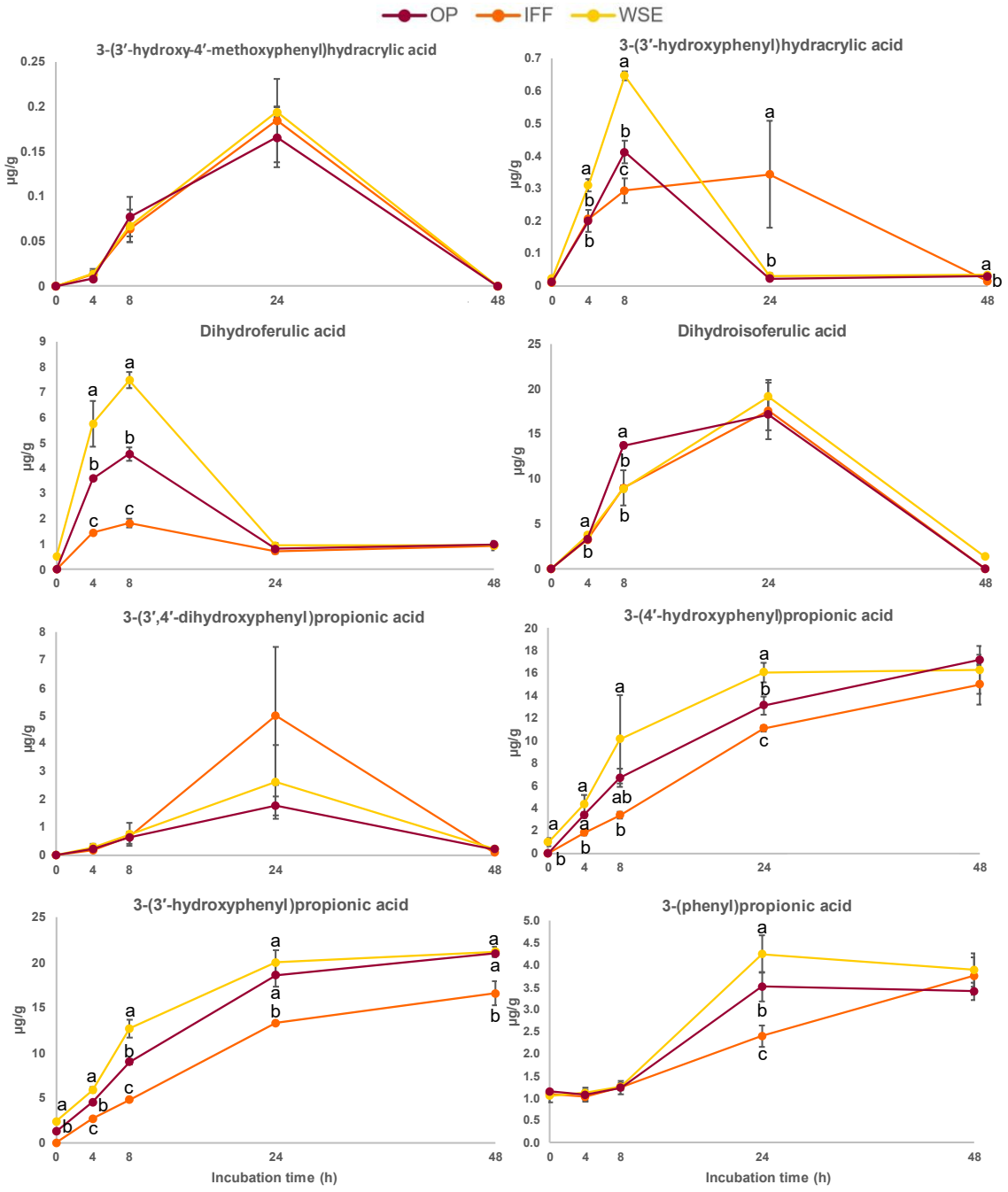


**Figure 49.** Phenylpropionic acid derivatives (µg/g of faecal slurry) found in fermentation slurries after 0, 4, 8, 24 and 48 h of *in vitro* fermentation with different samples of orange peel. Values are expressed as mean ± SD (n =3). Different letters (a–c) indicate significant differences ($p$<0.05) among the fractions (orange peel (●OP), insoluble fibre fraction (●IFF) and water-soluble extract (●WSE)) at the same fermentation time.

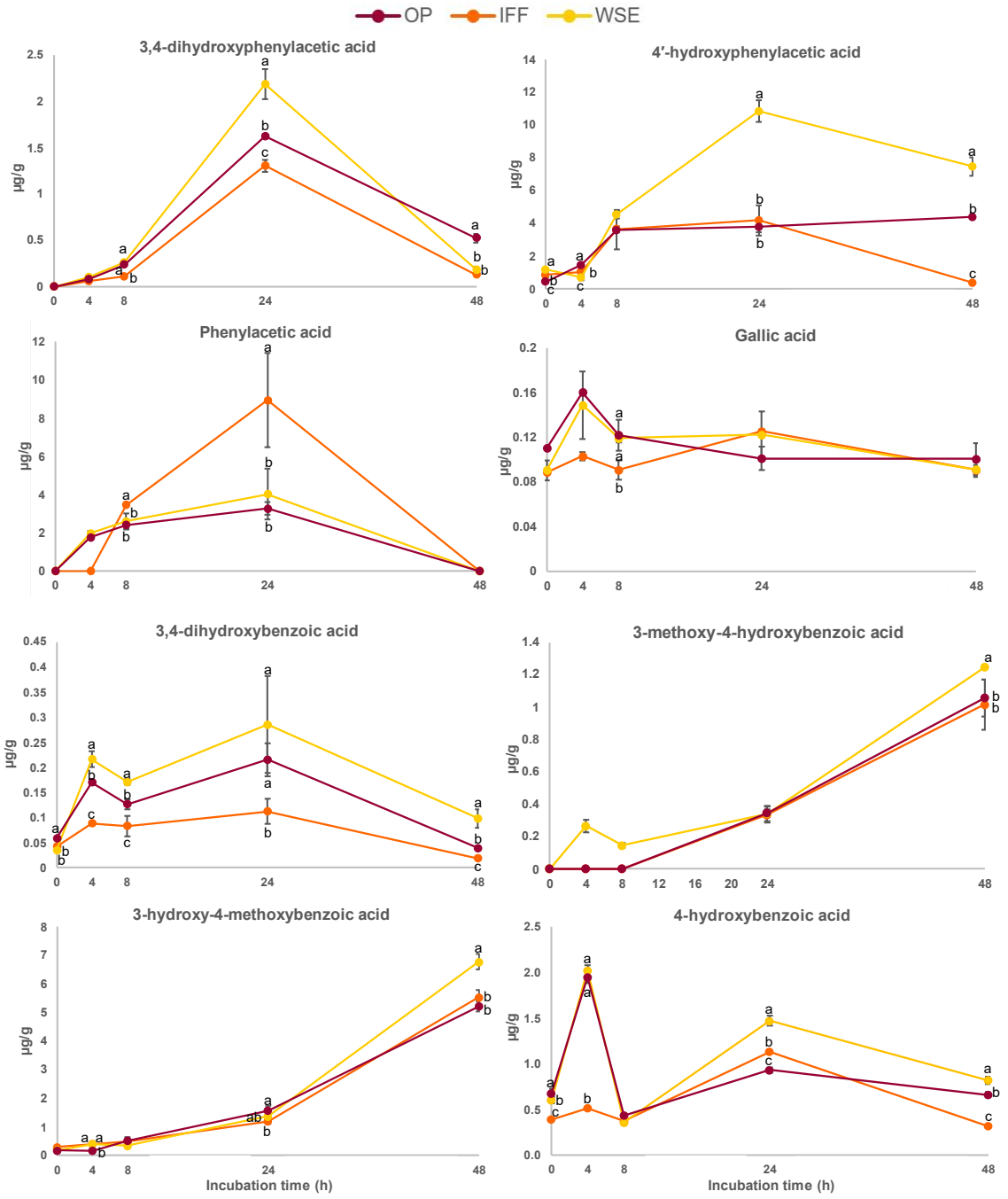


**Figure 50.** Phenylacetic acid derivatives and benzoic acid derivatives (µg/g of faecal slurry) found in fermentation slurries after 0, 4, 8, 24 and 48 h of *in vitro* fermentation with different samples of orange peel. Values are expressed as mean ± SD (n =3). Different letters (a–c) indicate significant differences ($p$<0.05) among the fractions (orange peel (●OP), insoluble fibre fraction (●IFF) and water-soluble extract (●WSE)) at the same fermentation time.

Related to the total phenylpropionic acid derivatives (Figure 51.a.), there was a gradual increase throughout fermentation, decreasing for all fractions after 28 h. The increase was significantly higher in WSE than in IFF. The production of these compounds

after 48 h of fermentation was significantly higher in WSE than in IFF due the larger amount of hydroxycinnamic acid derivatives and flavanones in this fraction compared with those present in the IFF fraction.

Figure 50 shows phenylacetic acid derivatives group, the second largest catabolites produced in our samples, the most remarkable compounds were 4′-hydroxyphenylacetic acid and phenylacetic acid, the first one, which is derived from 3-(4′-hydroxyphenyl)propionic acid, increased mainly after 8 h being highest in WSE fraction, reducing its concentration from 24 h onwards. This catabolite has been described also by Pereira-Caro, *et al*. (2015) from hesperetin, naringenin and ferulic acid metabolism. Regarding the second one, derived from 3-(phenyl)propionic acid and 3'-hydroxyphenylacetic acid, appeared at 4 h in OP and WSE and at 8 h in IFF, which was the fraction with the highest production, increasing notably at 8 h and then disappearing at 48 h. In terms of total phenylacetic acid derivatives content (Figure 51.b.), there was a gradual increase in all samples throughout the fermentation, being more noticeable at 8 and mainly at 24 h, after this time there was a decrease in the amount of these compounds. Regarding the different samples, the initial production was quite similar, except that the increase at 4 hours, being more pronounced in OP and WSE. At 24 h, it was WSE the one with the highest production and OP the significantly lowest. On the other hand, at 48 h the IFF was the fraction with the lowest production, and WSE was still the highest.

Although the benzoic acid derivatives group was produced in less quantity than the two previous ones, in our samples (Figure 50), three catabolites were remarkable, being 3-methoxy-4-hydroxybenzoic acid, 3-hydroxy-4-methoxybenzoic acid and 4-hydroxybenzoic acid. The 3-hydroxy-4-methoxybenzoic acid was the highest catabolite found in this group at 48 h of fermentation. This compound come from the degradation pathway of hesperetin, more specifically it come from the degradation of dihydroisoferulic acid, which may be degraded to 3'-hydroxy-4'-methoxyphenylacetic acid, which has been described as potential intermediate that accumulates in low amounts and which was not detected in our fermentation samples (Pereira-Caro *et al.*, 2016). Finally, this compounds by shortening of the side chain was converted in 3-hydroxy-4-methoxybenzoic acid, being produced mainly after 24 h and 48 h of fermentation and accumulating in high quantity in WSE fraction. 3-methoxy-4-hydroxybenzoic acid which comes from the degradation of dihydroferulic acid in naringenin pathway was produced after 4 h in WSE and after 24 in OP and WSE, being higher at 48 h without significant differences between the samples, showing WSE the highest production. The 4-

hydroxybenzoic acid was produced in OP and WSE in highest quantity at 4 h and in IFF at 24 h, being highest the content produced in WSE at 48 h of fermentation. Although benzoic acid did not outstand for the quantity produced (data not shown), the behaviour among the three samples analysed was different, being only produced at 24 h in the WSE fraction in a considerable amount (9.1 µg/g).

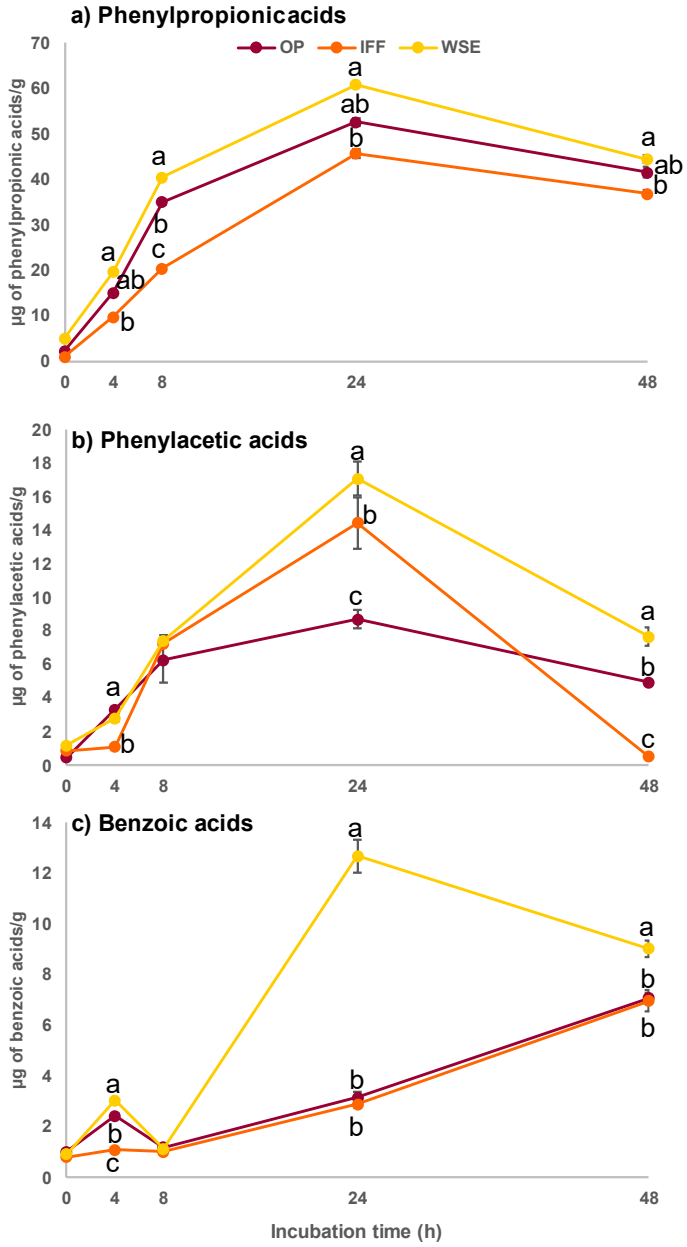


**Figure 51.** Total phenylpropionic acid derivatives (a), total phenylacetic acid derivatives (b) and total benzoic acid derivatives (c) (µg/g) produced in faecal slurries after 0, 4, 8, 24 and 48 h of *in vitro* fermentation with different samples of orange peel. Orange peel (●OP) and the fibre fractions, insoluble fibre fraction (●IFF) and water-soluble extract (●WSE). Values are expressed as mean ± SD (n =3). Different letters (a–c) indicate significant differences ($p$<0.05) among the fractions at the same fermentation time.

The behaviour of the total content of benzoic acid derivatives was similar in the three samples (Figure 51.c.), increasing between 0 and 4 h, decreasing at 8 h and increasing again after 24 h. The highest amounts for OP and IFF were recorded at 48 h and in WSE at 24 h, being the sample that showed the highest contents at 4 h, 24 h and 48 h of fermentation.

Only one compound has been found within the benzenetriol derivatives (data not shown), being phloroglucinol, this compound is derived from the A-ring fission of naringenin and hesperetin (Figure 46 and 47). It was found at 4 h in WSE, at 8 h in IFF and at 24 h in OP, appearing in all of them in similar concentrations but at different fermentation times. This could be due to the highest content of fibre in IFF and OP compared to WSE, which would affect its bioaccessibility, delaying the production of phloroglucinol.

Figure 52 shows the total catabolites production over 48 h of *in vitro* faecal fermentation. It can be seen that along the fermentation process, WSE was the fraction with the highest catabolites production at 8 h, 24 h and 48 h, reaching the highest value at 24 h of fermentation (91 µg/g), with the concomitant decrease of total flavanones in this fraction at that fermentation time (Figure 48).

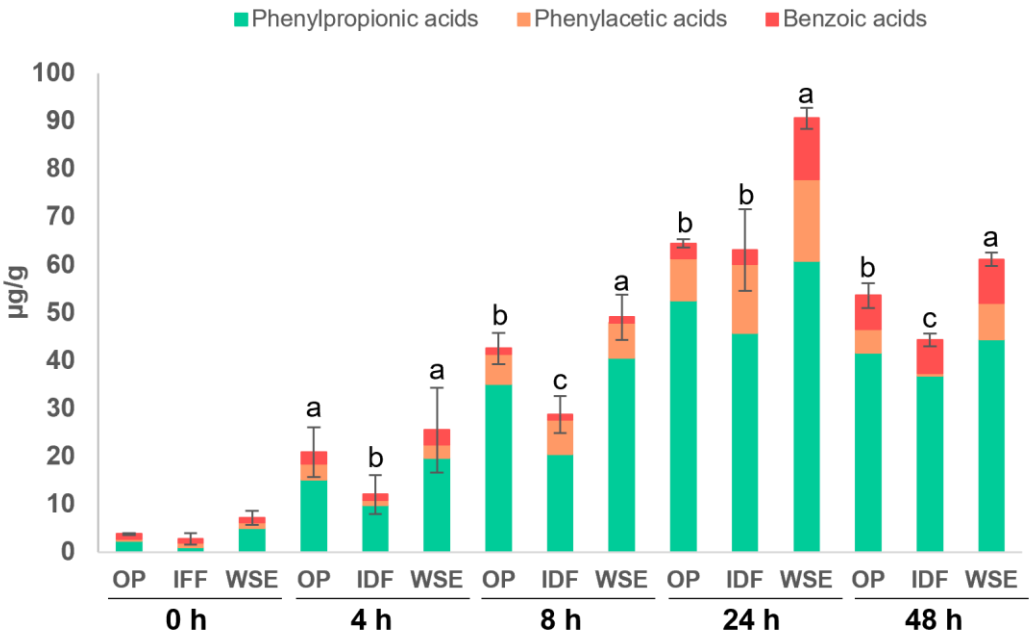


**Figure 52.** Total phenolic catabolites content (µg/g) as the sum of phenylpropionic (■), phenylacetic (■) and benzoic (■) acids derivatives. Different letters (a-c) indicate significant differences ($p$<0.05) between the different samples at the same fermentation time.

In summary, the extraction and separation of the two orange peel fractions with water, allowed to obtain a fraction with high fibre content (IFF) showing an important prebiotic effect, and another with a lower content of dietary fibre than IFF but with a higher content of (poly)phenols (WSE), which also exhibited a prebiotic effect. In addition, this water-soluble fraction showed a significant content of (poly)phenols, mainly flavanones, which may reach the colon where they are fermented by microbiota and led to the highest production of catabolites from phenolic origin. These results showed that orange peel is a by-product whose valorisation is feasible and interesting, as the characteristics of the fractions obtained allow its reintroduction into the food chain. Due to the differences in the chemical composition, both fractions could be used to design and develop functional ingredients, providing beneficial effects for human health, due to their dietary fibre and (poly)phenols content.

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# Chapter 8. General discussion

To achieve the general aim of this PhD Thesis different plant food by-products were selected according to the chemical composition: berries for their high content of (poly)phenols, broccoli stalks for their content of glucosinolates, and orange peel due to the presence of (poly)phenols and carotenoids. These raw materials were subjected to different extraction processes with the aim to isolate different fractions with high content of dietary fibre and bioactive compounds. Because the extraction processes led to obtain fibre-rich fractions with different composition, all of them were characterised, to know the nutritional value, the composition of the dietary fibre and the content of bioactive compounds evaluating the prebiotic effect and the production of catabolites from (poly)phenols, by using *in vitro* fermentation assays, to understand the bioavailability and the beneficial effect for human health, derived from the use of these fractions as ingredients with added-value.

In the scientific literature, different extraction methods have been applied to extract fibre-rich fractions (Tejada-Ortigoza *et al.,* 2016; Cruz-Requena *et al.,* 2019). Despite of the enzymatic extraction method led to a better separation of the fractions that comprise the dietary fibre, since enzymatic digestion eliminate starch and proteins, followed by the precipitation of the soluble fibre polysaccharides with ethanol. This method has some disadvantages because it requires long time, since is carried out in different steps, and it is very expensive due to the high prices of the enzymes (Tejada-Ortigoza *et al.,* 2016). This extraction method was applied to isolate the TDF, IDF and SDF from raspberry, whereas in the other three by-products a low-cost and green extraction procedures were used. The use of two phases extraction procedure, firstly with water and secondly with ethanol, allowed the isolation of two clearly differentiated fractions. Water extraction isolate mainly water-insoluble compounds of dietary fibre, and the subsequent addition of ethanol to water lead to the precipitation of the water-soluble polysaccharides. When the samples had a high content of soluble compounds, the ethanol precipitation step have to be included to isolate pectin and soluble hemicellulose, as have been applied in berries. However, this procedure is not profitable for broccoli stalks, due to its high content of insoluble fibre and the low proportion of soluble fibre. For this reason, the water-ethanol extraction could be considered a procedure to be used for the extraction of fibre from fruits, when the soluble fibre is represented in high proportion. Despite of the water-ethanol extraction use ethanol, the chemical characteristics of this extractant allow its recovery at the end of the process, and then the ethanol can be reused, and hence this method can be considered as a green extraction procedure. On the contrary, for the by-products from vegetables or other food matrix with reduced proportion of soluble fraction, the use of ethanol can make the processes expensive and unprofitable.

For the extraction of fibre-rich orange fractions from orange peels, ethanol was not used. Insoluble compounds were isolated with water, and due to the gelling properties of the sample, soluble compounds were physically separated after gelling, avoiding the use of ethanol. However, this procedure led to obtain a fraction with high content of IDF and other fraction with low content of SDF, since the non-digestible polysaccharides were not precipitated, but this fraction exhibited a high amount of (poly)phenols.

Our findings showed that for the extraction of dietary fibre, different procedures could be applied, but this fact determines the chemical characteristics of the fibre-rich fractions and hence the functional properties. In addition, the extraction process should be selected according to the composition of the by-products used as raw material, to obtain a high yield in the extractions. Taking into account the differences in the extraction processes, the chemical and physicochemical characterisation of the samples should be carried out to consider them as functional ingredients.

According to the results obtained regarding the linearity of the pectins, the pectin of the insoluble fractions from berries, broccoli stalks and orange peels showed a lower linearity than that of the soluble fractions. In addition, these fractions showed a low contribution of mannose to the hemicellulose structure, which indicates their high insoluble character, being the broccoli stalk fractions the ones that showed the lowest value for this parameter, since it is mainly composed by insoluble fibre. On the other hand, the pectin of the soluble fraction from berries showed a high linearity, but this effect was not observed in the soluble fraction from orange peel, which showed low linearity. This finding could be explained because this fraction was mainly constituted by soluble hemicellulose, with a high contribution of mannose. Despite of the soluble fraction of orange peel showed a high content of carbohydrates, only a 24% was dietary fibre, because during the extraction procedure the non-starch polysaccharides were not isolated with ethanol, as have been mentioned before. However, this fraction remains high content of hemicellulose and flavanones.

Moreover, should be taken into account that the composition and structure of the fibre of the different fractions determines their physicochemical properties, showing the fractions with the highest content of uronic acids, and hence, the highest pectin content, the best hydration properties, measured as water retention capacity and swelling capacity. However, the fat absorption capacity was not related to the chemical composition of fibre, although the variations may be due to other factors such as porosity and particle size, which have not been measured in our study (Mudgil and Barak, 2019). The physicochemical properties were measured to evaluate the technological role of

these fractions if they are used as ingredient for the development of functional foods. Those with high hydration properties, at the same time that provide soluble fibre, could contribute to avoid water loss and dehydration processes, improving the texture and viscosity of the developed fibre-rich products (Song *et al*., 2019; Liu *et al*., 2021). Because all samples showed a similar ability to absorb fat, this highlights its potential use to prevent fat loss during cooking. Moreover, hydration properties also are related to the physiological effects of dietary fibre, since soluble fibre may increase the stool bulk and bowel movements, as they are not previously fermented and reach the colon intact (McRorie and McKeown, 2017).

In terms of the content of bioactive compounds, and particularly for the content of (poly)phenols, in the fibre-rich extracted fractions depended both on the raw materials and on the methods used during the extraction. The enzymatic and water-ethanol extraction procedures, which use ethanol, removed and eliminated a proportion of the (poly)phenol compounds of the raw materials, but a significant amount remained in the samples. In this sense, the EPP were mainly removed during the extraction, in contrast to the NEPP, since they are bounded to the dietary fibre. Different type of (poly)phenols were detected in the fractions according to the by-products. The berry fractions showed anthocyanins, ellagitannins and ellagic acid derivatives in the EPP and NEPP, whereas only ellagic acid derivatives were found in the raspberry fractions, in contrast to the berry fractions, in which ellagic acid derivatives and hydroxycinnamic acids were found as NEPP. The fractions from broccoli stalk showed chlorogenic acid and sinapic acid derivatives in both EPP and NEPP, and hydroxycinnamic acid derivatives and flavanones were found in EPP and NEPP of the orange peel fractions.

It is worth mentioning the highest presence of NEPP than EPP in the fibre fractions, mainly in the insoluble fractions, which can be ought to two main reasons. Firstly, the isolation of soluble fractions, with the exception of water compounds of orange peel, was carried out with ethanol and hence the EPP were removed; secondly, the insoluble fractions contain a higher content of fibre than the soluble fractions and hence more NEPP. While the different berries fractions, from raspberry and mix of berries, exhibited a high content of NEPP, in the broccoli stalk, NEPP was ten-fold lower, because the content of (poly)phenols in this matrix or raw material was found at low proportion. On the other hand, the trend observed in the orange fractions was different, since only water was used in the extraction method, showing both fibre fractions a higher content of EPP than NEPP. It deserves to mention that the water-soluble fraction showed 5-fold more EPP than NEPP, since unlike the other extraction methods the water was not removed

and the gelled soluble fraction contained a low proportion of soluble fibre but a high amount of (poly)phenols, mainly flavanones which were extracted due to its water-soluble character (Vallejo *et al.,* 2010).

Other bioactive compounds (glucosinolates and carotenoids) were identified in the fibre-rich fractions from broccoli stalks and orange peels since they are naturally found in these by-products (Escobedo-Avellaneda *et al.,* 2014; Liu *et al.,* 2018). In this sense, both fractions obtained from broccoli stalks contained more than 50% of the glucosinolates of the raw material, being glucoraphanin and methoxyglucobrassicin the main compounds, mainly found in the insoluble fraction. This behaviour is explained because this fraction was isolated only with water, since in the total dietary fibre fraction, glucosinolates were removed with the ethanol during the precipitation of soluble non-starch polysaccharides. On the other hand, the orange peel fractions showed carotenoids, but they were mainly extracted in the insoluble fraction, because remained bounded to the insoluble part of the peel represented by the flavedo. The main carotenoids identified were violaxanthin and β-cryptoxanthin, and they were also found in the water-soluble extract of orange peel.

So, the extraction method not only determines the content of dietary fibre in the isolated fibre-rich fractions, but also the content of bioactive compounds, contributing to the intake of these antioxidants. However, depending on the extent to which they are bound to the fibre, this may also affect their bioavailability. For this reason, the presence of bioactive compounds is interesting from a nutritional point of view, because they provide biological activity to these fibre-rich fractions. The antioxidant capacity of the fibre-rich fractions was analysed to determine the functional properties to avoid oxidative reactions, by reducing iron (FRAP method) or by scavenging free radicals (ORAC method). All extracted fractions exhibited antioxidant capacity which was related to the (poly)phenol content (Del Rio *et al.,* 2013). Orange peel fractions showed a high values of antioxidant capacity because they were the fractions with the highest (poly)phenol content, followed by the berry fractions (raspberry and mixed of berries). On the contrary, the broccoli stalk fractions showed a lower antioxidant capacity as these fractions showed the lowest concentration of bioactive compounds. Therefore, the extraction of fibre-rich fractions from by-products also showed an interesting content of (poly)phenols that may act as antioxidant when they are used as ingredient in the development of functional foods, but also contribute to the intake of these antioxidants, as well as the intake of glucosinolates and carotenoids.

It is known that plant bioactive compounds can reach the colon intact where they are subjected to the action of the microbiota producing several catabolites, which may exert different beneficial effects (Ludwig *et al.,* 2018). They can reduce the oxidate stress in the intestinal lumen, but if the non-starch polysaccharides of the fibre fractions are fermented by the microbiota they are released and may be used also by the gut bacteria, leading to different catabolites than can be absorbed and then reach the liver where they are conjugated and distributed through the systemic circulation (Martínez-Meza. *et al.,* 2021). It is expected that the microbial catabolites derived from these fractions will be different, depending on the compounds naturally present in the by-products and the fibre-rich fractions, but also depend on the host microbiota. To evaluate the prebiotic effect of the non-starch polysaccharides and the (poly)phenols of the different fractions and their beneficial effect on the gut microbiota, *in vitro* fermentation assays were conducted, evaluating the microbiota metabolism by the analysis of SCFAs, and the production of catabolites from (poly)phenols with beneficial effect for human health.

All fibre fractions obtained from the different by-products showed production of SCFAs as a measure of the prebiotic effect (Pérez-Burillo *et al.,* 2021). Furthermore, the production of SCFAs from the (poly)phenol extracts prepared from the raspberry and berry mixture by-products was also observed, confirming the potential prebiotic-like effect previously attributed to these compounds by other authors (Jaquet *et al.*, 2009; Dueñas *et al.*, 2015), being similar to the production of SCFAs by the fibre fractions. It was observed that the majority of the SCFAs produced was acetate, followed by propionate and butyrate (Blaut, 2018). As for other minor SCFAs (isobutyrate, isovalerate, valerate, isocaproate, caproate and heptanoate) were produced to a lesser extent, since most of those present in this group are derived from the metabolism of proteins and amino acids, and in the case of the fibre fractions, polysaccharides predominate as carbon source to produce the major SCFAs, and metabolising to a lesser extent the proteins and amino acids (Shortt *et al.*, 2018). In this sense, ammonium was also measured as indicator of protein metabolism showing low values for all fractions. The differences observed in the production of SCFAs in the different experiments may be due to the different composition of the fibre-rich fraction and to the different inoculums used, since not all of the *in vitro* fermentations were carried out with the same inoculum. Furthermore, in the present PhD thesis the experiment 1 was carried out with individual inoculums, and experiments 2, 3 and 4 with pooled faeces from different volunteers. It has been observed that for the validation of the prebiotic effect of an ingredient it is more suitable to use a pool of faeces, since due to the greater homogeneity it is possible to observe more clearly the differences between the prebiotic effect of the different samples

(Aguirre *et al.,* 2014; Pérez-Burillo *et al.,* 2021). In this sense, these results indicate that after ingestion of these samples, the production of SCFAs after the fermentation in the colon might provide health benefits to the host, such as the reduction of pathogenic species, as well as anti-inflammatory effects, the modulation of glucose homeostasis and lipid metabolism, and the prevention of several types of cancer, including colon cancer, which is related to the production of butyrate (Morrison and Preston, 2016; Gill *et al*., 2018; Yegin *et al.*, 2020).

In addition to the production of SCFAs, the production of catabolites derived from the metabolism of (poly)phenols by the microbiota was analysed. In this regard, for the berries, the degradation of anthocyanins, ellagitannins and ellagic acid were monitored, and the production of urolithin A was evaluated as products from ellagic acid catabolism (González-Barrio *et al.*, 2012). In the first experiment using raspberries, a high production of urolithin was observed from the fractions with the highest amount of extractable ellagic acid, while in the second experiment, a high production was observed from the insoluble fraction with a high presence of non-extractable ellagic acid, which would indicate that its release by microbial activity allows a more prolonged production over time. After the fermentation of the broccoli stalk fractions, no catabolites were detected, as the concentration of (poly)phenols present in these fractions was low. However, phenolic acid catabolites were detected during *in vitro* fermentation of the orange peel fractions. In this case, the catabolites analysed were mainly from flavanones and hydroxycinnamic acids catabolism, producing mainly phenylpropionic acid derivatives, followed by phenylacetic and benzoic acid derivatives (Del Rio *et al.,* 2010). These catabolites were found in high proportion in the water-soluble fraction due to the highest presence of flavanones and hydroxycinnamic acids in this fraction. As the insoluble fraction had a high amount of fibre, a gradual production was observed due to the release of compounds bound to the fibre compared to the soluble fraction, where the fibre content was lower and therefore the compounds bounded were also lower.

The greater or lesser production of catabolites provides an estimation of the accessibility that the microbiota would have to the (poly)phenols that reach the colon intact, attached or not to fibre. It is well known that the production of these catabolites makes the ingested parent compounds more bioavailable after ring fission and the subsequent reduction, demethylation, and dehydroxylation reactions carried out by the microbiota on the (poly)phenols ingested (Pereira-Caro et al., 2016). Then, these breakdown products can be absorbed and rapidly subjected to an extensive phase II metabolism before entering the systemic circulation and providing important health

benefits (Martínez-Meza et al., 2021). Despite some authors reported that the presence of fibre might change the (poly)phenols metabolism (Palafox-Carlos *et al.,* 2011), our findings showed that, although the content of dietary fibre is different according to the extraction method used in berries and orange fraction, the fibre compounds did not interfere substantially in the ellagic acid, flavanones and hydroxycinnamic acids catabolism, producing urolithin A and a wide variety of catabolites. The results obtained showed that it is not the amount of fibre that determines the number of catabolites produced, but the amount of parent compounds present in the samples.

Based on this background, obtaining fractions with a high fibre content and an interesting profile of bioactive compounds is a tool for the valorisation of the by-products used, allowing the development of potential added-value ingredients. As described before, and according to their nutritional composition and their physicochemical properties they can be used as ingredient for the development of functional products, particularly fibre-enriched foods. In addition, due to the health benefits derived from the production of catabolites during *in vitro* fermentation, both from fibre metabolism and from the (poly)phenols present in these fractions, these fibre-rich fractions could also be used for the development of functional foods with improved nutritional properties, obtaining health benefits for consumers associated to the SCFAs and (poly)phenols catabolites (Mele *et al*., 2016; Pereira-Caro *et al*., 2016; Blaut, 2018). In this sense, they can be used for the development of juices and beverages, confectionery, jams, syrups, bakery products and candies, among others. However, taking into consideration that the fractions obtained would provide colour, taste and flavour, mainly due to the presence of the bioactive compounds, which may be desirable in some processes, but perhaps not in others, some disadvantage might appear for the application of them in the food industry.

Although *in vitro* fermentations give us a first approach about how these samples could be fermented in the colon, and hence which is their prebiotic effect and how they are used by the microbiota, leading to the formation of different microbial catabolites from non-starch polysaccharides and (poly)phenols, this method has some limitations. Different factors such as the total number of volunteers, sex, age and health condition may limit the results of the *in vitro* fermentation assays. We therefore suggest that to evaluate the prebiotic effect of different substrates, the use of faeces from a pool of volunteers (with similar physiological and health conditions) reduces the variability and allows to obtain consistent results. However, considering the differences in the microbiota according to sex, age and health condition and the occurrence of different

metabotypes, the number of volunteers should be increased to reduce the variability. Even though when a functional food is designed to a specific group of consumers, their characteristics should be taking into consideration to evaluate the prebiotic effect using *in vitro* fermentation assays, as part of personalised nutrition. As future lines of the present work, we highlight the analysis of the microbiota that will be carried out immediately after the completion of the PhD Thesis. The analysis of microbiota will provide more information about the prebiotic effect of these ingredients and how they can modulate the growing of specific beneficial bacteria from the gut.

# Chapter 9. Conclusions

The following conclusions have been obtained in the present PhD thesis:

**First**

Enzymatic methods allow a greater separation of the polysaccharides that make up the fibre, as well as the soluble and insoluble fractions, but they are expensive and laborious. The use of water-ethanol extraction has a good performance in separating the two fibre fractions (soluble and insoluble) when the samples have a high soluble fibre content, as in berries by-product. Water extraction led to a better separation of the insoluble fraction, obtaining a high fibre content in all by-products.

**Second**

The amount, composition and type of fibre (soluble and insoluble) present in the isolated fibre fractions depend on the by-product and the extraction method used. In this sense, these fibre-rich fractions show different physicochemical properties according to their composition, showing the fractions with high content of uronic acids and soluble hemicellulose the highest hydration capacity.

**Third**

The fibre fractions show antioxidant capacity directly related to their content of (poly)phenols. The EPP content of fibre samples depends on the major (poly)phenols present in the by-product and the extraction method used, since ethanol extraction removes these compounds. The content of NEPP is generally associated with high insoluble fibre content in the samples.

**Fourth**

Other bioactive compounds are associated with the fibre fractions according to the by-product. The total and insoluble fibre fractions from broccoli stalks contain glucosinolates from the starting material, being the main group indole glucosinolates, while the fibre-rich fractions from orange peel have carotenoids, being responsible for its colour.

**Fifth**

All fibre fractions extracted from the selected by-products show prebiotic effect, producing SCFAs during the *in vitro* faecal fermentation, depending on their content of fibre and chemical composition. For the berries fractions, the prebiotic effect is due to the presence of non-starch polysaccharides as well as (poly)phenols.

**Sixth**

Microbiota from human faeces used in the *in vitro* faecal fermentations produces catabolites from the (poly)phenols present in the fibre-rich fractions. The fermentation of the insoluble fraction from berries, containing ellagic acid, shows the production of urolithins. The orange peel fibres show the production of phenolic acids from the catabolism of flavanones and hydroxycinnamic acids. Fermentation of insoluble fibre from broccoli stalk do not allow the identification of any microbial catabolite from (poly)phenols might be due to its low content. The use of these fibre fractions to enrich foods may have beneficial effects on consumer health due to their fermentation by the microbiota and the subsequent production of more bioavailable compounds.

**Seventh**

The selected by-products can be valorised after the isolation of dietary fibre fractions, which can be used as ingredients for the design of functional foods. However, organoleptic properties should be taken into consideration for their application in different food products.

# Annexes

# Annex I

# INFORME DE LA COMISIÓN DE ÉTICA DE INVESTIGACIÓN DE LA UNIVERSIDAD DE MURCIA

Jaime Peris Riera, Catedrático de Universidad y Secretario de la Comisión de Ética de Investigación de la Universidad de Murcia,

CERTIFICA:

Que D.ª Vanesa Núñez Gómez ha presentado la memoria de trabajo de la Tesis Doctoral titulada "*Evaluación del efecto prebiótico de compuestos bioactivos y de la fibra dietética para la revaloración de subproductos de origen vegetal*", dirigida por : D.ª M.ª Jesús Periago Castón y D.ª Rocío González Barrio a la Comisión de Ética de Investigación de la Universidad de Murcia.

Que dicha Comisión analizó toda la documentación presentada, y de conformidad con lo acordado el día 11 de febrero de dos mil veinte[1], por unanimidad, se emite INFORME FAVORABLE, desde el punto de vista ético de la investigación.

Y para que conste y tenga los efectos que correspondan firmo esta certificación con el visto bueno del Presidente de la Comisión.

Vº Bº
LA PRESIDENTA DE LA COMISIÓN
DE ÉTICA DE INVESTIGACIÓN DE LA
UNIVERSIDAD DE MURCIA

Fdo.: María Senena Corbalán García

ID:2664/2019

[1]A los efectos de lo establecido en el art. 19.5 de la Ley 40/2015 de 1 de octubre de Régimen Jurídico del Sector Público (B.O.E. 02-10), se advierte que el acta de la sesión citada está pendiente de aprobación

# INFORME DEL COMITÉ DE BIOSEGURIDAD EN EXPERIMENTACIÓN DE LA UNIVERSIDAD DE MURCIA

Lucía Periago García, Jefa de Sección de Recursos Humanos de Investigación y del Plan Propio y Secretaria del Comité de Bioseguridad en Experimentación de la Universidad de Murcia.

CERTIFICA:

Que D. ª Vanesa Núñez Gómez ha presentado la memoria de trabajo de la Tesis Doctoral titulada *"Evaluación del efecto prebiótico de compuestos bioactivos y de la fibra dietética para la revaloración de subproductos de origen vegetal"*, dirigida por D. ª M.ª Jesús Periago Castón y D. ª Rocío González Barrio al Comité de Bioseguridad en Experimentación.

Que el Comité de Bioseguridad en Experimentación analizó toda la documentación presentada, y de conformidad con lo acordado el día tres de julio de dos mil veinte, por unanimidad, se emite INFORME FAVORABLE, desde el punto de vista ético de la bioseguridad en la investigación.

Y para que conste y tenga los efectos que correspondan, firmo esta certificación, con el visto bueno de la Presidenta de la Comisión.

Vº Bº
LA PRESIDENTA DEL COMITÉ
DE BIOSEGURIDAD EN EXPERIMENTACIÓN
DE LA UNIVERSIDAD DE MURCIA

Fdo.: María Senena Corbalán García

ID: 282/2019

# Annex II

HPLC-HR-MS based identification of (poly)phenols and phenolic acid catabolites in slurry from *in vitro* fermentation of orange peel samples.

| $t_R$ (min) | Compounds | Chemical formula | Mass (*m* / *z*) | | Δppm | MSIMI level* |
|---|---|---|---|---|---|---|
| | | | Theor | Expl | | |
| 1.95 | Naringenin-7-*O*-glucoside | $C_{21}H_{21}O_{10}$ | 433.1129 | 433.1126 | -0.79 | 1 |
| 1.98 | Hesperidin-7-*O*-rutinoside | $C_{28}H_{33}O_{15}$ | 609.1814 | 609.1821 | 1.2 | 1 |
| 5.37 | Naringenin | $C_{15}H_{11}O_5$ | 271.0601 | 271.0607 | 2.32 | 1 |
| 11.46 | Naringenin-7-*O*-rutinoside | $C_{27}H_{31}O_{14}$ | 579.1708 | 579.1715 | 1.17 | 1 |
| 12.61 | Hesperetin hexoside | $C_{16}H_{13}O_6$ | 463.1234 | 463.1247 | 0.89 | 2 |
| 13.72 | Isosakuranetin-7-*O*-rutinoside | $C_{28}H_{33}O_{14}$ | 593.1865 | 593.1876 | 1.92 | 1 |
| 15.93 | Hesperetin | $C_{16}H_{13}O_6$ | 301.0707 | 301.0712 | 1.78 | 2 |
| 8.67 | Ferulic acid hexoside | $C_{16}H_{19}O_9$ | 355.1023 | 355.1030 | 1.89 | 2 |
| 9.21 | Sinapic acid | $C_{11}H_{11}O_5$ | 223.0601 | 223.0606 | 2.02 | 1 |
| 10.91 | Caffeic acid | $C_9H_7O_4$ | 179.0339 | 179.0338 | 0.03 | 1 |
| 10.63 | 4′-hydroxycinnamic acid | $C_9H_7O_3$ | 163.0390 | 163.0388 | -0.68 | 1 |
| 11.33 | Ferulic acid | $C_{10}H_9O_4$ | 193.0495 | 193.0499 | 1.94 | 1 |
| 11.48 | 3’-hydroxycinnamic acid | $C_9H_7O_3$ | 163.0390 | 163.0388 | -1.05 | 1 |
| 11.61 | Isoferulic acid | $C_{10}H_9O_4$ | 193.0495 | 193.0497 | 0.70 | 1 |
| 7.77 | 3-(3′-hydroxyphenyl)hydracrylic acid | $C_9H_9O_4$ | 181.0493 | 181.0494 | -0.53 | 1 |
| 8.83 | 3-(3′,4′-dihydroxyphenyl)propionic acid | $C_9H_9O_4$ | 181.0495 | 181.0491 | -2.40 | 1 |
| 10.27 | 3-(4′-hydroxyphenyl)propionic acid | $C_9H_9O_3$ | 165.0546 | 165.0544 | -0.25 | 1 |
| 10.27 | 3-(3′-hydroxyphenyl)propionic acid | $C_9H_9O_3$ | 165.0546 | 165.0546 | -1.10 | 1 |
| 11.38 | 3-(3′-hydroxy-4′-methoxyphenyl)hydracrylic acid | $C_{10}H_{11}O_5$ | 211.0600 | 211.0606 | 2.32 | 2 |
| 10.91 | Dihydroferulic acid | $C_{10}H_{11}O_4$ | 195.0652 | 195.0657 | 2.59 | 1 |
| 11.40 | Dihydroisoferulic acid | $C_{10}H_{11}O_4$ | 195.0652 | 195.0656 | 2.02 | 2 |
| 14.50 | 3-(phenyl)propionic acid | $C_9H_9O_2$ | 149.0597 | 149.0597 | -0.18 | 1 |
| 7.37 | 3,4-dihydroxyphenylacetic acid | $C_8H_7O_4$ | 167.0338 | 167.0337 | -1.23 | 1 |
| 8.83 | 4′-hydroxyphenylacetic acid | $C_8H_7O_3$ | 151.0389 | 151.0389 | -0.80 | 1 |
| 8.85 | Phenylacetic acid | $C_8H_7O_2$ | 135.0440 | 135.0438 | -1.97 | 1 |
| 2.93 | Gallic acid | $C_7H_5O_5$ | 169.0132 | 169.0128 | -1.60 | 1 |
| 6.13 | 3,4-dihydroxybenzoic acid | $C_7H_5O_4$ | 153.0182 | 153.0174 | -5.59 | 1 |
| 8.25 | 4-hydroxybenzoic acid | $C_7H_5O_3$ | 137.0233 | 137.0235 | 1.31 | 1 |
| 9.45 | 3′-methoxy-4′-hydroxybenzoic acid | $C_8H_7O_4$ | 167.0339 | 151.0388 | -0.45 | 1 |
| 9.62 | 3-hydroxy-4-methoxybenzoic acid | $C_8H_7O_4$ | 167.0339 | 167.0339 | 0.03 | 1 |
| 12.28 | Benzoic acid | $C_7H_5O_2$ | 121.0284 | 121.0283 | -1.21 | 1 |
| 2.41 | Phloroglucinol | $C_6H_5O_3$ | 125.0233 | 125.0230 | -2.32 | 1 |

*Metabolite standards initiative metabolite identification levels. Reference compounds were available for all compounds identified at MSIMI level 1.

# Publications derived from this PhD thesis

## Scientific publications

**Nuñez-Gómez, V., Baenas, N., Navarro-González, I., García-Alonso, J., Moreno, D. A., González-Barrio, R., and Jesús Periago-Castón, M. J.** (2020). Seasonal Variation of Health-Promoting Bioactives in Broccoli and Methyl-Jasmonate Pre-Harvest Treatments to Enhance Their Contents. *Foods 2020, Vol. 9, Page 1371*, *9*(10), 1371.

**Baenas, N., Nuñez-Gómez, V., Navarro-González, I., Sánchez-Martínez, L., García-Alonso, J., Periago, M. J., and González-Barrio, R.** (2020). Raspberry dietary fibre: Chemical properties, functional evaluation and prebiotic *in vitro* effect. *LWT*, *134*, 110140.

**Núñez-Gómez, V., Periago, M. J., Navarro-González, I., Campos-Cava, M. P., Baenas, N., and González-Barrio, R.** (2021). Influence of Raspberry and Its Dietary Fractions on the *In vitro* Activity of the Colonic Microbiota from Normal and Overweight Subjects. *Plant Foods for Human Nutrition*, *76*(4), 494–500.

## Oral conference communications

**Núñez-Gómez, V., González-Barrio, R., Campos-Cava, M. P., Baenas, N., Sánchez-Martínez, L., García-Alonso, F. J., and Periago, M. J.** (2020). Formación de catabolitos colónicos a partir de frambuesa y sus fracciones de fibra dietética. I Congreso Universitario en Innovación y Sostenibilidad Agroalimentaria (CUISA). Escuela Politécnica Superior de Orihuela.

**Núñez-Gómez, V., González-Barrio, R., Baenas, N., and Periago, M. J.** (2021). Caracterización de ingredientes obtenidos a partir de la corteza de naranja como revalorización de subproductos generados en la industria de cítricos. VI Jornadas Doctorales, Escuela Internacional de Doctorado, Universidad de Murcia.

## Poster conference communications

**Núñez-Gómez, V., Periago, M. J., Baenas, N., and González-Barrio, R.** (2021). lnfluence of raspberry and its dietary fractions on the *in vitro* activity of the coloníc mícrobiota from normal and overweight subjects. X Food technology international symposium. Centro Tecnológico Nacional de la Conserva y Alimentación.

**Núñez-Gómez, V., Baenas, N., González-Barrio, R., and Periago, M. J.** (2021). Formación de urolitinas tras la fermentación in vitro de distintas fracciones de frutos rojos. XXV Jornadas Internacionales Nutrición Práctica. Sociedad Española de Dietética y Ciencias de la Alimentación.

**Núñez-Gómez, V., Baenas, N., González-Barrio, R., and Periago, M. J.** (2021). Revalorización del tronco del brócoli para la obtención de in ingrediente rico en fibra dietética. XXV Jornadas Internacionales Nutrición Práctica. Sociedad Española de Dietética y Ciencias de la Alimentación.