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et ses métabolites produits par des bactéries  
intestinales : études électrochimiques, analyses  
LC-MS/MS, biotransformation et études biologiques**

Gentiana Balaj

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Gentiana Balaj. L' acide 5-O-caféoylquinique, un polyphénol alimentaire et ses métabolites produits par des bactéries intestinales : études électrochimiques, analyses LC-MS/MS, biotransformation et études biologiques. Analytical chemistry. Université Rennes 1, 2022. English. NNT : 2022REN1S054 . tel-03951251

**HAL Id: tel-03951251**

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# THESE DE DOCTORAT DE

L'UNIVERSITE DE RENNES 1

Faculté des Sciences Pharmaceutiques et Biologiques

ECOLE DOCTORALE N° 596

*Matière, Molécules, Matériaux*

Spécialité : Chimie Analytique

Par

**Gentiana BALAJ**

## **5-O-caffeoylquinic acid, a dietary polyphenol, and its gut bacteria-derived metabolites: electrochemical, LC-MS/MS, biotransformation and biological studies**

Thèse présentée et soutenue à Rennes, le 22 juin 2022

Unité de recherche : UMR CNRS 6226 Equipe COInt

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# **General Introduction**



Polyphenols, natural secondary metabolites of plants, have drawn increasing attention from the scientific community due to their ability to decrease oxidative stress involved in many pathologies and their positive effects in modulating redox cellular signalling pathways (**Zhang and Tsao, 2016**). However, given that they are characterized by low bioavailability and considering the crucial role of microbiota in generating bioavailable and bioactive metabolites (**Pasinetti et al., 2018**), further insights are focused nowadays on their gut-derived metabolites as a promising approach to prevent and attenuate many pathologies such as cancer, diabetes, metabolic and neurodegenerative diseases (**Carregosa et al., 2019a; Cortés-Martín et al., 2020**). Indeed, a better understanding of the polyphenols-microbiota interplay will allow the development of novel nutraceutical strategies to prevent, and even more, alleviate various diseases. Considering that gut-derived metabolites have already shown interesting neuroprotective properties, we will do a focus on their impact on brain diseases.

This Ph.D. work was carried out in the Natural Products group of COrint (Chimie Organique aux Interfaces) team, whose main research activities concern lichens and their microflora as an original source of secondary metabolites and how these microorganisms can interact with their host. For my Ph.D. thesis, we have started a new topic thanks to the collaboration with one of the groups of NuMeCan team (Nutrition, métabolismes et Cancer), with the final objective of studying how diet can impact health and potentially prevent chronic disease.

In this context, our research focused on one of the main dietary phenolic compounds, 5-*O*-caffeoylquinic acid (5-CQA), also called chlorogenic acid. The objective was twofold: 1) to study the reciprocal interactions between this compound, 5-CQA, and three intestinal bacteria *Lactobacillus reuteri*, *Bacteroides fragilis* and *Bifidobacterium longum*, which are representative of the major phyla found in the gut; 2) to provide information on the potential effects of 5-CQA and its gut-derived metabolites on gut health.

In Chapter I, state-of-the-art scientific literature on polyphenols and the gut microbiota is presented. Since polyphenols have been widely reviewed, a brief bibliographic presentation is provided. Next, we present an overview of the gut microbiota which represents one of the main topics in the last decade. Finally, we discuss how the synergistic interactions between gut microbiota and polyphenols can alleviate brain disorders.

In Chapter II, the results of our study are presented, divided into subsections. The first part consist of electrochemical and enzymatic studies performed on 5-CQA and one of its gut-

derived metabolites, caffeic acid (CA). Then, the biotransformation process of 5-CQA carried out by mono- and co-culture using the resting cell method is presented. Furthermore, we will describe how we have investigated the potential effect of 5-CQA, when supplemented, to modulate the growth and metabolic profiles of *L. reuteri* and *B. fragilis*. Finally, using an original *in vitro* quadruple cell model, we describe the potential impact of 5-CQA and its gut-derived metabolites on modulating gut function as a target strategy to provide positive health effects.

The discussion and opening of new perspective are elaborated in Chapter III. Finally, the materials and methods section are detailed in the last chapter (IV) of this manuscript.

# Chapter I

## I.A. Polyphenols

### I.A.1. Chemistry and dietary sources

Polyphenols are secondary metabolites, also known as ‘specialized metabolites’, found in the plant kingdom (**Chikezie et al., 2015**). Notably, they are involved in plant defence, including protection against oxidative stress and ultraviolet radiation. In addition, they act as signalling compounds, that attract pollinators or animals for seed dispersal. They also contribute to the organoleptic characteristics of flowers, leaves, fruits and vegetables, such as bitterness, astringency, color and flavor (**Naczk and Shahidi, 2004**).

In addition to their beneficial effects on plants, these compounds are of great interest because of their impact on human health. Epidemiological, clinical and nutritional studies strongly support the suggestion that polyphenols consumption is associated with the prevention of many major chronic diseases such as cardiovascular, neurodegenerative and cancer diseases (**Del Bo’ et al., 2019; Fraga et al., 2019; Pandey and Rizvi, 2009; Scalbert et al., 2005b**). Polyphenols consists of at least one aromatic ring, containing one or more hydroxyl substituents (Figure 1). Indeed, they can range from simple phenolic acids (containing single rings) to highly polymerized compounds such as hydrolysable and condensed tannins (see Figure 2) (**Crozier et al., 2009**).

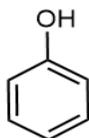


Figure 1. Representation of a phenolic structure

The most phenolic compounds occur naturally as conjugates with sugars or as esters with organic acids (**Manach et al., 2004**). More rarely, they are found as free molecules (aglycones). Owing to their structural diversity, a wide range of phenolic compounds is found in nature. Currently, more than 8000 phenolic compounds have been identified (**Vuolo et al., 2019**). Based on their chemical structures, they can be classified into two classes: flavonoids and non-flavonoid phenolic compounds, which are subdivided into different subgroups. The structures of the main flavonoid and non-flavonoid compounds are shown in Fig 2. (**Manach et al., 2004**).

#### I.A.1.1. Non-flavonoid compounds

The non-flavonoid polyphenol group consist of phenolic acids, stilbenes, lignans and hydrolysable tannins. The main dietary ones and their structures are shown in Figure 2. Chemically, phenolic acids have at least one aromatic ring in which at least one hydrogen atom is substituted by a hydroxyl group. They are divided into two major groups: hydroxybenzoic acids (HBAs) (gallic, protocatechuic, syringic and vanillic acids) and hydroxycinnamic acids (*p*-coumaric, caffeic, ferulic, and sinapic acids). Hydroxybenzoic acids are mainly present in plant food as conjugates, but they can be also free in some fruits (e.g., gallic acid in persimmon) or released during fruit or vegetable processing (**Tomás-Barberán and Clifford, 2000**). They constitute a smaller portion of edible plants and generally form components of a complex structure such as hydrolyzable tannins. An example of conjugated HBAs is gallic acid (tannin), which can be a dimer, trimer or tetramer (ellagic, tergallic and gallagic acids, respectively). Tannins consist of hydrolysable tannins (gallotannins and ellagitannins) and non-hydrolysable, condensed tannins (proanthocyanidins), which are derivatives of flavonoids (Figure 2). Strawberries, pomegranate and walnuts are good dietary sources of ellagic acid. Gallotannins, unlike ellagitannins, are rarely found in plant foods, and occur almost exclusively in mango, chestnuts and red sword beans (**Manach et al., 2004**).

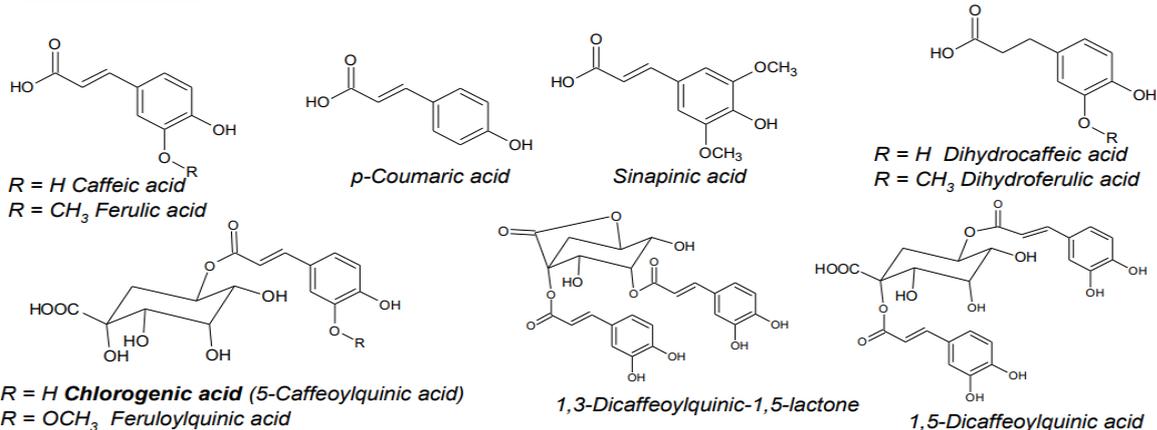
Hydroxycinnamic acids can be found in plants in their free and esterified forms e.g., with quinic acid (chlorogenic acids) or as glycosides (e.g with glucose in 1-*O* cinnamoylglucose and

with choline e.g., in sinapine). Coffee and some vegetables, fruits, and cereals are rich sources of hydroxycinnamic acids (**Crozier et al., 2009**).

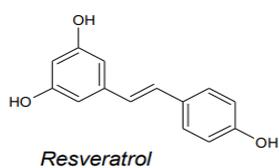
Lignans are commonly found as glycosides in many foods. Secoisolariciresinol, the main representative and its gut-derived metabolites enterolactone and enterodiol, have attracted the attention of scientists due to their potential biological effects (**Johnson et al., 2019; Raffaelli et al., 2002**).

Stilbenes are less present in the human diet. Resveratrol the main phenolic compound characteristic of this group, is found in grapes and red wine. It generally occurs in glycosylated forms rather than as *cis/trans* isomers, as well as in oligomers containing different resveratrol units, such as  $\delta$ -viniferin and  $\epsilon$ -viniferin (**Shen et al., 2009**).

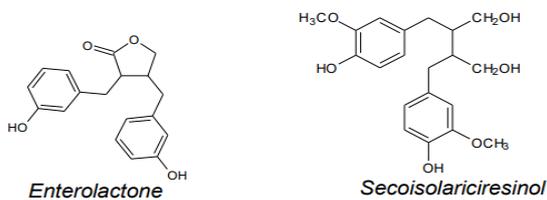
## Phenolic acids



## Stilbenes

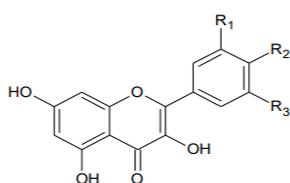


## Lignans



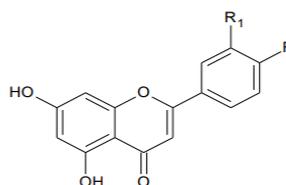
## Flavonoids

### Flavonols



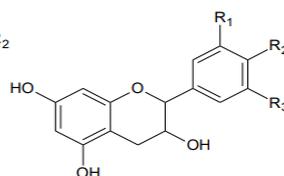
$R_1, R_3 = H, R_2 = OH$  Kaempferol  
 $R_1, R_2 = OH, R_3 = H$  Quercetin  
 $R_1, R_2, R_3 = OH$  Myricetin

### Flavones



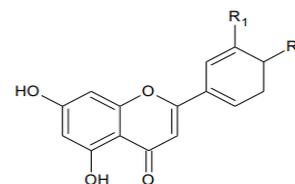
$R_1, R_2 = OH$  Luteolin  
 $R_1 = H, R_2 = OH$  Apigenin

### Flavanols



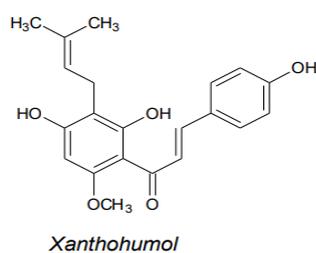
$R_1, R_2 = OH, R_3 = H$ : Catechins (catechin and epicatechin)  
 $R_1, R_2, R_3 = OH$  Gallocatechin

### Flavanones

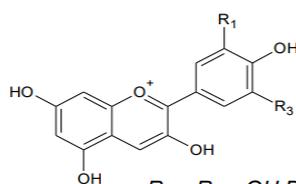


$R_1 = H, R_2 = OH$  Naringenin  
 $R_1 = R_2 = OH$  Eriodictyol  
 $R_1 = H, R_2 = OCH_3$  Hesperetin

## Chalcones

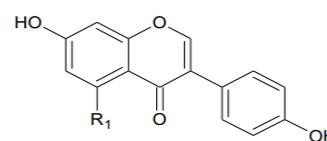


## Anthocyanidins



$R_1 = R_2 = OH$  Delphinidin  
 $R_1 = R_2 = H$  Pelargonidin  
 $R_1 = OH, R_2 = H$  Cyanidin  
 $R_1 = OCH_3, R_2 = OH$  Petunidin  
 $R_1 = R_2 = OCH_3$  Malvidin

## Isoflavones



$R_1 = H$  Daidzein  
 $R_1 = OH$  Genistein

Figure 2. Structures of main dietary polyphenols (adapted from Manach et al., 2004)

### I.A.1.2. Flavonoids

Flavonoids are the largest group of phenolic compounds. These include more than 6000 compounds present in dietary plants. Phenolic flavonoids have a basic structure with two benzene rings (A and B) linked to a heterocyclic pyrone C ring (Figure 2 ) (Tsao, 2010). Most of them are glycosylated, rather than aglycones (except flavan-3-ols and proanthocyanidins). Flavonoids are conjugated with mono- and polysaccharides (glucose, galactose, rhamnose, apiose), linked by  $\beta$ -glycosidic bonds to a hydroxyl group (*O*-glycosides) or to a carbon atom of the aromatic ring (*C*-glycosides). The main food sources are listed in Table 1.

Table 1. The main flavonoids and their food sources (Tomás-Barberán et al., 2020)

| Flavonoid class       | Main compounds  | Food sources   |
|-----------------------|---|--|
| <b>Flavanols</b>      | Catechin<br>Epicatechin<br>Epicatechin gallate<br>Gallocatechin | Apple, apricot, peach, grape, berries, cereals, chocolate, red wine, nuts, black and green tea   |
| <b>Flavones</b>       | Apigenin<br>Luteolin<br>Chrysin                                 | Parsley, celery, lettuce, artichoke, herbs (rosemary, thyme, oregano, etc.), citrus fruits, cereal grains, sweet peppers   |
| <b>Flavonols</b>      | Quercetin<br>Kaempferol<br>Myricetin<br>Isorhamnetin            | Yellow and red onion, caper, lettuce, parsley, berries, green and black tea, mango, carrot, pumpkin, kale, cabbage, broccoli, garlic   |
| <b>Flavanones</b>     | Naringenin<br>Hesperetin  | Orange, grapefruit, lemon, lime  |
| <b>Isoflavones</b>    | Daidzein<br>Genistein<br>Glycitein                              | Soybean, tofu, green bean, lentil, chickpea, pea, mung bean, broad bean, medicinal herbs   |
| <b>Anthocyanidins</b> | Cyanidin<br>Delphinidin<br>Pelargonidin<br>Peonidin             | Berries, currant, grape, aronia, cherries, plum, pomegranate, red wine, red cabbage, eggplant, red onion, radish, hazelnut, pistachio nut, black and red bean, medicinal herbs |

According to a study conducted by the European Prospective Investigation into Cancer and Nutrition (EPIC), the average intake of polyphenols in a healthy diet is 1 g/day. The most abundant polyphenols were phenolic acids (52.5–56.9 %), with chlorogenic acids being the most prominent compounds in this class, except in men from Mediterranean (MED) countries and in the United-Kingdom (UK) health-conscious group where the main compounds are flavonoids (49.1–61.7 %) (mainly flavanols (16–29%)). On the other hand, stilbenes and lignans are present to a lesser extent with percentages of 0.1–0.5% and 0.1–0.7%, respectively. 5-*O*-caffeoylquinic acid, 4-*O*-caffeoylquinic acid and proanthocyanidin polymers, were the three most consumed individual polyphenols (**Zamora-Ros et al., 2016**).

## I.A.2. Biological activities of polyphenols

It is well known that oxidative stress is closely related to mitochondrial dysfunctions and is one of the main factors associated to many pathological conditions including neurological disorders, cancer, metabolic diseases and others (**Valko et al., 2007**). We will first present the health risks of oxidative stress and ways to combat it, such as the use of polyphenols due to their properties.

### I.A.2.1. Oxidative stress: Risk on health and possible fights

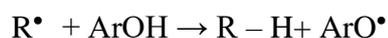
Free radicals are defined by the presence of one or more unpaired electrons in their atomic or molecular orbitals making these molecules highly reactive (**Valko et al., 2007**). These molecules, known as reactive oxygen species (ROS) and reactive nitrogen species (RNS), are produced during cellular respiration and normal metabolism (**Phaniendra et al., 2015**). The species mainly include superoxide anion free radicals ( $O_2^-$ ), hydroxyl free radicals ( $HO^\bullet$ ), peroxy radicals ( $ROO^\bullet$ ), nitrogen peroxide ( $\bullet NO$ ) and others (**Bergamini et al., 2004**). At low levels, ROS can function as signalling molecules that regulate basic cellular activities, such as cell growth and cellular adaptive responses (**Sies and Jones, 2020**). Under physiological conditions, there is a natural balance between the production of reactive species

and the antioxidant defence system of the body. When this balance is compromised, it causes an accumulation of ROS, and when this can occur on a short time scale, it is known as acute stress and ends with a return to redox balance. However, when oxidative stress persists over time, the body fails to rebalance its redox status (**Lushchak, 2012**). This causes so-called chronic, or pathological oxidative stress which can consequently lead to damage of cellular molecules such as DNA, proteins and lipids. There are two mechanisms through which oxidative stress can contribute to and trigger diseases. The production of reactive species during oxidative stress can directly oxidize macromolecules, leading to damage of cell function and death; second, it can cause misfolding of redox signalling (**Forman and Zhang, 2021**). In this context, polyphenols, have shown their ability to scavenge free radicals species and positively modulate redox signalling pathways, thereby contributing to reduced oxidative stress (**Gebicki and Nauser, 2021**).

#### I.A.2.2. Antioxidant properties

The antioxidant effects of polyphenols are attributed to their chemical structure. Polyphenols act as natural antioxidants thanks to their free radical-scavenging properties. They exert their antioxidant properties against reactive oxygen species (ROS) via hydrogen atom transfer (HAT) or electron transfer (ET) mechanisms. In the first mechanism, the antioxidant ArOH can give an hydrogen atom to a free reactive radical ( $R^\bullet$ ) leading to  $ArO^\bullet$ , a less harmful radical, which is firstly stabilized by resonance before being involved in non-toxic reactions (**Vuolo et al., 2019; Zhang and Tsao, 2016**).

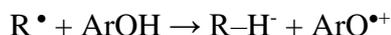
##### **HAT (H-atom transfer)**



In the second mechanism, the antioxidant can donate an electron to the free radical, which becomes a cation radical. In this mechanism, the lower the ionization potential (IP), the

easier the electron abstraction, which means a higher antioxidant activity. This electron transfer mechanism will be deeply developed in electrochemistry part.

### **Electron transfer**



The antioxidant potential of polyphenols is related to their structure. For example, in the case of flavonoids, the number and positions of the hydroxyl group (-OH), the presence of a double bond (C2=C3), glycosylation and the presence of substituents in the rings are the main factors that determine their antioxidant potential. It was revealed that the presence of the 3-OH group contributed to the increase of antioxidant properties compared to those with less OH groups in the ring. Thus, quercetin is considered as a strong antioxidant compound. The superior antioxidant properties are also related to the presence of a C2=C3 double bond in conjugation with the C4- carbonyl group. When considering glycosylation, the C-glycoside form has demonstrated greater antioxidant ability than the O-glycoside in chemical assays (**T. Wang et al., 2017**).

In the case of phenolic acids, a longer distance separating the carbonyl group and the aromatic ring of the phenolic acid, appears to increase antioxidant activity. For example, in the case of cinnamic acid and its derivatives compared to benzoic acids, hydroxyl groups on the benzoic ring at the *ortho*-position and/or *para*-position can lead to high antioxidant activity compared to those with other positions and to unsubstituted phenol (**Zhang and Tsao, 2016**).

#### I.B.2.3. Modulators of signalling pathway

Another mechanism of oxidative stress suppression by polyphenols is modulation of multiple signalling pathways, such as mitogen-activated protein (MAP) kinase and NF-κB, thus, suppressing the release of pro-inflammatory cytokines (TNF- α, IL-6, IL-8, and IL-1) (**Zhang and Tsao, 2016**). Many polyphenols have been reported to reduce oxidative stress

through the induction of *Nrf2*-dependent gene expression. For example, quercetin has shown a strong neuroprotective effect by repressing NF- $\kappa$ B. Likewise, catechin, increased cell survival by downregulating the NF- $\kappa$ B and MAP kinase pathways (**Di Meo et al., 2020**). The list of polyphenols as modulators of signalling pathways activated in neurodegenerative diseases also includes resveratrol, curcumin and epigallocatechin-3-gallate apigenin, kaempferol, genistein (**Jantan et al., 2021**).

### I.A.3. Chlorogenic acids compounds

We have focused our study on 5-*O*-caffeoylquinic acid, known as chlorogenic acid, one of the main phenolic compounds in the daily diet. Indeed, chlorogenic acids are a group of compounds formed by the esterification of *trans*-cinnamic acids such as caffeic acid (CA), *p*-coumaric acid or ferulic acid and quinic acid (**Clifford, 2000**). Depending on the type of ester substituent, the most common chlorogenic acids are caffeoylquinic acid (CQA), dicaffeoylquinic acid (diCQA), *p*-coumaroylquinic acid (*p*-CoQA), feruloylquinic acid (FQA) and caffeoylferuloylquinic acid (CFQA) (Figure 3) (**Lu et al., 2020**).

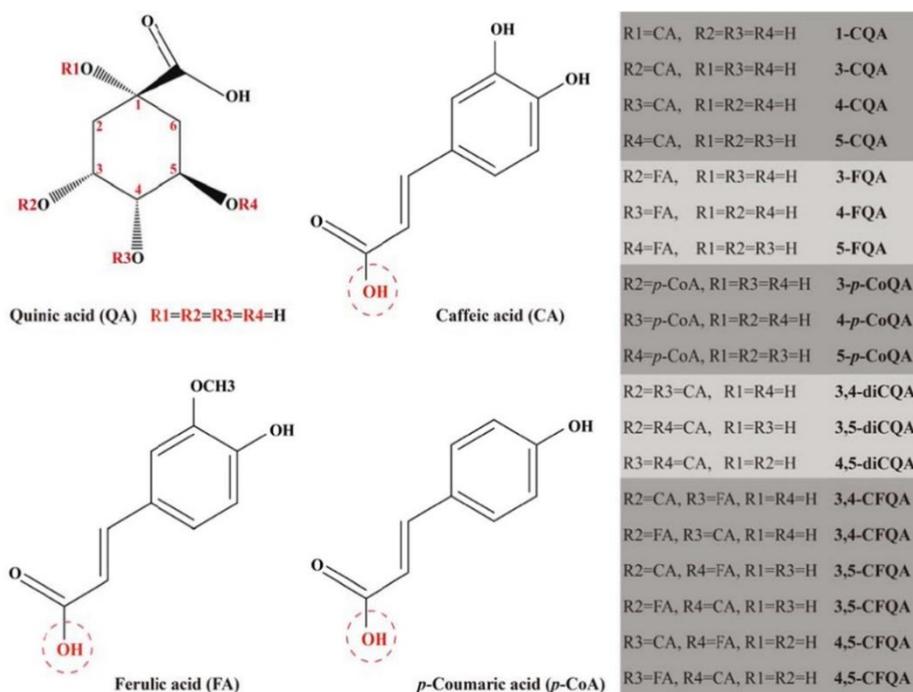
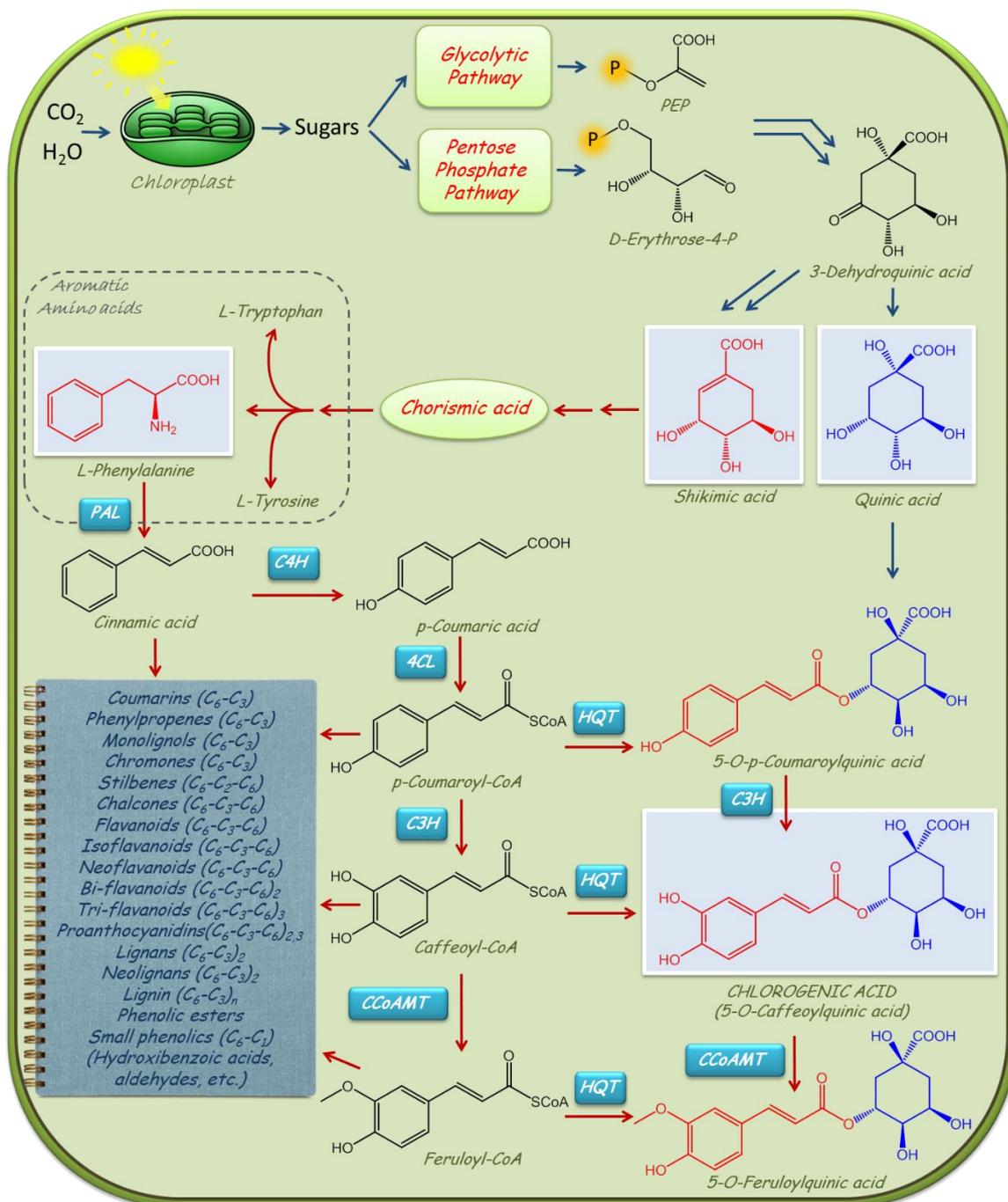


Figure 3. General structures of chlorogenic acids (CGAs) and its chemical variations. CQA, caffeoylquinic acid; diCQA, dicaffeoylquinic acid; *p*-CoQA, *p*-coumaroylquinic acid; FQA, feruloylquinic acid; CFQA, caffeoylferuloylquinic acid

### I.A.3.1. Biosynthesis of chlorogenic acids compounds

The biosynthesis of chlorogenic acids and the pathway that leads to 5-*O*-Caffeoylquinic acid (5-CQA) are well established. These types of compounds are formed from phenylalanine via shikimic acid pathway. Indeed, this pathway is a major biosynthetic route for both primary and secondary metabolism. It uses phosphoenolpyruvate and erythrose-4-phosphate as starting materials leading to shikimic acid which is then converted to chorismic acid as illustrated in Scheme 1. Chorismic acid is a pivotal compound in the formation of aromatic rings such as phenylalanine. This amino-acid is then converted to cinnamic acid by phenylalanine-ammonia-lyase (PAL). This pathway is responsible for the biosynthesis of a large number of diverse secondary metabolites such as lignin, flavonoids, isoflavonoids and chlorogenic acids as described in Scheme 1. The next step corresponds to the hydroxylation of cinnamic acid by

cinnamate 4-hydroxylase (C4H) leading to *p*-coumaric acid, which is then converted to *p*-coumaroyl-CoA thanks to 4-coumarate-CoA ligase (4CL). Then, *p*-coumaroyl-CoA can follow two pathways to produce 5-CQA: 1) It can be metabolized by the hydroxycinnamoyl CoA: quinate hydroxycinnamoyl transferase into 5-O-*p*-coumaroyl quinic acid, which subsequently undergoes a C3 hydroxylation by *p*-coumaroyl-3'-hydroxylase to give 5-CQA; 2) The other mechanism involves C3H- mediated hydroxylation of *p*-coumaroyl-CoA producing caffeoyl-CoA which is then converted to 5-CQA (**Clifford et al., 2017; El-Seedi et al., 2012**).



Scheme 1. Biosynthesis of 5-CQA acids and related phenolic compounds. Abbreviations: PEP (phosphoenolpyruvate), PAL (phenylalanine ammonia lyase), C4H (cinnamate 4'-hydroxylase), 4CL (4-cinnamoyl CoA ligase), HQT (hydroxycinnamoyl CoA:quinic acid hydroxycinnamoyl transferase), C3H (*p*-coumaroyl-3'-hydroxylase), and CCoAMT (caffeoyl-CoA-3-O-methyltransferase).

### I.A.3.2. 5-*O*-Caffeoylquinic acid (5-CQA): ubiquitous in the dietary sources

Currently, more than 400 chlorogenic acids have been identified. However, 5-CQA, is the major regio-isomer and the most studied compound among the chlorogenic acids family. In addition, 1-CQA, 3-CQA and 4-CQA are found in a small number of plants. It should be noted that previous studies have usually referred to as 3-CQA (Clifford et al., 2017). However, according to the IUPAC recommendation, it is now designated as 5-CQA (Kay et al., 2020). The former 3-CQA is referred to neochlorogenic acid (Figure 4). 5-CQA also exhibits geometric isomerization in the *cis* and *trans* forms (Clifford et al., 2017).

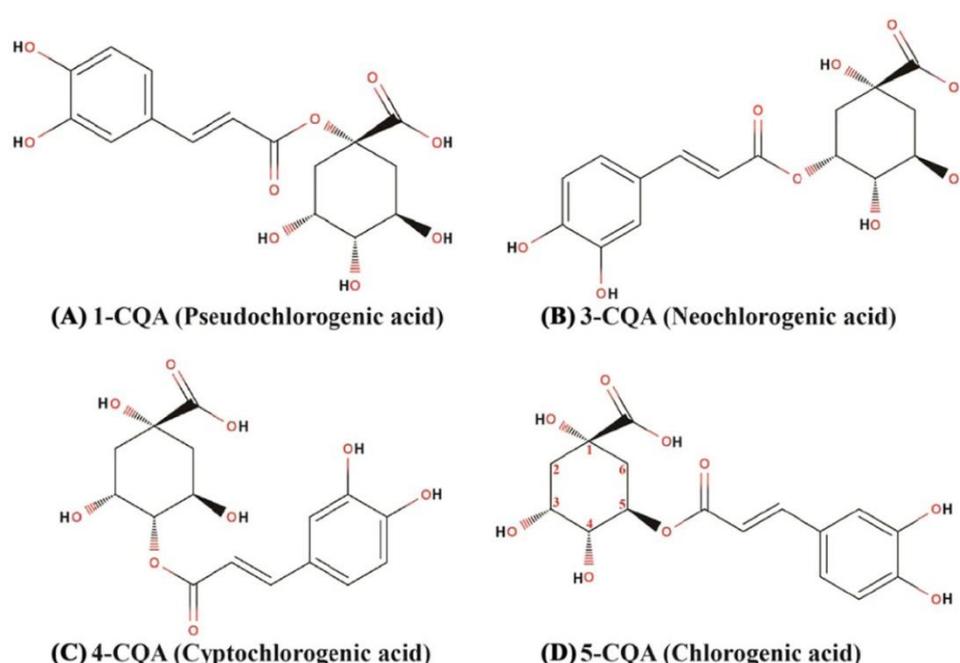


Figure 4. Chemical structures of chlorogenic acid stereoisomers of 5-CQA: A) 1-CQA; B) 3-CQA; C) 4-CQA; D) 5-CQA (Lu et al., 2020)

Many plants, including medicinal plants, fruits and vegetables produce this compound. For example, in coffee beans (100 mg/g) or green coffee beans it represents 76 to 84% of total CGAs. Its content varies in fresh ground coffee (8.19 to 23.78 mg/200 mL) or instant coffee (9.45 to 41.05 mg/200 mL) (Lu et al., 2020). Moreover, its content also depends on the coffee variety (Arabica vs. Robusta) and roasting time (Table 2). Indeed, coffee is the main dietary

source of 5-CQA (Farah and de Paula Lima, 2019). In addition, it has been reported that cider contains a high amount of 5-CQA (Guyot et al., 2009). Additionally to coffee and cider, fruits are also good dietary sources of 5-CQA including apples (0.41 to 1.16 mg/g), pears, plums, cherries (Crozier et al., 2009). Among vegetables, eggplant (1.4 to 28.0 mg/g), carrot (0.3 to 18.8 mg/g), artichoke (1.1 to 1.8 mg/g), and pepper (0.7 to 0.9 mg/g) also contribute to the dietary intake of 5-CQA (Lu et al., 2020).

Table 2. 5-CQA content in Arabica vs Robusta, compared to total chlorogenic acids and effects of roasting time on this content (Lu et al., 2020)

| Type of roasting | 5-CQA (mg/g) |         | Total CGAs (mg/g) |         | Roasting time periods (min at 205 °C) |         |
|------------------|--------------|---------|-------------------|---------|---------------------------------------|---------|
|                  | Arabica      | Robusta | Arabica           | Robusta | Arabica                               | Robusta |
| Green            | 45.34        | 49.66   | 68.8              | 88.0    | —                                     | —       |
| Light            | 12.08        | 13.87   | 26.9              | 35.4    | 7                                     | 5       |
| Medium           | 10.12        | 7.77    | 22.2              | 20.7    | 10                                    | 7       |
| Dark             | 3.25         | 2.07    | 7.71              | 6.15    | 13                                    | 14      |
| Vary dark        | 0.98         | 0.53    | 2.42              | 1.76    | 19                                    | 16      |

### I.A.3.3. Pharmacokinetic profiles of 5-CQA

Most human studies and *in vitro* investigations addressing the bioavailability of 5-CQA have been conducted using coffee due to the high levels of this polyphenol and considering that is the main daily dietary intake for most people (Clifford et al., 2017; Crozier et al., 2009). Additionally, some studies have used 5-CQA as the pure compound in their studies (Olthof et al., 2001).

Due to the low concentration in human plasma and urine after ingestion of 5-CQA, early studies suggested that its bioavailability is limited (Azuma et al., 2000; Stalmach et al., 2009). Later studies identified certain amounts of 5-CQA in the plasma, indicating that it is absorbed in humans. Lafay et al. detected 5-CQA in the plasma and urine of rats 1.5 h after the consumption of 5-CQA supplemental diet, suggesting that it is absorbed in intact form (Lafay et al., 2006). Coffee consumption resulted in two plasma concentration peaks of

chlorogenic acids at 0.5 to 1.0 and 1.5 to 4.0 h, where 5-CQA represented of 40% of total hydroxycinnamates identified in human plasma (**Monteiro et al., 2007**).

Furthermore, Olthof et al. reported that of a 2.8 mmol intake of 5-CQA by human ileostomized, approximately one-third was absorbed by the small intestine and the other portion reached the colon (**Olthof et al., 2001**). Similarly, in another study performed on ileostomists, from 385  $\mu\text{mol}$  of chlorogenic acids ingested through a cup of coffee, where 5-CQA accounted for the majority, around 30 percent was absorbed into the stomach and/or small intestine (**Stalmach et al., 2010**).

However, the majority of the dietary intake of 5-CQA, reaches the colon, largely unaltered, where the gut microbiota with cinnamoyl esterase (*e.g.*, *Escherichia coli*, *Bifidobacterium lactis*, *Lactobacillus gasseri* and *L. johnsoni*) hydrolyzes 5-CQA to caffeic acid (CA) and quinic acid (QA) (**Bel-Rhliid et al., 2013; Couteau et al., 2001a**), and subsequently to low-molecular-weight metabolites such as *m*-coumaric acid and hydroxylated derivatives of phenylpropionic, benzoic, and hippuric acids as shown in Figure 5 (**Tomas-Barberan et al., 2014**). There are two proposed colonic biotransformation pathways for QA (Figure 5): (1) a reductive route leading to cyclohexane carboxylic acid, and (2) an oxidative route leading to aromatic compounds (**Pinta et al., 2018**). However, the pharmacokinetic properties of chlorogenic acids have not been fully elucidated and further studies are encouraged to be undertaken (**Clifford et al., 2020**).

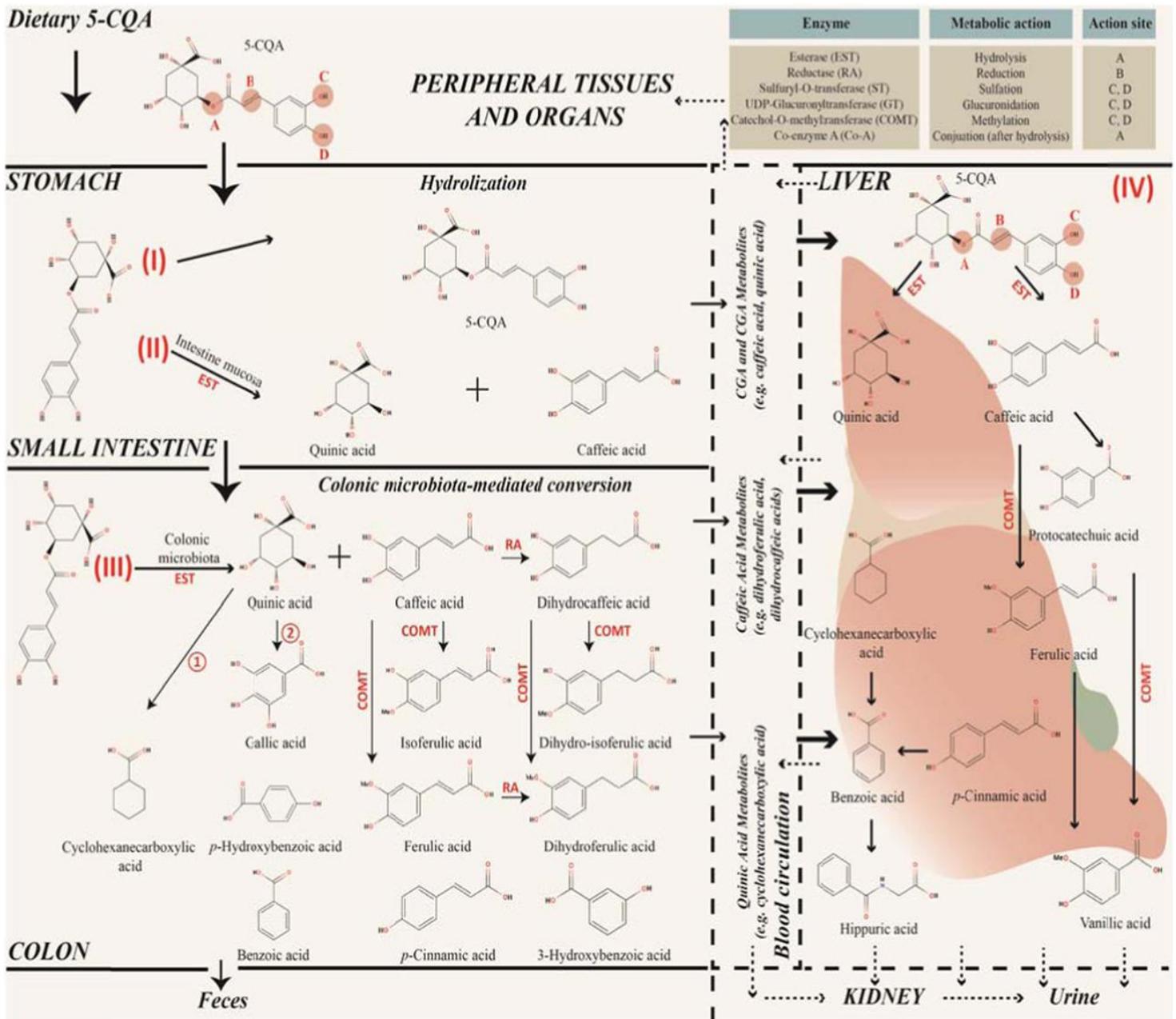


Figure 5. Representative scheme of absorption and metabolism pathway of 5-CQA in humans (I) almost one- third of 5-CQA intake is absorbed in upper digestive (stomach and/or small intestine, without hydrolysis and then enter to the circulatory system, (II) a small amount of the 5-CQA (around 7%) is hydrolysed into CA and QA and then absorbed via the small intestine. (III) the major part reaches the colon and is metabolised by gut bacteria; (IV) 5-CQA as native form and its metabolites are absorbed and/or are subjected to liver metabolism, including methylation, by COMT = catechol-O-methyltransferase (COMT), glucuronidation (GT) or sulfation (ST), (Lu et al., 2020)

#### I.A.3.4. The biological activities of 5-CQA

The biological activities of 5-CQA have been the subject of many studies ((reviewed by (Naveed et al., 2018)). More recently, Lu et al (Lu et al., 2020) has gathered all the scientific evidence of the potential health benefits of 5-CQA, mainly due its protective effects, in many tissues and organs, including the nervous, cardiovascular and gastrointestinal systems, lung, pancreas, kidney, liver, as presented in Figure 6.

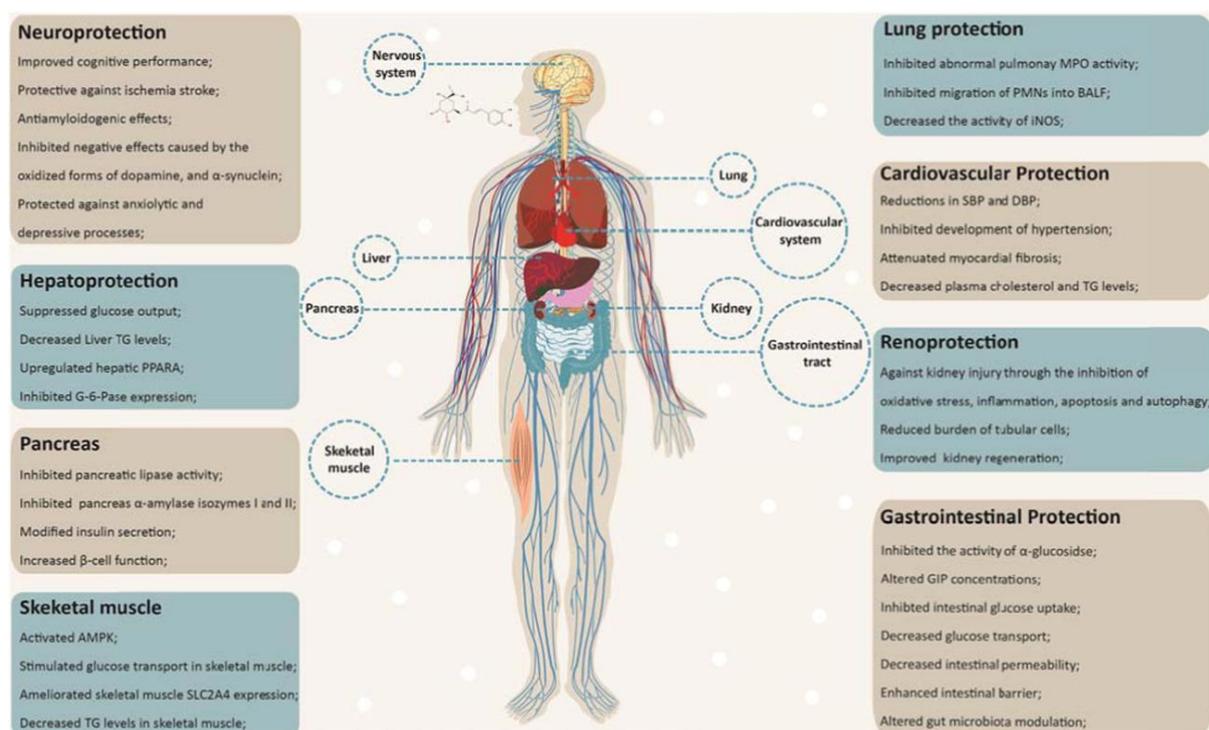


Figure 6. An overview of the biological activities of 5-CQA in humans (Lu et al., 2020)

In addition, despite the fact that some studies have demonstrated that 5-CQA exerts neuroprotective effects, it should be noted that this compound cannot cross the blood brain barrier (BBB) (Clifford et al., 2020). These findings put on the light the importance of regulating its bioavailability, for example, by encapsulating the compound (Feng et al., 2016)

but also to better understand the mechanism of action of 5-CQA *in vivo*, which will be of interest for the development of new nutraceutical uses of this compound.

Although the mechanism by which 5-CQA exhibits its beneficial effects has not been fully elucidated, it has been suggested that it mainly involves its free radical scavenging properties but also its targets in multiple signalling pathways, thus regulating transcription factors and protein kinases. Interestingly, Amano et al. (2019) revealed that 5-CQA and its metabolites were safe to use and could have beneficial effects as a pharmaceutical, by *in vitro* and *ex vivo* profiling assays, according to ICH S7A guidelines (Amano et al., 2019). However, studies suggest that further research should focus on 5-CQA, including its absorption and bioavailability in humans, interactions with microorganisms and evaluation of the benefits of 5-CQA and its metabolites (Lu et al., 2020).

## **I.B. The human gut microbiota**

### **I.B.1. The Microbiome: An overview**

Trillions of microorganisms, including bacteria, viruses, archaea and eukaryotes live in the human body (Thursby and Juge, 2017). These microbial organisms collectively referred to as microbiota, can be found in different body parts, including the skin, oral cavity, intestinal tract, urinary tract, respiratory tract, vagina (Lloyd-Price et al., 2016). Indeed, the term microbiota is followed by the location of the specific environment referred to; for example, the gut microbiota represents the entire collection of microorganisms in the gastrointestinal tract (Marchesi and Ravel, 2015). On the other hand, in the genomic context, microbiome refers to the all the genetic material (collective genomes) of the micro-organisms inhabiting a particular environment (Valdes et al., 2018). Thus, the microbiome represents the microbiota and their “theatre of activity” by which it includes microbial structures, metabolites, genetic material, and surrounding environment (Berg et al., 2020). Human microbiome research has become a topic of great interest among scientific community, medical research, and industry (Proctor, 2019). The differentiation between the microbiome and microbiota terms are depicted in Figure 7.

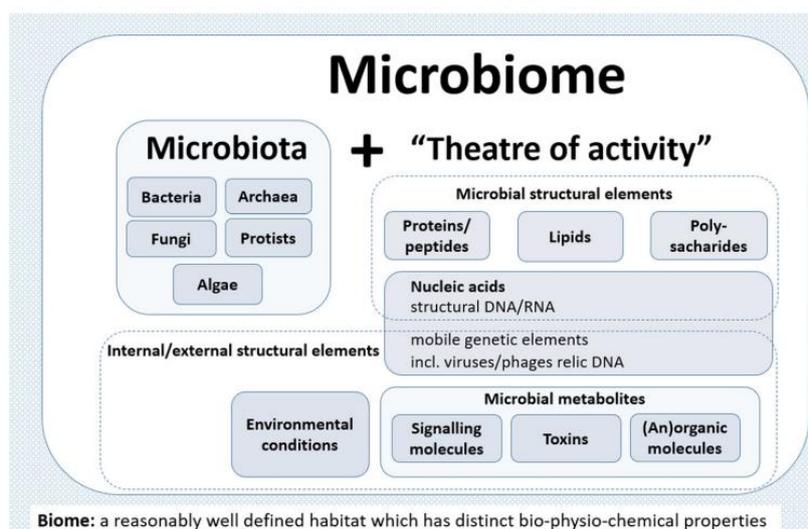


Figure 7. A schematic covering the microbiome nomenclature, highlighting the microbiota and their “theatre of activity” (Berg et al., 2020)

As a key component of microbiome, over the last years, bacteria are in the spotlight of microbiome research. The number of bacteria was initially estimated to outnumber human cells (~10 times more), representing the highest number of microorganisms present in the human body. However, Sender et al., found that this ratio is closer, and the number of bacteria in humans is similar to the number of host cells. It has been estimated that, in humans, the total number of bacteria in a person with a body weight of 70 kg is around  $3.0 \times 10^{13}$  (Sender et al., 2016).

Nonetheless, viral, fungal and archaeal communities, have recently aroused growing interest, since their dynamic presence impacts gut bacteria, and directly the host well-being (Matijašić et al., 2020).

### II.B.1. 2. Gut microbiota: Structure and diversity

Of all microorganisms that live in the human body, most of them are found in the intestine, and the gut encodes more than 3 million genes (150 times more than human genes). Thus, it is considered as a reservoir of genes in the human body (Qin et al., 2010; Tierney et

al., 2019). The gut microbiota is remarkably rich and diverse, predominantly by bacteria, classified taxonomically according to phylum, class, order, family, genus and species (Rinninella et al., 2019b). The highest amount of bacteria live within the colon, reaching a density of  $10^{12}$  bacteria per gram, with over 1000 species (Dieterich et al., 2018; Lozupone et al., 2012).

In regard to the microbiota, it is well known the tremendous interindividual variation and it has been established that microbiota profile is unique to each person (Huttenhower et al., 2012). Nevertheless, it is recognized that *Firmicutes*, *Bacteroidetes*, *Actinobacteria*, *Proteobacteria* and *Tenericutes*, are dominant phyla of human gut microbiota whereas *Fusobacteria*, *Saccharibacteria*, *Spirochaetes*, *Synergistetes* and *Verrucomicrobia* phyla are found in lower abundance (Almeida et al., 2019).

*Firmicutes* represents the most diverse and predominant phylum in the human gut, with more than 200 different Gram-positive bacteria; *Lactobacillus* spp, *Mycoplasma* spp, *Bacillus* spp and *Clostridium* spp, *Enterococcus* spp and *Ruminococcus* spp., among them (Rajilić-Stojanović and de Vos, 2014). Within the gut, these genera include microbial species that are considered the key players in gut homeostasis such as *Lactobacillus reuteri*, *Faecalibacterium prausnitzii*, *Roseburia intestinalis*, and *Clostridium* spp (Britton, 2017; He et al., 2021; Tamanai-Shacoori et al., 2017).

*Bacteroidetes* are Gram negative bacteria, which together with *Firmicutes*, represent the two predominant phyla in the healthy gut (more than 90%) (Eckburg et al., 2005). *Bacteroides*, *Alistipes*, *Parabacteroides* and *Prevotella* are the representative genera of *Bacteroidetes* (Gibiino et al., 2018). These are followed by *Actinobacteria* encompassing Gram-positive species, consisting of less than the 10% of total bacteria and mainly represented by *Bifidobacterium* ( e.g *B. adolescents*, *B. animalis*, *B. breve*, *B. bifidum*, *B. longum* and *B. angulatum*) (Binda et al., 2018). *Proteobacteria*, encompasses Gram-negative bacteria, including notable pathogens (e.g. *Escherichia*, *Helicobacter*, *Salmonella*, *Vibrio*) (Rizzatti et al., 2017). *Verrucomicrobia* are Gram negative bacteria, represented by *Akkermansia muciniphila*, the only known species of this phylum. *A. muciniphila* colonizes the mucus layer of the gut epithelium, consisting 3-5 % of the total microbiota community, and with a great interest related to host health and diseases (Xu et al., 2020).

Among non-bacterial communities in the gut, viruses are the most abundant, mainly corresponding to the order *Caudovirales* and *Microviridae* families. The human gut also harbours fungi such as the genera *Candida*, *Penicillium*, *Saccharomyces*, *Aspergillus*, *Cryptococcus*, *Malassezia* etc. Furthermore, *Methanobrevibacter smithii*, *Methanosphaera stadtmanae*, *Methanomassiliicoccus luminyensis* are the members of the archeal community identified in the human gut (**Matijašić et al., 2020**).

### II.B.1.3. The lifetime journey of human gut microbiota

#### a) Early life

The gut microbiota is a dynamic ecosystem, where development and maturation processes continue during several stages of life including infancy, adolescence, adulthood, and aging.

Over the years, the colonization process of the gut microbiota has been a topic of debate among academia (**Blaser et al., 2021**). Some scientists have suggested that colonization primarily may come through the placenta and *in utero* after detection of microbes and traces of bacterial DNA in the placenta, amniotic fluid and meconium (**Aagaard et al., 2014; Collado et al., 2016; Jiménez et al., 2008**). This hypothesis is questionable by others suggesting that these findings could be a result of contamination (**Lauder et al., 2016**).

Despite controversy, there is a consensus among the majority of scientists that humans are born sterile, and microbial colonization of the gut begins at birth. When birth is vaginally, a vertical transfer of bacteria from maternal genital and skin flora to infants through the birth canal initiates habitation, predominantly by *Lactobacillus*, *Prevotella* and *Sneathia* spp. (**Dominguez-Bello et al., 2010; Korpela, 2021**). After birth, the infant's gut is colonized by maternal gut sources (**Ferretti et al., 2018**) and is mainly dominated by facultative anaerobes, including Firmicutes (*Staphylococcus*, *Streptococcus*), Proteobacteria (*Enterobacteria*) and anaerobic species such as *Bifidobacterium*, *Bacteroides*, and *Clostridium* (**Korpela et al., 2018**). However, since Caesarean section (C-section) infants are exposed firstly to the skin

and hospital environment, they acquire *Staphylococcus*, *Corynebacterium*, and *Propionibacterium* spp. (Dominguez-Bello et al., 2010).

After birth, the microbiota composition of infants shows a low of diversity (Milani et al., 2017). Scientists consider that by the age of 1-3 years old, the bacterial diversity of the infant's gut microbiota gradually increases (Derrien et al., 2019).

However, the gut microbiota of infants and children is highly affected by different factors such as delivery mode, mode of feeding (breastfeeding versus formula), antibiotics, environment, and stress (Bokulich et al., 2016; Callaghan et al., 2020).

The delivery mode is the main factor affecting the gut microbiota composition at the earliest stage of life (1 months-1 year) (Shao et al., 2019). The gut microbiota of infants delivered by C-section is less diverse compared to vaginally born infants with low levels of *Bifidobacterium* and traces of *Bacteroides* species and an increase of pathogenic bacteria (Korpela and de Vos, 2018). Moreover, C-section born infants are predisposed to future diseases. As an illustration, scientific evidence has revealed that Bifidobacteria are the most negatively affected gut bacteria in early life, mainly by C-section birth, antibiotic exposure and formula feeding. Thus, early low abundance of this bacteria is associated with a higher risk of developing immune and metabolic diseases in the coming years Figure 8 (Korpela, 2021).

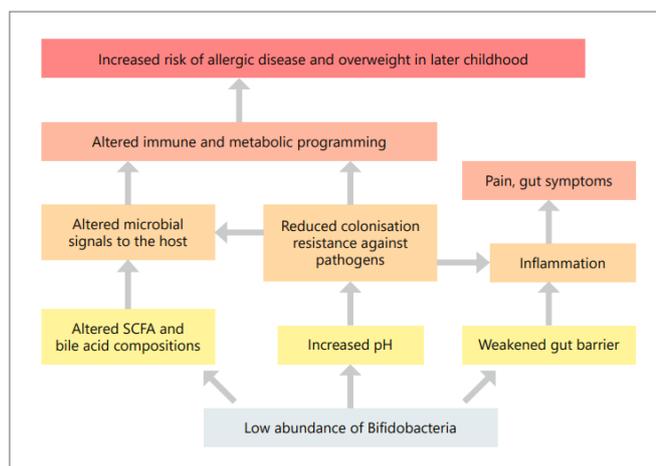


Figure 8. Consequences of low abundance of Bifidobacteria in infants (Korpela, 2021)

In addition to the delivery mode, other factors may affect gut microbiota, including diet (breastfeeding vs. formula feeding), physical illness, antibiotic use, and stress, which in turn may alter microbiota profile and consequently negatively influence long-term health (**Cryan et al., 2019b**). Emerging evidence suggests that imbalance (dysbiosis) of gut microbiota in early-life, is associated with upcoming diseases such as allergies, metabolic diseases (obesity, diabetes mellitus, type 1, cardiovascular diseases) and neuropsychiatric disorders, even with behavioural patterns (**Loughman et al., 2020; Sarkar et al., 2021; Skillington et al., 2021; H. Zhou et al., 2021**).

The importance of early life microbiota composition for shaping future health, with particular attention to brain development, is an emerging topic in the neuroscience field. Thus, the scientists are considering that taking care of and shaping the gut microbiota at early life stage may favorably affect health in the years to come (**S. Wang et al., 2018**).

#### *b) Adulthood*

However, even in pre-adolescents (children 7–12 years old) and adolescents, the microbiota does not reach adult microbiota profile maturation, including functional and taxonomic patterns (**Agans et al., 2011; Hollister et al., 2015**).

In adults, the gut microbiota reaches maturity and has long-term stability (**Faith et al., 2013**). Although is largely affected by infections, antibiotics, and diet it maintains its stable equilibrium state through the known resilience phenomena that characterize the gut microbiota (**Sommer et al., 2017**).

#### *c) Aging*

Studies have shown that aging is associated with the alternation of gut microbiota composition characterized by the decrease of diversity, particularly the reduction of short chain fatty acids (SCFA) producing species and an increase of opportunistic species (**Bana and**

**Cabreiro, 2019; Ragonnaud and Biragyn, 2021**). In a cross-sectional centenarian study, a decrease in anaerobic bacteria such as *Bifidobacterium* spp. and an increase in *Clostridium* and *Proteobacteria*, were observed among Japanese people (**Odamaki et al., 2016**). A study exploring age-related differences showed that the microbiota of centenarians was associated with a decrease in *Faecalibacterium prausnitzii* and an increase of pathobionts (**Biagi et al., 2010**). Given the roles of *Bifidobacterium* spp. and *Faecalibacterium prausnitzii* in the immune system and metabolic processes, these decreases may explain the low systemic inflammatory status and malnutrition in older adults (**Guigoz et al., 2008**).

Moreover, altered gut microbiota, caused by aging, contributes to several dysfunctions of the gut, including disruption of barrier integrity, which leads to `leaky gut` syndrome and translocation of pathogenic bacteria and endotoxins into the circulatory system which induces an inflammatory response resulting in the increased risk of brain and heart diseases (**Cryan et al., 2019a; Ragonnaud and Biragyn, 2021**). Hence, the gut microbiota is considered a key player to healthy ageing (**Claesson et al., 2012; Ragonnaud and Biragyn, 2021**). A cohort study conducted by Wilmanski et al. identified that the microbiome pattern reflects healthy aging, characterized by depletion of *Bacteroides* or high gut microbiome diversity (**Wilmanski et al., 2021**). Therefore, scientists have questioned whether taking care of our microbiota might be linked to longevity (**Biagi et al., 2016; Y. Zhou et al., 2021**) and beyond that, whether the fountain of youth lies in the gut microbiome (**Cryan et al., 2019a**).

## I.B.2. Factors shaping gut microbiota

In addition to delivery mode, it is well-known that both genetic and environmental factors contribute to microbiome composition and function (**Benson et al., 2010; Spor et al., 2011; Xie et al., 2016**). However, it has been established that the environment (diet, lifestyle, and medications) can overcome the heritage (**Qin et al., 2020; Rothschild et al., 2018; Scepanovic et al., 2019**).

### I.B.2.1. Genetics

Evidence obtained from mouse models suggests that host genetic contribute to shaping the gut microbiota (**Kemis et al., 2019; Suzuki et al., 2019; Ussar et al., 2015**). Twin studies have provided further insight and assessment of the role of genetics in determining the characteristics of the microbiome profile (**Goodrich et al., 2014; Turnbaugh et al., 2009; Xie et al., 2016**). Several gut microbial taxa have been found to be heritable in humans, and *Christensenellaceae* (belonging to the phylum Firmicutes) showed the highest heritability followed by Archaea (**Goodrich et al., 2014**). The same team of researchers, following a study with a larger sample size, detected a link between heritable bacteria and human genes related to diet, metabolism and immunity (**Goodrich et al., 2016**).

### I.B.2.2. Diet

Dietary patterns are one of the main contributing factors that shape the gut, and consequently affect health (**David et al., 2014; Moles and Otaegui, 2020; Sandhu et al., 2017**) and even mental health (**Firth et al., 2019; Taylor et al., 2020; Watson, 2020a**).

There is evidence that some popular diets, such as Mediterranean, vegetarian, vegan, Western, and gluten-free diets are associated with differences in the gut microbiota (**Rinninella et al., 2019a**). The intake of such known Mediterranean diet, which is rich in fruits, vegetables, coffee, wine, and olive oil fish, cereals, nuts, seeds and aromatic herbs, influences the gut microbiota (**Merra et al., 2020**). For example, consumption of the Mediterranean diet for one year by obese men (n = 20) significantly decreased the genera *Prevotella* and increased the *Roseburia* genus and *F. prausnitzii*, (**Haro et al., 2016**). Another study carried out in a cohort of Italian individuals (n = 153) reported that adherence to the Mediterranean diet was associated with increased levels of faecal (SCFAs) which correlated with the enrichment of Firmicutes and Bacteroidetes (**De Filippis et al., 2016**). Likewise, a study of a Spanish cohort (n = 150) also observed association of the Mediterranean diet with higher levels of butyrate-producing taxa such as *Roseburia faecis*, *Ruminococcus bromii* and *Oscillospira (Flavonifractor) plautii*

(Rosés et al., 2021). Garcia-Mantrana et al. highlighted the positive impact of this diet on the gut microbiota, particularly by enhancing the abundance of Bifidobacteria and levels of SCFAs (Garcia-Mantrana et al., 2018).

Moreover, emerging evidence suggests that the Mediterranean diet is associated with higher cognitive performance and a reduced incidence of brain diseases (Fresán et al., 2019; Karstens et al., 2019). A randomized controlled trial that evaluated dietary intervention in major depression (SMILES) confirmed that a poor diet was correlated with depression symptomatology and showed that the Mediterranean diet intervention improved depression scores (Jacka et al., 2017). The compounds present in the Mediterranean diet, mainly phenolic compounds, indicate that apart from their antioxidant effects, they can positively impact the human health by modulating the gut microbiota (Bucciantini et al., 2021; Selma et al., 2020).

On the other hand, consumption of a Western diet is characterized of high intake of processed high-fat meat, saturated fats, refined grains, sugar, salt, alcohol and corn-derived fructose, fast food, including French fries, and soft drinks. Recently, scientists have investigated the effects of long-term dietary patterns and specific nutrients on the composition and activity of gut microbiota. They found that the Western diet was accompanied by a high abundance of inflammatory bacteria, namely, *Clostridium bolteae*, *Ruminococcus obeum*, *R. gnavus* and *Blautia hydrogenotrophica* and a decrease in commensal bacteria such as Firmicutes (*Roseburia*, *Eubacterium rectale* and *Ruminococcus bromii*) (Bolte et al., 2021). Moreover, clinical studies support the hypothesis that a diet consisting of animal and processed foods, sugar and alcohol, by affecting the gut microbiota, may trigger obesity, metabolic diseases, cognitive impairment and increased anxiety (Attuquayefio et al., 2017; Peris-Sampedro et al., 2019; Zinöcker and Lindseth, 2018).

### I.B.2.3. Drug-gut microbiota interactions

Some studies have shown that the oral antibiotics additionally to their action on targeted microbes, also affect the composition and functions of the entire intestinal microbiota, consequently they can impact host health (Froehlich et al., 2016; Hagan et al., 2019; Maier et

al., 2021; Maurice et al., 2013; Mu and Zhu, 2019). In addition the antibiotics, growing evidence that other drugs also affect the intestinal microbiota (**Maier et al., 2018; Vila et al., 2020**), highlighting the importance of investigating the effects of drugs on the composition and metabolic function of the gut microbiota (**Guthrie and Kelly, 2019**).

From a pharmacological point of view, the stomach is not favourable absorbing environment for most orally administered drugs. Therefore, they pass into the small intestine and following their absorption undergo pharmacokinetic processes of distribution, metabolism, and excretion. In addition, some drugs, including drugs poorly absorbed in the small intestine and modified release formulations, can reach the colon (**Stillhart et al., 2020**). Once in the colon, a complex reciprocal interaction occurs between drugs and resident gut microbes (**Weersma et al., 2020**).

Several *in vitro* and *in vivo* studies in humans have shown that some drugs are associated with changes in gut microbiota, including decreased diversity and taxonomic changes (**Forslund et al., 2015; Jackson et al., 2016**). For example, when scientists screened 100 common drugs prescribed for various diseases, they found that a quarter of them had the antibiotic effects on 40 bacterial strains (**Maier et al., 2018**). Indeed, over the years, the strongest evidence of drug effects on the microbiota comes from human cohort studies. A recent investigation using metagenomic sequencing, found that 19 of 41 drugs were associated with the gut microbiome (**Vila et al., 2020**). A broad range of drugs that affect the gut microbiota, have been identified, including proton pump inhibitors (PPIs), metformin, statins, osmotic laxatives, inflammatory bowel disease (IBD) medications, female sex hormones, benzodiazepines, antidepressants, and antihistamines (**Falony et al., 2016; Zhernakova et al., 2016**). Furthermore, scientists have found that the use of multiple drugs (polypharmacy) can alter the gut microbiota differently from single drug administration (**Rogers and Aronoff, 2016**), with a significant negative correlation (**Ticinesi et al., 2017**).

Scientific data have revealed a complex dynamic interaction between drugs and the gut microbiota. As drugs affect the gut microbiota, the gut microbiota can also affect the pharmacokinetic profiles of drugs and consequently their efficacy and toxicity (**Weersma et al., 2020; Zhang et al., 2018**). The gut microbiota can extensively transform drugs using microbiome-derived enzymes (**Koppel et al., 2017; Zimmermann et al., 2019**). The microbial

drugs biotransformation's include several chemical reactions and mainly correspond to the first-phase reactions of reduction and hydrolysis. The enzymatic machinery of gut microbiota may contribute to the activation of prodrugs (e.g., sulfasalazine and prontosil, as shown in Figure 9), drug inactivation (e.g., L-Dopa, digoxin and methotrexate), and an increase in drug toxicity (e.g., sorivudine and brivudine) (**Spanogiannopoulos et al., 2016; Zhang and Chu, 2021; Zimmermann et al., 2021**).

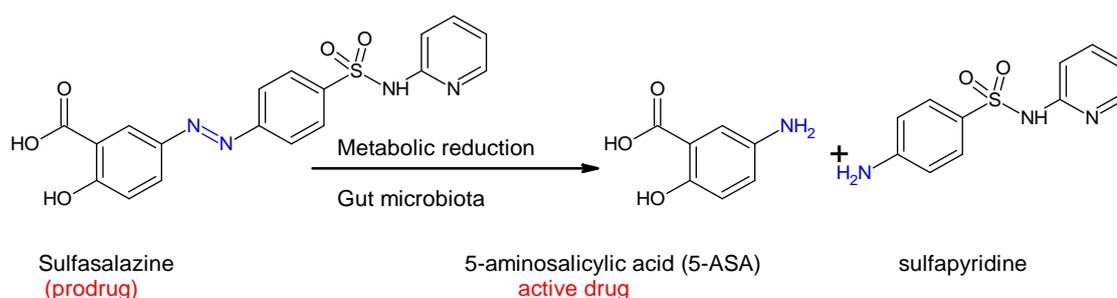


Figure 9. Bioactivation of sulfasalazine by intestinal bacterial metabolism

Drug-microbiota interactions are receiving increasing attention from scientists, particularly in the field of neuropharmacology (**Cussotto et al., 2019; Walsh et al., 2018**). Some evidence suggests that psychoactive drugs are subjected to gut microbiota metabolism (**Walsh et al., 2018**). In addition, effects of psychoactive drugs on the composition of gut microbiota have been observed. For example, citalopram, an antidepressant drug, was associated with a significant increase of the relative abundance of the Enterobacteriaceae family compared with that in patients not taking any medication (**Rogers and Aronoff, 2016**).

Moreover, the gut microbiota can influence individual responses to immunotherapy in treatment cancer treatment (**Gopalakrishnan et al., 2018; McCulloch et al., 2022; M. Wu et al., 2021; C.-B. Zhou et al., 2021**). Scientific data have shown that the gut microbiota should be considered in the framework of the drug discovery process, including its implications in pharmacokinetic and pharmacodynamic profiles. However, further research should be undertaken to explore drug-microbiota interactions with the aim of increasing the overall benefit of drug treatments, which would further contribute to new drug discovery and personalized medicine (**Javdan et al., 2020**).

### I.B.3. The gut microbiota and brain crosstalk signalling

The interactions between the Central Nervous System (CNS) and the intestinal tract have been studied over the years. The fact that gastrointestinal functions such as motility and secretion are closely controlled by the CNS is well documented (**Browning and Travagli, 2014**). In addition, the CNS can influence the composition and function of the gut microbiota, relying on changes in the brain physiology to modulate gut physiology (**Mayer, 2000**). Noteworthy, it is well established how the brain disturbs the gut microbiota in the case of stress and various emotional states (**De Palma et al., 2014**).

On the other hand, increasing evidence from animal models and human studies have shown that gut microbes can affect brain development and function (**Cryan and Dinan, 2012; Erny et al., 2015; Luczynski et al., 2016**). The first demonstrations came from studies on Germ-free (GF) mice which showed that the absence of the gut microbiota greatly affects the development of the nervous system, behaviour and gene expression in the brain (**Gareau et al., 2011; Heijtz et al., 2011; Neufeld et al., 2011; Weger et al., 2019**). Interestingly, the evidence provided by studies in mice have shown that the maternal gut microbiome influences fetal brain development (**Vuong et al., 2020**). Moreover, the maturation of gut microbiota at early life and the brain development go parallelly (**Dinan and Cryan, 2017**). Finally, brain imaging strongly supports the evidence that the gut microbiota communicates with and affects the human brain (**He et al., 2018; Labus et al., 2017**).

Further studies have revealed that microbiota greatly influences stress, eating and sexual behavior, cognition and addiction (**Cusotto et al., 2018; Foster and McVey Neufeld, 2013**). Specific gut microbes, such as *Enterococcus faecalis*, have shown a regulatory role insocial behaviour by modulating hypothalamic–pituitary–adrenal (HPA) axis, which mediates stress responses in the brain (**W.-L. Wu et al., 2021**). Studies have shown that the gut microbiota is also implicated in synaptic plasticity and greatly influences memory and learning (**Callaghan, 2020; Chu et al., 2019**). Furthermore, the changes of gut microbiota homeostasis

are linked with several brain states including mood (**Appleton, 2018; Liu and Zhu, 2018**) and other neurological diseases (**Cryan et al., 2020**).

The human brain constantly communicates with the gut in a bidirectional complex way, known as the gut-brain axis. However, due to the key regulatory role of microbiota on this axis, the microbiota-gut-brain axis is emerging as a concept in the field of neuroscience. These potential connections between the gut microbiota and the brain, include neuronal, immune, endocrine, and metabolic signalling (**Cryan et al., 2019b**), which will be developed in subsequent headings.

### I.B.3.1. Microbiota- brain immune signalling

One of the main routes that link the gut and brain is through immune mechanisms (**Fung, 2020**). The largest and the most complex immune system is found in the gut and is known as the gut-associated lymphoid tissue (GALT). GALT represents 70–80% of the body's immune cells, which constantly communicate with the gut microbiota, in turn influencing and shaping both the immune system at the intestinal and systemic levels (**Kasper et al., 2019**). The key role of GALT is to provide immune tolerance to commensal microorganisms while initiating immune responses against pathogenic ones (**Mörbe et al., 2021**). The intestinal epithelium separates and limits the contact of bacteria with the *lamina propria*, thus providing the defense against invading microbes and external factors. (**Chelakkot et al., 2018; Hooper, 2015**). Most of the GALT's organization in the colon is the same as that in the small intestine. Moreover, the colon has a dual mucus layer, with a thick largely sterile inner layer and a thinner outer layer. Below the gut epithelium, a layer of connective tissue called the *lamina propria* is located, where various immune cell types (e.g., dendritic cells, macrophages, and T and B lymphocytes) can be found (**Cader and Kaser, 2013**). The GALT organization of the colon is shown in Figure 10.

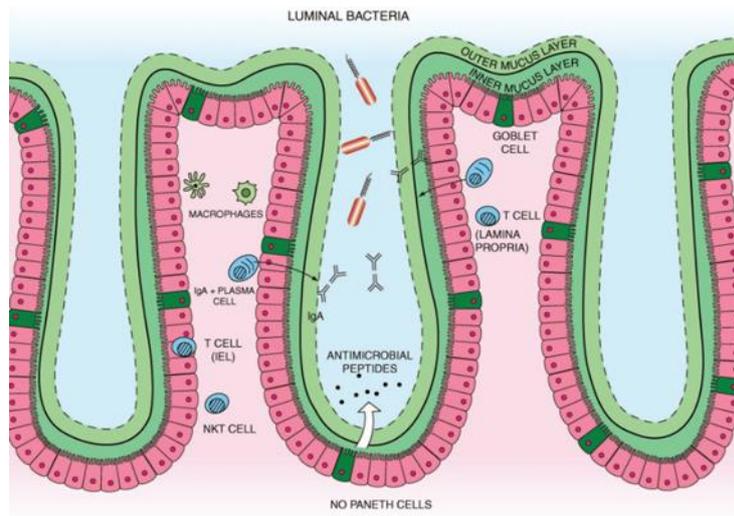


Figure 10. GALT organization of colon (Cader and Kaser, 2013).

The innate system of GALT, also known as the nonspecific immune system, provides a first line of defence (**Medzhitov, 2007**). Numerous mechanisms are involved to maintain the immune balance in the gut. Intestinal barrier, as an integral component of the innate immune system, represents the first line of physical and chemical protections (**Vancamelbeke and Vermeire, 2017**). The innate system provides the host defence by identifying the potential pathogens through Pattern Recognition Receptors (PRRs) expressed in epithelial cells, which detect molecular patterns specific to microorganisms known as Pathogen-Associated Molecular Patterns (PAMPs) (e.g, lipopolysaccharides, peptidoglycans, lipoteichoic acids and cell-wall lipoproteins) (**Medzhitov, 2007**). Furthermore, the innate immune system is mediated by crucial phagocytic cells including macrophages, dendritic cells, monocytes, whose role is to kill the invading microorganisms (phagocytosis) and induce a cascade of inflammatory response (**Charles A Janeway et al., 2001**).

The adaptive immune response is mediated by white blood cells called lymphocytes such are B and T cells (**Alberts et al., 2002**). When activated, T cells can differentiate into cytotoxic T cells, regulatory T cells (Treg) and various T helper cells, such are Th1, Th2, Th3, and Th17. Once stimulated T helper cells secrete different cytokines, which activate cytotoxic T cells and macrophages to kill infected cells. In addition, they stimulate the B cells to secrete

antibodies. The role of Treg is to control immune reactions (**Luckheeram et al., 2012**). Both systems closely collaborate to maintain immune homeostasis (**Jiao et al., 2020**)

At the intestinal level, immune homeostasis is maintained by a reciprocal and symbiotic relationship between immune system and gut bacteria (**Kasper et al., 2019; Mowat, 2018**). The gut microbiota represents an important factor that contributes to maintain intestinal epithelium integrity by strengthening the tight junction proteins, stimulating the production of the mucus layer and the secretion of antibacterial proteins (**Allam-Ndoul et al., 2020; Hayes et al., 2018; Paone and Cani, 2020**). Moreover, gut bacteria contribute in the regulation of the immune responses also during dysbiosis state (**Wang et al., 2019**).

In a dysbiosis state, Pathogen recognition by PRRs senses microbial signal which leads to the activation of innate cellular defence mechanism, thus inducing production of pro-inflammatory cytokines, by macrophages and DCs. DCs travel to the lymphoid nodes and drive the activation of T cells which in turn induce B- lymphocytes to secrete antigen-binding immunoglobulins (antibodies) and elicit antimicrobial immune responses. In addition, regulatory T-cells induce immune tolerance to commensal-derived antigens. This gut microbial balance avoids overreaction of the immune system (**Iwasaki and Medzhitov, 2015; Sansonetti, 2006**).

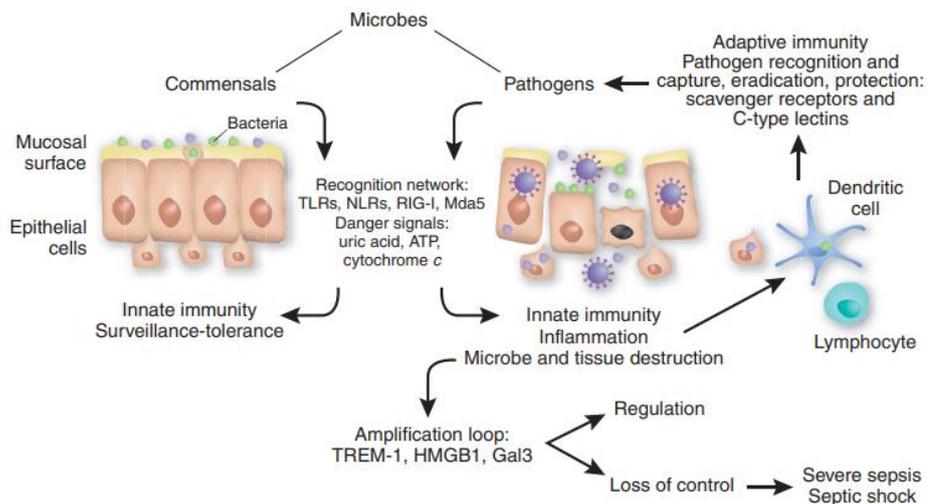


Figure 11. The Network between gut bacteria and innate and adaptive immune system (Sansonetti, 2006)

Furthermore, gut microbiota-immunity interaction is a key node in mediating communication between microbiota and brain (Zheng et al., 2020). However, when this immune balance is triggered by different external and internal factors, the gut barrier is compromised, called as ‘leaky gut. As a result, the pathogens and the bacterial toxins translocate into *lamina propria* (intracellular space) resulting in the activation of the immune system. Consequently, it induces an immune response in the gut followed by the secretion of various cytokines, chemokines, and immunoglobulins (Lobionda et al., 2019). Then, they send signals to the brain *via* vagus nerve or directly crossing BBB, in turn affecting brain functions and behavior. All these cascading events activate the hypothalamic-pituitary-adrenal axis (HPA) axis and stress response. In this regard, growing evidence links the disruption of the intestinal barrier integrity and the immune activation in gut with several pathological intestinal metabolic and even more neurological diseases (Schultz and Keita, 2020).

### I.B.3.2. Metabolic pathway

Another mechanism connecting the gut and brain is *via* microbiota-derived metabolites (Caspani and Swann, 2019). Human microbiota provides essential nutrients and bioactive metabolites which are involved in host biology processes. The metabolism by gut microbiota includes mostly direct biotransformation of ingested compounds, modification of host metabolites and *de novo* synthesis (Figure 12) (Silpe and Balskus, 2021).

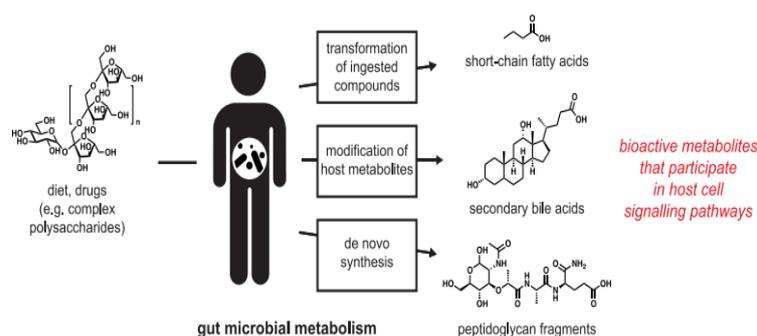


Figure 12. Main metabolic functions of gut microbiota, including biotransformation of dietary ingested compounds, modification of host metabolites and *de novo* synthesis of metabolites (Silpe and Balskus, 2021)

The main metabolites produced by the gut microbiota and the most studied ones are short-chain fatty acids (SCFAs) (Koh et al., 2016). They are fermented products of indigestible dietary polysaccharides by gut microbes, mostly into acetic (40–60%), propionic (20–25%) and butyric (15–20%) acids and smaller amounts of formic and valeric acids. The amount of production of SCFAs is estimated to be around 500–600 mmol per day. (Dalile et al., 2019a). The first step is the degradation of polymers (dietary fibers and resistant starch) into smaller monosaccharides by bacteria producing hydrolysis enzymes, followed by the subsequent biotransformation and cross-feeding processes as indicated in Figure 13 (Koh et al., 2016).

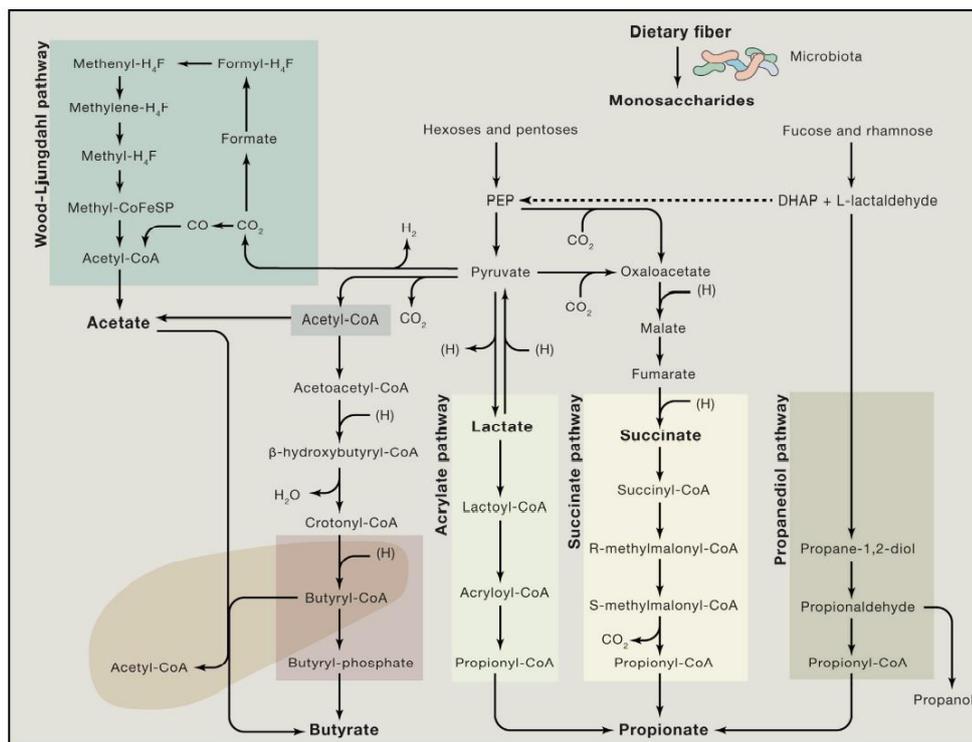


Figure 13. Gut microbial formations pathways of acetate, propionate and butyrate formations from dietary polysaccharide (Koh et al., 2016) ; abbreviations: PEP (phosphoenolpyruvate); DHAP, (dihydroxyacetonephosphate).

Most of the acetic acid amount is derived *via* acetyl-CoA by using pyruvate as a substrate. Another metabolic pathway includes Wood-Ljungdahl pathway, in which bacteria

can use  $H_2$  and  $CO_2$  or formic acid to synthesize it. Mainly enteric bacteria are involved in this process such are *Akkermansia muciniphila*, *Bacteroides* spp., *Bifidobacterium* spp., *Prevotella* spp., *Ruminococcus* spp (Koh et al., 2016). Propionic formation follows three pathways occurring by gut bacteria, such are the succinate, acrylate and propanediol pathway. Butyric acid is mostly produced by Gram-positive Firmicutes, such are *Faecalibacterium prausnitzii* and *Roseburia* spp. according to the acetate CoA-transferase pathway and the butyrate kinase pathway (Louis and Flint, 2017).

Moreover, SCFAs are also formed as end products of amino acids biotransformation. Indeed, proteins fermentation by gut microbiota produces amino acids, peptides, and tripeptides (Portune et al., 2016). Then, these fermented metabolites undergo to subsequent microbial transformation by certain bacteria to diverse metabolites and end-products such as SCFAs and branched chain fatty acids (*ie.*, isobutyric and isovaleric acids in particular) but also potentially toxic products (*ie.* sulfides and ammonia) (Zhao et al., 2019). Finally, consumption of fermented foods can contribute to SCFAs production (Annunziata et al., 2020).

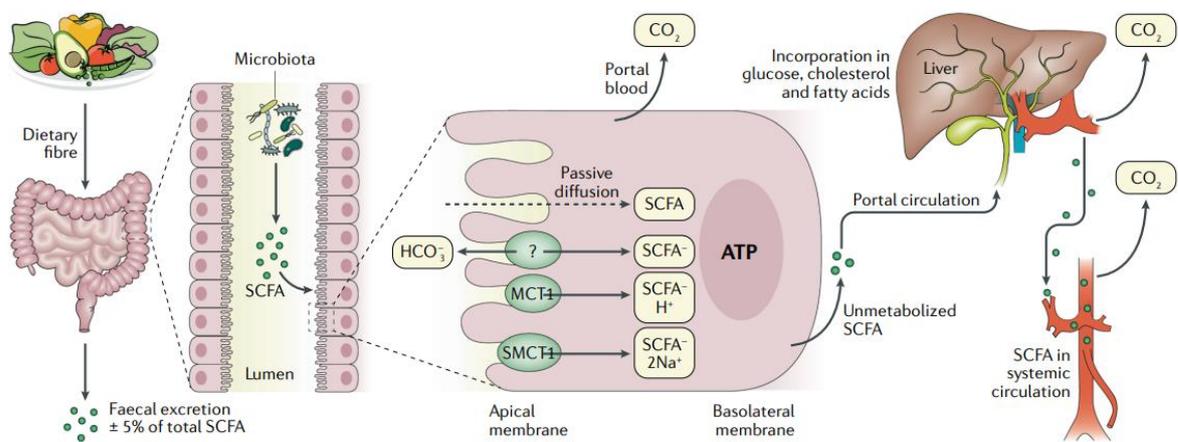


Figure 14. The journey of SCFAs in humans, including production, absorption, metabolism, and distribution (Dalile et al., 2019)

Once produced, SCFAs are absorbed into colonocytes, mainly by specific transporters or passive diffusion Figure 14. In cells, they are partially oxidized providing a source of energy

for colonocytes. The rest of the SCFAs through the portal circulation are transported to the liver where they are metabolized (**Bloemen et al., 2009**). Thus, only a small amount, precisely at  $\mu\text{M}$  concentrations, of these metabolites reaches the circulatory system and target organs, (**Stumpff, 2018; Verbeke, 2017**).

Given their ability to cross BBB and directly influence the brain, SCFAs are considered as key mediators in gut-brain crosstalk. In addition, they can indirectly influence immune, endocrine, and neuronal pathways (**Dalile et al., 2019**).

Some studies have found that SCFAs have immunomodulatory potential (**D'Souza et al., 2017; Ratajczak et al., 2019**). Thus, it has been reported that SCFAs modulate signalling pathways involved in oxidative stress, thereby restoring the intestinal barrier (**González-Bosch et al., 2021; Liu et al., 2021**). In addition, SCFAs can enhance the gut barrier function by stimulating mucus production. For example, butyric has been reported to stimulate mucin by affecting *muc2* gene expression in a mouse model (**Gaudier et al., 2009**), and among SCFAs, mainly butyric acid is involved in upregulating the expression of tight junction proteins and in increasing transepithelial electrical resistance (TEER) (**Huang et al., 2021; Peng et al., 2009; Wang et al., 2012**). Numerous studies have shown that SCFAs inhibit the activity of histone deacetylase, which leads to the inhibition of inflammatory response and induces anti-inflammatory activity of the immune cells (**Parada Venegas et al., 2019**). Microglia are among the innate immune cells in the CNS and their dysregulation was reported to be involved in several brain disorders. Studies have shown that SCFAs affect the morphology and function of microglia, thus modulating brain immunity (**Erny et al., 2015**).

SCFAs have been shown to stimulate production of gut hormones in enteroendocrine cells, such as glucagon-like peptide 1 (GLP1) and peptide YY (PYY) whose effects have been associated with anti-obesity and anti-diabetic actions (**Brooks et al., 2017; Greenhill, 2017; Tolhurst et al., 2012**).

Other studies have shown that SCFAs directly affect brain functions, although only acetate is present in cerebrospinal fluid (Nagashima et al., 2010). Interestingly, propionic acid at 1  $\mu$ M concentration positively impacts BBB function by inhibiting inflammatory response pathways (Hoyles et al., 2018).

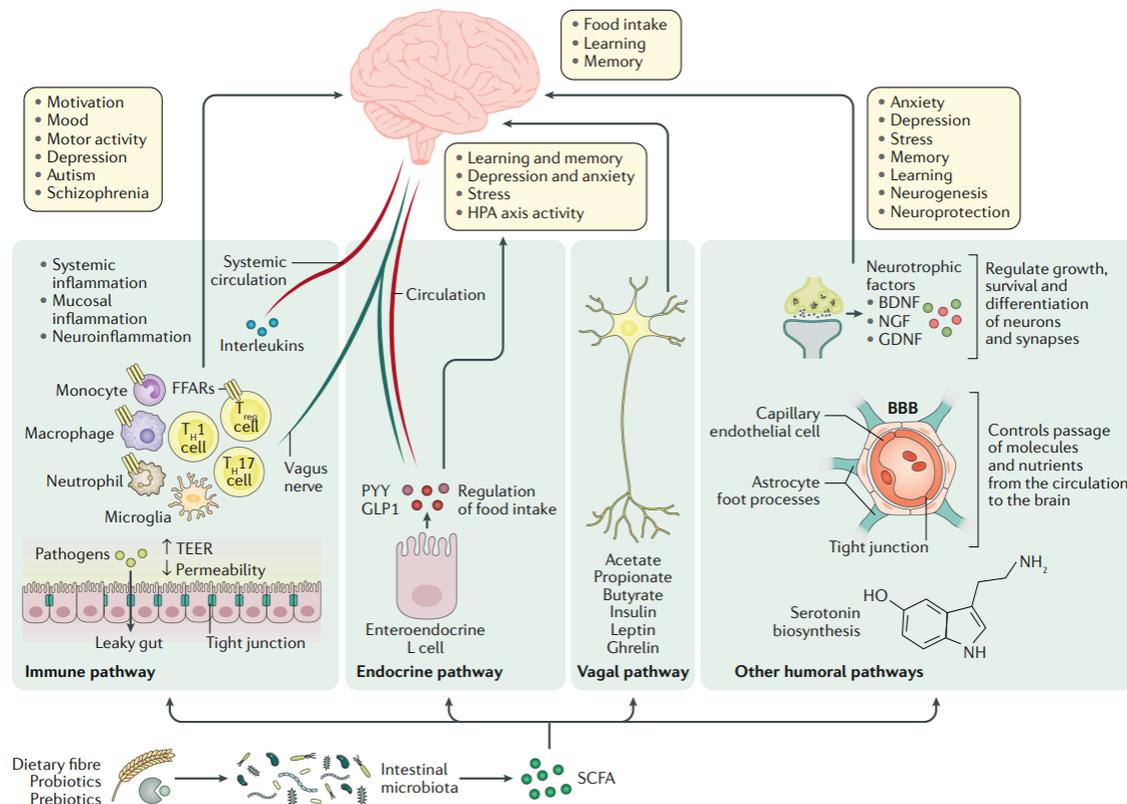


Figure 15. The role of SCFAs in human physiology function (Dalile et al., 2019)

### I.C.3.3. Endocrine pathway

The endocrine pathway is mediated through the hypothalamic-pituitary-adrenal (HPA) axis, comprising a major part of the neuroendocrine system, and through enteroendocrine cells (EECs). The HPA axis causes the secretion of adrenaline and noradrenaline, while EECs are distributed on mucosa and are responsible for the secretion of glucagon-like peptide-1 (GLP-1), cholecystokinin (CCK), peptide YY (PYY) and serotonin (5-HT) production (Latorre et

**al., 2016; Sherwin et al., 2016).** These two compartments, HPA and EECs, showed regulatory effects on the bidirectional communication between the gut and the brain.

It has been established that these gut hormones are involved in gut-brain axis crosstalk. More than 90 % of the neurotransmitter 5-HT is produced on the intestines by gut mucosal enterochromaffin cells (**Strandwitz, 2018**). It should be noted that 5-HT is unlike to reach the brain, but it can highly affect CNS function as a regulator of mood and sleep (**Sun et al., 2020**). First, tryptophan, as a precursor of 5-HT plays crucial role on CNS and ENS function, thereby is involved in gut-brain signalling. The availability of tryptophan in the CNS is largely affected by its metabolism via the kynurenine pathway in the peripheral tissues (**Evrensel et al., 2020**). In addition, 5-HT derived from enterochromaffin cells exerts critical effects on immune regulation. 5-HT released from the enterochromaffin cells leads to the alteration of the vagal afferent activity and then potentially affects the gut-brain signalling (**Sun et al., 2020**). In addition, gut peptides are also play role in signalling pathways. GLP-1, which is mainly involved in insulin secretion and CCK in food intake, are also involved in anxiety and stress responses (**Diz-Chaves et al., 2020; Lach et al., 2018**).

#### I.C.3.4. Neuronal pathway

The gut has 200–600 million neurons known as enteric nervous system (ENS). This system can function autonomously although it communicates with CNS via parasympathetic and sympathetic branches of the autonomous nervous system (ANS). Thus, brain exchange constantly information with the gut (**Furness et al., 2014**). Mainly, vagus nerve transmits information from ENS to brain. It is the 10<sup>th</sup> cranial nerve and consists of afferent (sensory) and efferent (motor). Vagal afferents are distributed and end in muscle layer and mucosa, thus they are not in contact with gut microbiota. However, when the homeostasis is break, microbiota interact with vagus nerve through metabolites, thereby stimulates vagus nerve (**Fülling et al., 2019**).

It is interesting to note that some gut bacteria have already shown ability to produce some neuroactive metabolites. For example, *Lactobacillus* spp. and *Bifidobacterium* spp. were

able to produce GABA; *Escherichia* spp., *Bacillus* spp. and *Saccharomyces* spp. to produce noradrenalin; *Streptococcus* spp., *Escherichia* spp. and *Enterococcus* spp. to produce serotonin; *Bacillus* spp. to produce dopamine; and *Lactobacillus* spp. to produce acetylcholine (**Cryan and Dinan, 2012**). However, since they are unlikely to cross BBB, they do not affect brain directly. The mechanism by which these neuroactive substances produced in the gut affect the brain is unknown. As a hypothesis, they may act in the enteric nervous system (**Sun et al., 2020**).

#### I.B.4. Gut microbiota in health and diseases

Understanding the profound role of microbiota in health and diseases, has become one the most intense and explosion topic in the past decades (**Fan and Pedersen, 2021; Lynch and Pedersen, 2016; B. Wang et al., 2017**). Indeed, studies have shown there is a mutually beneficial relationship between microorganisms reside with the host (**O'Hara and Shanahan, 2006**). It is well established that the gut microbiota is involved into numerous physiological processes of the human body. including digestion, protection, immune maturation and function, brain development and function, as well. Moreover it contributes to our well-being and mostly positively influence our health (**Adak and Khan, 2019**). In turn, the host provides major sources of carbon and energy for gut bacteria (**Bäckhed et al., 2005**).

In addition, altered gut microbiota composition and function has been linked with several metabolic and neuronal diseases (**Yinwei Chen et al., 2021**).(Wang et al., 2017).

#### I.B.5. Gut microbiota linked to the brain disorders

A large number of studies explained how gut dysbiosis can affect the brain and play a key role on the pathogenesis of neurodegenerative diseases (**Schächtle and Rosshart, 2021; Shabbir et al., 2021**). Moreover, an altered balance of gut microbiota has been linked to a range of brain disorders including Stress and Depression, Autism Spectrum disorders, Multiple Sclerosis (MS), Parkinson's disease (PD) and Alzheimer's disease (AD) (**Vijay and Valdes,**

**2021; Zhu et al., 2020).** Intriguingly, scientists detected differences of the gut microbiota profiles in patients with brain-related disorders compared to healthy controls (**Fung et al., 2017).**

#### I.B.5.1. Anxiety and Major depressive disorders (MDD)

Major depressive disorders (MDD) affect at least 4.4% of the world's population (**Flux and Lowry, 2019).** This disease is associated with the dysfunction of 5-hydroxytryptamine (5-HT, serotonin) and/or norepinephrine at specific synapses in the brain (**Foster et al., 2021).** Recently, numerous studies are supporting the concept that the gut microbiota is implicated in depression. For instance, several experimental *in-vivo* studies in animal models provide evidence for the involvement of the gut microbiota in stress-related disorders including anxiety and depression (**Dinan and Cryan, 2013; Foster and McVey Neufeld, 2013).**

Interestingly, when the scientists transplanted fecal microbiota from MDD patients to germ-free rat model, they observed that the host began to exhibit depressive features such as anhedonia and anxiety-like behaviors, as well as an alteration in tryptophan metabolism (which is in humans linked to a variety of neuropsychiatric diseases) by an increase of kynurenine/tryptophan ratio (**Kelly et al., 2016).** In the same way, Zheng et al., showed that a lack of microbiota induced depression-like behavior in germ-free (GF) mice (**Zheng et al., 2016).**

However, further studies shed the light on how the gut microbiota is linked to brain diseases. Those studies identified differences in the gut microbial composition of patients with anxiety and depression compared to controls (**Aizawa et al., 2016; Jiang et al., 2015; Lin et al., 2017; Naseribafrouei et al., 2014).** Anxiety has been characterized with overabundance of *Escherichia*, *Shigella*, *Fusobacterium* and *Ruminococcus gnavus* (**Jiang et al., 2018),** whereas *Prevotella* and *Klebsiella* were significantly increased in the fecal microbiota of patients with major depressive disorders (**Lin et al., 2017).** A large study from Flemish Gut Flora Project has shown further insights into the link between gut microbes and depression. The scientists analyzed the fecal microbiota of 1054 Belgian people and validated the results

in an independent cohort of 1,063 individuals from the Dutch LifeLines DEEP (LLD) and case-control studies previously published. These researchers found that the genera *Dialister* and *Coprococcus* were depleted in people with depression despite antidepressant treatments. Furthermore, the scientists described the correlation between a higher quality of life and the abundance of *Faecalibacterium* and *Coprococcus*. This study also revealed the ability of gut microbiota to produce particular neuroactive metabolites associated with mental health (Valles-Colomer et al., 2019).

#### I.C.5.2. Alzheimer's disease (AD)

Alzheimer's disease, the leading cause of dementia in the elderly, is characterized by memory loss and progressive neurocognitive dysfunction (Tiwari et al., 2019). Pathologically, it is associated with the deposition of amyloid-beta ( $A\beta$ ) protein into plaques in the spaces between nerve cells and hyperphosphorylation of tau protein on neurofibrillary tangles inside the cells (Selkoe and Hardy, 2016). Although the pathogenesis of AD is still unknown, the scientists suggest that the risk and triggering factors associated with AD are genetic susceptibility, aging and environmental influences. In addition, there is growing evidence suggesting that the gut microbiota may play a role in the pathogenesis and progression of AD (Harach et al., 2017; Pistollato et al., 2016; Seo et al., 2019; Szablewski, 2018). For example, Minter et al., showed that the perturbation of gut microbiota induced by antibiotics was associated with neuroinflammation and amyloidosis in a mouse model of AD (Minter et al., 2017). Moreover, a study in gut microbiota-free mice has shown reduced levels of cerebrospinal  $a\beta$  amyloid compared to controls (Harach et al., 2017). Three independent groups of scientists have explored the gut microbiota of AD patients, and they found differences at the taxonomic level in AD patients compared to control patients. These studies provided additional evidence that the gut microbiota plays a role in AD. Vogt et al., detected a decrease in the level of Firmicutes and Bifidobacterium (Actinobacteria) and an increase of Bacteroidetes in the microbiome of AD patients (Vogt et al., 2017). Similarly, Zhuang et al, demonstrated that the overall change in microbiota community between AD patients and healthy controls at taxonomic level, including Bacteroides, Actinobacteria, Ruminococcus,

Lachnospiraceae and Selenomonadales (**Zhuang et al., 2018**). In addition, a recent study further explored the differences between elderly AD patients including those without dementia and according to dementia types. In AD elders, a significant increase of *Bacteroides vulgatus*, *B. fragilis*, and *Eggerthella lenta* and a reduced level of *Butyrivibrio*, *Eubacterium*, *Clostridium* sp. strain SY8519, *Roseburia hominis*, and *F. prausnitzii* was observed. In the microbiome of these patients, higher abundance of bacteria causing proinflammatory states and lower prevalence of butyrate synthesizing bacteria were observed (**Haran et al., 2019**).

### I.B.5.3. Parkinson's disease

Parkinson's disease is the second most common neurodegenerative disease caused by the accumulation of the  $\alpha$ -synuclein protein known as Lewy bodies (LBs) in the brain's basal ganglia and the progressive degradation of dopaminergic neurons in the substantia nigra (**Balestrino and Schapira, 2020**). In neuronal cells, the neurotransmitter dopamine is mainly formed from the amino acid L-tyrosine, and plays a crucial role in motor control, while the  $\alpha$ -synuclein is related to neurotransmission (**Lebouvier et al., 2009**). Clinical features associated to PD are mainly motor deficit symptoms including tremors, bradykinesia, postural instability and gait disability (**Olson et al., 2019**). While it is known that the neuroinflammation contributes to PD pathogenesis (**Hirsch and Hunot, 2009**), there is also evidence that the gut microbiota may be involved, as well (**Sampson et al., 2016; D. Yang et al., 2019**). For example, when scientist fed rats with strain of *Escherichia coli* that produces a misfolding protein, similar to  $\alpha$ -synuclein, called curli, in the gut, they detected more  $\alpha$ -synuclein accumulation in the animals' brains. In addition, preclinical studies suggest that, the  $\alpha$ -synuclein in the gut can be transported to the brain *via* the vagus nerve (**Holmqvist et al., 2014**). Furthermore, the lipopolysaccharide (LPS), a gut microbial metabolite, has shown modulatory effects on the aggregation of  $\alpha$ -synuclein (**Bhattacharyya et al., 2019**).

Recently, based on different evidence, the scientists have postulated the ethology of PD. These authors divided PD into two subtypes: (1) the gut-phenotype which first starts in the peripheral nervous system (PNS) of the gut then enters to the brain *via* vagus nerve; (2) the

brain- phenotype which begins in the brain or in the olfactory epithelium and then spreads in the brain and the peripheral nervous system (PNS) (**Borghammer and Van Den Berge, 2019**).

The pathogenesis of PD is not yet fully elucidated. However, the evidence of the involvement of the microbiota, increases over the years. In addition, studies have detected several bacteria altered in PD patients compared to healthy individuals. Scheperjans et al. detected significantly lower levels of *Prevotellaceae* in the fecal samples from PD patients compared to controls. Furthermore, scientists revealed a positive correlation between the Enterobacteriaceae abundance and postural instability and gait difficulty in PD patients (**Scheperjans et al., 2015**). Another study put on the light that anti-inflammatory and butyrate-producing bacteria such as *Blautia*, *Coprococcus* and *Roseburia*, were depleted in PD patients, while *Oscillospira* and *Bacteroides* were significantly increased (**Keshavarzian et al., 2015**). Moreover, another study demonstrated that alteration of the gut microbiota was accompanied with a decrease of SCFAs levels in PD patients (**Unger et al., 2016**). Recently, by using a microbiome-wide association study (MWAS) with two large datasets of internal replication, a literature review highlighted an overgrowth of opportunistic *Corynebacterium* pathogens species in the gut microbiome of PD patients (**Wallen et al., 2021**).

#### I.B.5.4. Multiple sclerosis (MS)

MS is an immune-mediated and neurodegenerative disease that affects the CNS. Key pathological characteristics of MS are demyelination and axonal loss resulting in characteristic lesions in the brain and spinal cord (**Lubetzki et al., 2020**). The alteration on BBB allows infiltration of a wide range of immune cells to the CNS and is considered as the cause of demyelination (**Schepici et al., 2019**).

Despite of several studies that have identified the risk factors contributing to the development of MS including genetics, environmental factors, stress, vitamin D deficiency, Epstein-Barr virus and smoking (**Kasper et al., 2019**), the scientists have not been able to elucidate the real cause of MS. Recently, the gut microbiota draw attention as a potential risk factor of MS (**Freedman et al., 2018; Kirby and Ochoa-Repáraz, 2018; Schepici et al.,**

**2019).** Calvo-Barreiro et al. found that gut microbiota is implicated in modulation of the host's immune system, the BBB integrity and function, initiation of the autoimmune demyelination and the interactions with different types of CNS cell (**Calvo-Barreiro et al., 2018**). In addition, two significant studies support the role of the gut microbiota in the inflammatory response linked with MS disease (**Berer et al., 2017; Cekanaviciute et al., 2017**).

Moreover, several studies have observed differences in the gut microbiota profiles of MS patients compared to healthy controls. Jangi and colleagues reported the increased abundance of *Methanobrevibacter*, *Akkermansia* and the decrease of *Butyrivimonas* in MS patients. These authors noted also that *Collinsella*, *Sutterella* and *Slackia* were decreased in patients on disease-modifying therapy compared to untreated patients. In the following study, they found variations in the expression of genes involved in dendritic cell maturation, interferon signalling and NF-kB signalling pathways in circulating T-cells and monocytes (**Jangi et al., 2016**). A study conducted with the gut of patients with relapsing-remitting MS (RRMS), has detected increased levels of *Pseudomonas*, *Haemophilus*, *Blautia*, and *Dorea*, while healthy controls showed increased abundance of the genera *Parabacteroides*, *Adlercreutzia* and *Prevotella* (**Chen et al., 2016**). Another investigation on RRMS patients showed a decrease in the abundance of *Parabacteriodes distasonis* (Cekanaviciute et al., 2017). Interestingly, comparing the microbiome between treatment-naïve MS subjects through ethnicities including African Americans (AA), Hispanics (HA), and Caucasians (CA) scientists identified the increased abundance of the same genus among them *Clostridium* (**Ventura et al., 2019**).

However, further studies should be carried out in order to explore other aspects in addition to the correlation between microbiota and brain diseases. A fully picture of mechanism of how microbiomes could drive brain diseases will help scientists to develop therapeutical strategies to target brain diseases *via* gut microbiota (**Willyard, 2021**).

## **I.C. Polyphenols-gut microbiota interactions synergistically alleviate brain-related disorders**

Targeted therapy towards Central Nervous System (CNS) related disorders remains an ongoing challenge for the scientific community, and an area to be explored despite the existing treatments for the CNS related disorders (**Danon et al., 2019**). Over the years, the gut microbiota has received increasing attention through a large number of scientific articles, deciphering the complex and bidirectional communication between brain and gut, including neuronal, endocrine, immune and metabolic pathways (**Cryan et al., 2019b**). In addition, intriguing relationships and interactions between polyphenols and gut microbiota have been established so far (**Cardona et al., 2013; Cortés-Martín et al., 2020**). Thus, these complicated relationships brought about the idea of investigating the way by which polyphenols-gut-microbiota interactions could synergistically alleviate CNS related diseases (**Westfall and Pasinetti, 2019; Zhou et al., 2020**).

Therefore, the well-known mechanism of action of polyphenols, nowadays is not only limited to their antioxidant properties and their ability to modulate signalling pathways as was widely discussed (**Bucciantini et al., 2021; Crozier et al., 2009; Gebicki and Nauser, 2021; Silva and Pogačnik, 2020; Zhang and Tsao, 2016**). A growing number of data support the potential effects of polyphenols on gut-brain axis as targeting strategies to alleviate CNS related disorders via different mechanisms of actions (**Di et al., 2019a; Filosa et al., 2018a; Serra et al., 2018**). Firstly, a part of polyphenols, which is subjected to gut biotransformation, generates new metabolites which might reach and act into the brain (**Carregosa et al., 2019b**) or exert their activity in colon, thus contributing to the regulation of gut microbiota composition and metabolism, as well (**Iino et al., 2019; Parkar et al., 2013**). Secondly, polyphenols may modulate gut-microbiota diversity and functions, subsequently improving gut homeostasis and thereby contributing to attenuate CNS related diseases (**Di Meo et al., 2018**). Finally, by considering dynamic and bidirectional communication between gut and brain, polyphenols may improve health by modulating the well-established pathways such as immune, endocrine, metabolic and neuronal ones (**Leclerc et al., 2021**).

### I.C.1. Polyphenols-gut microbiota reciprocal interactions

Even though polyphenols are very abundant in the diet, their bioavailability is relatively low. Indeed, they are poorly absorbed in the stomach and small intestine in their native form, since they are conjugated to sugars or organic acids (**Manach et al., 2004**). Therefore, a significant amount of polyphenols reaches the colon. Once there, they are subjected to extensive biotransformation by gut microbiota, where the principal biotransformation reactions are hydrolysis (O-deglycosylations and ester hydrolysis), cleavage (C-ring cleavage, delactonization, demethylation), and reduction (dehydroxylation and double-bond reduction) reactions (Figure 16) (**Espín et al., 2017**). Moreover, studies have identified the specific enzymatic machinery of some bacterial species that is involved in the polyphenol's bioconversion into their low-weight metabolites (**Aura, 2008**). Firstly, microbial enzymes of the gut (e.g.,  $\alpha$ -rhamnosidase,  $\beta$ -glucuronidase,  $\beta$ -glucosidase, sulfatase, and esterases) can hydrolyse polyphenols to their corresponding aglycone form (**Aura, 2008**). For example, chlorogenic acid is biotransformed into caffeic acid (CA) and quinic acid by bacterial ester hydrolysis (**Tomas-Barberan et al., 2014**). *Lactobacillus*, *Bifidobacterium*, *Bacteroides*, *Enterococcus*, and *Enterobacter* strains have been reported to have rhamnosidase activity that leads to flavonoid O-rhamnosyl deconjugation and formation of hesperidin. The lignan secoisolariciresinol di-O-glucoside, is only hydrolyzed by some *Lactobacillus* and *Bifidobacterium* species, releasing secoisolariciresinol. The release of soy-derived polyphenol, isoflavone daidzein, leading to its glycoside form is promoted by elected probiotic strains of *Bifidobacterium*. The released aglycone form is readily absorbed into the circulatory system and/or further metabolized by the anaerobic gut microbiota into S-equol (Raimondi et al., 2009). Another example is the hydrolysis of the ester bonds of ellagitannins into hexahydroxydiphenic acid (HHDP) and glucose, by *Escherichia coli* through  $\beta$ -glucuronidase (**Tomás-Barberán and Espín, 2019**). These metabolites further undergo to reactions such as C-ring cleavage (Daidzein can lead to O-desmethylangolensin by a ring-cleavage reaction), delactonization (lactone ring opening and decarboxylation in ellagic acid which results in urolithin by *Gordonibacter urolithinifaciens* and *Gordonibacter pamelaee*, (**Selma et al., 2017**)) and demethylation. Other reactions mediated by gut bacteria are the hydrogenation of double bonds, carbonyl reductions, and the removal of hydroxyl groups (dehydroxylation)( see

Figure 16) (Espín et al., 2017). Subsequently, these biotransformation products undergo the Phase I and Phase II biotransformation and enter in the enterohepatic circulation, then reach tissues and brain where they exert their biological effects (Marhuenda-Muñoz et al., 2019).

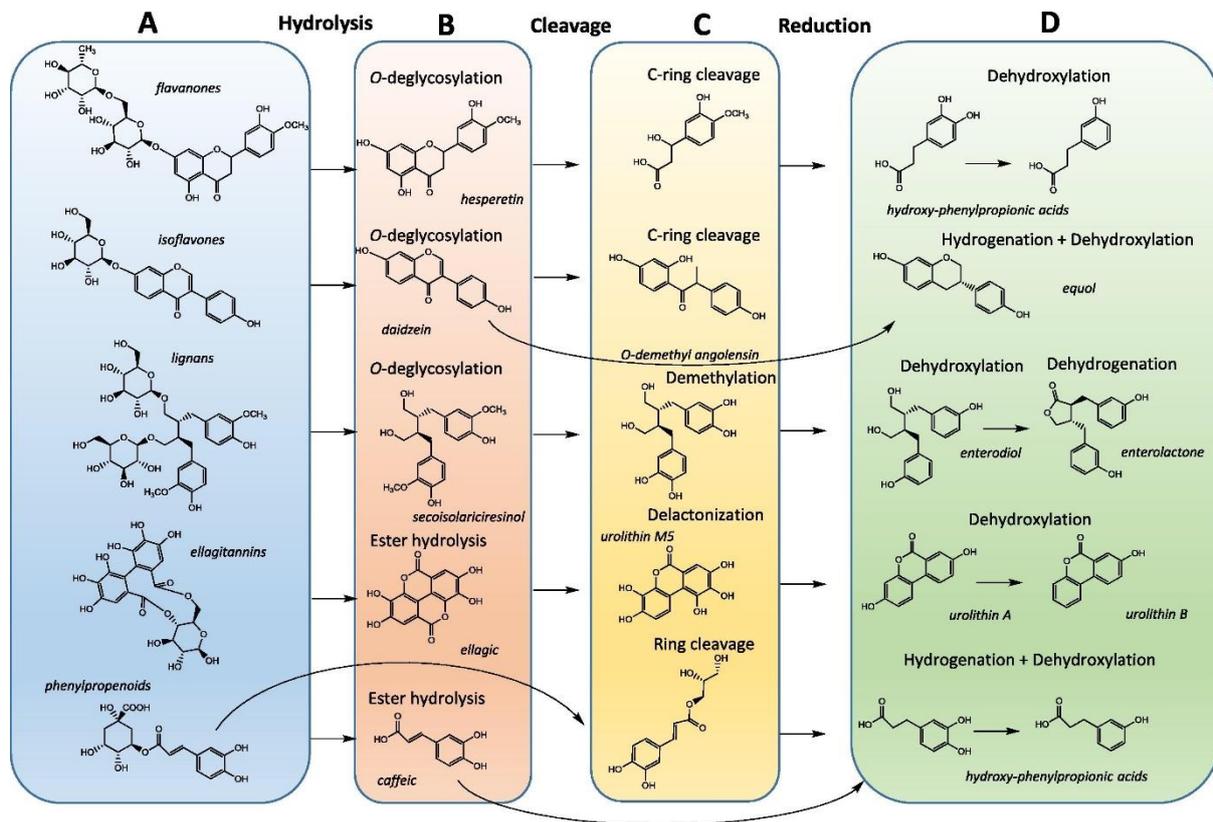


Figure 16. The illustration of gut microbiota biotransformation reactions of some of the polyphenols including: **Flavanones** (Hesperidin;Citrus), **isoflavones** (Daidzin;soy); **lignans** (secoisolariciresinol diglucoside; flaxseed); **ellagitannins** (hexahydroxydiphenic glucoside; nuts and berries); **phenylpropanoids** (chlorogenic acid; coffee). **A**) in their native form; **B**: Hydrolysis reactions mediated by gut microbiota; **C**: Ring cleavage and demethylation; **D**: Reduction reactions (dehydroxylation and hydrogenation) ( Espín et al., 2017)

### I.C.1.1. The positive effects of low weight metabolites generated by gut microbiota on brain functions

As we discussed previously, studies over the years have shown the impact of polyphenols on neuroprotection including cognitive function and neurodegenerative diseases (**Di Meo et al., 2020; Yang et al., 2021**). To exert such kinds of effects polyphenols is needed to reach the brain (**Figueira et al., 2017**). A limited number of parent polyphenols have shown ability to be presented in the brain ((-) epicatechin, hesperidin, naringenin, quercetin, and anthocyanins), since they have limitations due to poor absorption, extensive metabolism, rapid elimination, and limited ability to cross BBB (**Carregosa et al., 2019b**).

However, gut microbiota generates various low-weight metabolites, considered to be present in the circulatory system at higher concentrations (10–30  $\mu$ M) compared to parent compounds, and even more, being more able to potentially reach the brain (**Carregosa et al., 2019b**). Moreover, it has been shown that these metabolites persist in the human body with significant concentrations for longer period, up to 3-4 days after the intake (**González-Sarrías et al., 2017a**). Thus, nowadays, further insights are focused on biological effects of polyphenols gut-derived metabolites, making them as promising approach to prevent and attenuate neurodegenerative diseases (**Zhou et al., 2020**).

Indeed, there is body of evidence, including studies and reviews in the field, showing that such metabolites can exhibit biological activities including antioxidant, anti-inflammatory and anticarcinogenic properties (**Serreli and Deiana, 2019**). For example, Luca et al recently explored the biological activity of metabolites of resveratrol, curcumin, quercetin and rutin, genistein and daidzein, ellagitannins, proanthocyanins, which strongly support the fact that gut-derived metabolites are contributors of health benefits attributed to polyphenols (**Luca et al., 2020**). Moreover, such metabolites have shown ability to reach brain and there exert various biological effects (**Carecho et al., 2020**).

Researchers investigated neuroprotective properties of gut-derived polyphenols metabolites, mainly using different neuronal cell models. In a neuronal model study, gallic acid, ellagic acid, urolithins and 3,4-dihydroxyphenylpropionic acid, 3,4-

dihydroxyphenylacetic acid, exhibited neuroprotective activity *via* decreasing oxidative stress (González-Sarriás et al., 2017b). Similarly, Esteban-Fernández et al., investigated the neuroprotective effects of wine-derived gut metabolites, including 3,4-dihydroxyphenylacetic (3,4-DHPA), 3-hydroxyphenylacetic acids and salicylic  $\beta$ -D-O-glucuronide. They found that metabolites increased cell viability, by inhibiting neuronal MAPK p38 and ERK1/2, and reduced caspase 3 activity (Esteban-Fernández et al., 2017).

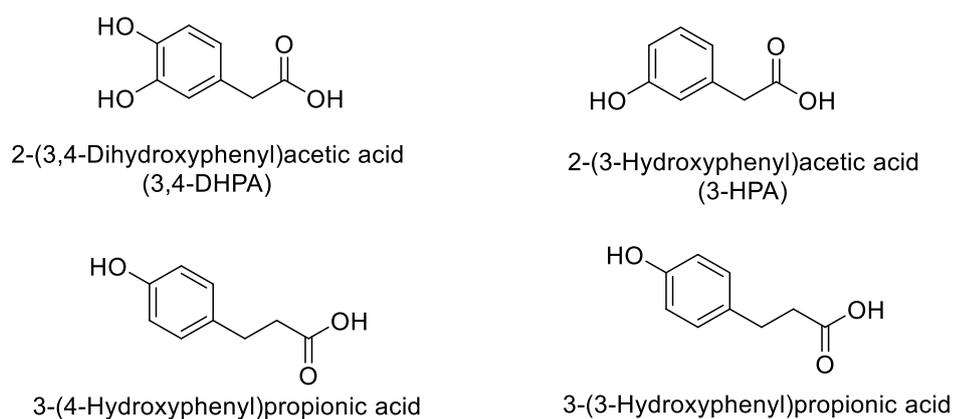


Figure 17. Structures of phenylacetic acids and phenylpropionic acids, evaluated for their neuroprotective effects

Benzene diols and triols and their conjugates (Figure 18) have been established as attenuators of neuroinflammation, by studies performed on microglia cells (Zheng et al., 2008). They are low-molecular weight compounds, and some are present in specific foods, e.g., catechol, guaiacol, pyrogallol, and 4-methylcatechol occurring in coffee, beer and cocoa.

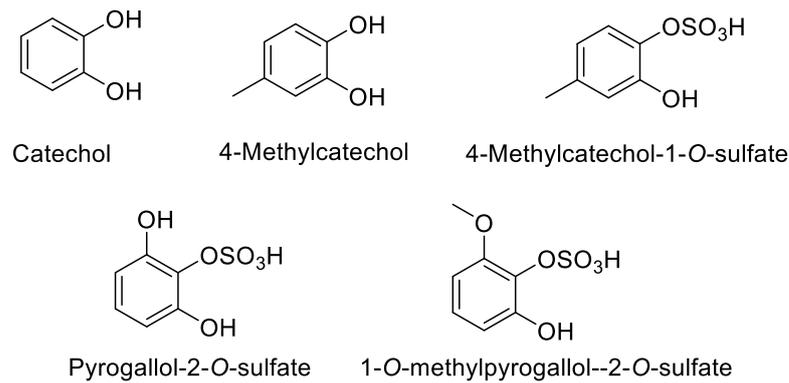


Figure 18. Structures of benzene diols and triols, evaluated for their neuroprotective effects on microglia cells.

In the same line, enterolactone, a gut microbial metabolite of lignans and equol derived from isoflavones, have shown anti-neuroinflammatory activity against microglia cells, by reducing the secretion of proinflammatory cytokines (IL-6 and TNF- $\alpha$ ), and decreasing nitric oxide (NO) production. These biological activities were not detected among parent compounds tested, such as daidzein and secoisolariciresinol (**Johnson et al., 2019**). The same group of authors has shown neuroprotective effects of equol against neurotoxins induced toxicity in human neuroblastoma, as well (**Johnson et al., 2020**).

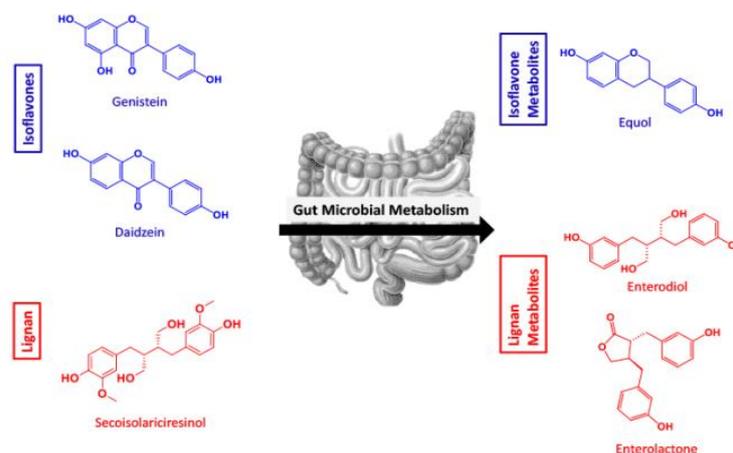


Figure 19. Illustration of the polyphenols, parents compounds; isoflavones (Genistein and daidzein) and lignan (secoisolariciresinol) and their gut-derived metabolites (equol and enterodiol) (Johnson et al., 2019).

Zhao et al., relying on preclinical/clinical studies, summarized the role of grape polyphenols on neuroprotection (**D. Zhao et al., 2019**). Likewise, the neuroprotective activity of curcumin (from turmeric) and its microbial metabolites has been recently explored as well. Tetrahydrocurcumin, demethoxycurcumin, octahydrocurcumin, bisdemethoxycurcumin, curcumin microbial metabolites, have been reported to be effective against neurodegenerative diseases *via* different modes of action (**Di et al., 2019b**). In Neuro-2a cells study, urolithin A, a gut-derived metabolite of ellagic acid and ellagitannins, has shown neuroprotective properties, mediated by cytoprotective properties, inhibition of oxidases (Monoamine Oxidase A and Tyrosinase) and action as a direct free radical scavenger (**Cásedas et al., 2020**).

Moreover, studies have shown the role of polyphenols on improving memory and cognitive function by modulating synaptic plasticity as well (**Yang et al., 2021**). For example, administration doses at 5–180 mg/kg of Grape-derived polyphenol has been shown to normalize brain-derived neurotrophic factor (BDNF) levels and reduce inflammation, in turn ameliorating depressive behaviour (**Caracci et al., 2019**). Grape-derived bioactive dietary polyphenol preparation (BDPP) supplementation was proven to improve cognitive damage in a sleep deprivation (SD) induced model. (**Frolinger et al., 2019**). Recently, a randomized study in middle-age and older subjects, demonstrated the favourable effects of daily consumption of catechins through green tea on cognitive functions (**Baba et al., 2020**).

Furthermore, studies have also shown the capacity of polyphenols metabolites to interfere with misfolded proteins, implicated as one of the main pathological mechanisms of brain diseases, including PD and AD. Ho et al, in an *in vitro* model, observed the ability of three phenolic acids, such are 3-hydroxybenzoic acid (3-HBA), 3,4-dihydroxybenzoic acid (3,4-diHBA) and 3-(3-hydroxyphenyl)propionic acid –(3-HPPA) to reach the brain among 15 biologically available flavanol-rich preparation (FRP)-derived metabolites (**Ho et al., 2019**). This data supports the evidence how the heterogenicity of gut microbiota may affect the generation of specific metabolites, thereby positively contributing or not to neurological conditions. In addition, the same team has demonstrated that the aforementioned phenolic metabolites attenuate seeding and spreading of  $\alpha$ -syn in a drosophila model of  $\alpha$ -synucleinopathy (**Yamasaki et al., 2020**).

Therefore, as polyphenols gut-derived metabolites are attenuators of neuroinflammation, studies should put them in the spotlight of investigations, since they could represent a promising strategy to positively modulate gut-brain axis, thus alleviating brain disorders.

#### I.C.2. Effects of polyphenols on growth and activity of gut bacteria: leading to positive impact on CNS related disorders

In fact, the scientific evidence put in the spotlight the existence of two kinds of interactions between polyphenols and microbiota (**Tomás-Barberán et al., 2016**). In one side, gut microbiota can extensively metabolize polyphenols into absorbable and often, potentially more bioactive phenolic metabolites than the parent compounds (**Carregosa et al., 2019b; Pasinetti et al., 2018a**). In another side, polyphenols can modulate the gut microbiota growth and activity (**Ozidal et al., 2016**).

The beneficial effects of polyphenols on the gut microbiota are attributed to their capacity to shape gut microbiota either by promoting the growth of beneficial bacteria or inhibiting potentially pathogens, which in turn positively impact human health (**Kumar Singh et al., 2019; Mithul Aravind et al., 2021**). Several significant investigations have shown the impact of polyphenols and their gut-derived metabolites on both, microbiota composition and its metabolic activity (**Firrman et al., 2018; Gwiazdowska et al., 2015; Henning et al., 2017; Pacheco-Ordaz et al., 2018; Sorrenti et al., 2020**).

##### I.C.2.1. Polyphenols acting as prebiotics

According to the International Scientific Association for Probiotics and Prebiotics consensus statement (ISAPP) the definition of prebiotics has been updated to ‘a substrate that is selectively utilized by host microorganisms conferring a health benefit’. This extension currently goes beyond non-digestible oligosaccharides concept, including polyphenols which have already exhibited potential prebiotic activity. However, it should be stressed that this

criterion excludes antibiotics, bacteriostatics, minerals and vitamins since they are not growth substrates, even though they might beneficially impact microbiota (**Gibson et al., 2017**).

The health effects of polyphenols as prebiotics are related to their capacity to promote commensal bacteria through several mechanisms of action. Polyphenols may facilitate electron transfer, thus provide carbon-sources, and contribute to the bacterial energy metabolism (**Hsueh et al., 2019**). In addition, they can act as electron acceptor, in turn ensuring the balance homeostasis (**Rodríguez-Daza et al., 2020**). Indeed, it is essential to keep a balance of intestinal redox state. The overproduction of ROS induces inflammatory responses and increases cell oxidative stress, which triggers a compromised barrier integrity, and consequently the invasion of pathogens. (**Campbell and Colgan, 2019**). Moreover, some studies demonstrated that increased redox status could drive changes of the gut microbiota community (**Million et al., 2016; Yardeni et al., 2019**). For example, Million et al. found a decrease of specifically anaerobic species of *Bacteroidaceae*, *Eubacteriaceae*, *Lachnospiraceae* and *Ruminococceae* families, whereas the prevalence of *Enterococcus faecalis*, *Escherichia coli* and *Staphylococcus aureus* were significantly increased (**Million et al., 2016**). Polyphenols can, in this context, directly neutralize free radicals, reactive oxygen species (ROS) and reactive nitrogen species (**Zhang and Tsao, 2016**), subsequently, improving the redox homeostasis of gut environment. For instance, in a high-fat diet model, sinapinic acid and resveratrol have demonstrated positive impact in the diversity of gut microbiota, through the suppression of oxidative stress (**C. Yang et al., 2019**). Another study, in a high-fat diet model, highlighted the effects of tea polyphenols in the alleviation of hyperlipemia, and those effects are potentially attributed to the maintaining of the intestinal redox system. Furthermore, scientists have observed that the presence of *Lachnospiraceae*, *Bacteroides*, *Alistipes*, and *Faecalibaculum* species was linked to oxidative stress (**Ma et al., 2019**).

Furthermore, prebiotic activity of polyphenols should be also attributed to the generation of gut-derived metabolites. Gut biotransformation can provide different polyphenols derived metabolites which either might have direct effects on gut microbiota or might be used by enzyme-producing bacteria in a cross-feeding process to improve their presence in the gut. Interestingly, a study with incubation of four polyphenols with fecal

microbiota showed that the growth of *Bifidobacterium* was only stimulated by biotransformed metabolites but not by the native polyphenols (Parkar et al., 2013). We can cite a study where urolithin A, as a gut-derived metabolite of ellagic acid, has stimulated the growth of *Lactobacilli*, *Bifidobacteria* and *Enterobacteria* in a rat model study (Larrosa et al., 2010). In addition, a randomized clinical trial showed that changes of gut microbiota composition after red wine and dealcoholized red wine intake were positively correlated with their gut derived metabolites such are syringic, *p*-coumaric, 4-hydroxybenzoic and homovanillic acids (all anthocyanin metabolites) (Iino et al., 2019).

Some *in vitro* reports demonstrated the beneficial effects on gut microbiota by polyphenols or polyphenols-rich foods/extracts. (Firman et al., 2018; Gwiazdowska et al., 2015; Hidalgo et al., 2012; Lee et al., 2006; Parkar et al., 2013; Sánchez-Patán et al., 2015; Tabasco et al., 2011; Tzounis et al., 2008). The consistent scientific evidence was also based on *in vivo* models and human clinical trials, where polyphenols were supplied in the diet as pure compounds or polyphenols-rich extracts. These studies shed more light on how polyphenols may improve health parameters, by modulating gut microbiota profile (Anhe et al., 2015; Guirro et al., 2020; López et al., 2018; Masumoto et al., 2016; Van Hul et al., 2018; Vendrame et al., 2011; L. Wang et al., 2018).

Some other examples of clinical trials supporting the evidence that consumption of polyphenols and rich- polyphenols foods are associated with the modulation of gut microbiota are presented in the following paragraph. More recently, Iino et al., (2019) observed that daidzein intake among Asian people (n = 1044), in the city of Hirosaki, increased the prevalence bacteria that are responsible of daidzein biotransformation to equol, such are *Asaccharobacter celatus* and *Slackia isoflavoniconvertens* (Iino et al., 2019). Tea polyphenols consumption was reported to increase portion of *Bifidobacterium* in a study with volunteers which were not green tea consumers (Jin et al., 2012). Likewise, Cocoa-derived polyphenols significantly increased *Bifidobacterium* spp. and *Lactobacillus* spp. abundance (Tzounis et al., 2011). In a same manner, moderate consumption of red wine was found to significantly increase the abundance of *Enterococcus*, *Prevotella*, *Bacteroides*, *Bifidobacterium* spp., and *Blautia coccoides*—an Eubacterium (Queipo-Ortuño et al., 2012). Another study dealing with the metabolic syndrome in obese patients related the stimulation effects of red wine

consumption. In this study, authors detected the increase of fecal abundance of *Bifidobacteria* and *Lactobacillus*, *Faecalibacterium prausnitzii* and *Roseburia*, while *Escherichia coli* and *Enterobacter cloacae* were reduced (Moreno-Indias et al., 2016). Another controlled clinical trial demonstrated the same tendency with the supplementation of orange juice in diet, which modulated the gut microbiota, including by the increase of *Lactobacillus* spp. and *Akkermansia* spp. (Fidélis et al., 2020). Overall, these studies have shown the capacity of polyphenols to beneficially modify gut microbiota mainly by promoting the growth of commensal bacteria such as *Lactobacillus*, *Bifidobacterium*, *Akkermansia*, *Faecalibacterium*, *Roseburia*. In addition some specific bacteria were modulated as well, including *Blautia*, *Eggerthella lenta*, *Clostridium coccooides*, *Enterobacter cloacae*, *Asaccharobacter celatus* and *Slackia isoflavoniconvertens*.

Beneficial effects of polyphenols can be also related to their ability to shape gut microbiota in favour to other specific gut microbial species that can provide health benefits. For instance, *Lactobacillus* and *Bifidobacterium* are known for their contribution in gut barrier function, suppressing proinflammatory cytokines and even inhibiting pathogenic bacteria (Azad et al., 2018). The increased abundance of *Roseburia*, known as butyrate producing bacteria, led to the maintenance of gut health and immune function as well (Tamanai-Shacoori et al., 2017). *Akkermansia muciniphila* is specialized in mucin degradation, making this bacterium an important contributor to maintain intestinal mucosal barrier function (Zhang et al., 2019; Zhou and Zhang, 2019). In the same way, *Faecalibacterium prausnitzii*, known as immunomodulatory bacteria, inhibits inflammation by modulating inflammatory cytokines and interleukins (He et al., 2021; Touch et al., 2022). Furthermore, the promoting of commensal bacteria in turn provides suppression of the growth of opportunistic bacteria, leading to the maintain of the gut homeostasis (Y. Guo et al., 2020).

#### I.C.2.2. Polyphenols acting as antimicrobial agents

Another means to regulate gut microbiota composition is to limit the number of pathogenic bacteria and polyphenols have been extensively studied as antimicrobial agents against several known pathogenic bacteria (Marín et al., 2015). *Escherichia coli*, *Helicobacter*

*pylori*, *Klebsiella pneumonia*, *Staphylococcus aureus*, *Listeria monocytogenes*, *Salmonella typhimurium*, *Pseudomonas aeruginosa* were significantly inhibited by polyphenols. A human study demonstrated that curcumin and turmeric decreased *Blautia* spp. and *Ruminococcus* spp. in the gastrointestinal tract (Peterson et al., 2018). Han et al. demonstrated that the strawberry decreased the levels of opportunistic bacteria such as *Dorea* and *Bilophila*, in a colitis induced model (Han et al., 2019). *Enterococcus caccae* was drastically inhibited by a flavanone naringenin treatment (Firrman et al., 2018). In another example, *Salmonella typhimurium*, *Listeria monocytogenes* and *Staphylococcus aureus* were inhibited by anthocyanins, flavan-3-ols, B-type proanthocyanidins, and ellagitannins, as phenolic extracts obtained from eight berries (Kawabata et al., 2019). A more recent systematic review gathered all the evidence on the antimicrobial activity of different classes of plant polyphenols against resistant and non-resistant Gram-positive bacteria. Scientists concluded that the significant antimicrobial effect of both, polyphenols and mixtures, was achieved at low  $\mu\text{g/mL}$  range of Minimal Inhibitory Concentration (MIC) values (Álvarez-Martínez et al., 2020).

Recently, a study based on Reliable Quantitative Structure-Activity Relationship (QSAR) models has investigated the antimicrobial activity of 35 dietary polyphenols against *S. aureus*, *B. subtilis*, *L. monocytogenes*, *E. coli*, *P. aeruginosa* and *Salmonella enteritidis*, and it was proposed a model for the prediction of their activity (Bouarab-Chibane et al., 2019).

The antimicrobial activity of polyphenols is not fully elucidated and requires further investigations. Nevertheless, studies have shown that different mechanisms of action potentially may be involved. The antibacterial activity of polyphenols could be mediated through their ability to form complexes with proteins involving nonspecific interactions such as hydrogen bonding and hydrophobic effects, as well as by covalent bond formation (Makarewicz et al., 2021). For example, kaempferol-3-rutinoside was demonstrated to inhibit *Streptococcus mutans* by affecting sortase A, a membrane enzyme that actively plays a crucial role in bacteria adhesion and the invasion of host cells (Kang et al., 2006). Furthermore, polyphenols may interact with both, bacterial cell wall and cell membrane and alternate their function (Makarewicz et al., 2021). For example, a study has shown that epigallocatechin gallate disrupts *E. coli* cell membrane (Nie et al., 2018). Moreover, polyphenols can influence gene expression regulation. They were found to disrupt DNA, by inhibiting both energy

metabolism and DNA synthesis (**Makarewicz et al., 2021**). In addition, they may act through inhibiting virulence factors. Incubation for 20 h of Cranberry pomace and the flavonol-rich fraction showed bactericidal effects against *Salmonella* strains, by reducing the expression of virulence genes and cell wall/membrane biogenesis (**Makarewicz et al., 2021**). Moreover polyphenols have shown inhibitory effects to biofilm formation through interfering with bacterial quorum sensing (**Matilla-Cuenca et al., 2020**).

This antimicrobial effect of polyphenols suggests that a diet enriched with polyphenols compounds could help to maintain or even restore gut homeostasis. Most of above-mentioned bacteria inhabited by polyphenols, are linked with the production of inflammatory cytokines. For example *Salmonella typhimurium* and *C. difficile*, causes their production (**Lombardi et al., 2018**). Moreover, as discussed previously, their abundance has been observed in brain diseases; for example, *Pseudomonas aeruginosa* with MS (**Chen et al., 2016**), *Escherichia* with anxiety (**Jiang et al., 2018**), *Prevotella* and *Klebsiella* species with MD (**Lin et al., 2017**), *Corynebacterium* spp with PD (**Wallen et al., 2021**).

### I.C.2.3. Polyphenols and modulation of host metabolism

In addition to the effects on gut composition, polyphenols and their gut-derived metabolites can shape the gut microbiota, indirectly, by affecting host metabolism. By contributing to the production of beneficial metabolites, they greatly affect the host health. *In vitro* studies have demonstrated the impact of polyphenols including 5-CQA, rutin, caffeic acid, quercetin or olive oil on the production of (SCFAs) (**Mosele et al., 2015**). SCFAs production leads to a further increase of beneficial bacteria, and therefore contributes to intestinal homeostasis. The role of SCFAs, in host health was discussed above and the role of polyphenols in their production will be described in the supplementation study.

#### I.C.2.4. Polyphenols and modulation of gut composition in brain diseases

Several studies have elegantly addressed the favourable biological effects of polyphenols in several metabolic diseases through the modulation of the composition of gut bacteria (Cheng et al., 2019; Li et al., 2019; Ushiroda et al., 2019; Wang et al., 2019; Yan et al., 2020; Yang et al., 2019).

A study with high-fat induced diet provides the evidence on how anthocyanidin extracts by modulating gut microbiota may improve neurologic complications of obesity patients (Marques et al., 2018). In a rat model study, Song and colleagues have shown that 5-CQA could significantly ameliorate depressive-like behaviours by modulating the abundance of specific bacteria. This study showed that treatment with 5-CQA decreased the abundance of *Desulfovibrionales*, *Desulfovibrio*, *Klebsiella*, *Burkholderiales* and increased *Bifidobacterium* in gut microflora (Song et al., 2019).

However, studies in the field of brain are scarce and further research should be encouraged to give more information on how reshaping gut microbiota through dietary polyphenols may provide positive effects in mental health and brain-related disorders.



# Chapter II



## II.A. Electrochemical studies on 5-CQA and CA

Our first objective was to identify the oxidized compounds arising from electrochemical and enzymatic oxidations of 5-CQA and its major gut- derived metabolites CA. Moreover, the generated MS/MS profile will help us to look for possible oxidized compounds occurring by polyphenols-gut microbiota interactions.

### II.A.1. Study of the electron transfer properties of phenolic compounds

Remarkably, polyphenols have drawn attention for their antioxidant properties as described above. Such compounds have shown activity towards inhibition of reactive oxygen/nitrogen species, and modulation of redox signalling pathways (**Bucciantini et al., 2021; Crozier et al., 2009; Hussain et al., 2016**). The antioxidant process is a complex system that involves electron, proton and radical transfers, among other modes of actions. The reactivity mechanisms brought into play by polyphenols are difficult to observe at the cell level. Thus, many methods for determining *in vitro* antioxidant capacity have been developed (**Apak, 2019**). Most of the tests developed are not close to physiological conditions. Nevertheless, even if the values obtained depend on the analysis method and conditions (solvent used, radical studied...) and cannot be extrapolated to the living environment, these *in vitro* tests have the advantage of highlighting the capacity of a molecule to participate in a radical and/or electron transfer mechanism.

Therefore, various *in vitro* methods have been applied to evaluate the antioxidant activities of these compounds (**Gulcin, 2020; Magalhães et al., 2008; Prior et al., 2005**). Some methods are based on radical hydrogen atom transfer mechanisms (HAT methods), while others on single electron transfer mechanisms (SET methods). Both types of reactivity can lead to identical reaction products but involve different reaction intermediates. The HAT mechanism of antioxidant relay on ability to transfer hydrogen atom (H) to a peroxy radical (Eq 1). It should be noted that a HAT process refers to simultaneous proton and electron transfer in the same atomic orbital. It also exists a "sub-HAT" mechanism, the Proton

Concerted Electron Transfer (PCET) mechanism in which the simultaneous proton and electron transfer takes place in two different atomic orbitals (**Apak et al., 2016; Meyer et al., 2007**). Mainly, the HAT-based tests are the Oxygen Radical Absorption Capacity (ORAC), Total Peroxyl Radical Trapping Antioxidant Parameter (TRAP) and Total Oxyradical Scavenging Capacity (TOSC) assays (**Munteanu and Apetrei, 2021**).

#### **HAT (H-atom transfer)**



#### **SET – PT (Sequential electron transfer-proton transfer)**



#### **SPLET (sequential proton loss–electron transfer)**



The SET mechanism leads to a reduction of the oxidized substrate in two steps: a first electron transfer step forms the cation radical  $\text{ArOH}^{\bullet+}$  (Eq 2). However, this reaction mechanism is unlikely between phenolic compounds and radical oxygen species (ROS) due to the high instability of the  $\text{ArOH}^{\bullet+}$  cation radical (**Di Meo et al., 2013**).

A third process involves the simultaneous transfer of an electron and a proton SPLET (Sequential Proton-Loss Electron Transfer) (Eq 3). This mechanism was notably observed during the study of the trapping of the DPPH<sup>•</sup> radical. The balance will be the same as during the HAT hydrogen atom transfer, but the sequential process involving the phenolate anion favors higher rate constants, because the  $\text{ArO}^-$  phenolate is a better electron donor than phenol  $\text{ArOH}$  (**Muñoz-Rugeles and Alvarez-Idaboy, 2015**). This process is particularly interesting

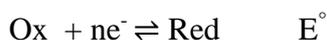
in physiological conditions. Indeed, at the physiological pH, due to their pKa range, the phenolic compounds lead to a non-negligible portion of phenolate forms which promote the transfer of electrons (**Dangles et al., 2016; Di Meo et al., 2013**).

It should be noted that the predominant mechanism for a given system depends on the structure, the properties of the antioxidant, its solubility in the solvent used and the study parameters (pH, temperature, concentration, and ratio antioxidant/ oxidant). Several studies have shown a difference in the antioxidant capacity of the compounds studied depending on the radical used (**Tabart et al., 2009**). Therefore, measured antioxidant capacity should always refer to the study context, as total antioxidant capacity may differ from antioxidant properties in biological systems. However, the diversity of antioxidant tests and radicals used, combined with a lack of standardization of these tests, makes it difficult to compare the results obtained between the different studies and the different compounds.

Electrochemistry commonly is a useful method which provide interesting elements, in terms of redox potential and also on the mechanisms by which the phenolic compounds could be oxidized. Hence, the oxidation potential values also reflect the antioxidant capacity of the phenolic compounds and have been correlated in some cases with values obtained by other methods. Moreover, they have been described as more accurate than values obtained by the DPPH test (**Arteaga et al., 2012; Gomes et al., 2008**).

#### II.A.1.2. Potential Redox

In the case of an oxidation reduction reaction:



where Ox and Red represent the oxidized and reduced forms, and n the number of exchanged electrons.

When a substance loses an electron, its oxidation state increases; thus, it is oxidized.  
When a substance gains an electron, its oxidation state decreases, thus being reduced.

The Nernst equation for a reversible redox couple is:

$$E = E^{\circ} + \frac{RT}{nF} \ln \frac{a_{ox}}{a_{red}}$$

R perfect gas constant (8.314 J. K<sup>-1</sup>. mol<sup>-1</sup>)  
F Faraday constant (96 485 Coulombs)  
T (K) absolute temperature  
n number of electrons involved

$$E = E^{\circ} + \frac{0.059}{n} \log \frac{a_{ox}}{a_{red}}$$

Expression at 25 °C (298 °K)  
ln = 2.303 x log10

E° (in volts) is the standard potential of the redox system considered, established under standard conditions. with an activity or fugacity (for gases) equal to 1 (a<sub>ox</sub> = a<sub>red</sub> = 1) at 25°C, following the recommendations IUPAC (International Union of Pure and Applied Chemistry).

When we are not in the standard conditions, the formal potential or apparent standard potential E'° is taken into account. This E'° is measured experimentally in solutions containing equal concentrations of the species Ox and Red species. E'° considers the ionic strength, pH, possible complexation and precipitation phenomena involving all the species in the Nernst equation.

$$E = E'^{\circ} + \frac{0.059}{n} \log \frac{[Ox]}{[Red]}$$

In solution, the reducing capacity of a molecule may be evaluated by its redox potential (E<sub>redox</sub>). Indeed, the lower the value of the potential, the lower the energy required to remove

an electron, thus the higher the reducing capacity of the compound. In fact, the redox potential of the couple phenol/phenoxy radical being relatively low, reactions of hydrogen atom transfers and/or rapid electrons with ROS can take place and constitute one of the main antioxidant mechanisms of phenolic compounds (**Cheng et al., 2002**).

The techniques for measuring these potentials are mainly based on the measurement of electron transfers such as pulsed radiolysis or by electrochemical techniques such as cyclic voltammetry (CV) or square wave differential voltammetry (Square Wave Voltammetry, SWV) (**Sochor et al., 2013; Ziyatdinova et al., 2013**). In all cases, the measurement of the potential depends on the medium in which the compounds are studied, on the pH and on the temperature.

#### II.A.1.3. Electrochemistry

Electrochemical techniques such as voltammetry, chronoamperometry and spectroelectrochemistry UV visible, are considered powerful analytical tools to study electron transfer reactions (**Sochor et al., 2013**). They can help to understand the reactivity *in vivo* of molecules with redox activity by studying the processes triggered by electron transfer, accessing certain thermodynamic (redox potential) and kinetic parameters and by creating physiological radicals (e.g.,  $O_2^{\bullet-}$ ) directly at the electrode (unlike the tests mentioned above such as DPPH), thus, allowing the analysis of the radicophilic properties of the substrate. Polyphenols being electroactive species, the study of their oxidation mechanism by the use of electrochemical tools is rapid, sensitive, easy to implement, reproducible, and does not use radicals, the electrode serving as a oxidant (**Cheng et al., 2002**).

#### ***Principles***

An electrochemical reaction is defined as a heterogeneous electron transfer occurring between an electrode and a species in solution. The electrochemical reaction occurs at the electrode when it is brought to a potential different from its equilibrium potential (overpotential

$\eta$ ). This overpotential can be related to the apparent normal potential by Nernst's law (see equation below). The redox system will then evolve towards a new equilibrium state by changing the concentrations of oxidant ( $C^{\text{sol}}_{\text{ox}}$ ) and reductant ( $C^{\text{sol}}_{\text{red}}$ ).

$$\eta = E - E^{\circ} - \frac{RT}{nF} \ln \frac{C^{\text{sol}}_{\text{Ox}}}{C^{\text{sol}}_{\text{Red}}}$$

The electron transfer reactions can be studied by three-electrode techniques (working or indicator electrode, reference electrode, auxiliary electrode) connected to a potentiostat-galvanostat which allows control of the potential (in the working- reference electrode circuit) and current measurement (in the working-auxiliary electrode circuit) (**Sochor et al., 2013**). The direction of the current generated depends on the value of the potential imposed on the working electrode in relation to the reference electrode. The production of an electrochemical reaction follows a quantitative law (Faraday's Law):

$$N_{\text{Ox}} = N_{\text{Red}} = \frac{Q}{nF}$$

N number of moles of oxidant or reductant electrolysed

Q the total electrical charge passed during electrolysis

F = 96485 C mol<sup>-1</sup>, the Faraday constant

n number of moles of electron exchanged

### ***Voltammetry***

In these techniques, voltammograms representing  $I = f(E)$  are generated by a potential sweep by gradually modifying the setpoint voltage controlled by the potentiostat and the measurement of the corresponding electrolysis current. In this system, electrolysis occurs in the low-volume diffusion layer ( $A \cdot \delta(t)$ ). The concentration of the species studied is therefore not modified in solution (Figure 20).

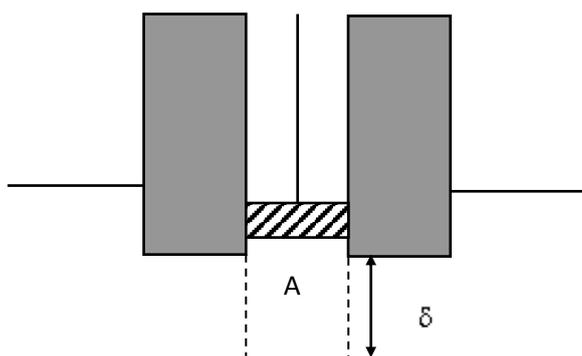


Figure 20. Representation of diffusion layer (A: area of the electrode;  $\delta$  (t): diffusion layer)

During an electrochemical process, different steps are observed: mass transfer (diffusion of the species to the electrode or to the solution), electron transfer between the electroactive species and the electrode, chemical reactions that can take place before or after the electrochemical reaction and the possible adsorption of the electroactive species, or of the product resulting from the reaction, on the electrode surface Figure 21. The latter type of reaction decreases the active surface area of the electrode, which leads to a reduction in charge transfer and measured current, and may also change the surface of the electrode, thus modifying the reaction (**Enache and Oliveira-Brett, 2011**). Electron transfer is rapid if the structural change is small and requires low activation energy (change in the solvation sphere or ionic atmosphere); the redox system is said to be rapid or reversible. The greater the structural modification, the greater the activation energy and the slower the electron transfer (a so-called slow or irreversible system).

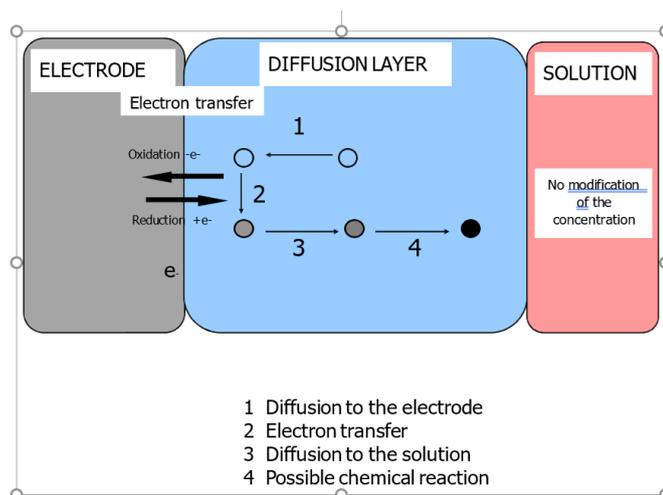


Figure 21. Representative scheme of steps occurring at the surface of an electrode during an electrochemical process

### *Cyclic voltammetry*

Cyclic voltammetry (CV) is widely used for the study of redox system. Indeed, it can provide information about the mechanism of reaction, to access thermodynamics (redox potential) as well as qualitative information, including the stability of these oxidation states and the electron transfer kinetics (Sochor et al., 2013).

In cyclic voltammetry the current is expressed by the Randles-Sevcik equations. For an electrochemical reversible system: the electron transfer kinetics are fast, diffusion is the limiting factor of the electrochemical reaction. The current equation is written as follows:

$$I_p = 0.4463 n^{3/2} F A C v^{1/2} D^{1/2} \frac{F^{1/2}}{(RT)^{1/2}}$$

$$I_p = (2.69 \cdot 10^5) n^{3/2} A D^{1/2} C v^{1/2}$$

A: area of the electrode (cm<sup>2</sup>)

I<sub>p</sub>: peak current intensity (A)

C: concentration of the solution

D: diffusion constant (cm<sup>2</sup>/s)

F: Faraday constant (F = 96484 C.mol<sup>-1</sup>)

v: scan rate (V.s<sup>-1</sup>)

R: perfect gas constant (R=8.31 J.K.mol)

n: number of electrons

For an irreversible system: the electron transfer kinetics are slow, the current is limited by this kinetics. The current equation is written:

$$I_p = 0.4958n(\alpha n_a)^{1/2} FACv^{1/2} D^{1/2} \frac{F^{1/2}}{(RT)^{1/2}}$$

$$\alpha = \frac{1.857RT}{\Delta E_{p/2} F n_a}$$

$$\Delta E_{p/2} = E_{p/2} - E_p$$

n: total number of electrons exchanged  
 n<sub>a</sub>: number of electrons involved in the kinetically decisive step  
 α charge transfer coefficient

### ***Chronoamperometry***

Chronoamperometry requires the same set-up as cyclic voltammetry. It is an electrochemical technique which is also part of the pure diffusion regime methods (no stirring of the solution during the measurement), implementing the measurement of a current as a function of time at a fixed imposed potential.

Chronoamperometry consists of one or more potential jumps. A starting potential E<sub>1</sub> is fixed at a value where no electrochemical reaction takes place between the electrode and the electroactive species in solution. The potential is then pulsed to a value E<sub>2</sub>, for which the electrochemical reaction occurs at the maximum rate (limited by the diffusion of species at the electrode), and held there for a time θ. In the case of double jump chronoamperometry (DPSC), it can then be pulsed to a potential E<sub>3</sub> and held there for the same time θ. These potentials are selected beforehand by a CV analysis. The current is measured during the time θ. The chronoamperogram obtained, i = f (t), follows Cottrell's law and the intensity of the current is proportional to the concentration of the species in solution:

$$i(t) = nFAC\left(\frac{D}{\pi t}\right)^{1/2}$$

n number of moles of electron exchanged

F = 96485 C mol<sup>-1</sup>, the Faraday constant

D diffusion coefficient of the species

i current (A)

t time (s)

A: area of the electrode (cm<sup>2</sup>)

C: initial concentration of the analyte  
(mol.cm<sup>-3</sup>)

### ***Spectroelectrochemistry***

This method has the advantage of combination of electrochemistry and spectroscopy techniques. Namely, electrochemical techniques involve cyclic voltammetry and chronoamperometry, while typically methods of spectroscopy are UV-VIS, Raman, infrared (IR) (**Kaim and Fiedler, 2009**). It provides (*in situ* real-time) molecular, kinetic, and thermodynamic information from the reactants, intermediates, and/or products involved in electron transfer processes (**Kaim and Fiedler, 2009**).

Phenolic compounds absorb in the UV-Visible wavelength range and have chromophore groups, or unsaturated groups corresponding with the types C=C, C=O. For example, flavonoids have absorption bands at 370 and 265 nm, characteristic of the conjugation between the nuclei B-C (cinnamoyl system) and A-C respectively (benzoyl system) (**Chalice, 1984**). Another example is the dimerization of hydroxytyrosol which can be monitored by spectrophotometry thanks to the appearance of an orange-yellow chromophore, absorbing at 390 nm (**Napolitano et al., 2010**). This tool is therefore particularly interesting for observing the redox properties of phenolic compounds through the displacement of the absorption bands in the UV-Visible range. Indeed, despite their relative stability, quinones and semiquinone radicals are unstable forms. Their detection requires rapid analysis methods.

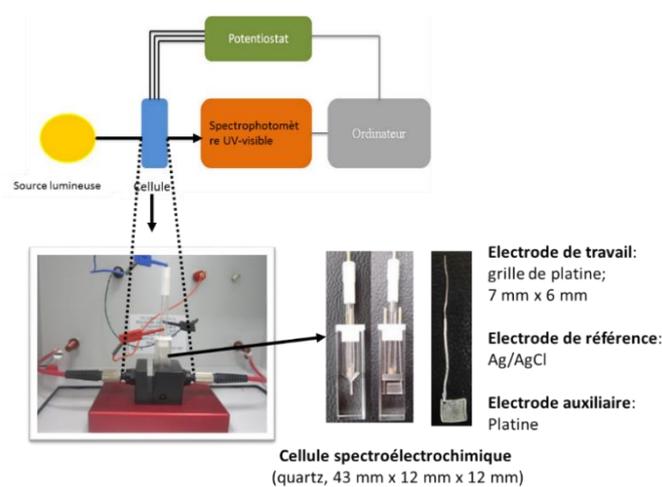


Figure 22. Spectroelectrochemistry UV-VIS setup

## Hypothesis

5-CQA and CA are well known for their antioxidant properties. So far, they have already been subjected to different electrochemical studies (Namazian and Zare, 2005; Šeruga and Tomac, 2014; Yardım, 2012). It has been shown that due to their *o*-diphenolic structure, 5-CQA and CA are oxidized by a 2-electron, 2-proton mechanism. Yet, there is a lack of information on the potential occurrence of this type of oxidation reaction *in vivo* or *in cellulo*, in contrast to hydrolysis, cleavage and reductions that are well known among the biotransformation reactions carried out by gut bacteria.

Enzymatic studies conducted by Guyot and his team highlighted the formation of oxidized products of 5-CQA, mainly dimers, identified by LC-MS/MS (Bernillon et al., 2004; Guyot et al., 2008). Similar MS<sup>2</sup> profiles were observed after electrochemical oxidation, carried out by the Abasq team (0.1M phosphate buffer solution at pH 3.7) [unpublished results]. In our biotransformation study, it would be interesting to explore metabolites that might result from similar oxidation mechanisms.

Hence, in this study, the objective was twofold. Electrochemical and enzymatic oxidations of 5-CQA and CA were performed with the aim to identify the oxidized compounds. The knowledge of the MS/MS data of the 5-CQA oxidized molecules obtained from the

oxidation processes will facilitate the search of these molecules in biotransformed extracts by comparison of their chromatographic and MS/MS patterns.

## II.A.2. Results and discussion

Solution of 5-CQA (2 mM) and CA (2 mM) were prepared in PBS (pH 5.8) with the aim of studying whether specific molecules of interest could arise from their oxidation *via* an electrochemical method. In a first step, the cyclic voltammetry was performed to access the anodic potential of 5-CQA and CA. In a second step, the electrolysis of the two phenolic compounds were performed by a chronoamperometry technique ( $i = f(t)$ ) at a fixed potential during 20 min. The electrolyzed samples were then analyzed by LC-MS<sup>2</sup>. For a better understanding of the mechanism involved, an enzymatic process was also carried out. Comparison of the resulting chromatographic profiles will allow the identification of the oxidized products.

### II.A.2.1. Cyclic voltammetry

The electrochemical behaviour of 5-CQA and CA was investigated by cyclic voltammetry in 0.1 M PBS at pH 5.8. The cyclic voltammogram of both phenolic compounds is shown in Figure 23, where one oxidation and one reduction peaks are observed. 5-CQA and CA possess in their structure a catechol moiety with two -OH groups in the *ortho*-position, which is known to oxidize into *ortho*-quinone form by a transfer of two-electron-two-proton mechanism (Namazian and Zare, 2005; Šeruga and Tomac, 2014; Yardım, 2012). As shown in Figure 23, 5-CQA exhibited an anodic peak at  $E_{pa} = + 0.55\text{V}/\text{Ag}/\text{AgCl}$  and CA one at  $E_{pa} = + 0.38\text{V}/\text{Ag}/\text{AgCl}$ . Then, on the reverse scan, the reversible reduction of the quinone its catechol form is observed ( $E_{pc} = + 0.11\text{V}/\text{Ag}/\text{AgCl}$   $E_{pc} = + 0.14\text{V}/\text{Ag}/\text{AgCl}$ , respectively for 5-CQA and CA). However, it can be noted here that under these experimental conditions at pH 5.8, the system is not electrochemically reversible, which is indicated by the large

separation of the potential peaks ( $\Delta E = E_{p_a} - E_{p_c}$ ) and by the current ratio  $i_c/i_a$ , which is less than 1, attesting to the instability of *ortho*-quinone.

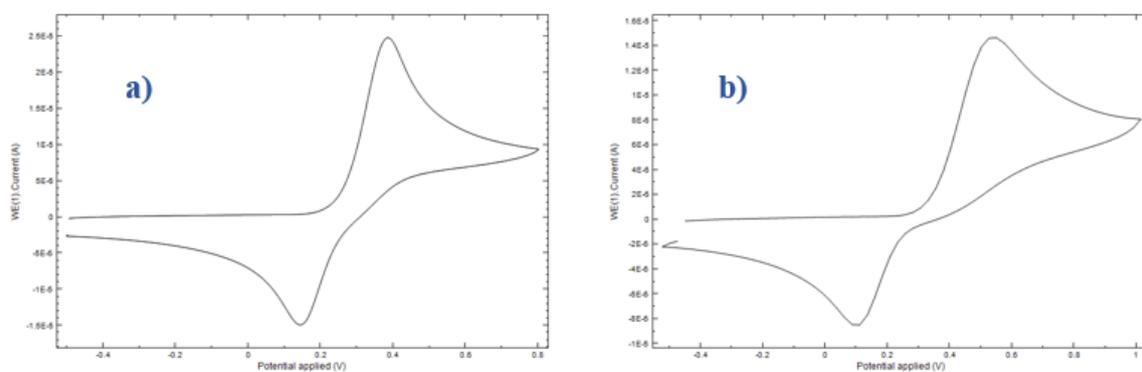


Figure 23. Cyclic voltammograms of 5-CQA (a) and CA (b) 2 mM in pH 5.8 at 0.1 M in phosphate buffer vs. Ag/AgCl reference electrode using a GCE working electrode, scan rate  $0.1 \text{ V}\cdot\text{s}^{-1}$ , room temperature.

#### II.A.2.2. Spectroelectrochemical analysis of CQA and CA

The UV-Visible spectroelectrochemistry study of 5-CQA and CA was performed in PBS at pH 5.8, in a modified UV cell at platinum electrode. First, the UV-Vis spectra were recorded during the linear sweep voltammetry (LSV) at different potential values. Both 5-CQA and CA remained in their native form up to + 0.75 V and + 0.52 V. Then, the electrolysis was performed for 20 min by chronoamperometry technique at a fixed potential, + 0.8 V and + 0.6 V, for 5-CQA and CA respectively. The UV-Vis spectra were recorded simultaneously over a spectral range of 200 to 600 nm. The appearance of a new absorption band was visible at 400 nm, indicating the formation of *o*-quinone form for both 5-CQA and CA as shown in Figure 24.

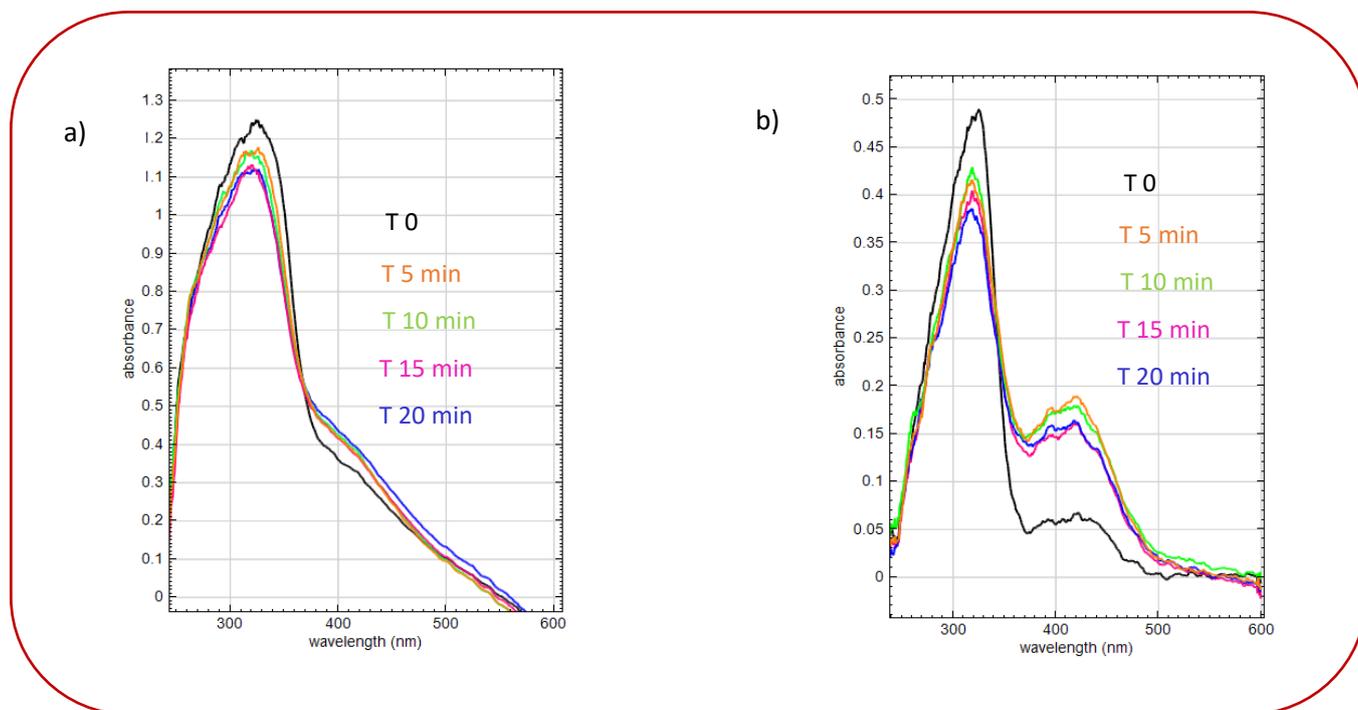


Figure 24. *In situ* thin layer UV vis spectra of a) 5-CQA and b) CA in function of the electrolysis time.

### II.A.2.3. LC-MS<sup>2</sup> analysis of electrochemical and enzymatic oxidations of 5-CQA and CA

The samples obtained after electrochemical and enzymatical oxidations of 5-CQA and CA were analysed by LC-MS<sup>2</sup> in negative mode. The oxidized compounds identified are listed in the Table 3 and peak labels,  $m/z$  ratios for the base peak corresponding mostly to the deprotonated ions  $[M-H]^-$ , retention times (RT min), and the corresponding MS/MS fragments and maximum absorbance ( $\lambda_{\max}$  (nm)) depicted.

#### *a) Characterization of oxidized products of 5-CQA after electrochemical and enzymatical oxidation*

The chromatograms indicated in Figure 25 exhibited 25 peaks. The list of peaks (numbered **1-25**) and the corresponding deprotonated ions detected are reported in Table 3.

The peak (7) at RT= 19.35 min corresponds to the residual 5-CQA, for both methods used. Indeed, it was easily identified based on spectral patterns (RT, UV  $\lambda_{\text{max}}$  at 325 nm), and mass spectrum ( $m/z$  353) and confirmed by comparison with an analytical standard. The comparison of LC-MS<sup>2</sup> chromatograms of 5-CQA, before and after electrolysis, highlighted the detection of numerous peaks, corresponding to oxidized metabolites of 5-CQA (Figure 25). The majority of them corresponded to ions with  $m/z$  705 (peaks **2, 5, 6, 10-24**). In addition, one peak **1** at  $m/z$  703 eluted before 5-CQA, at RT =8.58 min. Finally, peaks **3, 4** and **25** corresponded with ions at  $m/z$  723. We can note that most of the products formed by oxidation were less polar compounds than 5-CQA, except peaks **1-6**.

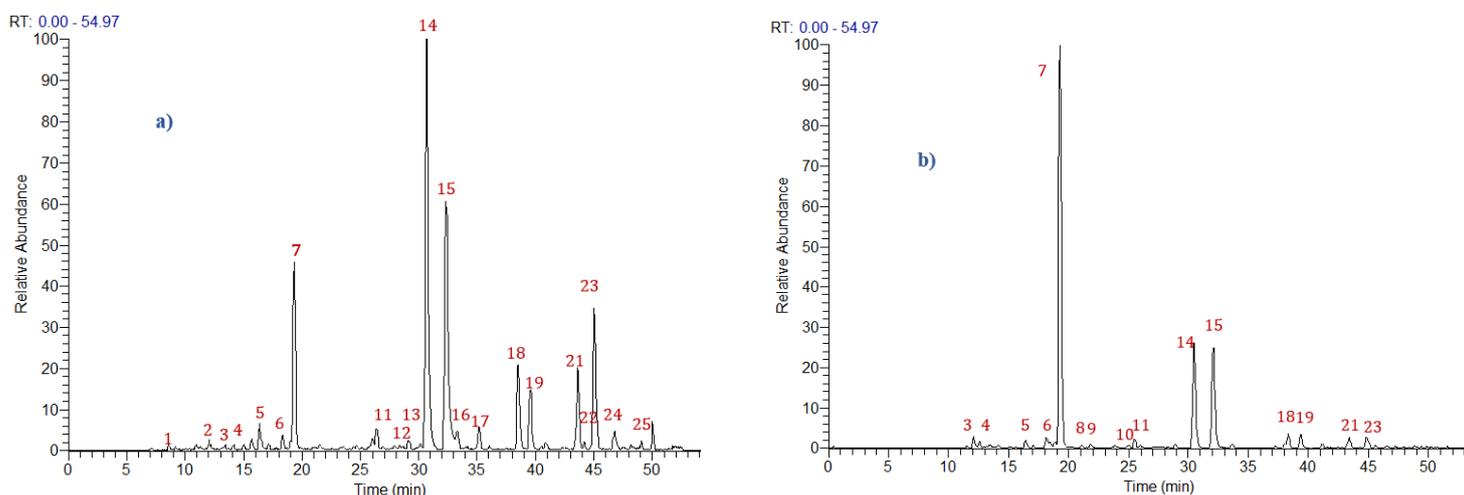


Figure 25. Base peak chromatograms of: a) electrolyzed solution of 5-CQA (2 mM); b) oxidized solution of 5-CQA by PPO

Table 3. LC-MS<sup>2</sup> analysis of electrochemical and enzymatic oxidized solution of 5-CQA. Green circles indicate the presence of peak and red ones the absence of peak

| ID n° | RT (min) | $\lambda_{\max}$ (nm) | m/z | MS <sup>2</sup> | Ox 5-CQA electrochemically | Ox 5-CQA enzymatically |
|-------|----------|-----------------------|-----|-----------------|----------------------------|------------------------|
| 1     | 8.58     | 306                   | 703 | 511             | ●                          | ●                      |
| 2     | 12.08    | /                     | 705 | 513             | ●                          | ●                      |
| 3     | 13.42    | 290                   | 723 | 705/513         | ●                          | ●                      |
| 4     | 14.16    | 290                   | 723 | 705/513         | ●                          | ●                      |
| 5     | 16.40    | 302/313               | 705 | 513             | ●                          | ●                      |
| 6     | 18.33    | 326                   | 705 | 513/311         | ●                          | ●                      |
| 7     | 19.35    | 325                   | 353 | 191/179         | ●                          | ●                      |
| 8     | 21.8     | /                     | 723 | 705/513         | ●                          | ●                      |
| 9     | 23.89    | 315/325               | 723 | 705/529/513     | ●                          | ●                      |
| 10    | 25.48    | 312                   | 705 | 513/321         | ●                          | ●                      |
| 11    | 26.35    | 288/305/319           | 705 | 513             | ●                          | ●                      |
| 12    | 28.31    | /                     | 705 | 513             | ●                          | ●                      |
| 13    | 29.10    | /                     | 705 | 513             | ●                          | ●                      |
| 14    | 30.66    | 327                   | 705 | 513             | ●                          | ●                      |
| 15    | 32.32    | 327                   | 705 | 513             | ●                          | ●                      |
| 16    | 33.25    | 287/312               | 705 | 513             | ●                          | ●                      |
| 17    | 35.17    | /                     | 705 | 513             | ●                          | ●                      |
| 18    | 38.23    | 290/319               | 705 | 513             | ●                          | ●                      |
| 19    | 39.6     | 298/319               | 705 | 513             | ●                          | ●                      |
| 20    | 40.88    | /                     | 705 | 513             | ●                          | ●                      |
| 21    | 43.63    | 301/319               | 705 | 513             | ●                          | ●                      |
| 22    | 44.17    | /                     | 705 | 513             | ●                          | ●                      |
| 23    | 44.97    | 300/317               | 705 | 513             | ●                          | ●                      |
| 24    | 46.43    | /                     | 705 | 513             | ●                          | ●                      |
| 25    | 49.38    | /                     | 723 | 705/513         | ●                          | ●                      |

The same ions were detected after oxidation (Figure 25 and Table 3). However, we noted that the enzymatic oxidation of 5-CQA showed slight differences compared to the electrochemical oxidation. Thus, even if peaks **14** and **15** were the most abundant compounds with the two methods, their relative intensity were higher than the control 5-CQA after 5-CQA electrochemical oxidation. Moreover, less ions at  $m/z$  705 were observed enzymatically vs electrochemically with the presence of 9 peaks (**5, 6, 10, 14, 15, 18, 19, 21, 23**) and the absence of the peaks **11-13, 16-17, 20-22** and **24**. In addition, two peaks **8** and **9** with  $m/z$  723 were

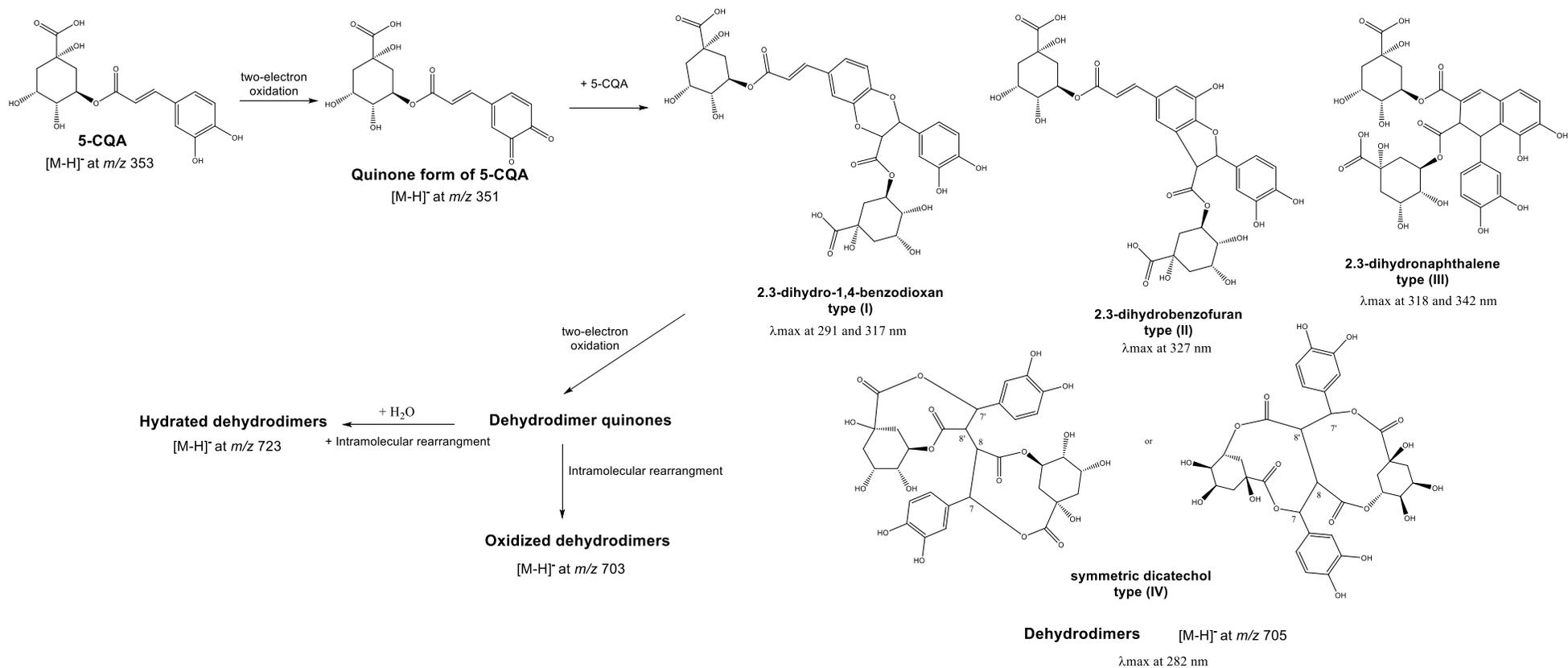
characteristic of enzymatic oxidation of 5-CQA, compared to others **3** and **4** which were also observed in the electrochemically oxidized sample.

The oxidized compounds formed both electrochemically and enzymatically have shown the same UV and MS/MS patterns (Scheme 2). UV spectrum of ions exhibited a maximum absorbance in 280-320 nm range for all oxidized compounds. Most of the ions at  $m/z$  705 have shown the MS/MS fragment with  $m/z$  513 corresponding to the loss of one quinic acid moiety. Only peak **10**, formed enzymatically, showed a different MS/MS spectrum, with the most intense fragment ion at  $m/z$  321, corresponding to the loss of both quinic acid moieties. The ion with  $m/z$  703 showed a MS/MS fragmentation with  $m/z$  511.

Previous studies carried out by Guyot et al. led to the identification of oxidized compounds after enzymatic oxidation of 5-CQA by PPO. About ten products at  $m/z$  705 (706 Da) corresponding to dehydrodimers of 5-CQA were observed. While the compounds with  $m/z$  703 (704 Da) were identified as reoxidized forms of 5-CQA dehydrodimers, ions at  $m/z$  723 were assigned to hydrated 5-CQA dehydrodimers (724 Da) (**Bernillon et al., 2004; Wong-Paz et al., 2015**). Moreover, the Guyot's team has recently purified using centrifugal chromatography partition (CPC), ten 5-CQA dehydrodimers (706 Da) (**Castillo-Fraire et al., 2019**), and has determined the structure by  $^1\text{H}$  and  $^{13}\text{C}$  one- and two-dimensional NMR spectroscopies of seven 5-CQA dehydrodimers with dihydrobenzofuran (type I), benzodioxane (type II) or dihydronaphthalene (type II) skeleton for six of them and with a symmetric dicatechol structure (type IV) for the last one Scheme 2 (**Castillo-Fraire et al., 2022**).

According to Guyot et al, the mechanism of enzymatic oxidation of 5-CQA, led to the formation of dimers. As a first step, CQA is oxidized in *ortho*-quinone, which is not stable and reactive. Then these *ortho*-quinones are involved in further oxidation reduction reactions and chemical addition. All these reactions lead to the formation of secondary *o*-quinones which can react with primary *o*-quinones and give semi-quinones (radical form). Quinones and semi-quinones can react and give a large number of products by addition, dimerization or water addition followed by intramolecular rearrangement. Polymerization seems to be minor (**Guyot and Poupard, 2011**).

By comparison of the LC-MS<sup>2</sup> data, the same oxidized molecules than those identified by Guyot's team, enzymatically, were detected by the electrochemical study of 5-CQA and were confirmed by spectral patterns comparison (Table 3). The UV spectra of these oxidation products were in accordance with those previously obtained by enzymatic oxidation of 5-CQA. In addition, MS/MS spectra were similar to previously data published by Guyot et al (**Castillo-Fraire et al., 2019; Guyot et al., 2008**). We have determined that the ions with  $m/z$  705 (MW, 706 Da) corresponded to dehydromers of 5-CQA (type I-III) (named also caffeicin-like dimers) (**Cilliers and Singleton, 1991**). According to the previous results, the didydronephthalene-type III which possess characteristic UV pattern (318 and 340 nm) appeared more polar than 5-CQA but were not similar to the UV pattern than the more polar compounds **5** and **6** found in our study. The compound **10** with characteristic MS/MS fragments at  $m/z$  513 and 321 and with a  $\lambda_{\max}$  at 312 nm seems not similar with the compound (**705-2**) described by Guyot et al as dehydromer type IV (**Castillo-Fraire et al., 2022**) which possess the same MS/MS data but with a  $\lambda_{\max}$  at 282 nm. We hypothesized that this compound possesses, in its structure, no conjugated propanoic moiety. The dihydrobenzofuran-type II dehydromers could be attributed to peaks **15** and **16** in accordance with their  $\lambda_{\max}$  at 327 and their medium polarity (RT at 30.66 and 32.32). Following their  $\lambda_{\max}$  (around 290 and 320 nm) the peaks **16**, **18-19**, **21** and **23** could be assigned to the dihydrobenzodioxan-type I which are the less polar dehydromers. Finally, the seven other peaks (**2**, **12-13**, **17**, **20**, **22** and **24**) were not assigned as we were not able to determine their UV pattern. The ion at  $m/z$  703 corresponding to compound **1** was assigned to reoxidized form of a 5-CQA dehydromer. The other products **3-4**, **8-9** and **25** with  $m/z$  723 were attributed to hydrated 5-CQA dehydromers. All these identifications need to be confirmed after separation using semi-preparative HPLC or CPC and structural identification using NMR. We can note the absence of trimers formed at  $m/z$  1057 showing that 5-CQA electrochemical and enzymatical oxidations do not favor their formation.



Scheme 2. Mechanisms of formation and structures of oxidized products derived from 5-CQA (adapted from (Castillo-Fraire et al., 2019) and (Castillo-Fraire et al., 2022))

*b) Characterization of oxidized products of CA after electrochemical and enzymatical oxidations*

An ion (peak **2**) with  $m/z$  525 eluted firstly at  $RT = 10.54$  min. Its fragmentation MS/MS data indicated a loss of water following by two subsequent decarboxylation reactions. As previously reported (**Pati et al., 2006**), this more polar compound than CA, putatively corresponding to hydroxylated species, could arise from nucleophilic addition of water to CA derivatives coming from typical coupling reactions. In a same manner, the peak **7** with  $m/z$  375 could be originated from a water addition to a CA dimer. Peaks **10** and **11** correspond to *trans* and *cis* CA geometric isomers, respectively, with both a specific MS/MS fragmentation corresponding to a decarboxylation (ion at  $m/z$  135). From *cis*-CA, with a mechanism which will be described in Biotransformation section, comes the ion at  $m/z$  177, which was identified as esculetin (peak **8**). Peaks **8**, **10** and **11** were also identified after enzymatic oxidation. Nevertheless, CA dimers with  $m/z$  357, previously identified and formed by either a Michael-1-4 type ring nucleophilic condensation (type a, like C-C dimerization) or by condensation between semi-quinone radicals (types b and caffeicin-like dimers c, d) (see Scheme 3) (**Pati et al., 2006; Weber et al., 2019**), were not detected, neither electrochemically nor enzymatically. However, some peaks (**17-23**) obtained electrochemically and those (**18, 21, 22**) afforded enzymatically exhibited  $m/z$  313, suggesting that they could be generated from CA dimers (**Pati et al., 2006**) and correspond to  $[M-CO_2-H]^-$  ions. Peaks **17, 18, 20** demonstrated a same MS/MS pattern at  $m/z$  269 arising from further decarboxylation and indicating their belong to C-C dimers of CA (type a). It is interesting to note that it is the dimer **18** which was the most abundant product in each oxidized sample. Peak **19** may also correspond to a C-C dimer as reported by Pati et al. Moreover, the same MS/MS spectra (269, 179, 177, 135 and 133) were detected for peaks **21-23**. These molecular fragmentations come either by  $CO_2$  loss, directly or combined with a retro Diels Alder and could lead to the determination of their structures as caffeicin-like dimers of CA (c) (**Pati et al., 2006**). Some peaks (**12-16**) detected in the enzymatic study corresponded to high molecular weight metabolites (e.g  $m/z$  383, 397, 427, 441, and 471) which are less polar than CA. Further purification steps will be interesting to carry out in order to determine their structures. An ion with  $m/z$  137 was detected at  $RT$  15.33 min and corresponds to a more polar compound than CA. In accordance to its  $\lambda_{max}$  at 280 nm,

this compound has lost the side chain double-bond conjugation and could come from decarboxylation of CA then reduction, leading to its identification as 4-ethylcatechol. This compound has been previously reported to be produced by *L. johnsonii* (Bel-Rhlid et al., 2013). Finally, peaks 3 and 4 characteristic of the enzymatic study have shown  $m/z$  251 and 265, respectively with a RT close to each other. In the same manner than previously, further purification steps will be required in order to determine the structures of these compounds.

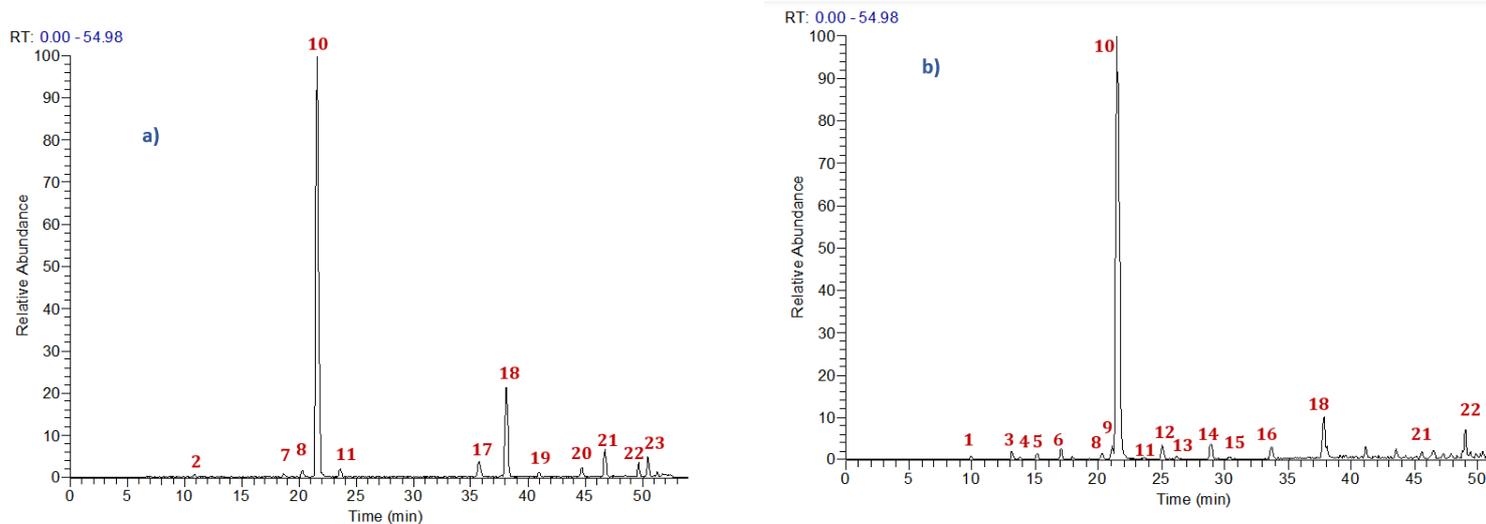
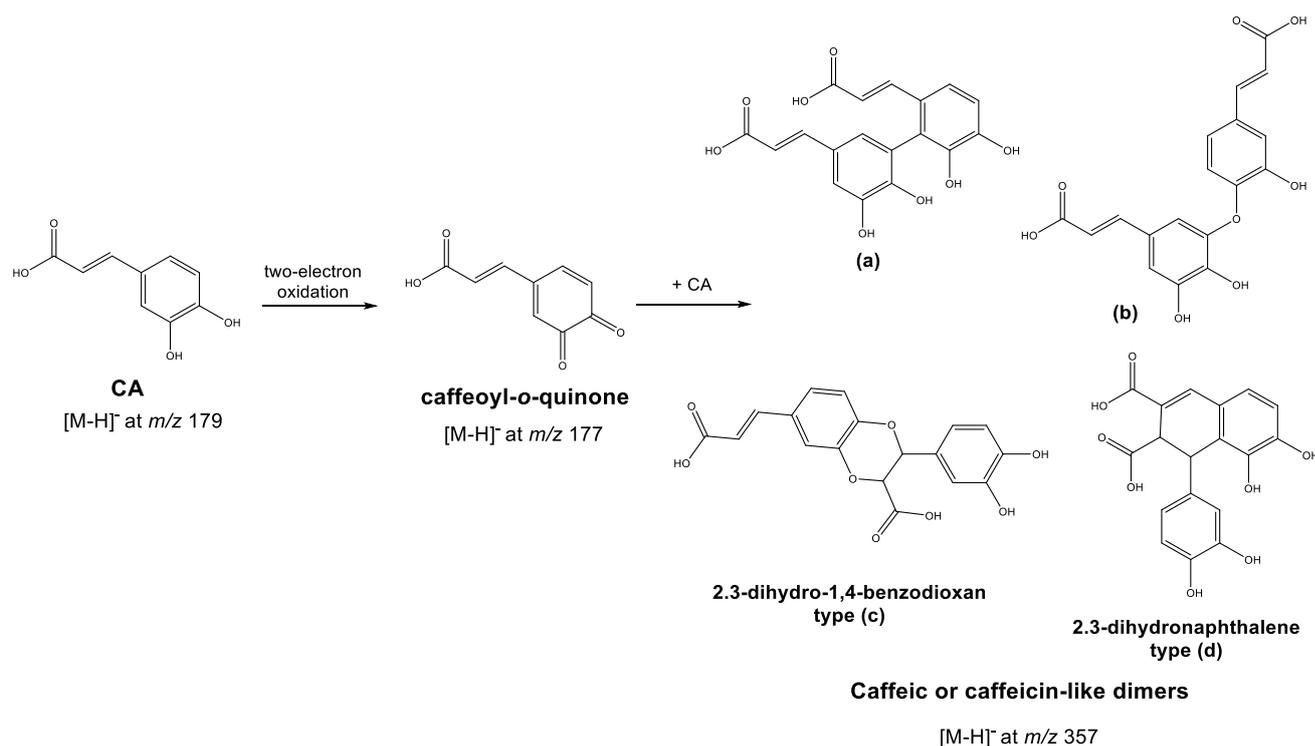


Figure 26. Base peak chromatograms of: a) electrolyzed solution of CA (2 mM); b) oxidized solution of CA by PPO

Table 4. LC-MS<sup>2</sup> analysis of electrochemical and enzymatic oxidized solution of CA: green cercles indicate the presence of peaks and red ones the absence of peaks. Starred values refer to ions different from the deprotonated molecular ion

| ID n° | RT (min) | $\lambda_{\max}$ (nm) | m/z  | MS <sup>2</sup> | Ox CA electrochemically | Ox CA enzymatically |
|-------|----------|-----------------------|------|-----------------|-------------------------|---------------------|
| 1     | 9.90     | /                     | 275  | 207             | ●                       | ●                   |
| 2     | 10.54    | /                     | 525  | 507/463/419     | ●                       | ●                   |
| 3     | 13.14    | /                     | 251  | 148/100/73      | ●                       | ●                   |
| 4     | 13.81    | /                     | 265  | 207/163         | ●                       | ●                   |
| 5     | 15.33    | 280                   | 137  | /               | ●                       | ●                   |
| 6     | 17.04    | /                     | 295  | 104/100         | ●                       | ●                   |
| 7     | 18.59    | /                     | 375  | 287/269         | ●                       | ●                   |
| 8     | 20.24    | 343                   | 177  | 133             | ●                       | ●                   |
| 9     | 21.10    | 295/316               | 339  | /               | ●                       | ●                   |
| 10    | 21.53    | 318/337               | 179  | 135             | ●                       | ●                   |
| 11    | 23.62    | 301/311               | 179  | 135             | ●                       | ●                   |
| 12    | 25.04    | /                     | 383  | /               | ●                       | ●                   |
| 13    | 26.17    | /                     | 397  | 339/295         | ●                       | ●                   |
| 14    | 28.88    | /                     | 427  | /               | ●                       | ●                   |
| 15    | 30.38    | /                     | 441  | /               | ●                       | ●                   |
| 16    | 33.66    | /                     | 471  | /               | ●                       | ●                   |
| 17    | 35.38    | 324                   | 313* | 269             | ●                       | ●                   |
| 18    | 38.11    | /                     | 313* | 269             | ●                       | ●                   |
| 19    | 40.95    | /                     | 313* | 269/251         | ●                       | ●                   |
| 20    | 44.55    | 336                   | 313* | 269             | ●                       | ●                   |
| 21    | 46.7     | 290/318               | 313* | 269/179/177/135 | ●                       | ●                   |
| 22    | 49.62    |                       | 313* | 269/179/177/135 | ●                       | ●                   |
| 23    | 50.47    |                       | 313* | 269/179/177/135 | ●                       | ●                   |



Scheme 3. Mechanisms of formation and structures of some oxidized products derived from 5-CA (adapted from (Pati et al., 2006))

LC-MS<sup>2</sup> analysis of electrochemical oxidation of 5-CQA and CA highlighted the formation of oxidized compounds such as various dimers. Moreover, these metabolites have shown the same spectral patterns with those obtained enzymatically. Interestingly, the fact that an antioxidant could exert its bioactivities through both its reduced and oxidized form but also through their oxidized metabolites, is emerging as depicted in Figure 27 (Hunyadi, 2019). Indeed, these compounds arising from the oxidation of polyphenols have already exhibited some interesting patterns towards human health (Bae and Kim, 2012; Bailly et al., 2013; D. Liu et al., 2019; Shingai et al., 2011). For example, our team has already demonstrated that mix of oxidized molecules of olive oil have demonstrated neuroprotective effects on cell assay (Lambert de Malezieu et al., 2019). Fási et al., in a study with various oxidized mixtures of *p*-coumaric acid methyl ester (pcm), revealed two metabolites with high antitumor potential (Fási et al., 2019). Yet, the studies are scarce in this field. Further investigations should be undertaken, since these metabolites of polyphenols issued from a redox pathway could be potentially bioactive compounds.

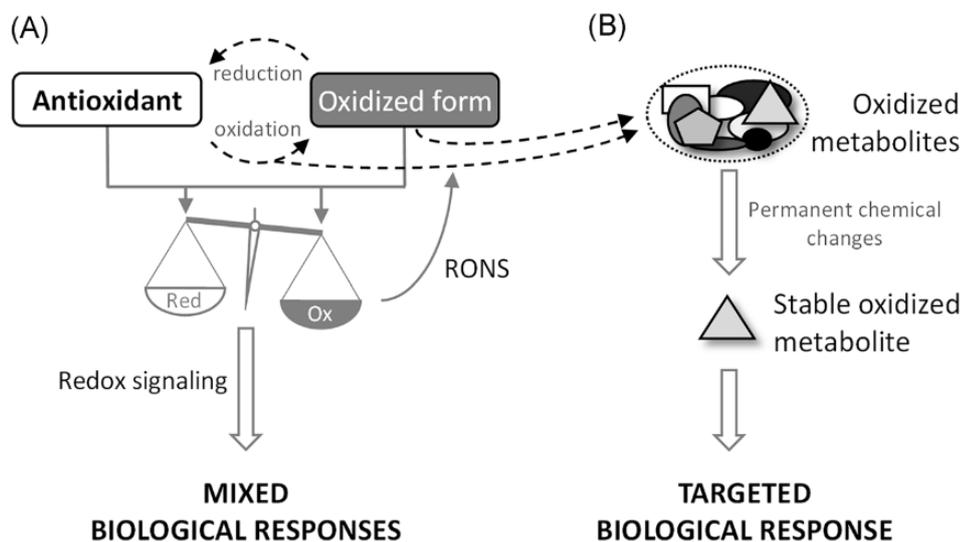


Figure 27. A) The well-known mechanism of antioxidants by modulating redox state and redox signalling pathways: B) potentially bioactivity of oxidized metabolites obtained via free radical scavenging (Hunyadi, 2019)

## II. B. Biotransformation of 5-CQA by gut bacteria in mono and co-culture

In this study, we have performed biotransformation of 5-CQA by three gut bacteria *L. reuteri*, *B. fragilis* and *B. longum* in monoculture. A first part is dedicated to the studied strains. The second part corresponds to an article accepted in Food and function journal. Next, we have investigated the biotransformation process using a co-culture of the two bacteria *L. reuteri* and *B. fragilis*.

## II.B.1. The characteristics and properties of three studied gut bacteria

### II.B.1.1. Taxonomy and ecology

#### a) *Lactobacillus reuteri*

*L. reuteri* (Figure 28) belongs to the phylum *Firmicutes*, class *Bacilli*, order *Lactobacillales* and family *Lactobacillaceae*. It is a Gram-positive, non-spore forming, non-motile, facultative anaerobic rod-shaped bacillus (**Britton, 2017**). The optimum growth temperature is between 37-42 °C and the optimal pH for growth is about 6.5 (in fact no growth occurs below pH 4.5). *L. reuteri* does not require anaerobic conditions for growth and is normally cultivated in oxygen-limited atmospheres (**Patman, 2015**).

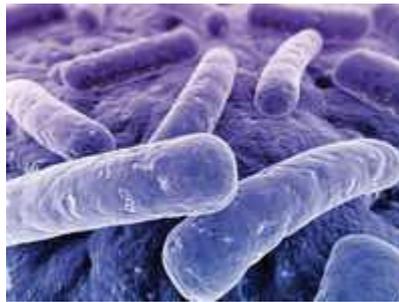


Figure 28. Scanning electron microscopy of *Lactobacillus reuteri* strain (Patman, 2015)

#### b) *Bacteroides fragilis*

*Bacteroides* (Figure 29) are the most predominant genera among intestinal microbiota. These Gram-negative species belonging to the phylum Bacteroidetes, are anaerobic, saccharolytic, non-spore-forming and bile resistant (**Kuwahara et al., 2004**).

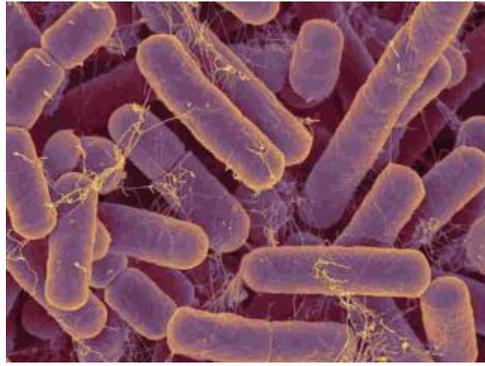


Figure 29. Scanning electron microscopy of *Bacteroides* strain

*B. fragilis*, is an anaerobic commensal constituting only 1–2% of it, and it represents the predominant anaerobic isolate in cases of intraabdominal sepsis. The *Bacteroides fragilis* group is one of the most important pathogens in polymicrobial infections and accounts for 63 % of all the group isolated during infection. It has been suggested that commensalism is a more appropriate description between *Bacteroides* and human host, thus implying that one partner benefits from the relationship and the other is not affected. Carbohydrates fermentation by *Bacteroides* results in the production of a various of volatile fatty acids that are reabsorbed through the large intestine (Wexler, 2007). *B. fragilis* are opportunist bacteria when the ecological conditions of the colon are no longer normal. This imbalance can be the cause of several infections such as septicemia. These infections are very difficult to treat due to the numerous resistances to antibiotics of these strains.

c) *Bifidobacterium longum*

*Bifidobacterium longum* belong to the type of genus *Bifidobacterium* which is the main representative of the Actinobacteria phylum and a part of the only family of bacteria in the order *Bifidobacteriales*, *Bifidobacteriaceae*. It is Gram-positive bacillus, anaerobic, non-motile, non-spore-forming, irregularly shaped rod and non-filamentous bacteria that survival in the large intestine (Schöpping et al., 2021). It is naturally found in oral and gastrointestinal

tract, particularly colon which can contain up to  $10^{12}$  bacterial cells/g (O'Callaghan and van Sinderen, 2016).

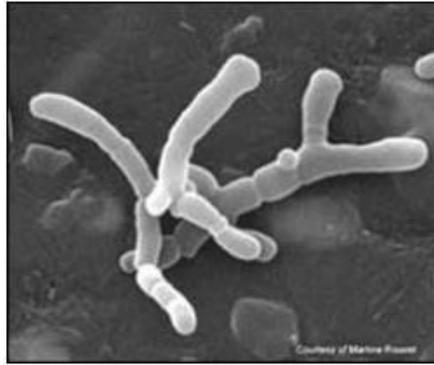


Figure 30. Scanning electron microscopy of *Bifidobacterium* strains (Rouvet, 2004).

Often, the approach used to identify *Bifidobacteria* involves the presence of a fructose-6-phosphoketolase (F6PPK) activity, a key enzyme of the genus *Bifidobacterium*. *Bifidobacteria* coexist with a large variety of bacteria in the human intestinal microflora (Biavati et al., 2000).

#### II.B.1.2. The roles in intestinal health of *L. reuteri*, *B. fragilis* and *B. longum*

The well-studied probiotic bacterium, *L. reuteri* is found in different body sites, including the gastrointestinal tract, urinary tract, skin and breast milk (Mu et al., 2018). The abundance of *L. reuteri* varies among different individuals. Several beneficial effects of *L. reuteri* have been reported. Due to its antimicrobial activity, *L. reuteri* can inhibit the colonization of pathogenic microbes and remodel the commensal microbiota composition in the host. Moreover, *L. reuteri* has shown immunomodulatory effects. For example, some strains can reduce the production of pro-inflammatory cytokines while promoting regulatory T cell development and function. Finally, considering its ability to enhance the intestinal barrier, the colonization of *L. reuteri* may decrease the microbial translocation from the gut lumen to the tissues (Mu et al., 2018).

In the micro-flora of the human gastrointestinal tract, *B. fragilis*, is an anaerobic commensal constituting only 1–2% of it, and it represents the predominant anaerobic isolate in cases of intra-abdominal sepsis and bacteraemia as mentioned above. *B. fragilis* can play two roles in the host (**Yekani et al., 2020**). As a friendly commensal, this strain must be able to establish itself in the host intestinal mucosa, to utilize and process polysaccharides for use by the host, and to resist to the noxious effects of bile salts. As pathogen, it must be able to attach itself to the site of infection, evade killing mechanisms implemented by host defense or with an antimicrobial treatment and produce factors that damage host tissue. The cell envelope of *B. fragilis*, likewise, must be able to be aggressor, defender and strategist in allowing the organism to establish itself in the host-whether as friend or foe (**Pumbwe et al., 2006**).

*Bifidobacteria* are gut commensals and are among the first microbes to colonize the human gastrointestinal tract. Among the probiotic features and functions that have been attributed to *Bifidobacteria* are the production and/or release of vitamins B, antioxidants and polyphenols; maturation of the immune system during early life and preservation of immune homeostasis during life; preservation of gut barrier functions and protection against pathogens by production of bacteriocins, decreasing luminal pH by acids production, and blocking the adhesion of pathogens to the intestinal mucosa (**Gagnon et al., 2015**). Another important function of the bifidobacterial genus is their contribution to gut homeostasis and host health in the production of acetic and lactic acids during carbohydrate fermentation, organic acids that in turn can be converted into butyric acid by other colon bacteria through cross-feeding interactions. In this context, *B. longum* species has a long history of safe use as probiotic adjunct in foods and in pharmaceutical (**O’Callaghan and van Sinderen, 2016**).

**Article status: Accepted on April 24, 2022, in Food and Function (IF 5.40)**

## An insight into an intriguing oxidative biotransformation pathway of 5-*O*-caffeoylquinic acid by a gut bacterium

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### Abstract

Microbiota is known to play a pivotal role to generate bioavailable and bioactive low molecular weight metabolites from dietary polyphenols. 5-*O*-caffeoylquinic acid (5-CQA), one of the main polyphenols found in diet, was submitted to a resting cells biotransformation study using three gut bacteria species *Lactobacillus reuteri*, *Bacteroides fragilis* and *Bifidobacterium longum*. These bacteria were selected in accordance to their belonging to the main phyla found in human gut microbiota. Our study highlighted the ability of only one of the strains studied, *L. reuteri*, to bioconvert 5-CQA into various metabolites due to the expression of cinnamoyl esterase enzyme as the first step. Interestingly, one natural known compound, esculetin, was for the first time described as a 5-CQA derived metabolite after conversion by a gut bacterium, the other metabolites were already reported. This evidence put in the spotlight an interesting oxidative pathway occurring in vivo by intestinal microbiota affording to esculetin. This molecule was also identified after electrochemical and enzymatic oxidations of caffeic acid.

The oxidation capacity of *L. reuteri* led to less diverse metabolites in comparison to those obtained either electrochemically and enzymatically where dimers and trimers were reported. Thus, esculetin may exert interesting and beneficial biological effects on gut microbiota, which must be further evaluated. Novel synbiotics could be formulated from the association of *L. reuteri* with chlorogenic acids.

## II.B.2. 1. Introduction

Polyphenols are known for their broad range of positive bioactivities in humans. These molecules have shown the ability to lower oxidative stress involved in many diseases and positively modulate redox signalling pathways (**Zhang and Tsao, 2016**). Luca et al. has recently described the notion of “low bioavailability/high bioactivity paradox of polyphenols” highlighting the interest of their intestinal metabolites on human health (**Luca et al., 2020**). Considering their superiority in terms of presence in systemic circulation compared to parent polyphenols, further insights focus nowadays on the pivotal role of microbiota to generate bioavailable low molecular weight metabolites (**Pasinetti et al., 2018a**). These gut-derived metabolites have exhibited biological activities and have shown ability to reach the brain (**Carregosa et al., 2019b; Johnson et al., 2019; Yamasaki et al., 2020**). Indeed, studies have put them in the spotlight as promising approach to prevent and attenuate many pathologies such as neurodegenerative diseases (**Carregosa et al., 2019b; Yamasaki et al., 2020**). Among dietary polyphenols, the hydroxycinnamate derivative 5-*O*-caffeoylquinic acid (5-CQA) according to IUPAC name, known as chlorogenic acid, is one of the main phenolic compounds in daily diet, being present in various fruits and vegetables (**Clifford, 1999; Lu et al., 2020**). This highly functional compound has been shown to have high benefits for human health (i.e. cardioprotective, gastrointestinal protective, hepatoprotective, neuroprotective properties etc..) (**Lu et al., 2020**). Pharmacokinetic profiles of 5-CQA have been already described in humans (**Clifford et al., 2017**). After intake, one third is absorbed in small intestine and majority reaches the colon in intact form. At this stage, intestinal microbiota with cinnamoyl esterase activity biotransform 5-CQA into caffeic acid (CA) and quinic acid, which further undergo their subsequent transformation into various microbial metabolites including *m*-coumaric acid

and hydroxylated derivatives of phenyl propanoic, benzoic, and hippuric acids (Clifford et al., 2017). These metabolites are bioavailable in the systemic circulation and achieve the targeting organs where they can exert their biological effects (Clifford et al., 2017; Lu et al., 2020). Indeed, the biotransformation of 5-CQA is dependent on the ability of bacterial species to produce this esterase as the first step. Hence, some bacteria species were already identified as producer of this specific enzyme such as *Escherichia coli*, *Bifidobacterium lactis*, *Lactobacillus gasseri* (Couteau et al., 2001b) or *L. johnsonii* (Bel-Rhlid et al., 2013).

In this work, we have investigated the biotransformation of 5- *O*-caffeoylquinic acid by three gut-bacteria species cultivated alone: *Lactobacillus reuteri* (Firmicutes), *Bacteroides fragilis* (Bacteroidetes) and *Bifidobacterium longum* (Actinobacterium), belonging to the dominant bacterial phyla of the gut microbiota. The biotransformed extracts were analysed by HPLC-DAD-ESIMS<sup>2</sup> and the data were subjected to MZmine 2.53 and a further treatment creating a molecular network (MN). It is interesting to note that gut microbiota polyphenol biotransformation encompassing hydrolysis, reduction and de- or di-hydroxylation reactions are well known (Espín et al., 2017; Vollmer et al., 2017) but there is a lack of information on possible oxidative pathways other than  $\alpha$ - or  $\beta$ -oxidations. For example, one study led to the characterization of  $\beta$ - oxidation of one metabolite of 5-CQA, 3-(4'- hydroxyphenyl) propanoic acid, into 4-hydroxybenzoic acid, using an in vitro experimental gastrointestinal dialysis model (Breynaert et al., 2015). Hence, electrochemical and enzymatic methods were adopted to generate oxidized compounds of 5-CQA and CA in order to characterize and compare their LC-MS profiles to those of the biotransformed extracts. This strategy allowed to highlight an intriguing oxidative pathway of cinnamic acids microbiota conversion.

## II.B.2. 2. Results and discussion

### a) Biotransformation studies of 5-CQA

The resting cell method (Julsing et al., 2012) was applied for the biotransformation of 5-CQA using three gut bacteria *L. reuteri*, *B. fragilis* and *B. longum*. Based on the growth curve of these bacteria monitored by the measure of OD<sub>600nm</sub>, the biotransformation reaction was

performed during 24 h when the stationary phase was reached. PBS was chosen as a solvent for 5-CQA and CA since it was previously described as closer to the intracytoplasmic bacterial composition (**Mortelé et al., 2019**). The biotransformation step was done at pH 5.8, close to the pH found in colon. 5-CQA was dissolved in PBS at 1 mM, a concentration close to those in the colon after coffee or fruits intake (**Clifford, 1999**). After centrifugation, the pellet and the supernatant were separately treated using ethyl acetate to obtain two extracts which were further analysed by LC-UV-ESI-MS<sup>2</sup> in negative mode. The base peak chromatograms of the control (5-CQA incubated at 37°C) and of the SN extracts after 5-CQA biotransformation by the three bacteria are reported in Figure 31. While *L. reuteri* was able to biotransform 5-CQA, none biotransformed metabolites were detected after bioconversion by *B. fragilis* and *B. longum* Figure 31. To validate these results, we searched in silico for enzyme responsible of CQA biotransformation named cinnamoyl esterase or feruloyl esterase (EC.3.1.1.73), which was already found in *L. johnsonii* (**Bel-Rhlid et al., 2013**) (accession number NC\_005362). A genomic comparison between the gene already described in *L. johnsonii* and those present in our strain *L. reuteri* DSM 20016 (GCA\_001436151 showed 70.7% of similarity with the geneLocus Tag named LREU\_RS08790 (Figure 32). Nonetheless, this gene was not found in *B. fragilis* and *B. longum*, suggesting that the results from biotransformation confirmed by LC-ES-MS<sup>2</sup> are in line with search in silico. Research on Pfam highlighted the presence of the same domain, hydrolase\_4, serine aminopeptidase, S33 in these two genes which belong to the Alpha/Beta hydrolase fold family (**El-Gebali et al., 2019**). This catalytic domain is found in a very wide range of enzymes like esterase. This cinnamoyl esterase led the hydrolysis of 5-CQA into CA and quinic acid. CA was well detected in LC-ESI-MS<sup>2</sup> chromatogram and quinic acid, being too polar, was either not extracted by ethyl acetate or not retained on the column (Figure 31) The peak 3 was identified as *cis*-5-*O*-caffeoylquinic acid as suggested by Clifford et al (**Clifford et al., 2008**) and confirmed after UV irradiation in a UV-quartz cell during 1 h of 5-CQA standard (Figure 33).

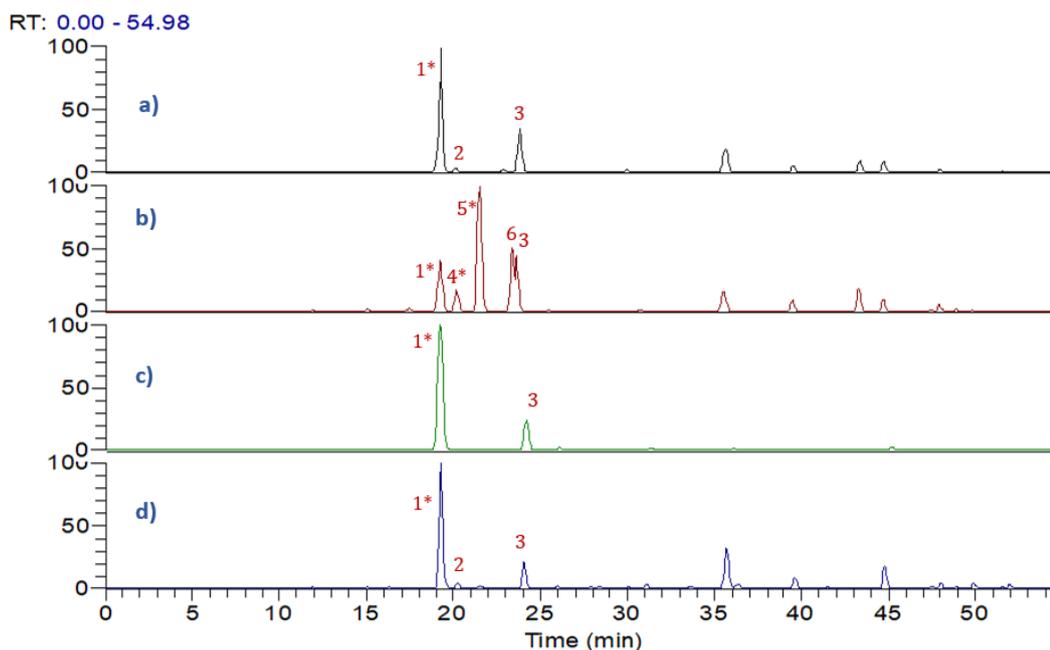


Figure 31. Base peak chromatograms in negative mode of **a)** 5-CQA incubated at 37°C in PBS at pH 5.8, **b)** SN extracts after 5-CQA biotransformation by *L. reuteri* in PBS, **c)** SN extracts after 5-CQA biotransformation by *B. fragilis* in PBS, **d)** SN extracts after 5-CQA biotransformation by *B. longum* in PBS. (1) 5-CQA, (2) isomer of 5-CQA (3) *cis*-5-*O*-caffeoylquinic acid, (4) esculetin, (5) *trans*-CA, (6) *cis*-CA. \*compounds with retention time (RT) confirmed after injection of analytical standards

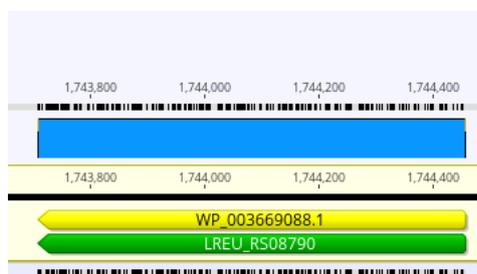


Figure 32. Genomic comparison of the gene of cinnamoyl esterase between *L. johnsonii* and *L. reuteri* (DSM 20016, GCA\_001436151)

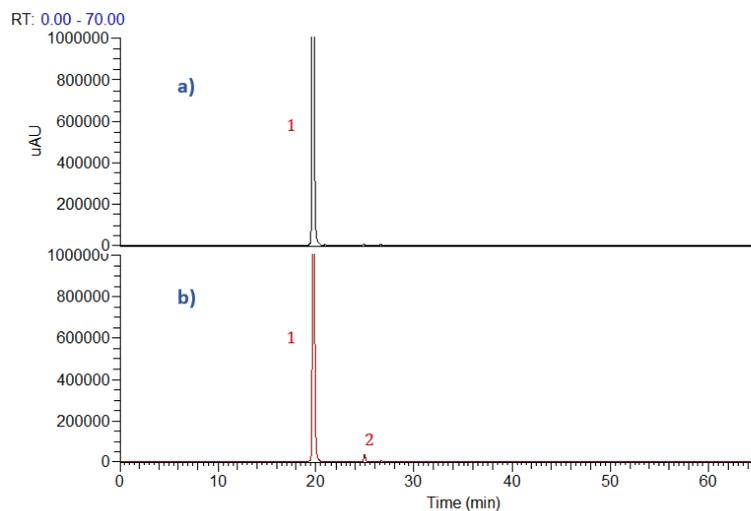


Figure 33. PDA chromatograms at 325 nm of a) 5-CQA 1 mM in water before UV irradiation, b) 5-CQA 1 mM in water after UV irradiation for 1 h. (1) 5-CQA (2) *cis*-5-*O*-caffeoylquinic acid

Data from *L. reuteri* extract were then subjected to MZMine 2.53 and further treatment in the Python v3.7 library matchms v0.9.2, for molecular networking analysis (Figure 34) where each tandem mass spectrum is described as a node and spectrum-to-spectrum alignments as edges defined by a cosine score ( $\cos$ ) (<http://gnps.ucsd.edu>). A high  $\cos$  means high similarity between two MS/MS spectra. The names of identified metabolites derived from 5-CQA were given in accordance to recent recommendations (Kay et al., 2020). Nevertheless, CA was preferred than 3',4'-dihydroxycinnamic acid for a better reading. The created molecular network (MN) highlighted 110 nodes. One cluster encompassed the majority of connected nodes and 45 nodes corresponded to self-loops, which represent unique feature. Specific biotransformed metabolites appeared in all the clusters but mostly as self-looped nodes. We then annotated MN by dereplication using GNPS library. Green nodes depicting metabolites derived from 5-CQA bioconversion by *L. reuteri* and showing a  $\cos > 0.9$  were displayed in Figure 34. Among them *cis*-CA, *trans*-CA, coumaroyl quinic acid and esculetin caught our attention linked to their structure close to 5-CQA. At this stage, we must underline that MN analysis led to the annotation of two nodes (labelled by an asterisk in Figure 33) and found either simultaneously in 5-CQA sample and in one biotransformed extract or in SN *L.*

*reuteri* biotransformed extract alone) as quinic acid after dereplication but with a very low score ( $\cos = 0.4$ ) (data not shown). CA and coumaroyl quinic acid have been already reported after 5-CQA in vitro biotransformation by gut microbiome (Mortelé et al., 2019; Tomas-Barberan et al., 2014).

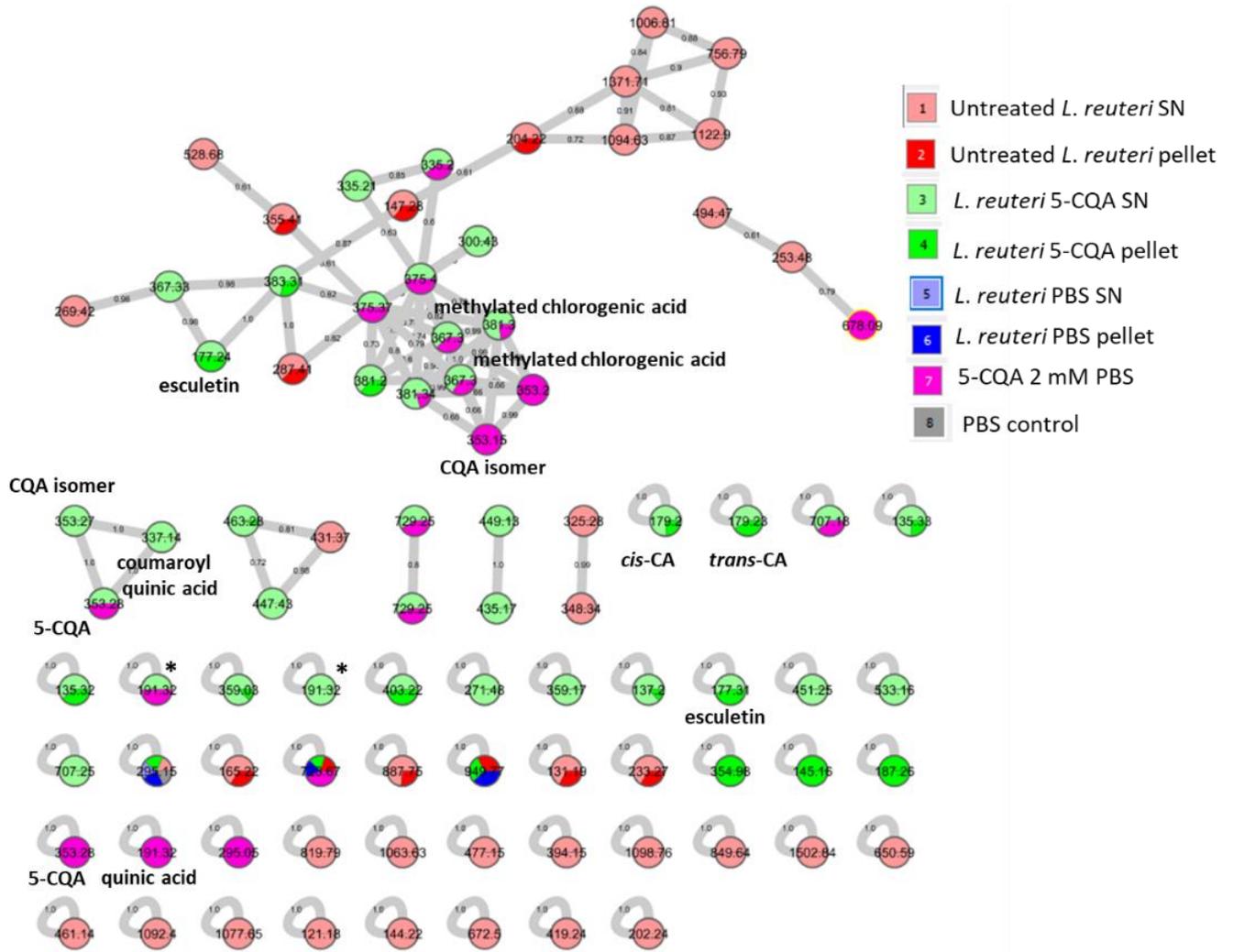


Figure 34. Molecular network analysis in negative mode of extracts and samples. 1 and 2: pellet and SN extracts from *L. reuteri* incubated in medium; 3 and 4: pellet and SN 5-CQA biotransformed extracts from *L. reuteri*; 5 and 6: pellet and SN extracts from *L. reuteri* incubated in PBS; 7: 5-CQA incubated at 37°C with PBS; 8: PBS control. Only nodes specific from biotransformed extracts (green color) with  $\cos > 0.9$  and related to 5-CQA structure were annotated

To validate some of these annotations, various analytical standards were injected as depicted in Table 5 and led to the identification of 5-CQA (ID 3), esculetin (ID 4) and *trans*-CA (ID 5). Surprisingly, both *cis* and *trans* geometric isomers of CA were identified in MN. To our knowledge, it is the first report of the presence of these two CA isomers after 5-CQA biotransformation either by bacteria or human gut microbiota. Recently Clifford expressed that only few studies were reported on metabolism of *cis* isomers in humans (Clifford et al., 2020). It is to note that MN analysis identified *cis*-CA at RT: 21.5 min highlighting a misidentification of this standard in GNPS library. Our results concerning the attribution of ID 5 and 8 as respectively *trans*-CA and *cis*-CA are in line with those previously published (Oniszczyk, 2016). The main point to highlight in our MN (Fig. 2) is the annotation of  $[M - H]^-$  ions at  $m/z$  177 as being esculetin. While Mortelé et al. (Mortelé et al., 2019) attributed this ion as caffeoyl quinone, we confirm the identification of esculetin using analytical standard (Table 5). Esculetin was reported only once time as an *in vivo* metabolite of *trans*-CA after oxidation through an isolated rat liver (Gumbinger et al., 1993). Our result underlines for the first time this specific mechanism by *L. reuteri* DSM 20016 strain leading to the formation of a coumarin, esculetin, after ring closure of *cis*-caffeoyl quinone, via an interesting oxidative pathway of 5-CQA.

Table 5. LC-DAD-ESI-MS<sup>2</sup> metabolites identified or detected in SN extract after 5-CQA biotransformation by *L. reuteri*.

| ID n° | Compound name   | RT (min) | m/z   | MS <sup>2</sup> |
|-------|---|----------|-------|-----------------|
| 1     | 3-Hydroxybenzoic acid <sup>a</sup>                          | 15.1     | 137.2 | 109 / 93        |
| 2     | Quinic acid <sup>d</sup>                                    | 19.3     | 191.3 | nd              |
| 3     | 5 CQA <sup>b,c</sup>  | 19.3     | 353.3 | 191 / 179 / 135 |
| 4     | Esculetin <sup>b,c</sup>                                    | 20.2     | 177.2 | 133 / 104       |
| 5     | <i>trans</i> -Caffeic acid <sup>b,c</sup>                   | 21.5     | 179.2 | 135             |
| 6     | Vinylcatechol <sup>d*</sup>                                 | 21.5     | 135.3 | nd              |
| 7     | 7-Phenylacetic acid <sup>d*</sup>                           | 23.4     | 135.3 | nd              |
| 8     | <i>cis</i> -Caffeic acid <sup>c</sup>                       | 23.5     | 179.2 | 135             |
| 9     | <i>cis</i> -5- <i>O</i> -Caffeoylquinic acid <sup>c,e</sup> | 23.6     | 353.3 | 191 / 179 / 135 |
| 10    | Quinic acid <sup>c</sup>                                    | 23.6     | 191.3 | nd              |
| 11    | Coumaroyl quinic acid <sup>c</sup>                          | 25.5     | 337.1 | 191 / 163       |
| 12    | 3-(3'-hydroxyphenyl)propanoic acid <sup>d</sup>             | 28.3     | 165.2 | nd              |
| 13    | Methylated chlorogenic acid                                 | 35.7     | 367.3 | nd              |
| 14    | Methylated chlorogenic acid                                 | 39.7     | 367.3 | nd              |

<sup>a</sup>. Not identified; <sup>b</sup>. Identified after injection of analytical standards; <sup>c</sup>. Identified by MN analysis; <sup>d</sup>. Detected in MN. <sup>e</sup>. Identified after comparison with data previously published; \* Metabolites which RT could be inverted. Quinic acid comes from fragmentation of 5-CQA or *cis*-5-*O*-caffeoylquinic acid in the source.

A putative 5-CQA biotransformation pathway by *L. reuteri* leading to esculetin is shown in Scheme 4. Due to the presence in the standard of all the two geometric 5-CQA isomers (ID 3 and 9), the first step could be, after ester cleavage, a geometric isomerization of *trans*-CA into its *cis* isomer which was already reported by photoisomerization (**Parrino et al., 2016**). This evidence could be supported by the detection of *cis*-CA (ID 8) and suppose the presence of a *cis*-*trans* isomerase in *L. reuteri* enzymatic machinery. This kind of enzyme has been already reported in *Lactobacillus* (**Kishino et al., 2013; Sun et al., 2020**) and after genomic comparison an ortholog sequence in *L. reuteri* studied strain (DSM 20016, GCA\_001436151) was found (Figure 35). The other possibility of the presence of *cis*-CA could come from hydrolysis of the residual *cis*-5-*O*-caffeoylquinic acid (ID 9) by cinnamoyl esterase. Similarly with the process of oxidative cyclization of *cis*-CA to esculetin after photocatalytic isomerization proposed previously (**Parrino et al., 2016**), the next step corresponds to the formation of caffeoyl quinone probably by an oxidase as the biotransformation was done under anaerobic conditions. Previous studies have reported the enzymatic conversion of *cis* CA into esculetin catalysed by an oxidase, previously named phenolase (**Sato et al., 1996; Satô, 1967**) for which we have found a sequence similarity with an ubiquinol oxidase in the genome of *L.*

*reuteri* DSM 20016 (Figure 36). The final step of ring closure can occur putatively spontaneously followed by classical re-aromatisation to afford esculetin.

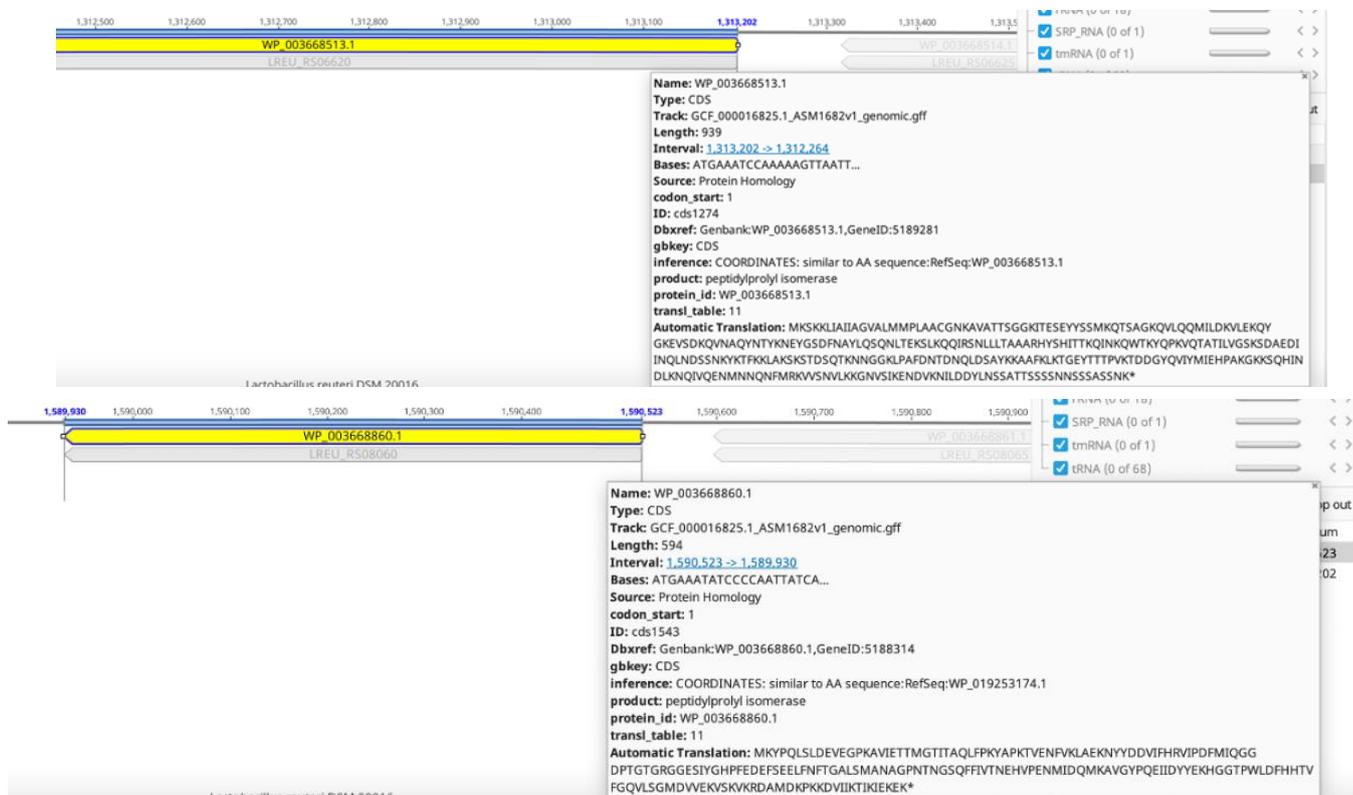
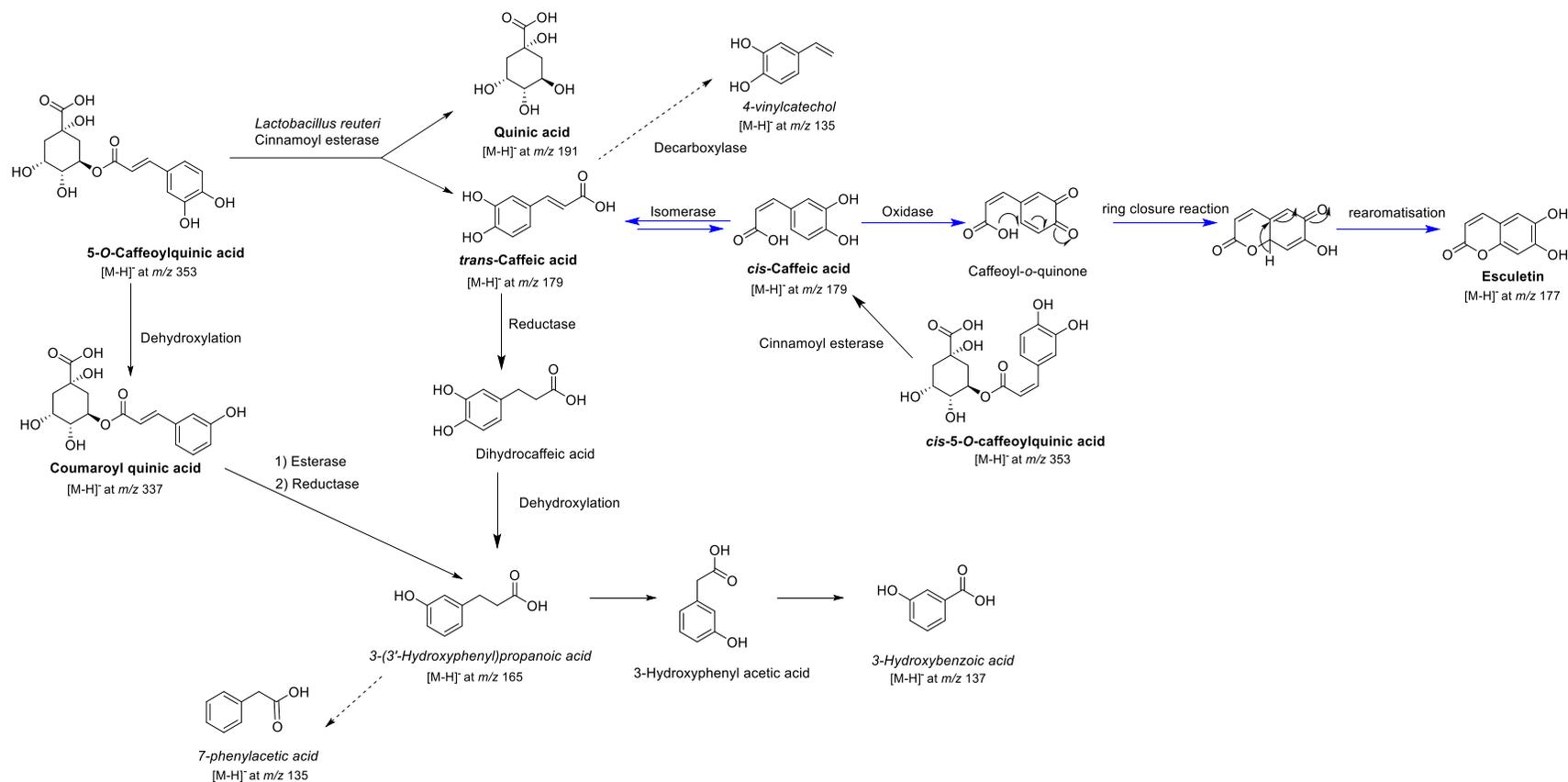


Figure 35. Genomic identification of peptidylprolyl isomerases (ortholog sequence of *cis-trans* isomerase EC 5.2.1.8) in *L. reuteri* strain (DSM 20016, GCA\_001436151)

|                |        |        |      |         |   |
|----------------|--------|--------|------|---------|---|
| WP_003667598.1 | 564223 | 565239 | 1017 | forward | cytochrome d ubiquinol oxidase subunit II |
| WP_011953418.1 | 562804 | 564222 | 1419 | forward | cytochrome ubiquinol oxidase subunit I    |

Figure 36. Genomic identification of cytochrome bd ubiquinol oxidase with two subunits I and II (EC 7.1.1.7) in *L. reuteri* strain (DSM 20016, GCA\_001436151)



Scheme 4. Putative biotransformation pathway of 5-CQA by *L. reuteri*. Black arrows: biotransformation already reported; blue arrows: putative mechanism of formation of the identified esculetin; hashed arrows: two possible pathways for ion at *m/z* 135. Compounds marked in bold were identified using analytical standards or by MN and in italic were detected and putatively identified by comparison data with previous results.

It is interesting to cite that Parrino et al. (Parrino et al., 2016) reported the no formation of esculetin at pH 3.5 in comparison to the possible conversion with pH > 6.30. These data suggest that esculetin can not be formed in stomach. To confirm the ability of *L. reuteri* to produce esculetin from CA after 5-CQA biotransformation, we used BHI instead of PBS at pH 5.8 in order to prevent CA autooxidation. This phenomenon was detected after CA incubation at 37°C in PBS at 5.8 but in a lesser degree than in biotransformed extracts (see Figure 37). CA autooxidation has not been checked so far in previous studies of 5-CQA and CA biotransformation. In our study, we confirm that *L. reuteri* was able to produce esculetin either from 5-CQA and CA in higher amount when compared to any controls. Moreover, the absence of *cis*-5-*O*-caffeoylquinic acid (peak 3) after 5-CQA incubation in BHI (Figure 37) confirm the major formation of *cis*-CA by *L. reuteri* from isomerisation of *trans*-CA.

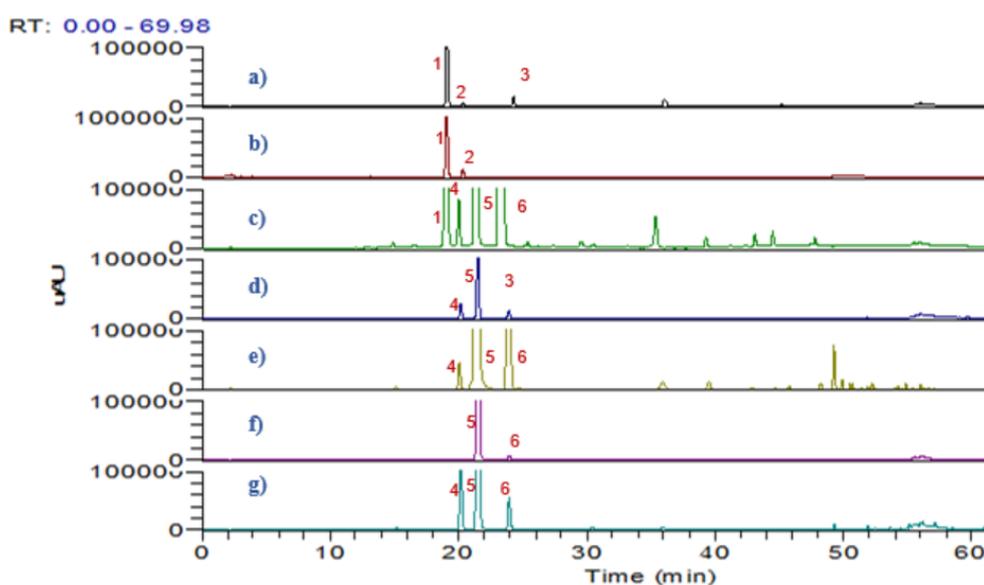


Figure 37. PDA chromatograms at 320 nm of a) 5-CQA incubated with PBS, b) 5-CQA incubated with BHI, c) SN extracts after 5-CQA biotransformation by *L. reuteri* in PBS, d) pellet extracts after 5-CQA biotransformation by *L. reuteri* in BHI, e) CA incubated with PBS, f) CA incubated with BHI, g) pellet extracts after CA biotransformation by *L. reuteri* in BHI. (1) 5-CQA, (2) isomer of 5-CQA (3) *cis*-5-*O*-caffeoylquinic acid, (4) esculetin, (5) *trans*-CA, (6) *cis*-CA.

Other green color nodes, displayed in Figure 34, could be highlighted as tentative metabolites coming from 5-CQA biotransformation by *L. reuteri* by comparison to m/z data previously reported. Regarding the node at m/z 165.2, it was annotated in our MN as phenyllactic acid with a cos = 0.84 and as caffeoyl alcohol with a cos = 0.72. While Mortelé et

al. (**Mortelé et al., 2019**) reported a metabolite with equivalent m/z ratio (165.0556) as 3-phenyllactic acid, other studies (**Gonthier et al., 2006; Li et al., 2021; Vollmer et al., 2017**) depicted this compound as 3-(3'-hydroxyphenyl) propanoic acid coming from in vivo metabolism of 5-CQA by fecal microbiota. This compound could come either from CA after reduction into 3-(3',4'-dihydroxyphenyl) propanoic acid then dihydroxylation (**Vollmer et al., 2017**) or from coumaroyl quinic acid, one of the metabolites identified in our MN, after reduction and ester bound hydrolysis (**Tomas-Barberan et al., 2014**) (Figure 34). This metabolite could be an intermediate leading to the formation of 3-hydroxybenzoic acid, already depicted in a recent publication (**Li et al., 2021**) describing the metabolism study of 5-CQA in rat.

The authors reported the identification of this metabolite by UHPLC-Q-TOF-MS in negative mode with a m/z at 137.023 ( $MS^2$ : 136, 109, 93) which seems similar to our  $[M - H]^-$  ion at m/z 137.2 in regard to their fragment ions (Table 5). Moreover, hydroxybenzoic acids were already reported from gut microbiota conversion of 5-CQA and CA (**Gonthier et al., 2006**) and confirm this pathway. MN annotation led also to the identification of two deprotonated molecular ions with m/z at 367.3 and at RT 35.7 and 39.7 min as already reported methylated chlorogenic with a  $\cos = 0.71$  confirming the possible methylation of 5-CQA in our study. Nevertheless, these two ions were also detected in 5-CQA sample dissolved in PBS suggesting that these compounds are artefacts. The formation of 5-CQA isomers during the incubation at 37°C was previously highlighted (**Tomas-Barberan et al., 2014**). Finally the two  $[M - H]^-$  ions at m/z 135.3 present in the two pellet and SN *L. reuteri* biotransformed extracts, could be putatively either vinylcatechol (**Li et al., 2021**) coming from decarboxylation of CA or 7-phenylacetic acid (**Tomas-Barberan et al., 2014**). Nevertheless, we were not able to determine their MS/MS product ions to confirm one of these two possibilities.

*b) Characterization of oxidized compounds of 5-CQA and CA by electrochemical and enzymatic methods*

For a better understanding of the oxidative biotransformed pathway leading to the esculetin formation, we have performed electrochemical and enzymatic oxidation of 5-CQA and CA.

*b-1) Electrochemical studies of 5-CQA and CA*

The electron transfer properties of the two dihydroxycinnamic acids, 5-CQA and CA, were investigated by cyclic voltammetry (CV) in 0.1 M PBS at pH 5.8, similar conditions to those of biotransformation studies. As expected, for both compounds, the ortho-diphenol function is oxidized in a two-electron-proton mechanism to form an ortho-quinone (visible on the anodic scan at  $E_{p_a} = +0.55$  V and at  $E_{p_a} = +0.38$  V, respectively for 5-CQA and CA), which is reduced back to the catechol form on the reverse cathodic scan ( $E_{p_c} = +0.11$  V and  $E_{p_c} = +0.14$  V, respectively for 5-CQA and CA) (see Figure 23 as previously reported). However, here, the system is not electrochemically reversible indicated by the large potential peak separation ( $E_{p_a} - E_{p_c}$ ) and by the current ratio  $i_c/i_a$ , which is below 1, attesting the instability of the orthoquinone. The electrochemical oxidation mechanisms of dihydroxycinnamic acids have been well described and Hapiot et al reported that the CV electrochemical parameters show that the caffeoyl quinone becomes less stable from pH 4, even at the timescale of CV (Giacomelli et al., 2002; Hapiot et al., 1996). Then, controlled potential electrolysis of 5-CQA and CA were achieved in pH 5.8 0.1 M PBS in a modified UV cell at a platinum grid electrode. As a first step, UV-Visible spectra were recorded during the linear sweep voltammetry LSV at different potential values. Up to +0.75 V and +0.52 V, 5-CQA and CA remained under their native form and the wavelength of maximum absorption  $\lambda_{max}$  were measured at 320 nm. Above these potentials, an absorption band was visible around 400 nm for both compounds, attesting their oxidation to their quinone form. With this knowledge, the electrolysis of 5-CQA and CA were conducted at +0.8 and +0.6 V, respectively. The electrolysis was stopped after 20 minutes (corresponding to an electrolysis yield of 15%), a time for which the quinone is still present in solution ( $\lambda_{max} = 404$  and 420 nm for 5-CQA and CA quinone respectively). All the electrolyzed samples were then analysed by LC-DAD-ESI-MS<sup>2</sup>.

*b-2) Comparison of LC-DAD-ESI-MS<sup>2</sup> of biotransformed, electrolyzed and enzymatic samples.*

CQA and CA were submitted to an enzymatic oxidation by PPO (**Cheyrier and Moutounet, 1992a**) and directly analysed by LC-DAD-ESI-MS<sup>2</sup>. These data were compared to those of 5-CQA biotransformed and CA electrolyzed samples (Table 6). Esculetin was each time identified independently of the method used underlining the stabilization of the oxidative CA product, caffeoyl quinone, into its coumarin form, but in far lesser amounts compared to the biotransformation process. Unsurprisingly, no traces of caffeoyl quinone were found in any samples studied.

Table 6. Comparison of spectral patterns of esculetin formed using different methods

| Study method   | Compound name | RT (min) | <i>m/z</i> | MS <sup>2</sup> | $\lambda_{\max}$ (nm) |
|--|---------------|----------|------------|-----------------|-----------------------|
| Oxidation of CA by PPO   | Esculetin     | 20.3     | 177.2      | 133             | 343/301/291           |
| Electrochemical oxidation of CA                                | Esculetin     | 20.2     | 177.2      | 133             | 342/301/295           |
| Biotransformation of 5-CQA by <i>L.reuteri</i> - (Supernatant) | Esculetin     | 20.2     | 177.3      | 133/104         | 343/300               |
| Biotransformation of 5-CQA by <i>L.reuteri</i> (Pellet)        | Esculetin     | 20.2     | 177.2      | 133/104         | 344/298/252           |

The value of  $\lambda_{\max}$  were in accordance to those in previous report (**Tattini et al., 2014**). Analysis of electrolyzed and enzymatic sample of 5-CQA did not lead to the detection of esculetin confirming that the first step of production of this coumarin is the cleavage of bond ester which was not permitted under electrochemical and PPO conditions. Moreover, the comparison of LC-DAD-ESI-MS<sup>2</sup> analysis of the oxidized samples of 5-CQA and CA, obtained electrochemically and enzymatically, has demonstrated that no other oxidative pathway than the one leading to esculetin occurs by biotransformation. Indeed, for example for 5-CQA, the oxidation by the electrode or by the PPO also involves dimerization pathways. Interestingly, the LC-MS/MS profiles of the electrolyzed and enzymatic solutions of 5-CQA

were very similar, leading to dimers involving mechanisms described by Guyot et al. (Guyot et al., 2008). The oxidation of CA enzymatically also led to dimers and trimers formation (Cheynier and Moutounet, 1992a; Pati et al., 2006).

### II.B.2.3. Conclusions

In summary, 5-CQA was differently biotransformed following the gut bacteria strains used. Molecular network analysis led to the identification, after 5-CQA biotransformation by *L. reuteri*, of one oxidized derivative of cis-CA, caffeoyl quinone, which was stabilized into esculetin its ring closure form. The inability of the two other studied gut bacteria to biotransform 5-CQA supports one more time the importance of interindividual differences of microbiota composition (Frolinger et al., 2019). The comparison with other oxidation methods such electrochemical and enzymatic ones confirm the formation of caffeoyl quinone followed by a ring closure into esculetin. The electrochemical and enzymatic studies also led to show that no other oxidation products of 5-CQA and CA were formed during biotransformation. These findings put also on the spotlight the interesting and specific enzymatic machinery of *L. reuteri* encompassing a cinnamoyl esterase, isomerase and oxidase leading to a coumarin compound. Further studies should be undertaken with the aim to identify the potential biological effects of esculetin on gut-microbiota axis and will give more insights whether this metabolite is more active than the parent one, an evidence that we have already demonstrated with olive oil phenol compounds (Lambert de Malezieu et al., 2019). Chlorogenic acids formulated as synbiotics (Swanson et al., 2020) with *L. reuteri* may be finally promising candidates in human health application.

### II.B.3. Biotransformation of 5-CQA by co-culturing *in vitro* two gut bacteria, *L. reuteri* and *B. fragilis*

The first set of questions aimed to identify the capacity of individual gut bacteria to biotransform 5-CQA which is specific to one species. Considering the complex interactions between bacteria in the gut, we studied the biotransformation of 5-CQA when two bacteria, *L.*

*reuteri* and *B. fragilis*, were co-cultured. Such studies will provide information on the potential influence of bacterial interactions on the community during biotransformation.

### II.B.3.1. Context of the study

According to the literature, the implications of the gut microbiota in health and disease nowadays is unquestionable (Cani, 2018; Codagnone et al., 2019; de Vos et al., 2022; Ding et al., 2019; Fan and Pedersen, 2021; Schächtle and Rosshart, 2021). It is well established that gut microbiota as a dynamic ecosystem communicates constantly with the host, providing the gut homeostasis (Bäckhed et al., 2005; Maynard et al., 2012; Perry and Arsenault, 2022). Indeed, the interaction network in the gut involves the triangle of: commensal–pathogen, commensal–host; and pathogen–host interactions (Khan et al., 2021). Most studies have focused on gut microbes and their host interactions and their outcomes related to health and diseases (Ayres, 2016; Yongyan Chen et al., 2021; Horne et al., 2019; Parker et al., 2018; Tsois and Bäuml, 2020). However, little is known about the complex interplay between bacteria in the gut microbial community (Khan et al., 2021). Notably, interaction patterns mainly include competition for nutrients and space, followed by exchange of nutrients, enzymes, and genetic materials (Kern et al., 2021) and/or the capacity of co-aggregation and involvement in biofilm formation (Palencia et al., 2022). Various modes of interaction there have been described among microorganisms in the gut, such as mutualism, which is a win-win interaction that benefits both participants; commensalism, where the commensal benefits without the partner being helped or harmed. On the other hand, negative interactions could be present such as amensalism, where one organism can inhibit or destroy the other; competition, where both the participants compete for the same resources; and parasitism, corresponding to a relationship in which one partner benefits from the other microorganism (Faust and Raes, 2012).

Remarkably, in terms of bacteria-bacteria interactions, commensal bacteria regulate pathogen colonization and activity, thus preventing pathogenic infections. This is mainly mediated by colonization resistance as well as the ability to inhibit pathogen adhesion to human cells. The colonization resistance consists directly by various ways such as nutrient and territory competition, toxic substances, and gut metabolites production (e.g., through commensal–pathogens/pathobionts interactions), or indirectly by modulating immune host functions and

expression of virulence factors (**Bäumler and Sperandio, 2016; Khan et al., 2021; Vogt et al., 2015**). For example, Gharbi et al. (2018) have shown that *Lactobacillus fermentum* and *Lactobacillus plantarum*, isolated from the human microbiota, were able to reduce the adhesion to HT29 cells of the pathogens such as *Escherichia coli*, *Cronobacter sakazaki* and *Salmonella enterica* (**Gharbi et al., 2018**).

Besides that, recently, specific interactions such as cross-feeding and quorum sensing have received increasing attention in bacterial interaction studies (**Jo et al., 2021**). Quorum sensing is a chemical communication between bacteria, in which individual bacteria in a community, coordinate their behavior in response to environmental changes of density and composition (**Mukherjee and Bassler, 2019**). Thus, depending on the circumstances, bacteria modify metabolic pathways, the pattern of virulence factors and/or the capacity of biofilm formation by self-regulating the expression of relevant genes (**Prescott and Decho, 2020**).

The interaction between bacterial strains, in which the metabolic by-products or end-products produced by one species can be nutrient resources and used by others, is termed cross-feeding. Cross-feeding is a specific form of mutualism and can be mediated via several forms including metabolites, substrates, mutual and augmented cross-feeding as depicted in Figure 38 (**Smith and Wissel, 2019**).

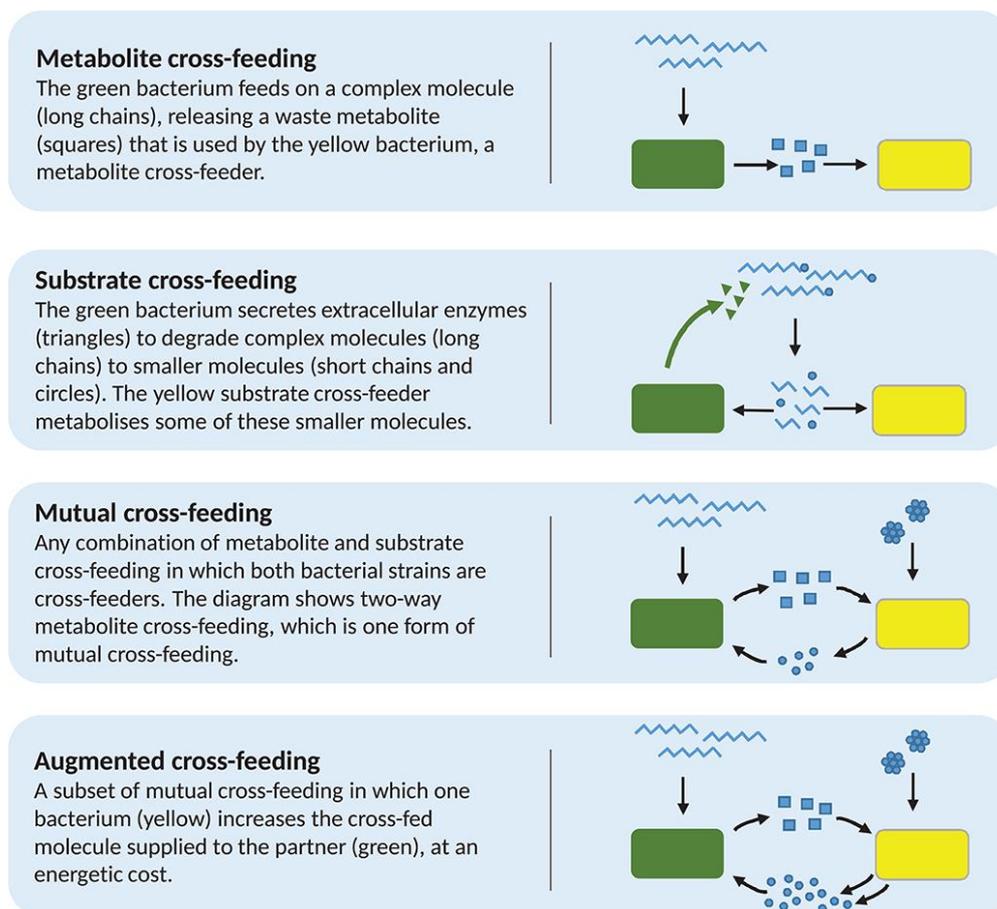


Figure 38. A schematic illustration of cross-feedings, including metabolite, substrate, mutualism, and augmented forms (Smith et al., 2019)

Some studies have investigated the cross-feeding process of the *Bifidobacterium* genus and butyrate-producing bacteria (Belenguer et al., 2006; Falony et al., 2006). Therefore, investigations of all aspects of teamwork in the gut, will help to better understand of the impact of thegut microbiota on human health (Faust et al., 2012; Kern et al., 2021).

Although most studies have focused on the above-mentioned bacterial interactions in the gut, there is lack of data on the influence of bacterial communities on biotransformation process. Most *in vitro* studies relied on conversion by single bacteria, as we have carried out in the chapter II.B.2., or using fecal microbiota (Aura, 2008; Tomas-Barberan et al., 2014). Indeed, the identification of specific gut microbes involved in the biotransformation of polyphenols into bioactive compounds, is crucial for a better understanding of the favorable impact of polyphenols on health. However, since the gut microbiota is a dynamic ecosystem, microorganisms do not function independently, but interact with each other and with the host. To move to these types of strategies and use more practical models to understand the bacterial

community at the laboratory scale, the cultivation of bacteria in the community in the form of co-cultures is used. Indeed, the complex cultivation of bacteria in co-culture and even isolation of gut bacteria in general, have impacted the limitations and available information related to this. Additionally, there is growing interest in co-culture engineering in the field of natural product discovery (**Scherlach and Hertweck, 2009**).

Although co-cultures still have limitations, especially in the case of anaerobic bacteria, they are a very useful approach to identify *in vitro* metabolic interactions between gut bacteria.

## **Hypothesis**

We hypothesized that cross-feeding could be involved in the biotransformation process. We have planned to investigate an anaerobic bacterial co-culture as a model of study based on the use of the two gut bacteria *L. reuteri* and *B. fragilis*. Based on our previous results, the cinnamoyl esterase capacity was only expressed by *L. reuteri*, and we questioned whether interactions between these bacteria can influence the biotransformation process. Indeed, we performed some studies by co-culturing *L. reuteri* and *B. longum*. However, because the former colonies were similar, it was difficult to distinguish them and we were not able to perform this study in a good manner because the time schedule of the thesis.

### II.B.3.1. Results and discussion

With the goal to give information on bacterial interactions in the gut community and their effects on the biotransformation of 5-CQA, we performed the resting cell biotransformation study using a co-culture of *B. fragilis* and *L. reuteri*. As a first stage, the two selected strains were co-cultured, for 30 hours ((corresponding to the stationary phase (data not shown)), with an equivalent number of bacteria, based on CFU determination at  $T_0$  as  $7.60 \times 10^7$  CFU.mL<sup>-1</sup> and  $8.14 \times 10^7$  CFU.mL<sup>-1</sup> for *L. reuteri* and *B. fragilis*, respectively. In parallel, the two bacteria were grown alone, which helped us to compare their culture evolution profile with those obtained in the co-culture. Then, we evaluated the impact of interactions between the two bacterial species on the biotransformation of 5-CQA. As described previously, biotransformation was performed using the resting cell method. When the stationary phase was reached (at  $T_{30}$ ), 5-CQA (1 mM) was added on the pellet obtained after co-culture or mono-

culture (*L. reuteri* alone). With all negative and blank controls, they were then incubated under anaerobic conditions using sealed jars and anaerobic bags for 24 h. Finally, the supernatant and pellet were separated and treated with ethyl acetate to obtain two extracts (SN and pellet) which were further subjected to LC-UV-ESI-MS<sup>2</sup>. Herein we present the results obtained on SN extracts as the metabolites are less concentrated in pellet ones. First studies have been undertaken and should be further investigated.

a) Culture evolution profile of *L. reuteri* and *B. fragilis* in co-culture

Firstly, we have analysed the interaction between *L. reuteri* and *B. fragilis* from a growth perspective. Their culture evolution in mono and co-culture, are presented in

Figure 39 .

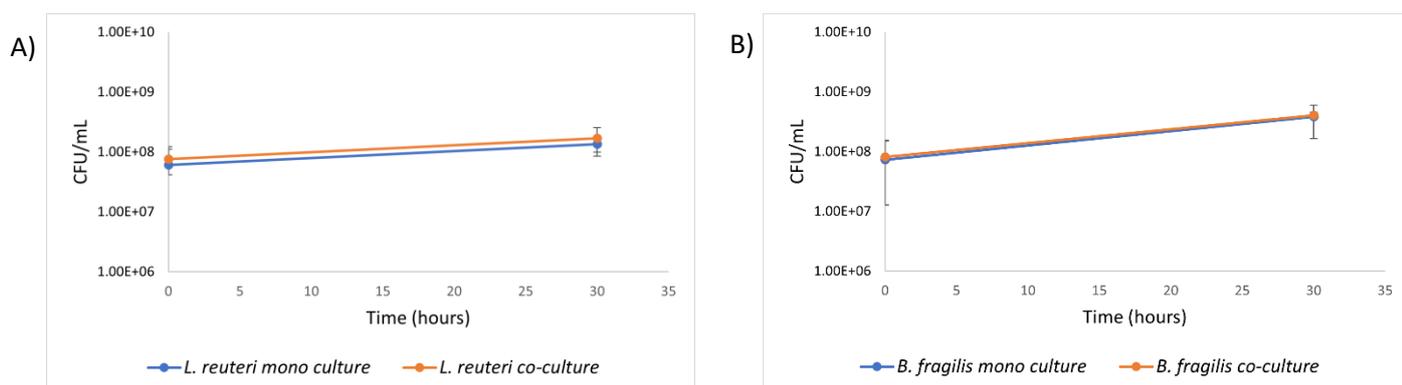


Figure 39. (A): Number of bacteria (CFU.mL<sup>-1</sup>) versus time: (A): culture evolution of *L. reuteri* in mono and co-culture; (B) *B. fragilis* in mono and co-culture; (C)

At T<sub>0</sub>, *L. reuteri* cultured alone was present at 6.05 x 10<sup>7</sup> CFU.mL<sup>-1</sup> (corresponding to OD<sub>600nm</sub> = 0.75) and in co-culture at 7.60 x 10<sup>7</sup> CFU.mL<sup>-1</sup>. As depicted in , *L. reuteri* and *B. fragilis* showed the same growth pattern in mono and co-culture during 30 h.

b) Impact of bacterial interactions on the biotransformation of 5-CQA

LC-MS<sup>2</sup> analysis of 5-CQA biotransformation by a co-culture of *L. reuteri* and *B. fragilis* compared to that by *L. reuteri*, highlighted the production of the same metabolites

(Figure 40), including, *trans*-CA (with RT: 21.5 min), esculetin at RT 20.2 min, *cis*-CA (RT: 23.5) and *cis*-5-CQA at 19.3 min but in a different ratio.

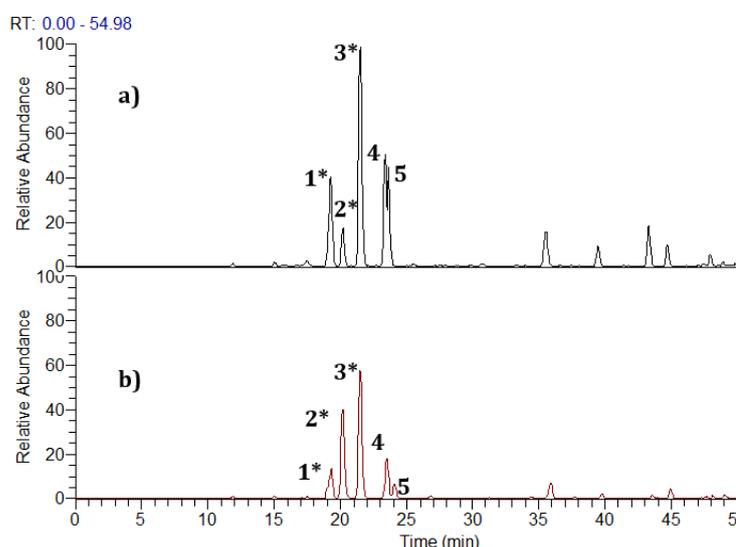


Figure 40. Base peak chromatograms in negative mode of **a)** 5-CQA biotransformation by *L. reuteri*: **b)** 5-CQA biotransformation by a co-culture of *L. reuteri* and *B. fragilis*. **(1)** 5-CQA, **(2)** esculetin **(3)** *trans*- CA, **(4)** *cis*-CA, **(5)** *cis*-5-CQA. \*Compounds with retention time (RT) confirmed after injection of analytical standards

Moreover, a calibration curve was determined for 5-CQA, CA and esculetin by LC-MS<sup>2</sup> and was used to estimate the concentration of each metabolite in both SN extracts, obtained after mono and co-culture. The calibration curves (

Figure 41) were linear in the of range from 2.5 µg/mL to 50 µg/mL, for 5-CQA and esculetin, with R<sup>2</sup> 0.9998 and R<sup>2</sup> 0.9968, respectively. The values for CA showed lower linearity with R<sup>2</sup> 0.9844 as depicted in

Figure 41.

The concentrations of metabolites found in each extract are shown in

Figure 42. We have observed that not equivalent amount of 5-CQA, CA and esculetin were formed when the two modes of biotransformation were compared, in mono or co-cultures. Interestingly, esculetin was found at higher amounts (438.4 µg/mL) in SN extracts after co-culture of *L. reuteri* and *B. fragilis* compared to esculetin formed when *L. reuteri* is alone (353.9 µg/mL) corresponding to a 23.9 % of increasing. Contrary, *trans*-CA was found to be present in lower amount (2220.0 µg/mL) in SN extracts from co-culture in comparison to CA

produced by *L. reuteri* in monoculture (4687.9  $\mu\text{g}/\text{mL}$ ), corresponding to a decrease of 52%. Indeed, this is a logical correlation with esculetin findings, since the majority of esculetin comes from *trans*-CA, after subsequent transformation as we described previously (see Scheme 4). In fact, CA was the major metabolite after biotransformation, in both mono and co-cultures (

Figure 42). Moreover, we can observe on the chromatograms (see Figure 40), a significant decrease of the *cis* form of both CA and 5-CQA, in the co-culture biotransformation study. Nevertheless, we can not give a precise concentration because they are not available as analytical standards in their *cis* form. In the same line, with *trans*-CA, 5-CQA was less present in co-culture SN extracts when compared to those of monoculture. Finally, the yield of conversion of 5-CQA into metabolites was slightly higher in the co-culture (89.7%) than in the monoculture (83.6%).

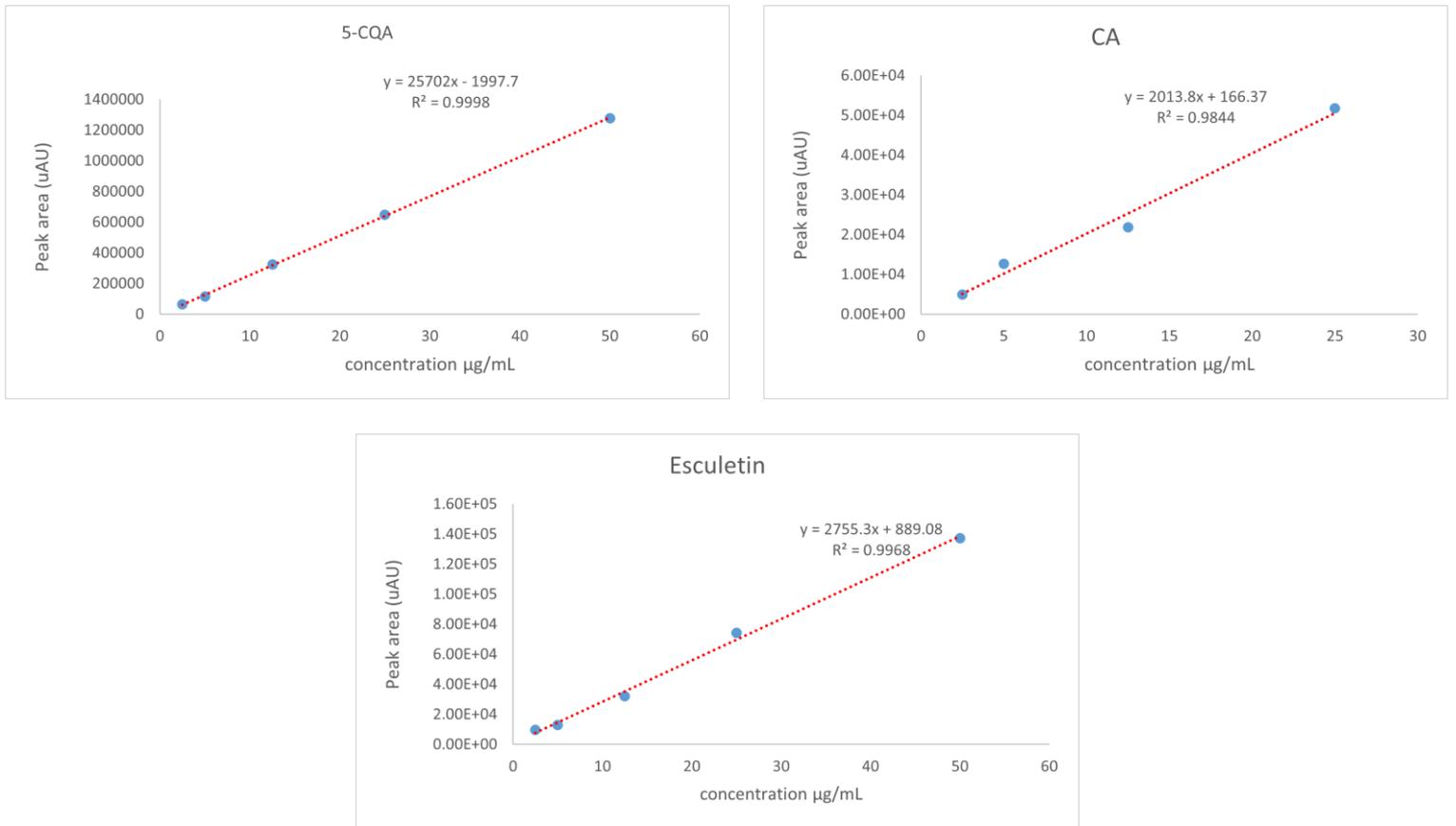


Figure 41. Calibration curves for 5-CQA, CA and esculetin in MeOH

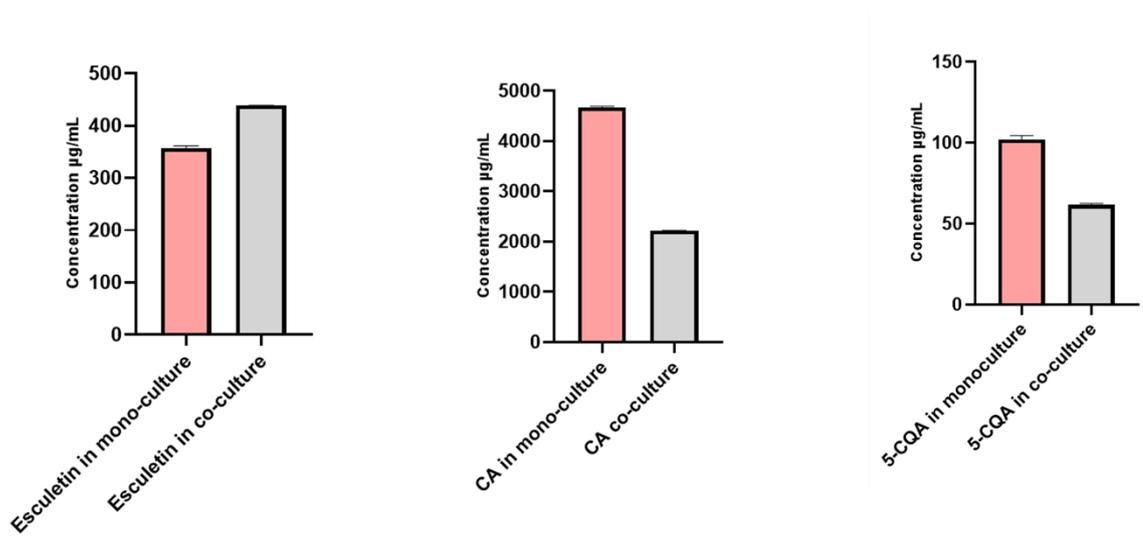


Figure 42. 5-CQA, CA, esculetin content (µg/mL) in SN extracts from mono and co-culture

From a growth perspective, the presence and/or metabolites of bacteria do not seem to play a role in their growth. Furthermore, this study highlighted no detection of any new metabolite, which may come from trophic networking between bacteria. The increase of esculetin in co-culture could be explained by cross-feeding process, when somehow *B. fragilis* helped its formation. For example, it could contribute to the production of its intermediate, *cis*-CA, and its oxidised form, as this species possesses the same type of isomerase and oxidase found in *L. reuteri*. Another hypothesis could be that *B. fragilis* provide to *L. reuteri* a source of energy, which could help this last strain to increase its biotransformation ability.

Herein, we highlight a particular pattern of 5-CQA biotransformation between the two bacteria studied in co-cultures (*B. fragilis* and *L. reuteri*) in comparison to *L. reuteri* alone. Interestingly, the formation of esculetin was higher in the co-culture biotransformation than when biotransformation was carried out only by *L. reuteri*. Contrary, *trans*-CA, and *trans*-5-CQA showed significant decreases in their amounts.

In perspective, we would like to conduct further studies using co-culture of the three bacteria studied (*L. reuteri*, *B. fragilis* and *B. longum*) to evaluate their potential impact on the formation of 5-CQA bioconverted metabolites. Besides the fact that bacteria metabolize polyphenols, one the other hand, they may modify the composition and function of gut bacteria. For this purpose, we investigated the impact of 5-CQA supplementation on the growth and metabolism of all strains studied.

## **II.C. Supplementation studies with 5-CQA**

### **II.C.1. Polyphenols: in frontiers of prebiotics**

Gut microbiota is involved in several functions of the human body. Among others, it provides essential nutrients and bioactive metabolites which are involved in host biology processes, including the production of (SCFAs), synthesis of vitamins and amino acids, biotransformation of dietary components (**Adak and Khan, 2019**). In addition, it is considered as a key node in the communication between gut and brain. Moreover, numerous studies have documented significant changes of gut microbiota composition and function associated to brain

diseases and other conditions such as obesity, diabetes, non-alcoholic liver diseases, cardio-metabolic diseases (**Cani, 2018; Cryan et al., 2020**) as already discussed before.

Given all of this, it is important to investigate how to regulate the microbiota composition and function as a potential approach to alleviate symptoms, and positively influence the treatment of neurodegenerative diseases (**Di Meo et al., 2018; Filosa et al., 2018b**). Indeed, the dietary patterns are one of the main factors shaping the gut microbiota (**Lombardi et al., 2018; Zmora et al., 2019**). Moreover, the link between diet, gut microbiota and health, including good mental health and positive impacts on brain diseases has been demonstrated (**Berding et al., 2021**). Furthermore, there is increasing awareness of the implications of eating habits on well-being (**Watson, 2020b**). Therefore, through diet and nutrition, scientific investigations aim to improve mood and mental health, enhance cognitive ability and have a positive effect on brain diseases (**Adan et al., 2019**).

In the context of diet, accumulating data suggest that polyphenols represent a more promising nutraceutical strategy than can beneficially reshape gut microbiota (**Bolte et al., 2021; Gutiérrez-Díaz et al., 2020; Plamada and Vodnar, 2021**), which is not surprising given highly concentrated dietary bioactive components possessing antioxidant properties (**Silva and Pogačnik, 2020; Tresserra-Rimbau et al., 2018**). As already mentioned, humans consume up to 1 g polyphenols per day, and as an illustration compared to other antioxidant molecules, this corresponds to ten times more than the amount of vitamin C and /or one hundred times more than those of vitamin E and carotenoids (**Scalbert et al., 2005a**).

Once polyphenols are consumed through the diet, most of them reach the colon in their native form, as discussed above. Reciprocal interactions occurring in the gut also involve the impact of polyphenols on gut microbiota. Thus, the unabsorbed part, as well as polyphenols gut-derived metabolites, greatly affect the gut microbiota composition and function (**Ozidal et al., 2016**).

The modulation of gut microbiota by polyphenols has been established so far. As discussed previously, the beneficial impact relies on the prebiotic effect capacity or inhibition of pathogens (**Plamada and Vodnar, 2021**).

Moreover, polyphenols can modulate the metabolism of gut microbiota by influencing the production of SCFAs and BCFAs (**Mosele et al., 2015**). Acetic, propanoic, butyric, formic and valeric acids are mainly produced from dietary fermentation by the gut microbiota (**Koh et al., 2016**). Specifically, polyphenols induce production of SCFAs by promoting the growth

of such known SCFAs producing bacteria of (Parkar et al., 2013; Unno and Osakabe, 2018). Moreover, animal polyphenol supplementation studies have shed more light on the hypothesis that by regulating gut microbiota, polyphenols could positively impact health, mainly in the context of metabolic diseases. These studies revealed an increase of SCFAs in addition to the changes in gut microbiota composition (Anhê et al., 2018; Li et al., 2019; J. Liu et al., 2019; Van Hul et al., 2018). In addition, these metabolites have been reported to exert several positive effects in the human health, due to their key role in gut-brain axis crosstalk. Some reports have highlighted the potential effects of SCFAs on neurological disorders (Arnoldussen et al., 2017; Chen et al., 2022; Gonzalez et al., 2010; Kim et al., 2020; van de Wouw et al., 2018) et al., 2017). Besides that, data on humans still are lacking. However, understanding how SCFAs are derived from polyphenols-gut interactions, could help develop a potential strategy to target the gut-brain axis through the dietary intervention (Dalile et al., 2019a). Contrary, high levels of BCFA are potentially prejudicial to gut health (Bloomgarden, 2018; Goffredo et al., 2017; Shi et al., 2021) as some correlations have been observed with diabetes (Bloomgarden, 2018), obesity (Goffredo et al., 2017), depression (Szczesniak et al., 2016) and Rett Syndrome (Borghi et al., 2017).

## Hypothesis

Our study investigated on the biotransformation of 5-CQA by three bacteria, *L. reuteri*, *B. fragilis* and *B. longum* highlighting its biotransformation into several metabolites only by *L. reuteri*, but with a higher quantity when *L. reuteri* was co-cultured with *B. fragilis*. In addition, the well-established interaction between the gut microbiota and polyphenols also has a direct impact on the gut microbiota composition. Following our objective of investigating 5-CQA, the aim of this study was to evaluate its effects on the growth and metabolism of selected species such as *L. reuteri* and *B. fragilis*, which presented direct or indirect play on 5-CQA biotransformation. We aimed to provide more information on the potential role of 5-CQA, as a natural compound, in positively modulating the microbiota in favor of specific gut bacteria species that can ensure intestinal homeostasis and, in turn, favorably affect human well-being.

## II.C.2. Results

### II.C.2.1. Impacts of 5-CQA on the bacterial growth

In this study, the impact of different concentrations of 5-CQA, 0.1, 2.5, 50 and 1000  $\mu\text{M}$  were evaluated on two intestinal bacterial strains *L. reuteri* and *B. fragilis*. This concentration range was selected from the lowest concentration to physiological ones that could potentially reach the intestine. 5-CQA was added at the beginning of the culture then bacteria were incubated in an anaerobic chamber for 30 hours at 37°C. The culture liquids were collected at the beginning (time 0) and after 6, 24, and 30 hours of incubation to measure the  $\text{OD}_{600 \text{ nm}}$  reflecting bacterial growth and to determine the bacterial concentration ( $\text{CFU.mL}^{-1}$ ). Cultures of the tested strains without 5-CQA were also performed as controls, referred to as untreated *L. reuteri* or untreated *B. fragilis*. The effect of 5-CQA on *L. reuteri* and *B. fragilis* growth was determined by comparing the growth parameters of the cultures treated with different 5-CQA concentrations to those of the untreated ones. These results correspond to the mean of values obtained from the three experiments. The size of the standard deviations shown can be explained by the cultural requirements of anaerobic bacteria. In fact, these cultures are very stringent in terms of the experimental conditions.

#### a) Effects of 5-CQA on *B. fragilis* growth

Untreated *B. fragilis* has exhibited a growth culture with a maximum reached at  $T_{6\text{h}}$  corresponding to  $6.56 \times 10^8 \text{ CFU.mL}^{-1}$ . After treatment using different 5-CQA concentrations, the growth curves of *B. fragilis* were still quite similar to controls, with a slight impact visualized at  $T_{6\text{h}}$  and  $T_{24\text{h}}$ . 5-CQA, at 2.5  $\mu\text{M}$ , led to a slightly increase of *B. fragilis* growth, reaching values, at  $T_{6\text{h}}$ , of  $8.65 \times 10^8$  versus control sample. The same trend was observed at  $T_{24\text{h}}$ , where *B. fragilis* have grown until  $8.56 \times 10^8 \text{ CFU.mL}^{-1}$  in presence of 5-CQA (2.5  $\mu\text{M}$ ) and until  $5.68 \times 10^8 \text{ CFU.mL}^{-1}$  in control. At  $T_{24\text{h}}$ , we observed a bacterial growth reduction (around 14%) using 5-CQA at 50  $\mu\text{M}$  compared to untreated bacteria. An increase of 51 % was noticed after treatment with 2.5  $\mu\text{M}$  of 5-CQA. At  $T_{30\text{h}}$  the effect of 5-CQA on the bacterial culture

disappeared to reach around the same value ( $5.46 \times 10^8$  CFU.mL<sup>-1</sup>) at different 5-CQA concentrations compared to untreated *B. fragilis*.

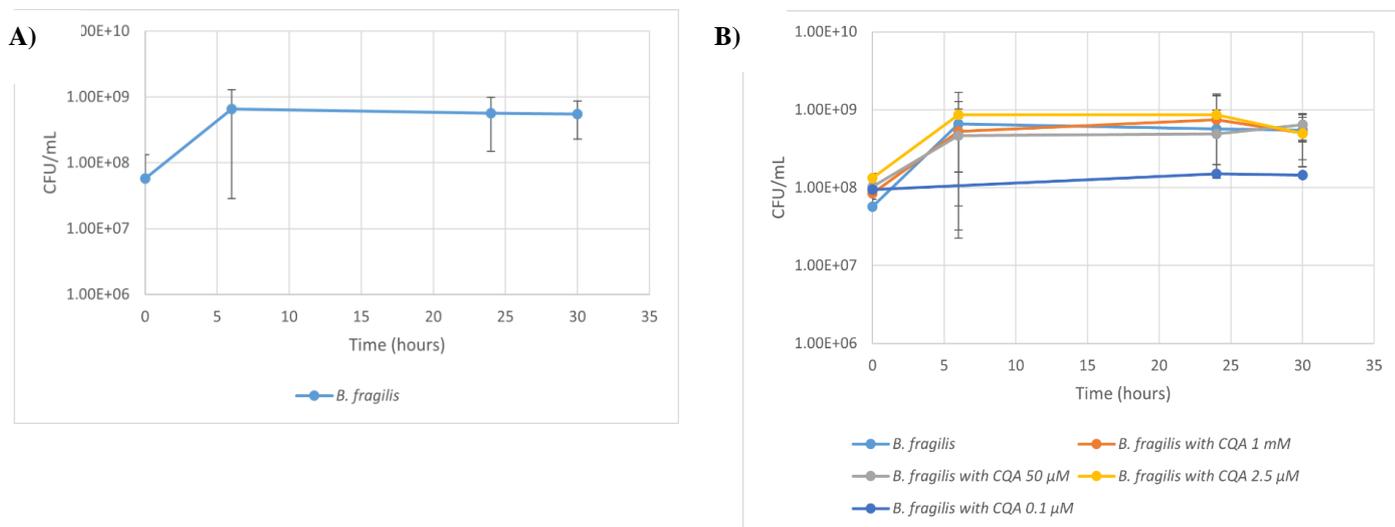


Figure 43. Number of *B. fragilis* (CFU.mL<sup>-1</sup>) versus time of culture (**B**): growth kinetics of *B. fragilis* in BHI standard medium or under different concentrations of 5-CQA. The values reported are means  $\pm$  SD of three independent experiments

The assay with 0.1  $\mu$ M of 5-CQA added on *B. fragilis* culture must be considered incomplete, due to the poor number of bacteria at T<sub>6h</sub>, probably owing to a handling error. Nevertheless, at T<sub>24h</sub> and T<sub>30h</sub> we noted a decrease (73% reduction) in the growth of *B. fragilis* in presence of 0.1  $\mu$ M 5-of CQA with a value of  $1.51 \times 10^8$  CFU.mL<sup>-1</sup> and  $1.45 \times 10^8$  CFU.mL<sup>-1</sup> compared to untreated bacteria ( $5.68 \times 10^8$  CFU.mL<sup>-1</sup> at T<sub>24h</sub> and  $5.46 \times 10^8$  CFU.mL<sup>-1</sup> at T<sub>30h</sub>).

#### b) Effects of 5-CQA on *L. reuteri* growth

According to the results depicted in the Figure 44, untreated *L. reuteri*, in our experimental conditions, has exhibited a growth culture with a maximum reached at T<sub>30h</sub> corresponding to  $6.61 \times 10^7$  CFU.mL<sup>-1</sup>. Nevertheless, bacteria in the presence of 5-CQA has displayed a smaller exponential growth phase compared to untreated bacteria, with the maximum reached at T<sub>6h</sub>. The effect of 5-CQA was concentration-dependent (Figure 44) although, not in a proportional correlation. In fact, 5-CQA at 0.1  $\mu$ M and 2.5  $\mu$ M had the most potent stimulation effect on growth of *L. reuteri* at T<sub>6h</sub> corresponding to  $1.78 \times 10^8$  CFU.mL<sup>-1</sup> (257% increasing) and  $1.19 \times 10^8$  CFU mL<sup>-1</sup> (138% increasing) respectively while the number of bacteria reached  $4.99 \times 10^7$  CFU mL<sup>-1</sup> for untreated samples. On the contrary, slightly effects

were observed on *L. reuteri* growth with 50  $\mu\text{M}$  and 1000  $\mu\text{M}$  of 5- CQA, where the values reached  $5.54 \times 10^7$  and  $5.27 \times 10^7$  CFU.mL<sup>-1</sup>, respectively. Moreover, the effectiveness of 5-CQA stimulation has been lost over time in each condition. *L. reuteri* culture at T<sub>30</sub>, reached a value of  $2.40 \times 10^7$  CFU mL<sup>-1</sup> under treatment with 0.1  $\mu\text{M}$  of 5-CQA (corresponding to a 64 % reduction). A significant decrease compared to control (untreated *L. reuteri*) was observed after treatment with 1000  $\mu\text{M}$  of 5-CQA. It is estimated to 84% of reduction, from  $6.61 \times 10^7$  CFU mL<sup>-1</sup> to  $1.09 \times 10^7$  CFU.mL<sup>-1</sup>. It was noted that 50  $\mu\text{M}$  of 5-CQA was found to be ineffective, and 5-CQA at 1 mM did not stimulate the growth of *L. reuteri*. Therefore, it could be hypothesized that these concentrations are too high for bacteria, affecting adversely their growth.

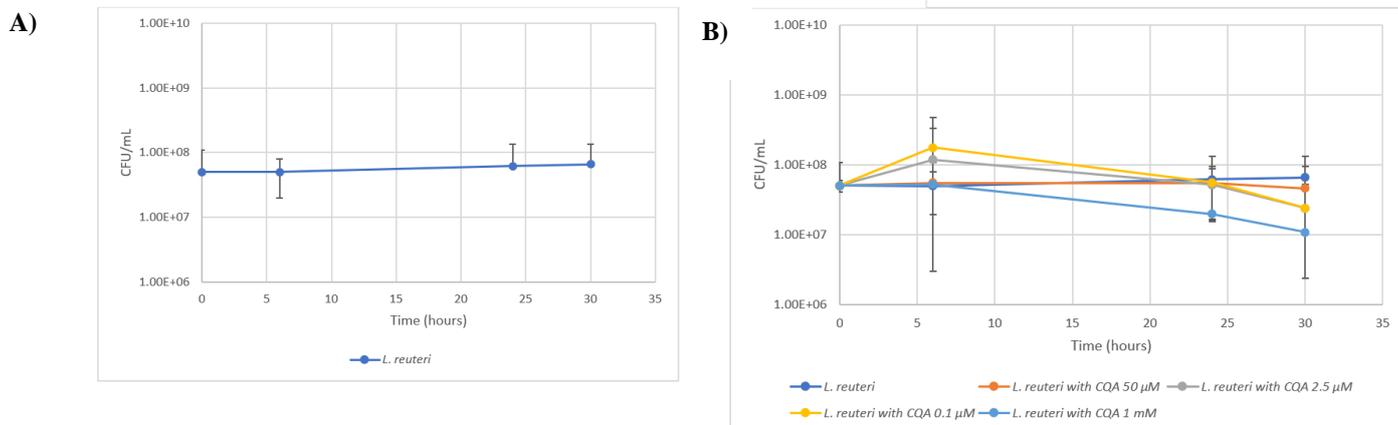


Figure 44. (A): Number of *L. reuteri* (CFU.mL<sup>-1</sup>) versus time of culture (B): growth kinetics of *L. reuteri* in BHI standard medium or under different concentrations of 5-CQA. The values reported are means  $\pm$  SD of three independent experiments

#### II.D.2.2. Effects of 5-CQA on metabolic production of *B. fragilis* and *L. reuteri*

To investigate the impact of different concentrations of 5-CQA on the metabolic production of the two bacterial species, such as SCFAs and BCFAs, cell-free supernatant cultures grown under standard conditions (BHI = 25  $\mu\text{g.mL}^{-1}$  of hemin) as well as in several 5-CQA concentrations were analysed by GC-MS. GC-MS is a combined system, in which thermally stable and volatile compounds are separated by a column and then eluting metabolites are detected by electron-impact (EI) mass spectrometer. GC-MS is considered the gold standard in metabolomics (Vernocchi et al., 2016). Besides that, GC-MS has several advantages, such as high efficiency, reproducibility, and sensitivity, it also shows some drawbacks. In fact, it can only be performed for volatile compounds, or those that can be volatile, or made stable by derivatization (Roessner et al., 2000).

##### a) Effects on SCFAs and BCFAs production by *B. fragilis*

The selected time for T<sub>30</sub> was based on the stationary phase of *B. fragilis*, a known phase of secondary metabolites production by bacteria. As shown in, eight metabolites were identified by GC-MS analysis of *B. fragilis* supernatant at T<sub>0</sub>. Acetic (2), propanoic (3), butyric

(= butanoic) (**6**) and isovaleric (**7**) acids were the main SCFAs produced by *B. fragilis*. Besides, *B. fragilis* produced, at a lower level, the known BCFAs isobutyric (**4**) and isocaproic acids (**8**). Moreover, we have observed the presence of hydroxyacetone (**1**). The last metabolite detected was butyrolactone (**5**) butyrolactone or GBL).

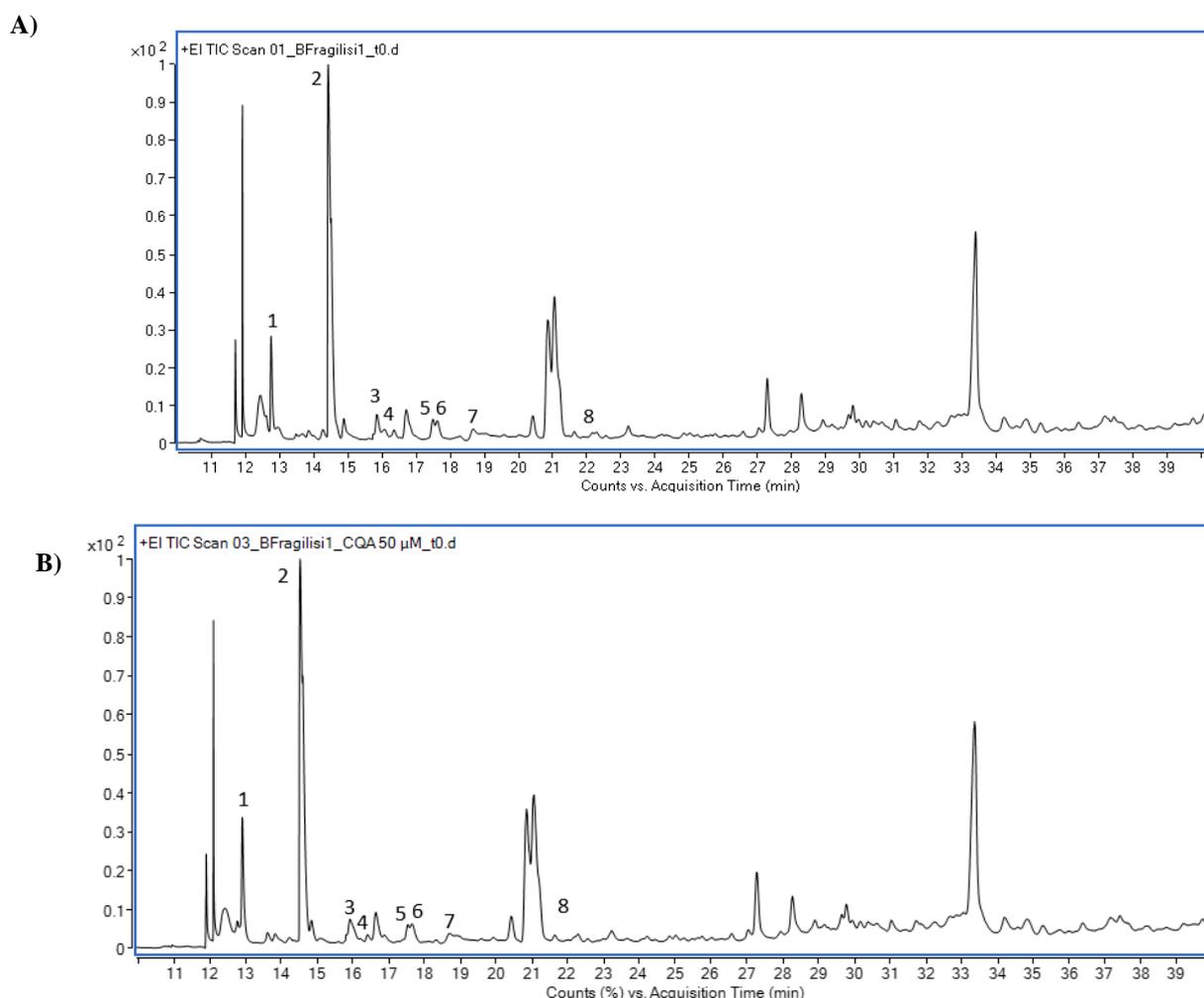
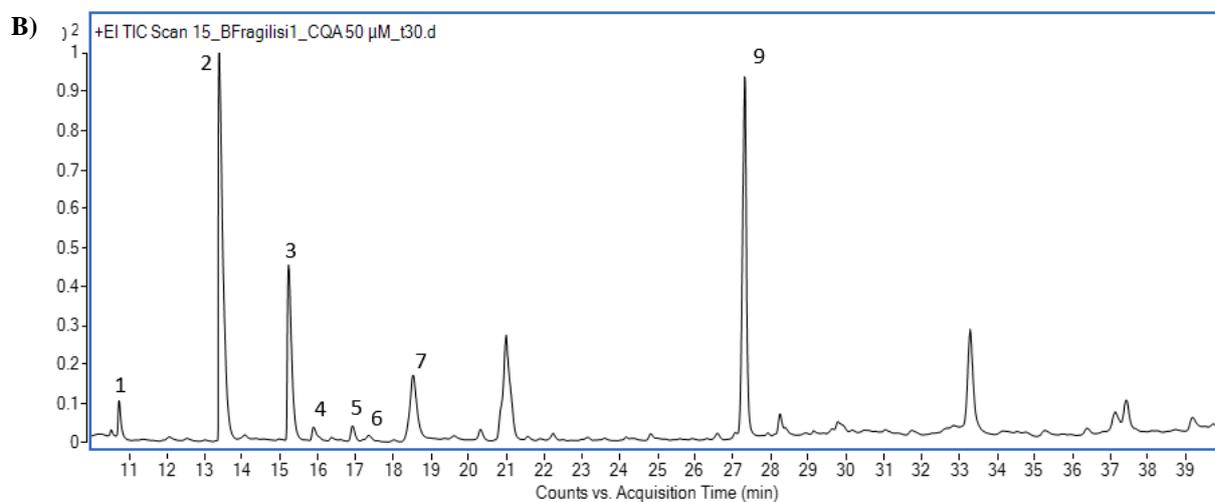
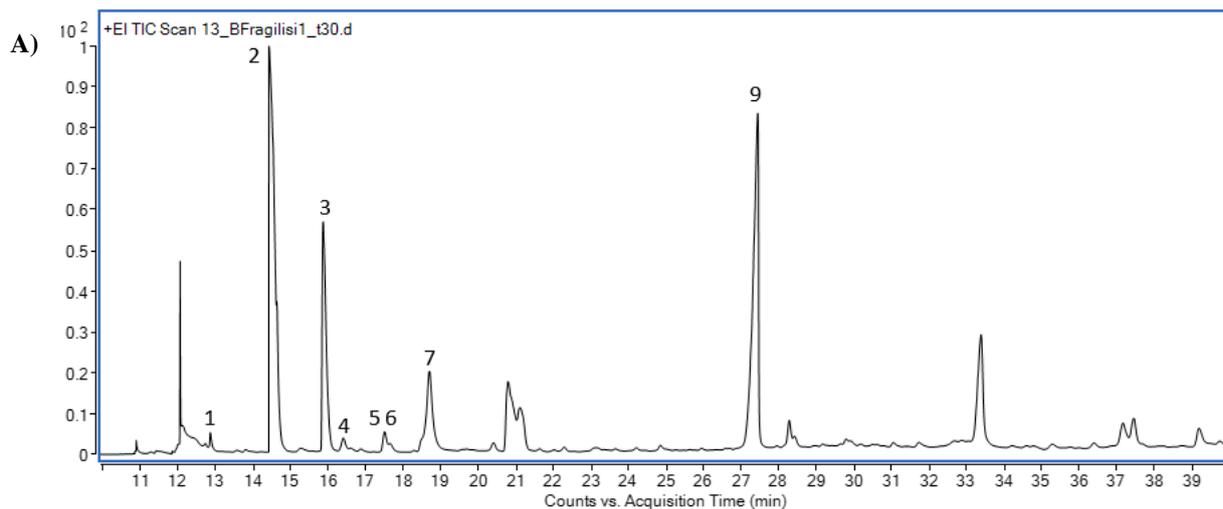


Figure 45. MS profiles obtained at  $T_0$  for *B. fragilis*: A) in standard condition (BHI = 25  $\mu\text{g}\cdot\text{ml}^{-1}$  of hemin); B) with 5-CQA at 50  $\mu\text{M}$ . (**1**) hydroxyacetone, (**2**) acetic, (**3**) propanoic, (**4**) isobutyric acids (**5**) butyrolactone, (**6**) butyric, (**7**) isovaleric, (**8**) isocaproic acids

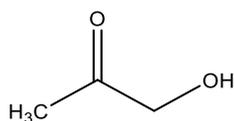
Compared to the standard conditions (Figure 45), *B. fragilis* produced the same metabolites during 30 h of anaerobic incubation, despite the absence of isocaproic acid (**8**). In the same manner, we observed an important decrease in the production of hydroxyacetone by *B. fragilis* under all conditions (data not shown). On the other hand, the production of isovaleric acid showed an increasing trend versus time Figure 46. Interestingly, we could note,

in the control sample at T<sub>6</sub>, the detection of a new metabolite  $\gamma$ -aminobutyric acid (**9**) (GABL) (= gamma-aminobutyric lactam) highlighting the evolution in the time of the production profile of *B. fragilis* independently to the 5-CQA supplementation. Indeed, GABL has been highly produced in T<sub>24h</sub>, both in presence of 5-CQA and in the control.

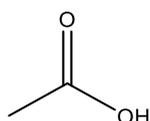
However, the highest production of this metabolite was at T<sub>30</sub>, and its peak area reached the values of  $1.3 \times 10^8$  uAU, compared to T<sub>6</sub> and T<sub>24</sub>, with peak area of  $9.7 \times 10^6$  and  $1.1 \times 10^7$ , respectively. Herein we present the effects of 5-CQA on bacteria dependent volatile organic compounds production by *B. fragilis*. Indeed, the same trend in *B. fragilis* metabolic production was observed in the presence of 5-CQA compared to the untreated bacteria, without any difference. The selected time of T<sub>30</sub> was based on the stationary phase of *B. fragilis*, a known phase of secondary metabolites production by bacteria. Indeed, no modification was observed at lower 5-CQA concentrations compared to the untreated samples. It is interesting to note that high levels of acetic acid were recorded at all sampling time.



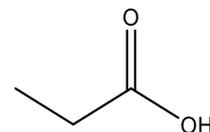
46. GC-MS profiles obtained at T<sub>30</sub> for *B. fragilis*: A) in standard condition (BHI = 25 μg.mL<sup>-1</sup> of hemin); B) with 5-CQA at 50 μM. (1) hydroxyacetone, (2) acetic, (3) propanoic, (4) isobutyric acids, (5) butyrolactone, (6) butyric, (7) isovaleric acids, (9) GABL



(1) Hydroxyacetone

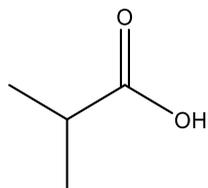


(2) Acetic acid



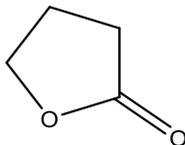
Propanoic acid

(3) Propanoic acid

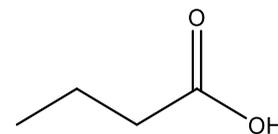


Isobutyric Acid

(4) Isobutyric Acid

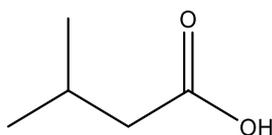


(5) Butyrolactone



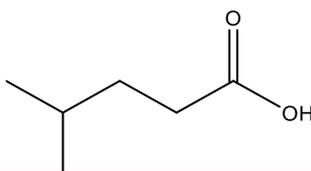
Butyric acid

(6) Butyric acid

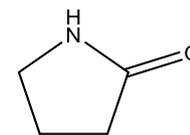


Isovaleric acid

(7) Isovaleric acid



(8) Isocaproic acid



(9) 2 pyrrolidone or GABL (= gamma-aminobutyric lactam)

Figure 47. Structures of metabolic profiles detected in our study

*b) Effects of 5-CQA on SCFAs and BCFAs production by L. reuteri*

The GC-MS data obtained for *L. reuteri* samples were less reproducible than those for *B. fragilis*. Although these results must be analyzed with caution, a trend has been reported. A total of eight compounds have been identified by GC-MS analysis in all the *L. reuteri* samples at T0. These metabolites correspond to those found for *B. fragilis*, such as acetic acid (2), propanoic acid (3), butyrolactone (5), butyric acid (6) and isovaleric acid (7). Indeed, formic acid was detected, specifically produced by *L. reuteri* with or without 5-CQA supplementation. A variation in the production of some metabolites was noted, because *L. reuteri* produced low levels of these metabolites. Two characteristics of *L. reuteri* metabolic profiles can be underlined independently of the supplementation: a) high levels of acetic acid

production in any sample analyzed and b) the appearance of 1,3-propanediol and 2-pyrrolidone production at T<sub>6</sub> (depicted in Figure 49 at T<sub>30</sub> as example). In fact, the 2-pyrrolidone was far less produced by *L. reuteri* in comparison with *B. fragilis* at each time sampling. In conclusion, no representative impact in terms of metabolites production was also noted when *L. reuteri* cultures were supplemented by 5-CQA.

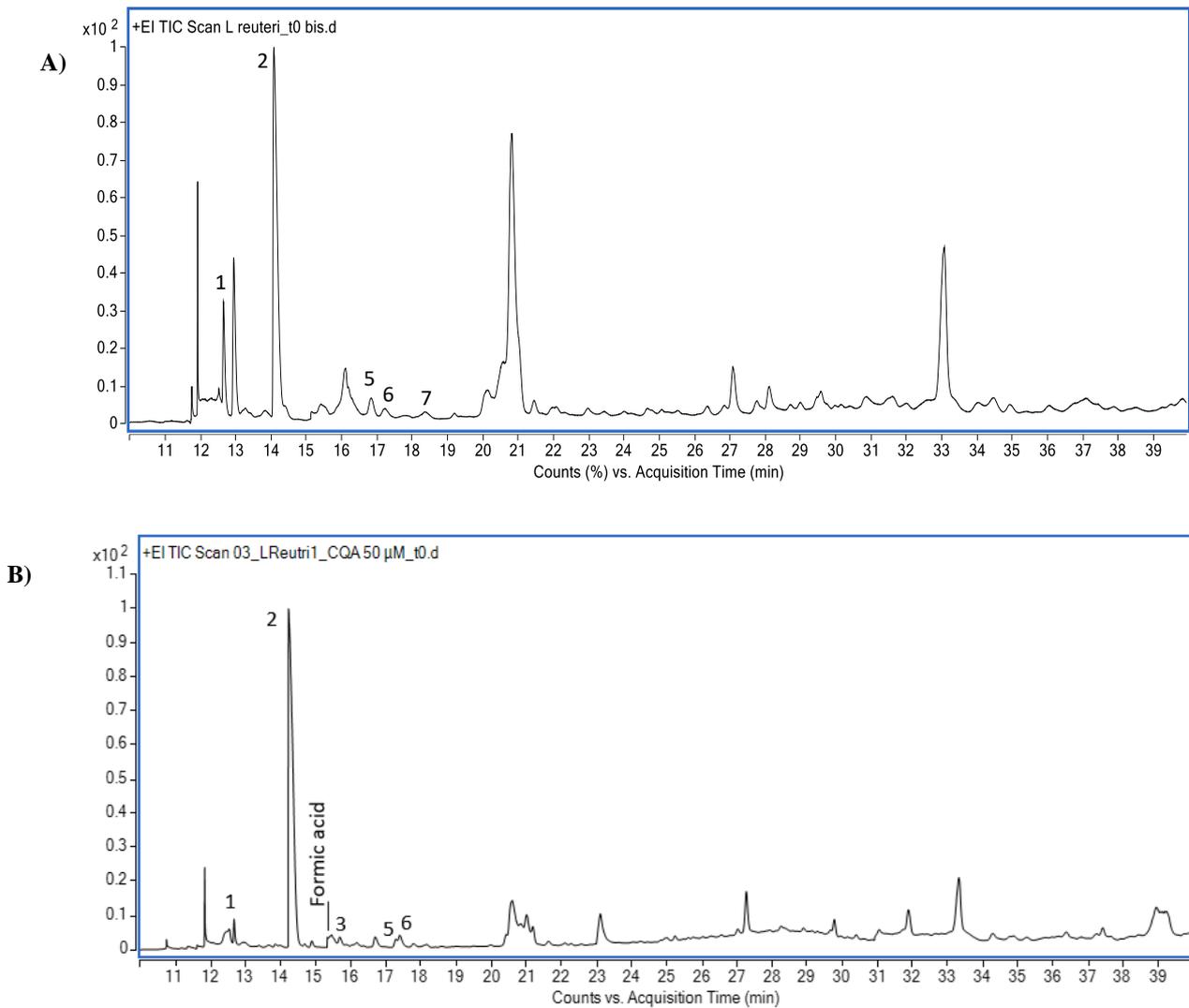


Figure 48. GC-MS profiles obtained at T<sub>0</sub> for *L. reuteri*: A) in standard condition (BHI = 25 µg.mL<sup>-1</sup> of hemin); B) with 5-CQA at 50 µM. (1) hydroxyacetone, (2) acetic, (3) propanoic, (4) isobutyric, (5) butyrolactone, (6) butyric acid

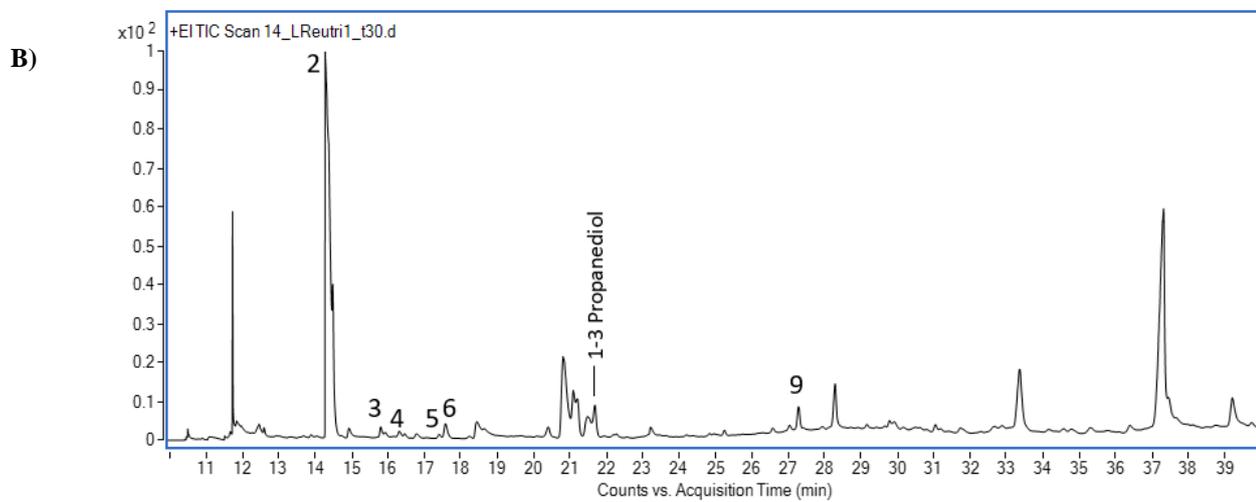
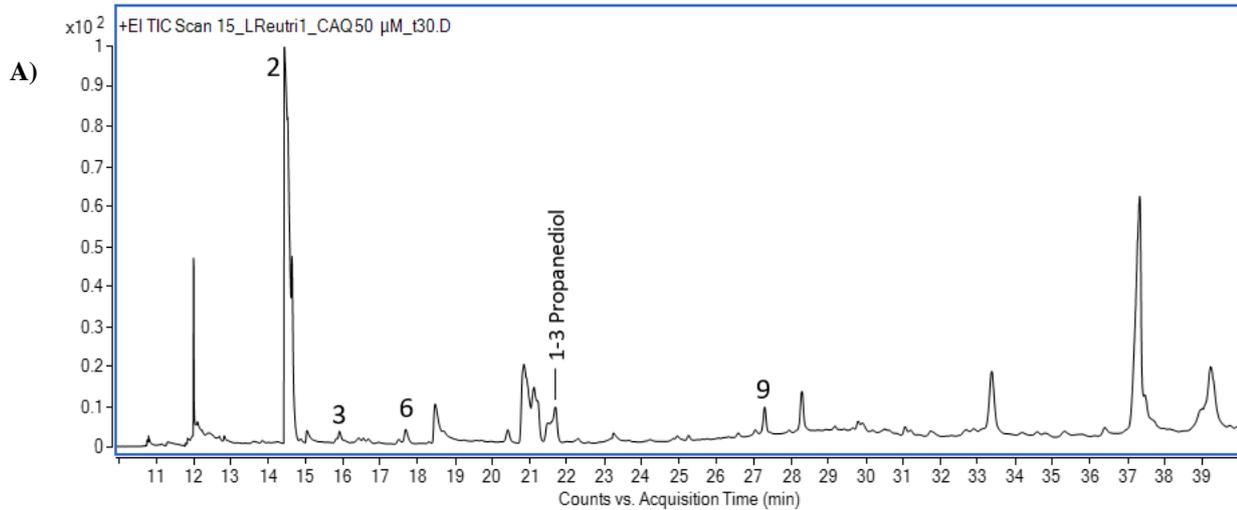


Figure 49. GC-MS profiles obtained at  $T_{30}$  for *L. reuteri*: A) in standard condition (BHI =  $25 \mu\text{g}\cdot\text{mL}^{-1}$  of hemin); B) with 5-CQA at  $50 \mu\text{M}$ . (2) acetic, (3) propanoic, (4) isobutyric acids, (5) butyrolactone, (6) butyric acid, (9) GABL, and 1,3 propanediol

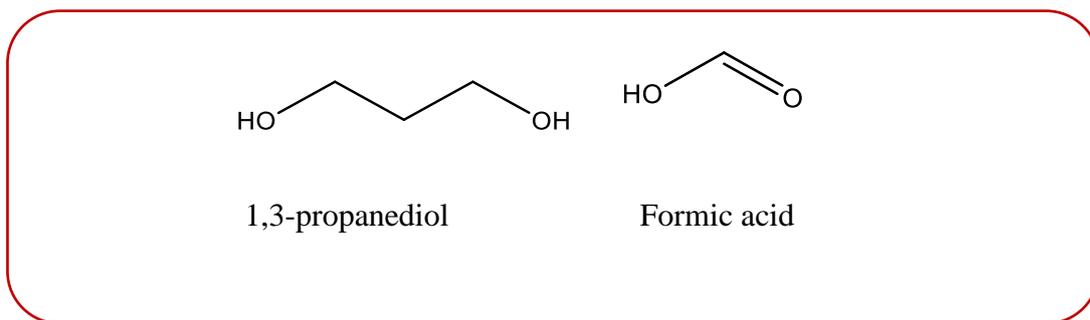


Figure 50. Chemical structures of metabolites, produced specifically by *L. reuteri*

## II.3. Discussion

### II.3.1. Metabolic profiles of *B. fragilis* and *L. reuteri* strains

GC-MS analysis of untreated bacterial cultures led to the characterization of the metabolic profile of each bacterium. Similar SFCAs and BFCAs were produced by the two bacteria except two metabolites, formic acid and 1,3-propanediol, which were specific of *L. reuteri*. Acetic acid, which has shown positive activity related to gut barrier integrity and function (**Barcelo, 2000; Feng et al., 2018**), was the main SCFA produced by both bacteria.

*L. reuteri* a lactic acid bacterium produces lactic acid as a major organic acid. However, this metabolite was not detected in our study, since lactic acid is a non-volatile compound, and that cannot be quantified by GC-MS analysis. Lactic acid is known for its beneficial properties, mainly its immunomodulatory effect (**Caslin et al., 2021**). Moreover, *L. reuteri* can produce various volatile metabolites with diverse biological activity such as 1,3-propanediol, detected in our samples, which is one of the oldest known fermentation products and has enormous applications in polymers, cosmetics, foods, adhesives, lubricants, laminates, solvents, antifreeze, and medicines industries (**Baeza-Jiménez et al., 2011**). *L. reuteri* is a promising probiotic bacterium. It has been found that metabolism of glycerol by *L. reuteri* can result in excretion of a metabolic intermediate, 3-hydroxypropionaldehyde known as reuterin, which has greater antimicrobial activity against potential pathogens such as *Salmonella*, *Clostridium*, and *Escherichia* species (**Mu et al., 2018**). Specifically, *L. reuteri* is able to bioconvert glycerol into 1,3-propanediol and 3-hydroxypropionaldehyde (Figure 51). As a first step, glycerol is transformed to 3-HPA (3-hydroxypropionaldehyde or reuterin) by glycerol dehydratase, which is further reduced to 1,3-propanediol by 1,3-propanediol oxidoreductase. This stage uses NADH and regenerates NAD<sup>+</sup>, which is considered a potential mechanism by which *L. reuteri* favors glycolysis by readjusting its redox status (**Britton, 2017**). Nonetheless, these intermediate metabolites were not detected under our experimental conditions.

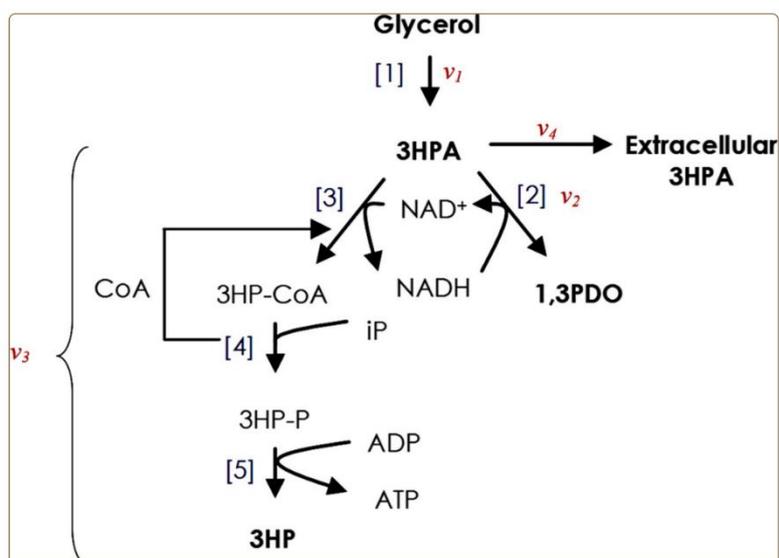


Figure 51. Biotransformation pathway of glycerol to 1,3-propanediol by *L. reuteri*. The enzymes involved in this process includes: [1] B12-dependent glycerol/diol dehydratase , [2] 1,3-propanediol oxidoreductase, [3] propionaldehyde dehydrogenase , [4] phosphotransacylase , and [5] propionate kinase (Dishisha et al., 2014) Abbreviations: 3HPA (3-hydroxypropionaldehyde), 1,3 PDO (1,3-propanediol), 3HP (3-hydroxypropionic acid)

The *L. reuteri* strain was also able to produce formic acid in our conditions even if we observed non-reproducibility of its presence in all samples studied. This compound, systematically named methanoic acid, is the simplest carboxylic acid, with the chemical formula  $\text{HCO}_2\text{H}$ . It is an important intermediate in chemical synthesis and occurs naturally, most notably in some ants. Indeed, the word "formic" comes from the Latin word for ant, *formica*, referring to its early isolation by the distillation of ant bodies. Moreover, it has been found that formic acid is able to reduce the growth of coliform and of the pathogenic *Salmonella typhimurium* (Luise et al., 2020).

The other metabolite, hydroxyacetone, is produced from methyl glyoxal, a pathway already described for lactic bacteria (Hatti-Kaul et al., 2018). Butyrolactones, such as GBL, are involved in quorum sensing, the cell-to-cell communication processes (Du et al., 2011). GBL production might also be correlated with the production of other metabolites as it has been reported that A-factor, another GBL, auto-induces morphological differentiation and production of secondary metabolites in *Streptomyces griseus* (Horinouchi and Beppu, 2007).

Moreover, 2-pyrrolidone, as the major metabolite of *B. fragilis*, is a precursor of gamma-aminobutyric acid (GABA), produced in the CNS as an inhibitory neurotransmitter

(Fasolato et al., 1988). In addition, pyrrolidone can cross cell membranes and BBB much more effectively than GABA, due to its chemistry, as it is an uncharged molecule in physiological conditions (Fasolato et al., 1988). Notably, a strain of *B. fragilis* was reported to produce a strong bioactive compound pyrrolopyrazine, which is known to exhibit antibacterial, antitumor, anti-angiogenesis and antioxidant activities (Ser et al., 2015); nevertheless, this metabolite was not detected in our analysis.

In conclusion, we have underlined that 5-CQA had no effect on the metabolic production (in terms of SFCAs and BFCAs) of the two bacterial strains studied.

*a) Impacts of 5-CQA supplementation in L. reuteri and B. fragilis strain as prebiotic*

These *in vitro* investigations *in vitro* of growth the rate and patterns of *L. reuteri* and *B. fragilis* under 5-CQA treatment showed that this natural compound can only influence the growth of *L. reuteri*.

After supplementation, 5-CQA at 0.1  $\mu\text{M}$  and 2.5  $\mu\text{M}$  has at  $T_{6\text{h}}$ , the strongest stimulation effect on *L. reuteri*, the known probiotic strain. On the contrary, the growth of *B. fragilis* was not stimulated by any 5-CQA concentrations, despite that, at  $T_{24\text{h}}$  and  $T_{30\text{h}}$ , we noted a slight decrease in its growth after treatment at 0.1  $\mu\text{M}$ . These data highlighted that 5-CQA can potentially stimulate the *L. reuteri* growth at the lowest concentrations evaluated.

Overall, polyphenols have shown the ability to positively shape gut microbiota composition, and consequently could have positive impact on intestinal barrier integrity, and finally on human health. The mechanism by which polyphenols can exert their prebiotic effects is not fully elucidated. However, some modes of action have been proposed (Rodríguez-Daza et al., 2021). As shown in Figure 52 prebiotic effect of polyphenols could be mediated through the activation of polyphenol-associated enzymes (PAZymes). Briefly, a gut bacterium can biotransform a polyphenol (M1) which could be subjected to metabolic utilization, as a source of energy. Then M1 could be converted in a trophic network by other PAZymes bacteria which could lead to the generation of new metabolites. Overall, all this cascade could have positive impact in intestinal barrier integrity, consequently in human health as shown in Figure 52 (Rodríguez-Daza et al., 2021).

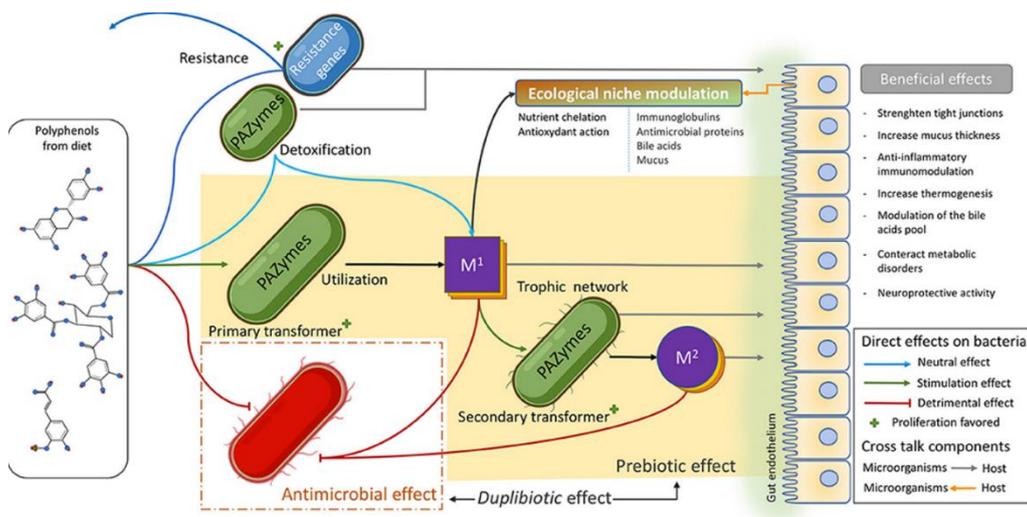


Figure 52. Overview of proposed duplibiotic nature of dietary polyphenols, including their prebiotic and antimicrobial effects on the gut microbiota (Rodríguez-Daza et al., 2021)

The prebiotic effects of polyphenols seem to be bacteria-dependent. In our study, we hypothesise that the effects of 5-CQA, only observed on *L. reuteri*, may be mediated through some PAZymes. As an example, only *L. reuteri* had shown, in our previous study, cinnamoyl esterase activity and capacity to biotransform 5-CQA. This hypothesis could be supported since by LC-MS/MS we have detected the same metabolites in this supplementation study than those detected in the biotransformation study (Figure 53). Moreover, this hypothesis is in line with findings from Li et al, (Li et al., 2015). They have shown that the pomegranate extract has promoted the growth of *A. muciniphila* only in participants for those their gut microbiota was able to produce urolithin- A, a gut-derived metabolites of ellagitannins and ellagic acid, the main polyphenols present in pomegranate (Li et al., 2015). The prebiotic effect seems decreased when the 5-CQA concentrations increase. Nevertheless, this impact could not be correlated with the SCFAs and BCFAs production by both intestinal bacteria as there were not significantly affected by the 5-CQA supplementation.

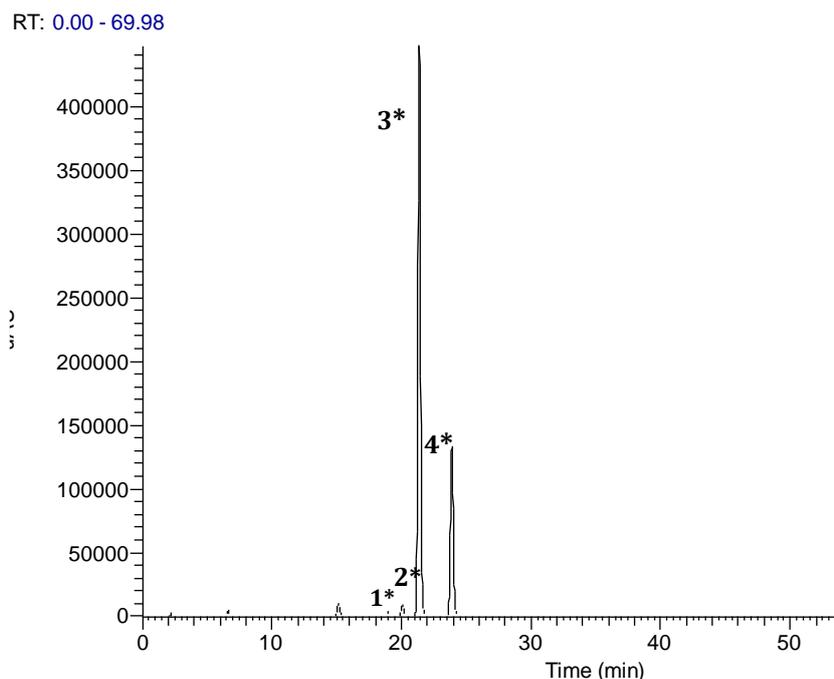


Figure 53. PDA at 320 nm of supplementation of 5-CQA (1 mM) in *L. reuteri* culture (SN extract) at T30: (1) 5-CQA (2) esculetin (3) *trans*-CA, (4) *cis*-CA. \*compounds with retention time (RT) confirmed after injection of analytical standards

#### II.D.4. Conclusion

Taken all together, this study provides information on how gut bacteria can be affected differently by the same dietary compound. It would be interesting to also investigate the impact of 5-CQA on the growth of bacterial community, through co-culture studies. Moreover, the potential of using *L. reuteri* with 5-CQA as synbiotic, would help to better understand the *in vivo* scenario of polyphenols-gut interactions, which could lead to positive effects on human health.

## **II.E. Biological study of 5-CQA and its gut-derived metabolites by quadruple original *in vitro* cell model**

Considering the biological effects of polyphenols on gut barrier integrity, we have evaluated the impact of 5-CQA, CA and esculetin on the intestinal epithelial cells by using an original *in vitro* model.

### **II.E.1. Roles of polyphenols on intestinal barrier**

The intestinal barrier integrity and function are essential for maintaining gut homeostasis, thereby host health (**Singh et al., 2019**). This intestinal barrier is a single layer consisting of various types of cells such as enterocytes, Goblet cells, enteroendocrine, Microfold (M) and Paneth cells. These cells are sealed by tight junction (TJ) transmembrane proteins (e.g., occludin, claudins, junctional adhesion molecules, and tricellulin) as well as peripheral membrane proteins (e.g., Zonula occludens (*Zo-1*) and cingulin). Their role is to prevent paracellular permeability and maintain the gut barrier function. In addition, the mucus layer prevents direct contact between the gut microbes and epithelial cells (**Chelakkot et al., 2018; Hooper, 2015**). The disruption of tight junctions leads to barrier dysfunction, followed by the translocation of pathogens and bacterial toxins at the systemic level and activation of the immune system and inflammation, thereby contributing to several pathological conditions (**Chelakkot et al., 2018**).

Evidence from *in vitro* and animal studies suggests that polyphenols may regulate and maintain intestinal integrity and function, through several mechanisms (**Bernardi et al., 2020b; Wan et al., 2021**). Most *in vitro* investigations have been performed using Caco-2 cell line, followed by T84 and HT29/B6 (**Bernardi et al., 2020b**). Some polyphenols have been shown to increase transepithelial electrical resistance (TEER) and expression of TJ proteins, including resveratrol (**Ling et al., 2016**) quercetin (**Amasheh et al., 2008; Suzuki and Hara, 2009**) epigallocatechin gallate (EGCG) (**Watson et al., 2004**), naringenin (**Noda et al., 2013**), kaempferol (**Suzuki et al., 2011**). In addition, some studies give more insights in the mechanism by which polyphenols regulate barrier integrity. As an example, urolithin A, a gut-derived metabolite of polyphenols from berries and its analogue were able to regulate gut epithelium by improving barrier function through activating Nrf2 and AhR signalling pathways (anti-oxidative pathways) and suppress inflammation (**Singh et al., 2019**). Similarly, in study

on a Dextran Sulphate sodium (DSS)-induced colitis model, hesperidin upregulated Nrf2 pathway and increased the Treg population **(K. Guo et al., 2020)**. Recently, some interesting evidence came from intervention studies in humans. The MaPLE (Microbiome Manipulation through Polyphenols for managing gut Leakiness in the Elderly) project has provided substantial evidence for the role of polyphenol-rich diet in improving the intestinal permeability with two independent phase I clinical trials **(Bernardi et al., 2020a; Guglielmetti et al., 2020)**.

Polyphenols may contribute to gut health also by maintaining or enhancing the protective mucus layer as a crucial component of barrier function **(Etienne-Mesmin et al., 2019)**. Cranberry proanthocyanins (anthocyanins, hydroxycinnamic acids, and flavonols) have been found to promote gut mucous layer morphology and function by stimulating goblet cell proliferation, as well as by increasing interleukin IL-4 and IL-13 levels and MUC2 production **(Pierre et al., 2013)**. In addition to gene regulation, polyphenols have been shown to indirectly affect mucin production by modulating the growth of specific bacteria related to the mucin-degrading process. For example, supplementation with 4% w/w powdered green tea leaves rich in flavanols led to an increase of *Akkermansia* spp, in a high-fat model, consequently improving intestinal barrier function by preserving of mucus layer thickness **(Peron et al., 2020)**. In a mice study with *Citrobacter rodentium* (*Cr*) induced dysbiosis, pomegranate peel extract (mainly containing punicalagin and ellagic acid) treatments have demonstrated positive modulatory effects by regulating the composition of the gut microbiota. In addition, this treatment inhibited the growth of *Citrobacter rodentium*, a mucus-degrading pathogen **(George et al., 2019)**. Furthermore, in two independent studies on a colitis model, ellagic acid and resveratrol have demonstrated the ability to increase mucus production **(Martín et al., 2004; Rosillo et al., 2011)**.

Most of the current knowledge of mechanistic insights of microbiome comes from model systems such as *in vitro*, *ex vivo* and animal **(Galloway-Peña and Hanson, 2020)**. Although animal models are frequently used, translation of data to humans has been questioned **(Bjornson-Hooper et al., 2022; Desmoulin-Canselier and Moutaud, 2019; Pound and Ram, 2020; Seok et al., 2013)**. In addition, they involve ethical issues and are quite expensive **(Fitzgerald et al., 2015)**.

Therefore, cell-based *in vitro* approaches can be an alternative to animal experimentation **(Bédard et al., 2020)**. Cell culture allows scientists to investigate the cellular

and molecular mechanisms in a relatively simple and reproducible manner (**Joseph et al., 2018**).

Several *in vitro* models have been widely used in pharmacology, nutrition and microbiology field (**Fedi et al., 2021; Pearce et al., 2018**). Indeed, the Caco-2 cell line is considered the gold standard intestinal model (**Fedi et al., 2021**). This human-derived cell line was isolated from a colorectal adenocarcinoma. After seeding, cells take 7 days reach confluence and then the cells start to differentiate spontaneously, a process that is completed within 21 days. It is then that the cells reach characteristics of enterocytes, polarized monolayer microvilli and connected to each other through tight junctions. Moreover it expresses a brush-border microvilli with typical cytoskeleton proteins (**Antunes et al., 2013**). Nevertheless, Caco-2 monoculture has some limitations. Despite its colonic origin, Caco-2 cell lines represent more to morphological and functional characteristics of small intestine. In addition, this model does not fully represent the intestinal barrier under normal physiological conditions.

To improve this model, scientists have developed a co-culture model consisting of two different types of cells. In this context, Caco-2 has been co-cultured with mucus-producing HT29-MTX cell lines, and this model represents both enterocytes and goblet cells. However, the intestinal epithelium contains several other cell types, that are not accurately represented by this model (**Fedi et al., 2021**).

In continuation, a triple co-culture model (3-D) has been developed, in which cells are grown in an extracellular matrix (ECM). Ussing Chamber and intestinal organoids have shown great potential to mimic the complex system *in vivo*. However, such models still require animal experiments (**Pearce et al., 2018**).

To try to get closer to the human intestine and to avoid use of animal models, Dr Latifa BOUSARGHIN and her team (NuMeCan), have developed an *in vitro* quadruple model (Figure 54). This original model is composed of enterocytes (Caco-2), mucus-secreting goblet cells (HT29-MTX), microfold cells (M cells) and enteroendocrine cells (NCI). Five cell types are used for the following, and therefore need to be cultured in specific conditions. The first types are NCI-H716 cells, floating cells mimicking enteroendocrine cells, obtained from a 33-year-old man with adenocarcinoma colorectal. Then Caco-2, mimicking enterocytes, are adherent cells from a 72-year-old man with of colorectal adenocarcinoma. The HT29-MTX line represents goblet cells, mucus producers obtained from HT29 colon cells of a 44-year-old patient with colorectal adenocarcinoma. The Raji-B cells, corresponding to floating cells, are

lymphoblasts obtained from an 11-year-old boy. They allow for the differentiation of Caco-2 cells into M cells through mediators. Finally, the cells of type THP-1 are monocytes, floating cells, from a one-year-old child with acute monocytic leukemia.

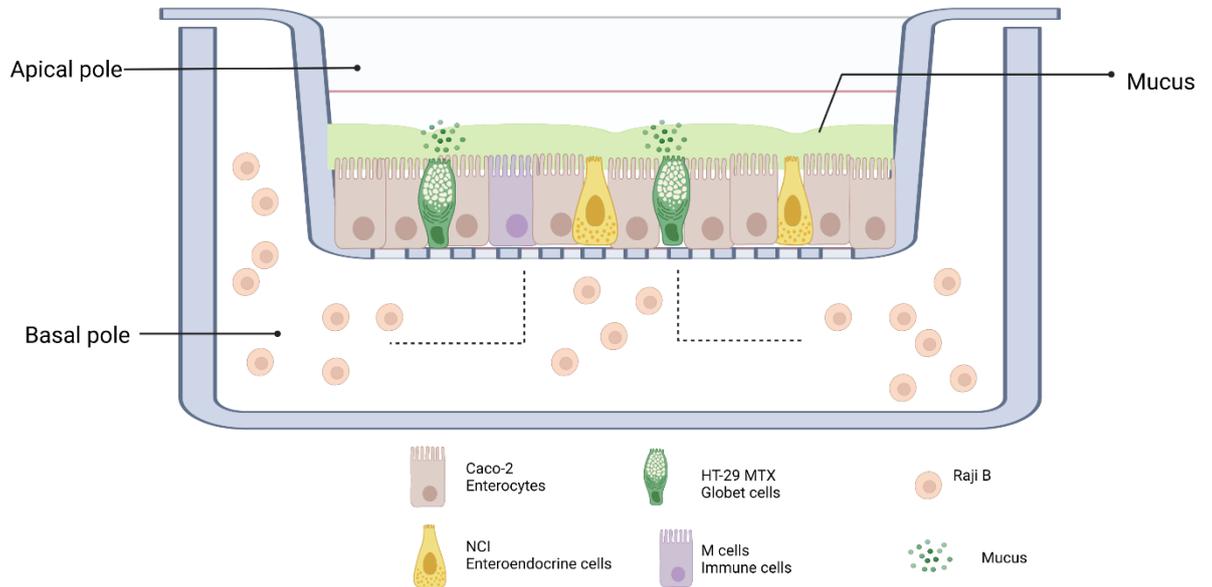


Figure 54. Illustrative picture of quadruple cellular model, used in our study

## Hypothesis

Given the importance of the intestinal barrier to health and considering the role of polyphenols in protecting its integrity and function, we aim to give information on the potential effects of 5-CQA and its gut-derived metabolites (CA and esculetin) on epithelial cells as a part of intestinal barrier integrity. Particular attention was paid to TEER, *occludin* and *Zo-1* gene expression. In this study, we used the original *in vitro* quadruple model described previously.

## II.E.2.

### Results and discussion

Considering that 5-CQA reaches the colon at  $\mu\text{M}$  concentration and its microbial metabolites such as CA and esculetin could be close to the gut epithelium when produced; we have investigated whether these compounds could have a direct impact on epithelial cell function. To address these issues, we have used a quadruple *in vitro* model in the presence of above-mentioned polyphenols at 1 mM, 50  $\mu\text{M}$  and 0.1  $\mu\text{M}$  (from the lowest concentration to physiologically ones that potentially could reach the intestine) in basal condition. Different measurements will be evaluated in the presence of the tested polyphenols including cell viability, transepithelial electrical resistance (TEER) and gene expression such as *occludin* and *Zo-1*.

#### II.E.2.1. Cell viability in presence of polyphenols

Cell cytotoxicity was determined using the LDH method. This method is based on the measuring of the activity of a cytoplasmic enzyme Lactate dehydrogenase (LDH) released by damaged cells (apoptosis, necrosis, and other forms of cellular damage) (Kumar et al., 2018).

Cytotoxicity was assessed based on the amount of LDH released by cells into the apical side in the presence of 5-CQA, CA and esculetin at 1 mM, 50  $\mu\text{M}$  and 0.1  $\mu\text{M}$  (

Figure 55). The mean values and SD were calculated from three independent measurements. As shown in Figure 55

Figure 55 no differences were observed in the presence of any polyphenols at all concentrations used compared to the untreated cells. These data indicated that of none polyphenols used at these concentrations induced cytotoxicity in the cell model used, suggesting that the conditions studied were appropriate for further investigation.

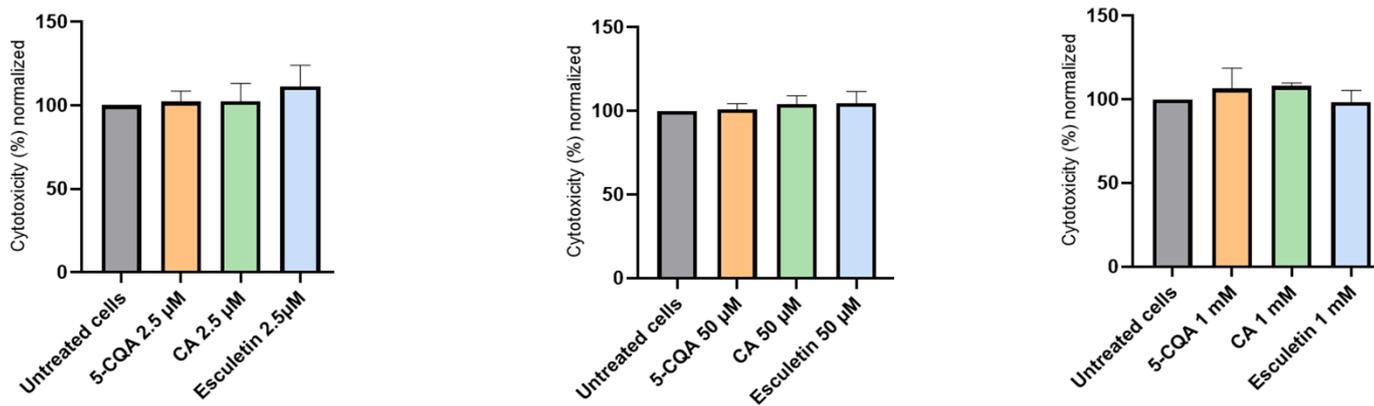


Figure 55. Effects of polyphenols at 2.5  $\mu$ M, 50  $\mu$ M and 1 mM on quadricellular cell viability measured by the release of LDH after incubation for 24 h

#### II.E.2.2. TEER evolution in presence of polyphenols

TEER is one of the main parameters used to investigate the barrier integrity function, *in vitro*. TEER represents the degree to which small, charged particles can cross the cell epithelium in a paracellular manner.

Intestinal barrier function was evaluated by monitoring the evolution of TEER over time. The TEER of cells was measured before the addition of the molecules at  $T_0$  and after 24 h, in the presence of different concentrations (2.5  $\mu$ M, 50  $\mu$ M and 1 mM) of 5-CQA, CA and esculetin.

Figure 56 reports the values of TEER in the presence of the tested polyphenols. The results clearly showed that none of the polyphenols studied significantly increased TEER. The differences, in TEER compared to previously results on polyphenols cited above, could be explained by the fact that the model used in this study is closer to intestinal physiology and is due to the presence of the mucus layer.

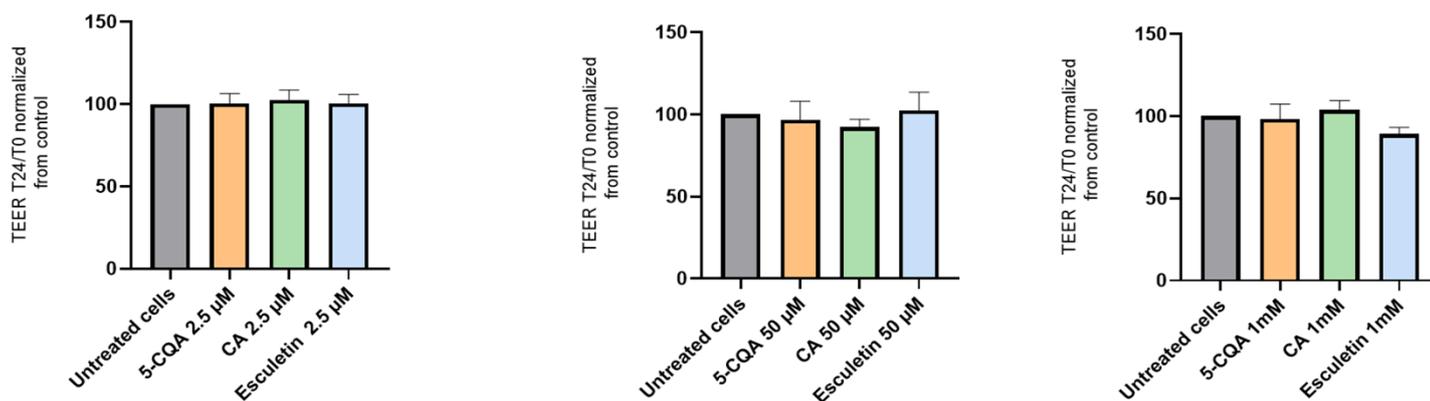


Figure 56. Effects of polyphenols at 2.5  $\mu\text{M}$ , 50  $\mu\text{M}$  and 1 mM on TEER of cells after incubation for 24 h

### II.2.3. Effects of polyphenols on *gene* expression

To study the effects of 5-CQA, CA and esculetin on intestinal tight junctions, real-time PCR was performed and the expression of selected gene were evaluated.

#### a) Effects of polyphenols on occludin expression

The relative expression of *occludin* in the presence of 5-CQA, CA and esculetin at different concentrations compared to that in untreated cells, is shown in Figure 57. *Occludin* expression was increased by esculetin in a dose- dependent manner. Interestingly, the highest effect of esculetin ( $p < 0,001$ ) was reached significantly at the lowest concentration, 2.5  $\mu\text{M}$ , compared to the other treatments used. Significant results were also observed with esculetin treatment at 2.5  $\mu\text{M}$  compared to those with CA ( $p < 0,01$ ) and untreated cells ( $p < 0,05$ ). However, a slight increase was observed with esculetin treatment at 50  $\mu\text{M}$  and 1 mM. In contrast, CQA and CA showed no significant effects on *occludin* expression, at these two concentrations. As none of the polyphenols tested were able to increase TEER, it is likely that the effects of esculetin on barrier integrity are due to the regulation of gene expression.

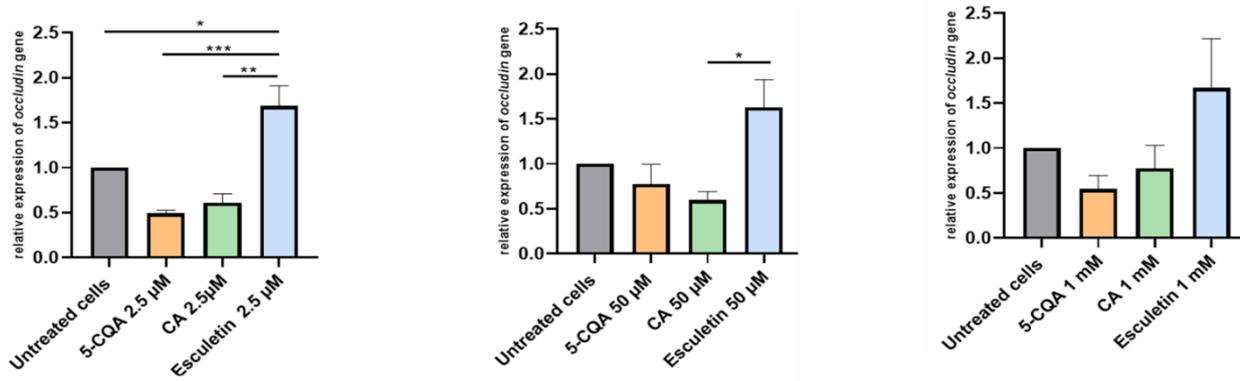


Figure 57. Effects of polyphenols at 2.5 μM, 50 μM and 1 mM on relative expression of *occludin* after incubation for 24 h. ns: not significantly different

*b) Effects of polyphenols on Zo-1 expression*

Interestingly *Zo-1* was differentially expressed in the presence of polyphenols compared to *occludin*. At 2.5 μM, only esculetin significantly induced *Zo-1* expression compared to CA and 5-CQA ( $p < 0,05$ ). However, higher effects were observed when the concentration of esculetin was increased. This coumarin has increased significantly ( $p < 0,01$ ), at 50 μM, *zo-1* expression compared to untreated cells and CA, and to a lesser extent than to 5-CQA ( $p < 0,05$ ). The highest effects of esculetin on *zo-1* were observed at a concentration of 1 mM, with  $p < 0,001$  compared to 5-CQA and CA and  $p < 0,01$  when compared to untreated cells.

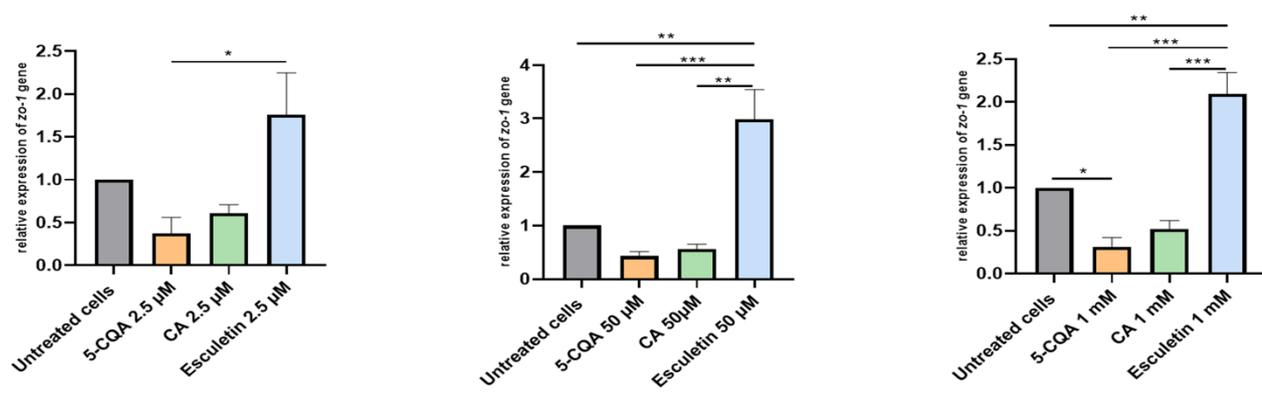


Figure 58. Effects of polyphenols at 2.5 μM, 50 μM and 1 mM on relative expression of *Zo-1* after incubation for 24 h

### E.3. Conclusion

In our study, 5-CQA, CA and esculetin have exhibited no effect on TEER when evaluated using a quadruple model under basal conditions. Nevertheless, the role of esculetin, as a gut-derived metabolite of 5-CQA, was highlighted. This compound significantly increased the relative mRNA expression of tight junction proteins such as *occludin* and *Zo-1*. In contrast, 5-CQA and CA did not affect in the regulation of gene expression. These differences among the polyphenols studied suggest that their effects on the intestinal barrier are related to their molecular structures.

Further studies are ongoing, in an inflammatory model, to investigate the role of these selected polyphenols on gut barrier integrity and the immune response that occurs during intestinal inflammation. The data generated *in vitro* will give us insight into the potential effects of polyphenols and their gut-derived metabolites on gut-microbiota crosstalk, which could open new era of targeting the gut-brain axis to improve and even treat brain disorders.



# Chapter III



### III. Conclusion and perspectives

A large body of research has indicated a profound role of polyphenols in human health (Del Bo' et al., 2019; Williamson, 2017). These compounds have shown a plethora of bioactivities, mainly mediated by their antioxidant properties and regulation of various redox signalling pathways. The French paradox has drawn the attention of the scientific community toward these molecules. However, their biological activity goes beyond classical exploration as antioxidant molecules (Filosa et al., 2018b; Serra et al., 2018). The different aspects of the study of these molecules will allow for a better understanding of their roles in humans. Electrochemistry is a useful tool for studying the antioxidant properties of polyphenols. Moreover, molecules that can arise in redox processes have attracted the attention of scientists, because of their potential positive bioactivities in humans (Lambert de Malezieu et al., 2019; D. Liu et al., 2019). However, there is still a lack of information on the presence of possible oxidized compounds from this type of reaction in the human body. Indeed, there is still no MS/MS database that could help in the identification of such compounds by comparing the MS/MS profiles.

With the main objective, of enriching this type of database, we set out to identify the oxidized metabolites obtained after electrochemical and enzymatic oxidation of one of the main dietary polyphenols, 5-CQA and its major gut-derived metabolite CA. The oxidation mechanism of these compounds involves two protons and two electrons is well known (Šeruga and Tomac, 2014). Moreover, some enzymatically generated oxidized compounds from 5-CQA and CA have been already identified (Castillo-Fraire et al., 2019; Pati et al., 2006). To the best of our knowledge, there is no data identifying these metabolites using electrochemical studies, which was our first objective. To this end, the UV-Vis spectroelectrochemistry of the two phenolic compounds was explored and the MS/MS profiles of the oxidized solutions were characterized. LC/MS<sup>2</sup> analysis of the electrolyzed solutions of 5-CQA led to the identification of one ion that was identified as a reoxidized form of 5-CQA dehydrodimers with  $m/z$  at 703, 17 ions with  $m/z$  705 (MW, 706 Da) corresponding to dehydrodimers of 5-CQA (type I-III) (also named caffeicin-like dimers) and three hydrated dehydrodimers of 5-CQA with  $m/z$  723. These findings are in line with previous data on the enzymatic oxidation of 5-CQA (Castillo-Fraire et al., 2022, 2019), with better reproducibility of the electrochemical method. Both the electrochemical and enzymatic oxidation of CA led to the formation of several oxidized molecules. The formation pathway is suggested to involve the formation of caffeoyl-*ortho*-quinone, followed by the formation of caffeicin-like dimers. The other peaks detected correspond to compounds that are more polar than CA and are

characterized by higher molecular weights. Further work is needed to isolate and characterize these oxidized molecules using semi-preparative HPLC or CPC and NMR, respectively. Further studies are encouraged to establish the biological activity of these oxidized compounds, either individually after isolation or directly as an oxidized solution. Finally, in future investigations, it would be interesting to use an on-line electrochemistry-LC-UV-Vis-ESI-MS<sup>2</sup> apparatus allowing the *in-situ* observation of less stable oxidized compounds than those we have identified.

Because most polyphenols have very low bioavailability, their role in humans has been questioned. This paradox between low bioavailability and high bioactivity was resolved when scientists looked insight what happens when they reach the colon (**Luca et al., 2020**). Interestingly, the gut microbiota biotransforms them, producing low-weight metabolites that are better absorbed and may have different biological activities, which are often superior to of those their parents and have favorable residence time in humans (**Carecho et al., 2020; González-Sarrias et al., 2017b**). Thus, nowadays, new knowledge is focused on the biological effects of the gut-derived metabolites of polyphenols, making them a promising approach to prevent and attenuate neurodegenerative diseases (**Carregosa et al., 2019b; Zhou et al., 2020**).

Therefore, there is great interest in studying the interaction of polyphenols with the gut microbiota. Indeed, identifying the specific bacteria responsible for the biotransformation of polyphenols into bioactive compounds, is crucial for understanding the impact of polyphenols and their metabolites on human health (**Pasinetti et al., 2018b**). Moreover, these findings can contribute to the development of a potential nutraceutical strategy that may beneficially regulate the gut-brain axis, which has a positive impact on human health (**Cortés-Martín et al., 2020; Frolinger and Pasinetti, 2019; Westfall and Pasinetti, 2019**).

Firstly, we have investigated the biotransformation of 5-CQA using the resting cell method, by three gut bacteria species in monoculture, *L. reuteri*, *B. fragilis* and *B. longum*, which are representative of the dominated bacterial phyla in the gut. LC/MS<sup>2</sup> analysis of the biotransformed extracts revealed that only *L. reuteri* had the ability to biotransform 5-CQA into various metabolites related to the presence of cinammoyl esterase in its enzymatic machinery. Nevertheless, no metabolites were formed after biotransformation by *B. fragilis* and *B. longum*, indicating the absence of this enzyme in these bacterial species. Molecular networking analysis of 5-*O*-caffeoylquinic acid (5-CQA) biotransformation by *L. reuteri* highlighted for the first time its bioconversion to esculetin by an oxidative pathway, as well as other well-known gut-derived 5-CQA metabolites. The first step in the biosynthesis of this coumarin involves cinnamoyl esterase activity

to hydrolyse the ester bond between caffeic and quinic acids. Then, *cis*-CA is putatively formed by an isomerase present in the studied *L. reuteri* strain and, then it can be converted by an oxidase into caffeoyl-*o*-quinone. In the end, this latter is stabilized to esculetin. This coumarin was also identified after the electrochemical or enzymatic oxidations of CA, but in much smaller amounts compared to the biotransformed extracts. Indeed, these results provide insights into the biotransformation pathway of a natural compound, 5-CQA, which is highly ingested by the diet. Moreover, esculetin, is a molecule of great interest in medical research (**Garg et al., 2020**). It has already shown some biological activities such as inhibition of oxidative stress and inflammation; it is also known to possess anticancer properties (**Zhang et al., 2022**). Some coumarins have been approved by the FDA as drugs, and warfarin is an example. It blocks vitamin K reductase and thereby disrupting the clotting mechanism. Much work is needed to bring these types of compounds, particularly esculetin, in nutraceutical forms to the clinical trial stage.

In addition, since there is interplay between bacteria in the gut, we studied whether such interactions could influence the biotransformation process. For this purpose, we have co-cultured *L. reuteri* species that is involved in the biotransformation of 5-CQA with *B. fragilis*, which did not show the ability to biotransform it. As a preliminary result, esculetin formation was favorably increased by 23.9 % by co-culture of these two bacteria compared to *L. reuteri* alone. The next step will be to subject 5-CQA to the biotransformation process involving the three bacteria studied, *L. reuteri*, *B. fragilis* and *B. longum*.

Another question that needs to be addressed is how polyphenols and their gut-derived metabolites also modulate the composition and function of the gut microbiota, which has a positive impact on human health (**Selma et al., 2020**). Thus, we have studied the impact of 5-CQA on the growth and metabolic profiles of *L. reuteri* and *B. fragilis*. Interestingly, only *L. reuteri* was affected by the presence of 5-CQA, with a positive correlation of growth at 0.1 and 2.5  $\mu$ M. The mechanism by which 5-CQA have stimulated growth, could involve PAZymes, a fact of presence of this kind of enzymes was revealed in the biotransformation process. Nevertheless, 5-CQA did not show any effect on metabolism in either *L. reuteri* or *B. fragilis*.

On the other hand, maintaining the homeostasis in the gut or even more, restoring it, could be the key to alleviate and treat brain disorders. In this research context, we have investigated the role of 5-CQA and its gut-derived metabolites, CA and esculetin, on barrier function by using an *in vitro* quadruple model. Interestingly, esculetin has increased the relative mRNA expression of tight

junction proteins such as *occludin* and *Zo-1*. Our study supports the evidence of the role of this coumarin as a natural compound to positively impact their intestinal barrier. Nevertheless, neither CA and nor CQA, exhibited effects on the expression of these selected genes. These data are in basal condition, and further studies are underway, using an inflammatory model, with the aim of providing more information on the potential effects of these phenolic compounds on inflammatory pathways that may be involved in the gut-brain axis crosstalk.

From a general point of view, it is clear, that polyphenols can modulate the gut microbiota and consequently impact host health, making them a promising approach to improve brain health. Nevertheless, it should be emphasized that more research needs to be conducted, first to elucidate the complexity of the gut microbiota related to brain disease. It remains still unclear whether alteration of the gut microbiota plays an initial role in the pathogenesis and progression of brain disorders or whether it is a consequence (**Yinwei Chen et al., 2021**). Furthermore, interventions to modify the microbiota should be considered specifically for each disease. To date, the case of altered profiles of *Akkermansia spp.* is very intriguing. Low gut levels of *Akkermansia muciniphila* have been detected in metabolic diseases, including diabetes and obesity (**Cani, 2018**), whereas MS is characterized by an overabundance of this bacterium (**Kasper et al., 2019**). Therefore, it can be assumed that there is a range of balance in the presence of this bacterium which is link to gut health. Indeed, this type of microorganism that is present in small amounts in the gut microbiota could be the key to understanding the link between gut bacteria and host health and disease. Studies should consider the bacteria-bacteria complex, for successful polyphenol intervention, as bacteria do not function individually in the gut but in a complex ecosystem (**Khan et al., 2021**). It is not yet fully established what is a 'healthy gut microbiota', but scientists consider a stable and diverse of microbiota to be present in the gut (**Rinninella et al., 2019b**). Besides, humans may respond differently to polyphenols ingestion, due to the high inter-individual variability in their gut microbiota profile, which supports the development of personalized treatment strategies based on an individual's metotype (**Blasco et al., 2021**).

Nevertheless, larger intervention studies should be carried out, to move from proposals of personalized polyphenol-based dietary strategies to global ones. Recently, the combination of a prebiotic, e.g. polyphenols, and a suitable probiotic, has been proposed as new tool giving rise to the concept of synbiotics (**Swanson et al., 2020; Westfall and Pasinetti, 2019**). In this context, our findings could be of great interest for the development of novel synbiotics related to *L. reuteri* and 5-CQA, in favor of esculetin production. It is now known that regular

consumption of dietary polyphenols can maintain or restore the balance of the gut microbiota, thus contributing to the general human well-being. Therefore, understanding the complex interactions between polyphenols and the microbiota is pivotal to promote the overall health benefits of dietary polyphenol consumption and, in addition, represents a promising strategy to identify a potential approach toward a modifiable gut microbiota, which could lead to positive effects on brain function.

In addition, multiple pathways are involved between gut microbiota and brain crosstalk: *neural* (via vagus nerve, enteric nervous system and spinal nerves) *immune* (via cytokines and chemokines secretion), *endocrine* (via HPA axis, tryptophan, serotonin) and *metabolic* (due to short-chain fatty acids and microbial metabolites) pathways. However, the fully picture how microbes communicate with the brain is not yet fully elucidated and further investigations are undertaken to address unresolved questions, which will help for the development of intervention strategy based on the gut microbiota (**Chakrabarti et al., 2022**). Thus, the polyphenols-gut-microbiota brain axis may be an intriguing pathway by which phenolic compounds, as natural products, modify brain activity and thus alleviate brain disorders, going beyond the classical antioxidant activity.



# Chapter IV



## IV. Materials and methods

### IV.A. Generalities

#### IV.A.1. Reagents and chemicals

All commercial reagents were purchased from Carlo Erba Reactifs and /or from Merck\_Sigma Aldrich (Val de Reuil, France and St Quentin Fallavier, France). 5-CQA was acquired from Acros Organics (Italia) (CAS number 327-97-9) and *trans*-CA, esculetin, were acquired from Merck\_Sigma Aldrich. BHI (Brain Heart Infusion) broth was purchased from Bio-Rad (France). Ethyl acetate (reagent grade) and acetonitrile ( $\geq 99.9\%$ , LC-MS grade) was obtained from Merck\_Sigma Aldrich. Ultrapure water was obtained using an EasyPure (Barnstead, USA) water purification system. Phosphate buffer solution (PBS) was prepared at 0.1 M by mixing 95% of 1.36% w/v  $\text{KH}_2\text{PO}_4$  and 5% of 1.78% w/v  $\text{Na}_2\text{HPO}_4 \cdot 2\text{H}_2\text{O}$  and the pH was adjusted to 5.8. diethylacetic acid (= 2-ethylbutyric acid) (Sigma).

#### IV.A.2. Bacterial strains

The bacterial strains used in our study were *Lactobacillus reuteri* DSM 20016, *Bacteroides fragilis* ATCC 25285 and *Bifidobacterium longum* subsp *infantis* DSM 20088.

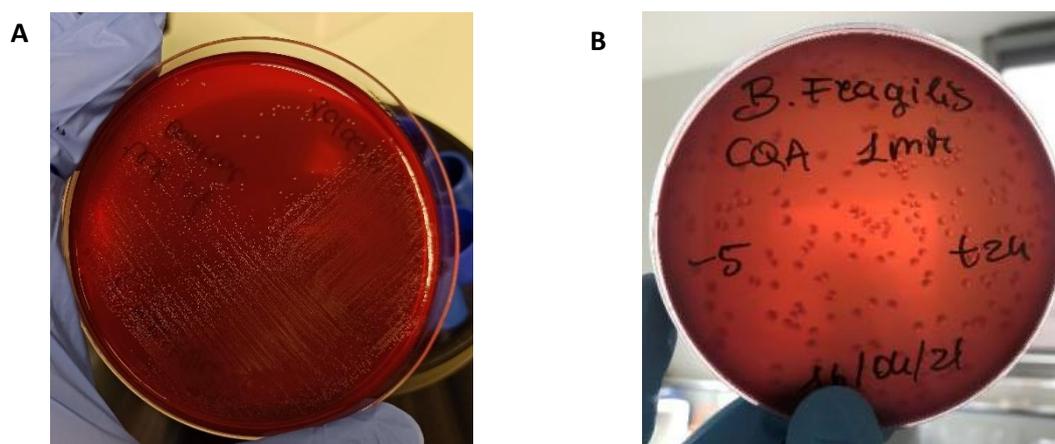


Figure 59. Aspect on the Columbia agar plate of *L. reuteri* (A) *B. fragilis* (B) and colonies after 48 h incubation at 37°C under anaerobic condition

#### IV.A.3. Growth conditions of *L. reuteri*, *B. fragilis* and *B. longum*

Bacteria were grown in BHI broth under anaerobic conditions using an anaerobic chamber (Macs- VA500, Don Whitley) flooded with 80% N<sub>2</sub>, 10% H<sub>2</sub> and 10% CO<sub>2</sub>). Kinetics of cultures were monitored by measuring absorbance (optical density (OD) at 600 nm) and colony-forming units (CFU.mL<sup>-1</sup>). The cultures of bacteria (150 mL) were established by suspending the fresh bacterial strains isolation colonies (from a 48h culture in anaerobic conditions at 37°C) in BHI standard medium in order to obtain an appropriate inoculum of each bacterium; for *L. reuteri* 10<sup>7</sup> CFU.mL<sup>-1</sup> while for *B. fragilis* and *B. longum* 10<sup>8</sup> CFU.mL<sup>-1</sup>. Then, cultures and a flask containing only BHI standard used as a control of the purity, were incubated in anaerobic chamber for 30 h.

### IV.B. Electrochemical Procedures

Before each measurement, a 2 mM 5-CQA or CA solution was freshly prepared in 0.1 M PBS pH 5.8 and nitrogen saturated during 5 min at room temperature. All potentials reported herein are *vs.* Ag/ AgCl reference electrode.

#### IV.B.1. Cyclic voltammetry (CV)

CV experiments were carried out on a dual potentiogalvanostat PGSTAT100 (Autolab instrument, Eco Chemie B.V., Utrecht, The Netherlands). Measurements were performed with a three-electrode cell at room temperature. A glassy carbon disk working electrode (Ø2 mm), a platinum wire counter electrode and a reference electrode, Ag/AgCl/KCl 3 M, were used. Before each measurement, the glassy carbon disk working electrode was polished on a waterproof Silicon Carbide Paper 4000 (Struers) using a 0.3 µm alumina suspension, washed with distilled water and dried. The CV were recorded at a scan rate 0.1 V.s<sup>-1</sup> under nitrogen atmosphere.  $E_{p_a}$  and  $E_{p_c}$  are relative to anodic and cathodic potential, respectively;  $i_a$  and  $i_c$  to anodic and cathodic current respectively.

#### IV.B.2. Spectroelectrochemical measurements

Spectroelectrochemical studies were conducted using a combined potentiostat/galvanostat (AUTOLAB PGSTAT 204, Metrohm, Switzerland) and spectrometer (Avantes, AvaSpec-ULS20 48) equipped with a UV-Visible deuterium/ halogen light source (200 – 2500 nm). This system was controlled by NOVA 1.11 software. A three- electrode cell was used comprising a platinum grid working electrode (7 x 6 mm, wire diameter 0.3 mm), a platinum wire counter electrode and an Ag/ AgCl/ KCl 3M reference electrode placed in a spectrophotometric quartz cuvette (SEC-C thin layer quartz cristal spectroelectrochemical cell, 1 mm optical path length (ALS CO., Ltd)). Before each measurement, the platinum grid working electrode was washed with H<sub>2</sub>SO<sub>4</sub> 0.2 M and sonicated for 5 minutes. Then, a cleaning was done by recording 200 cyclic voltammograms in H<sub>2</sub>SO<sub>4</sub> 0.2 M between - 0.5 V and + 1.5 V (scan rate 0.5 V.s<sup>-1</sup>). In a second step, the platinum grid was cleaned recording 200 cyclic voltammograms in PBS pH 5.8 between -1.2 and + 1.5 V (scan rate 0.5 V.s<sup>-1</sup>).

#### III.B.3. Linear Sweep Voltammetry (LSV)

The LSV ( $i = f(E)$ ) were firstly recorded at a scan rate of 0.005 V.s<sup>-1</sup>. UV-Vis spectra were simultaneously recorded between 200 and 600 nm.

#### III.B.4. Electrolysis

Electrolysis were performed by a chronoamperometry technique  $i = f(t)$  at a fixed potential during 1200 s for 5-CQA and CA. The applied potential was fixed at + 0.8 V for the 5-CQA electrolysis and + 0.6 V for CA. The UV-Vis spectra were simultaneously recorded. During electrolysis, nitrogen bubbling was maintained, first to avoid any oxidation derived from oxygen and also to mix the solution (the whole cell volume is 600  $\mu$ L, whereas the optic way compartment holds approximately 200  $\mu$ L of the solution). After electrolysis, the whole solution was retrieved, saturated with argon in a sealed vial and stored at - 20°C.

#### **IV.C. Enzymatic procedures by polyphenol oxidase (PPO)**

The procedure were adapted from Le Bourvellec et *al.*(Le Bourvellec et al., 2004) and Cheynier et *al.*(Cheynier and Moutounet, 1992b) 4.5 ml of 5-CQA or CA 5 mM dissolved in malate buffer 30 mM at pH 4.8 reacted with 500  $\mu$ L polyphenol oxidase (PPO) malate buffer solution. The whole reaction was stirred during 5 sec then filtered on PVDF 0.45  $\mu$ m just before LC-DAD-ESI-MS<sup>2</sup> analysis.

#### **IV.C. Biotransformation study**

##### **IV.C.1. Biotransformation method**

Resting cells biotransformation studies were carried out when bacteria reached stationary phase (at 30 h of growth), by adding solution of 5-CQA (1 mM) in PBS (50 mL; pH 5.8) on biomass (pellet) of each bacterial strain separated from the culture medium by centrifugation (3500 rpm, 4°C, 15 min). In parallel, as controls (50 mL, pH 5.8), PBS alone or with 5-CQA, CA (1 mM) were incubated without bacteria. In addition, two more controls were prepared either incubating bacteria in BHI or in PBS. Other biotransformation experiments were performed using the same condition, except BHI instead of PBS at 5.8. They were incubated at 37°C, 120 rpm under anaerobic condition using sealed jars and anaerobic bags. After 24 h of incubation, the samples were centrifugated at 3500 rpm, at 4°C for 15 min. The extraction process was further performed on pellet and on supernatant (SN) using ethyl acetate (2 x 10 mL for pellet and 2 x 25 mL for SN).

In addition, we do co-culture *L reuteri* with *B. fragilis* at approximately equimolar concentration, based on OD<sub>600</sub> and CFU results. The first contact of bacteria with polyphenols refered as T<sub>0</sub>. The biotransformation study by co-culture was carried out in the same condition as describe above.

#### IV.C.2. In silico search of cinnamoyl esterase activity

The sequence of cinnamoyl esterase from *L. jnhsonii* was searched in the sequenced genomes of *L. reuteri* DSM\_20016 (GCA\_001436151), using Geneious Prime 2020.2, (<https://www.geneious.com>) and corresponded to Locus Tag named LREU\_RS08790

#### IV.C.3. UV irradiation of samples

5-CQA standard at 1 mM dissolved in water (1 mL) placed in a UV-quartz cell was irradiated at 365 nm using a UV-lamp for 1 h.

#### IV.C.4. LC-DAD-ESI-MS<sup>2</sup> analysis

All the samples (bacterial extracts, electrolyzed, enzymatic) were analysed under the same conditions. After evaporation of organic solvents, the resulting supernatant and pellet extracts were analysed by LC-DAD-ESI-MS<sup>2</sup>.

The mass spectrometry analysis was carried out using Liquid Chromatography–Electrospray Ionization–Tandem Mass Spectrometry (LC-ESI-MS<sup>2</sup>). The system equipped with a SCM1000 degasser (ThermoScientific, San Jose, CA, USA), a 1100 series binary high-pressure pump (Agilent Technologies, Palo Alto, CA, USA), and a Surveyor autosampler thermostated at 4 °C (ThermoFinnigan, San Jose, CA, USA). Two detectors were connected in series: a UV-visible diode array detector (model UV6000 LP, ThermoFinnigan) and an Ion Trap Mass Spectrometer equipped with an electrospray ionization source (model LCQ Deca, ThermoFinnigan). Chromatographic separations were performed on a Purospher® STAR RP-18 endcapped (3 µm) Hibar® HR (Merck, Kenilworth, New Jersey, 2.1 × 150 mm) column, equipped with a pre-column EclipseXDB-C8 (Agilent Technologies, 2.1 × 12.5 mm, 5 µm). The bacterial extracts were injected with a concentration of 2 mg/mL while enzymatic and electrochemical solutions were directly analysed without treatment. The volume injection is of 2 µL. The solvent system was a gradient of A (0.1% formic acid in ultrapure water) and B (0.1% formic acid in acetonitrile), which were filtered on a 0.45 µm GH Polypro membrane (VWR, Fontenay sous Bois, France), respectively, before being used. An elution gradient was applied as follows with a flow rate of 0.2 mL/min: initial, 3% B; 0–3 min, 7% B, linear; 3–21

min, 13% B, linear; 21–27 min, 13% B isocratic; 27–39 min, 38% B, linear; 39–47 min, 50% B, linear; 47–58 min, 90% B, linear; 58–61 min, 90% B; followed by washing and reconditioning the column. UV-visible detection covered the 240–600 nm wavelength range. The whole effluent from the UV-Visible detector was injected in an ESI (Electrospray Ionization) source. The source parameters were negative ion mode with the following parameters: spray voltage (4.2 kV), capillary voltage (−41 V), sheath gas (66 arbitrary units), auxiliary gas (10 arbitrary units), and capillary temperature (250 °C). The nebulizing gas was nitrogen, and the damping gas was helium. MS spectra were acquired in full scan, negative ionization mode in the  $m/z$  50–2000 range. For the MS<sup>2</sup> experiment, collision energy of 35 eV was used. Data obtained were processed with the Xcalibur 1.2 software (Finnigan Corp., San Jose, CA, USA).

#### IV.C.5. Mzmine and GNPS data treatment

Data obtained from low resolution mass spectrometry were first processed with MZmine 2.53. The Agilent files were converted into mzXML format with the software MSconvert, part of the ProteoWizard package. After uploading into the MZmine workflow, the detection mass was performed with a noise level at  $1.10^5$  for MS<sup>1</sup> and 1 for MS<sup>2</sup>. Molecular features were detected using the centroid algorithm followed by a chromatogram building step. The chromatograms were built with the ADAP algorithm with a minimum group size of 2, a group intensity threshold of  $1.10^5$ , a minimum highest intensity at 1. and an  $m/z$  tolerance at 0.8  $m/z$ . The ADAP wavelet algorithm was then used for deconvolution with the following settings: S/N threshold: 10, S/N estimator: Intensity window SN, min feature height: 200, coefficient/area threshold: 10, peak duration range: 0.01-10, RT wavelet range: 0.-0.50. The isotopic peak grouper algorithm was then used with an  $m/z$  tolerance at 0.8  $m/z$ , an RT tolerance at 0.5 min and a maximum charge of 2. The peaks were aligned in a unique peak list using the join aligner algorithm with the following parameters:  $m/z$  tolerance: 0.8, weight for  $m/z$ : 1, retention time tolerance: 0.5 min, weight for RT: 1, require same charge state: unchecked, require same ID: unchecked. Finally, the peak list was filtered using the peak list rows filter by keeping only the peaks with MS<sup>2</sup> scan (GNPS). The peak list was exported to a MGF file and a csv file gathering the metadata. The MGF file was imported using the Python v3.7 library matchms v0.9.2, a MN was generated and dereplicated using the GNPS spectral libraries. Clustering threshold was set at 0.6, and the dereplication parameters were the following: cosine

threshold = 0.4, minimum matched peaks = 3, precursor and fragment mass tolerance = 0.3, top hits kept = 3. The network was opened and visualized using Cytoscape (3.8.0).

#### IV.C.6. Determination of calibration curves for 5-CQA, CA, esculetin by LC-MS/MS

Standard solutions of 5-CQA, CA and esculetin at 1 Mm were prepared in MeOH and were further diluted to obtain working standard solutions at several concentration levels, in the range of 0.6- 100  $\mu\text{g}/\text{mL}$ . Then, the calibration curve was obtained in the concentration range of 2.5- 50  $\mu\text{g}/\text{mL}$ .

#### IV.D. Supplementation studies

The strains were maintained on Columbia 3 agar plates. With the aim to evaluate the impact of 5-CQA in growth and/or metabolism of *B. fragilis* and *L. reuteri*, the experiments were carried out in presence of 5-CQA at different concentrations: 1 mM, 50  $\mu\text{M}$ , 2.5  $\mu\text{M}$  and 0.1  $\mu\text{M}$ . An aliquot of 29 mL initial appropriate inoculum of each bacteria  $\sim 10^8$  CFU $\text{mL}^{-1}$ ,  $\text{OD}_{600\text{nm}} = 0.1$  for *B. fragilis* and  $\sim 10^7$  CFU $\text{mL}^{-1}$   $\text{OD}_{600\text{nm}} = 0,7$  for *L. reuteri* were established by suspending fresh bacterial isolation colonies

In addition, a stock solution of 5-CQA (2 mM) was prepared in PBS at pH 5.8. Then, from this stock solution, adequate dilutions were done and then 1 mL of each was added at in 29 mL of each working culture. At each time, a control containing only bacteria (without 5-CQA) was incubated with all samples.

The kinetic bacterial growth curve was established by absorbance measurement  $\text{OD}_{600\text{nm}}$  and appreciated by bacterial enumeration expressed in CFU. $\text{mL}^{-1}$  at different time ( $T_{0\text{h}}$ ,  $T_{6\text{h}}$ ,  $T_{24\text{h}}$  and  $T_{30\text{h}}$ ). The incubation of cultures was carried out for overnight at 37°C in anaerobic chamber.

At each sampling time, a count is carried out: this was done by cascade dilutions to 1:10 of the sample, in sterile water; indeed, 100  $\mu\text{L}$  of each culture were taken and then diluted 10 times and plated on Columbia 3 agar for bacterial enumeration. Therefore, 100  $\mu\text{L}$  of  $10^{-4}$ ,  $10^{-5}$ ,  $10^{-6}$  and  $10^{-7}$  are inoculated in duplicate by spreading on agars. The dishes were incubated

for approximately 72 hours in anaerobic chamber or under anaerobic condition using sealed jars in presence of the anaerobic sachets (AnaeroGen AN0025) which absorbs rapidly all the oxygen in the jar and simultaneously generates carbon dioxide (Figure 60) at 37 ° C.



Figure 60. Jars with anaerobic sachet for inoculated petri dish incubation

The colonies are then counted in the dishes in order to enumerate the viable and cultivable bacteria. For the control of purity, the dishes were prepared by spreading bacteria from every culture in each time.

Then, at each stage, 5 mL of each sampling time was taken and then centrifuged for 15 minutes at 3500 rpm in 4° C. Thereafter, the supernatant separated from the pellet, was used to study the bacterial metabolites by evaluation of the metabolic profile after extraction. Each sample has been afterwards analysed by gas chromatography - mass spectrometry (GC-MS) for (SCFAs) and branched chain fatty acids (BCFAs).

#### IV.D.2. Analysis of SCFAs, BCFAs,

##### IV.D.2.1. Extraction methods

The extraction of SCFAs, BCFAs, and AA was performed in a chemical fume hood from 1 mL of bacterial cell-free supernatants obtained after treatment with different 5-CQA concentrations and in each sampling time for both bacteria. The supernatants were mixed with, 1/100 methanol (80%) and 1/100 acetonitrile (5%), and 1/100 of 2 mg.mL<sup>-1</sup> of diethylacetic acid (= 2-ethylbutyric acid) used as an internal standard (IS), then centrifuged for 5 min at 4000 rpm in 4°C in order to precipitate proteins then filtered with 0.2 µm filter before being

stored in glass vials at -80 °C for further analysis. Similarly, a solution containing only BHI, extraction solvents and IS was used as a control.

#### IV.D.2.2. Gas chromatography-mass spectrometry (GC-MS) analysis to determine SCFAs or BCFAs production

##### a) GC-MS analysis

Firstly, we have optimized and developed different metabolites extraction methods to have the maximum yield of these metabolites. Chromatographic conditions were tested (column type, composition of the mobile phases, pressure level, temperature, and elution gradient) to achieve a maximum of compounds separation quality and purity of chromatograms profile.

The quantification and identification of these metabolites was carried out using a7020A-5975N GC-MS system (Agilent Technologies, Inc., Palo Alto, CA, USA) Data were collected with Enhance ChemStation G1701DA (Agilent software). Briefly, 1 µL of each extracted sample were directly injected in a ZB-WAX capillary column (30 mm length by 250 µm diameter with 0.25 µm thicknesses) using helium as a gas carrier with a constant flow rate of 1.5 mL/min. The temperature of the injector was kept at 220°C, and the injection was performed in splitless mode. The following oven temperature protocol was used as follows: an initial oven temperature of 50°C, 5°C/min up to 180°C, 1 min at 180°C, and 20°C/min up to 220 °C for cleaning the column. The column was directly connected to the mass spectrometer which was operated at 70 eV and 260°C in the electron impact ionization mode with a full scan from 25 to 220 *m/z*. SCFA were identified by comparing their mass spectra with those held in the NIST MS Data Centre and 2HP-Wiley 138 library (Agilent) and by comparing their retention times with those of corresponding standards (Volatile Free fatty acid mixture CRM46975) purchased from Sigma. The peaks were quantified as the relative total ionic count abundance with respect to the internal standard.

## IV.E. Intestinal cell study

### IV.E.1. Cell lines and growth culture

Human NCI-H716 cells line, derived from a poorly differentiated adenocarcinoma of human cecum (Park et al., 1987), was purchased from ATCC (LGC Standards, Molsheim, France). Cells were maintained and grown in suspension in Roswell Park Memorial Institute medium (RPMI-1640, Gibco, Life Technologies) supplemented with 10 % of fetal bovine serum (FBS), 1% of L-glutamine, 1% of 100 UI/mL penicillin, and 100 µg/mL streptomycin in a humidified incubator at 37°C with 5% of CO<sub>2</sub>. Cell adhesion and differentiation into endocrine cells were initiated by growing cells for 48 h in 24-well culture plate coated with Matrigel Matrix (Corning®) (**Gagnon and Brubaker, 2015**); (Reimer et al., 2001). Caco-2 cells, obtained from American Type Culture Collection (ATCC), were cultivated in complete medium consisting of Dulbecco's modified Eagle medium (DMEM) supplemented with 20% fetal bovine serum, 1% L-glutamine and 1% penicillin and streptomycin. HT29-MTX cells were kindly provided by CRB *CelluloNet* (SFR Biosciences, CNRS UMS 3444, Inserm US 8, Université Claude-Bernard, Lyon, France) and were grown in the same medium as Caco-2 with only 10% of fetal bovine serum under a 5% CO<sub>2</sub> water saturated atmosphere (**Lesuffleur et al., 1991**). The Raji B (ECACC 85011429), issued from human Burkitt's lymphoma cell-line, were grown in RPMI 1640 medium supplemented with 10% fetal bovine serum, 1% non-essential amino acids, 1% L-glutamine and 1% penicillin and streptomycin, at 37 °C in a 5% CO<sub>2</sub> water saturated atmosphere.

### IV.E.2. Cellular model composed of four cells (quadricellular model)

Human NCI-H716 cells were added to apical chamber of polycarbonate Transwell® inserts containing 25µg/cm<sup>2</sup> of matrigel and incubated at 37°C during 48h to obtain endocrine differentiated NCI-H716 cells. Upon confluence, Caco-2 and HT29-MTX cells were harvested with trypsin–EDTA and a predetermined amount of cells of each type were mixed prior to seeding to yield cell ratio of 8:1(Caco-2:HT29-MTX) on this

insert containing endocrine differentiated NCI-H716 cells. After 14 days of culture, Raji B cells are added to the basolateral chamber to induce the differentiation of Caco-2 cells into M

cells (Vernay et al., 2020). Caco-2/HT29-MTX/ endocrine differentiated NCI-H716 cells and RajiB co-culture were maintained for 7 days in DMEM.

#### IV.E.3. Evaluation of the activity of esculetin, 5-CQA and CA on the quadricellular model

Stock solution (2 mM) of 5-CQA, CA were prepared in PBS, while esculetin was firstly diluted in DMSO then in PBS in order to obtain a final concentration of DMSO as 0.12%. Subsequent dilutions were done to obtain two solutions at 300  $\mu$ M and 15  $\mu$ M. Then, 50 $\mu$ L of the corresponding concentrations (2 mM, 300  $\mu$ M, 15 $\mu$ M) were diluted in culture medium (250 $\mu$ L DMEM) in order to obtain each compound at the following concentrations: 1 mM, 50  $\mu$ M and 0.1  $\mu$ M. These solutions were added to the apical side of the culture system and incubated for 24 h. Simultaneously, 50 mL of PBS and 50 mL of (DMSO+PBS) were added in cells as controls. After 24 h both, apical and basolateral contents were collected, and then 350  $\mu$ L of lysis buffer were added to the cells for RNA harvesting. All samples were stored at -80°C for further analysis.

#### IV.E.4. Transepithelial electrical resistance (TEER) measurements and paracellular permeability study

The integrity of the polarized epithelial co-culture (Caco-2/HT29-MTX/M cells) was evaluated by measuring the transepithelial electrical resistance (TEER) using an Ohm/voltmeter (EVOM2; World Precision Instruments). The resistance obtained from a cell-free culture insert was subtracted from the resistance measured across each well and resistance values were calculated in Ohms ( $\Omega$ ).cm<sup>2</sup> by multiplying the resistance values by the filter surface area.

#### III.E.5. Cytotoxicity analysis by LDH (Lactate Deshydrogenase) release

20  $\mu$ L of apical middle of each condition is taken after the 24 hours of incubations and put in duplicates on 96 wells plate. Cytotoxicity detection Kit (LDH) (Ref 11644793001, Sigma-Aldrich) contains solution 1 (Catalyst: Diaphorase/NAD<sup>+</sup> mixture) and solution 2 (Dye

solution: INT and sodium lactate) has been prepared before. 100  $\mu$ L of solution 1 and 5 mL of solution 2 were mixed. 100  $\mu$ L of this mix was added on the 20  $\mu$ L of each apical middle. 96 well plate was incubated in the dark during 30 min at ambient temperature. After incubation the well lecture was performed using a spectrophotometer AMR-100 Microplate reader using 492 nm wavelength which is corresponded to the red formazan chromogen phenomenon observation. Value absorbances have been subtracted to those of controls (apical middle without cells).

### III.E.6. Expression level of selected genes by quantitative reverse transcription PCR (RT-qPCR)

After 21 days of co-culture, RNA was extracted from the upper chamber using Total RNA and Protein isolation kit (Macherey-Nagel) according to the manufacturer's instructions. Afterward, High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems) was used to reverse-transcribe the RNA into cDNA. Then, the genes that were selected specifically for each cells, were relatively quantified using StepOnePlus (Applied Biosystems) with the SYBR Green PCR Master Mix (Applied Biosystems) (Le Bars et al., 2012). To investigate genes of tight-junction components, we have selected occludin and Zonula occludens (Zo-1) genes.

Each gene was normalized to the TBP (TATA box binding protein) mRNA expression level before calculating the fold-change values. Relative gene expression was calculated by the  $2^{-\Delta\Delta CT}$  method (Livak and Schmittgen, 2001).

### III.E.7. Statistical Analysis

All experiments were performed at least in triplicates. After normality of data, distribution was confirmed using the Shapiro–Wilk test, differences within and between groups were calculated using a two-way analysis of variance (ANOVA). Data presented as mean  $\pm$  SD and p-value less than 0.05 was considered as significant.



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**Titre :** L'acide 5-O-caféoylquinique, un polyphénol alimentaire, et ses métabolites produits par des bactéries intestinales : études électrochimiques, analyses LC-MS/MS, biotransformation et études biologiques

**Mots clés :** polyphénol, microbiote intestinal, biotransformation, LC-MS/MS, électrochimie, intégrité de la barrière intestinale

### Résumé :

Les polyphénols sont connus pour leurs propriétés antioxydantes et comme modulateurs des voies de signalisation redox, ce qui a un effet positif sur la santé humaine. Compte tenu de leur supériorité en termes de présence dans la circulation systémique et de leur bioactivité souvent plus élevée que celle des polyphénols parents, on s'intéresse aujourd'hui davantage au rôle central du microbiote intestinal dans la production de métabolites de faible poids moléculaire. Ainsi, nous avons concentré notre étude sur l'acide 5-O-caféoylquinique (5-CQA), un polyphénol alimentaire, soumis à une étude de biotransformation en utilisant *Lactobacillus reuteri*, *Bacteroides fragilis* et *Bifidobacterium longum*. Notre étude a mis en évidence la capacité de *L. reuteri* à bioconvertir le 5-CQA en plusieurs métabolites, et notamment en un composé naturel connu, l'esculetine. Cette capacité de biotransformation a été également évaluée en co-culture. Cette étude a mis en lumière, pour la première fois, une voie oxydative intéressante réalisée par les bactéries intestinales. Des études électrochimiques et enzymatiques ont permis d'identifier, après analyse par LC-MS/MS, les composés oxydés du 5-CQA et de l'acide caféique. De plus, l'esculetine a montré un effet bénéfique sur l'intégrité de la barrière épithéliale par une étude originale réalisée *in vitro* sur un modèle intestinal constitué de quatre types de cellules.

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**Title:** 5-O-caffeoylquinic acid, a dietary polyphenol, and its gut bacteria-derived metabolites: electrochemical, LC-MS/MS, biotransformation and biological studies

**Keywords:** polyphenol, gut microbiota, biotransformation, LC-MS/MS, electrochemistry, gut barrier integrity

### Abstract:

Polyphenols are known for their antioxidant properties and as modulators of redox signalling pathways, which have a positive effect on human health. Given their superiority in terms of presence in systemic circulation and their often higher bioactivity than parent polyphenols, there is now more interest in the gut microbiota's pivotal role in the production of low molecular weight metabolites. Thus, we have focused our study on 5-O-caffeoylquinic acid (5-CQA), a dietary polyphenol, subjected to a resting cell biotransformation study using *Lactobacillus reuteri*, *Bacteroides fragilis* and *Bifidobacterium longum*. Our study highlighted the ability of only *L. reuteri* to bioconvert 5-CQA into several metabolites, including a known natural compound esculetin. This biotransformation capacity was also evaluated in co-culture. This study put in the spotlight, for the first time, an interesting oxidative pathway carried out by gut bacteria. Electrochemical and enzymatic studies led to identify, after LC-MS/MS analysis, the oxidized compounds of 5-CQA and caffeic acid. In addition, esculetin has shown a beneficial effect on the integrity of the epithelial barrier using an original *in vitro* quadruple intestinal model.

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Les polyphénols sont connus pour leurs propriétés antioxydantes et comme modulateurs des voies de signalisation redox, expliquant en partie leur effet positif sur la santé humaine (**Zhang and Tsao, 2016**). Comme la plupart des polyphénols ont une biodisponibilité très faible, leur rôle chez l'homme a été remis en question. Ce paradoxe entre faible biodisponibilité et forte bioactivité a été résolu lorsque les scientifiques ont examiné ce qui se passe lorsqu'ils atteignent le colon (**Luca et al., 2020**). Il est intéressant de noter que le microbiote intestinal les biotransforme, produisant des métabolites de faible poids moléculaire qui sont mieux absorbés. Ces derniers peuvent avoir des activités biologiques différentes, souvent supérieures à leurs précurseurs, et ont un temps de demie-vie supérieur chez l'homme (**Carecho et al., 2020; González-Sarrías et al., 2017**). Actuellement, de nouvelles études se concentrent sur les effets biologiques des métabolites de polyphénols dérivés de l'intestin, ce qui en fait une approche prometteuse pour prévenir et atténuer les maladies neurodégénératives (**Carregosa et al., 2019; Zhou et al., 2020**). Par conséquent, l'étude de l'interaction des polyphénols avec le microbiote intestinal suscite un grand intérêt. En effet, identifier les bactéries spécifiques responsables de la biotransformation des polyphénols en composés bioactifs est crucial pour comprendre l'impact des polyphénols et de leurs métabolites sur la santé humaine (**Pasinetti et al., 2018**). De plus, ces résultats peuvent contribuer au développement d'une stratégie nutraceutique d'intérêt susceptible de réguler de manière bénéfique l'axe intestin-cerveau, et ainsi avoir un impact positif sur la santé humaine (**Cortés-Martín et al., 2020; Frolinger and Pasinetti, 2019; Westfall and Pasinetti, 2019**).

Parmi les polyphénols alimentaires, l'acide 5-*O*-caféoylquinique (5-CQA), également connu sous le nom d'acide chlorogénique, a montré des avantages potentiels pour la santé humaine. Dans ce contexte, nous avons soumis le 5-CQA à une étude de biotransformation en utilisant *Lactobacillus reuteri*, *Bacteroides fragilis* et *Bifidobacterium longum* par la méthode de "resting cells". Ces bactéries ont été sélectionnées en fonction de leur appartenance aux principaux phyla présents dans le microbiote intestinal humain. Sur la base de la courbe de croissance de ces bactéries obtenue par la mesure de la densité optique à 620 nm, la réaction de biotransformation a été réalisée pendant 24 h lorsque la phase stationnaire était atteinte. Le tampon phosphate (PBS) a été choisi pour solubiliser le 5-CQA et son métabolite l'acide caféique (CA) car ce tampon a été décrit précédemment comme ayant des caractéristiques proches de la composition bactérienne intracytoplasmique (**Mortelé et al., 2019**). L'étape de biotransformation a été réalisée à pH 5,8, proche du pH mesuré dans le côlon. Le 5-CQA a été

dissous dans du PBS à 1 mM, une concentration proche de celles mesurées dans le côlon après l'ingestion de café ou de fruits (Clifford, 1999). Après centrifugation, le culot et le surnageant ont été traités séparément à l'acétate d'éthyle pour obtenir deux extraits qui ont été analysés par LC-UV-ESI-MS<sup>2</sup> en mode négatif. Alors que *L. reuteri* était capable de biotransformer le 5-CQA, aucun métabolite biotransformé n'a été détecté après bioconversion par *B. fragilis* et *B. longum*. Pour valider ces résultats, nous avons recherché *in silico*, dans le génome de ces bactéries, l'enzyme responsable de la biotransformation du 5-CQA, nommée cinnamoyl esterase ou feruloyl esterase (EC.3.1.1.73), qui a déjà été trouvée chez *L. johnsonii* (accession number NC\_005362) (Bel-Rhlid et al., 2013). Une comparaison génomique entre le gène déjà décrit chez *L. johnsonii* et ceux présents dans notre souche *L. reuteri* DSM 20016 (GCA\_001436151 a montré 71 % de similarité avec le gène Locus Tag nommé LREU\_RS08790. Néanmoins, ce gène n'a pas été trouvé dans le génome de *B. fragilis* et *B. longum*, ce qui suggère que les résultats de biotransformation obtenus par LC-ES-MS<sup>2</sup> sont conformes aux analyses *in silico*. La recherche sur Pfam a mis en évidence la présence du même domaine, hydrolase\_4 serine aminopeptidase S33 dans ces deux gènes qui appartiennent à la famille de Alpha/Beta hydrolases (El-Gebali et al., 2019). Ce domaine catalytique se retrouve dans une très large gamme d'enzymes comme les estérases. Cette cinnamoyl esterase a permis l'hydrolyse du 5-CQA en CA et en acide quinique. Le CA a effectivement été détecté dans le chromatogramme obtenu par LC-ESI-MS<sup>2</sup>. Par contre, l'acide quinique, étant trop polaire, n'a pas été extrait par l'acétate d'éthyle ou n'a pas été retenu sur la colonne C-18 utilisée. Les données LC-MS/MS de l'extrait de *L. reuteri* ont ensuite été traitées par le logiciel MZMine 2.53 et par Python v3.7 matchms v0.9.2, pour une analyse en réseau moléculaire où chaque spectre de masse en tandem est décrit comme un nœud et les alignements entre spectres comme des liens définis par un score de cosinus (cos) (<http://gnps.ucsd.edu>). Un cos élevé signifie une grande similarité entre deux spectres MS/MS. Le réseau moléculaire (MN) créé a mis en évidence 110 nœuds. Un cluster englobait la majorité des nœuds connectés et 45 nœuds correspondaient à des “auto-boucles”, ce qui représentent une caractéristique spectral unique. Des métabolites spécifiques de cette biotransformation sont apparus dans tous les clusters, mais principalement sous forme de nœuds “auto-bouclés”. Nous avons ensuite annoté les MN par déréplication en utilisant la bibliothèque GNPS. Parmi les métabolites issus de la bioconversion du 5-CQA par *L. reuteri*, le *cis*-CA, le *trans*-CA, l'acide coumaroyl quinique et l'esculetine ont attiré notre attention en raison de leur structure proche du 5-CQA. Pour valider certaines de ces annotations, divers témoins analytiques ont été injectés et ont conduit à l'identification du 5-CQA, de l'esculetine et du *trans*-CA. De manière surprenante, les isomères géométriques *cis* et

*trans* du CA ont été identifiés dans le MN. À notre connaissance, il s'agit de la première indication de la présence de ces deux isomères de CA après biotransformation du 5-CQA par des bactéries ou par le microbiote intestinal humain. L'analyse du réseau moléculaire de la biotransformation du 5-CQA par *L. reuteri* a mis en évidence pour la première fois sa bioconversion en esculetine par une voie oxydative. D'autres métabolites de 5-CQA bien connus ont également été identifiés.

Nous avons proposé une voie de biosynthèse de l'esculetine à partir du 5-CQA. La première étape de la biosynthèse de cette coumarine implique une activité de type cinnamoyl estérase qui hydrolyse la liaison ester entre les acides caféique et quinique. Ensuite, le *cis*-CA est formé par une isomérase présente dans la souche de *L. reuteri* étudiée, puis il peut être converti par une oxydase en caféoyl-*o*-quinone. Finalement, cette dernière est stabilisée en esculetine. Ainsi, ces résultats donnent un aperçu de la voie de biotransformation d'un composé naturel, le 5-CQA, qui est fortement ingéré par l'alimentation. Il est à noter que l'esculetine est une molécule de grand intérêt pour la recherche médicale (Garg et al., 2020). Elle a ainsi montré certaines activités biologiques telles que l'inhibition du stress oxydatif et de l'inflammation ; elle est également connue pour posséder des propriétés anticancéreuses (Zhang et al., 2022).

Pour mieux comprendre la voie de biotransformation oxydative menant à l'esculetine, nous avons étudié l'oxydation du 5-CQA et du CA par voie électrochimique et par voie enzymatique. Dans un premier temps, le comportement électrochimique du 5-CQA et du CA a été étudié par voltampérométrie cyclique dans du PBS 0,1 M à pH 5,8 dans des conditions similaires à celles des études de biotransformation. Comme attendu pour les deux composés, la fonction *ortho*-diphénol est oxydée selon un mécanisme à deux électrons-protons pour former une *ortho*-quinone (visible sur le balayage anodique à  $E_{pa} = +0,55$  V et à  $E_{pa} = +0,38$  V, respectivement pour le 5-CQA et le CA), qui est réduite en sa forme catéchol sur le balayage cathodique retour ( $E_{pc} = +0,11$  V et  $E_{pc} = +0,14$  V, respectivement pour le 5-CQA et le CA). Ensuite, l'électrolyse à potentiel contrôlé de 5-CQA et CA a été réalisée dans du PBS 0,1 M à pH 5,8 sur grille de platine dans une cellule spectrophotométrique UV-Visible. Dans un premier temps, les spectres UV-Visible ont été enregistrés en temps réel au cours du tracé de la voltampérométrie à balayage linéaire LSV à différentes valeurs de potentiel. Jusqu'à +0,75 V et +0,52 V respectivement, le 5-CQA et le CA sont restés sous leur forme native et la longueur d'onde d'absorption maximale  $\lambda_{max}$  a été mesurée à 320 nm. Au dessus de ces valeurs de potentiels, une bande d'absorption était visible autour de 400 nm pour les deux composés, attestant de leur oxydation sous leur forme quinone. Dans un deuxième temps, la technique de

chronoampérométrie a été appliquée pendant 20 min à un potentiel fixe, + 0,8 V et + 0,6 V, pour le 5-CQA et le CA respectivement pour réaliser leur électrolyse. Les spectres UV-Vis ont été enregistrés simultanément sur une plage spectrale de 200 à 600 nm. L'apparition d'une nouvelle bande d'absorption était visible à 400 nm, indiquant la formation de la forme *o*-quinone pour le 5-CQA et le CA. L'électrolyse a été arrêtée après 20 minutes (correspondant à un rendement d'électrolyse de 15%), temps au bout duquel la quinone est encore présente en solution ( $\lambda_{\text{max}} = 404$  et 420 nm pour la quinone 5-CQA et CA respectivement).

Parallèlement, le 5-CQA et le CA ont été soumis à une oxydation enzymatique par l'enzyme PPO (**Cheynier and Moutounet, 1992**). Les échantillons obtenus après les oxydations électrochimique et enzymatique du 5-CQA et du CA ont été analysés par LC-DAD-ESI-MS<sup>2</sup>. Cette analyse des solutions électrolysées et enzymatiques de 5-CQA a conduit à l'identification d'un ion avec un  $m/z$  à 703 comme étant une forme réoxydée de déshydrodimères de 5-CQA, d'ions avec  $m/z$  705 (MW, 706 Da) correspondant à des déshydrodimères de 5-CQA (type I-III) (également appelés dimères de type caféine) et de déshydrodimères hydratés de 5-CQA correspondant à  $m/z$  723. Ces résultats sont en accord avec les données précédentes sur l'oxydation enzymatique du 5-CQA (**Castillo-Fraire et al., 2022, 2019**), avec une meilleure reproductibilité de la méthode électrochimique. L'oxydation électrochimique et enzymatique du CA a conduit à la formation de plusieurs molécules oxydées. Il a été suggéré la formation de caféoyl-*ortho*-quinone, suivie de la formation de dimères de type caféicine. Les autres pics détectés correspondent à des composés plus polaires que le CA et sont caractérisés par des poids moléculaires plus élevés. Des travaux supplémentaires sont nécessaires pour isoler et caractériser ces molécules oxydées en utilisant respectivement la CLHP semi-préparative ou la CPC (Chromatographie de Partage Centrifuge) et la spectroscopie par résonance magnétique nucléaire (RMN). D'autres études sont à réaliser pour établir l'activité biologique de ces composés oxydés, soit individuellement après isolement, soit directement sous forme de solution oxydée. Ces données ont été comparées à celles du 5-CQA biotransformé. L'esculétine a été identifiée à chaque fois indépendamment de la méthode utilisée, soulignant la stabilisation du produit oxydatif du CA, la caféoyl quinone, dans sa forme coumarine, mais en quantité bien moindre par rapport au processus de biotransformation. Sans surprise, aucune trace de caféoyl quinone n'a été trouvée dans les échantillons étudiés.

Comme il existe une interaction entre les bactéries dans l'intestin, nous avons étudié, dans une autre partie, si ces interactions pouvaient influencer le processus de biotransformation. À cette fin, nous avons co-cultivé l'espèce *L. reuteri* qui est impliquée dans la biotransformation du 5-CQA avec *B. fragilis*, qui n'a pas montré la capacité de le biotransformer. Comme résultat préliminaire, la formation d'esculetine a été favorablement augmentée de 24 % par la co-culture de ces deux bactéries par rapport à *L. reuteri* seule. La prochaine étape consistera à soumettre le 5-CQA au processus de biotransformation impliquant les trois bactéries étudiées, *L. reuteri*, *B. fragilis* et *B. longum*.

Une autre question qui doit être abordée est de savoir comment les polyphénols et leurs métabolites issus de l'intestin modulent également la composition et la fonction du microbiote intestinal, ce qui pourrait avoir un impact positif sur la santé humaine (Selma et al., 2020). Ainsi, nous avons étudié l'impact du 5-CQA sur la croissance et les profils métaboliques de *L. reuteri* et *B. fragilis*. Dans cette étude, l'impact de différentes concentrations de 5-CQA, à 0,1, 2,5, 50 et 1000  $\mu\text{M}$  ont été évaluées sur les deux souches bactériennes intestinales *L. reuteri* et *B. fragilis*. Cette gamme de concentrations a été choisie de la concentration la plus faible aux concentrations physiologiques pouvant potentiellement atteindre l'intestin. Le 5-CQA a été ajouté au début de la culture puis les bactéries ont été incubées dans une chambre anaérobie pendant 30 heures à 37°C. Les surnageants de culture ont été recueillis au début (temps 0) et après 6, 24 et 30 heures d'incubation pour mesurer la DO à 620 nm reflétant la croissance bactérienne et pour déterminer la concentration bactérienne (CFU.mL<sup>-1</sup>). Des cultures des souches testées sans 5-CQA ont également été réalisées en tant que témoins, appelés *L. reuteri* non traité ou *B. fragilis* non traité. L'effet du 5-CQA sur la croissance de *L. reuteri* et de *B. fragilis* a été déterminé en comparant les paramètres de croissance des cultures traitées avec différentes concentrations de 5-CQA à ceux des cultures non traitées.

Pour étudier l'impact de différentes concentrations de 5-CQA sur la production métabolique de ces deux espèces bactériennes, comme les acides gras à chaînes courtes (SCFA) et les ramifiés (BCFA), les surnageants des cultures cultivées dans des conditions standard (BHI = 25  $\mu\text{g.mL}^{-1}$  d'hémine) ainsi que dans des concentrations de 5-CQA supérieures ont été analysées par GC-MS. Nous avons finalement observé que le 5-CQA n'avait aucun effet ni sur la croissance, ni sur les productions métaboliques (SCFAs et BFCAs) des deux souches bactériennes étudiées.

L'intégrité et la fonction de la barrière intestinale sont essentielles au maintien de l'homéostasie intestinale, et donc de la santé de l'hôte (**Singh et al., 2019**). Cette barrière intestinale est une couche unique constituée de différents types de cellules telles que les entérocytes, les cellules de Goblet, les entéroendocrines, les cellules de Microfold (M) et de Paneth. Ces cellules sont scellées par des protéines transmembranaires de jonction serrée (TJ) (par exemple, occludine, claudines, molécules d'adhésion jonctionnelle et tricelluline) ainsi que par des protéines membranaires périphériques (par exemple, Zonula occludens (Zo-1) et cinguline). Leur rôle est d'empêcher la perméabilité paracellulaire et de maintenir la fonction de barrière intestinale. En outre, la couche de mucus empêche le contact direct entre les microbes intestinaux et les cellules épithéliales (**Chelakkot et al., 2018; Hooper, 2015**). La perturbation des jonctions serrées entraîne un dysfonctionnement de la barrière, suivi de la translocation des agents pathogènes et des toxines bactériennes au niveau systémique et de l'activation du système immunitaire et de l'inflammation, contribuant ainsi à plusieurs conditions pathologiques (**Chelakkot et al., 2018**). Des preuves issues d'études *in vitro* et animales suggèrent que les polyphénols peuvent réguler et maintenir l'intégrité et la fonction intestinales, par le biais de plusieurs mécanismes (**Bernardi et al., 2020; Wan et al., 2021**). La plupart des études *in vitro* ont été réalisées à l'aide de la lignée cellulaire Caco-2, puis T84 et HT29/B6 (**Bernardi et al., 2020**). La plupart des connaissances actuelles sur les mécanismes du microbiome proviennent de systèmes modèles tels que les systèmes *in vitro*, *ex vivo* et animaux (**Galloway-Peña and Hanson, 2020**). Bien que les modèles animaux soient fréquemment utilisés, la transposition des données à l'homme a été remise en question (**Bjornson-Hooper et al., 2022; Desmoulin-Canselier and Moutaud, 2019; Pound and Ram, 2020; Seok et al., 2013**). En outre, elles impliquent des problèmes éthiques et sont assez coûteuses (**Fitzgerald et al., 2015**). Par conséquent, les approches *in vitro* à base de cellules peuvent constituer une alternative à l'expérimentation animale (**Bédard et al., 2020**). La culture cellulaire permet ainsi aux scientifiques d'étudier les mécanismes cellulaires et moléculaires d'une manière relativement simple et reproductible (**Joseph et al., 2018**).

Pour tenter de se rapprocher de l'intestin humain et éviter l'utilisation de modèles animaux, le Dr Latifa BOUSARGHIN et son équipe (NuMeCan), ont développé un modèle *in vitro* quadruple. Ce modèle original est composé d'entérocytes (Caco-2), de cellules de gobelets sécrétant du mucus (HT29-MTX), de cellules de micro-plis (cellules M) et de cellules entéroendocrines (NCI). Cinq types de cellules ont été utilisés pour ce qui suit, et doivent donc être

cultivés dans des conditions spécifiques. Les premiers types sont les cellules NCI-H716, des cellules flottantes imitant les cellules entéroendocrines, obtenues à partir d'un homme de 33 ans atteint d'un adénocarcinome colorectal. Ensuite, les Caco-2, qui imitent les entérocytes, sont des cellules adhérentes provenant d'un homme de 72 ans atteint d'un adénocarcinome colorectal. La ligne HT29-MTX représente les cellules gobelets, productrices de mucus, obtenues à partir des cellules HT29 du côlon d'un patient de 44 ans atteint d'un adénocarcinome colorectal. Les cellules Raji-B, correspondant aux cellules flottantes, sont des lymphoblastes obtenus à partir d'un garçon de 11 ans. Elles permettent la différenciation des cellules Caco-2 en cellules M par l'intermédiaire de médiateurs. Enfin, les cellules de type THP-1 sont des monocytes, cellules flottantes, provenant d'un enfant d'un an atteint de leucémie monocyttaire aiguë.

Étant donné l'importance de la barrière intestinale pour la santé et compte tenu du rôle des polyphénols dans la protection de son intégrité et de sa fonction, nous avons voulu donner des informations sur les effets potentiels du 5-CQA et de ses métabolites issus de l'intestin (CA et esculetine) sur les cellules épithéliales dans le cadre de l'intégrité de la barrière intestinale. Une attention particulière a été accordée à la mesure de la résistance électrique transépithéliale (TEER) et de l'expression des gènes *occludine* et *Zo-1*. Dans cette étude, nous avons utilisé le modèle original *in vitro* quadruple décrit précédemment.

Étant donné que le 5-CQA atteint le côlon à une concentration  $\mu\text{M}$  et que ses métabolites microbiens tels que le CA et l'esculetine pourraient être proches de l'épithélium intestinal lorsqu'ils sont produits, nous avons cherché à savoir si ces composés pouvaient avoir un impact direct sur la fonction des cellules épithéliales. Pour répondre à ces questions, nous avons utilisé le modèle *in vitro* quadruple en présence des polyphénols sus-mentionnés à 1 mM, 50  $\mu\text{M}$  et 0,1  $\mu\text{M}$  (de la concentration la plus faible à celles physiologiques qui pourraient potentiellement atteindre l'intestin) en condition basale. Différentes mesures ont été évaluées en présence des polyphénols testés, notamment la viabilité cellulaire, la résistance électrique transépithéliale (TEER) et l'expression de gènes tels que *occludin* and *Zo-1*.

Le parameter TEER est l'un des principaux paramètres utilisés pour étudier la fonction d'intégrité de la barrière, *in vitro*. Le parameter TEER représente le degré auquel de petites particules chargées peuvent traverser l'épithélium cellulaire de manière paracellulaire. Ainsi, la fonction de barrière intestinale a été évaluée en surveillant l'évolution du TEER dans le temps. Le TEER des cellules a été mesuré avant l'ajout des molécules à T0 et après 24 h, en

présence de différentes concentrations (2,5  $\mu$ M, 50  $\mu$ M et 1 mM) de 5-CQA, CA et esculetine. Les résultats ont clairement montré qu'aucun des polyphénols étudiés n'augmentait significativement le TEER.

Pour étudier les effets du 5-CQA, du CA et de l'esculetine sur les jonctions serrées intestinales, une PCR en temps réel a été réalisée et l'expression de certains gènes a été évaluée. L'expression du gène *occludine* a été augmentée par l'esculetine de manière dose-dépendante. De manière intéressante, l'effet le plus élevé de l'esculetine ( $p < 0,001$ ) a été atteint de manière significative à la plus faible concentration, de 2,5  $\mu$ M, par rapport aux autres traitements utilisés. Des résultats significatifs ont également été observés avec le traitement à l'esculetine à 2,5  $\mu$ M par rapport à ceux obtenus avec le CA ( $p < 0,01$ ) et aux cellules non traitées ( $p < 0,05$ ). Cependant, une légère augmentation a été observée avec le traitement à l'esculetine à 50  $\mu$ M et 1 mM. En revanche, le 5-CQA et le CA n'ont montré aucun effet significatif sur l'expression de l'*occludine*, à ces deux concentrations. Comme aucun des polyphénols testés n'a été en mesure d'augmenter le TEER, il est probable que les effets de l'esculetine sur l'intégrité de la barrière soient dus à la régulation de l'expression des gènes.

Il est intéressant de noter que *Zo-1* était différentiellement exprimé en présence de polyphénols par rapport à l'*occludine*. À 2,5  $\mu$ M, seul l'esculetine a induit de manière significative l'expression de *Zo-1* par rapport au CA et au 5-CQA ( $p < 0,05$ ). De plus, des effets plus importants ont été observés lorsque la concentration d'esculetine a été augmentée. Cette coumarine a augmenté de manière significative ( $p < 0,01$ ), à 50  $\mu$ M, l'expression de *Zo-1* par rapport aux cellules non traitées et au CA, et dans une moindre mesure par rapport au 5-CQA ( $p < 0,05$ ). Les effets les plus importants de l'esculetine sur la *zo-1* ont été observés à une concentration de 1 mM, avec  $p < 0,001$  par rapport au 5-CQA et au CA et  $p < 0,01$  par rapport aux cellules non traitées.

L'esculetine a donc montré *in vitro* un effet bénéfique sur l'intégrité de la barrière épithéliale en utilisant un modèle original quadruple mimant l'intestin.

D'autres études sont en cours, dans un modèle inflammatoire, pour étudier le rôle de ces polyphénols sélectionnés sur l'intégrité de la barrière intestinale et la réponse immunitaire qui se produit pendant l'inflammation intestinale. Les données générées *in vitro* nous donneront un aperçu des effets potentiels des polyphénols et de leurs métabolites issus de l'intestin sur l'axe intestin-microbiote, ce qui pourrait ouvrir une nouvelle ère de ciblage de l'axe intestin-cerveau pour améliorer et même traiter les troubles cérébraux.

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