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Mutual interactions of stilbenoids, colon microbiota and intestinal epithelium

Doctoral thesis

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Declaration

I declare that given Ph.D. thesis is solely my own work unless otherwise referenced or acknowledged.

Prague, September 4, 2020

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Ing. Veronika Jarošová

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1

General Introduction

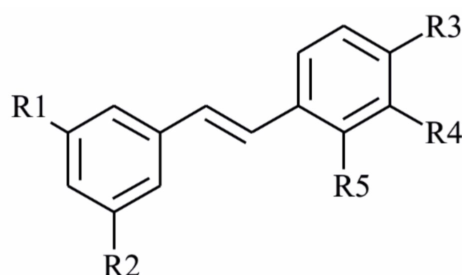
1.1.1 Phenolic compounds

Phenolic compounds are major plant secondary metabolites, generally derived from phenylalanine or tyrosine (Soto-Vaca et al. 2012). They represent a diverse group of molecules that contain an aromatic group and one or more hydroxyl groups on the aromatic ring (Soto-Vaca et al. 2012). According to their chemical structure phenolics are usually divided into four main classes: flavonoids, phenolic acids, stilbenes, and lignans (Pérez-Jiménez et al. 2010). In plants, they are involved in protection against biotic and abiotic stresses, such as UV radiation, vegetal body injuries or infection by pathogens, and they are also attractants for pollinators and seed-dispersing animals (Babbar et al. 2015; Baião et al. 2017). In higher or lower concentrations phenolics are present in all foods of plant origin (Pérez-Jiménez et al. 2010). In food, they contribute to the bitterness, astringency, colour, flavour, odour and oxidative stability (Naczk & Shahidi 2006).

The epidemiological, clinical and nutritional studies from recent years show significant biological effects of polyphenols such as oxidative stress protection, prevention of cancer, cardiovascular or neurodegenerative diseases (Schaefer et al. 2006; Han et al. 2007; Parkar et al. 2008; Tsao 2010; Zhang & Tsao 2016). Very interesting is also their influence on colon microbiota composition in an *in vitro* and *in vivo* assays (Anhê et al. 2015; Ritchie et al. 2015). This could be the consequence of their selective antimicrobial properties.

1.2 Stilbenoids

Stilbenoids are a group of non-flavonoid polyphenolic compounds, which share a common backbone stilbene structure (1,2-diphenylethylen) but differ in the type and position of substituents on the ring (***Figure 1***). They are naturally occurring as both *cis*- and *trans*-isomers, of which the *trans*- form is the most common configuration (Rivière et al. 2012). Stilbenoids exist in the form of monomers or oligomers, which are arising from the oxidative coupling of *trans*-resveratrol or other monomeric stilbenoids, such as oxyresveratrol, isorhapontigenin or piceatannol, by C-C or C-O-C bond in either two, four, six or eight linkage points (Shen et al. 2009).



	R1	R2	R3	R4	R5
stilbene	H	H	H	H	H
resveratrol	OH	OH	OH	H	H
piceatannol	OH	OH	OH	OH	H
pterostilbene	OMe	OMe	OH	H	H
oxyresveratrol	OH	OH	OH	H	OH
pinostilbene	OH	OMe	OH	H	H

Figure 1. Chemical structures of stilbene and its selected derivatives.

Up to date, more than 400 derivatives of stilbene have been identified in various edible and medicinal plants, of which the most important dietary sources are grapes, red wine, peanuts and some berries (El Khawand et al. 2018). Some of them have been recognized as phytoalexins and associated with defence mechanisms. In grapes the synthesis of stilbenoids is induced by fungal infection (*Botritis cinera*), injury, UV radiation and wilting, and it is also affected by such factors as grape cultivar, developmental stage of berry and soil cultivation practices (Naczk & Shahidi 2006). Stilbenoids present in the plants provide the protection against many pathogens such as grapevine downy mildew (*Plasmopara viticola*) (Bavaresco & Fregoni 2001).

In plants, stilbenoids are synthesized via the phenylpropanoid pathway (shown in ***Figure 2***). The key enzymes in this pathway are phenylalanine ammonia-lyase (PAL), cinnamic acid 4-hydroxylase (C4H) and 4-coumarate: CoA ligase (4CL). PAL genes are transcriptionally activated after infection or plant damage and it catalyses the conversion of phenylalanine to *trans*-cinnamic acid by nonoxidative deamination. Thereafter the *trans*-cinnamic acid is hydroxylated to 4-coumaric acid, catalysed by C4H, a cytochrome P450 monooxygenase, which is induced by light, elicitors, and wounding, and it is often closely coordinated with the induction of PAL (Lu et al. 2012). The 4-coumaric acid or cinnamic acid are converted to their CoA thioester by 4CL, which then can diverge into different pathways, leading to the formation of a wide range of phenolic metabolites, including lignin or flavonoids (Lu et al. 2012). Stilbenoids are formed by the addition of three acetate units from malonyl-CoA

by stilbene synthase (STS), and can be further prenylated, geranylated, methylated or glycosylated (Rivière et al. 2012; Akinwumi et al. 2018).

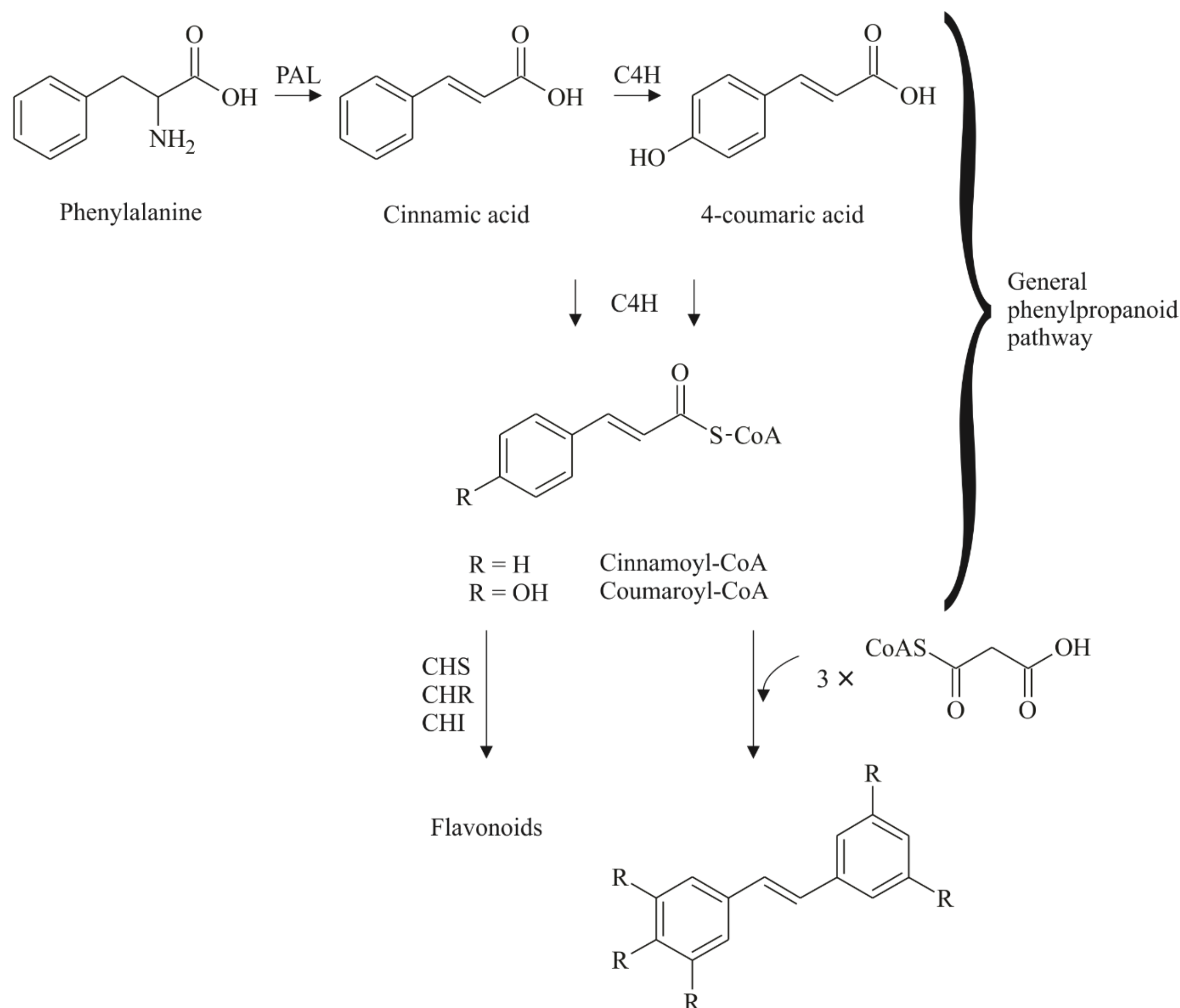


Figure 2. The biosynthesis pathway of stilbenes (Lu et al. 2012).

1.2.1 Resveratrol

The most well-known stilbenoid is resveratrol (3,4',5-trihydroxystilbene; ***Figure 1***). It was first detected in roots of *Veratrum grandiflorum* and later isolated from *Polygonum cupsidatum*, which is currently the richest known source of resveratrol, with concentrations 150 - 1770 µg/g (Beňová et al. 2008). The dry root of *P. cuspidatum* is commonly used in traditional Chinese medicine, as well as Korean or Japanese folk medicine, as analgesic, antipyretic, diuretic,

expectorant, and antitussive agent and also used for the treatment of chronic bronchitis, infectious hepatitis, diarrhoea, cancer, hypertension, atherosclerosis, hyperlipidaemia, leucorrhoea, dysmenorrhea, trauma with blood stasis, burn, snake bites, and allergic inflammatory diseases (Shan et al. 2008). Important dietary sources of resveratrol are grapes (*Vitis vinifera*), where its average concentration, depending on the variety, is 35–172 μg/g (Pascual-Martí et al. 2001). During the wine fermentation, resveratrol is released and its concentration in red wine could be, according to some authors up 20 μg/mL (Tian & Liu 2019). Other food sources of resveratrol include peanuts (*Arachis hypiogaea*; 0.03 – 1.92 μg/g; Tokuşoğlu et al. 2005), pistachios (*Pistacia vera*; 0.09 – 1.67 μg/g; Tokuşoğlu et al. 2005), cocoa (*Theobroma cacao*; 1.4 – 2.3 μg/g; Hurst et al. 2008), wide range of berries (Genus *Vaccinium*; 3.75 - 30 μg/g; Ehala et al. 2005) and passion fruit seeds (*Passiflora edulis*; 0.1 mg/g of raw seed; Matsui et al. 2010).

The metabolism of natural compounds before they reach the bloodstream plays a crucial role in their bioactivity. When reaching the colon, stilbenoids are intensively metabolized by colon microbiota. Up to date, three metabolites of *trans*-resveratrol, dihydroresveratrol, 3,4'-dihydroxy-*trans*-stilbene, and 3,4'-dihydroxybibenzyl (lunularin), have been detected in an *in vitro* faecal fermentation system (Bode et al. 2013). Subsequently, resveratrol is transported through the enterocytes. *In vivo* and *in vitro*, using Caco-2 cell line model, some resveratrol metabolites have been identified, including resveratrol-4′-Oglucuronide, resveratrol 3-O-glucuronide, resveratrol-3-O-sulphate, and resveratrol-4′-O-sulphate, in both *cis*- and *trans*- forms (Kaldas et al. 2003; Maier-Salamon et al. 2006; Urpi-Sarda et al. 2007; Boocock et al. 2007). For *trans*-resveratrol the monosulphate was identified as predominant metabolite (Kaldas et al. 2003; Maier-Salamon et al. 2006). According to the study of Sabolovic et al. (2006) the glucuronidation rate of *cis*-resveratrol was about 90-fold higher than that of *trans*-resveratrol. A part of resveratrol and its metabolites is pumped back to the lumen by efflux proteins, such as the ATP-binding cassette (ABC) transporters. Multidrug resistance-associated protein (MRP2) and breast cancer resistance protein (BCRP) were identified as two transporters involved in the efflux of resveratrol conjugates (Juan et al. 2010).

Trans-resveratrol is often connected with so-called French paradox, a low incidence of coronary heart disease despite high saturated fat intake in a French sample population (Renaud & de Lorgeril 1992; Kopp 1998). Since then, *trans*-resveratrol has been tested in numerous clinical trials. The database of clinical studies (http://clinicaltrials.gov/) reveals a total

of 170 studies involving resveratrol, connected mostly with the treatment of diabetes mellitus, cancer, cardiovascular and neurodegenerative diseases.

However, despite its high pharmacological potential, *trans*-resveratrol displays very low bioavailability and only trace amounts of unchanged *trans*-resveratrol are detected in plasma after oral administration (Walle et al. 2004). This high bioactivity but low bioavailability is often referred to as the "Resveratrol paradox" and might have few possible explanations. *In vivo* studies in mice (Lagouge et al. 2006) and obese humans (Timmers et al. 2011) showed a significant biological effect of resveratrol even at low plasma concentrations 10–120 ng/mL and 231 ng/mL, respectively. These studies showed that resveratrol administration increased mitochondrial function through the activation of SIRT1 (silent information regulator) and PGC-1α (peroxisome proliferator-activated receptor γ coactivator), which in mice translated into an increase in energy expenditure, improved anaerobic capacity, enhanced sensorimotor function, and, in humans, a reduction in sleep and in the resting metabolic rate. Chen et al. (2013) in their study showed that resveratrol is transported to the vascular endothelial cells by both passive diffusion and an SGLT1-mediated process, and its concentration in the thoracic aorta samples declined more slowly compared to that in blood. This suggested the important role of the intracellular pool of resveratrol, which might be even more important than serum level *in vivo*.

Other studies focused on the role of resveratrol metabolites. The conjugation of resveratrol leads to the higher water solubility of metabolites and allows easier elimination from the organism by urine. Walle et al. (2004) showed that after an oral dose of 25 mg of *trans*-resveratrol sulphate and glucuronide conjugates the amount excreted in the urine accounts for around 24% and 13% of the dose, respectively. The serial blood samples taken over 72 hours from six healthy volunteers showed very low concentrations of unchanged *trans*-resveratrol (<5 ng/mL) in plasma, nevertheless, the concentration of its metabolites was as high as 491 ± 90 ng/mL (mean ± SEM) with a plasma half-life of 9.2 ± 0.6 hours (Walle et al. 2004). One of the hypothesis is that resveratrol metabolites act as inactive reservoirs for resveratrol generation (Maier-Salamon et al. 2013). Most of the recent studies, however, focused on the biological activity of resveratrol conjugates. It has been shown that some resveratrol metabolites are more cytotoxic towards tumour cells (HT-29, Caco-2, and MCF-7) compared to parental resveratrol, and less toxic towards noncancerous HEK-239 cell lines (Storniolo et al. 2014; Falomir et al. 2016). Some metabolites of resveratrol were also shown to inhibit

angiogenesis, and telomerase production (Falomir et al. 2016). Piceid, a glucuronidated metabolite of *trans*-resveratrol, exhibits greater scavenging activity against hydroxyl radicals than *trans*-resveratrol *in vitro* (Su et al. 2013).

1.2.2 Other important stilbenoids

1.2.2.1 Piceatannol

Piceatannol (3,3′,4′,5-tetrahydroxystilbene, see ***Figure 1***), also known as astrininin (Rimando et al. 2004), is one of the most studied resveratrol analogues. It exists in both, *cis*- and *trans*-isomers, however, *trans*-piceatannol is more stable than its *cis*- form (Kukreja et al. 2014). Important sources of piceatannol in the human diet are grapes (*Vitis vinifera*) and wine, where it is present in about four times lower concentrations than resveratrol (Piotrowska et al. 2012). (Buiarelli et al. (2007) in their study analysed 22 wine samples, where the concentration of *trans*-resveratrol ranged from 0.8 to 3.4 µg/mL, *cis*-resveratrol from 0.4 to 5 µg/mL, and piceatannol from 0.5 to 5.2 µg/mL. A high concentrations of piceatannol were also detected in passion fruit (*Passiflora edulis*) seeds 2.2 mg/g of raw seed (Matsui et al. 2010). It has been also detected in tea, rhubarb (*Rheum* spp.), *Vaccinium* berries, or Japanese knotweed (*P. cuspidatum*) (Piotrowska et al. 2012).

Piceatannol possesses similar biological properties as resveratrol, which accounts, among others, antioxidant, antiproliferative, and anti-inflammatory activity (Piotrowska et al. 2012). Ovesná et al. (2006) in their study shows piceatannol as a strong antioxidant with a significantly higher protective effect against DNA damage caused by ˙OH radicals than resveratrol. Piceatannol has been also shown to exert positive effects on cultured dermal cells (melanin-producing MNT-1 human melanoma cells) and yield higher melanin inhibitory and a collagen synthesis effects compared to resveratrol (Matsui et al. 2010). Piceatannol is known as Janus kinase (JAK1) inhibitor, which activates STAT3 (signal transducer and activator of transcription 3), a common future of prostate cancer (Barton et al. 2004). It has been shown to inhibit proliferation of Caco-2 and HCT-116 human colon carcinoma cells (Wolter et al. 2002), and T24 and HT1376 human bladder cancer cells (Kuo & Hsu 2008). Kim et al. (2008) reported, that piceatannol induced apoptosis of the human leukaemia cell line U937. Results of Ashikawa et al. (2002) demonstrate that piceatannol suppresses NF-κB activation induced by various inflammatory agents through inhibition of IκBα kinase and p65 phosphorylation.

1.2.2.2 Pterostilbene

Pterostilbene (3,5-dimethoxy-4'-hydroxystilbene, see ***Figure 1***) is a dimethylated analogue of resveratrol. It was originally isolated from the heartwood of red sandalwood (*Pterocarpus santalinus*, Lin et al. 2009), but later it has been also found in blueberries (99 – 520 ng/g, Rimando et al. 2004). The concentrations of pterostilbene in berries are significantly lower than those of resveratrol, however, due to presence of two methoxy groups, its bioavailability is considerably higher, up to 95% (Lin et al. 2009; McCormack & McFadden 2013).

Pterostilbene has also been identified as the main phenolic compound in Darakchasava, fermented grape juice, traditionally used in Indian Ayurvedic medicine for the treatment of several diseases such as piles, loss of appetite, heart diseases, anaemia, epistaxis, tumour, worm infestation, tuberculosis etc. (Paul et al. 1999). Pterostilbene has also been found in a heartwood of Indian Kino Tree (*Pterocarpus marsupium*), traditionally used for treatment of diabetes in the Ayurvedic system of medicine (Manickam et al. 1997) or in a bark of bubinga tree (*Guibourtia tessmanii*), well-known in African traditional medicine for its antimicrobial, antifungal or anthelmintic effects (Fuendjiep et al. 2002).

In vitro pterostilbene has been shown to decrease MDA-MB-231 and MCF-7 breast cancer cells viability, increased caspase-3/7 activity and apoptosis in both cell lines (Alosi et al. 2010). Pterostilbene also shows anti-obesity and cholesterol-lowering activity, which might be possibly caused by its strong influence on colon microbiota, namely *Verrucomicrobia* phylum, as it has been shown in an *in vivo* study in Zucker rats (Etxeberria et al. 2017)

1.2.2.3 Oxyresveratrol

Oxyresveratrol (2',3,4',5-tetrahydroxystilbene, see ***Figure 1***), another natural hydroxystilbene, is present in mulberry (*Morus* spp.) fruits and twigs (Matencio et al. 2017). It is abundantly present in the heartwood of *Artocarpus lakoocha* Roxb. (Moraceae) in a concentration of 23.4 to 69.6 mg/g (Maneechai et al. 2009). In Thailand, a dried aqueous extract of *A. lakoocha* is used as traditional drug Puag-Haad, an effective anthelmintic, containing about 800 mg of oxyresveratrol per gram (Maneechai et al. 2009).

Oxyresveratrol has been shown to possess strong antioxidant activity, and to be even more effective scavenger of DPPH (2,2-diphenyl-1-picrylhydrazlyl) than resveratrol (Lorenz et al. 2003). It has also been shown as strong tyrosinase inhibitor, the key enzyme for melanin

biosynthesis and has an effect on skin depigmentation. Therefore, it might have a potential as a skin-whitening agent or as an anti-browning agent for food products of plant origin (Likhitwitayawuid et al. 2006). Similarly to resveratrol, also oxyresveratrol possesses antioxidant, antivirotic, anti-inflammatory, antiapoptotic, antiherpetic, hepatoprotective and neuroprotective or photoprotective activity (Likhitwitayawuid et al. 2006; Matencio et al. 2017).

1.2.2.4 Pinostilbene

Another analogue of resveratrol, pinostilbene (3,4'-dihydroxy-5-methoxystilbene, ***Figure 1***), was originally found in the bark of Siberian pine (*Pinus sibirica*) (Tyukavkina et al. 1972). It has been also found in stem wood of *Dracena loureiri*, used in traditional Thai medicine as an antipyretic and an analgesic (Likhitwitayawuid et al. 2002).

Pinostilbene has been shown as inhibitor of dopamine-induced cell death through extracellular signal-regulated kinase (ERK 1/2) activation in an *in vitro* study and was shown to alleviate the loss of motor function seen on aging *in vivo* (Allen et al. 2018). It is also an effective neuroprotective agent against free-radical mediated oxidative stress triggered by 6-hydroxydopamine in SH-SY5Y cells with a wider effective concentration range than resveratrol (Allen et al. 2018). Pinostilbene is also a potent anti-inflammatory agent, showing strong inhibitory activity against COX-1 and COX-2 enzymes (Likhitwitayawuid et al. 2002). The study of Sun et al. (2016) showed that pinostilbene significantly inhibited the growth of HCT116 and HT29 human colon cancer cells and at physiologically relevant concentration, caused cell cycle arrest at S phase and induced apoptosis in colon cancer cells.

1.3 Gastrointestinal tract

The functions of the gastrointestinal tract (GIT) include ingesting food, digesting and absorbing nutrients, and removing waste products (Sanders et al. 2006). The GIT wall consists of four layers: mucosa, submucosa, muscular layer, and serosa. This general structure is conserved within the whole GIT, however, the function of each organ and even specific regions within an organ, is specialized for efficient nutrient assimilation (Thompson et al. 2018). GIT consists of the oral cavity, oesophagus, stomach, small intestine, and colon. The stomach and small intestine are principally responsible for digestion and absorption, while the large intestine is

primarily concerned with desiccation and compaction of waste and its storage prior to elimination (Cheng et al. 2010).

1.3.1 Colon microbiota

Since the development of culture-independent sequencing methods, which combine isolation and sequencing of nucleic acids and bioinformatic analyses, the knowledge on the human gut microbiota is quickly increasing (Mosca et al. 2016). The results of culture-based studies have suggested that healthy adults share a core microbiota with most of the same gut bacterial species, however, culture-independent sequencing studies have demonstrated a vast microbial diversity (Lozupone et al. 2012). The gut microbial community is reaching 10^{12}–10^{14} of microorganisms, including many bacterial species and other microorganisms such as methanogenic archaea (mainly *Methanobrevibacter smithii*), eukaryotes (mainly yeasts), and viruses (mainly phage) (Lozupone et al. 2012). In healthy human adults, the bacteria are dominated by two phyla Firmicutes (mainly *Clostridium*, *Enterococcus*, *Lactobacillus* and *Faecalibacterium* genera) and Bacteroidetes (mainly *Bacteroides* and *Prevotella* genera), the others include Actinobacteria (notably *Bifidobacterium* genera), Proteobacteria, Verrucomicrobia, Fusobacteria and limited number of Archaea, mainly methanogens (Shortt et al. 2018; Senghor et al. 2018).

The composition of colon microbiota is changing within the whole life. It has been generally believed that *in utero* environment is sterile and the microbial colonisation of the neonatal gut starts during the birth. However, in recent years this dogma has been challenged, when some studies have shown the presence of bacteria in the placenta, umbilical cord, and amniotic fluid in healthy full-term pregnancies (Jiménez et al. 2005; Stout et al. 2013; Collado et al. 2016; Milani et al. 2017). The mode of infant delivery plays a crucial role in the early bacterial colonisation. Children delivered by natural vaginal birth are colonised by bacteria present in labour channel, with predominant genera of *Lactobacillus*, *Prevotella* or *Sneathia* spp., while children delivered by caesarean section are primarily colonised by environmental microorganisms from maternal skin, the hospital staff, or the hospital environment, dominated by *Staphylococcus*, *Corynebacterium*, and *Propionibacterium* spp. (Dominguez-Bello et al. 2010; Milani et al. 2017) and the colonisation by *Bifidobacterium*-like bacteria can be delayed by up to one month (Isolauri 2012). Recent studies have shown that the delivery by Caesarean

section increases the risk of obesity (Huh et al. 2012; Mueller et al. 2017), asthmatic symptoms (Krzych-Falta et al. 2018) or allergic rhinitis (Brandão et al. 2016).

In adulthood, the microbiota becomes a stable system, although long-term changes resulting from diet, lifestyle, gastrointestinal infections, antibiotic treatments or surgery as well as geographical provenance can modify the composition (Houghton et al. 2016; Senghor et al. 2018). The high colon microbial genes content, and thus the colon microbiota richness, is associated with improved metabolic health (Cotillard et al. 2013; Le Chatelier et al. 2013). Diet plays a crucial role in our microbiota composition. Dietary components provide nutrients for bacteria, which produce metabolites involved in energy balance, metabolism, immune response and development of some non-communicable diseases (Houghton et al. 2016; Shortt et al. 2018). The colon microbiota can be therefore modulated by the change of eating habits, modifying dietary components (fats, proteins, and carbohydrates), or introducing probiotics and prebiotics (Senghor et al. 2018).

In an *in vivo* animal model using humanized gnotobiotic mice it has been shown that switch from a low-fat, plant polysaccharide-rich diet to a high-fat, high-sugar "Western" diet leads to the change of colon microbiota within a single day, changed the representation of metabolic pathways in the microbiome, and altered microbiome gene expression (Turnbaugh et al. 2009). In the study of David et al. (2014) the effect of animal-based and a plant-based diet on human colon microbiota has been investigated. The results have shown that animal-based diet increased the quantity of bile-tolerant bacteria (*Alistipes*, *Bilophila*, and *Bacteroides*) and decrease the abundance of Firmicutes, that metabolize dietary plant polysaccharides (*Roseburia*, *Eubacterium rectale* and *Ruminococcus bromii*). The abundance and activity of *Bilophila wadsworthia*, inflammatory bowel disease-causing bacteria, significantly increased on the animal-based diet (David et al. 2014).

The Western diet, high in fat and carbohydrates, is associated with an altered colon microbiota and an increased risk of developing obesity and non-alcoholic fatty liver disease (Houghton et al. 2016). The study of De Filippo et al. (2010) compared the microbial composition in the faecal samples from European children and children from the Mossi ethnic group living in the small African village of Boulpon in Burkina Faso, where the diet is similar to that of early human settlements. The microbiota of African children seemed to be adapted to the polysaccharide-rich diet, allowing them to maximize energy intake from fibres while also

protecting them from inflammations and non-infectious colonic diseases, by significant increase in Bacteroidetes to Firmicutes ratio, with a unique abundance of bacteria from the genus *Prevotella* and *Xylanibacter*, known to contain a set of bacterial genes for cellulose and xylan hydrolysis, completely lacking in the European children (De Filippo et al. 2010). The study of Moreno-Indias et al. (2016) showed significant changes of microbiota composition in obese metabolic syndrome patients, after two 30-day intervention periods of red wine and de-alcoholised red wine consumption. Significant positive effects on the colon microbiota composition (increase in the abundance of intestinal barrier protectors and butyrate-producing bacteria and decrease in lipopolysaccharide producers) and a reduction in the metabolic syndrome risk markers (decrease in blood pressure, glucose, triglycerides, total cholesterol, C-reactive protein and lipopolysaccharides and a significant increase in the serum level of HDL cholesterol) have been observed (Moreno-Indias et al. 2016).

Colon microbiota plays an important role in the normal functioning of the host organism. It significantly contributes to human health and may play an important role in the development and/or prevention of some non-communicable diseases (Shortt et al. 2018). Dysbiosis has been shown to be associated with the development of some intestinal disorders, such as inflammatory bowel disease, irritable bowel syndrome (IBS), and coeliac disease and some extra-intestinal disorders such as allergy, asthma, metabolic syndrome, cardiovascular disease, and obesity (Carding et al. 2015).

1.4 Models of GIT

A large number of studies is demonstrating a wide range of biological effects of different natural compounds. However, many of these studies are conducted *in vitro* and do not take into account the metabolism, dose or co-existence of several metabolites. Therefore, many published claims were not able to be confirmed *in vivo* (Aragonès et al. 2017). The transformations of the natural compound while passing through the GIT, including microbial metabolism by colon microbiota and intestinal epithelium cells, inclusive of accumulation of compounds in tissues, plays a crucial role in natural compounds studies.

To understand the fate of food and pharmaceutics in GIT, ideally, it should be studied *in vivo* on humans. However, ethical and financial obstacles do not always allow it (Dupont et al. 2019). Therefore many *in vitro* models simulating the conditions in GIT have been developed and are widely used. The *in vitro* models have many advantages compared to *in vivo* models,

they are more rapid, less expensive, less labour intensive, and do not have ethical restrictions (Minekus et al. 2014). On the other side, any *in vitro* method is not able to match the accuracy of real *in vivo* conditions, due to the inherent complexity of the process, and some compromise is needed between accuracy and ease of utilisation of any *in vitro* model (Hur et al. 2011).

To mimic the digestion in an *in vitro* condition many various models have been developed. Standard *in vitro* digestion typically includes oral, gastric and small intestine phases, occasionally also large intestinal fermentation. These models are taking into account, among others, the presence of digestive enzymes and their concentrations, pH, digestion time, and salt concentrations (Minekus et al. 2014). For the more accurate simulation of *in vivo* conditions, the dynamic models have been developed, that allow pH regulation, the flow of the food and injection in real-time of digestive enzymes in the different compartments of the gastrointestinal tract, by diverse control systems (Dupont et al. 2019). However, due to the high cost of the dynamic models, most of the experiments is still performed using static models. Digestion models described in literature strongly vary in digestion parameters, complicating the comparison across research groups. Therefore, a standardised static *in vitro* digestion method has been developed in a framework of COST-INFOGEST action (Minekus et al. 2014)

For the investigation of colon microbial metabolism, typically the *in vitro* faecal fermentation system inoculated by human faecal slurries is used. This model uses the faecal samples from donors to reach the complexity of real *in vivo* situation. The most widely used batch fermentation models are generally closed systems using sealed bottles or reactors containing a suspension of faecal material with carefully selected medium without the further addition of nutrients and are maintained under anaerobic conditions (Payne et al. 2012). The other models used for mimicking the colon microbial metabolism include continuous fermentation models or artificial digestive systems, which mimic the conditions found *in vivo* by continuous flow (Payne et al. 2012).

The *in vitro* cell culture models are commonly used to study the mechanistic effects of probiotics or drug absorption and transport (Payne et al. 2012). Effective *in vitro* culture models often use immortalized cell lines that can form an adherent monolayer reflecting certain characteristics of the epithelium of the GI tract (Lefebvre et al. 2015). The most commonly used human colorectal carcinoma cell lines for *in vitro* models are for example Caco-2, HT-29, T-84,

or DLD-1 (Pedersen 2015). Classical *in vitro* culture models consist from only single-cell population and they not fully recapitulate normal tissue architecture, segment specificity, biology, molecular signalling including paracrine and autocrine cross-talk between different cell types in the intestinal epithelium, or susceptibility to human pathogens (Blutt et al. 2017). Due to their properties, Caco-2 cell lines become the most widely used model system for simulation of intestinal mucosal processes. However, one of the biggest disadvantages of this cell line is the inability to produce mucin. The co-culture of Caco-2/HT29-MTX, seeded in the ratio of 9 : 1 (Caco-2 : HT29-MTX) provides an advanced model, mimicking the real ratio of Goblet cells to absorbing epithelial cells in the healthy GIT, and producing mucin (Volstatova et al. 2017).

For modelling of some processes, such as prediction of absorption, distribution, metabolism and renal elimination of compounds, the *in vivo* animal models might be used. Animal models strongly vary from humans in metabolizing of food or pharmaceutics and are increasingly criticized for their limited ability to predict the efficacy of new chemical entities, safety and toxicity in humans (McGonigle & Ruggeri 2014). Hence, novel pre-clinical animal models have been developed, which are using genetically modified animal models (knockout mice, transgenic mice with humanized drug-metabolizing enzymes, transporters or nuclear receptors and chimeric mice with transplanted human hepatocytes) (Jaiswal et al. 2014). These models are very close to real human-like drug metabolism and might help to bridge the gap between *in vitro* tests and clinical trials and alleviate the probability of clinical failure of a drug (Jaiswal et al. 2014).

1.5 References

Akinwumi BC, Bordun KAM, Anderson HD. 2018, Biological activities of stilbenoids. International Journal of Molecular Sciences. **19**:792.

Allen EN, Potdar S, Tapias V, Parmar M, Mizuno CS, Rimando A, Cavanaugh JE. 2018. Resveratrol and pinostilbene confer neuroprotection against aging-related deficits through an ERK1/2-dependent mechanism. Journal of Nutritional Biochemistry **54**:77–86.

Alosi JA, McDonald DE, Schneider JS, Privette AR, McFadden DW. 2010. Pterostilbene Inhibits Breast Cancer In Vitro Through Mitochondrial Depolarization and Induction of Caspase-Dependent Apoptosis1. Journal of Surgical Research **161**:195–201.

Anhê FF et al. 2015. A polyphenol-rich cranberry extract protects from diet-induced obesity, insulin resistance and intestinal inflammation in association with increased Akkermansia spp. population in the gut microbiota of mice. Gut **64**:872–883.

Aragonès G, Danesi F, Del Rio D, Mena P. 2017. The importance of studying cell metabolism when testing the bioactivity of phenolic compounds. Trends in Food Science and Technology **69**:230–242.

Ashikawa K, Majumdar S, Banerjee S, Bharti AC, Shishodia S, Aggarwal BB. 2002. Piceatannol Inhibits TNF-Induced NF-κB Activation and NF-κB-Mediated Gene Expression Through Suppression of IκBα Kinase and p65 Phosphorylation. The Journal of Immunology **169**:6490–6497.

Babbar N, Oberoi HS, Sandhu SK. 2015. Therapeutic and Nutraceutical Potential of Bioactive Compounds Extracted from Fruit Residues. Page Critical Reviews in Food Science and Nutrition. **55**:319–337.

Baião D, Defreitas C, Gomes L, Silva D, Correa ACNTF, Pereira P, Delaguila E, Paschoalin VMF. 2017. Polyphenols from root, Tubercles and grains cropped in brazil: Chemical and nutritional characterization and their effects on human health and diseases. Nutrients **9**: 1044.

Barton BE, Karras JG, Murphy TF, Barton A, Huang HF-S. 2004. Signal transducer and activator of transcription 3 (STAT3) activation in prostate cancer: Direct STAT3 inhibition induces apoptosis in prostate cancer lines. Molecular Cancer Therapeutics. **3**:11–20.

Bavaresco L, Fregoni C. 2001. Physiological Role and Molecular Aspects of Grapevine Stilbenic Compounds. Pages 153–182 Molecular Biology & Biotechnology of the Grapevine. 153–182.

Beňová B, Adam M, Onderková K, Královský J, Krajíček M. 2008. Analysis of selected stilbenes in *Polygonum cuspidatum* by HPLC coupled with CoulArray detection. Journal of Separation Science **31**:2404–2409.

Blutt SE, Broughman JR, Zou W, Zeng XL, Karandikar UC, In J, Zachos NC, Kovbasnjuk O, Donowitz M, Estes MK. 2017. Gastrointestinal microphysiological systems. Experimental Biology and Medicine **242**:1633–1642.

Bode LM, Bunzel D, Huch M, Cho GS, Ruhland D, Bunzel M, Bub A, Franz CM, Kulling SE. 2013. In vivo and in vitro metabolism of trans-resveratrol by human gut microbiota. The American Journal of Clinical Nutrition **97**:295.

Boocock DJ et al. 2007. Phase I Dose Escalation Pharmacokinetic Study in Healthy Volunteers of Resveratrol, a Potential Cancer Chemopreventive Agent. Cancer Epidemiology Biomarkers & Prevention **16**:1246–1252.

Brandão HV, Vieira GO, de Oliveira Vieira T, Camargos PA, de Souza Teles CA, Guimarães AC, Cruz AA, Cruz CMS. 2016. Increased risk of allergic rhinitis among children delivered by cesarean section: A cross-sectional study nested in a birth cohort. BMC Pediatrics **16**:57.

Buiarelli F, Coccioli F, Jasionowska R, Merolle M, Terracciano A. 2007. Analysis of some stilbenes in Italian wines by liquid chromatography/tandem mass spectrometry. Rapid Communications in Mass Spectrometry **21**:2955–2964.

Carding S, Verbeke K, Vipond DT, Corfe BM, Owen LJ. 2015. Dysbiosis of the gut microbiota in disease. Microbial Ecology in Health & Disease **26**:26191.

Chen M et al. 2013. Absorption of resveratrol by vascular endothelial cells through passive diffusion and an SGLT1-mediated pathway. The Journal of Nutritional Biochemistry **24**:1823–1829.

Cheng LK, O'Grady G, Du P, Egbuji JU, Windsor JA, Pullan AJ. 2010. Gastrointestinal system. Wiley Interdisciplinary Reviews: Systems Biology and Medicine **2**:65–79.

Collado MC, Rautava S, Aakko J, Isolauri E, Salminen S. 2016. Human gut colonisation may be initiated in utero by distinct microbial communities in the placenta and amniotic fluid. Scientific Reports **6**:23129.

Cotillard A et al. 2013. Dietary intervention impact on gut microbial gene richness. Nature **500**:585–588.

David LA et al. 2014. Diet rapidly and reproducibly alters the human gut microbiome. Nature **505**:559–563.

De Filippo C, Cavalieri D, Di Paola M, Ramazzotti M, Poullet JB, Massart S, Collini S, Pieraccini G, Lionetti P. 2010. Impact of diet in shaping gut microbiota revealed by a comparative study in children from Europe and rural Africa. Proceedings of the National Academy of Sciences of the United States of America **107**:14691–14696.
Dominguez-Bello MG, Costello EK, Contreras M, Magris M, Hidalgo G, Fierer N, Knight R. 2010. Delivery mode shapes the acquisition and structure of the initial microbiota across multiple body habitats in newborns. Proceedings of the National Academy of Sciences of the United States of America **107**:11971–11975.
Dupont D et al. 2019. Can dynamic in vitro digestion systems mimic the physiological reality? Critical Reviews in Food Science and Nutrition **59**:1546–1562.
Ehala S, Vaher M, Kaljurand M. 2005. Characterization of phenolic profiles of Northern European berries by capillary electrophoresis and determination of their antioxidant activity. Journal of Agricultural and Food Chemistry **53**:6484–6490.
El Khawand T, Courtois A, Valls J, Richard T, Krisa S. 2018. A review of dietary stilbenes: sources and bioavailability. Phytochemistry Reviews **17**:1007–1029.
Etxeberria U, Hijona E, Aguirre L, Milagro FI, Bujanda L, Rimando AM, Martínez JA, Portillo MP. 2017. Pterostilbene-induced changes in gut microbiota composition in relation to obesity. Molecular Nutrition & Food Research **61**:1500906.
Falomir E, Lucas R, Peñalver P, Martí-Centelles R, Dupont A, Zafra-Gómez A, Carda M, Morales JC. 2016. Cytotoxic, Antiangiogenic and Antitelomerase Activity of Glucosyl- and Acyl- Resveratrol Prodrugs and Resveratrol Sulfate Metabolites. ChemBioChem **17**:1343–1348.
Fuendjiep V, Wandji J, Tillequin F, Mulholland DA, Budzikiewicz H, Fomum ZT, Nyemba AM, Koch M. 2002. Chalconoid and stilbenoid glycosides from Guibourtia tessmanii. Phytochemistry **60**:803–806.
González-Barrio R, Edwards CA, Crozier A. 2011. Colonic catabolism of ellagitannins, ellagic acid, and raspberry anthocyanins: In vivo and in vitro studies. Drug Metabolism and Disposition **39**:1680–1688.
Han X, Shen T, Lou H. 2007. Dietary Polyphenols and Their Biological Significance. International Journal of Molecular Sciences **8**:950–988.
Houghton D, Stewart CJ, Day CP, Trenell M. 2016, March 25. Gut microbiota and lifestyle interventions in NAFLD. International Journal of Molecular Sciences. **17**:447.
http://clinicaltrials.gov/
Hubatsch I, Ragnarsson EGE, Artursson P. 2007. Determination of drug permeability and prediction of drug absorption in Caco-2 monolayers. Nature Protocols **2**:2111–2119.
Huh SY, Rifas-Shiman SL, Zera CA, Rich Edwards JW, Oken E, Weiss ST, Gillman MW. 2012. Delivery by caesarean section and risk of obesity in preschool age children: A prospective cohort study. Archives of Disease in Childhood **97**:610–616.
Hur SJ, Lim BO, Decker EA, McClements DJ. 2011. In vitro human digestion models for food applications. Food Chemistry. **125**:1–12.
Hurst WJ, Glinski JA, Miller KB, Apgar J, Davey MH, Stuart DA. 2008. Survey of the trans-resveratrol and trans-piceid content of cocoa-containing and chocolate products. Journal of Agricultural and Food Chemistry **56**:8374–8378.
Isolauri E. 2012. Development of healthy gut microbiota early in life. Journal of Paediatrics and Child Health. **48**:1–6.
Jaganath IB, Mullen W, Lean MEJ, Edwards CA, Crozier A. 2009. In vitro catabolism of rutin by human fecal bacteria and the antioxidant capacity of its catabolites. Free Radical Biology and Medicine **47**:1180–1189.
Jaiswal S, Sharma A, Shukla M, Vaghasiya K, Rangaraj N, Lal J. 2014. Novel pre-clinical methodologies for pharmacokinetic drug-drug interaction studies: Spotlight on

"humanized" animal models. Drug Metabolism Reviews. **46**:475–493.
Jarosova V, Vesely O, Marsik P, Jaimes JD, Smejkal K, Kloucek P, Havlik J. 2019. Metabolism of stilbenoids by human faecal microbiota. Molecules **24**:1155.
Jiménez E, Fernández L, Marín ML, Martín R, Odriozola JM, Nueno-Palop C, Narbad A, Olivares M, Xaus J, Rodríguez JM. 2005. Isolation of commensal bacteria from umbilical cord blood of healthy neonates born by cesarean section. Current Microbiology **51**:270–274.
Juan ME, González-Pons E, Planas JM. 2010. Multidrug Resistance Proteins Restrain the Intestinal Absorption of trans-Resveratrol in Rats. The Journal of Nutrition **140**:489–495.
Kaldas MI, Walle UK, Walle T. 2003. Resveratrol transport and metabolism by human intestinal Caco-2 cells. The Journal of pharmacy and pharmacology **55**:307–12.
Kim YH, Park C, Lee JO, Kim GY, Lee WH, Choi YH, Ryu CH. 2008. Induction of apoptosis by piceatannol in human leukemic U937 cells through down-regulation of Bcl-2 and activation of caspases. Oncology Reports **19**:961–967.
Kopp P. 1998. Resveratrol, a phytoestrogen found in red wine. A possible explanation for the conundrum of the'French paradox'? European journal of endocrinology **138**:619–620.
Krzych-Falta E, Furmanczyk K, Lisiecka-Bielanowicz M, Sybilski A, Tomaszewska A, Raciborski F, Wojas O, Walkiewicz A, Samel-Kowalik P, Samolinski B. 2018. The effect of selected risk factors, including the mode of delivery, on the development of allergic rhinitis and bronchial asthma. Postepy Dermatologii i Alergologii **35**:267–273.
Kukreja A, Wadhwa N, Tiwari A, Gandhi R, Vishwavidyalaya P. 2014. Therapeutic Role of Resveratrol and Piceatannol in Disease Prevention. J Blood Disorders Transf **5**:240.
Kuo PL, Hsu YL. 2008. The grape and wine constituent piceatannol inhibits proliferation of human bladder cancer cells via blocking cell cycle progression and inducing Fas/membrane bound Fas ligand-mediated apoptotic pathway. Molecular Nutrition and Food Research **52**:408–418.
Lagouge M et al. 2006. Resveratrol Improves Mitochondrial Function and Protects against Metabolic Disease by Activating SIRT1 and PGC-1α. Cell **127**:1109–1122.
Le Chatelier E et al. 2013. Richness of human gut microbiome correlates with metabolic markers. Nature **500**:541–546.
Lefebvre DE et al. 2015. Utility of models of the gastrointestinal tract for assessment of the digestion and absorption of engineered nanomaterials released from food matrices. Nanotoxicology **9**:523–542.
Likhitwitayawuid K, Sawasdee K, Kirtikara K. 2002. Flavonoids and stilbenoids with COX-1 and COX-2 inhibitory activity from Dracaena loureiri. Planta Medica **68**:841–843.
Likhitwitayawuid K, Sornsute A, Sritularak B, Ploypradith P. 2006. Chemical transformations of oxyresveratrol (trans-2, 4, 3′, 5′-tetrahydroxystilbene) into a potent tyrosinase inhibitor and a strong cytotoxic agent. Bioorganic & medicinal chemistry letters **16**:5650–5653.
Lin HS, Yue B De, Ho PC. 2009. Determination of pterostilbene in rat plasma by a simple HPLC-UV method and its application in pre-clinical pharmacokinetic study. Biomedical Chromatography **23**:1308–1315.
Lorenz P, Roychowdhury S, Engelmann M, Wolf G, Horn TFW. 2003. Oxyresveratrol and resveratrol are potent antioxidants and free radical scavengers: Effect on nitrosative and oxidative stress derived from microglial cells. Nitric Oxide - Biology and Chemistry **9**:64–76.
Lozupone CA, Stombaugh JI, Gordon JI, Jansson JK, Knight R. 2012. Diversity, stability and resilience of the human gut microbiota. Nature **489**:220–230.
Lu D, Zhao W, Zhu K, Zhao S. 2012. Relevant Enzymes, Genes and Regulation Mechanisms in Biosynthesis Pathway of Stilbenes. Open Journal of Medicinal Chemistry **02**:15–23.
Maier-Salamon A, Böhmdorfer M, Riha J, Thalhammer T, Szekeres T, Jaeger W. 2013.

Interplay between metabolism and transport of resveratrol. Annals of the New York Academy of Sciences **1290**:98–106.
Maier-Salamon A, Hagenauer B, Wirth M, Gabor F, Szekeres T, Jäger W. 2006. Increased transport of resveratrol across monolayers of the human intestinal Caco-2 cells is mediated by inhibition and saturation of metabolites. Pharmaceutical Research **23**:2107–2115.
Maneechai S, Likhitwitayawuid K, Sritularak B, Palanuvej C, Ruangrungsi N, Sirisa-Ard P. 2009. Quantitative analysis of oxyresveratrol content in Artocarpus lakoocha and 'Puag-Haad.' Medical Principles and Practice **18**:223–227.
Manickam M, Ramanathan M, Farboodniay Jahromi MA, Chansouria JPN, Ray AB. 1997. Antihyperglycemic activity of phenolics from Pterocarpus marsupium. Journal of Natural Products **60**:609–610.
Matencio A, García-Carmona F, López-Nicolás JM. 2017. The inclusion complex of oxyresveratrol in modified cyclodextrins: A thermodynamic, structural, physicochemical, fluorescent and computational study. Food Chemistry **232**:177–184.
Matsui Y, Sugiyama K, Kamei M, Takahashi T, Suzuki T, Katagata Y, Ito T. 2010. Extract of passion fruit (Passiflora edulis) seed containing high amounts of piceatannol inhibits melanogenesis and promotes collagen synthesis. Journal of Agricultural and Food Chemistry **58**:11112–11118.
McCormack D, McFadden D. 2013. A review of pterostilbene antioxidant activity and disease modification. Oxidative Medicine and Cellular Longevity. **2013**.
McGonigle P, Ruggeri B. 2014. Animal models of human disease: Challenges in enabling translation. Biochemical Pharmacology. **87**:162–171.
Milani C et al. 2017. The First Microbial Colonizers of the Human Gut: Composition, Activities, and Health Implications of the Infant Gut Microbiota. Microbiology and Molecular Biology Reviews **81**.
Minekus M et al. 2014. A standardised static in vitro digestion method suitable for food - an international consensus. Food & function **5**:1113–24.
Moreno-Indias I, Sánchez-Alcoholado L, Pérez-Martínez P, Andrés-Lacueva C, Cardona F, Tinahones F, Queipo-Ortuño MI. 2016. Red wine polyphenols modulate fecal microbiota and reduce markers of the metabolic syndrome in obese patients. Food and Function **7**:1775–1787.
Mosca A, Leclerc M, Hugot JP. 2016. Gut microbiota diversity and human diseases: Should we reintroduce key predators in our ecosystem? Frontiers in Microbiology **7**:455.
Mueller NT, Mao G, Bennet WL, Hourigan SK, Dominguez-Bello MG, Appel LJ, Wang X. 2017. Does vaginal delivery mitigate or strengthen the intergenerational association of overweight and obesity? Findings from the Boston Birth Cohort. International Journal of Obesity **41**:497–501.
Naczk M, Shahidi F. 2006, August 28. Phenolics in cereals, fruits and vegetables: Occurrence, extraction and analysis. Journal of Pharmaceutical and Biomedical Analysis. **41**:1523–1542.
Ovesná Z, Kozics K, Bader Y, Saiko P, Handler N, Erker T, Szekeres T. 2006. Antioxidant activity of resveratrol, piceatannol and 3,3′,4,4′, 5,5′-hexahydroxy-trans-stilbenein three leukemia cell lines. Oncology Reports **16**:617–624.
Parkar SG, Stevenson DE, Skinner MA. 2008. The potential influence of fruit polyphenols on colonic microflora and human gut health. International journal of food microbiology **124**:295–298.
Pascual-Martí MC, Salvador A, Chafer A, Berna A. 2001. Supercritical fluid extraction of resveratrol from grape skin of vitis vinifera and determination by HPLC. Talanta **54**:735–740.
Paul B, Masih I, Deopujari J, Charpentier C. 1999. Occurrence of resveratrol and pterostilbene

in age-old darakchasava, an ayurvedic medicine from India. Journal of Ethnopharmacology **68**:71–76.

Payne AN, Zihler A, Chassard C, Lacroix C. 2012, January. Advances and perspectives in in vitro human gut fermentation modeling. Trends in Biotechnology. **30**:17-25.

Pedersen IG. 2015. Development, validation and implementation of an in vitro model for the study of metabolic and immune function in normal and inflamed human colonic epithelium. Dan Med J **62**:298–306.

Pérez-Jiménez J, Neveu V, Vos F, Scalbert A. 2010. Systematic Analysis of the Content of 502 Polyphenols in 452 Foods and Beverages: An Application of the Phenol-Explorer Database. Journal of Agricultural and Food Chemistry **58**:4959–4969.

Piotrowska H, Kucinska M, Murias M. 2012. Biological activity of piceatannol: Leaving the shadow of resveratrol. Mutation Research - Reviews in Mutation Research **750**:60–82.

Renaud S, de Lorgeril M. 1992. Wine, alcohol, platelets, and the French paradox for coronary heart disease. The Lancet **339**:1523–1526.

Rimando AM, Kalt W, Magee JB, Dewey J, Ballington JR. 2004. Resveratrol, pterostilbene, and piceatannol in Vaccinium berries. Journal of Agricultural and Food Chemistry **52**:4713–4719.

Ritchie LE, Sturino JM, Carroll RJ, Rooney LW, Azcarate-Peril MA, Turner ND. 2015. Polyphenol-rich sorghum brans alter colon microbiota and impact species diversity and species richness after multiple bouts of dextran sodium sulfate-induced colitis. FEMS microbiology ecology **91**.

Rivière C, Pawlus AD, Mérillon JM. 2012. Natural stilbenoids: Distribution in the plant kingdom and chemotaxonomic interest in Vitaceae. Natural Product Reports **29**:1317–1333.

Sabolovic N, Humbert AC, Radominska-Pandya A, Magdalou J. 2006. Resveratrol is efficiently glucuronidated by UDP-glucuronosyltransferases in the human gastrointestinal tract and in Caco-2 cells. Biopharmaceutics and Drug Disposition **27**:181–189.

Sanders KM, Koh D, Ward SM. 2006. Interstitial Cells of Cajal As Pacemakers in the Gastrointestinal Tract. Annu. Rev. Physiol **68**:307–343.

Schaefer S, Baum M, Eisenbrand G, Dietrich H, Will F, Janzowski C. 2006. Polyphenolic apple juice extracts and their major constituents reduce oxidative damage in human colon cell lines. Molecular Nutrition and Food Research **50**:24–33.

Senghor B, Sokhna C, Ruimy R, Lagier JC. 2018, April 1. Gut microbiota diversity according to dietary habits and geographical provenance. Human Microbiome Journal. **7**:1–9.

Shan B, Cai Y-Z, Brooks JD, Corke H. 2008. Antibacterial properties of Polygonum cuspidatum roots and their major bioactive constituents. Food Chemistry **109**:530–537.

Shen T, Wang XN, Lou HX. 2009. Natural stilbenes: An overview. Natural Product Reports. **26**:916–935.

Shortt C et al. 2018. Systematic review of the effects of the intestinal microbiota on selected nutrients and non-nutrients. European Journal of Nutrition. **57**:25-49.

Soto-Vaca A, Gutierrez A, Losso JN, Xu Z, Finley JW. 2012. Evolution of phenolic compounds from color and flavor problems to health benefits. Journal of Agricultural and Food Chemistry **60**:6658–6677.

Storniolo CE, Quifer-Rada P, Lamuela-Raventos RM, Moreno JJ. 2014. Piceid presents antiproliferative effects in intestinal epithelial Caco-2 cells, effects unrelated to resveratrol release. Food Funct. **5**:2137–2144.

Stout MJ, Conlon B, Landeau M, Lee I, Bower C, Zhao Q, Roehl KA, Nelson DM, MacOnes GA, Mysorekar IU. 2013. Identification of intracellular bacteria in the basal plate of the human placenta in term and preterm gestations. American Journal of Obstetrics and Gynecology **208**: 226.e1-226.e7.

Su D, Cheng Y, Liu M, Liu D, Cui H, Zhang B, Zhou S, Yang T, Mei Q. 2013. Comparision of Piceid and Resveratrol in Antioxidation and Antiproliferation Activities In Vitro. PLoS ONE **8**:e54505.

Sun Y et al. 2016. Identification of pinostilbene as a major colonic metabolite of pterostilbene and its inhibitory effects on colon cancer cells. Molecular Nutrition and Food Research **60**:1924–1932.

Thompson CA, DeLaForest A, Battle MA. 2018. Patterning the gastrointestinal epithelium to confer regional-specific functions. Developmental Biology. **435**:97–108.

Tian B, Liu J. 2019. Resveratrol: a review of plant sources, synthesis, stability, modification and food application. Journal of the Science of Food and Agriculture.

Timmers S et al. 2011. Calorie Restriction-like Effects of 30 Days of Resveratrol Supplementation on Energy Metabolism and Metabolic Profile in Obese Humans. Cell Metabolism **14**:612–622.

Tokuşoğlu Ö, Ünal MK, Yemiş F. 2005. Determination of the phytoalexin resveratrol (3, 5, 4 ‘-trihydroxystilbene) in peanuts and pistachios by high-performance liquid chromatographic diode array (HPLC-DAD) and gas chromatography− mass spectrometry (GC-MS). Journal of Agricultural and Food Chemistry **53**:5003–5009.

Tsao R. 2010. Chemistry and biochemistry of dietary polyphenols. Nutrients **2**:1231–1246.

Turnbaugh PJ, Ridaura VK, Faith JJ, Rey FE, Knight R, Gordon JI. 2009. The effect of diet on the human gut microbiome: A metagenomic analysis in humanized gnotobiotic mice. Science Translational Medicine **1**:6ra14-6ra14.

Tyukavkina NA, Gromova AS, Lutskii VI, Voronov VK. 1972. Hydroxystilbenes from the bark of Pinus sibirica. Chemistry of Natural Compounds **8**:570–572.

Urpi-Sarda M, Zamora-Ros R, Lamuela-Raventos R, Cherubini A, Jauregui O, De La Torre R, Covas MI, Estruch R, Jaeger W, Andres-Lacueva C. 2007. HPLC-tandem mass spectrometric method to characterize resveratrol metabolism in humans. Clinical Chemistry **53**:292–299.

Volstatova T, Marsik P, Rada V, Geigerova M, Havlik J. 2017. Effect of apple extracts and selective polyphenols on the adhesion of potential probiotic strains of Lactobacillus gasseri R and Lactobacillus casei FMP. Journal of Functional Foods **35**:391–397.

Walle T, Hsieh F, DeLegge MH, Oatis JE, Walle UK. 2004. High absorption but very low bioavailability of oral resveratrol in humans. Drug Metabolism and Disposition **32**:1377–1382.

Wolter F, Clausnitzer A, Akoglu B, Stein J. 2002. Piceatannol, a Natural Analog of Resveratrol, Inhibits Progression through the S Phase of the Cell Cycle in Colorectal Cancer Cell Lines. The Journal of Nutrition **132**:298–302.

Zhang H, Tsao R. 2016. Dietary polyphenols, oxidative stress and antioxidant and anti-inflammatory effects. Current Opinion in Food Science **8**:33–42.

Scientific Hypothesis and Objectives

The dietary intake of plant polyphenols plays an important role in human health. While passing through the gastrointestinal tract, polyphenols are transformed by colon microbiota and colon epithelial cells, which considerably affect their pharmacokinetics. However, this relationship also works the other way around, polyphenols affect the colon microbiota, either by direct inhibitory activity against individual taxa, or as a selective substrate for the growth and progression of microorganisms, or by affecting adhesion and thereby colonisation of colon. Stilbenoids are an important and interesting group of polyphenols. The most well-known stilbenoid, resveratrol, has been intensively studied for its connection with French paradox, low incidence of cardiovascular disease and influence on longevity. Therefore, the following hypothesis has been set: I) Some of the selected stilbenoids are more resistant to the metabolism of colon microbiota, II) These stilbenoids are transported through the colon epithelial cells and present in the plasma as active conjugates, III) The presence of selected stilbenoids affects the colon microbial composition and reduces the ability of lactobacilli to adhere.

The aim of this doctoral thesis is to find out, how the polyphenols from the group of stilbenoids, which are naturally occurring in the diet, are transformed in the gastrointestinal tract and how their presence affects the composition of colon microbiota.

Therefore, the objectives of the thesis were:

1. Investigation whether selected stilbenoids undergo metabolic transformation by human colon microbiota.
2. Determination of stilbenoids transformation during the transport through the colon enterocytes in the Transwell^{TM} cellular system.
3. Assessment of the effect of selected stilbenoids on the colon microbiota composition at dietary relevant concentrations.
4. Determination of the effect of selected stilbenoids on the adhesion of lactobacilli in the colon epithelial cell model.

The structure is based on a collection of scientific papers, which I authored or in which I had a major contribution. Copyright policy of the journals where our work has been published allows me to reprint them as chapters of the thesis.

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Abstract

Stilbenoids are dietary phenolics with notable biological effects on humans. Epidemiological, clinical, and nutritional studies from recent years have confirmed the significant biological effects of stilbenoids, such as oxidative stress protection and the prevention of degenerative diseases, including cancer, cardiovascular diseases, and neurodegenerative diseases. Stilbenoids are intensively metabolically transformed by colon microbiota, and their corresponding metabolites might show different or stronger biological activity than their parent molecules. The aim of the present study was to determine the metabolism of six stilbenoids (resveratrol, oxyresveratrol, piceatannol, thunalbene, batatasin III, and pinostilbene), mediated by colon microbiota. Stilbenoids were fermented in an in vitro faecal fermentation system using fresh faeces from five different donors as an inoculum. The samples of metabolized stilbenoids were collected at 0, 2, 4, 8, 24, and 48 h. Significant differences in the microbial transformation among stilbene derivatives were observed by liquid chromatography mass spectrometry (LC/MS). Four stilbenoids (resveratrol, oxyresveratrol, piceatannol and thunalbene) were metabolically transformed by double bond reduction, dihydroxylation, and demethylation, while batatasin III and pinostilbene were stable under conditions simulating the colon environment. Strong inter-individual differences in speed, intensity, and pathways of metabolism were observed among the faecal samples obtained from the donors.

Keywords: bacteria colon model; fecal fermentation; metabolites; phenolics; polyphenols; stilbenoids; liquid chromatography high resolution mass spectrometry

3.1 Introduction

Stilbenoids are dietary phenolics that occur in a wide range of edible fruits and seeds, such as grapes (*Vitis vinifera*), peanuts (*Arachis hypogaea*), sorghum (*Sorghum bicolor*), and some tree species (*Pinus* spp. and *Picea* spp.) (Reinisalo et al. 2015). Resveratrol (3,4',5-trihydroxystilbene) is the most studied stilbenoid, and is commonly associated with the French paradox, where resveratrol is thought to lower the incidence of coronary heart disease, despite a high intake of saturated fat in the French population (Renaud & de Lorgeril 1992). In mice studies, both *in vitro* and *in vivo*, resveratrol has shown strong anti-inflammatory activity by a reduction of tumor necrosis factor alpha (TNF-α) and interleukin 1 beta (IL-1β), an increase of interleukin 10 (IL-10), and a reduced expression of prostaglandin E synthase-1 (PGES-1), cyclooxygenase-2 (COX-2), and inducible nitric oxide synthase (iNOS) (Sánchez-Fidalgo et al. 2010; Leláková et al. 2019). Therefore, stilbenoid could act as an anti-inflammatory agent through some of the same mechanisms as nonsteroidal antiphlogistic drugs. Resveratrol has also been reported to affect various factors and metabolic targets associated with oxidative stress (Brasnyó et al. 2011; Kairisalo et al. 2011; Reinisalo et al. 2015). Among these, resveratrol has a strong affinity to quinone reductase 2, with a dissociation constant as low as 35 nM, making it the strongest known inhibitor so far, which, in turn, may regulate the expression of cellular antioxidant enzymes and cellular resistance to oxidative stress (Buryanovskyy et al. 2004). Resveratrol further interacts with a large number of receptors and enzymes that could plausibly make major contributions to its biological effects. Both *in vitro* and *in vivo*, resveratrol treatment upregulates mammalian target of rapamycin (mTOR), sirtuin 1 (SIRT1), and adenosine monophosphate-activated protein kinase (AMPK), which influence the regulation of metabolism in multiple tissues (Liu et al. 2010; Smoliga & Blanchard 2014). However, the *in vivo* importance is rather more relevant to insects than to mammals. Structural analogs of resveratrol are present in medicinal plants and show significant bioactivity. For instance, piceatannol (3,3',4',5-tetrahydroxystilbene) is found in plants such as grapes (*V. vinifera*), passion fruit (*Passiflora edulis*), Japanese knotweed (*Polygonum cuspidatum*), and Norway spruce (*P. abies*) (Piotrowska et al. 2012). Compared to resveratrol, piceatannol shows greater biological activity as an inhibitor of COX-2 and of the constitutive photomorphogenesis 9 signalosome (CSN)-associated kinase (Seyed et al. 2016), possibly due to its better solubility in H_2O. Moreover, piceatannol inhibits the activation of p40 and p56 protein tyrosine kinases

and NF-κB (Murias et al. 2004). Another analog of resveratrol, pinostilbene (3,4'-dihydroxy-5-methoxystilbene), found in Siberian pine (*P. sibirica*), showed an inhibition of dopamine-induced cell death through extracellular signal–regulated kinase (ERK 1/2) activation in an *in vitro* study, was shown to alleviate the loss of motor function seen on aging *in vivo* (Allen et al. 2018). Oxyresveratrol (2',3,4',5-tetrahydroxystilbene), found in white mulberry (*Morus alba*), exhibited, among other effects, the inhibition of TNF-α production and stronger antioxidant activity than resveratrol (Xu et al. 2014). Many of these molecules have been subjected to clinical trials and are being investigated as clinical drugs (Qiang et al. 2018; Sattarinezhad et al. 2018; Kantartzis et al. 2018).

Phenolics are intensively metabolically transformed in intestinal epithelial cells and transported through the basolateral membrane in the form of conjugates (Wang & Sang 2018). Some typical intestinal epithelium metabolites include O-β-glucuronides, 3-O-sulfates, or their methoxy-derivatives. However, a large portion of the phenolic compounds escape intestinal absorption and undergo their microbial metabolic conversion in the colon (van Duynhoven et al. 2011). Colon catabolism is an important phase of the pharmacokinetics of chemical entities in the human body. Its knowledge is an important prerequisite for bioassay validation or for the development of more active substituents. Colonic catabolites might be more biologically relevant forms of the compounds, and their use in bioassays is a more realistic reflection of the compound's bioactivity. To date, only resveratrol and its fate in the human colon has been investigated, finding three metabolites: dihydroresveratrol, 3,4'-dihydroxy-trans-stilbene, and 3,4'-dihydroxybibenzyl (lunularin) (Bode et al. 2013). The bioactivity of dihydroresveratrol, found by *in vivo* and *in vitro* studies, includes antioxidant (Tsang et al. 2016) and anti-inflammatory (Lin et al. 2016) activity.

Thus, the aim of the present study was to investigate whether six selected stilbenoids (batatasin III, oxyresveratrol, piceatannol, pinostilbene, resveratrol, thunalbene) undergo metabolic transformation by human colon microbiota and thereafter detect their main metabolites by liquid chromatography mass spectrometry (LC/MS).

3.2 Material and Methods

3.2.1 *In vitro* Faecal Fermentation System

A slightly modified fermentation model, previously described by other authors (Jaganath et al. 2009; González-Barrio et al. 2011), was used to mimic the conditions in the human colon.

3.2.1.1 Fermentation Medium

The fermentation medium was prepared as a solution of 225 mL distilled water, 1.12 g tryptone, 56.25 µL of micromineral solution (2.64 g $CaCl_2{*}2H_2O$, 2 g $MnCl_2{*}4H_2O$, 0.20 g $CoCl_2{*}6H_2O$, 1.60 g $FeCl_3{*}6H_2O$, and distilled water up to 20 mL), 112.5 mL of macromineral solution (7.14 g of $Na_2HPO_4{*}2H_2O$, 6.20 g KH_2PO_4, 0.60 g $MgSO_4{*}7H_2O$, and distilled water up to 1 L), 112.5 mL of CO_3 buffer (1 g NH_4HCO_3, 8.75 g $NaHCO_3$, and distilled water up to 250 mL), and 562.5 µL of 0.1% resazurin solution. All chemicals were obtained from Merck (Darmstadt, Germany) and stored at 4 °C for up to 1 month. The prepared fermentation medium was covered with aluminum foil and stored at 4 °C until the next day.

3.2.1.2 Sodium Phosphate Buffer and Reducing Solution

The sodium phosphate buffer for preparation of faecal slurries was made of 1.7702 g KH_2PO_4 in 195 mL distilled water, and 3.6222 g of Na_2HPO_4 in 305mL distilled water (both 1/15M). Afterwards, the buffer's pH was modified to 7.0 by hydrochloric acid and stored at 4 °C up to one month. Reducing solution was prepared just before the experiment from 125 mg cysteine hydrochloride, 0.8 mL 1M NaOH, 125 mg Na_2S and distilled water up to 20 mL.

4.1.3. Stilbenoid Preparation

Batatasin III, piceatannol, thunalbene, and pinostilbene were purchased from ChemFaces (China) in 98% purity; resveratrol, oxyresveratrol and [$^{13}C_6$] *trans*-resveratrol were obtained from Merck (Germany) in 98% purity. Stock solutions for fermentation experiments were prepared at a concentration of 10 mg/mL in DMSO (dimethylsulfoxide; Sigma-Aldrich, Czech Republic) and kept at 4 °C. Analytical standard stock solutions for LC/MS were prepared in methanol (1 mg/mL) and stored at -80 °C.

3.2.1.3 Faecal Samples and Ethics Statement

Human faecal samples were collected in October and November 2016, at the Czech University of Life Sciences in Prague, Czech Republic from 5 healthy volunteers. These volunteers were aged 23 to 29 years, with a mean BMI of 24.6, no history of gastrointestinal disease and no antibiotic treatment for at least 3 months prior to the experiment. Female volunteers were neither pregnant nor lactating. All donors followed an omnivorous diet in their daily life and a two-day low polyphenol diet before the sample collection. All subjects gave their informed consent for inclusion before they participated in the study. The study was conducted in accordance with the Declaration of Helsinki, and the protocol was approved by the Ethics Committee of the Czech University of Life Sciences in Prague (ZEK/22/09/2017). Samples were collected into 1 L plastic container, tied in a plastic bag with GENbag anaer (Biomérieux, Lyon, France) and kept at 37 °C for 2 h maximum. Fresh faeces were homogenized in a stomacher bag for 30 s with a sodium phosphate buffer and the obtained 32% faecal slurry was filtrated through a nylon mesh.

3.2.1.4 *In Vitro* Incubations

The fermentation medium and sodium phosphate buffer were boiled with cotton cups and cooled to approximately 37 °C while they were purged with nitrogen gas free of oxygen (approximately 30 min). In the end, the color of the medium changed from blue to pink. The medium's pH was adjusted to pH 7.0 using HCl. The fermentation bottles (20 mL) were filled with 16.8 mL of medium and sealed with PTFE/aluminum caps. The reducing solution (0.8 µL) was added through the septa and, after full decolorization of resazurin, 20 µL of the tested compound and 2 mL of the faecal slurry were added. 20 µL of DMSO, instead of the tested compound, was added to the negative control vials, and 2 mL of the sodium phosphate buffer, instead of the faecal slurry, was added to the positive control vials. The incubation was carried out in a shaking water bath at 37 °C, at 100 strokes per minute. Samples (3 mL) were collected at 0, 2, 4, 8, 24, and 48 h with a syringe through the septa and stored at -80 °C until analysis.

3.2.2 LC/MS analyses

3.2.2.1 Standards

Standards were prepared as 1% Methanol/Formic Acid solution. Stock solutions were prepared at a concentration of 10 mg/mL in DMSO (Sigma-Aldrich, Stribrna Skalice, Czech Rep) and kept at 4 °C.

3.2.2.2 Sample Purification

A liquid-liquid extraction was used for the purification of samples. The samples from fermentations were centrifuged (5 min; 15,000 rpm/min), and 400 μL of supernatant was diluted with 2 mL of ultra-pure water (Milipore, Bedford, MA, United States of America); 20 μL of [$^{13}C_6$] *trans*-resveratrol solution in methanol (2 μg/mL) was added as an internal standard. Then, the samples were extracted three times by 2.5 mL ethyl acetate (VWR Chemicals, Stribrna Skalice, Czech Republic). After purification, the combined organic phase was dried under nitrogen gas and re-dissolved in 1 mL of methanol (VWR Chemicals, Stribrna Skalice, Czech Republic) with 1% formic acid (Fisher Scientific, Merelbeke, Belgium). Final samples were analyzed by LC/MS.

3.2.2.3 LC/MS Analysis of Metabolites

Analyses were performed on a LC/MS system consisting of an UHPLC chromatograph Ultimate 3000 Thermofisher Scientific (Sunnyvale, CA, USA) coupled with quadrupole time of flight (Q-TOF) mass spectrometer with ultra-high resolution and high mass accuracy (HRAM) Impact II (Bruker Daltonics, Bremen, Germany) equipped with an electrospray ionization (ESI) source.

Chromatography was carried out on a Kinetex 1.7 μm F5 100 Å 100 × 2.1 mm column (Phenomenex, Torrance, CA, USA) using a mobile phase consisting of 0.1% formic acid (solvent A) and methanol (solvent B). The binary gradient was run as follows: 0–3 min isocratic at 20 % B, 3–6 min from 20 % to 50 % B, 6–15 min from 50% to 100 % B, and 15–20 min isocratic at 20 % B. The flow rate was kept at 0.2 mL/min and the column oven was adjusted to 35 °C. The injected volume was 5 μL.

The ESI source was operated in the negative mode with parameters listed in Appendix C, Table A1. The identity of each detected compound was confirmed by MS/MS fragmentation spectra

collected at three collision energy levels (20, 30 and 50 eV). Data acquisition was performed using HyStar 3.2 SR4, QTOF series 4.0 (Bruker Daltonics–Germany), and Chromeleon Xpress (Thermo Fisher Scientific) software, and the obtained data were processed by DataAnalysis 4.3. and TASQ 1.4 (both Bruker Daltonics–Germany). For calibration, commercially available standards of resveratrol, dihydroresveratrol, oxyresveratrol, piceatannol, thunalbene, batatasin III and pinostilbene were used each at 6 concentration levels in the range of 20–1000 ng/mL. As an internal standard, 20 µL of *trans*-[$^{13}C_6$] resveratrol at a concentration of 2 µg/mL was used.

3.2.3 Statistical Evaluation

Resveratrol, thunalbene, piceatannol and pinostilbene were used in five biological repetitions. Oxyresveratrol and batatasin III were used in three and four repetitions, respectively. All samples were measured by LC/MS in triplicates. Values are expressed as a mean ± standard error. Microsoft Excel and SPSS (IBM corp.) version 25 were used for basic statistical analysis and graph creation.

3.3 Results

The *in vitro* faecal fermentation system, using fresh faeces from five different donors (D) as inoculum, was performed to analyze the metabolism of selected stilbenoids (batatasin III, oxyresveratrol, piceatannol, pinostilbene, resveratrol, thunalbene) in the human colon. As seen in Figure 1, significant differences in the microbial transformation among stilbene derivatives were observed. Four stilbenoids (resveratrol, oxyresveratrol, piceatannol and thunalbene) were metabolically transformed to new products, while batatasin III and pinostilbene were stable in the colon environment.

The only metabolite of resveratrol detected in our model was dihydroresveratrol, which was not further metabolized and remained stable in the colon environment. Amounts of resveratrol and dihydroresveratrol were monitored: after 48 h, the concentration of resveratrol decreased to 3.2 ± 0.9 µg/mL (mean ± SD) from the initial 9.1 ± 4.4 µg/mL. The concentration of dihydroresveratrol rose gradually to a final concentration of 0.7 ± 0.4 µg/mL after 48 h of fermentation. The metabolism of oxyresveratrol was similar to resveratrol, and one metabolite, 2′,3,4′,5-tetrahydroxybibenzyl, formed by double bond reduction, was detected. It reached its maximum level after 24 h and then was further degraded to still unknown products.

The metabolism of piceatannol was more complex. The main metabolic pathway was the double bond reduction of the connective chain, forming dihydropiceatannol, which reached its maximum level after 4 h and then was also further degraded to still unknown products. In some donors, piceatannol was dehydroxylated at one of the *meta* positions on ring A, forming trihydroxystilbene different than resveratrol or isoresveratrol (see Appendix A, Figure A1), and was further metabolized to 3,3′,4′-trihydroxybibenzyl. This reaction was much slower than the formation of dihydropiceatannol and occurred between 4 to 8 h of fermentation. Even though thunalbene was not observed in the samples, its demethylated metabolite, isoresveratrol (3,3’,5-trihydroxystilbene), was detected. This metabolite was stable and was not further metabolized or degraded. Batatasin III and pinostilbene did not form any metabolites and were found to be stable in the colon environment.

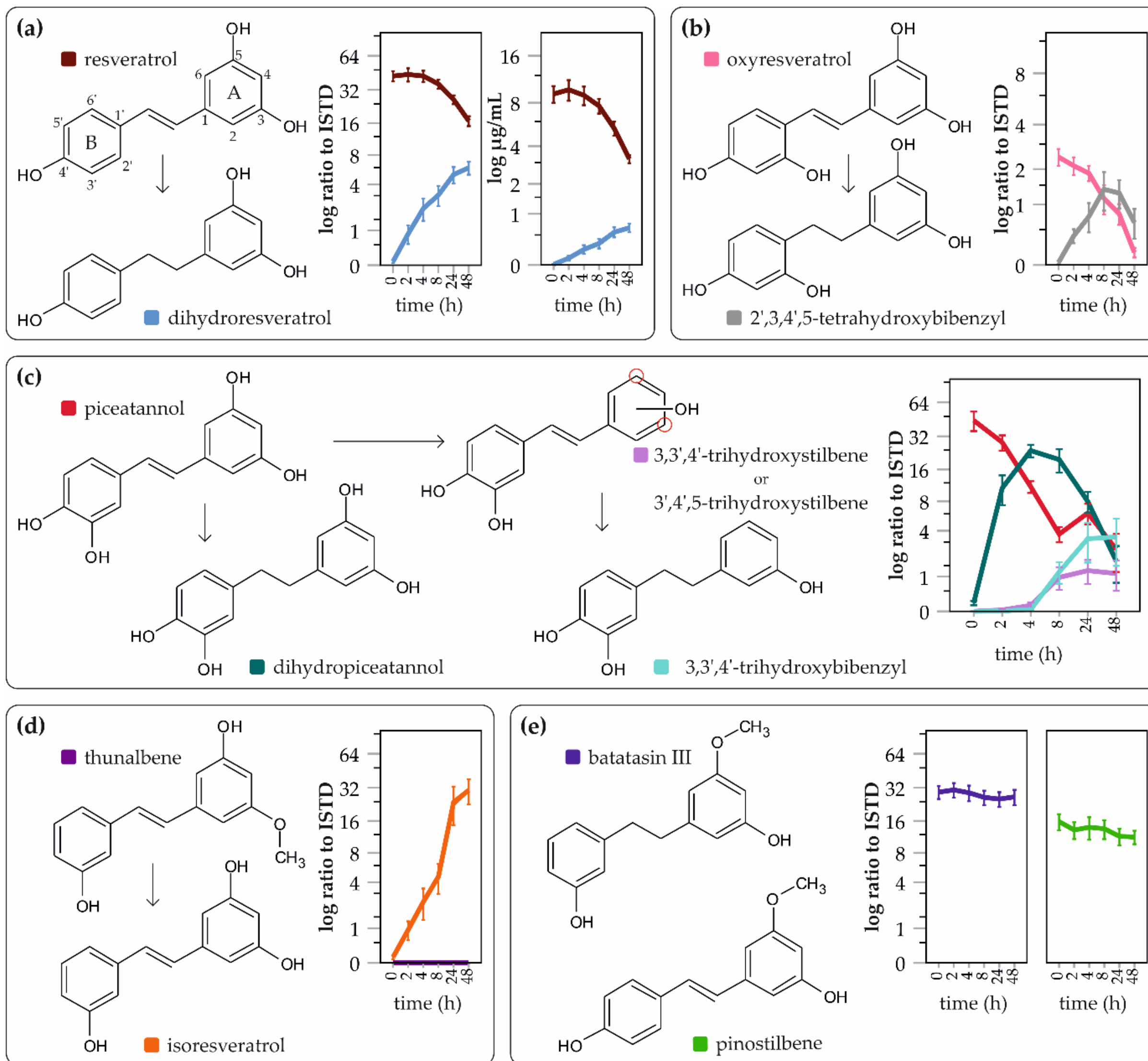

Figure 1. The effect of bacterial metabolism on (a) resveratrol (N = 5); (b) oxyresveratrol (N = 3); (c) piceatannol (N = 5); (d) thunalbene (N = 5); (e) batatasin III (N = 4); and pinostilbene (N = 5); values obtained from LC/MS are expressed as ratios of produced metabolite to internal standard (ISTD) as means ± 1 SE ($p < 0.05$); N represents the number of donors analyzed.

Strong inter-individual differences were observed among the donors, as seen in Appendix B Figure A2. Resveratrol was gradually metabolized in all samples to dihydroresveratrol, but the final concentrations varied among the donors from 77 ± 1% (mean ± SD; D2) to 11 ± 1% (D5). Oxyresveratrol was metabolized in each sample at different intensities, 100 ± 0% (D1), 84 ± 1% (D3), and 98 ± 2% (D5). Piceatannol, after 48 h of fermentation, was more than 99.6% metabolized in four of the samples, except for sample D2, where 12 ± 1% of piceatannol was still present at the end of the fermentation. Piceatannol was metabolized to dihydropiceatannol in all faecal samples, but in two out of the five cases (D4 and D5) it was

metabolized to 3,3’,4’-trihydroxystilbene or 3’,4’,5-trihydroxystilbene and further to 3,3’,4’-trihydroxybibenzyl. Similarly, thunalbene was metabolized to isoresveratrol in only three out of the five faecal samples (D2, 3 and 4), while in the others no metabolites were detected.

3.4 Discussion

The current study provides new information about the biotransformation of six stilbenoids by human gut microbiota, depending on their structural molecular properties. The obtained data show that stilbenoids differ in their stability in a colonic environment. Whereas batatasin III and pinostilbene did not produce any metabolites, resveratrol, oxyresveratrol, piceatannol, and thunalbene were intensively metabolized by colon microbiota. Three main reactions were found to be ongoing in our human colon model: double bond reduction, dihydroxylation, and demethylation. An important factor in the course of the reactions was the location of the hydroxyl and methyl groups. Differences in the intensity, rate, and spectrum of metabolites were also observed among the faecal samples obtained from different donors.

The only resveratrol metabolite detected in this study was dihydroresveratrol, which is in agreement with other *in vitro* and *in vivo* studies (Walle et al. 2004; Wang et al. 2005; Jung et al. 2009). Another study, using a similar model, described two more metabolites, 3,4’-dihydroxy-*trans*-stilbene and 3,4’-dihydroxybibenzyl (lunularin), which were detected in six out of seven faecal samples (Bode et al. 2013). Their absence in our study might be caused by a different composition of bacterial species in faecal samples or different initial concentrations of stilbenoids, which might saturate some enzymatic catabolic pathways and change the method of metabolite formation. It is evident that the course of the catabolic reaction was affected by the inter-individual differences in the bacterial composition of the faecal samples. In our study, resveratrol had been gradually catabolized, and its final concentration, after 48 h of fermentation, ranged from 77 ± 1% (D2) to 11 ± 1% (D5). This gradual decrease contrasts with another study (Bode et al. 2013) that reported a complete degradation of resveratrol in a time frame of 2 to 24 h. This might be partly caused by different initial concentrations of resveratrol (80 µM vs. 44 µM in our study), lower inoculum, medium composition, or simply differences in the donor’s microbial composition. Previously, we reported bacterial composition in a subset of samples reported here (time points 0 and 24 h; and donors D1-D4), showing major differences between this study (Jaimes et al. 2019)

and the study from Bode et al. (2013). While Faecalibacterium (12–21% of DNA) and Bacteroides (9–16% of DNA) were the most abundant group in (Bode et al. 2013), our fermentations were dominated by Clostridia at time points 0 h and 24 h (Jaimes et al. 2019). Mean *Faecalibacteium prausnitzii* abundance at time point 0 h was only 2.01 ± 1.01% and Bacteroides were only 0.06 ± 0.06%. In a previous study, 43 bacteria, mostly gut-associated strains, were screened for their capacity to catabolize resveratrol (Jung et al. 2009), from which 11 strains were capable of metabolizing resveratrol by more than 20%, among them *Escherichia coli* ATCC 25922, *Bacillus cereus* NCTR-466, and *Achromobacter denitrificans* NCTR-774 had transformed resveratrol almost completely within 24 h.

Similar to resveratrol and oxyresveratrol, piceatannol was metabolized to dihydropiceatannol by colon microbiota via hydrogenation of the ethylene bridge. However, another pathway has also been detected. Faecal bacteria from some donors were able to cleave the hydroxyl group on ring A in one of the *meta* positions and form trihydroxystilbene, different from resveratrol or isoresveratrol, which was further dehydroxylated to 3,3’,4’-trihydroxybibenzyl.

In the present study, three derivatives of stilbenoids formed by double bond reduction were detected. Their further fate in the colon model was dependent on the position of the hydroxyl group. Dihydroresveratrol, with only one hydroxyl group on ring B in *para* position, was not further metabolized or degraded, and it was the end-product of resveratrol colon metabolism. The catabolite of oxyresveratrol, 2,3,3’,5’-tetrahydroxystilbene, has two hydroxyl groups in *para* and *ortho* positions on ring B. Concentration change of this metabolite was more dynamic in this model, reaching its maximum concentration at 24 h and then further degrading to still unknown products. The least stable of these metabolites was dihydropiceatannol, which had two hydroxyl groups in *para* and *meta* position on ring B. It has been shown that dihydroresveratrol could be produced as an end-product or transient intermediate, depending on the donor (Bode et al. 2013), so the high persistence of dihydroresveratrol in the present study might only be an observed effect particular to our donors. The marked influence of colon microbiota composition on the metabolism of polyphenols has also been well reported by other authors (van Duynhoven et al. 2011).

Thunalbene was not observed as a parent compound in the samples, possibly because of binding to the matrix or polymerization. However, its demethylated catabolite, isoresveratrol, was detected in three out of the five donors (D2, 3, and 4). Interestingly, batatasin III

and pinostilbene, other methylated stilbenes, were demonstrably stable in the colon model. This matches results obtained in a study of pterostilbene (3,5-dimethoxy-4'-hydroxystilbene) metabolism in the colons of mice, where pinostilbene was found as its main product; however, no metabolites of pterostilbene with a reduced double bond were identified (Sun et al. 2016; Wang & Sang 2018). This result indicates that the position of the methoxy group could play an important role in its demethylation, as well as in the reduction of the ethylene bridge by intestinal microbiota.

In our previous study (Jaimes et al. 2019) the microbiota composition of donors D1-4 had been observed. Compared to the others, donors D1 and D2 seemed to be very atypical, with a higher representation of class Bacilli (*Streptococcaceae* family) in donor D1 and a higher representation of the *Enterobacteriaceae* family in donor D2. Due to the lack of the compounds, the full set of all six stilbenoids was fermented only by samples from donors D1, D3, and D5. Microbiota from the faecal sample of donor D1 was able to metabolize about half of the resveratrol and completely metabolize oxyresveratrol and piceatannol within 48 h, but it was not metabolizing thunalbene. Microbiota from donor D3 were effectively metabolizing resveratrol (20 ± 1% occurred after 48 h), and demethylating thunalbene to isoresveratrol, but was the least effective in metabolizing oxyresveratrol (84 µ 1% occurred after 48 h). Microbiota from donor D5 were the most effective in metabolizing resveratrol (11 ± 1% occurred after 48 h), and were able to dehydroxylate piceatannol. However, similar to donor D1, these microbiota were not able to metabolize thunalbene to isoresveratrol. Microbiota from donor D2 were the least effective in metabolizing resveratrol (77 ± 1% occurred after 48 h), and piceatannol (12 ± 1% occurred after 48 h) but seem to be the most effective in metabolizing thunalbene. Microbiota of donor D4 were not very effective in metabolizing either resveratrol or thunalbene but were as efficient as an inoculum of donor D5 by dehydroxylating piceatannol.

3.5 Conclusion

In conclusion, it has been shown that some stilbenoids are intensively catabolized by the colon microbiota, whereas others seem to be stable in the colon environment. In our model, the degree of substitution played an important role on the level of molecular fragility. Derivatives with the hydroxy group in the para position were less fragile than the others, and further study of their behavior in the colon is needed. The rate, intensity, and the pathways of metabolism are closely associated with colon microbiota composition. However, the role of particular bacterial

species on the metabolism of stilbenoids is not clear, and thus future research should also be focused on inter-individual differences and work with a larger number of donors. To our knowledge, this is the first study investigating the metabolic fate of stilbenoids other than resveratrol in the faecal human colon model. This study has also implication on future screening assays for biological activities, so that relevant metabolites can be included.

3.6 Acknowledgments

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Appendix A

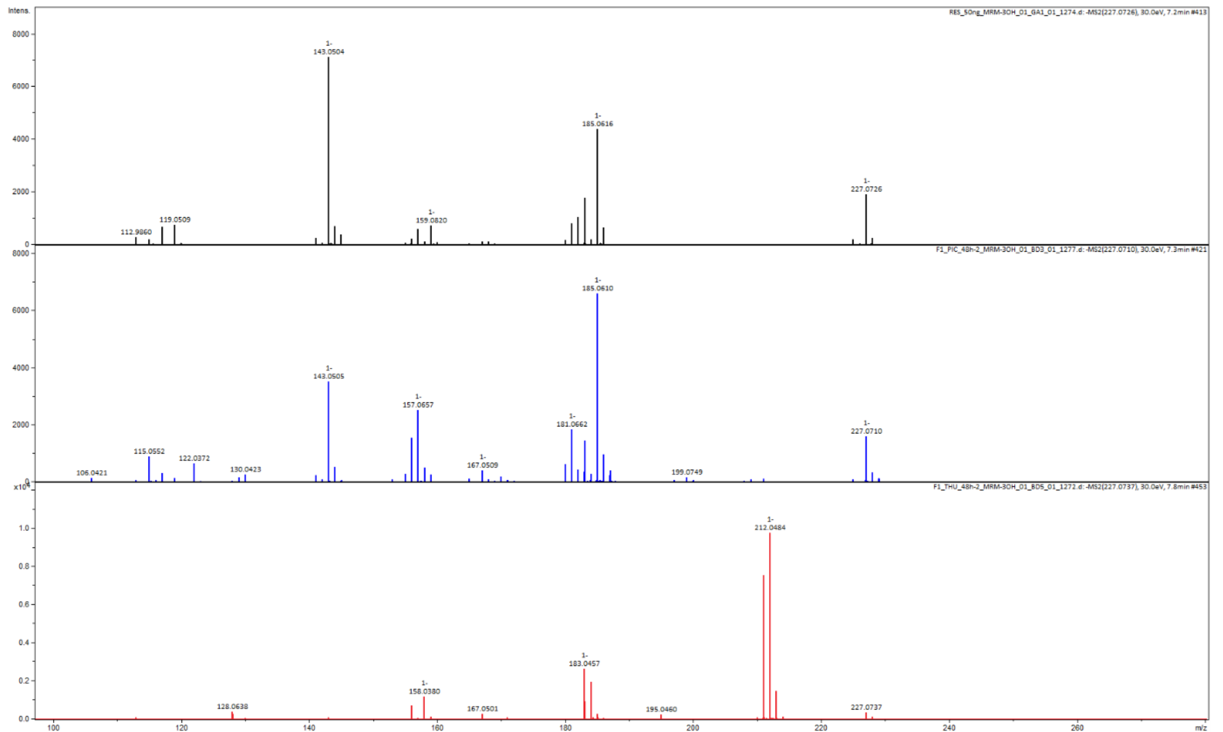

Figure A1. MS/MS spectra of resveratrol, 3,3’,4’-trihydroxystilbene/3’,4’,5-trihydroxystilbene and isoresveratrol.

Appendix B

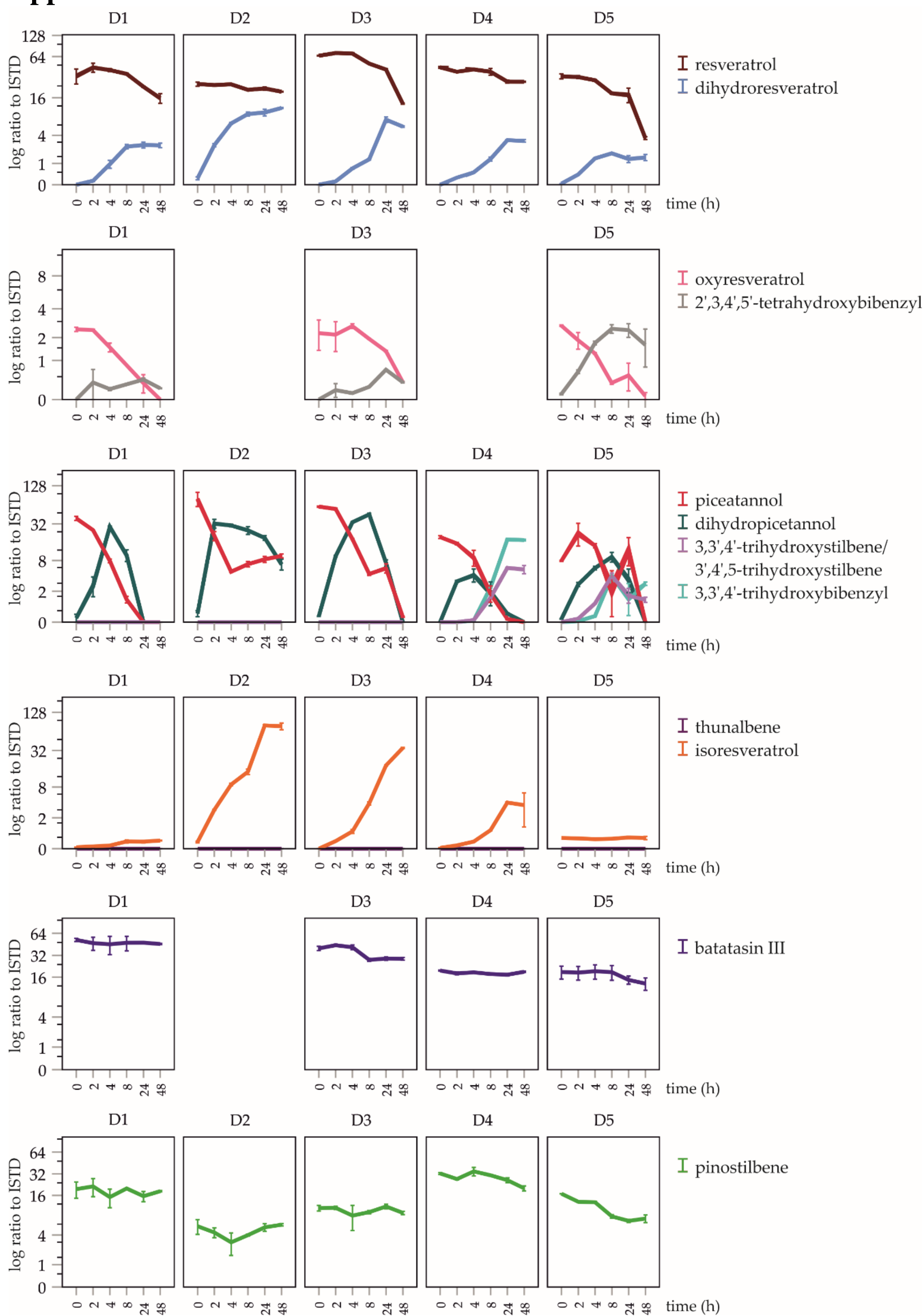

Figure A2. Comparison of metabolism between donors. Values are expressed as Means ± 1 SE, n = 3, technical replicates.

Appendix C

Table A1. List of the stilbenoids monitored and detected in the samples by LC/MS

Name	Molecular formula	Neutral molecule exact mass:	Measured [M -H]- exact mass	Comparison with standard
monitored:				
thunalbene	$C_{15}H_{14}O_3$	242.0943	241.0865	YES
pinostilbene	$C_{15}H_{14}O_3$	242.0943	241.0865	YES
piceatannol	$C_{14}H_{12}O_4$	244.0736	243.0657	YES
oxyresveratrol	$C_{14}H_{12}O_4$	244.0736	243.0657	YES
batatasin III	$C_{15}H_{16}O_3$	244.1099	243.1021	YES
resveratrol	$C_{14}H_{12}O_3$	228.0786	227.0708	YES
lunularin	$C_{14}H_{14}O_2$	214.0994	213.0916	YES
detected:				
dihydroresveratrol	$C_{14}H_{14}O_3$	230.0943	229.0865	YES
2,3',4,5'-tetrahydroxybibenzyl	$C_{14}H_{14}O_4$	246.0892	245.0814	NO
dihydropiceatannol	$C_{14}H_{14}O_4$	246.0892	245.0814	NO
trihydroxystilbene	$C_{14}H_{12}O_3$	228.0786	227.0708	NO
3,3',4-trihydroxybibenzyl	$C_{14}H_{14}O_3$	230.0943	229.0865	NO
isoresveratrol	$C_{14}H_{12}O_3$	228.0786	227.0708	NO

3.7 References

Allen EN, Potdar S, Tapias V, Parmar M, Mizuno CS, Rimando A, Cavanaugh JE. 2018. Resveratrol and pinostilbene confer neuroprotection against aging-related deficits through an ERK1/2-dependent mechanism. Journal of Nutritional Biochemistry 54:77–86.

Bode LM, Bunzel D, Huch M, Cho GS, Ruhland D, Bunzel M, Bub A, Franz CM, Kulling SE. 2013. In vivo and in vitro metabolism of trans-resveratrol by human gut microbiota. The American Journal of Clinical Nutrition 97:295.

Brasnyó P et al. 2011. Resveratrol improves insulin sensitivity, reduces oxidative stress and activates the Akt pathway in type 2 diabetic patients. British Journal of Nutrition 106:383–389.

Buryanovskyy L, Fu Y, Boyd M, Ma Y, Hsieh TC, Wu JM, Zhang Z. 2004. Crystal structure of quinone reductase 2 in complex with resveratrol. Biochemistry 43:11417–11426.

González-Barrio R, Edwards CA, Crozier A. 2011. Colonic catabolism of ellagitannins, ellagic acid, and raspberry anthocyanins: In vivo and in vitro studies. Drug Metabolism and Disposition 39:1680–1688.

Jaganath IB, Mullen W, Lean MEJ, Edwards CA, Crozier A. 2009. In vitro catabolism of rutin by human fecal bacteria and the antioxidant capacity of its catabolites. Free Radical Biology and Medicine 47:1180–1189.

Jaimes JD, Jarosova V, Vesely O, Mekadim C, Mrazek J, Marsik P, Killer J, Smejkal K, Kloucek P, Havlik J. 2019. Effect of selected stilbenoids on human fecal microbiota. Molecules 24:1155.

Jung CM, Heinze TM, Schnackenberg LK, Mullis LB, Elkins SA, Elkins CA, Steele RS, Sutherland JB. 2009. Interaction of dietary resveratrol with animal-associated bacteria. FEMS Microbiology Letters 297:266–273.

Kairisalo M, Bonomo A, Hyrskyluoto A, Mudò G, Belluardo N, Korhonen L, Lindholm D. 2011. Resveratrol reduces oxidative stress and cell death and increases mitochondrial antioxidants and XIAP in PC6.3-cells. Neuroscience Letters 488:263–266.

Kantartzis K et al. 2018. Effects of resveratrol supplementation on liver fat content in overweight and insulin-resistant subjects: A randomized, double-blind, placebo-controlled clinical trial. Diabetes, Obesity and Metabolism 20:1793–1797.

Leláková V et al. 2019. Parallel in vitro and in silico investigations into anti-inflammatory effects of non-prenylated stilbenoids. Food Chemistry. 285:431–440.

Lin Z-S, Ku CF, Guan Y-F, Xiao H-T, Shi X-K, Wang H-Q, Bian Z-X, Tsang SW, Zhang H-J. 2016. Dihydro-Resveratrol Ameliorates Lung Injury in Rats with Cerulein-Induced Acute Pancreatitis. Phytotherapy Research 30:663–670.

Liu M, Wilk SA, Wang A, Zhou L, Wang RH, Ogawa W, Deng C, Dong LQ, Liu F. 2010. Resveratrol inhibits mTOR signaling by promoting the interaction between mTOR and DEPTOR. Journal of Biological Chemistry 285:36387–36394.

Murias M, Handler N, Erker T, Pleban K, Ecker G, Saiko P, Szekeres T, Jäger W. 2004. Resveratrol analogues as selective cyclooxygenase-2 inhibitors: synthesis and structure-activity relationship. Bioorganic & medicinal chemistry 12:5571–8.

Piotrowska H, Kucinska M, Murias M. 2012. Biological activity of piceatannol: Leaving the shadow of resveratrol. Mutation Research - Reviews in Mutation Research 750:60–82.

Qiang L, Di Y, Jiang Z, Xu J. 2018. Resveratrol improves efficacy of oral amoxicillin against childhood fast breathing pneumonia in a randomized placebo-controlled double blind clinical trial. Microbial Pathogenesis 114:209–212.

Reinisalo M, Kårlund A, Koskela A, Kaarniranta K, Karjalainen RO. 2015. Polyphenol stilbenes: Molecular mechanisms of defence against oxidative stress and aging-related diseases. Oxidative Medicine and Cellular Longevity 2015:1–24.

Renaud S, de Lorgeril M. 1992. Wine, alcohol, platelets, and the French paradox for coronary heart disease. The Lancet 339:1523–1526.

Sánchez-Fidalgo S, Cárdeno A, Villegas I, Talero E, de la Lastra CA. 2010. Dietary supplementation of resveratrol attenuates chronic colonic inflammation in mice. European Journal of Pharmacology 633:78–84.

Sattarinezhad A, Roozbeh J, Shirazi Yeganeh B, Omrani GR, Shams M. 2018. Resveratrol reduces albuminuria in diabetic nephropathy: A randomized double-blind placebo-controlled clinical trial. Diabetes and Metabolism 45:53–59.

Seyed MA, Jantan I, Bukhari SNA, Vijayaraghavan K. 2016. A Comprehensive Review on the Chemotherapeutic Potential of Piceatannol for Cancer Treatment, with Mechanistic Insights. Journal of Agricultural and Food Chemistry 64:725–737.

Smoliga JM, Blanchard O. 2014. Enhancing the delivery of resveratrol in humans: If low bioavailability is the problem, what is the solution? Molecules 19:17154–17172.Sun Y et al. 2016. Identification of pinostilbene as a major colonic metabolite of pterostilbene and its inhibitory effects on colon cancer cells. Molecular Nutrition and Food Research 60:1924–1932.

Tsang SW, Guan YF, Wang J, Bian ZX, Zhang HJ. 2016. Inhibition of pancreatic oxidative damage by stilbene derivative dihydro-resveratrol: Implication for treatment of acute pancreatitis. Scientific Reports 6. 22859.

van Duynhoven J et al. 2011. Metabolic fate of polyphenols in the human superorganism. Proceedings of the National Academy of Sciences 108:4531–4538.

Walle T, Hsieh F, DeLegge MH, Oatis JE, Walle UK. 2004. High absorption but very low bioavailability of oral resveratrol in humans. Drug Metabolism and Disposition 32:1377–1382.

Wang D, Hang T, Wu C, Liu W. 2005. Identification of the major metabolites of resveratrol in rat urine by HPLC-MS/MS. Journal of Chromatography B: Analytical Technologies in the Biomedical and Life Sciences 829:97–106.

Wang P, Sang S. 2018. Metabolism and pharmacokinetics of resveratrol and pterostilbene. BioFactors 44:16–25. John Wiley & Sons, Ltd.

Xu L, Liu C, Xiang W, Chen H, Qin X, Huang X. 2014. Advances in the study of oxyresveratrol. International Journal of Pharmacology 10:44–54.

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Effect of Selected Stilbenoids on Human Fecal Microbiota

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Abstract

Dietary phenolics or polyphenols are mostly metabolized by the human gut microbiota. These metabolites appear to confer the beneficial health effects attributed to phenolics. Microbial composition affects the type of metabolites produced. Reciprocally, phenolics modulate microbial composition. Understanding this relationship could be used to positively impact health by phenolic supplementation and thus create favorable colonic conditions. This study explored the effect of six stilbenoids (batatasin III, oxyresveratrol, piceatannol, pinostilbene, resveratrol, thunalbene) on the gut microbiota composition. Stilbenoids were anaerobically fermented with fecal bacteria from four donors, samples were collected at 0 and 24 h, and effects on the microbiota were assessed by 16S rRNA gene sequencing. Statistical tests identified affected microbes at three taxonomic levels. Observed microbial composition modulation by stilbenoids included a decrease in the Firmicutes to Bacteroidetes ratio, a decrease in the relative abundance of strains from the genus *Clostridium*, and effects on the family *Lachnospiraceae*. A frequently observed effect was a further decrease of the relative abundance when compared to the control. An opposite effect to the control was observed for *Faecalibacterium prausnitzii*, whose relative abundance increased. Observed effects were more frequently attributed to resveratrol and piceatannol, followed by thunalbene and batatasin III.

Keywords: phenolics; polyphenols; stilbenoids; human gut microbiota; 16S rRNA gene sequencing; batatasin III; oxyresveratrol; piceatannol; pinostilbene; resveratrol; thunalbene; fermentation; human colon model; *Lachnospiraceae*; Firmicutes; Bacteroidetes; *Clostridium*; *Faecalibacterium prausnitzii*

4.1 Introduction

Stilbenoids are a subclass of plant-derived phenolic compounds often consumed in the diet as components from red grapes, peanuts, certain berries, and many others. Their average dietary intake is 1 g/day (Pérez-Jiménez et al. 2011, Ozdal et al. 2016, Edwards et al. 2017). The most well studied stilbenoid is resveratrol, which came into the spotlight with the so-called French paradox, where it was attributed in reducing coronary heart disease mortality among the sample population despite the strong presence of risk factors (Akinwumi et al. 2018, WHO Monica Project 1989). Further studies have attributed many other potential health benefits to resveratrol, as well as to various other phenolics, such as potent antioxidant activity, cardio-protection, neuroprotection, anti-inflammatory effects, cancer prevention, and others (Akinwumi et al. 2018).

In plants, phenolics are usually conjugated to sugars, organic acids, and macromolecules (e.g., dietary fiber and proteins) and most of them are not properly released and absorbed in the small intestine, reaching the colon for further microbial fermentation; at colonic level, they are fermented by the resident gut microbiota (GM) (Tomás-Barberán et al. 2016). It is the resulting metabolites that are attributed the health benefits as bioactive compounds. Evidence shows that 90–95% of ingested dietary phenolics, usually in their glycosylated form, are not absorbed in the upper part of the digestive tract. Most of them reach the colon, where the GM metabolize them into lower molecular weight-phenolic compounds, such as phenolic acids, that can be more easily absorbed by intestinal epithelial cells and enter the liver for further biotransformation or systemic circulation (Monagas et al. 2010, van Duynhoven et al. 2011, Bode et al. 2013, Cueva et al. 2013, Etxeberria et al. 2013, Carrera-Quintanar et al. 2018, Pasinetti et al. 2018). These microbial bio-transformations are grouped into three major catabolic processes: hydrolysis (O-deglycosylations and ester hydrolysis), cleavage (C-ring cleavage; delactonization; demethylation), and reductions (dehydroxylation and double bond reduction) (Espín et al. 2017). Reciprocal to these bio-transformations by the GM, phenolics appear to modulate the GM composition by favoring/disfavoring certain microbial strains, thus establishing a two-way relationship between the GM and phenolics (Larrosa et al. 2009, Dueñas et al. 2015, Tomás-Barberán et al. 2016, Cueva et al. 2017, Ding et al. 2018). The undigested phenolics, along with diet-independent substrates like endogenous host secretions, are the main substrates of gut bacterial metabolism, and may affect the GM in a similar manner as prebiotics, shape microbial composition by antimicrobial action, and/or influence bacterial adhesion

(Kemperman et al. 2013, Marín et al. 2015, Braune & Blaut 2016, Ozdal et al, 2016, Volstatova et al. 2017, Havlik & Edwards 2018, Tomas-Barberan at al. 2018). For example, chlorogenic acid, resveratrol, catechin, and certain quercetin derivatives have exhibited prebiotic-like effects by increasing the proportional representation of *Bifidobacterium* strains (Tzounis et al. 2008, Ozdal et al. 2016, Carrera-Quintanar et al. 2018, Catinean et al. 2018, Mayta-Apaza et al. 2018). Antimicrobial action has been shown by inoculation with resveratrol and certain ellagitannins by inhibiting the growth of several *Clostridia* species (Bialonska et al. 2009, Larrosa et al. 2009, Etxeberria et al. 2013, Ozdal et al. 2016). Bacterial adhesion effects by procyanidin and chlorogenic acid have been noticed through adhesion enhancement of certain *Lactobacillus* strains to intestinal epithelial cells (Volstatova et al. 2017, Havlik & Edwards 2018).

To our knowledge, except for resveratrol and a few studies with piceatannol, both of which are well-recognized plant-derived phenolics, there is not much information regarding the effects of stilbenes on the GM. Other than a 2016 study on the effects of repeated stilbenoid administration on the GM, the rest have mostly focused on evaluating a single dose effect, mainly on culturable microbial strains. The findings from the former showed a strong change in the GM composition after application of resveratrol and viniferin, especially in the enrichment of the order Enterobacteriales, and a decrease of Bifidobacteriales (Giuliani et al. 2016) Observations from the single dose studies showed changes in the GM composition; for example, increases for species *Akkermansia muciniphila* and *Faecalibacterium prausnitzii* by resveratrol, and in the genus *Lactobacillus* by piceatannol (Hervert-Hernández & Goñi 2011, Bode et al. 2013, David et al. 2014, Ozdal et al. 2016, Etxeberria et al. 2017, Carrera-Quintanar et al. 2018, Catinean et al. 2018, Rowland et al. 2018, Scarano et al. 2018).

The objective of this study was to assess the effect of six stilbenoid phenolics (batatasin III (Bat), oxyresveratrol (Oxy), *trans*-resveratrol (Res), piceatannol (Pic), pinostilbene (Pino), and thunalbene (Thu); the corresponding chemical structures are given in Figure 1) on the GM at dietary relevant concentrations. Using an *in vitro* fecal fermentation (FFM) system, these stilbenoids were fermented with human fecal bacteria from four donors. Effects on the GM composition were based on 16S rRNA gene sequencing results.

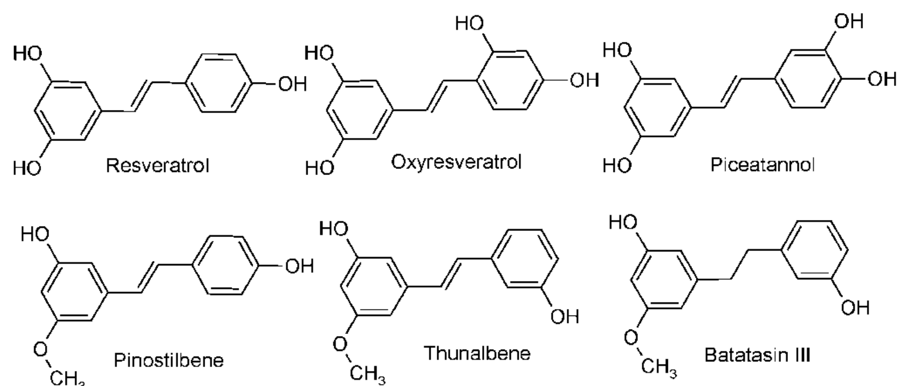

Figure 1. Molecular structures of stilbenoids studied. All stilbenoids have a C6-C2-C6 structure.

4.2 Material and Methods

4.2.1 Study Design

Using an *in vitro* fecal fermentation (FFM) system, a set of six stilbenoid phenolics were fermented in vials via inoculation with human fecal bacteria obtained from four donors. The vials were sampled at 0 hour (0 h) and 24 h (24 h) time points, and the effect of the stilbenoids on human GM was assessed by 16S rRNA gene sequencing. Both parametric and non-parametric statistical tests were used to identify potentially affected strains at the phylum, family, and species taxonomic levels.

4.2.2 Donors and Ethics Statement

The fecal samples originated from four volunteer donors, all of whom consented for their samples to be used for research purposes by signing a consent form. The ethical agreement for stool collection was obtained by the ethical committee (ZEK/22/09/2017) of the Czech University of Life Sciences in Prague. The donors were two males and two females ages 23, 28 (Donors 1 and 3) and 26, 29 (Donors 2 and 4) respectively. Their respective body mass index (BMI) were 23.0, 24.7, 26.0, and 26.5. To reduce potential interference from other dietary phenolics, all donors followed a low-phenolic diet for at least 48 hours prior to providing the fecal sample. Also, none had taken any antibiotics for at least 6 months prior to sampling. They described themselves as being in good health, and none reported any chronic conditions or diseases. They followed an omnivorous diet in their daily life. Females were neither pregnant nor lactating. The samples were collected in October and November 2016, at the Czech University of Life Sciences in Prague, Czech Republic.

4.2.3 *In vitro* Fecal Fermentation (FFM) System

4.2.3.1 Standard Compounds and Chemicals

The chemicals used for preparation of the fermentation medium were obtained from Merck (Darmstadt, Germany). The stilbenoids batatasin III, piceatannol, thunalbene, and pinostilbene were purchased from ChemFaces (Wuhan, China) in 98% purity; *trans*-resveratrol, oxyresveratrol were obtained from Merck in 98% purity. Standards were prepared as 1% methanol/formic acid. Methanol and ethyl acetate were of analytical grade and purchased from VWR Chemicals (Stribrna Skalice, Czech Republic). Dimethyl sulfoxide (DMSO) was obtained from VWR Chemicals. Formic acid was obtained from Fisher Scientific (Merelbeke, Belgium) in >98% purity. Ultra-pure water (MilliQ) was obtained from a Millipore system (Bedford, MA, USA).

4.2.3.2 Fermentation Medium

Fermentation medium was prepared from the following solutions based on previous fecal fermentation studies (Edwards et al. 1996, Jaganath et al. 2009, González-Barrio et al. 2011). Micromineral solution was prepared from 2.64 g $CaCl_2$, 2 g $MnCl_2$, 0.2 g $CoCl_2$, 1.6 g $FeCl_3$, and up to 20 mL distilled water. Macromineral solution was prepared from 7.14 g of Na_2HPO_4, 6.2 g KH_2PO_4, 0.6 g $MgSO_4$, and up to 1 L distilled water. Carbonate buffer was made of 1 g NH_4HCO_3, 8.75 g $NaHCO_3$, and distilled water up to 250 mL (stored max. 1 month). The fermentation medium was prepared from 225 mL distilled water and 1.125 g of tryptone, 56.25 µL of micromineral solution, 112.5 mL of CO_3 buffer, 112.5 mL of macromineral solution, and 562.5 µL of 0.1% resazurin solution.

4.2.3.3 Phosphate Buffer, Reducing Solution

Sodium phosphate buffer for the preparation of fecal slurries was made of 1.7702 g KH_2PO_4 in distilled water (195 mL), and 3.6222 g Na_2HPO_4 in 305 mL distilled water (both 1/15 M). Afterwards, the buffer's pH was modified to 7.0 by hydrochloric acid. Reducing solution was prepared from 125 mg cysteine hydrochloride, 0.8 mL 1 M NaOH, 125 mg Na_2S and distilled water up to 20 mL.

4.2.3.4 Fermentations Using Human Fecal Microbiota

Each tested stilbenoid was dissolved in DMSO to reach a concentration of 10 mg/mL. The fermentation medium and sodium phosphate buffer were boiled and cooled to approximately 37 °C while they were purged with oxygen free nitrogen gas (approx. 30 min). The medium's pH was adjusted to pH 7.0 using HCl. For each vial, 16.8 mL of medium was transferred to the corresponding fermentation bottle and 0.8 mL of reducing solution was added. Per each donor, freshly obtained feces were homogenized in a stomacher bag with the sodium phosphate buffer to make a 32% fecal slurry. This slurry was then filtered through a mesh, from which 2 mL of the resulting filtrate was mixed with the fermentation medium in each of the fermentation bottles. 20 µL of tested compound solution (or DMSO alone for the controls) was also added. The bottles were incubated at 37 °C for 48 hours in a shaking bath at 100 strokes per minute. Four aliquots of fecal suspensions were prepared in 1.5 mL Eppendorf tubes by transferring from 20 mL glass bottles, collected at 0, 2, 4, 8, 24 and 48 h, and stored at −80 °C until further analysis. These timepoints were used for a related metabolomic study. For this particular study, only 0 and 24 timepoints were used.

4.2.4 Microbial Analysis

4.2.4.1 DNA Extraction

Bacterial DNA was isolated from the fecal samples according to the manufacturer's instructions using the Quick-DNA Fecal/Soil Microbe Miniprep Kit (Zymo Research, Irvine, CA, USA). The purified DNA was eluted in 100 µL of elution buffer and stored at −20 °C until further use.

4.2.4.2 16.S rDNA amplification: Nested PCR

During this nested PCR, two genes were amplified and targeted by two different pairs of primers in two successive reactions of PCR. The first PCR was done to amplify almost full length bacterial 16S rRNA gene fragments using the universal bacterial primers 616V (5′(5′ AGA GTT TGA TYM TGG CTC 3′) and 630R (5′ CAK AAA GGA GGT GAT CC 3′) (Juretschko et al. 1998). The thermal cycling was carried out with an initial denaturation step of 94 °C for 5 min, followed by 32 cycles of denaturation at 94 °C for 45 s, annealing at 52 °C for 1 min, and elongation at 72 °C for 1 min and 30 s; cycling was completed by a final elongation step of 72 °C for 6 min. Using the purified PCR product from the first PCR, the second PCR was performed as described by Fliegerova et al. (2014) to amplify the V4-V5 region of the 16S

rRNA gene by the primer pair: BactBF (GGATTAGATACCCTGGTAGT) and BactBR (CACGACACGAGCTGACG). The used thermal cycling program was: initial denaturation for 5 min at 95 °C, followed by 35 cycles of 30 s at 95 °C, 30 s at 57 °C and 30 s at 72 °C, ending by final elongation for 5 min at 72 °C. The PCR amplicons (300 bp) were checked at 1.5% agarose electrophoresis (30 min at 100 V), purified by QIAquick PCR Purification Kit (Qiagen, Venlo, The Netherlands) according to the protocol and quantified by Nanodrop (Thermo Fisher, Waltham, MA, USA).

4.2.4.3 Semi-conductor Based Next Generation Sequencing

Obtained PCR products were used to prepare libraries for diversity analyses by next generation sequencing (NGS) approach on Personal Genome Machine (Life Technologies, Carlsbad, CA, USA) according to Milani et al. (2013). 200 ng of DNA from each sample was used to prepare sequencing libraries by NEBNext® Fast DNA Library Prep Set kit (New England Biolabs, Ipswich, MA, USA) according to manufacturer's protocol. The Ion Xpress Barcode adapters (Thermo Fisher Scientific, Waltham, MA, USA) were used to label each sample. The adaptor ligated libraries were purified and simultaneously size-selected using AMPure XP bead sizing (Beckman Coulter, Brea, CA, USA). The barcoded libraries were pooled in equimolar amount (about 26 pM). The pool of libraries was used to prepare sequencing template by emulsion PCR on Ion Sphere Particles (ISPs) using Ion PGMTM Hi-QTM View OT2 400 Kit (Thermo Fisher Scientific) in Ion OneTouchTM 2 instrument. The enrichment of the template positive ISPs were performed on Ion OneTouchTM ES instrument. The enriched template positive ISPs were then loaded in Ion 316TM Chip v2 BC (Thermo Fisher Scientific). The sequencing was then performed on an Ion Torrent PGM sequencer (Thermo Fisher Scientific, Waltham, MA, USA) using Ion PGMTM Hi-QTM View Sequencing solutions kit (Thermo Fisher Scientific).

4.2.4.4 Data Analysis

The sequences obtained in FASTQ format were processed by QIIME analyses pipeline (Caporaso et al. 2010). The chimeras were removed by USEARCH tool (Edgar 2010). Remaining sequences were clustered and identified by performing open-reference OTU picking against the Greengene database (DeSantis et al. 2006). Diversity index analysis and unweighted and weighted UniFrac distance metrics analyses were generated using QIIME and expressed by principle coordinate analysis (PCoA).

4.2.4.5 Statistical Analysis

Using SPSS version 25 (IBM Corp., Armonk, NY, USA), both parametric and non-parametric statistical tests were used to identify taxa of interest at the phylum, family, and species level by the following comparisons: (1) Using the relative abundance of the control fermentation with stool samples at 24 h with DMSO only as our baseline for comparison, we identified taxa from the fermentations with stilbenoids (each comparison done separately) that had *p* values <0.05 for the Paired sample t-test, and/or <0.075 for the Wilcoxon signed-rank test when compared to our baseline. (2) The magnitude of change (growth or decline) in relative abundance between the control fermentation with only stool sample at 0 h (Ctrl0 h) and our control fermentation with samples with DMSO only at 24 h was calculated, and this value became our baseline for comparison against the magnitude of change from 0 h to 24 h for the fermentations with stilbenoids. Selected taxa had *p* values <0.05 for the Paired sample t-test, and/or <0.075 for the Wilcoxon signed-rank test. Only 5 stilbenoids were tested. Pinostilbene was excluded since samples for it were only available for two out of the four donors. Similarly, any pair that had $n \leq 2$ was excluded. Since the data was in percent, the magnitude of change was obtained by obtaining the percentage change of the given percentages. Values of 0% at 0 h were excluded, even if they were detectable at higher percentages. This was done due to the ambiguity of whether they were low values that were undetectable or whether they were simply not present.

4.3 Results and Discussion

4.3.1 Firmicutes to Bacteroidetes (F/B) Ratio

The most abundant phyla in human gut microbiota are Firmicutes and Bacteroidetes, which often account for more than 90% of the total gut microbiota (Healey et al. 2017). However, that was not the case in this study. Firmicutes were the most abundant, followed by Actinobacteria, with Bacteroidetes coming in at either fourth or fifth place depending on the donor. One possibility may be that one of the kits used during processing may have been more sensitive to phyla other than Bacteroidetes, or perhaps these bacteria progress to a higher relative abundance during *in vitro* cultivation compared to what would normally be found in stool alone. Nevertheless, the ratio of these two phyla can still be evaluated.

An increased F/B ratio in both human and mouse gut microbiota has consistently been associated with higher obesity and disease occurrence (Turnbaugh et al. 2006, Koliada et al. 2017). Resveratrol has been previously shown to decrease this ratio (Ozdal et al. 216, Qiao et al. 2014, Catinean et al. 2018), and our findings support this. Similarly, the other tested stilbenoids also decreased the F/B ratio as can be seen in Figure 2. Res, Bat, and Thu reached lower ratios (61 ± 23, 49 ± 22, 96 ± 53 respectively) than the control at 24 h (121 ± 73). Interestingly, Pino showed an increase (227 ± 127), while Pic stayed approximately equal (131 ± 98) to the control at 24 h. The response is a result of a decrease in the relative abundance of Firmicutes and an increase of Bacteroidetes, which is consistent with findings from other studies (Ozdal et al. 216, Carrera-Quintanar et al. 2018, Catinean et al. 2018). For Firmicutes, after treatment with all tested stilbenoids, the relative abundance decrease (−2.9% ± 0.03%) was lower than the control at 24 h (−4.6% ± 0.03%), with the least decrease observed under Oxy and Pino (−1.5% ± 0.03% and −0.7% ± 0.02%, respectively). For Bacteroidetes, after treatment with all tested stilbenoids except for Pino (51.0.2% ± 0.00%), the growth in relative abundance (Bat 278.0% ± 0.02%; Oxy 198.1% ± 0.00%; Pic 86.0% ± 0.05%; Res 195.6% ± 0.04%; Thu 300.3% ± 0.01%) was greater than that of the control at 24 h (68.0% ± 0.04%).

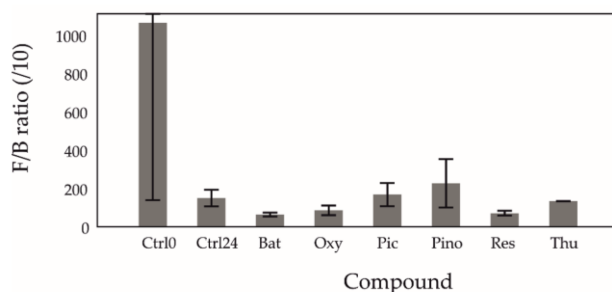

Figure 2. Mean Firmicutes/Bacteroidetes ratio (/10) in fermentations. Error bars represent the 95% CI. Ctrl0 = control at 0 h; Ctrl24 = control at 24 h; Bat = batatasin III; Oxy = oxyresveratrol; Pic = piceatannol; Pino = pinostilbene; Res = *trans*-resveratrol; Thu = thunalbene. All stilbenoids at 24 h.

4.3.2 Most and Least Abundant Species

A total of 230 bacterial species entities were detected in the tested fecal samples. This number includes unidentified species that could only be categorized as part of a higher taxonomic level. For example, an unidentified species, from an unidentified genus, that belongs to the *Clostridiaceae* family. The lowest detected relative abundance was 0.00047% for an unidentified species of the *Christensenella* genus.

The five species with the highest relative abundance per each of the tested samples were identified. These accounted for 53% to 66% of the total relative abundance and, in total, comprised 11 distinct species (Table 1). Therefore, there appears to be certain consistency, and not much variability, among the most abundant taxa.

Table 1. The most abundant species obtained by identifying the five species with the highest relative abundance for Control 0 h and 24 h, and per each of the six tested stilbenoid samples at 24 h. Gen. = unnamed genus, sp. = unnamed species.

Phylum	Class	Order	Family	Genus	Species
Actinobacteria	Actinobacteria	Bifidobacteriales	*Bifidobacteriaceae*	*Bifidobacterium*	*sp.*
Firmicutes	Bacilli	Lactobacillales	*Streptococcaceae*	*Streptococcus*	*sp.*
	Clostridia	Clostridiales	*Lachnospiraceae*	*Blautia*	*sp.*
				Gen.	*sp.*
				Gen.	*sp.*
			Ruminococcaceae	*Faecalibacterium*	*prausnitzii*
				Ruminococcus	*sp.*
				Gen.	*sp.*
				Gen.	*sp.*
			Unnamed	*Gen.*	*sp.*
Proteobacteria	Gammaproteobacteria	Enterobacteriales	*Enterobacteriaceae*	*Gen.*	*sp.*

Focusing on the inverse, in the five species with the least relative abundance, there is less consistency and greater variability since it comprised 27 distinct species (Appendix A, Table A1). It's important not to ignore the least abundant species since their low abundance may not necessarily correlate with the importance of their function. As stated in Cueva et al. (2017), the microorganisms present in smaller quantities, but developing specific functions, could be the key to understanding the individual response to consumption of bioactive compounds (i.e., phenolics). Some metabolic functions seem to be achieved by a wide variety of species, while other functions are only done by a specific few. For example, *Ruminococcus bromii*, identified within the 27 species, has been noted to be a butyrate (a short-chain fatty acid) producer, which is a function that appears to be found in fewer species than those for acetate (Morrison & Preston 2016).

4.3.3 Changes in Relative Abundance (Phylum, Family, Species)

Both parametric and non-parametric statistical tests were used to identify taxa of interest at the phylum, family, and species level based on two comparisons. The statistical tests were used as a tool to identify potential significantly affected taxa, and should not be interpreted as a portrayal of definite statistical significance (for those with p values in the range) due to the small sample size (four donors). The identified taxa reported $p < 0.075$ for at least one p value (paired sample t-test and/or Wilcoxon signed-rank test) for both comparisons

1 and 2. Comparison 1 used as a baseline the relative abundance of the 24 h control, and compared this value to each of the six stilbenoid fermentations. Comparison 2 used as a baseline the magnitude of change (growth or decline) in relative abundance between Control 0 h and Control 24 h, and compared this value to the magnitude of change between Control 0 h and each of the six stilbenoid fermentations.

Figure 3 displays these identified taxa in the form of a phylogenetic tree sorted by phylogenetic distance. The corresponding *p* values are listed in Appendix A, Table A2, and the corresponding relative abundance box plots are shown in Figure 4. Each comparison (1&2) is shown separately in Appendix A, Table A3 and Table A4, and list additional taxa. Clustered bar graphs of bacterial composition at the phylum and family levels can be seen in Appendix A, Figure A1 and Figure A2. Table 2 displays how our study compares to findings and observations from other studies regarding the effect of the selected stilbenoids on a specific taxon.

4.3.3.1 Decrease in Relative Abundance

A decrease in relative abundance was observed for several taxa under some of the tested stilbenoids. The most frequently observed response was a further decrease of the relative abundance of a specific taxon as compared to the 24 h control by either Res, Pic or Thu. For example, for *Clostridium sp.* there was a decrease of −54.2% ± 28.8% for Ctrl24, while the decrease caused by Pic and Thu were of a greater magnitude, −62.9% ± 28.0% (t(3) = 3.960, p = 0.029) and −79.3% ± 22.6% (t(3) = 3.901, p = 0.030), respectively. Similar responses were observed, albeit at different magnitudes, for family *Lachnospiraceae,* and species *Coprococcus sp.*, *Collinsella aerofaciens,* and *Lachnospiraceae Gen. sp*. At the genus level, *Clostridium* decreased under all tested stilbenoids in our study. Previous findings, as listed in Table 2, observed that several species from the genus *Clostridium*, which includes both commensal and deleterious species, had been shown to decrease with resveratrol (Etxeberria et al. 2013, Ozdal et al. 2016).

A second observed response was a decrease in relative abundance while the 24 h control increased. This was observed by three species, *Ruminococcus sp.* (−3.2% ± 69.1%, t(2) = 4.448, p = 0.047 under Bat; −7.0% ± 69.4%, t(3) = 8.253,p = 0.004 under Pic; −41.1% ± 50.9%, t(3) = 1.953, p = 0.146 under Thu)*, Ruminococcus sp.* (−3.3% ± 12.7%, t(3) = 3.947, p = 0.029 under Res), and *Coriobacteriaceae Gen. sp.* (−0.9% ± 94.2%, t(2) = 6.272, p = 0.024 under Oxy; −3.7% ± 90.6%, t(3) = 3.261,p = 0.047 under Pic; −39.2% ± 10.0%, t(3) = 1.726, p = 0.183 under Thu)*,* while they increased in the 24 h control (27.8% ± 80.6%, 32.2% ± 68.5%, 15.5%

± 20.8%, respectively). Regarding *Ruminococcus,* this may not be a favorable response according to recent research that points to a high proportion of long-chain dietary fibers degraders, butyrate producing bacteria such as *Ruminococcus, Eubacterium,* and *Bifidobacterium* as being part of healthy gut microbiota (Benson et al. 2003, Sayers et al. 2012, Biagi et al. 2016, Pasinetti et al. 2018). The *Ruminococcus* genus has previously been identified as one of the three taxa, besides *Bacteroides* and *Prevotella*, that define the enterotype concept, which could help in explaining variability in responders/non-responders in intervention studies (Kong et al. 2012). In regards to *Coriobacteriaceae*, it has been noted that many species that metabolize phenolics belong to this family, however, its potential health implications are still poorly understood (Tomás-Barberán et al. 2016). Nevertheless, one important aspect of this family is that all identified *S*-equol-producing bacteria, except for the genus *Lactococcus,* belong to it (Ramakrishna 2013, O'Mahony 2015).

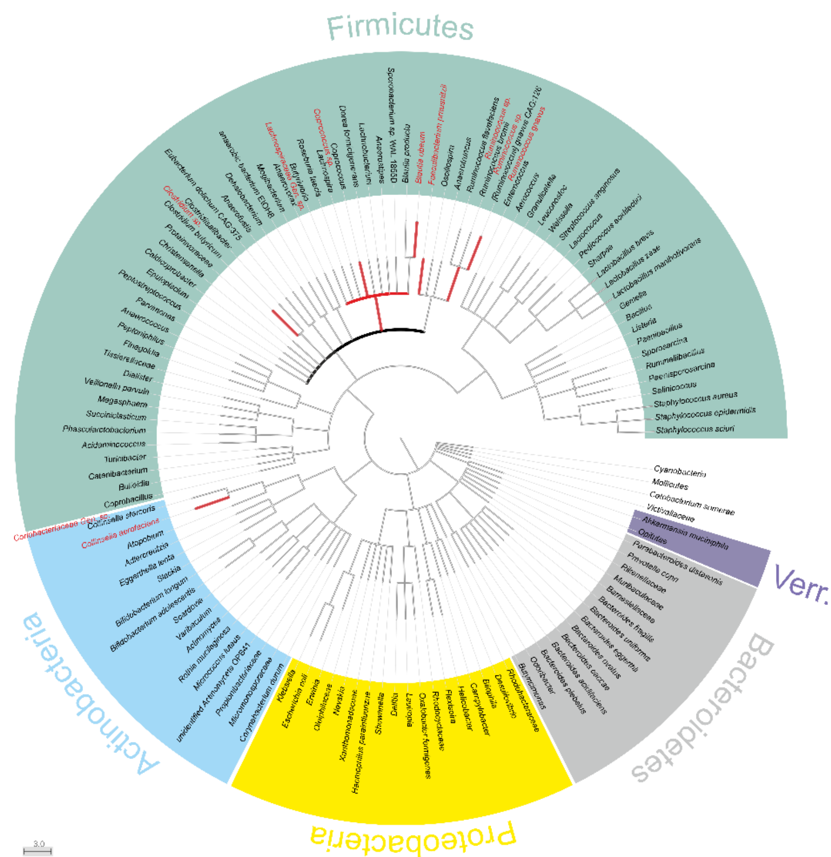

Figure 3. Phylogenetic tree of all identified bacterial entitites. The tree is sorted by phylogenetic distance, therefore the closer they are on the tree, the closer they are genetically [41,42]. Taxa shown in red displayed p < 0.075 for at least one p value (Paired sample t-test and/or Wilcoxon signed-rank test) for both comparisons 1 and 2. Bolded black line refers to family Lachnospiraceae. Verr. = Verrucomicrobia. Gen. = unnamed genus, sp. = unnamed species.

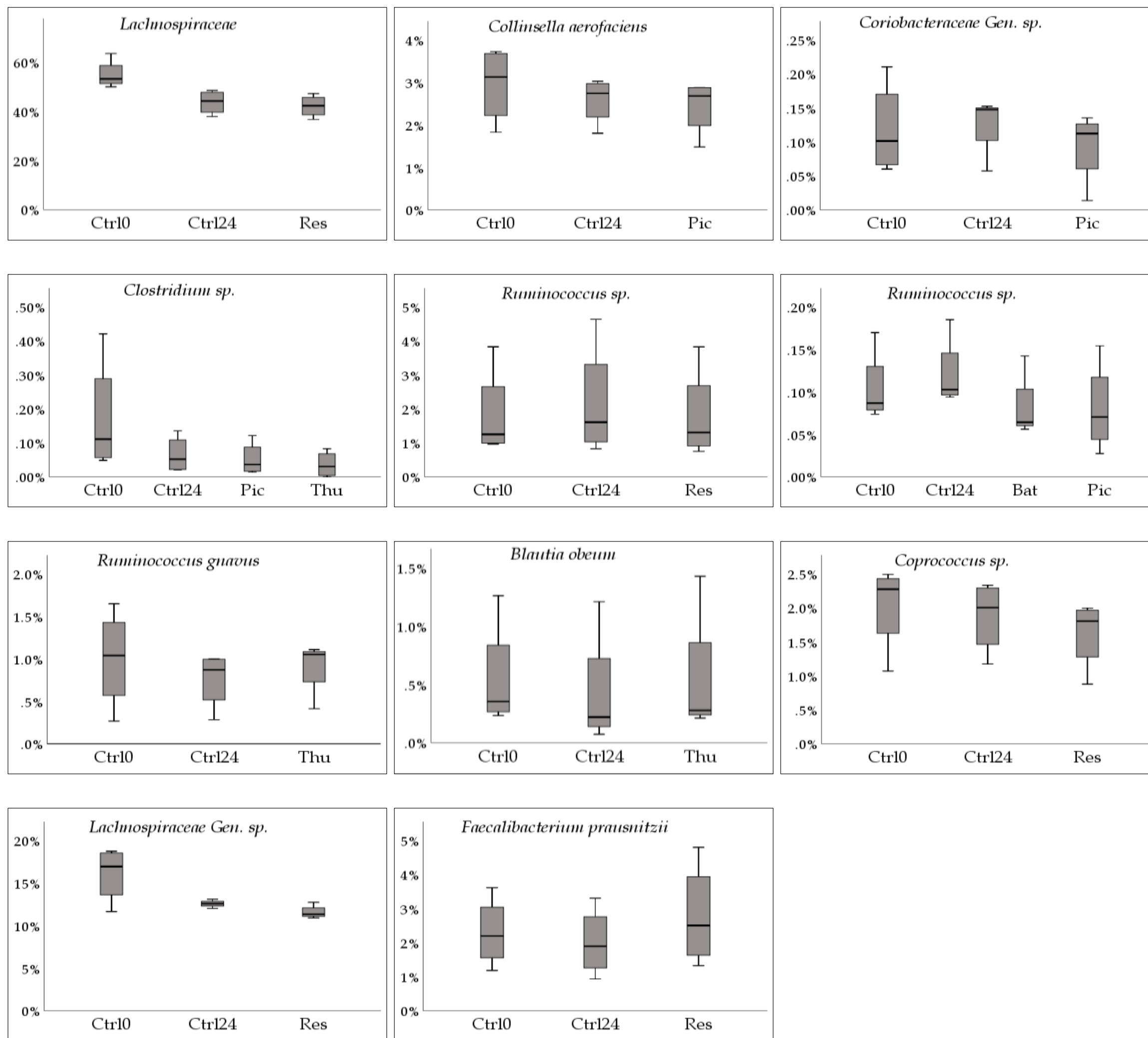

Figure 4. Box plots corresponding to the identified species and stilbenoids in Table A2. The y-axis displays relative abundance (%). The x-axis shows Ctrl0, control at 0 h; Ctrl24, control at 24 h; as well as the stilbenoid(s) corresponding to the observation (Bat, batatasin III; Oxy, oxyresveratrol; Pic, piceatannol; Pino, pinostilbene; Res, trans-resveratrol; Thu, thunalbene (all stilbenoids at 24 h). Gen. = unnamed genus, sp. = unnamed species.

A third observed response was a decrease in relative abundance while the 24 h control also decreased, but with a larger magnitude. This was observed for *Blautia obeum*, which was recently reclassified, its former name being *Ruminococcus obeum* (Uchiyama et al. 2007). *Blautia* has been considered one of the major representatives of the Firmicutes phylum due to its relatively high abundance (Cueva et al. 2017). This species experienced a decrease in relative abundance by thunalbene (−5.6% ± 32.1%, ($t(3) = 3.763$, $p = 0.033$), but at a lower magnitude than the control at 24 h (−29.8% ± 35.6%). A decrease of *Blautia,* at the genus level, was also reported in a study conducted on mice fed a phenolic-enriched tomato diet, as well as in a study of human fecal fermentation study after consumption of phenolics from tart cherries (Mayta-Apaza et al. 2018, Scarano et al. 2018). These findings, along with our study,

suggest that certain phenolics may cause a decrease in this genus, but at a lesser magnitude than without it. This taxon also appears to be a butyrate-producing microbe whose reduction has been correlated with decreased production of butyrate (Gaya et al. 2011).

Eight of the identified taxa belonged to the family *Lachnospiraceae*. There was no consistent response from the tested stilbenoids within this family however, the most frequent response was a decrease in relative abundance. This decrease was also observed in a study where rats were supplemented with the stilbenoid pterostilbene in their diet. In that study, *Lachnospiraceae* was significantly reduced in each tested group when compared to baseline levels (Etxeberria et al. 2017).

4.3.3.2 Increase in Relative Abundance

An increase in relative abundance with no change in the 24 h control was observed for *Faecalibacterium prausnitzii* under Res (36.6% ± 88.0%, $t(3) = -2.806$, $p = 0.068$ under Res), 24 h control (−0.5% ± 62.5%). This species has been previously identified as a butyrate producing bacterium and is regarded as being beneficial. Butyrate production appears to be key in maintaining the colonic epithelium by inducing proliferation of healthy colonocytes. Fiber-poor diets, such as the one our donors were subject to prior to sample donation, have been associated with low butyrate production. One study showed a strong positive correlation between the proportion of *F. prausnitzii* and that of butyrate in individuals on a normal diet, and the reduction in *F. prausnitzii* on switching to a fiber-free or fiber-supplemented diet correlated with the reduction in fecal butyrate (Summaries 2011, Gaya et al. 2016). The gut epithelium is the main body site for butyrate sequestration, and low butyrate production has been connected to inflammatory diseases such as ulcerative colitis (Jeffery et al. 2012, Morrison & Preston 2016). Unlike acetate producing bacteria, which are widely distributed, there appear to be fewer butyrate producing bacteria such as *S. prausnitzii, E. rectale, E. hallii,* and *R. bromii* (Qiao et al. 2014). It was observed to increase in plant-based, fiber-rich, diets, thus, stilbenoids being phytochemicals, were expected to increase their abundance. Our findings support this with resveratrol.

An increase in relative abundance with a decrease in the 24 h control was observed for *Ruminococcus gnavus* under Thu (8.2% ± 40.6%, $t(3) = -2.244$, $p = 0.111$ under Thu), 24 h control (−12.9% ± 30.7%). The observed p value, along with the box plot in Figure 4, show that *R. gnavus'* increase was not as pronounced as that of *F. prausnitzii*. Both of these taxa tend

to be quite reduced in inflammatory bowel diseases such as Crohn's disease (Lawson & Finegold 2015, Shortt et al. 2018).

Although it was detected in only one of our donors, *Akkermansia muciniphila* was observed to be enhanced by resveratrol. This species has been previously observed to be enhanced by pterostilbene, which has shown to exhibit similar cellular effects to resveratrol. One of these is that both phenolics have been hypothesized to mimic caloric restriction effects at the molecular level, thus modifying the gut microbiota, especially enhancing *A. muciniphila* (Etxeberria et al. 2017).

These findings emphasize the importance of trying to get to the lowest possible taxonomic level to better characterize the gut microbiota. As can be seen from our study, species within the same family level are not all uniform in their responses. Higher taxonomic levels are quite useful, and can make experiments and data processing much more manageable; however, care must be taken in generalizing for every member of a taxon.

Whether the microbiota response is a decrease or an increase in relative abundance, effects are more frequently attributed to resveratrol and piceatannol, followed by thunalbene and batatasin III. This difference may be related to their chemical moieties. All stilbenoids share a basic C6-C2-C6 structure, differing only in the presence or absence of a C-C double bond on - 2-, and on the type and position of functional groups, mainly hydroxyl (-OH) and o-methoxyl (-OCH_3) groups on the aromatic rings. In phenolics, -OH groups play an important role on their bioactivity, and their substitution by -OCH_3 groups has been shown to reduce their bioactivity (Karamać & Amarowicz 1996, Al Shukor et al. 2013, Goncalves & Romano 2017). -OH groups are good hydrogen donors, are considered very reactive and potent radical scavengers, are key in the general antioxidant mechanism of resveratrol, and it has been shown that phenolics with more -OH groups exhibit higher capacity for enzyme inhibition than those with -OCH_3 groups (Karamać & Amarowicz 1996, McMurry 1998, Iuga 2012, Buchholz & Melzig 2015, Goncalves & Romano 2017). Enzyme inhibition capacity has also been shown to be affected by hydrogenation of the C-C double bond on -C2-, which decreased enzyme inhibition (Tadera et al. 2006, Lo Piparo et al. 2008, Xiao et al. 2013, Goncalves & Romano 2017). This suggests that phenolics with -OH moieties and C-C double bond on -C2- may be more bioactive than those with -OCH_3 moieties and lacking a C-C double bond on -C2-. Resveratrol and Piceatannol have three and four -OH groups respectively, as well as a C-C double-bond on -C2-. They were the two stilbenoids that were most frequently attributed effects

on the GM in this study. These were followed by thunalbene, which is O-methylated and has a C-C double bond on -C2-, and by batatasin III, which is O-methylated and lacks a C-C double bond on -C2-. Regarding demethylation, a recent study reported a demethylated colonic metabolite of the phenolic curcumin by *Blautia sp.* MRG-PMF1 (Burapan et al. 2017). Thunalbene is O-methylated and, as reported earlier, *Blautia sp.* experienced a decrease in relative abundance under thunalbene, but at a lower magnitude than that of the control. Regarding C-C double bond reduction, Bode et al. (2013) showed that *Slackia equalifaciens* and *Adlercreutzia equolifaciens* were able to metabolize resveratrol to dihydroresveratrol by reduction of the C-C double bond, but could not identify any bacteria for the -OH cleavage that produced two other metabolites. Reduction of the C-C double bond by GM has also been shown for other phenolics such as isoflavones and hydroxycinnamates, while -OH cleavage for lignans and phenolic acids (Wang et al. 2000, Blaut et al. 2003, Jin et al. 2007, Braune & Blaut 2016). How chemical moieties affect metabolite production by microbial strains and bioactivities such as antioxidant activity, enzyme inhibition, quorum sensing, and others is outside the scope of our study; nevertheless, it's an important avenue for ongoing and future research.

The interpretation of the results from GM studies such as this one should take into consideration the concept of inter-individual variability. This concept is well known in the literature, the most well-known example being the difference between individuals whose microbiota are either producers or non-producers of the *S*-equol phytoestrogen. Oral administration of S-equol results in improvement of certain cardiovascular disease biomarkers, but only on those who are producers (Gaya et al. 2016, Tomas-Barberan et al. 2018). Although our sample size is small, differences among donor GM composition can be visualized in Appendix Figures A1 and A2. Donor D2, for example, appears to have a very atypical microbial composition when compared to the other three donors.

Table 2. Observations from previous studies regarding the effect of select stilbenoids on specific taxa compared to observations in this study [2,7,8,27,30–34,65]. From the literature, ↑ or ↓ indicate a reported abundance increase or decrease of the strain. From this study, S, NS, Un, ND, signify, respectively, supported, not supported, undefined, not detected. Gen. = unnamed genus, sp. = unnamed species.

Stilbenoid	Effect	Phylum	Family	Genus	Species		Notes
Resveratrol	↑	Actinobacteria	*Bifidobacteriaceae*	*Bifidobacterium*	*sp.*	NS	
		Firmicutes	*Clostridiaceae*	*Clostridium*	*XB90*	S	
				Faecalibacterium	*prausnitzii*	S	Won't grow without acetate in pure culture.
			Lactobacillaceae	*Lactobacillus*	*sp.*	Un.	
	↓	Bacteroidetes	*Tannerellaceae*	*Parabacteroides*	*distansonis*	NS	Only detected in one donor.

Stilbenoid	Effect	Phylum	Family	Genus	Species		Notes
		Firmicutes	*Clostridiaceae*	*Clostridium*	*aldenense*	S	Species not identified, responsesignificn at genus level.
					C9	S	
					hathewayi	S	
					MLG661	S	
			Enterococcaceae	*Enterococcus*	*faecalis*	ND	
			Gracilibacteraceae	*Gracilibacter*	*thermotolerans*	ND	
		Proteobacteria	*Enterobacteriaceae*	*Proteus*	*mirabilis*	ND	
		Firmicutes to Bacteroidetes (F/B) ratio				S	
	Other	Actinobacteria	*Coriobacteriaceae*	*Slackia*	*equolifaciens*	Other	Dihydroresveratrol producers. Identified at genus level only. Slackia's abundance highest for Res, and not detectable at Ctrl0.
				Adlercreutzia	*equolifaciens*	Other	Adlercreutzia's abundance highest for Ctrl24, and lowest for Ctrl0.
Phenolic mix, includes Resveratrol	↑	Verrucomicrobia	*Verrucomicrobiaceae*	*Akkermansia*	*muciniphila*	S	Mice study. Detected in one of our donors.
	↓	Firmicutes	*Lachnospiraceae*	*Blautia*	*sp.*	Un.	Mice study.
			Ruminococcaceae	*Oscillospira*	*sp.*	S	Mice study. Has never been cultured, but always detected.
Piceatannol	↑	Firmicutes	*Lactobacillaceae*	*Lactobacillus*	*sp.*	NS	Mice study.
			Unnamed	*Gen.*	*sp.*	NS	Mice study.
	↓	Bacteroidetes	*Unnamed*	*Gen.*	*sp.*	NS	Mice study. Decrease was observed, but at a lower magnitude than Ctrl24.
	Other		*Bacteroidaceae*	*Gen.*	*sp.*	S	Mice study. Abundance change.
Fiber	↑	Bacteroidetes	*Prevotellaceae*	*Prevotella*	*sp.*	S	Stilbenoids associated with fiber-containing food.
		Firmicutes	*Clostridiaceae*	*Faecalibacterium*	*prausnitzii*	S	Saccharolytic microbes.
			Lachnospiraceae	*Roseburia*	*sp.*	NS	Saccharolytic microbes.
Plant-based diet	↓	Proteobacteria	*Desulfovibrionaceae*	*Bilophila*	*sp.*	ND	Putrefactive microbes. Less abundance expected in a plant-based diet.
		Bacteroidetes	*Bacteroidaceae*	*Bacteroides*	*sp.*	NS	Putrefactive microbes. Less abundance expected in a plant-based diet.

4.4 Conclusion

From the surveyed literature, none of the tested stilbenoids, other than resveratrol and piceatannol, had been tested on their effect on the human GM. Our findings suggest that the tested stilbenoids, at physiological concentrations of 10 µg/mL, modulate the GM as observed in a fecal fermentation human colon model. Some of these effects are similar to other studies that have also assessed the effects of dietary phenolics on the GM. Some of our observed effects include a decrease in the Firmicutes to Bacteroidetes ratio, a consistent decrease in the relative abundance of strains from the genus *Clostridium*, and responses from several strains from the family *Lachnospiraceae*. A frequently observed effect on the identified taxa was a further decrease of the relative abundance when compared to the control. An opposite effect to the control was observed for *Faecalibacterium prausnitzii*, which, contrary to the control, increased in relative abundance. This strain has been previously considered

beneficial for health. Looking at specific stilbenoids, observed responses were more frequently attributed to resveratrol and piceatannol, followed by thunalbene and batatasin III.

The use of 16S rRNA gene sequencing, in combination with a fecal fermentation human colon model, appears to be a very useful tool to characterize the human GM, especially to identify unculturable strains. It is important to note that studies such as this one are expected to increase in precision as the sensitivity of the detection technology, as well as the taxonomical reference databases, are refined and expanded. The tested stilbenoids appear to support the well-observed view of the potential positive impact of phenolics through the modulation of human GM, and thus further studies are recommended to characterize this microbial environment and its function more precisely.

4.5 Acknowledgments

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Appendix A

Table A1. 27 least abundant species. Obtained by identifying the five species with the lowest relative abundance for Control 0 and 24 and per each of the six stilbenoids. Gen. = unnamed genus, sp. = unnamed species.

Phylum	Class	Order	Family	Genus	Species
Actinobacteria	Actinobacteria	Bifidobacteriales	*Bifidobacteriaceae*	*Bifidobacterium*	*adolescentis*
					longum
	Coriobacteriia	Coriobacteriales	*Coriobacteriaceae*	*Eggerthella*	*lenta*
				Gen.	*sp.*
Firmicutes	Bacilli	Bacillales	*Staphylococcaceae*	*Staphylococcus*	*sp.*
		Lactobacillales	*Lactobacillaceae*	*Lactobacillus*	*sp.*
			Leuconostocaceae	*Weissella*	*sp.*
			Streptococcaceae	*Lactococcus*	*sp.*
				Streptococcus	*sp.*
	Clostridia	Clostridiales	*[Mogibacteriaceae]*	*Gen.*	*sp.*
			Clostridiaceae	*Gen.*	*sp.*
			Lachnospiraceae	*[Ruminococcus]*	*gnavus*
				Blautia	*sp.*
					sp.
				Lachnospira	*sp.*
				Roseburia	*faecis*
					sp.
			Ruminococcaceae	*Ruminococcus*	*bromii*
					sp.
			Veillonellaceae	*Dialister*	*sp.*
				Phascolarctobacterium	*sp.*
				Succiniclasticum	*sp.*
	Erysipelotrichi	Erysipelotrichales	*Erysipelotrichaceae*	*[Eubacterium]*	*biforme*
				Gen.	*sp.*

Phylum	Class	Order	Family	Genus	Species
Proteobacteria	Betaproteobacteria	Burkholderiales	*Alcaligenaceae*	*Sutterella*	*sp.*
	Deltaproteobacteria	Desulfovibrionales	*Desulfovibrionaceae*	*Bilophila*	*sp.*
	Gammaproteobacteria	Enterobacteriales	*Enterobacteriaceae*	*Gen.*	*sp.*

Table A2. Taxa that displayed $p < 0.075$ for at least one p value (Paired sample t-test and/or Wilcoxon signed-rank test) for both comparisons 1 and 2. Bolded p values are those <0.05 for the t-test, and ≤0.068 for the signed-rank test. Results from pairs with $n \leq 2$ were excluded, which includes all pinostilbene samples. See materials and methods section for more details. Bat, batatasin III; Oxy, oxyresveratrol; Pic, piceatannol; Pino, pinostilbene; Res, trans-resveratrol; Thu, thunalbene. *Gen.* = unnamed genus, *sp.* = unnamed species.

Phylum	Class	Order	Family	Genus	Species	Stilbenoid	Magnitude Change from 0 h to 24 h				Rel. Abundance at 24 h	
							Mean(%) ± SD	df	Paired-T	Wilcoxon	Paired-T	Wilcoxon
Firmicutes	Clostridia	Clostridiales	*Lachnospiraceae*			Control	−20.1 ± 9.7					
						Res	−22.9 ± 10.2	3	0.025	0.068	0.045	0.068
Actinobacteria	Coriobacteriia	Coriobacteriales	*Coriobacteriaceae*	*Collinsella*	*aerofaciens*	Control	−1.0 ± 51.5					
						Pic	−6.2 ± 51.2	3	0.075	0.068	0.097	0.068
				Gen.	*sp.*	Control	27.8 ± 80.6					
						Pic	−3.7 ± 90.6	3	0.047	0.068	0.020	0.068
Firmicutes	Clostridia	Clostridiales	*Clostridiaceae*	*Clostridium*	*sp.*	Control	−54.2 ± 28.8					
						Pic	−62.9 ± 28.0	3	0.029	0.068	0.098	0.068
						Thu	−79.3 ± 22.6	3	0.030	0.068	0.043	0.068
			Lachnospiraceae	*[Ruminococcus]*	*sp.*	Control	32.2 ± 68.5					
						Bat	−3.2 ± 69.1	2	0.047	0.109	0.004	0.109
						Pic	−7.0 ± 69.4	3	0.004	0.068	0.021	0.068
					sp.	Control	15.5 ± 20.8					
						Res	−3.3 ± 12.7	3	0.029	0.068	0.110	0.068
					gnavus	Control	−12.9 ± 30.7					
						Thu	8.2 ± 40.6	3	0.111	0.068	0.057	0.068
				Blautia	*obeum*	Control	−29.8 ± 35.6					
						Thu	−5.6 ± 32.1	3	0.033	0.068	0.061	0.068
				Coprococcus	*sp.*	Control	−5.3 ± 11.9					
						Res	−19.9 ± 3.6	3	0.063	0.068	0.030	0.068
				Gen.	*sp.*	Control	−19.0 ± 18.4					
						Res	−25.5 ± 16.7	3	0.041	0.068	0.040	0.068
				Faecalibacterium	*prausnitzii*	Control	−0.5 ± 62.5					
						Res	36.6 ± 88.0	3	0.068	0.068	0.062	0.068

Table A3. Identified taxa based on Comparison 1, which used as a baseline the relative abundance of the 24 h control, and compared that to each of the six stilbenoid fermentations at 24 h. Taxa displayed Paired-T p value <0.05 and/or Wilcoxon Signed Rank p value <0.075. Bolded values are those within these ranges. Gen. = unnamed genus, sp. = unnamed species.

										Paired-T	Wilcoxon
Phylum	**Class**	**Order**	**Family**			**Stilbenoid**	**Mean(%) ± SD**	**t**	**df**	**P < 0.05**	**P < 0.075**
Actinobacteria	Actinobacteria	Actinomycetales	*Actinomycetaceae*	-		Control	0.16 ± 0.28				
						Pic	0.11 ± 0.20	1.352	3	0.269	0.068
	Coriobacteriia	Coriobacteriales	*Coriobacteriaceae*	-		Control	3.68 ± 0.93				
						Pic	3.48 ± 1.12	2.114	3	0.125	0.068
Firmicutes	Bacilli	Lactobacillales	*Aerococcaceae*	-		Control	0.04 ± 0.06				
						Res	0.02 ± 0.04	1.417	3	0.252	0.068
			Leuconostocaceae	-		Control	0.11 ± 0.19				
						Res	0.13 ± 0.21	−1.733	3	0.182	0.068
	Clostridia	Clostridiales	*Lachnospiraceae*	-		Control	43.72 ± 4.93				
						Res	42.15 ± 4.53	3.312	3	0.045	0.068
			Ruminococcaceae	-		Control	23.70 ± 7.06				
						Pic	27.30 ± 2.77	−1.606	3	0.207	0.068
						Res	25.39 ± 5.64	−2.062	3	0.131	0.068
	Unnamed	Unnamed	*Unnamed*	-		Control	0.01 ± 0.00				
						Pic	0.00 ± 0.00	4.303	3	0.023	0.068
Phylum	**Class**	**Order**	**Family**	**Genus**	**Species**	**Stilbenoid**	**Mean(%) ± SD**	**t**	**df**	**P < 0.05**	**P < 0.075**
Actinobacteria	Coriobacteriia	Coriobacteriales	*Coriobacteriaceae*	*Gen.*	*sp.*	Control	0.42 ± 0.33				
						Pic	0.48 ± 0.39	−1.746	3	0.179	0.068
						Res	0.48 ± 0.39	−1.821	3	0.166	0.068
				Adlercreutzia	*sp.*	Control	0.13 ± 0.09				
						Pic	0.09 ± 0.07	1.554	3	0.218	0.068
				Collinsella	*sp.*	Control	0.14 ± 0.07				
						Thu	0.09 ± 0.03	1.194	3	0.148	0.068
					aerofaciens	Control	2.58 ± 0.55				
						Pic	2.43 ± 0.66	2.391	3	0.097	0.068
				Gen.	*sp.*	Control	0.13 ± 0.05				
						Pic	0.09 ± 0.05	4.546	3	0.020	0.068
						Thu	0.07 ± 0.05	2.061	3	0.131	0.068
Bacteroidetes	Bacteroidia	Bacteroidales	*Bacteroidaceae*	*Bacteroides*	*sp.*	Control	0.06 ± 0.06				
						Bat	0.10 ± 0.07	−26.712	2	0.001	0.109

Phylum	Class	Order	Family	Genus	Species	Stilbenoid	Mean(%) ± SD			t	df	Paired-T P < 0.05	Wilcoxon P < 0.075
Firmicutes	Bacilli	Lactobacillales	*Aerococcaceae*	*Gen.*	*sp.*	Control	0.03	±	0.06				
						Res	0.02	±	0.03	1.430	3	0.248	0.068
	Clostridia	Clostridiales	*[Mogibacteriaceae]*	*Gen.*	*sp.*	Control	0.40	±	0.30				
						Res	0.49	±	0.35	−2.088	3	0.128	0.068
			Clostridiaceae	*Clostridium*	*sp.*	Control	0.07	±	0.05				
						Pic	0.05	±	0.05	2.378	3	0.098	0.068
						Res	0.01	±	0.01	1.808	3	0.168	0.068
						Thu	0.04	±	0.04	3.390	3	0.043	0.068
				SMB53	*sp.*	Control	0.04	±	0.01				
						Pic	0.02	±	0.02	2.222	3	0.113	0.068
						Thu	0.02	±	0.02	2.008	3	0.138	0.068
				Gen.	*sp.*	Control	0.28	±	0.05				
						Pic	0.19	±	0.08	2.926	3	0.061	0.068
			Lachnospiraceae	*[Ruminococcus]*	*sp.*	Control	0.12	±	0.04				
						Bat	0.09	±	0.05	16.420	2	0.004	0.109
						Pic	0.08	±	0.05	4.482	3	0.021	0.068
						Thu	0.06	±	0.04	2.193	3	0.116	0.068
					sp.	Control	2.17	±	1.71				
						Res	1.80	±	1.39	2.251	3	0.110	0.068
					gnavus	Control	0.76	±	0.34				
						Thu	0.91	±	0.33	-3.012	3	0.057	0.068
				Anaerostipes	*sp.*	Control	0.05	±	0.07				
						Pic	0.04	±	0.07	2.485	3	0.089	0.068
						Res	0.09	±	0.09	−2.516	3	0.086	0.068
				Blautia	*obeum*	Control	0.43	±	0.52				
						Thu	0.55	±	0.59	−2.929	3	0.061	0.068
					producta	Control	0.01	±	0.01				
						Res	0.00	±	0.00	2.185	3	0.117	0.068
				Coprococcus	*sp.*	Control	1.88	±	0.54				
						Res	1.62	±	0.52	3.895	3	0.030	0.068
				Dorea	*sp.*	Control	0.07	±	0.04				
						Res	0.07	±	0.04	-4.817	2	0.040	0.715
					formicigenerans	Control	0.36	±	0.16				
						Thu	0.49	±	0.28	−2.143	3	0.121	0.068

Phylum	Class	Order	Family	Genus	Species	Stilbenoid	Mean(%)	±	SD	t	df	Paired-T P < 0.05	Wilcoxon P < 0.075
				Lachnospira	*sp.*	Control	0.29	±	0.44				
						Pic	0.33	±	0.48	−1.723	3	0.183	0.068
				Roseburia	*sp.*	Control	0.19	±	0.11				
						Pic	0.23	±	0.09	−1.555	3	0.218	0.068
				Gen.	*sp.*	Control	12.58	±	0.44				
						Res	11.59	±	0.80	3.468	3	0.040	0.068
			Ruminococcaceae	*Faecalibacterium*	*prausnitzii*	Control	2.01	±	1.01				
						Res	2.79	±	1.53	−2.912	3	0.062	0.068
			[Mogibacteriaceae]	*Gen.*	*sp.*	Control	0.02	±	0.03				
						Res	0.49	±	0.35	−2.088	3	0.128	0.068
						Thu	0.01	±	0.02	1.431	3	0.248	0.068
	Unnamed	Unnamed	*Unnamed*	*Gen.*	*sp.*	Control	0.01	±	0.00				
						Pic	0.00	±	0.00	4.303	3	0.023	0.068
Proteobacteria	Gammaproteobacteria	Enterobacteriales	*Enterobacteriaceae*	*Gen.*	*sp.*	Control	0.01	±	0.01				
						Thu	0.00	±	0.00	1.884	3	0.156	0.068

Table A4. Identified taxa based on Comparison 2, which used as a baseline the magnitude of change (growth or decline) in relative abundance between Control 0 h and Control 24 h, and compared that to the magnitude of change between Control 0 h and each of the 6 stilbenoid fermentations. Taxa displayed Paired-T p value <0.05 and/or Wilcoxon Signed Rank p value <0.075. Bolded values are those within these ranges. Gen. = unnamed genus, sp. = unnamed species.

Phylum	Class	Order	Family		Stilbenoid	Mean(%)	±	SD	t	df	Paired-T P < 0.05	Wilcoxon P < 0.075
Actinobacteria	Coriobacteriia	Coriobacteriales	*Coriobacteriaceae*	-	Control	13.01	±	51.37				
					Pic	6.73	±	52.38	2.465	3	0.090	0.068
Firmicutes	Clostridia	Clostridiales	*Lachnospiraceae*	-	Control	−20.13	±	9.71				
					Res	−22.88	±	10.24	4.197	3	0.025	0.068
			Ruminococcaceae	-	Control	4.68	±	35.06				
					Pic	21.94	±	27.47	−1.604	3	0.207	0.068
					Res	12.17	±	28.65	−1.894	3	0.155	0.068

Phylum	Class	Order	Family	Stilbenoid		Mean(%) ± SD	t		df	Paired-T			Wilcoxon
										P < 0.05			P < 0.075
Actinobacteria	Coriobacteria	Coriobacteriales	*Coriobacteriaceae*	*Gen.*	*sp.*	Control	43.71	±	85.20				
						Pic	59.03	±	95.62	-2.563	3	0.083	0.068
						Res	58.79	±	92.21	-2.608	3	0.080	0.068
				Collinsella	*Sp.*	Control	24.32	±	63.97				
						Thu	−21.06	±	13.23	1.722	3	0.183	0.068
					aerofaciens	Control	−1.03	±	51.47				
						Pic	−6.22	±	51.19	2.685	3	0.075	0.068
				Gen.	*sp.*	Control	27.75	±	80.59				
						Oxy	−0.93	±	94.16	6.272	2	0.024	0.109
						Pic	−3.70	±	90.64	3.261	3	0.047	0.068
						Thu	−39.16	±	10.03	1.726	3	0.183	0.068
Firmicutes	Clostridia	Clostridiales	*[Mogibacteriaceae]*	*Gen.*	*sp.*	Control	72.05	±	96.46				
						Res	121.96	±	121.92	−2.783	3	0.069	0.068
			Clostridiaceae	*Clostridium*	*sp.*	Control	−54.19	±	28.78				
						Pic	−62.90	±	27.96	3.960	3	0.029	0.068
						Res	−90.28	±	15.89	1.908	3	0.152	0.068
						Thu	−79.31	±	22.65	3.901	3	0.030	0.068
				Gen.	*sp.*	Control	122.65	±	206.83				
						Pic	6.93	±	40.25	1.353	3	0.269	0.068
			Lachnospiraceae	*[Ruminococcus]*	*sp.*	Control	32.18	±	68.47				
						Bat	−3.23	±	69.11	4.448	2	0.047	0.109
						Pic	−7.02	±	69.37	8.253	3	0.004	0.068
						Thu	−41.13	±	50.91	1.953	3	0.146	0.068
					sp.	Control	15.46	±	20.76				
						Res	−3.29	±	12.72	3.947	3	0.029	0.068
					gnavus	Control	−12.89	±	30.72				
						Thu	8.24	±	40.57	−2.244	3	0.111	0.068
				Blautia	*obeum*	Control	−29.83	±	35.61				

										Paired-T			Wilcoxon
Phylum	Class	Order	Family	Stilbenoid		Mean(%) ± SD				t	df	P < 0.05	P < 0.075
						Thu	−5.56	±	32.11	−3.763	3	0.033	0.068
				Coprococcus	*sp.*	Control	−5.31	±	11.92				
						Res	−19.86	±	3.60	2.883	3	0.063	0.068
				Dorea	*sp.*	Control	16.18	±	75.27				
						Oxy	59.34	±	95.43	−9.591	2	0.011	0.109
					formicigenerans	Control	−5.41	±	27.27				
						Thu	30.22	±	52.23	−2.397	3	0.096	0.068
				Lachnospira	*sp.*	Control	69.70	±	26.12				
						Pic	128.21	±	95.39	−1.274	3	0.292	0.068
				Roseburia	*sp.*	Control	−63.94	±	38.85				
						Pic	−56.62	±	37.62	−1.597	3	0.209	0.068
				Gen.	*sp.*	Control	−19.04	±	18.36				
						Res	−25.50	±	16.67	3.433	3	0.041	0.068
			Ruminococcaceae	*Faecalibacterium*	*prausnitzii*	Control	−0.51	±	62.49				
						Res	36.58	±	87.95	−2.806	3	0.068	0.068

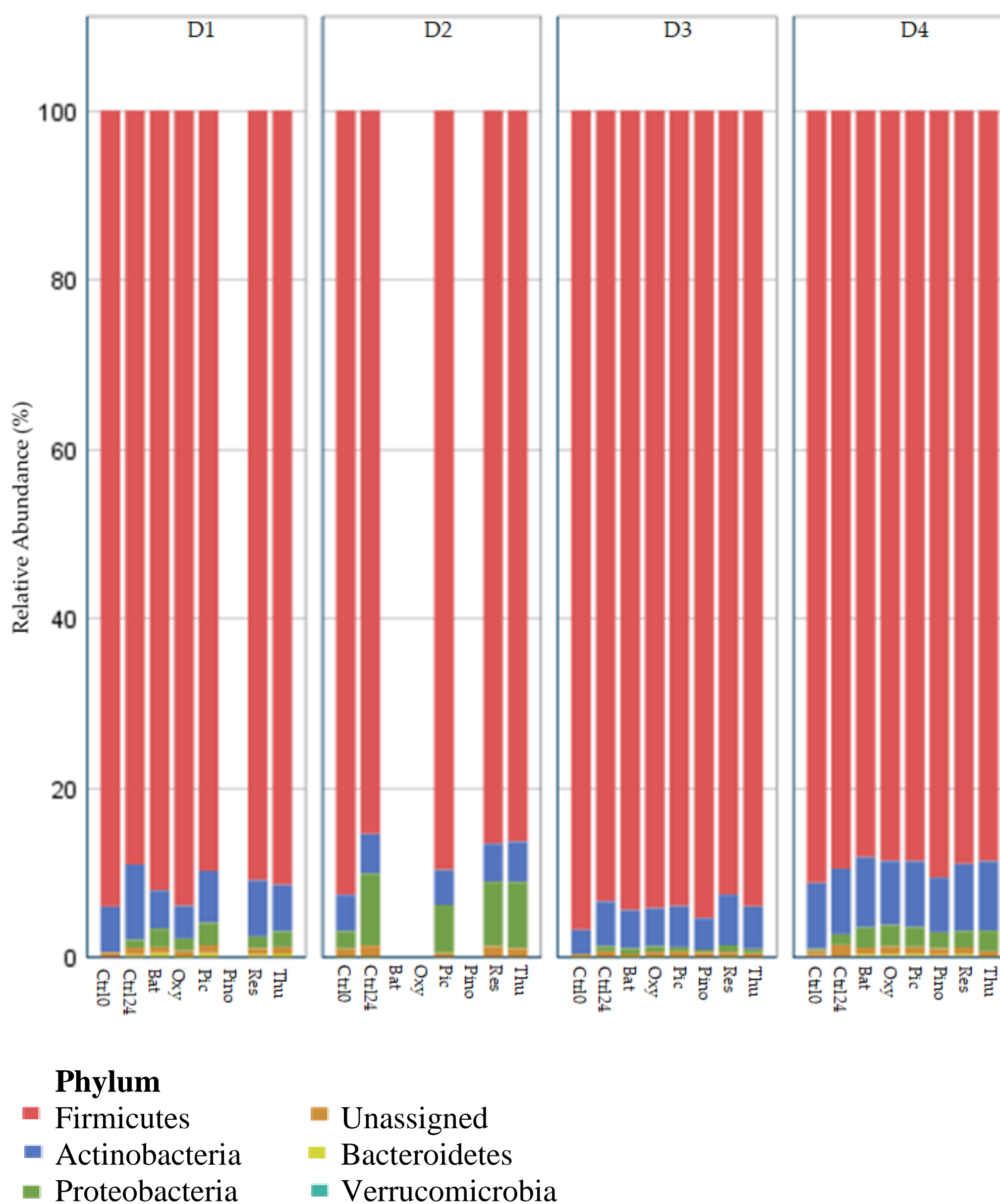


Figure A1. Bacterial composition at the phylum level, per donor. D# denotes the donor; Ctrl0, control at 0 h; Ctrl24, control at 24 h; Bat, batatasin III; Oxy, oxyresveratrol; Pic, piceatannol; Pino, pinostilbene; Res, trans-resveratrol; Thu, thunalbene (all stilbenoids at 24 h).

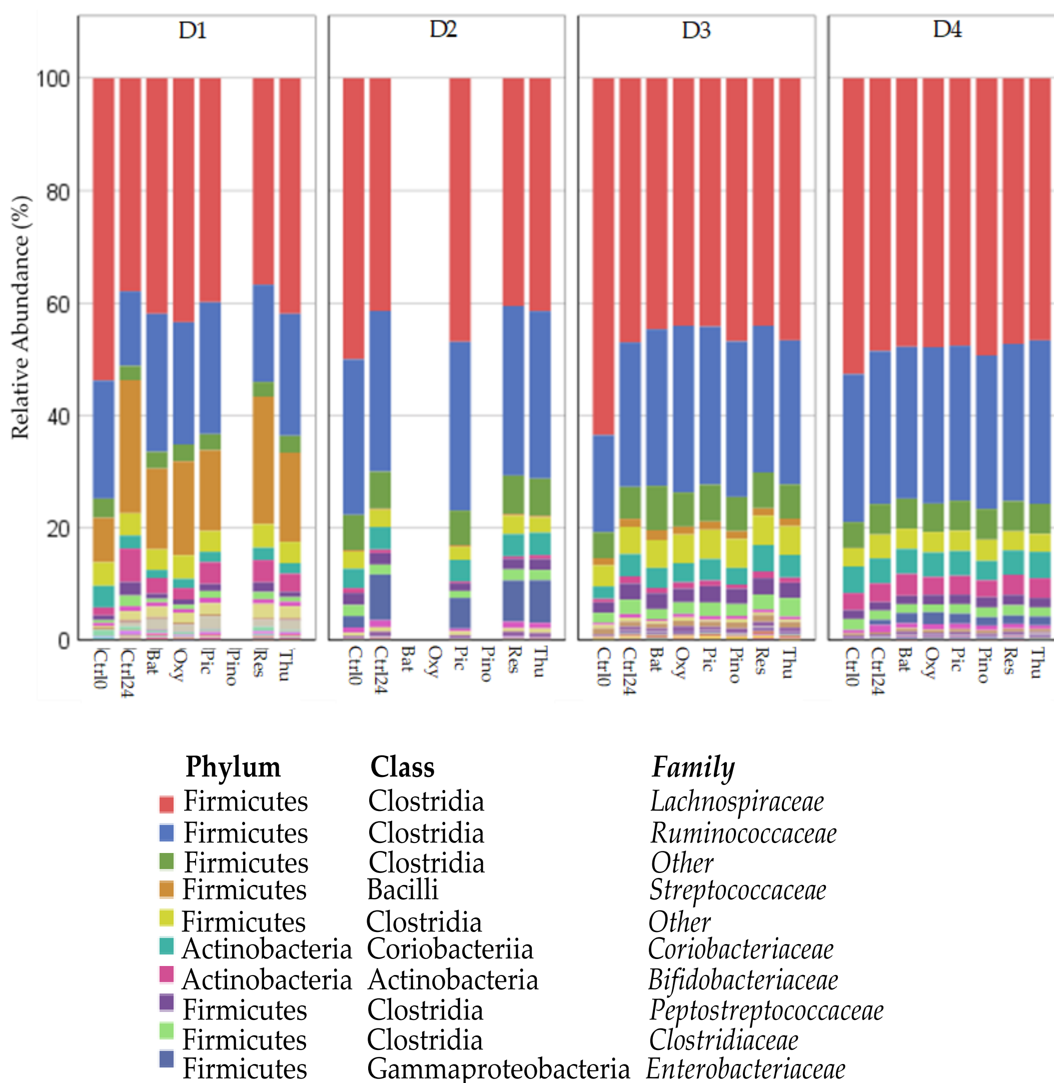


Figure A2. Bacterial composition at the family level, per donor, for the 10 most abundant taxa. D# denotes the donor; Ctrl0, control at 0 h; Ctrl24, control at 24 h; Bat, batatasin III; Oxy, oxyresveratrol; Pic, piceatannol; Pino, pinostilbene; Res, trans-resveratrol; Thu, thunalbene (all stilbenoids at 24 h).

4.6 References

Akinwumi BC, Bordun KAM, Anderson HD. 2018. Biological activities of stilbenoids. International Journal of Molecular Sciences. 19. 1–26.

Al Shukor N, Van Camp J, Gonzales GB, Staljanssens D, Struijs K, Zotti MJ, Raes K, Smagghe G. 2013. Angiotensin-converting enzyme inhibitory effects by plant phenolic compounds: A study of structure activity relationships. Journal of Agricultural and Food Chemistry. 61. 11832–11839.

Benson DA, Karsch-Mizrachi I, Lipman DJ, Ostell J, Wheeler DL. 2003. GenBank. Nucleic acids research. 31. 23–27.

Biagi E et al. 2016. Gut Microbiota and Extreme Longevity. Current Biology. 26. 1480–1485.

Bialonska D, Kasimsetty SG, Schrader KK, Ferreira D. 2009. The effect of pomegranate (punica granatum l.) byproducts and ellagitannins on the growth of human gut bacteria. Journal of Agricultural and Food Chemistry. 57. 8344–8349.

Blaut M, Schoefer L, Braune A. 2003. Transformation of Flavonoids by Intestinal Microorganisms. International Journal for Vitamin and Nutrition Research. 73, 79–87.
Bode LM, Bunzel D, Huch M, Cho GS, Ruhland D, Bunzel M, Bub A, Franz CM, Kulling SE. 2013. In vivo and in vitro metabolism of trans -resveratrol by human gut. American Journal of Clinical Nutrition. 97. 295–309.
Braune A, Blaut M. 2016. Bacterial species involved in the conversion of dietary flavonoids in the human gut. Gut Microbes. 7. 216–234.
Buchholz T, Melzig MF. 2015. Polyphenolic Compounds as Pancreatic Lipase Inhibitors. Planta Medica. 81. 771–783.
Burapan S, Kim M, Han J. 2017. Curcuminoid Demethylation as an Alternative Metabolism by Human Intestinal Microbiota. Journal of Agricultural and Food Chemistry. 65. 3305–3310.
Caporaso JG et al. 2010. Correspondence QIIME allows analysis of high- throughput community sequencing data Intensity normalization improves color calling in SOLiD sequencing. Nature. 7. 335–336.
Carrera-Quintanar L, López Roa RI, Quintero-Fabián S, Sánchez-Sánchez MA, Vizmanos B, Ortuño-Sahagún D. 2018. Phytochemicals That Influence Gut Microbiota as Prophylactics and for the Treatment of Obesity and Inflammatory Diseases. Mediators of Inflammation. 2018.
Catinean A, Neag MA, Muntean DM, Bocsan IC, Buzoianu AD. 2018. An overview on the interplay between nutraceuticals and gut microbiota. PeerJ. 6. e4465.
Cueva C, Sánchez-Patán F, Monagas M, Walton GE, Gibson GR, Martín-Álvarez PJ, Bartolomé B, Moreno-Arribas MV. 2013. In vitro fermentation of grape seed flavan-3-ol fractions by human faecal microbiota: Changes in microbial groups and phenolic metabolites. FEMS Microbiology Ecology. 83. 792–805.
Cueva C, Gil-Sánchez I, Ayuda-Durán B, González-Manzano S, González-Paramás AM, Santos-Buelga C, Bartolomé B, Victoria Moreno-Arribas M. 2017. An integrated view of the effects of wine polyphenols and their relevant metabolites on gut and host health. Molecules. 22(1). 99.
David LA et al. 2014. Diet Rapidly and Reproducibly Alters the Human Gut Microbiome. Nature. 505, 559–563.
DeSantis TZ, Hugenholtz P, Larsen N, Rojas M, Brodie EL, Keller K, Huber T, Dalevi D, Hu P, Andersen GL. 2006. Greengenes, a chimera-checked 16S rRNA gene database and workbench compatible with ARB. Applied and Environmental Microbiology. 72. 5069–5072.
Ding S, Jiang H, Fang J. 2018. Regulation of Immune Function by Polyphenols. Journal of Immunology Research. 2018, 1–8.
Dueñas M, Muñoz-González I, Cueva C, Jiménez-Girón A, Sánchez-Patán F, Santos-Buelga C, Moreno-Arribas MV, Bartolomé B. 2015. A survey of modulation of gut microbiota by dietary polyphenols. BioMed Research International. 2015.
Edgar R.C. 2010. Search and clustering orders of magnitude faster than BLAST. Bioinformatics. 26. 2460–2461.
Edwards CA, Gibson G, Champ M, Jensen B-B, Mathers JC, Nagengast F, Rumney C, Quehl A. 1996. In Vitro Method for Quantification of the Fermentation of Starch by Human Faecal Bacteria. Journal of the Science of Food and Agriculture. 71. 209–217.
Edwards CA, Havlik J, Cong W, Mullen W, Preston T, Morrison DJ, Combet E. 2017. Polyphenols and health: Interactions between fibre, plant polyphenols and the gut microbiota. Nutrition Bulletin. 42. 356–360.
Espín JC, González-Sarrías A, Tomás-Barberán FA. 2017. The gut microbiota: A key factor in the therapeutic effects of (poly)phenols. Biochemical Pharmacology. 139. 82–93.

Etxeberria U, Fernández-Quintela A, Milagro FI, Aguirre L, Martínez JA, Portillo MP. 2013. Impact of polyphenols and polyphenol-rich dietary sources on gut microbiota composition. Journal of Agricultural and Food Chemistry. 61. 9517–9533.

Etxeberria U, Arias N, Boqué N, Macarulla MT, Portillo MP, Martínez JA, Milagro FI. 2015. Reshaping faecal gut microbiota composition by the intake of trans -resveratrol and quercetin in high-fat sucrose diet-fed rats. Journal of Nutritional Biochemistry. 26. 651–660.

Etxeberria U, Hijona E, Aguirre L, Milagro FI, Bujanda L, Rimando AM, Martínez JA, Portillo MP. 2017. Pterostilbene-induced changes in gut microbiota composition in relation to obesity. Molecular Nutrition & Food Research. 61. 1500906.

Fliegerova K et al. 2014. Effect of DNA extraction and sample preservation method on rumen bacterial population. Anaerobe. 29. 80–84.

Gaya P, Medina M, Sánchez-Jiménez A., Landete J. 2016. Phytoestrogen Metabolism by Adult Human Gut Microbiota. Molecules. 21. 1034.

Giuliani C, Marzorati M, Innocenti M, Vilchez-Vargas R, Vital M, Pieper DH, Van De Wiele T, Mulinacci N. 2016. Dietary supplement based on stilbenes: A focus on gut microbial metabolism by the: In vitro simulator M-SHIME®. Food & Function. 7. 4564–4575.

González-Barrio R, Edwards CA, Crozier A. 2011. Colonic catabolism of ellagitannins, ellagic acid, and raspberry anthocyanins: In vivo and in vitro studies. Drug Metabolism & Disposition. 39. 1680–1688.

Havlik J, Edwards CA. 2018. Non-extractable Polyphenols and the Gut Microbiome. Pages 241-262 in Saura-Calixto F, Pérez-Jiménez J. Non-extractable Polyphenols and Carotenoids: Importance in Human Nutrition and Health. RSC Publishing: Cambridge, UK.

Healey GR, Murphy R, Brough L, Butts CA, Coad J. 2017. Interindividual variability in gut microbiota and host response to dietary interventions. Nutrition Reviews. 75. 1059–1080.

Hervert-Hernández D, Goñi I. 2011. Dietary polyphenols and human gut microbiota: A review. Food Reviews International. 27. 154–169.

Iuga C, Alvarez-Idaboy JR, Russo N. 2012. Antioxidant Activity of trans -Resveratrol toward Hydroxyl and Hydroperoxyl Radicals: A Quantum Chemical and Computational Kinetics Study. Journal of Organic Chemistry. 77. 3868–3877.

Jaganath IB, Mullen W, Lean MEJ, Edwards CA, Crozier A. 2009. In vitro catabolism of rutin by human fecal bacteria and the antioxidant capacity of its catabolites. Free Radical Biology and Medicine. 47. 1180–1189.

Jeffery IB, Claesson MJ, O′toole PW. 2012. Categorization of the gut microbiota: enterotypes or gradients? Grouping the microbiota of individual subjects into compositional categories, or enterotypes, based on the dominance of certain genera may have oversimplified a complex situation. Nature Reviews Microbiology. 10. 591–592.

Jin JS, Zhao YF, Nakamura N, Akao T, Kakiuchi N, Min BS, Hattori M. 2007. Enantioselective Dehydroxylation of Enterodiol and Enterolactone Precursors by Human Intestinal Bacteria. Biological and Pharmaceutical Bulletin. 30. 2113–2119.

Juretschko S, Timmermann G, Schmid M, Schleifer K, Pommerening-ro A. 1998. Combined Molecular and Conventional Analyses of Nitrifying Bacterium Diversity in Activated Sludge: Nitrosococcus mobilis and Nitrospira-Like Bacteria as Dominant Populations. Applied and Environmental Microbiology. 64. 3042–3051.

Karamać M, Amarowicz R. 1996. Inhibition of Pancreatic Lipase by Phenolic Acids-Examination in vitro. Zeitschrift für Naturforschung C. 51. 903–906.

Kemperman RA, Gross G, Mondot S, Possemiers S, Marzorati M, Van de Wiele T, Doré J, Vaughan EE. 2013. Impact of polyphenols from black tea and red wine/grape juice on a gut model microbiome. Food Research International. 53. 659–669.

Koliada A et al. 2017. Association between body mass index and Firmicutes/Bacteroidetes ratio in an adult Ukrainian population. BMC Microbiology. 17. 4–9.

Kong F, Hua Y, Zeng B, Ning R, Li Y, Zhao J. 2016. Gut microbiota signatures of longevity. Current Biology. 26. R832–R833.

Larrosa M, Yañéz-Gascón MJ, Selma MV, González-Sarrías A, Toti S, Cerón JJ, Tomás-Barberán F, Dolara P, Espín JC. 2009. Effect of a low dose of dietary resveratrol on colon microbiota, inflammation and tissue damage in a DSS-induced colitis rat model. Journal of Agricultural and Food Chemistry. 57. 2211–2220.

Lawson PA, Finegold SM. 2015. Reclassification of Ruminococcus obeum as Blautia obeum comb. nov. International Journal of Systematic and Evolutionary Microbiology. 65. 789–793.

Lo Piparo E, Scheib H, Frei N, Williamson G, Grigorov M, Chou CJ. 2008. Flavonoids for controlling starch digestion: Structural requirements for inhibiting human α-amylase. Journal of Medicinal Chemistry. 51. 3555–3561.

Marín L, Miguélez EM, Villar CJ, Lombó F. 2015. Bioavailability of dietary polyphenols and gut microbiota metabolism: Antimicrobial properties. Biomed Research International. 2015.

Mayta-Apaza AC, Pottgen E, De Bodt J, Papp N, Marasini D, Howard L, Abranko L, Van de Wiele T, Lee SO, Carbonero F. 2018. Impact of tart cherries polyphenols on the human gut microbiota and phenolic metabolites in vitro and in vivo. Journal of Nutritional Biochemistry. 59. 160–172.

McMurry J. 1998. Fundamentals of Organic Chemistry, 4th Ed., Brooks/Cole Publishing Company, Pacific Grove, CA, USA.

Milani C et al. 213. Assessing the Fecal Microbiota: An Optimized Ion Torrent 16S rRNA Gene-Based Analysis Protocol. PLoS One. 8. e68739.

Monagas M, Urpi-Sarda M, Sánchez-Patán F, Llorach R, Garrido I, Gómez-Cordovés C, Andres-Lacueva C, Bartolomé B. 2010. Insights into the metabolism and microbial biotransformation of dietary flavan-3-ols and the bioactivity of their metabolites. Food & Function. 1. 233–253.

Morrison DJ, Preston T. 2016. Formation of short chain fatty acids by the gut microbiota and their impact on human metabolism. Gut Microbes. 7. 189–200.

O'Mahony L. 2015. Host-microbiome interactions in health and disease. Clinical Liver Disease. 5. 142–144.

Ozdal T, Sela DA, Xiao J, Boyacioglu D, Chen F, Capanoglu E. 2016. The reciprocal interactions between polyphenols and gut microbiota and effects on bioaccessibility. Nutrients. 8. 78.

Pasinetti GM et al. 2018. The Role of the Gut Microbiota in the Metabolism of Polyphenols as Characterized by Gnotobiotic Mice. Journal of Alzheimer's Disease. 63(2). 409-421.

Pérez-Jiménez J, Fezeu L, Touvier M, Arnault N, Manach C, Hercberg S, Galan P, Scalbert A. 2011. Dietary intake of 337 polyphenols in French adults. American Journal of Clinical Nutrition. 93. 1220–1228.

Qiao Y, Sun J, Xia S, Tang X, Shi Y, Le G. 2014. Effects of resveratrol on gut microbiota and fat storage in a mouse model with high-fat-induced obesity. Food & Function. 5. 1241–1249.

Ramakrishna, B.S. Role of the gut microbiota in human nutrition and metabolism. J. Gastroenterol. Hepatol. **2013**, 28, 9–17.

Rowland I, Gibson G, Heinken A, Scott K, Swann J, Thiele I, Tuohy K. 2018. Gut microbiota functions: Metabolism of nutrients and other food components. European Journal of Nutrition. 57. 1–24.

Goncalves S, Romano A. 2017. Inhibitory Properties of Phenolic Compounds Against Enzymes Linked with Human Diseases. In Phenolic Compounds-Biological Activity, Marco Soto-Hernandez, Mariana Palma Tenango, R.G.-M., Ed., InTech: London, UK, Volume 2, pp. 581–770.

Sayers EW et al. 2012. Database resources of the National Center for Biotechnology Information. Nucleic Acids Research. 40. 5–15.

Scarano A, Butelli E, De Santis S, Cavalcanti E, Hill L, De Angelis M, Giovinazzo G, Chieppa M, Martin C, Santino A. 2018. Combined Dietary Anthocyanins, Flavonols, and Stilbenoids Alleviate Inflammatory Bowel Disease Symptoms in Mice. Frontiers in Nutrition. 4. 1–10.

Shortt C et al. 2018. Systematic review of the effects of the intestinal microbiota on selected nutrients and non-nutrients. European Journal of Nutrition. 57. 25–49.

Summaries S. 2011. Dysbiosis of the Faecal Microbiota in Patients with Crohn's Disease and Their Unaffected Relatives. Gut. 9. 166–168.

Tadera K, Minami Y, Takamatsu K, Matsuoka T. 2006. Inhibition of .ALPHA.-Glucosidase and α-Amylase by Flavonoids. Journal of Nutritional Science and Vitaminology. 52. 149–153.

The WHO Monica Project. 1989. A Worlwide Monitoring System for Cardiovascular Diseases: Cardiovascular Mortality & Risk Factors in Selected Communities. World Health Statistics Annual. WHO: Geneva, Switzerland. pp. 27–149.

Tomas-Barberan FA, Selma MV, Espin JC. 2016. Interactions of gut microbiota with dietary polyphenols and consequences to human health. Current Opinion in Clinical Nutrition and Metabolic Care. 19. 471–476.

Tomas-Barberan FA, Selma MV, Espin JC. 2018. Polyphenols' Gut Microbiota Metabolites: Bioactives or Biomarkers? Journal of Agricultural and Food Chemistry. 66. 3593–3594.

Turnbaugh PJ, Ley RE, Mahowald MA, Magrini V, Mardis ER, Gordon JI. 2006. An obesity-associated gut microbiome with increased capacity for energy harvest. Nature. 444. 1027–1022.

Tzounis X, Vulevic J, Kuhnle GGC, George T, Leonczak J, Gibson GR, Kwik-Uribe C, Spencer JPE. 2008. Flavanol monomer-induced changes to the human faecal microflora. British Journal of Nutrition. 99. 782–792.

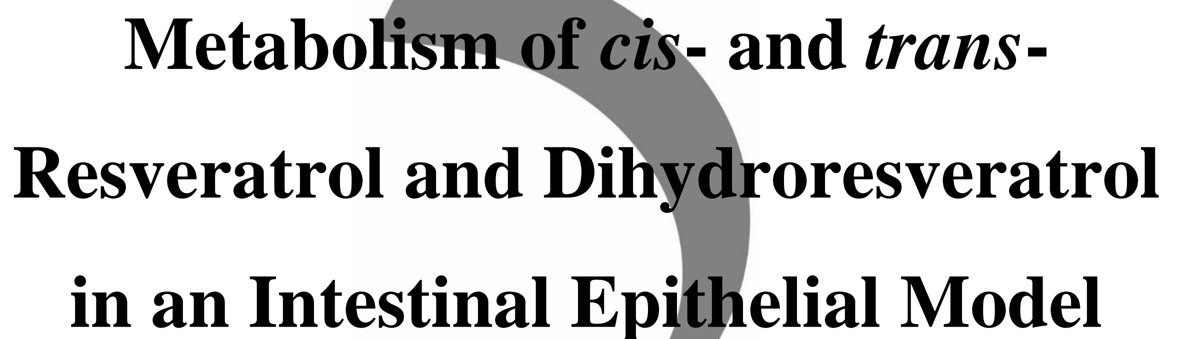
Uchiyama S, Ueno T, Suzuki T. 2007. Identification of a newly isolated equol-producing lactic acid bacterium from the human feces. Journal of Intestinal Microbiology. 21. 217–220.

van Duynhoven J et al. 2011. Metabolic fate of polyphenols in the human superorganism. Proceedings of the National Academy of Sciences. 108. 4531–4538.

Volstatova T, Marsik P, Rada V, Geigerova M, Havlik J. 2017. Effect of apple extracts and selective polyphenols on the adhesion of potential probiotic strains of Lactobacillus gasseri R and Lactobacillus casei FMP. Journal of Functional Foods. 35. 391–397.

Wang L-Q, Meselhy MR, Li Y, Qin G-W, Hattori, M. Human intestinal bacteria capable of transforming secoisolariciresinol diglucoside to mammalian lignans, enterodiol and enterolactone. Chemical and pharmaceutical bulletin. 48. 1606–1610.

Xiao J, Ni X, Kai G, Chen X. 2013. A review on structure-activity relationship of dietary polyphenols inhibiting α-amylase. Critical Reviews in Food Science and Nutrition. 53. 497–506.



5 Metabolism of *cis*- and *trans*-Resveratrol and Dihydroresveratrol in an Intestinal Epithelial Model

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Abstract

Trans-resveratrol, a well-known plant phenolic compound, has been intensively investigated due to its association with the so-called French paradox. However, despite its high pharmacological potential, *trans*-resveratrol has shown relatively low bioavailability. *Trans*-resveratrol is intensively metabolized in the intestine and liver, yielding metabolites that may be responsible for its high bioactivity. The aim of this study was to investigate and compare the metabolism of *trans*-resveratrol (tRes), *cis*-resveratrol (cRes) and dihydroresveratrol (dhRes) in an in vitro epithelial model using Caco-2 cell lines. Obtained metabolites of tRes, cRes and dhRes were analyzed by LC/MS Q-TOF, and significant differences in the metabolism of each compound were observed. The majority of tRes was transported unchanged through the Caco-2 cells, while cRes was mostly metabolized. The main metabolite of both *cis*- and *trans*-resveratrol observed as a result of colon microbial metabolism, dhRes, was metabolized almost completely, with only traces of the unchanged molecule being found. A sulphate conjugate was identified as the main metabolite of tRes in our model, while a glucuronide conjugate was the major metabolite of cRes and dhRes. Since metabolism of simple phenolics and polyphenols plays a crucial role in their bioavailability, detailed knowledge of their transformation is of high scientific value.

Keywords: Caco-2 cell lines; glucuronidation; phenolics; stilbenoids; sulphatation; UHPLC-MS-Q-TOF

5.1 Introduction

Stilbenoids are a group of plant phytoalexins occurring in various edible and medicinal plants. Currently, more than 400 derivatives of stilbene have been identified (El Khawand et al. 2018). Among these, the most well-known is *trans*-resveratrol, which is found in grapes (*Vitis vinifera* L.) and therefore red wine, peanuts (*Arachis hypiogaea* L.), and in a wide range of berries (genus *Vaccinium* L.) (Moreno et al. 2008; Berman et al. 2017). Resveratrol occurs in both *trans*- and *cis*- form; *trans*-resveratrol is believed to be the bioactive form (Moreno et al. 2008). *Trans*-resveratrol has been intensively studied due to its connection with the French paradox, where low mortality from coronary heart disease was observed despite high intake of saturated fat in a French sample population (Renaud & de Lorgeril 1992; Kopp 1998). Up to now, *trans*-resveratrol has been tested in more than 160 clinical trials, connected mostly with the treatment of diabetes mellitus, cancer, cardiovascular and neurodegenerative diseases. *Trans*-resveratrol displays great pharmacological potential, but in parallel, very low bioavailability (Walle et al. 2004). After oral administration of *trans*-resveratrol (25 mg), its plasma concentration was detected to be lower than 5 ng/mL, however, total concentration of resveratrol metabolites was as high as 491 ± 90 ng/mL (Walle et al. 2004; Wenzel & Somoza 2005).

Both *trans*- and *cis*- *r*esveratrol are intensively metabolized in the intestine and liver. Similar to most of the other polyphenols, resveratrol undergoes microbial metabolism in the colon (Jarosova et al. 2019). *Ex vivo* studies have shown that resveratrol metabolites differ among individuals, and thus far three of its metabolites have been identified in an *in vitro* faecal fermentation system: dihydroresveratrol, 3,4′-dihydroxy-*trans*-stilbene, and 3,4′-dihydroxybibenzyl (lunularin) (Bode et al. 2013; Jarosova et al. 2019). Resveratrol and its catabolites are absorbed by epithelial cells; once in the enterocyte, resveratrol is conjugated into glucuronides or sulphates and partly transported back to the intestinal lumen (Maier-Salamon et al. 2006; Juan et al. 2010b). *In vivo* and *in vitro*, using a Caco-2 cell line model, some resveratrol metabolites have been identified including resveratrol-4′-*O*-glucuronide, resveratrol 3-*O*-glucuronide, resveratrol-3-*O*-sulphate, and resveratrol-4′-*O*-sulphate, in both *cis*- and *trans*- forms (Maier-Salamon et al. 2006; Urpi-Sarda et al. 2007; Boocock et al. 2007). A part of resveratrol and its metabolites is pumped back to the lumen by efflux proteins, such as the ATP-binding cassette (ABC) transporters. Multidrug resistance-associated protein (MRP2) and breast cancer resistance protein (BCRP)

were identified as two transporters involved in the efflux of resveratrol conjugates (Juan et al. 2010b). Transport of resveratrol into the vascular endothelial cells is either by passive diffusion or a sodium-dependent glucose transporter 1 (SGLT1)-mediated pathway (Chen et al. 2013).

The high bioactivity but low bioavailability of resveratrol is often referred to as the "Resveratrol paradox," which has several potential explanations. A recent study (Chen et al. 2013) mentioned the crucial role of an intracellular resveratrol pool, which might be even more important than the serum level in vivo. It has also been suggested that metabolites of resveratrol might act as inactive reservoirs for resveratrol generation (Maier-Salamon et al. 2013). *In vivo* studies in mice (Lagouge et al. 2006) and in obese humans (Timmers et al. 2011) showed a significant biological effect of resveratrol even at low plasma concentrations 10–120 ng/mL and 231 ng/mL, respectively. These studies showed that resveratrol administration increased mitochondrial function through the activation of SIRT1 (silent information regulator) and PGC-1α (peroxisome proliferator-activated receptor γ coactivator), which in mice translated into an increase in energy expenditure, improved anaerobic capacity, enhanced sensorimotor function, and, in humans, a reduction in sleep and in the resting metabolic rate. The conjugation also leads to higher water solubility of metabolites and their easier elimination from the organism by urine. It has been shown that after oral dose of sulphate and glucuronide conjugates the amount excreted in the urine accounts for around 24% and 13%, respectively (Walle et al. 2004).

Therefore, in recent years, studies have focused more on the biological activity of resveratrol metabolites. It has been shown that some resveratrol metabolites are more cytotoxic towards tumor cells (HT-29, Caco-2, and MCF-7) compared to parental resveratrol, and less toxic towards noncancerous HEK-239 cell lines (Storniolo et al. 2014; Falomir et al. 2016). Some metabolites of resveratrol were also shown to inhibit angiogenesis, and telomerase production (Falomir et al. 2016). Piceid, a glucuronidated metabolite of *trans*-resveratrol, exhibits greater scavenging activity against hydroxyl radicals than *trans*-resveratrol *in vitro* (Su et al. 2013).

While the transport and metabolism of *trans*-resveratrol in an intestinal model is well known, sufficient data are lacking for its *cis*-isomer (present with tRes in wines, although at a lower abundance) and its main colon catabolite. This knowledge is very important to fully understand the biological activity of these compounds, as well as the bioactivity of stilbenoids in general. Therefore, we aimed to investigate the phase II metabolism of *trans*-resveratrol (tRes),

cis-resveratrol (cRes), and dihydroresveratrol (dhRes) (Figure 1) in a standard model using a Caco-2 cell line in a Transwell™ cellular system.

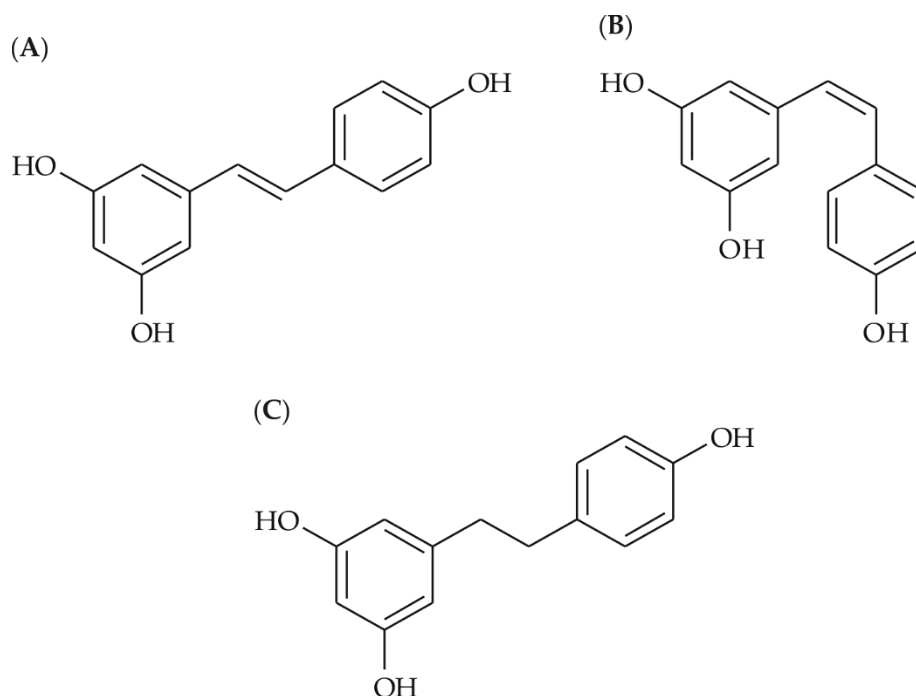

Figure 3. Structures of parent compounds (A) *trans*-resveratrol, (B) *cis*-resveratrol, (C) dihydroresveratrol.

5.2 Material and Methods

5.2.1 Preparation of Compounds

Trans-resveratrol (tRes) was obtained from Merck (Darmstadt, Germany), *cis*-resveratrol (cRes) and dihydroresveratrol (dhRes) were obtained from ChemFaces (Hubei, China), all in purity of 98%. Resveratrol-3-*O*-glucuronide and resveratrol-3-*O*-sulphate, both in purity of 95% were obtained from Cayman chemical company (Michigan, USA). All samples were diluted in DMSO, and then HBSS (Hanks' Balanced Salt solution) at the day of experiment. The final concentration of 20 μM was based on previous MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) cytotoxicity tests, published before (Jarosova et al. 2018). The concentration of DMSO in the final solution did not exceeded 2% to assure no effect on Caco-2 monolayer.

5.2.2 Cell Cultures

The human epithelial intestinal cell line Caco-2 was obtained from American Type Tissue Collection (Rockville, MD, USA), and 25th passage of Caco-2 cells was used in the present experiment. Conditions for growing and passaging of the cells were previously described here (Jarosova et al. 2018).

5.2.3 Permeability Assay

The permeability assay protocol was conducted according to Hubatsch et al. (2007).

5.2.3.1 Preparation of Inserts with Caco-2 Cells

Cells were cleaned from the medium and re-suspended in DMEM (-F12, Dulbecco's modified Eagle's medium) supplemented with 10% FBS (fetal bovine serum), 1% non-essential amino acids, 1% penicillin and streptomycin, all obtained from Sigma-Aldrich (Prague, Czech Republic) at a concentration of 0.6×10^6 cells/mL. The inserts in 24-well cell culture clusters were pre-wetted with 50 µL of medium for at least 2 min before cell seeding. The cells were applied to the apical side (Figure 2) in seeding density of 2.6×10^5 cells/cm^2. The basolateral chamber was filled with 1 mL of DMEM and incubated at 37 °C, 5% CO_2-humidified atmosphere. To remove non-adherent cells the apical medium was removed after 6 h of incubation and replaced with 0.5 mL of fresh DMEM. The medium was changed daily, firstly aspirated from the basolateral and then from the apical side, fresh DMEM was added first to the apical and then to the basolateral side. Cells were grown for 21 to 25 days to create a fully confined monolayer. Last change of the medium was carried out 16 h before the experiment.

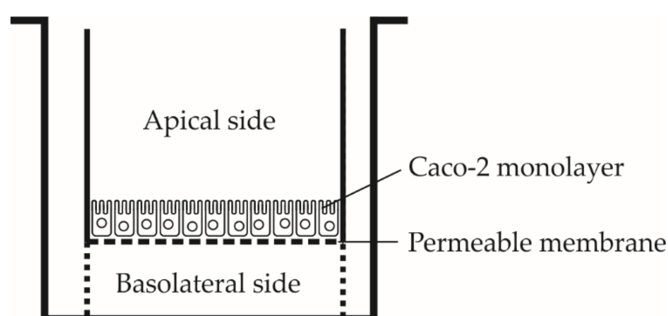

Figure 2. A schema of Transwell^{TM} cellular system.

5.2.3.2 Measuring of the Monolayer Integrity

The filter inserts with a monolayer of Caco-2 cell line were washed three times with HBSS pre-warmed to 37 °C and at pH 7.4. Transepithelial electrical resistance (TEER) was required to be at least 600 Ω, to ensure the integrity of the cellular barrier. Then, a Lucifer yellow dye at a concentration of 25 μM was added and the plates were incubated at 37 °C, and 5% CO_2 atmosphere for 1 h while shaking (150 rpm). The plates were measured in a Tecan Infinite M200 reader (Excitation/Emission wavelength 480 nm/530 nm). Only the inserts with integrity higher than 95% were used.

5.2.3.3 Metabolism and Absorption of Tested Compounds

Inserts were washed three times, and 500 μL of solution of parental compounds in a concentration of 20 μM was added to the apical side, 1000 μl of HBSS was added to the basolateral side. The samples from the apical side (50 μL) were taken immediately (time point 0 h). Plates with inserts were incubated on an orbital shaker (150 rpm) in a CO_2 incubator (37 °C, 5% CO_2-humidified atmosphere). Samples from the basolateral compartment were collected at time points 0.5, 1, 1.5, 2, 3, and 4 h, respectively. 500 μL of HBSS was removed and replaced with 500 μL of fresh HBSS. At the end, the samples from the apical side were collected and inserts were washed three times with HBSS. TEER was measured to make sure that the integrity of the cellular barrier was not broken (>500 Ω). To evaluate the intracellular contents of the tested compounds, the remaining cells on inserts were extracted with 100% methanol. All samples were stored at −80 °C until the analysis.

5.2.4 LC/MS Analysis

5.2.4.1 Standards

Standards of tRes and dhRes were kept in dry form, with exception of cRes, which was provided as a solution in ethanol, and stored at −18 °C up to one year. Due to a relative instability of standards in solvent, especially tRes and cRes, fresh stock solutions were prepared before each measurement. Calibration samples and quality control (QC) samples were prepared by diluting of stock solutions in methanol/formic acid (99/1) to make calibration series in the range of 1–1000 ng/mL, and kept at 4 °C.

5.2.4.2 Sample Purification

All samples from the permeability assay were centrifuged (5 min, 15,000× *g*; Rotanta 460R, Hettich, Germany). The samples from 0 h time point were diluted in 450 μL in methanol/formic acid (99/1) (1:9), while all other samples were diluted 1:1. Each sample was spiked with 20 μL of [$^{13}C_6$] *trans*-resveratrol solution in methanol (2 μg/mL) used as an internal standard.

5.2.4.3 LC/MS Analysis of Metabolites

Analyses were performed on a LC/MS system consisting of a UHPLC chromatograph Ultimate 3000 Thermo Fisher Scientific (Sunnyvale, CA, USA) coupled with a quadrupole time-of-flight (Q-TOF) mass spectrometer with ultra-high resolution and a high mass accuracy (HRAM) Impact II (Bruker Daltonics, Bremen, Germany) equipped with an electrospray ionization (ESI) source. Chromatography was carried out on a Kinetex 1.7 μm F5 100 Å 100 × 2.1 mm column (Phenomenex, CA, USA). Detailed description of analysis was previously published by Jarosova et al. (2019). The list of all searched and detected compounds is shown in Appendix A, Table A1. After each five sample injections the QC (50 ng/mL of each analyte in the mixture) injection was performed. The validation parameters are shown in Appendix B, Table A2. Briefly, accuracy of the LC/MS measurement was calculated from repeated injections of standard solution and was in the range of 0.12–3.15% RSD and limit of detection calculated as signal to noise ratio 3:1 was in the range of 7.1–17.4 ng/mL. For metabolites, where analytical standards were not available, abundance was expressed as intensity, referring to the peak area.

5.2.5 Statistical Analysis

Because of reduction in donor concentration on the basolateral side after every sampling, the actual concentrations at each time point were counted according to following equation:

$$C_A = \frac{\Sigma C_P}{2} + C_M$$

where C_A is the actual concentration at the time point, C_P are the previous concentrations, and C_M is the concentration measured at the time point. Values are expressed as a mean ± standard deviation. Microsoft Excel, SPSS (IBM corp., Armonk, NY, USA) version 25, and Statistica12 (StatSoft, Tulsa, OK, USA) were used for basic statistical analysis and graph creation. Quantitative data were normalized to 20 μM to correct the minor dilution

errors. The experiments for tRes and cRes were carried out in four biological replicates, dhRes in five biological replicates. Each of them was prepared in three technical repetitions.

5.3 Results

The fate of tRes, cRes, and dhRes in the intestinal model significantly differed for each test compound (Figure 3). From the initial 20 μM, tRes was mostly transported through the membrane to the basolateral side (57.2 ± 2.9%) while 22.1 ± 4.5% either remained or was pumped back to the apical side, and 20.3 ± 7.2% was transformed or metabolized. Its isomer, cRes, was transformed or metabolized by 62.1 ± 2.4%; 32.2 ± 1.7% was transported unchanged to the basolateral side; and only 5.5 ± 2.2% was detected on the apical side at the end of the experiment. On the contrary, only traces of their metabolite, dhRes, were detected on the apical or basolateral side after 4 h of incubation. Most dhRes, 99.4 ± 0.06%, was metabolized or transformed by Caco-2 cells, and only 0.6 ± 0.3% was detected unchanged on the basolateral side and 0.01 ± 0.01% on the apical side. Less than 0.5% of all the parent compounds (0.4 ± 0.1% for tRes, 0.1 ± 0.0% for cRes, dhRes not detected), accumulated in the cells.

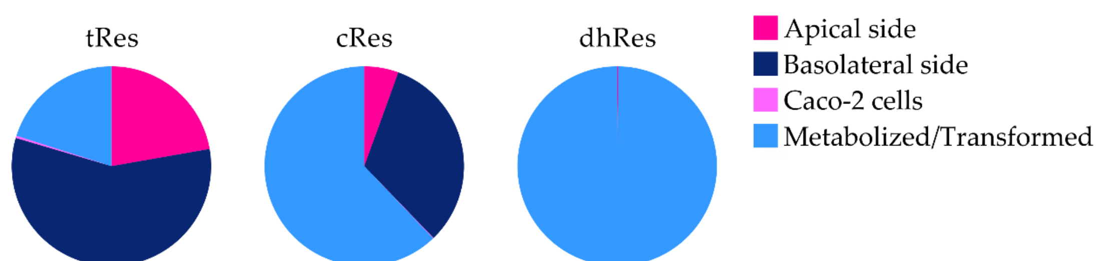

Figure 3. Fate of parent compounds in Transwell^{TM} cellular system (% mol).

As seen in Figure 4, three metabolites of tRes were detected and identified as tRes-sulphate and two tRes-glucuronides. Compared to the standards, these metabolites were identified as tRes-3-*O*-sulphate, tRes-3-*O*-glucuronide, and tRes-4′-*O*-glucuronide. Sulphate was the dominant metabolite of tRes. After 4 h of experiment, 3.96 ± 0.84 μM of tRes-3-*O*-sulphate was detected on the basolateral side, 2.75 ± 0.53 μM on the apical side, and 0.09 ± 0.02 μM in the cells, respectively. Metabolites tRes-3-*O*-glucuronide, and tRes-4′-*O*-glucuronide were detected on the basolateral side at 1.15 ± 0.15 μM and 0.39 ± 0.06 μM, respectively, and on the apical side at 0.52 ± 0.09 μM and 0.18 ± 0.05 μM, respectively. Similarly, three

metabolites, cRes-sulphate and two cRes-glucuronides, were detected for cRes. However, contrary to tRes, cRes-glucuronide seemed to be the dominant metabolite of cRes. Two metabolites, dhRes-sulphate and dominant dhRes-glucuronide, were detected for dhRes. No isomeric transformations were detected for any compound during the incubation as well as during the storage of the samples.

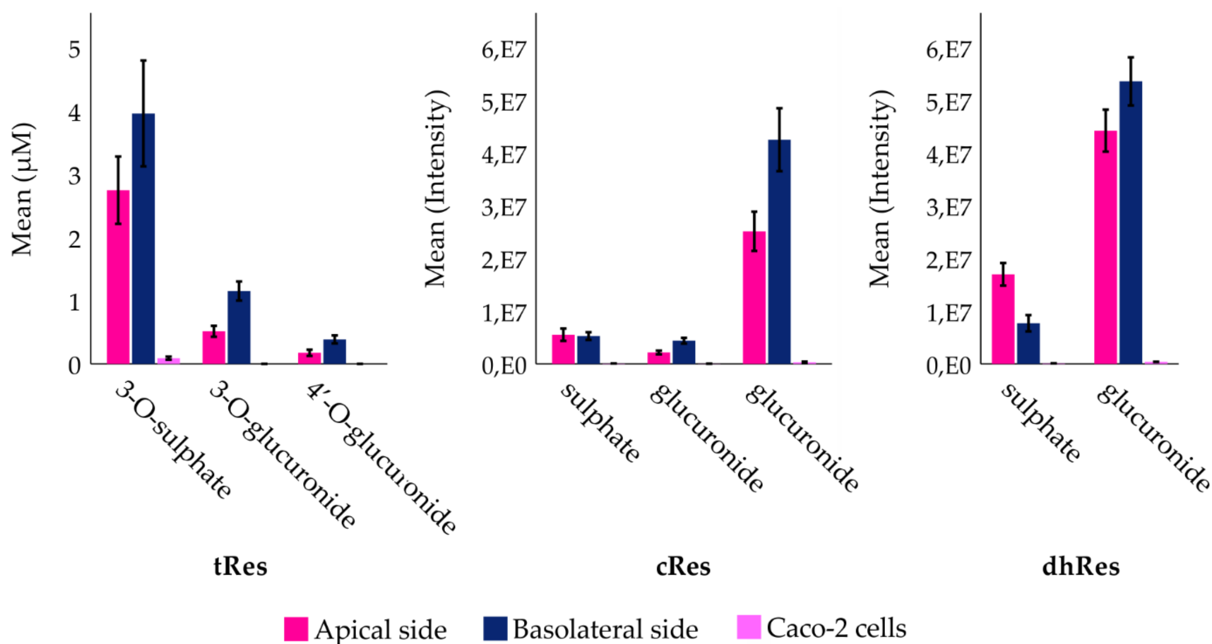

Figure 4. Metabolites observed after 4 h of incubation in Transwell^{TM} cellular system. Values obtained from LC/MS for tRes are expressed as mean concentration ± standard deviation, $n = 4$; for cRes and dhRes values are expressed as mean intensity ± standard deviation, $n = 4$ and $n = 5$, respectively. Steric positions of bonded conjugated units on cRes and dhRes cannot be specified, due to lack of confirmed standards.

The time-dependent changes of metabolism are shown in Figure 5. The concentration of tRes on the basolateral side increased continuously and slowed down after 3 h of incubation. On the contrary, the increment in concentration of its main metabolite, tRes-sulphate, sped up after 2 h of incubation. The concentration of both tRes-glucuronides rose slowly but continuously, during the entire incubation. Contrary to the pattern observed for tRes, the intensity of cRes on the basolateral side increased rapidly during the first hour of incubation and reached a plateau after 2 h of incubation. Regarding the three metabolites of cRes, they all continuously increased in intensity during the entire incubation. Only traces of unchanged dhRes were found on the basolateral side, and both of its metabolites slowed down in their intensity increase after 2 h of incubation.

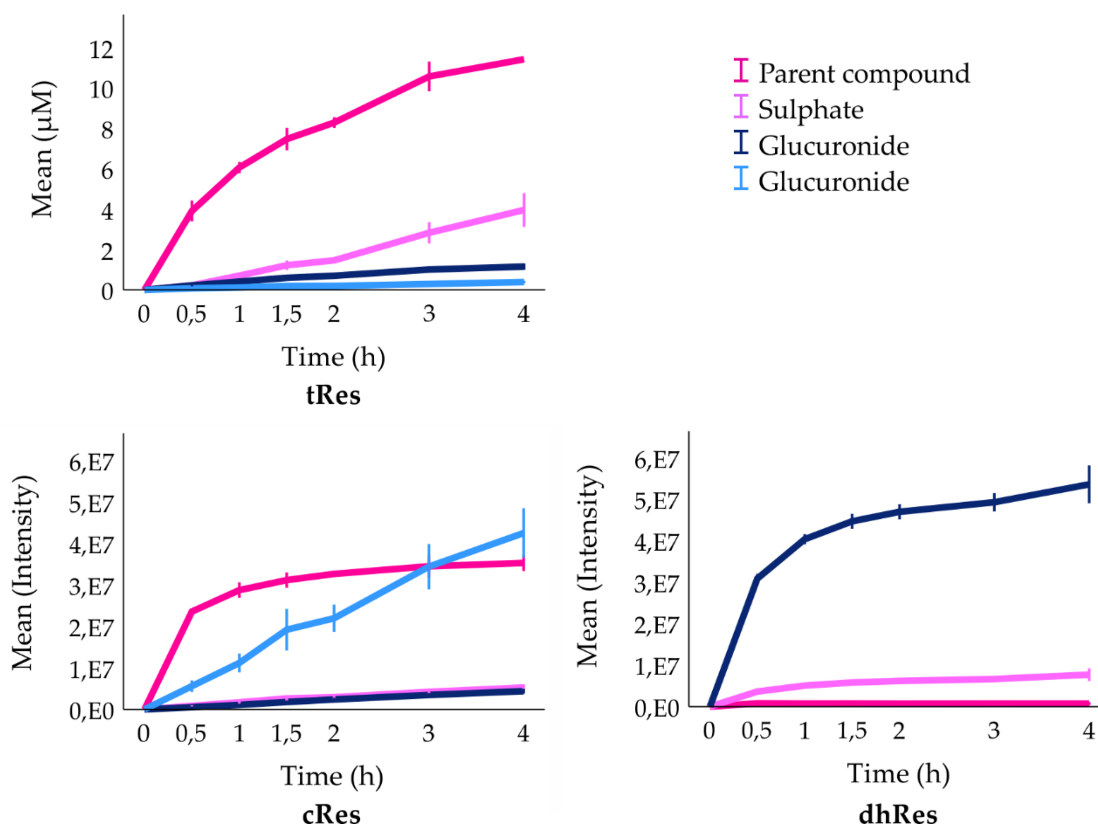

Figure 5. The changes of parent compounds and their metabolites on the basolateral side of inserts in Transwell^{TM} cellular system. Values obtained from LC/MS for tRes are expressed as mean concentration ± standard deviation, n = 4; for cRes and dhRes values are expressed as mean intensity ± standard deviation, n = 4 and n = 5, respectively (see Materials and methods).

5.4 Discussion

The metabolism of natural compounds before they reach the bloodstream plays a crucial role in their bioactivity. When reaching the colon, stilbenoids are intensively metabolized by colon microbiota and transported through the enterocytes, possibly mainly in the form of conjugates. The aim of our experiment was to find the most important metabolites and compare the metabolic fate of tRes, cRes and dhRes in the intestinal epithelium using an epithelial Caco-2 cell line model. We found major differences among each of the parent compounds.

The majority of tRes (57.2%) was transported unchanged to the basolateral side; this was shown in a similar study (Li et al. 2003), where after 4 h of incubation, 53% of unchanged tRes appeared on the basolateral side. This indicates that metabolic degradation of tRes during the intestinal absorption may not be an important factor influencing its bioavailability.

However, only trace amounts of unchanged tRes were detected *in vivo* in plasma after oral administration, as shown in studies (Walle et al. 2004; Wenzel & Somoza 2005). As described in a previous study (Jarosova et al. 2019), tRes is metabolized by gut microbiota, at different intensities per individual donors (77–11% of unchanged tRes appeared after 48 h of incubation), which together with intensive metabolism in the liver might contribute to its low plasma concentration *in vivo*. Another factor responsible for tRes low bioavailability could be its bidirectional transport through epithelial cells. After 4 h of incubation, 22.1% of unchanged tRes was found in the apical chamber. Interestingly, only traces of tRes have been detected intracellularly after 4 h. This could be explained by intensive active transport of tRes, which was previously observed and MRP2 was identified as the responsible apical efflux transporter (Henry et al. 2005). On the other hand, another study (Kaldas et al. 2003) showed completely different results by detecting high intracellular concentrations of tRes. However, the analysis of the cells was conducted after one hour of incubation, which might have, together with a slightly different extraction method, caused the divergence from our results, which were obtained after 4 h. In our study, 20.3% of tRes was metabolized or differently transformed, and three metabolites of tRes, dominant tRes-3-*O*-sulphate, tRes-3-*O*-glucuronide, and tRes-4′-*O*-glucuronide, were detected. The intensity of tRes-3-*O*-sulphate was about three times higher than the sum of both glucuronides. A similar study (Kaldas et al. 2003) of tRes detected two metabolites, tRes-monoglucuronide and tRes-monosulphate, with a trend similar to the one observed by us. In a different study using a rat small intestine model (Kuhnle et al. 2000), a glucuronide conjugate of tRes was detected as a major metabolite. An *in vivo* study in pigs also showed a tRes-glucuronide as a main metabolite in fluids and organs (Azorín-Ortuño et al. 2011). This might be caused by interspecies differences of intestinal conjugation enzymes. The concentrations of all metabolites grew during the entire incubation, and after 4 h they were also detected on the apical side of inserts. No *cis* isomers or hydrogenated metabolites were found during the passage of tRes through Caco-2 cells.

The metabolism of cRes differed significantly from its *trans* isomer. The majority of cRes was metabolized and only 32.2% passed unchanged to the basolateral side. Only 5.5% of cRes was detected on the apical side after 4 h of incubation, indicating bidirectional transport with strong predominance from the apical to the basolateral side, which is an important factor affecting its bioavailability. Similar to tRes, only traces of cRes were detected intracellularly after 4 h of incubation, indicating the efficient active transport of these compounds out of the cells. Three metabolites of cRes were detected, cRes-sulphate, and two cRes-glucuronides. In contrast

to tRes, which was mainly conjugated with a sulphate, cRes was shown to conjugate mainly with glucuronic acid. The intensity of cRes glucuronides was about seven times higher than that of sulphate. The glucuronidation of tRes and cRes by Caco-2 cell lines was demonstrated in an earlier study (Sabolovic et al. 2006) where the rate of cRes glucuronidation was up to 90-fold higher than that of tRes. Similar to tRes, no *trans* isomers or hydrogenated metabolites were found in our model.

Dihydroresveratrol is the main gut microbiota metabolite of resveratrol as was observed in our previous study (Jarosova et al. 2019). After application on Caco-2 cells, the vast majority (99.4%) of dhRes was conjugated and only traces of unchanged dhRes were found on both the apical and basolateral side of the Caco-2 cells. Two metabolites of dhRes were detected in our model, dhRes-sulphate and dominant dhRes-glucuronide. The intensity of dhRes-glucuronide was about four times higher than that of dhRes-sulphate. As mentioned earlier, in the Caco-2 cells cRes forms glucuronides at a higher rate than tRes. In the gastrointestinal tract, UDP-glucuronosyltransferases (UGT) are active in glucuronidation of tRes or cRes, and it has been shown that UGT has a greater substrate specificity towards cRes than to tRes (Sabolovic et al. 2006). The presence of a single bond in a dhRes molecule allows configuration changes that can make it more similar to either the *cis* or *trans* isomer of resveratrol. This bond arrangement allows dhRes to comply with different active site positions of UGT present in cells and it might explain its prevalent glucuronidation similar to cRes. Interestingly, dhRes-sulphate was the only metabolite detected at a higher intensity (2-fold) on the apical side than on the basolateral side and only traces of it were detected intracellularly. This indicates efficient active transport of this metabolite to the apical side, which may decrease its absorption into the blood stream and simultaneously prohibit the potentially positive effect within enterocytes. In an in vivo study in rats (Juan et al. 2010a), 30 min after oral administration of 60 mg/kg of dhRes, a glucuronide conjugate was most abundant in plasma (33.5 μM), and a sulphate conjugate was also present at lower intensities (6.4 μM). Unchanged dhRes was also detected in plasma 30 min after the oral administration at very low intensities (0.88 μM). During the passage of dhRes through the Caco-2 cells no dehydrogenated analogues were found, showing that dhRes is not a source of resveratrol.

5.5 Conclusion

In conclusion, the permeability of tRes, cRes and dhRes in a Transwell^{TM} system using Caco-2 cell lines has been explored, detecting altogether eight principal metabolites. Our results showed significant differences in the metabolism of resveratrol configurational isomers. The compounds differed in a degree of metabolism, tRes was metabolized by 20%, followed by cRes (62%) and dhRes (99%) A conjugate with sulphate was identified as the main metabolite of tRes, whilst a glucuronide was a major metabolite of cRes and of dhRes.

5.6 Acknowledgments

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Appendix A

Table A1. List of the stilbenoids monitored and detected in the samples by LC/MS.

Compound	Molecular formula	Neutral molecule exact mass:	Measured [M -H]- exact mass	Comparison with standard	Detected
trans-resveratrol	$C_{14}H_{12}O_3$	228.0786	227.0708	YES	YES
cis-resveratrol	$C_{14}H_{12}O_3$	228.0786	227.0708	YES	YES
dihydroresvaratrol	$C_{14}H_{14}O_3$	230.0943	229.0865	YES	YES
trans-resveratrol-*O*-sulphate	$C_{14}H_{12}O_6S$	308.3064	307.0276	YES	YES
cis-resveratrol-*O*-sulphate	$C_{14}H_{12}O_6S$	308.3064	307.0276	NO	YES
dihydroresvaratrol-*O*-sulphate	$C_{14}H_{14}O_6S$	310.3223	309.0433	NO	YES
trans-resveratrol-*O*-glucuronide	$C_{20}H_{20}O_9$	404.3674	403.1029	YES	YES
cis-resveratrol-*O*-glucuronide	$C_{20}H_{20}O_9$	404.3674	403.1029	NO	YES
dihydroresvaratrol-*O*-glucuronide	$C_{20}H_{22}O_9$	406.3832	405.1186	NO	YES
dihydroxymethylstilbene	$C_{15}H_{14}O_2$	226.0994	225.0916	NO	NO
methylresveratrol	$C_{15}H_{14}O_3$	242.0943	241.0865	NO	NO
dihydroxymethylstilbene	$C_{15}H_{14}O_2$	212.0837	211.0759	NO	NO
hydroxystilbene	$C_{14}H_{12}O$	196.0888	195.0810	NO	NO
stilbene	$C_{14}H_{12}$	180.0939	179.0861	NO	NO
dihydroxymethyldihydrostilbene	$C_{15}H_{16}O_2$	226.0994	225.0916	NO	NO
methyl-dihydroresveratrol	$C_{15}H_{16}O_3$	242.0943	241.0865	NO	NO
dihydroxydihydrostilbene	$C_{14}H_{14}O_2$	212.0837	211.0759	NO	NO
hydroxydihydrostilbene	$C_{14}H_{14}O$	196.0888	195.0810	NO	NO
dihydrostilbene	$C_{14}H_{14}$	180.0939	179.0861	NO	NO
phendiol	$C_6H_6O_2$	110.0368	109.0290	NO	NO

phenol	C_6H_6O	94.0419	93.0340	NO	NO
ethylenphenol	$C_8H_{10}O$	122.0732	121.0653	NO	NO
ethylphenol	$C_8H_{10}O_2$	138.0681	137.0603	NO	NO

Appendix B

Table B1. List of validation parameters

Compound	Calibration curve equation	R^2	Linear range [ng/mL]	LOD [ng/mL]	LOQ [ng/mL]	RSD [%] of injection triplicate
Res	Y=1570.9X – 11845	0.9961	5-1000	8.2	9.8	1.68
dhRes	Y=612.13X – 5494.6	0.9906	5-800	10.4	13.6	3.15
tRes-sulphate	Y=543.14X – 8201.7	0.9902	100-600	17.4	22.6	2.41
tRes-glucuronide	Y=398.29X – 2733.6	0.9961	100-1000	9.4	15.4	2.98

5.7 References

Azorín-Ortuño M, Yáñez-Gascón MJ, Vallejo F, Pallarés FJ, Larrosa M, Lucas R, Morales JC, Tomás-Barberán FA, García-Conesa MT, Espín JC. 2011. Metabolites and tissue distribution of resveratrol in the pig. Molecular Nutrition & Food Research 55:1154–1168.

Berman AY, Motechin RA, Wiesenfeld MY, Holz MK. 2017. The therapeutic potential of resveratrol: a review of clinical trials. npj Precision Oncology **1**:1–9.

Bode LM, Bunzel D, Huch M, Cho GS, Ruhland D, Bunzel M, Bub A, Franz CM, Kulling SE. 2013. In vivo and in vitro metabolism of trans-resveratrol by human gut microbiota. The American Journal of Clinical Nutrition **97**:295.

Boocock DJ et al. 2007. Phase I Dose Escalation Pharmacokinetic Study in Healthy Volunteers of Resveratrol, a Potential Cancer Chemopreventive Agent. Cancer Epidemiology Biomarkers & Prevention **16**:1246–1252.

Chen M et al. 2013. Absorption of resveratrol by vascular endothelial cells through passive diffusion and an SGLT1-mediated pathway. The Journal of Nutritional Biochemistry **24**:1823–1829.

El Khawand T, Courtois A, Valls J, Richard T, Krisa S. 2018. A review of dietary stilbenes: sources and bioavailability. Phytochemistry Reviews **17**:1007–1029.

Falomir E, Lucas R, Peñalver P, Martí-Centelles R, Dupont A, Zafra-Gómez A, Carda M, Morales JC. 2016. Cytotoxic, Antiangiogenic and Antitelomerase Activity of Glucosyl- and Acyl- Resveratrol Prodrugs and Resveratrol Sulfate Metabolites. ChemBioChem **17**:1343–1348.

Henry C, Vitrac X, Decendit A, Ennamany R, Krisa S, Mérillon JM. 2005. Cellular uptake and efflux of trans-piceid and its aglycone trans-resveratrol on the apical membrane of human intestinal Caco-2 cells. Journal of Agricultural and Food Chemistry **53**:798–803.

Hubatsch I, Ragnarsson EGE, Artursson P. 2007. Determination of drug permeability and prediction of drug absorption in Caco-2 monolayers. Nature Protocols **2**:2111–2119.

Jarosova V, Doskocil I, Volstatova T, Havlik J. 2018. Adhesive Property of Different Strains of Lactobacilli in the Presence of Resveratrol. Scientia Agriculturae Bohemica **49**:291–296.

Jarosova V, Vesely O, Marsik P, Jaimes JD, Smejkal K, Kloucek P, Havlik J. 2019. Metabolism of stilbenoids by human faecal microbiota. Molecules **24**:1155. Multidisciplinary Digital Publishing Institute.

Juan ME, Alfaras I, Planas JM. 2010a. Determination of dihydroresveratrol in rat plasma by HPLC. Journal of Agricultural and Food Chemistry **58**:7472–7475.

Juan ME, González-Pons E, Planas JM. 2010b. Multidrug Resistance Proteins Restrain the Intestinal Absorption of trans-Resveratrol in Rats. The Journal of Nutrition **140**:489–495.

Kaldas MI, Walle UK, Walle T. 2003. Resveratrol transport and metabolism by human intestinal Caco-2 cells. The Journal of pharmacy and pharmacology **55**:307–12.

Kopp P. 1998. Resveratrol, a phytoestrogen found in red wine. A possible explanation for the conundrum of the'French paradox'? European journal of endocrinology **138**:619–620.

Kuhnle G, Spencer JPE, Chowrimootoo G, Schroeter H, Debnam ES, Srai SKS, Rice-Evans C, Hahn U. 2000. Resveratrol is absorbed in the small intestine as resveratrol glucuronide. Biochemical and Biophysical Research Communications **272**:212–217.

Lagouge M et al. 2006. Resveratrol Improves Mitochondrial Function and Protects against Metabolic Disease by Activating SIRT1 and PGC-1α. Cell **127**:1109–1122.

Li Y, Shin Y, Yu C, Kosmeder J, Hirschelman W, Pezzuto J, van Breemen R. 2003. Increasing the Throughput and Productivity of Caco-2 Cell Permeability Assays Using Liquid Chromatography-Mass Spectrometry: Application to Resveratrol Absorption and Metabolism. Combinatorial Chemistry & High Throughput Screening **6**:757–767.

Maier-Salamon A, Böhmdorfer M, Riha J, Thalhammer T, Szekeres T, Jaeger W. 2013. Interplay between metabolism and transport of resveratrol. Annals of the New York Academy of Sciences **1290**:98–106.

Maier-Salamon A, Hagenauer B, Wirth M, Gabor F, Szekeres T, Jäger W. 2006. Increased transport of resveratrol across monolayers of the human intestinal Caco-2 cells is mediated by inhibition and saturation of metabolites. Pharmaceutical Research **23**:2107–2115.

Moreno A, Castro M, Falqué E. 2008. Evolution of trans- and cis-resveratrol content in red grapes (Vitis vinifera L. cv Mencía, Albarello and Merenzao) during ripening. European Food Research and Technology **227**:667–674.

Renaud S, de Lorgeril M. 1992. Wine, alcohol, platelets, and the French paradox for coronary heart disease. The Lancet **339**:1523–1526.

Sabolovic N, Humbert AC, Radominska-Pandya A, Magdalou J. 2006. Resveratrol is efficiently glucuronidated by UDP-glucuronosyltransferases in the human gastrointestinal tract and in Caco-2 cells. Biopharmaceutics and Drug Disposition **27**:181–189.

Storniolo CE, Quifer-Rada P, Lamuela-Raventos RM, Moreno JJ. 2014. Piceid presents antiproliferative effects in intestinal epithelial Caco-2 cells, effects unrelated to resveratrol release. Food Funct. **5**:2137–2144.

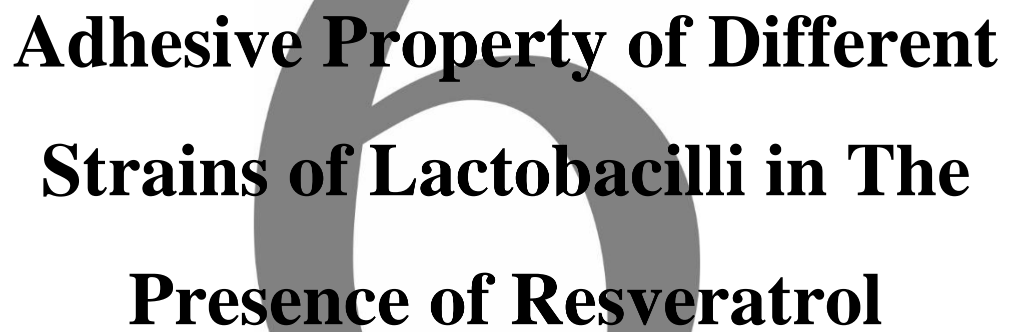
Su D, Cheng Y, Liu M, Liu D, Cui H, Zhang B, Zhou S, Yang T, Mei Q. 2013. Comparision of Piceid and Resveratrol in Antioxidation and Antiproliferation Activities In Vitro. PLoS ONE **8**:e54505. Public Library of Science.

Timmers S et al. 2011. Calorie Restriction-like Effects of 30 Days of Resveratrol Supplementation on Energy Metabolism and Metabolic Profile in Obese Humans. Cell Metabolism **14**:612–622. Cell Press.

Urpi-Sarda M, Zamora-Ros R, Lamuela-Raventos R, Cherubini A, Jauregui O, De La Torre R, Covas MI, Estruch R, Jaeger W, Andres-Lacueva C. 2007. HPLC-tandem mass spectrometric method to characterize resveratrol metabolism in humans. Clinical Chemistry **53**:292–299.

Walle T, Hsieh F, DeLegge MH, Oatis JE, Walle UK. 2004. High absorption but very low bioavailability of oral resveratrol in humans. Drug Metabolism and Disposition **32**:1377–1382.

Wenzel E, Somoza V. 2005. Metabolism and bioavailability of trans-resveratrol. Molecular Nutrition and Food Research 49:472–481.



Adhesive Property of Different Strains of Lactobacilli in The Presence of Resveratrol

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Abstract

The ability of bacteria to adhere to the intestinal epithelial cells is one of the main criteria for selection of new probiotic strains. Some dietary polyphenols have been proven to affect bacterial adhesion, providing a rationale for the use of mixtures of polyphenols and probiotics. Resveratrol, a naturally occurring stilbene in plants, has been shown to have a number of beneficial biological effects. The adhesion ability of four *Lactobacillus* strains (*Lactobacillus brevis*, *L. fermentum*, *L. gasseri*, and *L. plantarum*), in the presence of resveratrol, has been investigated in an *in vitro* model based on mixed co-culture of Caco-2 and HT29-MTX intestinal epithelial cells. The effective concentration of resveratrol, used in the adhesion experiment, has been selected based on cytotoxicity test. Resveratrol in three physiologically low concentrations (4.5, 2.25, and 1.125 μg/mL), added together with the bacterial suspension, had no statistically significant influence on the adhesion of any strain ($P < 0.05$). Since the health benefits of polyphenols are often associated with the composition of gut microbiota, the knowledge of interactions between known bacteria and polyphenols would be of high scientific value.

Keywords: bacterial adherence, Caco-2, HT29-MTX, microbiota, stilbenes, polyphenols

6.1 Introduction

Stilbenes are phenolic compounds characterized by the presence of a 1,2diphenylethylene nucleus with hydroxyls substituted on the aromatic rings (Fresco et al. 2006; Han et al. 2007). The most studied and well-known is resveratrol (3,4′,5-trihydroxystilbene), which is found in a wide range of plants including wine grape skin (*Vitis vinifera*), peanuts (*Arachis hypogaea*), and several medicinal plants (Calamini et al. 2010; Lin et al. 2011; Piotrowska et al. 2012). Historically, resveratrol from red wine was associated with the French paradox (Renaud & de Lorgeril 1992), a low incidence of cardiovascular disease despite high saturated fat intake in mediterranean diet. Currently, many studies have confirmed its antioxidant, antibacterial, antifungal, anti-atherogenic, cardioprotective, neuroprotective, and antitumor or chemopreventive activity *in vitro* (Frémont 2000; Park et al. 2001; Piotrowska et al. 2012; Biais et al. 2017). Moreover, resveratrol may have a neuroprotective effect against Alzheimer's disease due to its ability to inhibit the aggregation of amyloid-β peptide (Aβ) (Rivière et al. 2007). Currently, resveratrol is marketed as various food supplements.

Based on rodent studies, resveratrol intake has been associated with healthy body weight, visceral adipose weights, and blood glucose and lipid levels (Qiao et al. 2014; Bird et al. 2017). It has been shown to change the microbiota composition, inhibit growth of non-beneficial bacteria, and potentiate the growth of probiotic bacteria (Queipo-Ortuno et al. 2012). As the health effects of polyphenols are often associated with the composition of gut microbiota (Bustos et al. 2012; Cueva et al. 2017), any additional knowledge of interaction between known bacteria and polyphenols would be of high scientific value.

Diet has an important influence on intestinal microbiota and polyphenols are an abundant group of phenylpropanoid constituents in plant-based food. Several studies in recent years indicate the effect of polyphenols on compositional changes of intestinal microbiota *in vitro* and *in vivo* (Parkar et al. 2008; Anhê et al. 2015; Ritchie et al. 2015; Valdes et al. 2015; Volstatova et al. 2017). Parkar et al. (2008) hypothesized that one of the mechanisms could be selective influence on the adhesion of microorganisms, and he reported increased adhesion in an *in vitro* model in the presence of apple polyphenols phloridzin and rutin. Similarly, epigallocatechin and epigallocatechin gallate were shown to increase adherence of lactobacilli in Caco-2 model (Bustos et al. 2012).

The primary objective of this study was to determine the effect of resveratrol on the adhesion of four potential probiotic *Lactobacillus* strains (*Lactobacillus plantarum*, *L. gasseri*, *L. fermentum*, and *L. brevis*) in colon epithelial cell model. The secondary objective was to assess the adherence potential of the four potentially probiotic strains in a Caco-2/HT29-MTX model. To our knowledge, this is the first study focused on bacterial adhesion in this model in presence of resveratrol.

6.2 Material and Methods

6.2.1 Bacterial strains

Bacterial strains, obtained from Czech Collection of Microorganisms, were: *L. plantarum* (MILCOM 195; unknown), *L. gasseri* (DSMZ 20243; human), *L. fermentum* (CCM 91; unknown), and *L. brevis* (CCM 3805; human faeces).

6.2.2 Cell cultures

Of the human epithelial intestinal cell lines, Caco-2 cell line was obtained from the American Type Tissue Collection (Rockville, Maryland, USA), and the HT29-MTX cell line was purchased from Sigma-Aldrich (Prague, CZ). Caco-2 cells were used in experiment at passages 20-25 and the HT29-MTX cells were used at passage 35-40. Both cell lines were grown in DMEM-F12 (Dulbecco's modified Eagles medium) supplemented with 10% FBS (foetal bovine serum), 1% nonessential amino acids, 100 U/mL penicillin, and 100 μg/mL streptomycin, all obtained from Sigma-Aldrich (Prague, CZ). Cell culture bottles were kept at 37 °C in a thermostat with humidified atmosphere containing 5 % (v/v) CO_2. The medium was changed every two to three days.

6.2.3 Preparation of bacterial suspension

The lactobacilli were grown overnight in Wilkins-Chalgren anaerobe broth (Oxoid Ltd., Basingstoke, UK) at 37 °C under anaerobic conditions. Prior to the assay, bacteria were centrifuged (2000 × rpm; 10 min) and washed three times with PBS (phosphate buffer saline; Sigma–Aldrich, Prague, CZ). The bacteria were then re-suspended in PBS at concentration of 10^8 CFU/mL determined from their optical density at 600 nm (Infinite M200; Tecan Austria GmbH, Grödig, Austria).

6.2.4 Preparation of resveratrol

Resveratrol was obtained from Sigma-Aldrich (Prague, CZ) and the stock solution of concentration 10 mg/mL was prepared in DMSO (dimethylsulfoxide; Sigma–Aldrich, Prague, CZ). Prior to the adhesion experiment, working solutions of resveratrol were prepared in DMEM, without any supplement, at concentrations of 5, 2.5, and 1.25 μg/mL, and aliquots were applied to the cells. The final concentrations of resveratrol after addition of bacterial suspension have been 4.5, 2.25, and 1.125 μg/mL.

6.2.5 MTT cytotoxicity assay

The viability of the mixed co-culture Caco-2/HT29-MTX was evaluated using the MTT cytotoxicity assay. Cell lines were seeded in 96-well plates at a density of 4×10^4 cells/well and incubated for 24 h at 37 °C in a 5 % CO_2-humidified atmosphere. Two-fold serial dilution of resveratrol (0.125–256 μg) was applied for 72 h. Subsequently, the medium was replaced by MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; Sigma–Aldrich, Prague, CZ) reagent (1 mg/mL) in DMEM, and plates were incubated for an additional 2 h. The culture supernatants were then aspirated and the formazan product was dissolved in DMSO. Absorbance was measured at 555 nm using the Tecan Infinite M200 reader (Tecan Austria GmbH). The absorbance values were then plotted against cell mortality and used to determine the IC_{50} and IC_{10} (inhibition concentration) values.

6.2.6 Adhesion assays

The adhesion assay was performed according to Volstatova et al. (2017) with slight modifications. Mixed co-culture of Caco-2 and HT29-MTX cell lines was seeded in 24-well culture plate at concentration of 3.6×10^4 Caco-2 cells and 0.4×10^4 HT29-MTX cells per well. They were grown for 14 ± 1 days at 37 °C in a 5 % CO_2-humidified atmosphere in DMEM supplemented with 10% FBS, 1% nonessential amino acids, 100 U/mL penicillin, and 100 μg/mL streptomycin. The culture medium was changed every second or third day. Before the experiment, the medium was washed three times with PBS, without disrupting the monolayer. Thereafter, the monolayer was overlaid with 900 μL of resveratrol solution (5, 2.5, and 1.25 μg/mL), or medium (without supplements) as a control, and 100 μL of bacterial suspension in PBS at concentration of 10^8 CFU/mL. For each bacteria and concentration six replicates were set. The plates were incubated for 2 h at 37 °C and 5 % CO_2-atmosphere.

After the incubation, wells were gently washed three times with PBS to remove non-attached bacteria, and trypsinised by the addition of 300 µL of 1 % Triton-X100 (Sigma–Aldrich, Prague, CZ) per well for 30 s, followed by 700 µL PBS. The remaining suspensions, with viable, adhered bacteria, were diluted (*L. brevis* and *L. fermentum* 50× - 500×, *L. gasseri* and *L. plantarum* 500× - 2500×) and seeded on petri dishes with Rogosa agar (Oxoid Ltd., Basingstoke, UK). The colony-forming units (CFU) were counted after 72 h of aerobic incubation at 37 °C. The adhesion was determined as a percentage of adherent bacteria over the total bacteria added.

6.2.7 Statistical analysis

Statistical analysis for MTT tests was performed using Magellan^{TM} software (Tecan Group, Männedorf, Switzerland). Two-way analysis of variance (ANOVA; $P < 0.05$) was employed using Statistica 12 program to evaluate the statistical influence of resveratrol to bacterial adhesion. Data are expressed as mean ± standard error and the posteriori comparison was analysed by the Scheffe's test.

6.3 Results

Resveratrol exhibited high cytotoxicity towards Caco-2/HT29-MTX co-culture, as seen from IC_{50} and IC_{10} values of 13.60 ± 0.16 and 3.56 ± 0.36 µg/mL, respectively (Figure 1); these values have been used to design adhesion experiments with effective concentrations. There were significant differences in the adhesive properties of the tested strains (Figure 2). The highest ability to adhere was showed by *L. gasseri* followed by *L. plantarum*; from the initial 10^7 cells/mL applied on the monolayer, average adhesion was 18.96 and 7.26%, respectively. The other two strains, *L. brevis* and *L. fermentum*, showed very low adhesion of 1.09 and 0.36%, respectively.

Resveratrol in three physiologically low concentrations of 4.5, 2.25, and 1.125 µg/mL, added together with the bacterial suspension, had no statistically significant influence on the adhesion of any strain ($P < 0.05$).

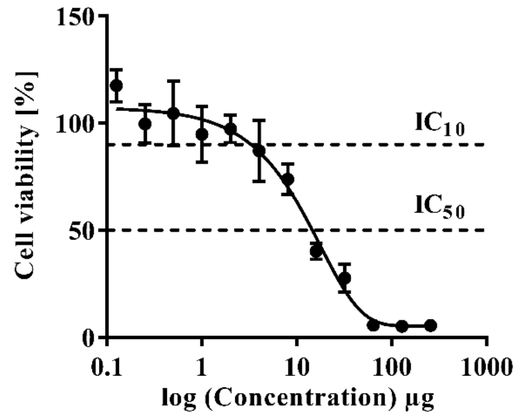

Figure 1. Dose-response cytotoxicity curve showing inhibitory concentrations IC_{10} and IC_{50} of resveratrol towards mixed co-culture of Caco-2 and HT29-MTX cell lines. Values are expressed as mean of six repetitions ± standard deviation ($P < 0.05$)

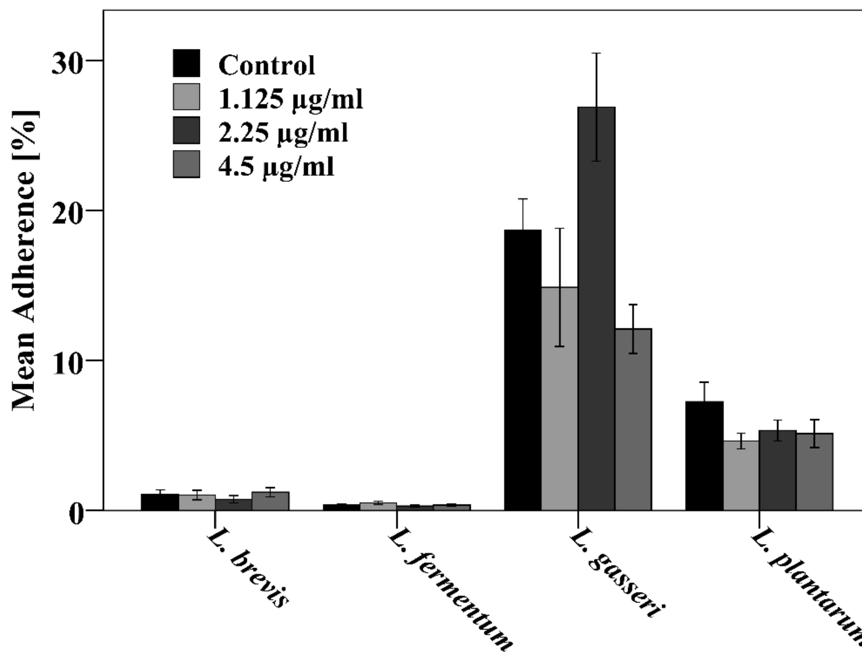

Figure 2. Adhesion of four lactobacilli to the mixed co-culture of Caco-2/HT29-MTX cell lines in the presence of three concentrations of resveratrol. Values are expressed as percentage of bacterial adherence, after a 2-hour exposition of three concentrations of resveratrol, compared to the control. Values are expressed as means ± standard error of three independent assays ($P < 0.05$)

6.4 Discussion

The ability of bacteria to adhere to the intestinal epithelial cells is one of the main criteria for selection of new probiotic strains, together with other features, such as survival in simulated gastrointestinal conditions or production of antimicrobial substances (Makinen et al. 2012). *In vitro* cell models form a widely established platform for screening of bacterial adhesion capabilities, prior to *in vivo* trials. Different cell lines, used as an *in vitro* model of human colon, include Caco-2, HT 29, T-84, and others. However, the co-culture of Caco-2/HT29-MTX, seeded in the ratio of 9:1 (Caco-2:HT29-MTX) provides an advanced model, mimicking the real ratio of Goblet cells to absorbing epithelial cells in the healthy tract (Laparra & Sanz 2009; Volstatova et al. 2015; Volstatova et al. 2017), and producing mucin. Presence of this glycoprotein results in spatially rich network of binding sites and substrate moieties for commensal microbiota (Carrière et al. 1995), similar to that in the gut.

In our study, both *L. gasseri* and *L. plantarum* showed high ability to adhere to an *in vitro* Caco-2/HT29-MTX co-culture. This is in agreement with numerous *in vivo* studies investigating the capabilities of Lactobacillus strains to colonize human intestinal mucosa after oral administration. Both strains, *L. gasseri* (Fujiwara et al. 2001) and *L. plantarum* (Johansson et al. 1993, 1998), were among the species with high colonising properties, and were consistently found in faeces after withdrawing the oral administration.

Previously, *L. gasseri* had shown moderate adhesion in an *in vitro* model based on HT-29 cell line (12.05 ± 1.34 %) (Wang et al. 2008). In the same model, *L. plantarum* and *L. brevis* showed poor adhesion ability of 6.14 ± 0.85% and 3.75 ± 0.50%, respectively. Adhesion of *L. plantarum* to Caco-2 cell line model has been shown to be either 6.7 ± 1.4% (Tuomola & Salminen 1998) or 8.51 ± 2.46% (Bianchi et al. 2004).

The adhesion of probiotic strains can be significantly affected by the presence of dietary polyphenols in their administration. Recent studies have suggested that the health benefits of polyphenols might arise from their interactions with the gut microbiota (Bustos et al. 2012; Cueva et al. 2017). It is well proven that some dietary polyphenols and/or their metabolites can stimulate the growth, proliferation, and adhesion ability of commensal bacteria and inhibit the growth and adhesion of gut pathogens (Parkar et al. 2008; Laparra & Sanz 2010).

A study by Bustos et al. (2012) showed that the effect of polyphenols is inconsistent, thereby suggesting high specificities. Most of them do not show any effect, but some seem to stimulate adhesion of selected strains to certain types of cell lines. For example, epigallocatechin increased the adhesion of *L. casei* to Caco-2 cells while procyanidins B1 and B2 increased its adhesion to HT-29 cells. The adhesion of *L. acidophilus* to Caco-2 cells has been shown to be increased by epigallocatechin gallate. This implies that the effect of dietary polyphenols to bacterial adhesion depends on the *Lactobacillus* strain and the cell line in consideration. The lack of resveratrol activity, seen in our study, might be valid for the strains used, and therefore, the effect on other strains, not included in our panel, cannot be ruled out. A recent study (Celebioglu et al. 2017) has investigated the effect of some common dietary polyphenols, including resveratrol on adhesive capacity of *L. acidophilus* toward mucin and HT-29 cells. In their model, resveratrol significantly increased adhesion of this *Lactobacillus* sp. to both mucin and HT-29 cells at 100 µg/mL, and has been proven to be one of the most effective among all polyphenols tested, owing to the possible changes in surface protein expression. However, concerns remain about the physiological relevance of the concentrations used.

6.5 Conclusion

In conclusion, our results have shown significant differences in the adhesion ability between *Lactobacillus* strains, however the main hypothesis that resveratrol increases bacterial adhesion was not confirmed. Among the tested lactobacilli, the highest adherence was shown by *L. gasseri*, followed by *L. plantarum*, thereby suggesting their potential for use in probiotic supplements. Even though resveratrol has not shown any effect on adhesion of our tested bacteria, many *in vitro* and rodent studies from last years have already shown that resveratrol intake may provide some health benefits through modulating gut microbiota and consequently contribute to the prevention of selected non-communicable diseases. The future research should be aimed at exploring this effect.

6.6 Acknowledgments

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6.7 References

Anhê FF et al. 2015. A polyphenol-rich cranberry extract protects from diet-induced obesity, insulin resistance and intestinal inflammation in association with increased Akkermansia spp. population in the gut microbiota of mice. Gut **64**:872–883.

Biais B, Krisa S, Cluzet S, Da Costa G, Waffo-Teguo P, Mérillon J-M, Richard T. 2017. Antioxidant and Cytoprotective Activities of Grapevine Stilbenes. Journal of Agricultural and Food Chemistry **65**:4952–4960.

Bianchi MA, Del Rio D, Pellegrini N, Sansebastiano G, Neviani E, Brighenti F. 2004. A fluorescence-based method for the detection of adhesive properties of lactic acid bacteria to Caco-2 cells. Letters in Applied Microbiology **39**:301–305.

Bird JK, Raederstorff D, Weber P, Steinert RE. 2017. Cardiovascular and Antiobesity Effects of Resveratrol Mediated through the Gut Microbiota. Advances in nutrition **8**:839–849.

Bustos I, Garcia-Cayuela T, Hernandez-Ledesma B, Pelaez C, Requena T, Martínez-Cuesta MC. 2012. Effect of flavan-3-ols on the adhesion of potential probiotic lactobacilli to intestinal cells. Journal of agricultural and food chemistry **60**:9082–9088.

Calamini B, Ratia K, Malkowski MG, Cuendet M, Pezzuto JM, Santarsiero BD, Mesecar AD. 2010. Pleiotropic mechanisms facilitated by resveratrol and its metabolites. The Biochemical journal **429**:273–82.

Carrière V, Lesuffleur T, Barbat A, Rousset M, Dussaulx E, Costet P, de Waziers I, Beaune P, Zweibaum A. 1995. Expression of cytochrome P-450 3A in HT29-MTX cells and caco-2 clone TC7. [FEBS Letters 355 (1994) 247-250] (PII:0014-5793(94)01199-0). FEBS Letters **362**.

Celebioglu HU, Delsoglio M, Brix S, Pessione E, Svensson B. 2017. Plant Polyphenols Stimulate Adhesion to Intestinal Mucosa and Induce Proteome Changes in the Probiotic *Lactobacillus acidophilus* NCFM. Molecular Nutrition & Food Research:1700638.

Cueva C, Gil-Sánchez I, Ayuda-Durán B, González-Manzano S, González-Paramás AM, Santos-Buelga C, Bartolomé B, Victoria Moreno-Arribas M. 2017. An integrated view of the effects of wine polyphenols and their relevant metabolites on gut and host health. Molecules **22**.

Frémont L. 2000. Biological effects of resveratrol. Life Sciences **66**:663–673.

Fresco P, Borges F, Diniz C. 2006. New insights on the anticancer properties of dietary polyphenols. Medicinal Research Reviews **26**:747–766.

Fujiwara S, Seto Y, Kimura A, Hashiba H. 2001. Establishment of orally-administered Lactobacillus gasseri SBT2055SR in the gastrointestinal tract of humans and its influence on intestinal microflora and metabolism. Journal of Applied Microbiology **90**:343–352.

Han X, Shen T, Lou H. 2007. Dietary Polyphenols and Their Biological Significance. International Journal of Molecular Sciences **8**:950–988.

Johansson ML, Molin G, Jeppsson B, Nobaek S, Ahrné S, Bengmark S. 1993. Administration of different Lactobacillus strains in fermented oatmeal soup: in vivo colonization of human intestinal mucosa and effect on the indigenous flora. Applied and environmental microbiology **59**:15–20.

Johansson ML, Nobaek S, Berggren A, Nyman M, Björck I, Ahrné S, Jeppsson B, Molin G. 1998. Survival of Lactobacillus plantarum DSM 9843 (299v), and effect on the short-chain fatty acid content of faeces after ingestion of a rose-hip drink with fermented oats. International Journal of Food Microbiology **42**:29–38.

Laparra JM, Sanz Y. 2009. Comparison of in vitro models to study bacterial adhesion to the intestinal epithelium. Letters in Applied Microbiology **49**:695–701.

Laparra JM, Sanz Y. 2010. Interactions of gut microbiota with functional food components and

nutraceuticals. Pharmacological Research **61**:219–225.
Lin HY, Tang HY, Davis FB, Davis PJ. 2011. Resveratrol and apoptosis. Annals of the New York Academy of Sciences **1215**:79–88.
Makinen K, Berger B, Bel-Rhlid R, Ananta E. 2012. Science and technology for the mastership of probiotic applications in food products. Journal of Biotechnology **162**:356–365.
Park J-W, Choi Y-J, Jang M-A, Lee Y-S, Jun DY, Suh S-I, Baek W-K, Suh M-H, Jin I-N, Kwon TK. 2001. Chemopreventive agent resveratrol, a natural product derived from grapes, reversibly inhibits progression through S and G2 phases of the cell cycle in U937 cells. Cancer Letters **163**:43–49.
Parkar SG, Stevenson DE, Skinner MA. 2008. The potential influence of fruit polyphenols on colonic microflora and human gut health. International journal of food microbiology **124**:295–298.
Piotrowska H, Kucinska M, Murias M. 2012. Biological activity of piceatannol:leaving the shadow of resveratrol. PubMed **750**:60–82.
Qiao Y, Sun J, Xia S, Tang X, Shi Y, Le G. 2014. Effects of resveratrol on gut microbiota and fat storage in a mouse model with high-fat-induced obesity. Food and Function **5**:1241–1249.
Queipo-Ortuño MI, Boto-Ordóñez M, Murri M, Gomez-Zumaquero JM, Clemente-Postigo M, Estruch R, Cardona Diaz F, Andrés-Lacueva C, Tinahones FJ. 2012. Influence of red wine polyphenols and ethanol on the gut microbiota ecology and biochemical biomarkers. American Journal of Clinical Nutrition **95**:1323–1334.
Renaud S, de Lorgeril M. 1992. Wine, alcohol, platelets, and the French paradox for coronary heart disease. The Lancet **339**:1523–1526.
Ritchie LE, Sturino JM, Carroll RJ, Rooney LW, Andrea Azcarate-Peril M, Turner ND. 2015. Polyphenol-rich sorghum brans alter colon microbiota and impact species diversity and species richness after multiple bouts of dextran sodium sulfate-induced colitis. FEMS Microbiology Ecology **91**:fiv008.
Rivière C, Richard T, Quentin L, Krisa S, Mérillon J-M, Monti J-P. 2007. Inhibitory activity of stilbenes on Alzheimer's β-amyloid fibrils in vitro. Bioorganic & Medicinal Chemistry **15**:1160–1167.
Tuomola EM, Salminen SJ. 1998. Adhesion of some probiotic and dairy Lactobacillus strains to Caco-2 cell cultures. International Journal of Food Microbiology **41**:45–51.
Valdés L, Cuervo A, Salazar N, Ruas-Madiedo P, Gueimonde M, González S. 2015. The relationship between phenolic compounds from diet and microbiota: impact on human health. Food Funct. **6**:2424–2439.
Volštátová T, Havlík J, Doskočil I, Geigerová M, Rada V. 2015. Effect of hydrolyzed milk on the adhesion of Lactobacilli to intestinal cells. Scientia Agriculturae Bohemica **46**:21–25.
Volstatova T, Marsik P, Rada V, Geigerova M, Havlik J. 2017. Effect of apple extracts and selective polyphenols on the adhesion of potential probiotic strains of Lactobacillus gasseri R and Lactobacillus casei FMP. Journal of Functional Foods **35**:391–397.
Wang B, Wei H, Yuan J, Li Q, Li Y, Li N, Li J. 2008. Identification of a surface protein from Lactobacillus reuteri JCM1081 that adheres to porcine gastric mucin and human enterocyte-like HT-29 cells. Current Microbiology **57**:33–38.

Summary discussion

The dietary intake of plant polyphenols plays an important role for human health. It has been shown that consumption of polyphenols may be protective against some non-communicable diseases. For example, resveratrol has been shown to possess high pharmacological potential to treat diabetes mellitus, cancer, cardiovascular and neurodegenerative diseases. However, its bioavailability is very low and it is often referred to as the "Resveratrol paradox". Metabolism of natural compounds while passing through the GIT plays a crucial role for their pharmacokinetics. A lot of studies do not consider the transformations of natural compounds in GIT and therefore molecules that would have never appear *in vivo* are applied in *in vitro* models (Aragonès et al., 2017).

The concentration of trans-resveratrol in the bloodstream after oral administration of 25 mg, was detected to be lower than 5 ng/mL (Walle et al., 2004). In our study we have shown that resveratrol is intensively metabolized by colon microbiota and in epithelial cell models. In agreement with other studies (Walle et al., 2004; Wang et al., 2005; Jung et al., 2009) we have determined dihydroresveratrol as main metabolite of *trans*-resveratrol. Bode et al. (2013) in a similar study described two more metabolites of *trans*-resveratrol: 3,4'-dihydroxy-*trans*-stilbene and 3,4'-dihydroxybibenzyl (lunularin). The intensity, rate, and spectrum of metabolites differed among the faecal samples obtained from different donors. The final concentrations of *trans*-resveratrol after 48 h fermentation differed among the donors from 11 ± 1% to 77 ± 1%. In the study of Bode et al. (2013) *trans*-resveratrol has been metabolized in a different rate among the faecal cultures and has been completely degraded after 2 to 24 h of fermentation, possibly due to lower initial concentration. In the epithelial model, majority (57.2 ± 2.9%) of *trans*-resveratrol has been transported unchanged to the basolateral side, similarly to study of Li et al. (2012) where 53% of *trans*-resveratrol has been transported unchanged. Therefore, the low bioavailability of *trans*-resveratrol *in vivo* is most probably not caused by its transformation in the epithelial cells during the transport but by microbial metabolism together with metabolism in liver. during the transport through Caco-2 cells *trans*-resveratrol has been metabolized to mainly *trans*-resveratrol-3-*O*-sulphate and to two glucuronides *trans*-resveratrol-3-*O*-glucuronide and *trans*-resveratrol-4'-*O*-glucuronide, as in the study of (Li et al., 2003) where sulphate was also detected as a main metabolite in Caco-2 cells. In contrast with our results, in a study using a rat small intestine and *in vivo* in pigs, *trans*-resveratrol-glucuronide was identified as the main metabolite, which might have been caused by interspecies differences of intestinal conjugation enzymes. The fate of *cis*-resveratrol differed from that one of *trans*-resveratrol and only 32.2% passed unchanged

to the basolateral side, while majority has been metabolised. The metabolites detected were *cis*-resveratrol-sulphate and two *cis*-resveratrol-glucuronides, while the intensity of glucuronide metabolites was about seven times higher than that of sulphate. In earlier study of Sabolovic et al. (2006) was shown that the rate of *cis*-resveratrol glucuronidation is up to 90-fold higher than that of *trans*-resveratrol. The main metabolite of resveratrol delivered from microbial metabolism, dihydroresveratrol, was almost completely metabolised by Caco-2 cells (99.4%), and two metabolites have been detected, dominant dihydroresveratrol-glucuronide and dihydroresveratrol-sulphate. The intensity of dihydroresveratrol-glucuronide was about four times higher than that of dihydroresveratrol-sulphate. Dihydroresveratrol-glucuronide was also detected as the most abundant metabolite of dihydroresveratrol in an *in vivo* study in rats (Juan et al., 2010).

In our study we have also shown for the first time the fate of five other stilbenes: oxyresveratrol, piceatannol, thunalbene, batatasin III and pinostilbene, in the colon microbiota model. While batatasin III and pinostilbene did not form any metabolites and seem to be stable in the colon environment, thunalbene, oxyresveratrol and piceatannol has been metabolically transformed to new products. We have detected three metabolic reactions to be ongoing in our human colon microbiota model: double bond reduction, dihydroxylation, and demethylation. Similarly to *trans*-resveratrol, oxyresveratrol formed one metabolite by double bond reduction, 2’,3,4’,5-tetrahydroxybibenzyl, which was after 24 hours of fermentation further degraded to still unknown products. Thunalbene was demethylated and formed one metabolite: isoresveratrol. Interestingly the other two tested methylated analogues, batatasin III and pinostilbene, were stable in our model, indicating the crucial role of the methoxy group position. Three metabolites of piceatannol were detected in our model: dihydropiceatannol, formed by double bond reduction and trihydroxystilbene, formed by cleavage of one hydroxyl group on the ring A, which was further dehydroxylated to 3,3’,4’-trihydroxybibenzyl. The intensity, rate, and spectrum of metabolites differed among the faecal samples obtained from different donors and further experiments with higher number of donors is needed to fully understand the fate of these stilbenoids.

Dietary polyphenols are metabolised by colon microbiota and reciprocally they can modulate the microbial composition. In our study, the influence of six stilbenoids to colon microbiota composition have been observed. One of the important indicators of healthy microbiota is Firmicutes to Bacteroidetes ratio, which, when increased, is associated with higher obesity and

occurrence of diseases (Turnbaugh et al., 2006). Previously resveratrol has been shown to decrease this ratio (Qiao et al., 2014), which is in agreement with our results. Compared to control also batatasin III and thunalbene decrease the ratio, however, pinostilbene showed an increase. While treated with all stilbenoids, decrease in relative abundance of genus *Clostridium* has been observed in our study, similarly as in the study of Etxeberria et al. (2015) after treatment of resveratrol. The presence of stilbenoids also affected the family *Lachnospiraceae*, where the response was not consistent, however, the most frequent response was a decrease in relative abundance, as well as in another study after treatment of pterostilbene, dimethylated analogue of resveratrol (Etxeberria et al., 2017). The relative abundance of *Ruminococcus sp*. and *Coriobacteriaceae Gen. sp.* decreased in a presence of some stilbenoids while the increased in the control. Interesting is an observed effect of resveratrol to increase of *Faecalibacterium prausnitzii*, a butyrate producing bacterium, which has been previously shown as an important component of healthy human microbiota. The same effect has been observed by Moreno-Indias et al. (2016) in an *in vivo* study in obese patients after consumption of red wine.

The administration of probiotics in and adequate amounts, have a positive effect on human health. One of the main criteria for new probiotic strains selection is their ability to adhere (Makinen et al., 2012). Dietary polyphenols have been shown to significantly affect the adhesion of probiotic strains. In a study of Celebioglu et al. (2018) the presence of tannic acid improved adhesion of *Lactobacillus acidophilus* to HT29 cell lines, and presence resveratrol and ferulic acid stimulated adhesion to both mucin and HT29 cell lines. The results of Volstatova et al. (2017) show significant effects of apple extracts to adhesion of *Lactobacillus gasseri* and *Lactobacillus casei*, when adhesion has been improved in presence of apple peal extracts and decreased in presence of apple pulp. Our results have not shown any effect of resveratrol on adhesion ability of four tested lactobacilli (*L. gasseri*, *L. plantarum*, *L. fermentum* and *L. brevis*), however, this might be valid only for these particular bacterial strains and cell lines, and other strains not included in this study, cannot be ruled out.

7.1 Conclusion and future perspectives

In conclusion, this thesis has shown that some stilbenoids are intensively metabolically transformed by human microbiota and during the transport through the epithelial cells, while

the others are resistant to the metabolism in colon. Reciprocally, at physiologically relevant concentrations, some stilbenoids affected the colon microbiota composition. The increase of some beneficial bacteria e.g. butyrate-producing *Faecalibacterium prausnitzii*, has been observed under stimulated conditions in the presence of some stilbenoids. Three main metabolic pathways have been observed under simulated colon conditions: double bond reduction, dihydroxylation, and demethylation. While passing through the epithelial cells the sulphate and glucuronide conjugates have been detected in different ratios for each parent compound.

In vitro models of GIT used in these studies have been shown to be useful for future research as they do not stand ethical restrictions, they are more rapid, reproductible, less expensive, and less labour intensive, compared to *in vivo* models. Therefore, these *in vitro* models can be very suitable for studies focused on pharmacokinetics of natural delivered compounds and subsequently for hypothesis building.

I hope that my doctoral thesis contributed to the understanding of metabolic transformations of polyphenols in the human gastrointestinal tract. The results of this thesis can also be useful for future research, where relevant metabolites can be included in screening assays for biological activities.

7.2 References

Aragonès G, Danesi F, Del Rio D, Mena P. 2017. The importance of studying cell metabolism when testing the bioactivity of phenolic compounds. Trends in Food Science and Technology **69**:230–242.

Bode LM, Bunzel D, Huch M, Cho GS, Ruhland D, Bunzel M, Bub A, Franz CM, Kulling SE. 2013. In vivo and in vitro metabolism of trans-resveratrol by human gut microbiota. The American Journal of Clinical Nutrition **97**:295.

Celebioglu HU, Delsoglio M, Brix S, Pessione E, Svensson B. 2018. Plant Polyphenols Stimulate Adhesion to Intestinal Mucosa and Induce Proteome Changes in the Probiotic Lactobacillus acidophilus NCFM. Molecular Nutrition & Food Research:1700638.

Etxeberria U, Arias N, Boqué N, Macarulla MT, Portillo MP, Martínez JA, Milagro FI. 2015. Reshaping faecal gut microbiota composition by the intake of trans-resveratrol and quercetin in high-fat sucrose diet-fed rats. Journal of Nutritional Biochemistry **26**:651–660. Elsevier Inc.

Etxeberria U, Hijona E, Aguirre L, Milagro FI, Bujanda L, Rimando AM, Martínez JA, Portillo MP. 2017. Pterostilbene-induced changes in gut microbiota composition in relation to obesity. Molecular Nutrition & Food Research **61**:1500906.

Juan ME, Alfaras I, Planas JM. 2010. Determination of dihydroresveratrol in rat plasma by HPLC. Journal of Agricultural and Food Chemistry **58**:7472–7475.

Jung CM, Heinze TM, Schnackenberg LK, Mullis LB, Elkins SA, Elkins CA, Steele RS, Sutherland JB. 2009. Interaction of dietary resveratrol with animal-associated bacteria. FEMS Microbiology Letters **297**:266–273.

Li Y, Shin Y, Yu C, Kosmeder J, Hirschelman W, Pezzuto J, van Breemen R. 2003. Increasing

the Throughput and Productivity of Caco-2 Cell Permeability Assays Using Liquid Chromatography-Mass Spectrometry: Application to Resveratrol Absorption and Metabolism. Combinatorial Chemistry & High Throughput Screening **6**:757–767.

Makinen K, Berger B, Bel-Rhlid R, Ananta E. 2012. Science and technology for the mastership of probiotic applications in food products. Journal of Biotechnology **162**:356–365.

Moreno-Indias I, Sánchez-Alcoholado L, Pérez-Martínez P, Andrés-Lacueva C, Cardona F, Tinahones F, Queipo-Ortuño MI. 2016. Red wine polyphenols modulate fecal microbiota and reduce markers of the metabolic syndrome in obese patients. Food and Function **7**:1775–1787.

Qiao Y, Sun J, Xia S, Tang X, Shi Y, Le G. 2014. Effects of resveratrol on gut microbiota and fat storage in a mouse model with high-fat-induced obesity. Food and Function **5**:1241–1249. The Royal Society of Chemistry.

Sabolovic N, Humbert AC, Radominska-Pandya A, Magdalou J. 2006. Resveratrol is efficiently glucuronidated by UDP-glucuronosyltransferases in the human gastrointestinal tract and in Caco-2 cells. Biopharmaceutics and Drug Disposition **27**:181–189.

Turnbaugh PJ, Ley RE, Mahowald MA, Magrini V, Mardis ER, Gordon JI. 2006. An obesity-associated gut microbiome with increased capacity for energy harvest. Nature **444**:1027–1031.

Volstatova T, Marsik P, Rada V, Geigerova M, Havlik J. 2017. Effect of apple extracts and selective polyphenols on the adhesion of potential probiotic strains of Lactobacillus gasseri R and Lactobacillus casei FMP. Journal of Functional Foods **35**:391–397.

Walle T, Hsieh F, DeLegge MH, Oatis JE, Walle UK. 2004. High absorption but very low bioavailability of oral resveratrol in humans. Drug Metabolism and Disposition **32**:1377–1382.

Wang D, Hang T, Wu C, Liu W. 2005. Identification of the major metabolites of resveratrol in rat urine by HPLC-MS/MS. Journal of Chromatography B: Analytical Technologies in the Biomedical and Life Sciences **829**:97–106.