Universidad de la Sabana

Doctorado en Biociencias

Noviembre de 2019

Study on the Interaction Between Diet, Quercetin and Intestinal Microbiota

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Acknowledgements

I am deeply thankful to the following people because without their care and help this work wouldn't have been possible

To my adviser, Federico Rey, for always listening to me and being so generous and supportive

To my coadviser, Alejandro Acosta, for always believing in me and pushing me to keep going

To my daughter Eva for her love and understanding of every sacrifice

To my parents for their love and unconditional support

To my partner, Alejandro Caro, for his constant advice and his vision of the full potential of this work

And to the PhD Program in Biosciences Committee for their constant support and understanding

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Abstract

Flavonoids are secondary metabolites of plants which have anti-cancer, anti-inflammatory, and antioxidant properties. However, the intestinal microbiota can change the bioactivity and bioavailability of these compounds, which may trigger different levels of response to a treatment. In order to expand our understanding of the capacity of the gut microbiota to modify these therapeutic compounds, we explored the microbial degradation of quercetin, one the most abundant flavonoids in the human diet. First, we revealed that a non-quercetin degrader (*Bacteroides thetaiotaomicron*) can provide, via cross-feeding, substrates to a quercetin-degrader (*Eubacterium ramulus*) for the cometabolization of the flavonoid. Second, through a metataxonomic analysis of fecal communities exposed to the flavonoid, we detected two variants related to the quercetin degrader, *Flavonifractor plautii*, that presented a negative correlation in their relative abundances upon incubation with quercetin. Lastly, a bioinformatic analysis of the genome of the closest relatives of these variants showed that they are discordant for the catabolism of an important substrate in the gastrointestinal tract, ethanolamine, which it is formed from bacterial and intestinal cell membranes and is abundant even in the absence of dietary compounds due to the constant washing away of these cells in the intestinal mucus. Overall, these observations indicate that flavonoid-degrading bacteria can be differentially affected by dietary and host's substrates and interactions with different microbial species. Thus, the community structure and metabolic capacity of each individual's gut microbiota may impact the health-related effects of these compounds.

Resumen

Los flavonoides son metabolitos secundarios de plantas que tienen propiedades anticancerígenas, antiinflamatorias y antioxidantes. Sin embargo, la microbiota intestinal puede cambiar la bioactividad y la biodisponibilidad de estos compuestos, lo que puede desencadenar diferentes niveles de respuesta a un tratamiento. Con el fin de ampliar nuestra comprensión de la capacidad de la microbiota intestinal para modificar estos compuestos terapéuticas, exploramos la degradación microbiana de la quercetina, uno de los flavonoides más abundantes en la dieta humana. Primero, demostramos que un no degradador de quercetina (*Bacteroides thetaiotaomicron*) puede proporcionar, mediante alimentación cruzada, sustratos a un degradador quercetina (*Eubacterium ramulus*) para la cometabolización del flavonoide. Segundo, a través de un análisis metataxonómico de comunidades fecales expuestas al flavonoide, detectamos dos variantes del degradador de quercetina, *Flavonifractor plautii*, quienes presentaban una correlación negativa en sus abundancias relativas tras la incubación con quercetina. Por último, un análisis bioinformático del genoma de los parientes más cercanos de estas variantes mostró que son discordantes para el catabolismo de un sustrato importante en el tracto gastrointestinal, la etanolamina, que se forma a partir de las membranas celulares bacterianas e intestinales y es abundante incluso en la ausencia de compuestos dietarios debido al lavado constante de estas células en el moco intestinal. En general, estas observaciones indican que las bacterias que degradan flavonoides pueden verse afectadas de manera diferente por los sustratos dietarios y del huésped junto a las interacciones con diferentes especies microbianas. Por lo tanto, la estructura de las comunidades y las capacidades metabólicas de la microbiota intestinal de cada individuo podría influenciar los efectos relacionados con la salud de estos compuestos.

I. Introduction

The concept of the human microbiota, as first described by Joshua Lederberg, is defined as "the ecological community of commensal, symbiotic, and pathogenic microorganisms that literally share our body space" (1). The gut microbiome of healthy individuals varies significantly and only dominant bacterial phyla have been consistently described, these are Firmicutes, Bacteroidetes and Actinobacteria, with Proteobacteria and Verrucomicrobia also present in lower abundance.

The gut microbiota plays a very important role in host metabolism. Among the mechanisms in which the microbiota is involved, an important one is the modulation of inflammatory pathways in the body. For instance, butyrate produced by some intestinal bacteria has anti-inflammatory effects, mainly by the suppression of the pro-inflammatory pathway of Nuclear Factor kappa beta (NF-κβ) (2). There are also probiotic strains that can reduce oxidative stress levels, among these, *Lactobacillus curvatus* HY7601, *L. plantarum* KY1032, and *L. fermentum* ME-3 stand out (3–5). Oxidative radicals are normally produced in high concentrations during food digestion and are also generated during cigarette smoking and exposure to contaminants, if the number of oxidative radicals surpasses the body's capacity to neutralize them, these radicals start to accumulate and exert cellular damage (6). Oxidative stress also increases the activity of the PI 3-kinase and the myosin light chain kinase promoter that regulate the opening of the intestinal tight junction barrier. Thus, oxidative stress mediates the enlargement of the spaces in the gut epithelium allowing the translocation of normally non-invasive bacteria or their toxic products and components (e.g. Lipopolysaccharides [LPS]), that induce the activation of NF-κβ, perpetuating a vicious cycle of NF-κβ activation and impairment of the tight junction barrier (7,8). The activation of NFκβ in parts of the body different from the gastrointestinal tract also elicits an inflammatory response that can also alter the permeability of the intestinal epithelium, facilitating the translocation of luminal materials which will exacerbate the inflammation state.

An approach that can counteract these harmful effects is the supplementation of dietary compounds like dietary fiber and phenolic compounds. Non-digestible fiber promotes the growth of beneficial microorganisms in the gastrointestinal tract. Some examples are: inulin, fructooligosaccharides, resistant starch, pectin, among others. These are metabolized to short-chain fatty acids (SCFAs), like butyrate, which as mentioned earlier exert many beneficial health outcomes. SCFAs activate the SCFA receptor GPR43 that reduces insulin sensitivity in adipose tissue and hence its fat accumulation, thereby reducing the uptake, synthesis, and oxidation of toxic fatty acids in other tissues (9,10). They also increase proliferation and inhibit apoptosis of intestinal cells (11), hinders intestinal secretion of chylomicron into the circulation (12), and limits inflammation perhaps through inhibition of the NF-κβ pathway (13). Meanwhile, polyphenols can be found in wine, cocoa, cranberry, grape, curcumin, propolis, coffee, and tea; they function as antioxidants (14), strengthen intestinal barrier function (15), prevent endotoxemia (presence of LPS in the blood), the loss of some beneficial bacterial strains, and the development of diabetes (16–18). It is possible that the beneficial effects of some of these dietary compounds are exerted through the modulation of the microbiota. For example, the administration of cranberry extract and grape polyphenols is associated with an increased abundance of a genus *Akkermansia* that has been associated with beneficial metabolic effects even under a high sucrose and/or high fat diet (18–21).

The ingestion of foods rich in polyphenolic antioxidants can control the exposure to oxidative radicals. Flavonoids are an important group of natural polyphenolic substances with variations in their 3-ring basic structure. They are found in many fruits, vegetables, grains, pollen, among others, and are well known for their beneficial effects on health. However, some clinical studies have shown the health benefits of flavonoids, while others have not observed significant differences. It is possible that the differences observed lie in the capacity of absorption, distribution, excretion, response of each individual and the type of treatment, therefore, the type of flavonoid used, the dose, the frequency and route of the administration can be decisive for the success of the treatment. In addition to these factors intrinsic to the treatment and to the patient, there is another under-studied factor: the ability of the intestinal microbiota to metabolize flavonoids. Microorganisms have a great versatility to degrade compounds that reach the lower part of the gastrointestinal tract, in some cases, more than 90% of the flavonoid is degraded. The gut microbiota transforms these phenolic compounds in chemical species that might have a different bioactivity and/or bioavailability. Thus, the therapeutic use of flavonoids requires the understanding of the role of the gut microbiota as a modifier of these compounds. Specifically, the nature and rate of this modification may be affected by dietary substrates and interaction between bacteria. In the present study, we studied ecological interactions of flavonoid-degrading bacteria in simple (cocultures with non-flavonoid degrading bacteria) and complex (*in vitro* incubations of fecal matter) communities, using quercetin as a flavonoid type and different carbon sources, including several dietary fibers. The results of this research establish the importance of studying the interactions carried out by flavonoid-degrading bacteria with dietary compounds and with flavonoid degrading and non-degrading bacteria, revealing factors that can affect the bioavailability and bioactivity of these compounds and therefore their health effects.

Note: This introduction includes sections of the article 'Advances in Gut Microbiome research, opening new strategies to cope with a Western lifestyle'. A complete copy can be found in Appendix 4.

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II. Objectives

Characterize the degradability of flavonoid quercetin by the gut microbiota.

Specific objective 1

To evaluate the effect of different dietary fibers on the degradation of quercetin in an *in vitro* model of simplified intestinal community (co-cultures of quercetin-degrading bacterium, *E. ramulus* and fiber-degrading bacterium, *B. thetaiotaomicron*).

Specific objective 2

To study the capacity of degrading quercetin by fecal microbial communities of different individuals in an animal model of humanized microbiota.

Specific objective 3

To describe the functional potential in the intestine of the quercetin-degrading model microorganism, *E. ramulus* strain ATCC 29099.

III. Conceptualization

The interest in the gut microbiota as an influencing factor on human health is currently a hot topic and is considered in the first review article 'Advances in Gut Microbiome Research, Opening New Strategies to Cope with a Western Lifestyle' (Introduction and Appendix 4). In this review, the inflammatory effect of the western lifestyle is discussed as well as approaches that can counteract the threat of an ongoing oxidative state. The role of flavonoids as compounds that can exert anti-inflammatory effects and potentially as modifiers of the gut microbiota is remarked. However, there is a gap in the scientific literature regarding the role of the gut microbiota as a modifier of these compounds. Members of the gut microbiota can change the bioavailability and bioactivity of flavonoids, thus altering their health-related effects. Flavonoids may not be used as sole carbon sources by the gut microbiota but might be cometabolized with other carbon sources and in fact, the literature mentions that fermentable dietary fibers can greatly affect the bioavailability and degradability of flavonoids (production of smaller phenolic compounds). In this line, we propose to study the relationship between gut microbiota, flavonoids, and diet. Quercetin was selected as a type flavonoid since it is an aglycone flavonoid common in human diet, thus, it has no sugar that could be deglycosylated and used by the microbial community obscuring the results. Meanwhile, dietary fiber was chosen as the dietary component because it is a common accompanying substrate of flavonoids and influences their degradability.

The starting point of the experimental approach of this project was the observation that the well-recognized quercetin degrader *E. ramulus* consistently failed to colonize the gastrointestinal tract of germ-free mice when rodents were fed a diet low in fiber. Meanwhile, *E. ramulus* was able to colonize when mice were fed a diet with a high content of fiber. These germ-free mice were gavaged a synthetic microbial community of known microorganisms, among these, only *E. ramulus* failed to colonize under a fiber-restricted diet. This was an indication that fiber could be important for the establishment of *E. ramulus,* and therefore for the metabolization of flavonoids carried out by this bacterium. The relationship between fiber and flavonoid-degrading bacteria was studied by two strategies, simple (pure cultures and co-cultures) and complex (feces as inoculum for *in vitro* cultures) communities. In the first approach, we evaluated which dietary fiber, among arabinogalactan, fructooligosaccharides (FOS), galactomannan, inulin, pectin, gum arabic, and starch, promoted the cometabolization of quercetin by *E. ramulus*, and whether the metabolization of fiber by another bacterium stimulated *E. ramulus.* In the second approach, we studied the degradation of quercetin by fecal communities from mice fed two diets with different fiber contents.

In simple communities, *E. ramulus* used arabinogalactan, FOS, galactomannan, inulin, and pectin as carbon sources for the cometabolization of quercetin, in both pure cultures as well as in cocultures with *B. thetaiotaomicron.* Interestingly, we observed that only in cocultures*, E. ramulus* was able to metabolize quercetin when starch was the only carbon source. This kind of pattern between bacteria is rarely document in the scientific literature, thus we highlighted these results obtained with starch in our first

original article titled '*Bacteroides thetaiotaomicron* Starch Utilization Promotes Quercetin Degradation and Butyrate Production by *Eubacterium ramulus*' (Chapter 1).

In complex communities, we expected faster rates of quercetin degradation when *in vitro* cultures were inoculated with feces from mice fed a high-fiber diet. However, in many cases incubations inoculated with feces from mice fed a low-fiber diet had a faster degradation of the flavonoid. We opted for looking for correlations between members of the microbial community with either diet and the rate of degradation of quercetin. Still, no significant associations were found, a possible explanation could be the high variability of the gut microbiota between individuals. Nevertheless, during the analysis of the microbial communities we observed that two species whose relative abundances increased during incubation with quercetin and are related to a known quercetin degrader, *F. plautii*, presented an interesting pattern of negative correlation (Chapter 2). Again, this finding has biological importance for the field and given its relevance, close relatives of *F. plautii* were included as part of the third objective, which aimed to describe through a bioinformatic approach the functional potential in the intestine of *E. ramulus* (Chapter 3). The important observations obtained for species related to *F. plautii* were then the focus of our second original article titled 'Gut-derived *Flavonifractor* species variants are differentially enriched during *in vitro* incubation with quercetin' which is under review in Plos One. Overall our results highlight the importance of the ecological interactions that flavonoid-degrading bacteria have with dietary substrates and with other flavonoid-degrading bacteria and members of the gut microbiota that are involved in fiber fermentation. Notably, these interactions could impact the anti-inflammatory properties of flavonoids.

IV. Results

Chapter 1. *Bacteroides thetaiotaomicron* Starch Utilization Promotes Quercetin Degradation and Butyrate Production by *Eubacterium ramulus*

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Keywords: Quercetin degradation, butyrate, *Eubacterium ramulus*, *Bacteroides thetaiotaomicron,* cross-feeding, starch.

Abstract

Consumption of flavonoids has been associated with protection against cardiovascular and neurodegenerative diseases. Most dietary flavonoids are subjected to bacterial transformations in the gut where they are converted into biologically active metabolites that are more bioavailable and have distinct effects relative to the parent compounds. While some of the pathways involved in the breakdown of flavonoids are emerging, little it is known about the impact of carbon source availability and community dynamics on flavonoid metabolism. This is relevant in the gut where there is a fierce competition for nutrients. In this study, we show that the metabolism of one of the most commonly consumed flavonoids, quercetin, by the gut-associated bacterium *Eubacterium ramulus* is dependent on interspecies cross-feeding interactions when starch is the only energy source available. *E. ramulus* can degrade quercetin in the presence of glucose but is unable to use starch for growth or quercetin degradation. However, the starch-metabolizing bacterium *Bacteroides thetaiotaomicron*, which does not metabolize quercetin, stimulates degradation of quercetin and butyrate production by *E. ramulus* via cross-feeding of glucose and maltose molecules released from starch. These results suggest that dietary substrates and interactions between species modulate the degradation of flavonoids and production of butyrate, thus shaping their bioavailability and bioactivity, and likely impacting their health-promoting effects in humans.

Introduction

Flavonoids are phenolic compounds produced by the secondary metabolism of plants. They are present in fruits, grains, and vegetables. Their basic structure consists of 15 carbon atoms arranged in three rings (A, B and C). Their consumption is associated with a lower risk of suffering from cardiovascular and neurodegenerative diseases [1]–[4]. Most polyphenols are poorly absorbed in the upper gastrointestinal tract (stomach, duodenum, jejunum, and ileum) and reach the colon where they are

metabolized by the gut microbiota into more readily absorbable phenolic acids, increasing bioavailability of these biologically active compounds [5], [6].

Among the more than 8,000 different flavonoids characterized to date, quercetin is one of the most common in nature. It is found in apples, onions, red wine, tea, lettuce, and tomatoes and is extensively metabolized in the gastrointestinal tract (>90 %; Chen et al. 2005; Hertog et al. 1992). Carbon dioxide is a major metabolite derived from quercetin metabolism in humans; a process that starts with the cleavage of the flavonoid's C-ring by intestinal bacteria [9]. Most members of the gut microbiota that are known to cleave the C-ring of quercetin belong to the Clostridia class; these include *Flavonifractor plautii, Eubacterium ramulus,* and *Eubacterium oxidoreducens* [10]–[12]. *E. ramulus* is a prevalent bacterial species commonly found in healthy subjects at levels ranging from 10^7 to 10^9 cells/ g of dry feces [13]. *E. ramulus* ferments glucose to butyrate, a major energy source of colonocytes that inhibits colon inflammation and carcinogenesis, and it has systemic effects lowering diet-induced insulin resistance [14]–[17]. *In vitro* studies indicate that *E. ramulus* requires glucose for the co-metabolization of quercetin [18]. Degradation of this flavonoid results in the production of 3,4-dihydroxyphenylacetic acid (DOPAC), which has antiproliferative activity in colon cancer cells [18]–[20]. Nevertheless, glucose is rapidly absorbed in the small intestine and negligible amounts reach the colon, where *E. ramulus* resides [21].

Dietary compounds that can impact microbial flavonoid metabolism in the colon are those that resist digestion in the upper gastrointestinal tract. Most carbohydrates that reach the lower gastrointestinal tract are of plant origin, including plant cell-wall and storage polysaccharides [22]. Among these, the fraction of starch that escapes digestion in the small intestine; i.e., resistant starch, represents an important fermentation substrate that boosts bacterial flavonoid metabolism [23] and butyrate production [24]. Starch that is incompletely digested in the upper digestive tract is naturally present in many foods, including bananas, rice, maize, and potatoes. For example, around 3 % of hot potato starch and 12 % of cold potato starch are resistant to digestion. It has been estimated that for individuals following a modern diet, the quantity of starch entering the colon is about 10 % of starch intake, around 8-40 g/d [25]. Previous work indicates that undigested starch enhances bacterial metabolism of daidzein, a soy isoflavone [23]. Furthermore, the abundance of the flavonoid-degrading bacterium, *E. ramulus,* is positively influenced by consumption of resistant starch [26].

In order to get insights into the fate of flavonoids in the presence of polysaccharides in the multi-species environment of the gut, we evaluated in a simplified model of the gut microbiota the interactions between *E. ramulus,* which has a limited capacity to utilize polysaccharides, and *Bacteroides thetaiotaomicron*, which has the capacity to degrade many polysaccharides but it is unable to degrade the flavonoid, quercetin. We found that *B. thetaiotaomicron* liberates glucose and maltose from starch at levels that support the growth of *E. ramulus* and degradation of quercetin by this bacterium. Our results illustrate how cross-feeding between bacterial taxa can impact the metabolic fate of flavonoids in the gut.

Methods and Materials

Chemicals. Ammonium formate, 98% crystalline (Alfa Aesar), EDTA ≥98.5% w/w (Sigma-Aldrich), methanol HPLC-grade (Fisher Scientific), ultrapure grade water purified to 18.1 MΩ·cm using a Barnstead

water filtration system (Thermo Fisher Scientific), potato starch (Sigma; 102954), and quercetin dihydrate, 97% w/w (Alfa Aesar).

Bacterial Strains and Culture Conditions. Frozen stocks of *E. ramulus* strain ATCC 29099 and *B. thetaiotaomicron* VPI-5482 were diluted 1:50 in 7N minimal medium, consisting of 50 mM MOPS·KOH (pH 7.2), 0.2 % resazurin, 2 mM tricine, 0.025 % tween 80, 20 mM $C_2H_3NaO_2$, 20 mM NaCl, 14 mM NH_4Cl, 0.25 mM K_2SO_4, 0.5 mM $MgCl_2{\cdot}6H_2O$, 0.5 mM $CaCl_2{\cdot}2H_2O$, 10 µM $FeSO_4{\cdot}7H_2O$, 20 mM $NaHCO_3$, 1 mM KH_2PO_4, 1µg ml^{-1} vitamin K_3, 1.9 µM hematin, 0.2 mM histidine, 8 mM L-cysteine, 1× ATCC trace minerals, 1× ATCC vitamin supplement, amended with 40 mM glucose. Media were filter-sterilized (0.22 µm pore diameter). Cultures were incubated with constant agitation at 37° C overnight (OD_{600} = 1.5-1.7 for *E. ramulus*, 1.3-1.5 for *B. thetaiotaomicron*), then washed thrice with 10 ml of anaerobic 7N medium without glucose inside an anaerobic chamber. All centrifugations steps were done in Hungate tubes at 3,000 rpm 5 min. After the final centrifugation, *E. ramulus*'s cell suspension was resuspended in a sixth of the initial volume and *B. thetaiotaomicron* in half of the initial volume to produce concentrated cell suspensions with equivalent number of cells for both (an overnight culture of *B. thetaiotaomicron* has about 3 times more cells than *E. ramulus*). For a typical assay, about 150 µl of cell suspension was added to a 10 ml medium (this corresponds to about 10^9-10^{10} genome equivalents ml^{-1}), cultures were grown at 37° C with agitation. Glucose was filter-sterilized and added at a final concentration of 40 mM. Starch was autoclaved and added at a final concentration of 1 %. Quercetin dihydrate was used at a final concentration of 0.25 mg ml^{-1} in MilliQ water, autoclaved for 20 min, let cooled for 1 h with stirring and dispensed with stirring. Bacteria were handled inside an anaerobic chamber under an atmosphere of nitrogen (75%), carbon dioxide (20%), and hydrogen (5%).

DNA preparation. DNA extraction was performed as previously described with modifications [27]. Briefly, 300 µl aliquot of cultures with starch as sole carbon source was mixed with a solution containing 500 µl of extraction buffer (200 mM Tris pH 8.0, 200 mM NaCl, 20 mM EDTA), 200 µl of 20 % SDS, 500 µl of a mixture of phenol:chloroform:isoamyl alcohol (25:24:1, pH 7.9) and 1.2 mg of 0.1-mm diameter zirconia/silica beads (BioSpecProducts). The suspension was then subjected to 3 min of bead beating (BioSpec Products) at room temperature (RT), spun at 8,000 rpm for 5 min at RT, and then 750 µl of the top layer was transferred to a 15 ml tube (BD Falcon 12 x 75 mm, #352063) for immediate column purification (Nucleospin, Macherey-Nagel). Column binding buffer NTl was used at 2.5 vol, 3 washes with washing buffer NT3 were performed and final elution was done with 25 µl of low $T_{(10)}E_{(0.1)}$ buffer.

Real-time Quantitative PCR (qPCR). qPCRs were performed using the SsoAdvanced universal SYBR Green supermix (2X) (BioRad, 172-5270-5275) and the BioRad CFX96 Real-Time PCR Detection System. Species-specific primers for the 16S rRNA gene were used at a final concentration of 0.4 mM. Primer sequences for *B. thetaiotaomicron* were 5'-GCAAACTGGAGATGGCGA-3' and 5'-AAGGTTTGGTGAGCCGTTA-3' (Tm 62.5°C) [28] and for *E. ramulus*, 5'-CGGTACCTGACTAAGAAGC-3' and 5'-AGTTTCATTCTTGCGAACG-3' (Tm: 55°C) [29]. Each culture was analyzed in triplicate. DNA extractions from pure cultures of *B. thetaiotaomicron* or *E. ramulus* were used to generate standard curves using 7 serial dilutions ranging from 442 ng ml^{-1} to 4.42×10^{-4} ng ml^{-1} and 235 mg ml^{-1} to 2.35×10^{-4} ng ml^{-1}, respectively (quantified by the Qubit dsDNA HS assay). The qPCR run consisted of a denaturation step (95°C for 30

sec), an amplification step (35 cycles of 95°C for 10 sec, Tm for 15 sec and 60°C for 30 sec), and a melting cycle (65-95°C 0.5°C increment 2-5 sec/step).

HPLC analyses of Quercetin and metabolites. Samples from 0 and 22 h cultures were thawed in ice, vortexed extensively and 400 µl were mixed with 1000 µl HPLC-grade methanol + 20 µM genistein as internal standard, the suspension was subjected to bead beating (BioSpecProducts) for 2 min at RT, then heated to 56 °C for 20 min and spun for 10 min at 18, 000 *g* at RT. Then 1 ml of the supernatant was transferred to an HPLC vial and 200 µl of 10 mM ammonium formate/0.5 M EDTA buffer (pH 3.5) was added. Quercetin and its metabolites were analyzed using a Dionex UltiMate 3000 HPLC equipped with an LPG-3400 quaternary pump, a WPS-3000 analytical autosampler, a DAD-3000 diode array detector, and a FLD-3100 fluorescence detector. Separations were performed on a Kinetex 5 µm EVO C18, 100 Å, 250 × 4.6 mm column (Phenomenex, Torrance, CA, USA). Injection volumes were 5 µL. A flow rate of 1 ml min^{-1} was used throughout the 59 min run. The mobile phase was a binary gradient of (A) 10 mM ammonium formate and 0.3 mM ethylenediaminetetraacetic acid in water adjusted to pH 3.5 using concentrated HCl and (B) methanol. Solvents were vacuum filtered with 0.20 µm nylon membrane filters (Merk Millipore Ltd, Cork, Ireland). The gradient began at 5 % B for 5 min, increased to 30 % B over 30 min, increased to 95 % B over 10 min, remained constant at 95 % B for 5 min, decreased to 5 % B over 2 min, and then re-equilibrated at 5 % B for 7 min. Three-dimensional absorbance data were collected using the diode array detector and chromatograms of 280 nm absorbance were analyzed. Reportable values are shown in Table S1.1 (Appendix 1) and an example chromatogram is shown in Fig. S1.1 (Appendix 1).

Samples were quantitated based on external calibration with injections of 10 µL over the linear range 1-125 µM for protocatechuic acid; 3,4-dihydroxyphenylacetic acid; 3,4-dihydroxyphenylpropionic acid; 3-hydroxybenzoic acid; 3-hydroxyphenylacetic acid; 3-(3-hydroxyphenyl) propionic acid; phenylacetic acid; quercetin; and genistein and 5-125 µM for benzoic acid. Some compounds could not be resolved by this method, namely 3-(3-hydroxyphenyl) propionic acid and phenylacetic acid.

Gas chromatography-mass spectrometry measurements of butyrate. Samples were processed as described before [30]. Briefly, an aliquot of 50 µl of cultures incubated for 12 h with starch and quercetin and *E. ramulus* monocultures with glucose and quercetin (control) were mixed with 20 mM of a butyric-d7 acid, 99.5 atom % D, CDN isotopes #D-171 as internal standard, acidified with 5 µl of 33 % HCl, extracted twice with Diethyl Ether, then 60 µl of each sample was mixed with 2 µl of derivatizing reagent (N-Methyl-N-tert-butyldimethylsilyltrifluoroacetamide, MTBSTFA) and incubated at RT for 2 h. For detection, 1 µl of each sample was injected in a gas chromatography–mass spectrometry (GC-MS) instrument (Agilent 7890B/5977A GC/MSD), and an Agilent DB-1ms column was used. Oven program was: initial temperature, 40°C for 2.25 min; then 20°C min^{-1} to 200°C; next 100°C min^{-1} to 300°C, maintained for 7 min.

Colorimetric assay for determination of Glucose and Maltose. An aliquot of each culture with starch as sole carbon source was centrifuged at 11,000 g 5 min and glucose and maltose levels were determined by a colorimetric method (Maltose and Glucose Assay Kit; Sigma-Aldrich, MO, USA) at 0, 4 and 8 h of incubation following the manufacturer's instructions. Standard curve: 2-10 nmoles of glucose per well.

Statistical analysis. Data was analyzed using analysis of variance (ANOVA – Minitab 18.1). Differences considered significant at $p < 0.05$.

Results

***Eubacterium ramulus* requires an energy source to metabolize quercetin.** Glucose and starch were evaluated for their capacity to promote quercetin degradation by *E. ramulus.* The structure of quercetin is shown in Fig. 1.1A. We used media with no addition of a carbon source as a negative control. We found that glucose stimulates the degradation of quercetin by *E. ramulus* and the production of 3,4-dihydroxyphenylacetic acid (DOPAC) (Fig. 1.1B) as the main metabolite derived from quercetin (Fig. 1.2 and Table S1.1 in Appendix 1), as previously described [18]. In the presence of glucose *E. ramulus* also generates high levels of butyrate (Table 1.1). We assessed different concentrations of glucose in order to determine the lowest concentration of this monosaccharide required for the co-metabolization of 0.8 mM of quercetin. Quercetin degradation was checked by visual inspection; i.e., disappearance of the quercetin from the test tube, which is insoluble and has a yellow color. We also quantified quercetin and the main degradation product, DOPAC, by HPLC at the end of the experiment (22 h). We found that concentrations of glucose above 0.3 mM are required to stimulate detectable quercetin degradation (Fig. S1.2). Additionally, we observed a decrease in the population of *E. ramulus* when no carbon source was added but quercetin (Table S1.2 in Appendix 1), suggesting cell death. Accordingly, under these conditions little production of DOPAC was detected (Fig. 1.2). Monocultures of *E. ramulus* supplemented only with starch did not show growth (Fig. 1.3A and Table S1.2 in Appendix 1), as expected they accumulated little butyrate (Table 1.1) and DOPAC after 22 h of incubation (Fig. 1.2). There were no signs of quercetin degradation after 4 days of incubation (data not shown). *E. ramulus* did not grow on starch with or without quercetin (Fig. S1.3).

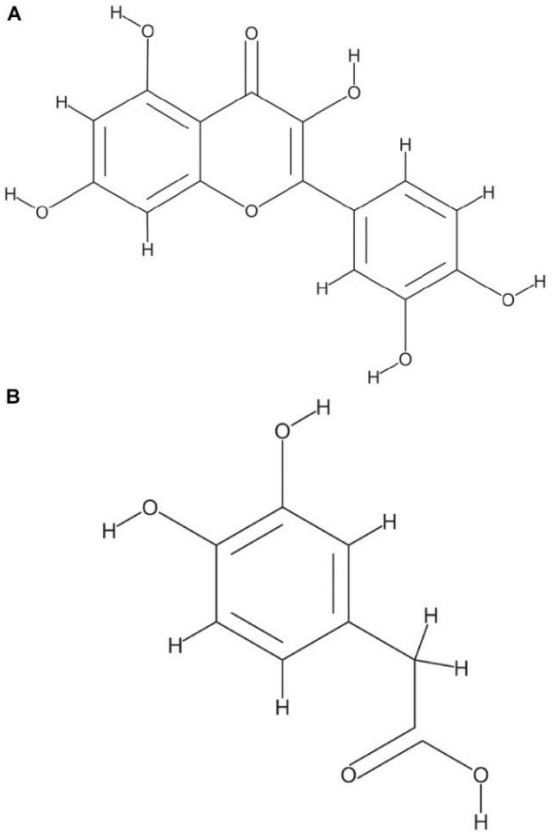

Figure 1. 1

Structure of Quercetin (A) and 3,4-Dihydroxyphenylacetic acid (DOPAC), the main metabolite generated by *E. ramulus* from quercetin degradation (B) (National Center for Biotechnology Information. PubChem Database, compound 5280343 and 547, respectively [https://pubchem.ncbi.nlm.nih.gov/].

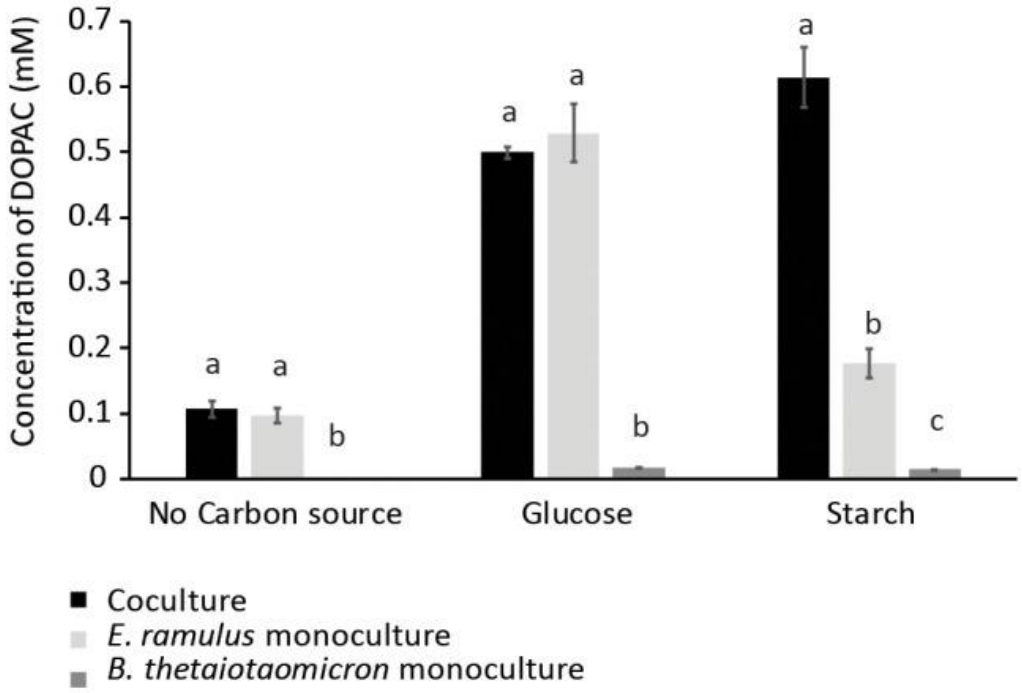

Figure 1. 2

DOPAC concentration as measured by HPLC in cultures with no carbon source, glucose (0.7 %), and starch (1 %). Culture tubes were inoculated with only *B. thetaiotaomicron* or *E. ramulus* or both and incubated for 22 h. Error bars corresponds to 3 replicates. Different letters above bars indicate significant difference between type of culture at p <0.05 according to LSD.

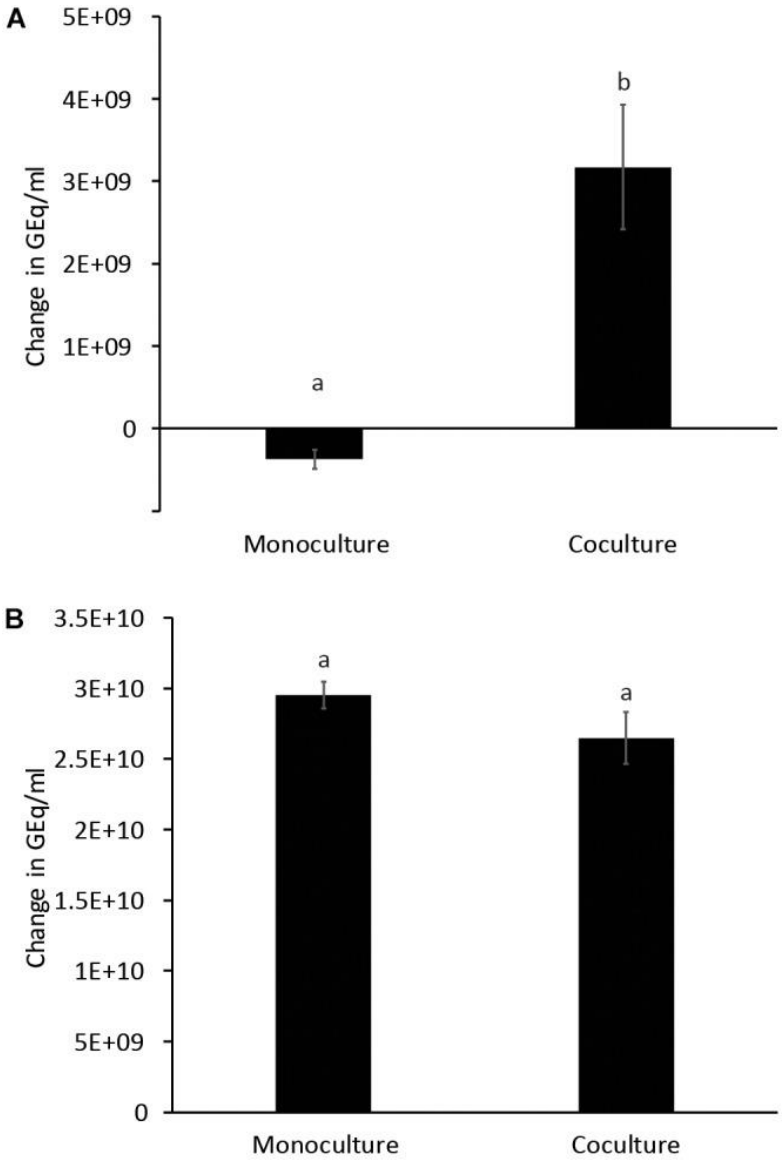

Figure 1. 3

Growth of *E. ramulus* (A) and *B. thetaiotaomicron* (B) in cultures with 1 % starch as carbon source. GEq, Genome equivalents. Change in Geq/ml between time 0 h and 8 h. Results from independent experiments are presented in Table S1.2 (Appendix 1). Different letters above bars indicate significant differences between type of culture at p <0.05 according to LSD, data from 2 independent experiments (with 2-3 replicates each).

Table 1. 1.

Butyrate concentrations in monocultures and cocultures of *E. ramulus* and *B. thetaiotaomicron.*

Culture and Carbon source	Butyrate (mM)
E. ramulus monoculture with Glucose	7.09 ± 0.40
E. ramulus monoculture with Starch	0.22 ± 0.07[a]
*B. thetaiotaomicro*n monoculture with Starch	0.01 ± 0.0005[b]
Coculture with Starch	3.19 ± 0.06[c]

Average values for 3 replicates. Different letters after standard deviation indicate significant difference between type of culture (only starch cultures) at p <0.05 according to LSD.

***Bacteroides thetaiotaomicron* starch utilization enhances butyrate production and quercetin degradation by *Eubacterium ramulus*.** We examined whether the presence of the versatile polysaccharide-metabolizing bacterium, *B. thetaiotaomicron*, influenced the degradation of quercetin and production of butyrate by *E. ramulus*. We incubated both species with quercetin and starch individually and in coculture. We did not observe significant degradation of quercetin by either species in monoculture (Fig. 1.2). However, a marked appearance of DOPAC (Fig. 1.2) and enhanced growth of *E. ramulus* was observed in cocultures (Fig. 1.3A and Table S1.2 in Appendix 1). Growth of *E. ramulus* was not enhanced in cocultures when glucose was the sole carbon source (Fig. S1.4, Appendix 1). Additionally, while butyrate was not produced by either species in monoculture incubated with starch, cocultures accumulated high levels of butyrate, ~44 % of the amount produced by *E. ramulus* monocultures with 40 mM glucose (Table 1.1). On the other hand, the presence of *E. ramulus* did not change significantly the growth yield of *B. thetaiotaomicron* incubated with 1 % starch (Fig. 1.3B and Table S1.2 in Appendix 1).

***Bacteroides thetaiotaomicron* releases glucose and maltose from starch.** Lastly, we hypothesized that *B. thetaiotaomicron* promotes *E. ramulus* metabolism by releasing free glucose from starch. We quantified free glucose in cultures with starch as the sole carbon source (Table 1.2). We found that *B. thetaiotaomicron* releases free glucose at levels that are higher than what we found is necessary to stimulate quercetin degradation (Fig. S1.2 in Appendix 1), whereas cocultures accumulated approximately 50 % of the glucose of *B. thetaiotaomicron* monocultures ($p<0.05$) (Table 1.2). Additionally, we detected maltose in monocultures of *B. thetaiotaomicron* incubated with starch at a concentration of 0.2 mM. (±0.04 mM) at 4 h and 0.8 mM (±0.1 mM) at 8 h of incubation. In cocultures, there were lower concentrations of maltose, 0.08 mM (±0.02 mM) after 4h of incubation and 0.4 mM (±0.2 mM) after 8 h. Accordingly, maltose also stimulated the degradation of the flavonoid by *E. ramulus* in monoculture (data not shown). Altogether these results suggest that *E. ramulus* uses glucose and maltose released by *B. thetaiotaomicron* from starch to grow, produce butyrate and to degrade the flavonoid.

Table 1. 2.

Concentration of glucose liberated from starch by *E. ramulus* or *B. thetaiotaomicron* (monocultures) or both organisms in coculture.

	Free Glucose (mM)		
Culture	0 h	4 h	8 h
E. ramulus monoculture	0.02 ±0.004[a]	0.02 ±0.01[a]	0.06 ±0.05[a]
B. thetaiotaomicron monoculture	0.03 ±0.01[a]	1.29 ±0.43[b]	8.29 ±2.76[b]
Coculture	0.02 ±0.01[a]	0.68 ±0.33[c]	4.62 ±1.70[c]

Average values for 2 independent experiments with 2 replicates each. Different letters after parenthesis indicate significant difference between treatments at each time point at $p < 0.05$ according to LSD.

Discussion

Here we evaluated how the utilization of a common carbohydrate in human diet, starch, by a member of the Bacteriodetes phylum changes the dynamics of flavonoid degradation and production of butyrate by *E. ramulus*. We found that metabolization of starch by *B. thetaiotaomicron* enhanced these processes in the Firmicute via cross-feeding of glucose and maltose released from the carbohydrate. Mahowald and coworkers observed in gnotobiotic mice co-colonized with *Eubacterium rectale* and *B. thetaiotaomicron* that *E. rectale* is better able to access nutrients and upregulates genes in the central carbon and nitrogen pathways in the presence of the *B. thetaiotaomicron* [31]. One explanation for this is the observation that *B. thetaiotaomicron* releases simple saccharides when digesting complex carbohydrates that then *Eubacterium* can access. We have observed that *B. thetaiotaomicron* can enhance the growth of *E. ramulus* when growing on different oligo and polysaccharides (e.g., inulin, FOS, and arabinogalactan; data not shown), however the most striking stimulation was on starch. *B. thetaiotaomicron* possess membrane-associated amylase activity, encoded by *susG*, which may allow the release of products of starch breakdown to the extracellular medium [32], however, not all bacteria can access these public goods [33]. Cross-feeding of starch-derived metabolites has also been reported for *Bifidobacterium adolescentis*, this bacterium generates resources that can be used by butyrate producers including *Roseburia sp*., *Eubacterium hallii,* and *Anaerostipes caccae.* This cross-feeding involves end-products (lactate or acetate) of *B. adolescentis* starch fermentation and potentially products released by partial hydrolysis of starch likely to be malto-oligosaccharides [34], however it is not clear whether glucose is released from starch by *B. adolescentis*. In our studies, supplementation of acetate to the media (20 mM), in the absence of a usable carbohydrate, did not prompt the degradation of quercetin or the production of butyrate by *E. ramulus*.

In this work, we evaluated a soluble form of starch. Soluble starch could reach the colon entrapped in non-soluble cell-wall particles which can be released when cellulose-degrading microorganisms (e.g. *Ruminococcus* spp. or *Enterococcus* spp.) act on them releasing the soluble part. It is also possible that cellulose-degrading microorganisms could release free glucose, however, the capacity to degrade cellulose in humans seems to be limited [22], [35]. *B. thetaiotaomicron* has a great ability to ferment soluble starch while its ability to ferment resistant starch depends on the type of resistant starch

and treatment. *In vitro* experiments show an efficiency of >90 % for autoclaved or boiled high amylopectin corn starch but <1 % for raw high amylose corn starch. Thus, in the gut other players that ferment resistant starch more efficiently may potentially play an important role in providing resources for cross-feeding. It is worth noting that several butyrate-producers including *E. ramulus* do not have the capacity to degrade starch; nonetheless resistant starch is recognized as a butyrogenic substrate [24], thus we suggest that the acquisition of luminal products of starch breakdown (e.g., glucose and maltose) by butyrate-producing species may be important for the production of butyrate.

Several studies indicate that quercetin has antibacterial activity [36], [37]. Quercetin increases the permeability of certain bacteria to ions [38]. Some species of bacteria have defense mechanisms against quercetin. For example, the plant root-colonizer, *Pseudomonas putida,* has an efflux pump, TtgABC, that has a high affinity for quercetin, as well as for certain antibiotics [39]. Cellular targets also include enzymes like DNA gyrase and D-alanine:D-alanine ligase, essential for DNA replication and the assembly of peptidoglycan precursors, respectively, in these enzymes quercetin recognizes the conserved ATP-binding region and compete with ATP [40], [41]. Some sugar transporters depend on ATP [42] thus the ability of using starch when quercetin was not present was evaluated for *E. ramulus*. Under these conditions *E. ramulus* was also unable to use starch. Thus, our data indicates that *E. ramulus* is unable to perform the initial breakdown of starch but possess the ability of using public goods generated by the breakdown of this substrate [33]. In the intestine, the ability to degrade quercetin may create a temporal niche where *E. ramulus* can access nutrients (e.g., luminal glucose) that are in the vicinity of flavonoids and that other flavonoid-sensitive bacteria cannot access while the concentration of the flavonoid is still high.

Microbial-derived biologically active metabolites have a major impact on host's health. Quercetin is one of the most abundant flavonoids, however it is still not clear whether its colonic degradation is beneficial for the host since the parent compound and bacterial products derived from its degradation all have bioactivity. Studies that explore the extent to which degradation of quercetin is beneficial for the host are needed. For this goal, carbohydrates that promote more or less the degradation of the flavonoid can be useful. For example, it has been shown that fructooligosaccharides administered to the diet accelerate the use of the flavonoids rutin, quercitrin and quercetin in cecal contents of rats relative to animals supplemented with non-fermentable fiber [43]. The characterization of gut microbes able to metabolize flavonoids will make possible in the future to stratify subjects in clinical trials based on the flavonoid-degrading capacity of their gut microbiotas. Furthermore, understanding how interpersonal or disease-associated differences in gut microbial metabolism of flavonoids impact the potential benefits associated with their consumption, and identifying biomarkers for these processes will help nutritionists formulate dietary recommendations that are matched by the metabolic potential of a subject's gut microbiota with the ultimate goal of optimizing food function efficacy.

Acknowledgments

We would like to thank Dr. Robert Kerby (University of Wisconsin, Madison, USA) for his support and Dr. Wilson Teran (Javeriana University, Bogota, Colombia) for his help providing resources for the

qPCR. We would like to thank the Doctorate program in Biosciences of La Sabana University and PhD scholarship 647 (Colciencias, Colombia) for supporting Gina Paola Rodriguez.

Funding Disclosure

This research is supported by the National Institute of Food and Agriculture, U.S. Department of Agriculture, award number 2016-67017-24416.

Author Contributions Statement

GR and FR conceived and planned the experiments. GR, MD, and XL carried out the experiments. GR, AA, BB, and FR contributed to the interpretation of the results. AA and FR helped supervise the project. GR wrote the manuscript with input from all authors. All authors discussed the results and contributed to the final manuscript.

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Chapter 2. Gut-derived *Flavonifractor* species variants are differentially enriched during *in vitro* incubation with Quercetin and persist in diets with different fiber contents

Abstract

While the health-promoting properties of flavonoids are widely studied little is known about the ecology of the gut bacteria that are able to transform these phenolic compounds influencing their health benefits. These bacteria are often studied in pure culture; however, they reside in the heterogenous colon environment where multiple interactions between microbes happen. In this study, a comparative metataxonomic analysis of complex fecal communities (n=15) supplemented with the flavonoid quercetin led us to describe a potential ecological phenomenon between two variants related to a flavonoid degrading species, *Flavonifractor plautii*. Fecal pellets retrieved from germ-free mice transplanted with human fecal samples and fed 2 diets with different fiber contents were used for fecal slurries with quercetin, during incubation the relative abundance of these two variants related to *Flavonifractor* sp. were inversely correlated, one variant (ASV_65f4) increased in relative abundance in half of the libraries and the other variant (ASV_a45d) in the other half. Mouse's diet did not change the pattern of dominance of either variant, and initial relative abundances did not predict which one ended up dominating in the presence of the flavonoid. This pattern was also observed with 9 additional fecal samples that were used directly as an inoculum in *in vitro* cultures. Potential distinct metabolic properties of these two *Flavonifractor*-related species was evidenced, as only one species became consistently enriched in complex communities supplemented with acetate. Additionally, co-abundance patterns with other taxa showed that when one of the *Flavonifractor*-related species was dominant, the relative abundance of *Desulfovibrio* was significantly higher whereas the other variant was associated with higher levels of the succinate-utilizing genus *Phascolarctobacterium*, suggesting potentially distinct interactions of these variants within the gut microbial community and/or its metabolites. Here, we showed that the *Flavonifractor* genus harbors variants whose relative abundances may fluctuate according to the dietary substrates present and the interactions with the microbial community. Whether this behavior affects the dynamic of quercetin degradation warrants further investigation.

Introduction

Flavonoids are 3-ring phenolic compounds found in fruits and vegetables, their regular consumption is associated with health benefits (1,2). Among these, quercetin is one of the most abundant in human diet. It exerts effects in the immune, digestive, endocrine, nervous, and cardiovascular systems (3–6). Some gut bacteria have the capacity to cleave the central ring in the flavonoid skeleton by a process known as C-ring fission which generates smaller phenolic products. In the case of quercetin biodegradation, phloroglucinol and 3,4-dihydroxyphenylacetic acid (DOPAC) are formed (7). It is not clear to what extent do the health effects of flavonoids depend on their transformation to biologically active compounds by the gut microbiota. Vissiennon and collaborators showed that the anxiolytic activity of quercetin is induced by DOPAC and not by the parent compound, evidencing a case in which the microbial metabolite induces the beneficial effect (8). DOPAC has also antiproliferative activity in colon cancer cells (9) and anti-platelet aggregation activity (10). Additionally, the well-recognized quercetin-degraders, *F.*

plautii (formerly *Clostridium orbiscindens*) and *E. ramulus,* are also butyrate-producers, a short-chain fatty acid that is the preferred source of energy for colonocytes and essential for colon health (11–14).

Most commonly, these flavonoid degrading bacteria are studied in pure culture, however, they reside in the colon where more than 10^{13} bacterial cells inhabit (15). *In vitro* fecal incubation systems provide a scenario where complex microbial communities can be studied in order to reveal important ecological interactions that can impact the biotransformation of flavonoids. *In vitro* fecal incubations with quercetin have revealed the metabolites formed during the transformation of this flavonoid; however, ecological interactions between quercetin-degraders and the rest of the community have been overlooked. Additionally, most studies have analyzed fecal samples from one donor (16,17) or pooled fecal samples from different donors (18–20), dismissing the importance of the gut microbiota at the individual level. In order to characterize key players in quercetin degradation that are common or unique among subjects, the evaluation of *in vitro* fecal incubation experiments with individual fecal samples is needed. However, fecal samples taken directly from individuals may be influenced by the subject's diet, the donor's handling of the stool sample, and transport conditions. Therefore, murine models of humanized microbiota provide a strategy that can minimize the interference of factors such as genetics, lifestyle and the diet of the individual, and can be used to study the effect of a controlled diet on the microbial communities.

In this study, microbial community dynamics from different subjects were analyzed individually by *in vitro* incubations of feces with quercetin, 6 fecal samples were inoculated in human microbiota-associated mice (HMAM) and 9 that were directly assayed. Species related to *Flavonifractor, Eubacterium*, and *Intestinimonas* became enriched under these conditions. A more detailed analysis revealed that two species variants related to the genus *Flavonifractor* were present in every library but after quercetin incubation one or the other variant but not both became enriched. Given the metabolic differences observed between these variants, we propose that their role in the gut microbiome is differentially mediated by carbon sources in the diet and interactions with other members of the community and may have distinct roles in the degradation of the flavonoid.

Methods and Materials

Sample collection and processing. Stool samples were collected from subjects participating in the Wisconsin Longitudinal Study (WLS) between November 2014 and February 2015 as previously described (21). Briefly, participants collected stool samples directly in sterile containers, then samples were kept at ~4 °C until arrival to the processing laboratory within 48 hours of collection. Upon arrival, sterile straws were filled with the fecal material and stored at -80˚ C as previously described (22). The use of WLS fecal microbiota was approved by the Institutional Review Board at the University of Wisconsin-Madison.

***In vitro* incubations with fecal samples from human microbiota-associated mice (HMAM) under different diets.** Experiments involving mice were performed using protocols approved by the University of Wisconsin-Madison Animal Care and Use Committee. Six female C57BL/6 (B6) germ-free mice were gavaged with ~200 µl of fecal slurry which were prepared under anaerobic conditions in Hungate tubes using a 1 cm piece of straw containing the frozen fecal material and 5 ml mega media (23). Mice were

maintained on a chow diet for 2 weeks after humanization, then, a diet high in fiber (Teklad 2018S) was administered for two weeks, and then switched to a low fiber diet for two weeks (Teklad TD.97184). Three fecal pellets were collected for each mouse after each experimental diet (high and low in fiber) and used separately as inoculum for *in vitro* incubations. Fecal pellets were weighted and then resuspended at 0.15 mg ml^{-1} final concentration with vigorous vortexing in anaerobic 7N minimal medium supplemented with 20 mM sodium acetate (filter-sterilized through a 0.22-m pore diameter) (24). Quercetin dihydrate, 97 % w/w (Alfa Aesar) concentration was 0.25 mg ml^{-1} (0.8 mM) in the corresponding medium. Sample processing was done in an atmosphere of nitrogen (75%), carbon dioxide (20%), and hydrogen (5%) and incubation was also done under anaerobic conditions at 37° C statically. Sampling was done at 0 and 7 days of incubation.

***In vitro* incubations with human fecal samples and quercetin.** Human fecal samples were directly used for *in vitro* incubations with quercetin. Samples from 9 subjects kept in frozen straws were aliquoted (~50 mg) on dry ice, weighted, and resuspended at 0.5 mg ml^{-1} final concentration with heavy vortexing in anaerobic 7N minimal medium supplemented with 20 mM sodium acetate (24). Quercetin concentration was 0.125 mg ml^{-1} (0.4 mM) in the corresponding medium. Controls consisted of the same medium plus fecal sample. Anaerobic bottles were kept statically at 37° C. When quercetin disappearance was visible, 10 % of the culture was transferred to another anaerobic bottle with the same medium. Treatments with quercetin had three replicates and controls one replicate. Sample processing was done in an anaerobic chamber under anaerobic conditions. Sampling was done at the end of the first and second incubation time once quercetin degradation was completed across all samples (72 h of incubation). In a second experiment, combinations of fecal matter from different subjects were tested. Two fecal samples used in the previous *in vitro* incubation experiment (from subjects #9 and #10) were selected based on their enrichment of one or the other *Flavonifractor*-related variant (ASV_65f4 or ASV_a45d). Three combination of these fecal samples were done as follows (#9/#10): 0/0.1, 0.1/0.1, 0.1/0 mg ml^{-1}. Incubations were done again in anaerobic 7N minimal medium supplemented with 20 mM sodium acetate with and without quercetin using three replicates. Sampling was done at 0 and 72 h of microbial growth.

HPLC analyses of Quercetin and metabolites. Samples were processed as previously described (24). Briefly, 200 µl samples were mixed with 1000 µl HPLC-grade methanol plus 20 µM genistein as internal standard, the suspension was bead beated (2 min), heated (56 °C for 20 min) and spun (10 min at 18, 000 *g*). Then 1 ml of the supernatant was mixed with 200 µl of 10 mM ammonium formate/0.5 M EDTA buffer (pH 3.5). Separations were performed on a Kinetex 5 µm EVO C18, 100 Å, 250 × 4.6 mm column (Phenomenex, Torrance, CA, USA). Injection volumes were 10 µL. Flow rate was 1 ml min^{-1}. Run time was 59 min run. The mobile phase was a binary gradient of (A) 10 mM ammonium formate and 0.3 mM ethylenediaminetetraacetic acid in water adjusted to pH 3.5 using concentrated HCl and (B) methanol. Gradient was: 5 % B for 5 min, increased to 30 % B over 30 min, increased to 95 % B over 10 min, remained constant at 95 % B for 5 min, decreased to 5 % B over 2 min, and then re-equilibrated at 5 % B for 7 min. Chromatograms at 280 nm absorbance were analyzed.

DNA preparation. A 300 µL aliquot of each culture was mixed with a solution containing 500 µl of 200 mM Tris (pH 8.0), 200 mM NaCl, 20 mM EDTA, 200 µl of 20 % SDS, 500 µl of phenol:chloroform:isoamyl alcohol (25:24:1, pH 7.9) and 1.2 mg of 0.1-mm diameter zirconia/silica beads (BioSpecProducts). The

suspension was bead beated (3 min), spun at 8,000 rpm (5 min), and then top layer was transferred to a 15 ml tube for immediate column purification with 2.5 vol of NTl buffer, 3 washes with NT3 and final elution with 25 µl of elution buffer (Clontech, Machery-Nagel 740609.250).

16S rRNA gene V4 amplification and sequencing. PCR was performed using primers 515F and 806R for the variable 4 (V4) region of the bacterial 16S rRNA gene (25). PCR reactions contained 1 ng μl^{-1} DNA, 10 µM each primer, 12.5 µl 2X HotStart ReadyMix (KAPA Biosystems, Wilmington, MA, USA), and water to 25 µl. PCR program was 95 °C for 3 min, then 30 cycles of 95 °C for 30 s, 55 °C for 30 s, and 72 °C for 30 s, final step was 72 °C for 5 min. PCR products were purified by gel extraction from a 1.5 % low-melt agarose gel using a Zymoclean Gel DNA Recovery Kit (Zymo Research, Irvine, CA). Samples were quantified using the Qubit dsDNA HS assay (Invitrogen, Carlsbad, CA, USA) and equimolar concentrations pooled. The pool was sequenced with the MiSeq 2x250 v2 kit (Illumina, San Diego, CA, USA). All DNA sequences generated in this study will be deposited in NCBI's Short Read Archive.

16S rRNA Sequence Analysis. Sequences were demultiplexed on the Illumina MiSeq, and sequence clean-up was completed in Qiime2 Core 2018.11 (https://qiime2.org). Quality control, including removal of chimeras was performed with the DADA2 pipeline. The first 10 nucleotides were trimmed, and reads were truncated to 220 bases. DADA2 generates high-resolution tables of amplicon sequence variants (ASVs) which represent biological sequences in the sample differing by as little as one nucleotide (26). Taxonomy was assigned to ASVs using the feature-classifier classify-sklearn. The abundance of the resulting taxonomy assignments of ASVs was analyzed using STAMP 2.1.3 (statistical analysis of taxonomic and functional profiles) (27), with statistical comparisons between groups (e.x. control *vs*. quercetin treatment) performed by two-sided Welch'st-test within 95% confidence interval. A subset of 6 ASVs whose abundance increased in quercetin treatments were further analyzed using SILVA ACT (Alignment, Classification and Tree Service) (28), and 10 closest neighbors were downloaded from this analysis. ASVs that lack neighbors with a defined taxonomy at the genus level in SILVA ACT were subjected to BLASTn (https://blast.ncbi.nlm.nih.gov/) using the Whole-genome shotgun contigs (WGS) database for Clostridia (taxid:186801), 16s rRNA partial sequences from the most similar genomes with a defined taxonomy at the genus level were used for the phylogenetic analysis. This group of sequences were aligned and analyzed in MEGA 6.06 (29), the alignment file was used to construct a phylogenetic tree using the UPGMA method and a Distance Matrix for estimating evolutionary divergence between sequences (29–31). Complete ids for ASV enriched in quercetin treatments and accession numbers for reference sequences are listed in Fig. 2.3 (only the first 4 letters of each ASV are going to be mentioned throughout the text). Correlations (Spearman's rs and Bonferroni correction) and Principal component analysis (PCA) were done using PAST 3.23 (PAleontological STatistics) (32).

Results

Degradation of quercetin did not correlate with fiber content of diet. Germ-free mice were inoculated with fecal samples from 6 human subjects (HMAM mice) and fed diets with different fiber content, after a period of acclimatization to the diet, fecal pellets were retrieved and used for *in vitro* incubations with quercetin. The degradability of quercetin by fecal communities did not correlate with whether the fecal community came from mice fed a high or low content diet as shown in Table 2.1. For

some subjects, fecal communities that came from mice fed a diet high in fiber had a more pronounced quercetin degradation at day 7th of incubation and for others fecal communities that came from mice fed a diet low in fiber had a faster degradation. However, a PCA analysis showed that diet affected the composition of the microbial community (Fig. 2.1 A). Component 2 in PCA was explained by diet at 0 days of incubation (Fig. 2.1 A) but did not explain the grouping of component 2 at day 7th of incubation (Fig. S2.1 B in Appendix 2). An analysis of whether there were ASVs that explained the grouping at day 7th of incubation showed that the enrichment of two ASVs, ASV_65F4 and ASV_a45d, explained the grouping of component 2 at day 7th (Fig. 2.1 B) and not at day 0 of incubation (Fig. S2.1 A in Appendix 2), indicating an importance of these two ASVs after incubation with quercetin.

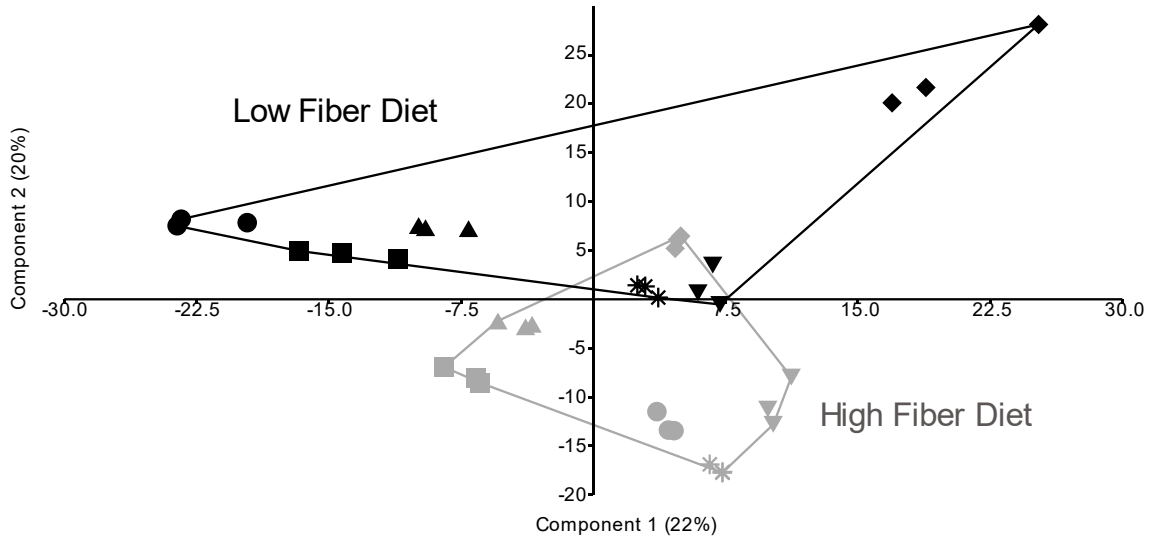
Table 2. 1

Concentration (mM) in culture of Quercetin for *in vitro* incubations with fecal samples from human microbiota-associated mice fed diets with different fiber content (day 7th of incubation).

Subject #	High Fiber Diet	Low Fiber Diet	Enrichment of *Flavonifractor*-related variant
Subject 1	0.004 ± 0.005	0.04 ± 0.02	ASV_a45d
Subject 2	0.01 ± 0.01	0.48 ± 0.07	ASV_65f4
Subject 3	0.61 ± 0.04	0.43 ± 0.01	ASV_65f4
Subject 4	0.48 ± 0.03	0.44 ± 0.05	ASV_a45d
Subject 5	0.38 ± 0.07	0.40 ± 0.22	ASV_65f4
Subject 6	0.14 ± 0.05	0.03 ± 0.03	ASV_a45d

Note: Average quercetin concentration for all samples at day 0 of incubation was 0.76 mM

A



B

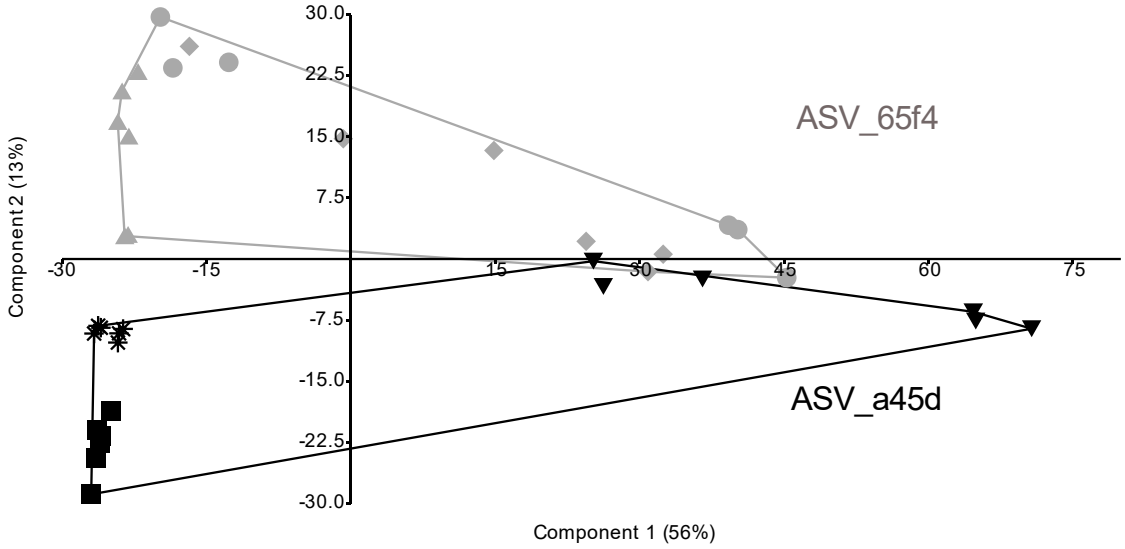


Figure 2. 1

Principal component analysis (PCA) plot of the libraries from *in vitro* incubations with fecal samples from human microbiota-associated mice (HMAM) at 0 (A) and 7 days of incubation (B). In the top panel, libraries from HMAM mice fed a high fiber diet are shown in gray and libraries from HMAM mice fed a low fiber diet in black. In bottom panel, libraries that were enriched in ASV_65f4 are shown in gray and libraries enriched in ASV_a45d are shown in black. Each symbol represents a library (n=6, 3 replicates), different shapes represent libraries from a different subject: #1, star (∗); #2, diamond (♦); #3, dot (●); #4, inv. triangle (▼); #5, triangle (▲); and #6, square (■).

Amplicon Sequence Variants ASV_65F4 and ASV_a45d were differentially enriched in *in vitro* incubations with fecal samples. After quercetin treatment, it was observed that ASV_65F4 and ASV_a45d dominated in different libraries (Fig. 2.2). The dominance of each ASV during incubation with quercetin could not be explained by their initial abundances (Fig. S2.2 A and B in Appendix 2). Even though ASV_a45d was lower in abundance (undetectable in most cases) than ASV_65f4, it dominated in half of the libraries after quercetin treatment. This pattern of dominance of ASV_65F4 over ASV_a45d or vice versa was not affected by diet (Fig. 2.2), however, the relative abundance of ASV_65F4 seemed to be more affected by the diet (Fig. S2.3). Additionally, the dominance of ASV_65F4 or ASV_a45d did not explained the rate of degradation of quercetin (Table 2.1).

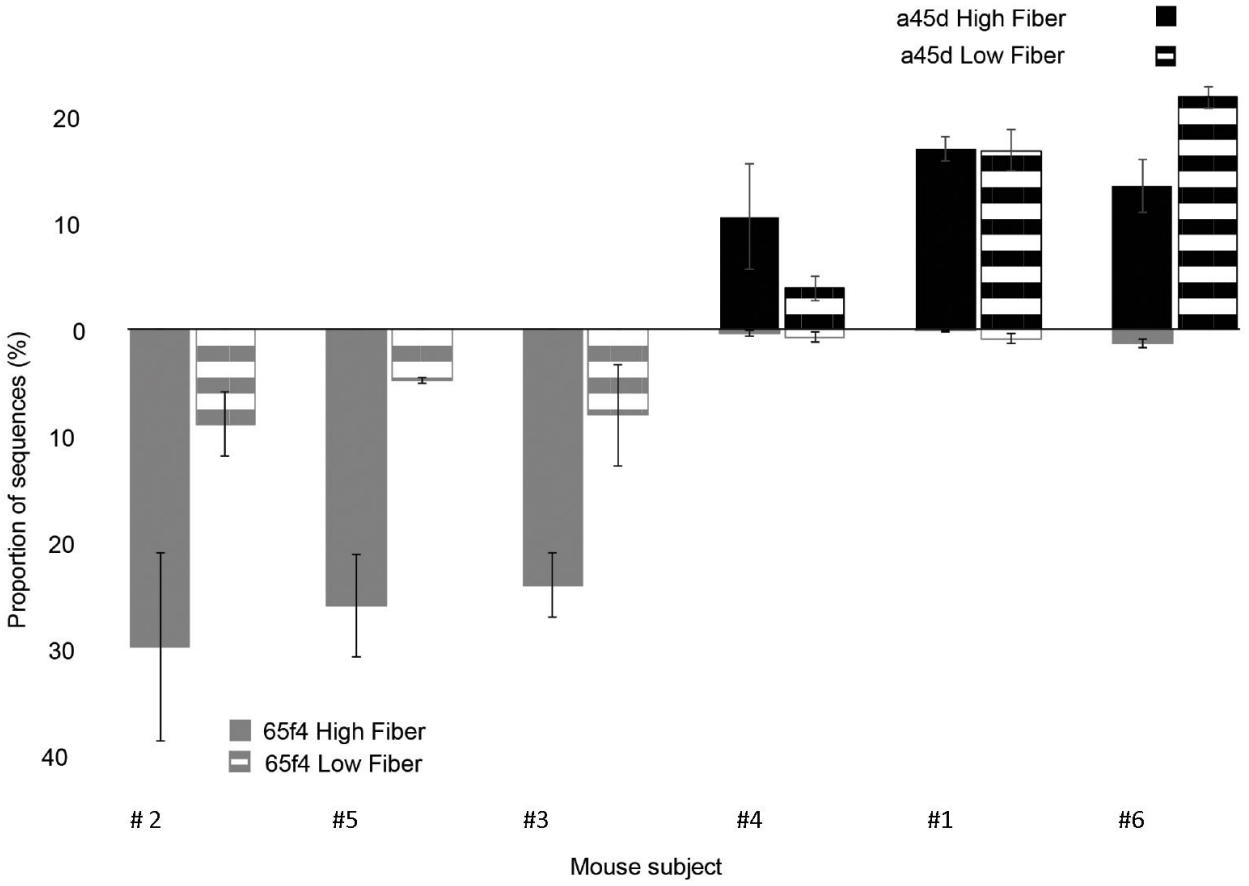


Figure 2. 2

Relative abundance of bacterial ASV_65f4 and ASV_a45d in HMAM mice fecal samples. ASV_65f4 is represented in gray and ASV_a45d in black. Libraries from HMAM mice fed a diet high in fiber are shown in solid color and mice fed a diet low in fiber are shown with line pattern. Error bars correspond to 3 incubations done with fecal matter from the same donor individually sampled. Relative abundances obtained after 7 days of incubation with quercetin are shown.

Amplicon Sequence Variants ASV_65F4 and ASV_a45d were most related to *Flavonifractor* spp.

After *in vitro* incubations with feces from HMAM mice, 9 human fecal samples different from the previous ones were directly inoculated in *in vitro* cultures with and without quercetin and two successive incubations were done. In this experiment, the main objective was not to compare between diets since the diet of the donors is not known but to identify bacteria that became enriched in two successive incubations with quercetin and whether ASV_65F4 and ASV_a45d were differentially enriched again. In all quercetin treatments, the main metabolite produced was DOPAC (Table S2.1 in Appendix 2). STAMP statistical analysis showed that 6 ASVs were identified as being enriched in one or more libraries when comparing quercetin treatments *vs* controls with no quercetin (Table S2.2 in Appendix 2), among these 6 ASVs were ASV_65f4 and ASV_a45d. A phylogenetic analysis revealed that the closest relative for ASV_65f4 was *F. plautii* (100 % identical in the 16S rRNA region V4) and ASV_a45d was most related to the *Flavonifractor* genus (98.6 % identical to *Flavonifractor* sp. An4 and An82) (Fig. 2.3 and Table S2.3). These ASVs were the ones that increased the most in abundance when quercetin was present, together with another one related to *E. ramulus* (ASV_c588) (Fig. 2.4). The other 3 ASVs increased slightly in relative abundance and were related to the genera *Eubacterium* (Lachnospiraceae) and *Intestinimonas* (Ruminococcaceae) (Fig. 2.3 and 2.4). Although these ASVs related to *Eubacterium* and *Intestinimonas* genera were enriched significantly in 1 or 2 libraries, *Flavonifractor*-related ASVs were found to be more ubiquitous after quercetin incubation, with at least one variant significantly enriched in every library (Table S2.2 in Appendix 2). It should be noted that one of these *Flavonifractor*-related variants, ASV_65f4, showed also a significant increase when no quercetin was present in the medium (Fig. S2.4 A in Appendix 2), indicating that this ASV is favored by the culture conditions used. Nevertheless, its relative abundance increased significantly more when quercetin was present (Fig. S2.4 B in Appendix 2). This behavior did not change when higher concentrations of fecal matter were tested (1 and 10 mg/ml). The other *Flavonifractor* variant, ASV_a45d, showed no enrichment in media with no quercetin. An important observation was that libraries that came from human fecal samples showed a differentially enrichment of ASV_65F4 and ASV_a45d after quercetin treatment (Fig. 2.5), as it happened with libraries that came from HMAM fecal samples (Fig. 2.2). We then further examined this pattern.

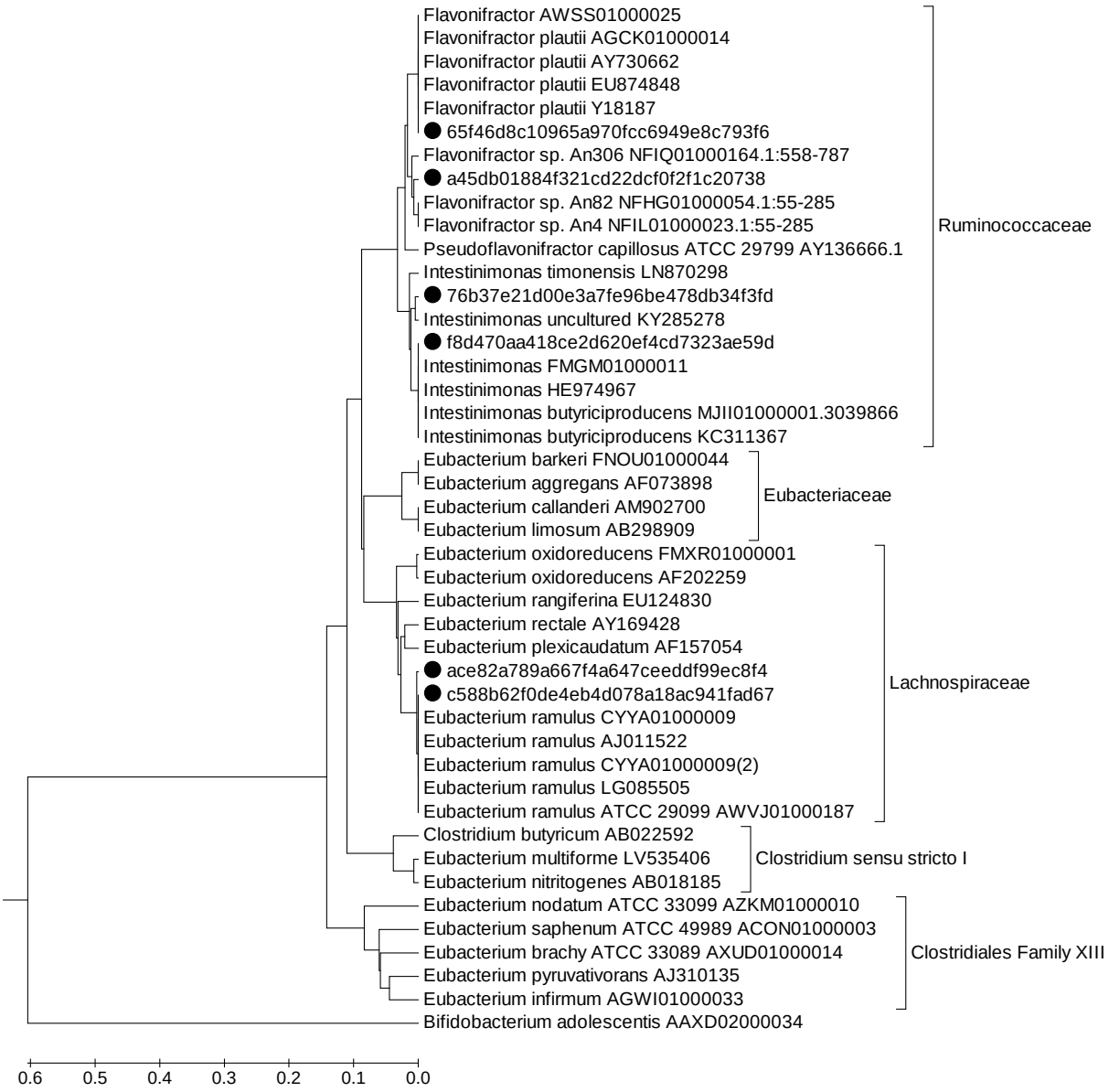


Figure 2. 3

Phylogenetic analysis of taxa enriched in the presence of quercetin. Phylogenetic tree shows six ASVs (black dots) whose abundance increased in the presence of quercetin; distances in the tree were inferred using the UPGMA method (31). The optimal tree with the sum of branch length = 2.18101115 is shown. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Maximum Composite Likelihood method (30) and are in the units of the number of base substitutions per site. The analysis involved 44 nucleotide sequences. All positions containing gaps and missing data were eliminated. There were a total of 225 positions in the final dataset. Evolutionary analyses were conducted in MEGA6 (29).

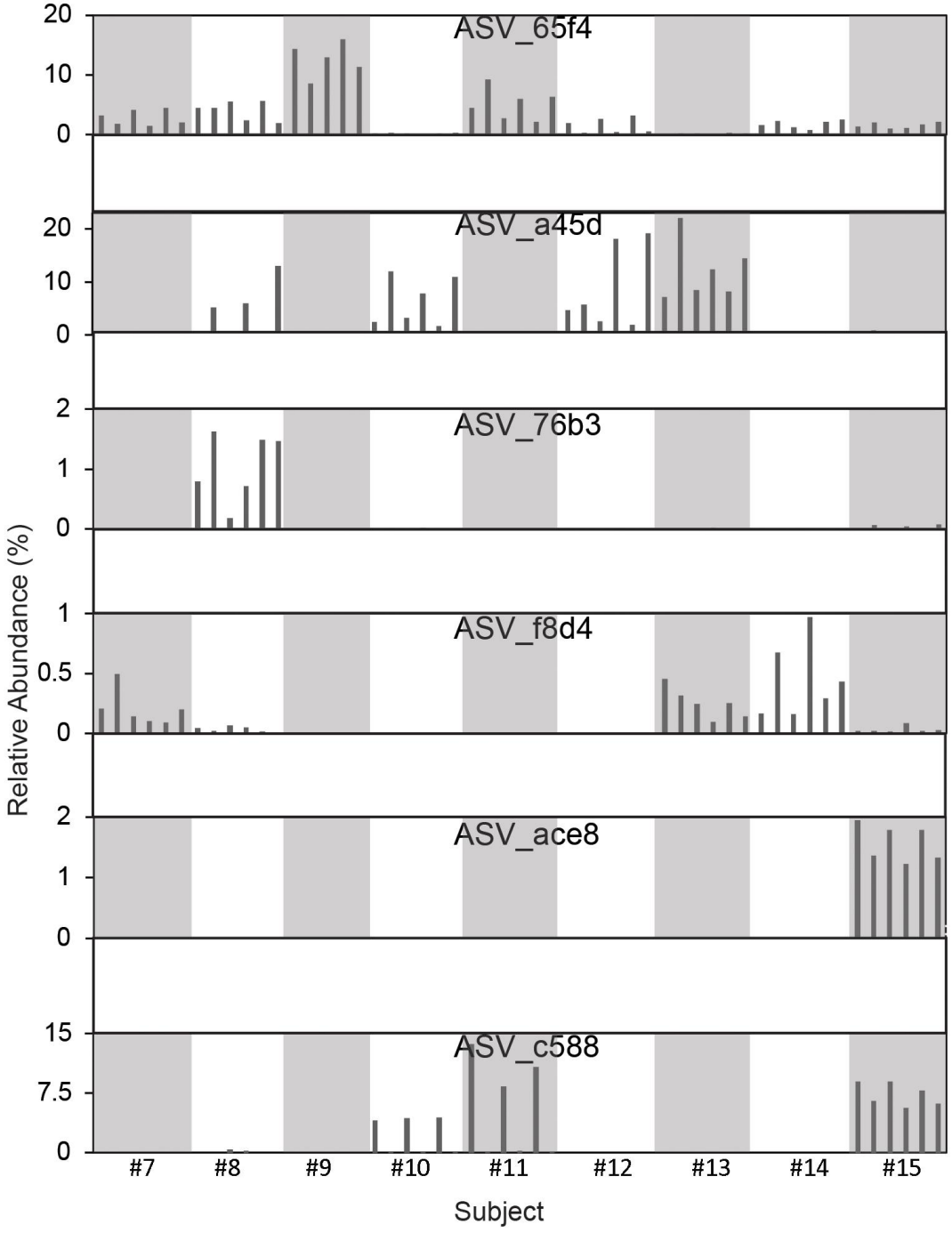


Figure 2. 4

Bar plots for the relative abundances of the 6 ASVs enriched in quercetin. Human subjects are labeled as Subject #7, #8, #9, #10, #11, #12, #13, #14, #15 in bar plots, for better visualization intercalated libraries are highlighted in gray. Each library has 6 bars, 3 corresponds to replicates from first incubation and 3 from second incubation (Subject #9 had only 2 replicates for second incubation).

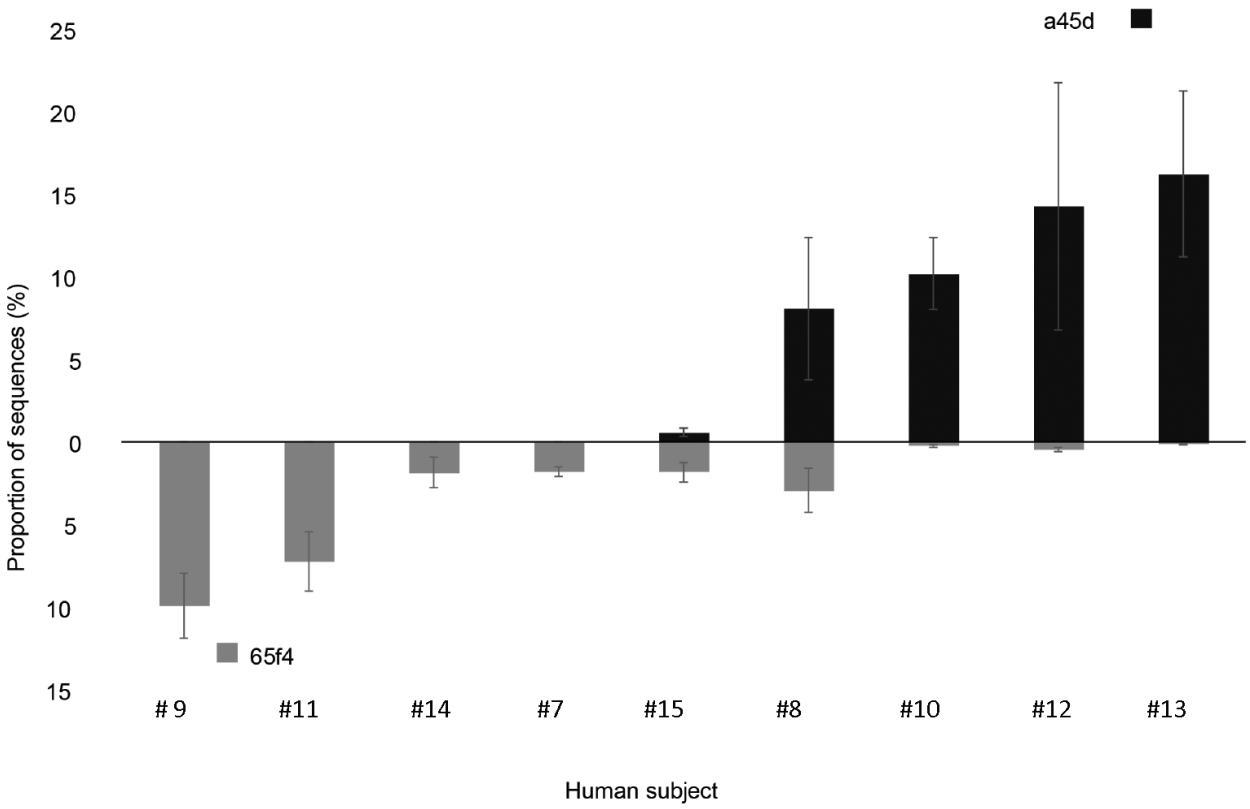


Figure 2. 5

Relative abundance of bacterial ASV_65f4 and ASV_a45d in human fecal samples (n=9). ASV_65f4 is represented in gray and ASV_a45d in black. Error bars correspond to 3 incubations done with fecal matter from the same donor individually sampled. Relative abundances obtained for the second incubation are shown.

Amplicon Sequence Variants (ASV) related to *Flavonifractor* spp. were negative correlated in *in vitro* incubations with fecal samples. After observing that ASV_65f4 and ASV_a45d were differentially enriched in *in vitro* incubations done with both types of fecal samples, HMAM mice and human feces, a correlation analysis across the libraries of the abundances of the 6 ASVs enriched in quercetin treatments was performed. This analysis showed a strong negative correlation between ASV_65f4 and ASV_a45d after quercetin incubation (Fig. S2.5 in Appendix 2) and was not present in incubations without quercetin.

Fecal sample combinations showed dominance of ASV_65f4 over ASV_a45d. The biological phenomenon between ASV_65f4 and ASV_a45d was observed in fecal samples from 15 different subjects, thus, we aimed to continue studying this pattern in cocultures. Unfortunately, only ASV_65f4 was isolated in pure culture while ASV_a45d could not be isolated. Thus, an experiment combining fecal samples that were previously enriched in ASV_65f4 or ASV_a45d was carried out (combination of fecal samples from subjects #9 and #10 previously enriched in ASV_65f4 and ASV_a45d, respectively, Fig. 2.5). In this experiment, we expected that if the combination of fecal specimens had no effect on the *Flavonifractor*-related variants, we should observe a reduction in their relative abundance corresponding only to the dilution factor. This means that when samples #9 and #10 were combined, the relative abundances of ASV_65f4 and ASV_a45d should reach 50 % of the one reached when fecal samples are not combined. However, it was observed that when samples #9 and #10 were combined (50:50), ASV_65f4 dominated reaching a relative abundance comparable to the one reached when fecal samples #9 and #10 were not mixed (Fig. 2.6). Meanwhile, the relative abundance of ASV_a45d was severely affected by the combination of fecal samples reaching a relative abundance below 1 % (Fig. 2.6).

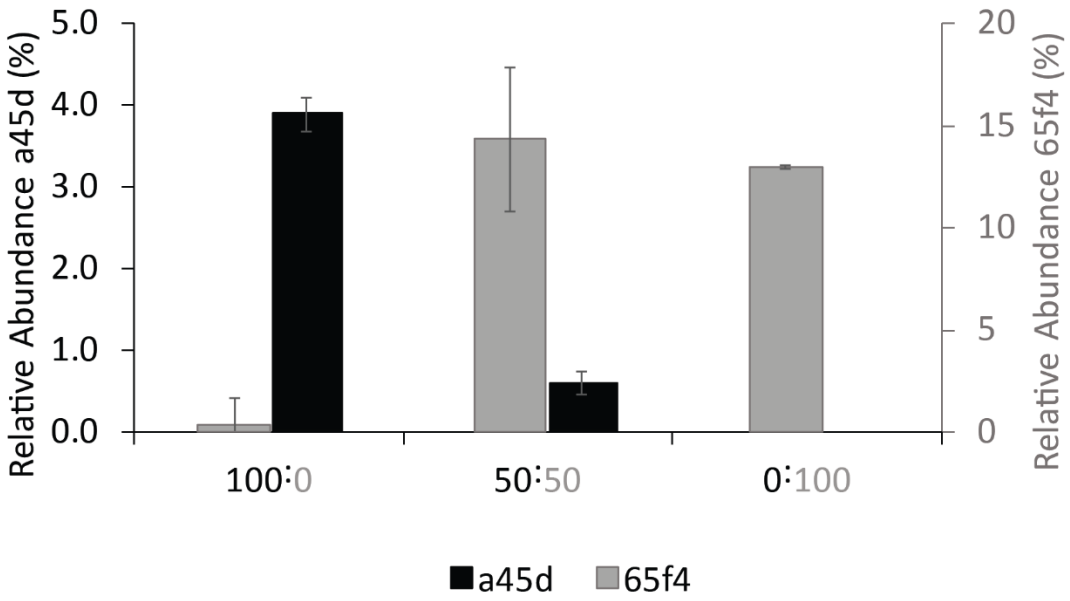


Figure 2. 6

Relative abundance of bacterial ASV_65f4 and ASV_a45d in *in vitro* incubations with fecal samples from subjects #9 and #10 combined (50:50) or not (100:0 and 0:100). *In vitro* incubations with fecal sample from subject #9 (enriched in ASV_65f4) are shown in gray (right) and from subject #10 (enriched in ASV_a45d) are shown in black (left).

Dominance of ASV_a45d or ASV_65f4 was associated with genera *Desulfovibrio* and *Phascolarctobacterium.* We also searched for organisms whose relative abundance were associated with the two variants. We found that when ASV_a45d dominated after incubation with quercetin, the relative

abundance of *Desulfovibrio* was significantly higher ($p<0.05$ in all 3 experiments) (Fig. 2.7 A). When present, *Desulfovibrio* sp. increased in relative abundance with or without quercetin, indicating its metabolic resilience under the culture conditions. Meanwhile, when ASV_65f4 was dominant, the relative abundance of the genus *Phascolarctobacterium* was significantly higher ($p<0.05$ in all 3 experiments) (Fig. 2.7 B). This genus increased in relative abundance with or without quercetin as well.

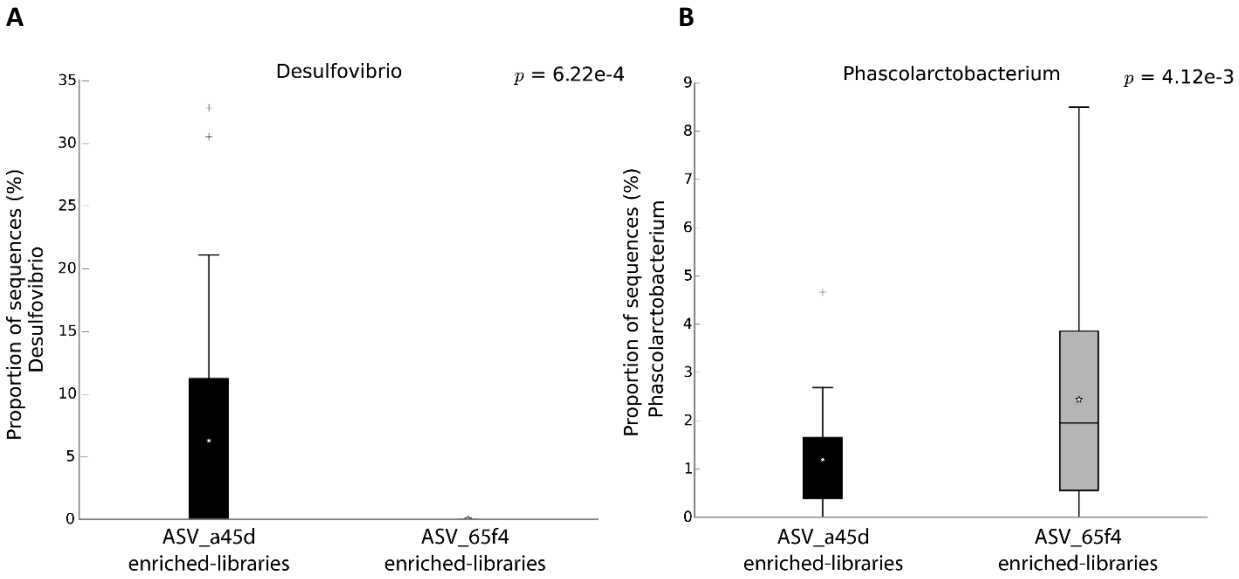


Figure 2. 7

Box plots for the relative abundances of the genera *Desulfovibrio* (A) and *Phascolarctobacterium* (B) in *in vitro* incubations with quercetin. Analysis for libraries from human subjects #7-#15 grouped by their enrichment in ASV_a45d (black) or ASV_65f4 (gray) is shown. Box plots were calculated with Statistical Analysis of Taxonomic and Functional Profiles (STAMP).

Discussion

Quercetin is present in most fruits and its degradation produces biological active metabolites with effects on the host. Extending our knowledge of quercetin-degrading communities is important for predicting the health outcomes of flavonoid consumption. To study this matter, we used *in vitro* fecal incubation system which can isolate the effect of the microbiota on the flavonoid. In order to limit the enrichment of non-quercetin degraders in these incubations, a medium low in carbon sources was used (20 mM of acetate). Under these conditions, mostly *Flavonifractor*-related sequences were enriched across libraries, specifically ASV_65f4 and ASV_a45d. These ASVs belong to different species, sharing 96.4 % of identity in their 16S rRNA variable region V4 (33). ASV_65f4 was 100 % identical to *F. plautii* in this region, while ASV_a45d was 98.6 % identical to *Flavonifractor* sp. An4 and An82, which were isolated by Medvecky and collaborators from chicken cecum, both are described as *Flavonifractor* sp. and have whole genome assembly projects (34). Several of the *Flavonifractor* strains isolated from the chicken cecum, including strain An4 and An82, have a predicted phloretin hydrolase gene in their genomes which catalyzes the hydrolytic C-C cleavage of phloretin, a flavonoid structurally similar to quercetin (35), generating phloroglucinol and 3-(4-hydroxyphenyl)propionic acid, this enzyme is well characterized for another quercetin-degrader, *E. ramulus* (36). This suggests that strains An4 and An82 may harbor the enzymatic machinery necessary for the cleavage of the C-ring in quercetin as well, as *F. plautii* and *E. ramulus* do (37,38). Since ASV_a45d was closely related to *Flavonifractor* sp. strains An4 and An82 and *F. plautii*, and was enriched in quercetin treatments with DOPAC production, even in the absence of ASV_65f4 and *E. ramulus*-related ASVs, this may indicate its metabolic capability for degrading quercetin.

ASVs related to *E. ramulus* were detected only in 4 out of 9 human fecal samples and none of the mice samples. *E. ramulus*-related ASV were significantly increased by quercetin treatment only in one sample. This bacterium was enriched in a previous study that supplemented with quercetin the diet of healthy volunteers under a flavonoid-free intervention (39). However, under the culture conditions of this study, ASVs related to *Flavonifractor* were more prevalent. Another genus related to two of the ASVs enriched by quercetin treatments was *Intestinimonas*. Despite the relatedness of this bacterium with *Flavonifractor* sp., the ability to degrade quercetin was not detected in *Intestinimonas butyriciproducens* (40). However, it is not known whether this bacterium can use metabolites derived from quercetin degradation like phloroglucinol. In some of our incubations, the genus *Coprococcus,* which is reported to use phloroglucinol, increased in relative abundance (41).

Correlations can be useful for identifying potential ecological interactions between microbial species. An interesting pattern of negative correlation was observed for *Flavonifractor*-related variants, ASV_65f4 and ASV_a45d. This may indicate a potential antagonistic interaction between these two species. Antagonism is more prevalent among phylogenetically and metabolically similar species. A study that screened 2,211 competing bacterial pairs from 8 different environments found that antagonism increased significantly between closely related strains and between strains that had a greater overlap in growth on the 31 carbon sources screened through the Biolog assay (42). Since both ASVs from our study are phylogenetically related and both may have the capacity to degrade quercetin, this could explain the observed pattern of dominance of one or the other but not both. In competition assays, it is often the species that starts at high initial abundance the one that dominates (43). In our experiments, initial

relative abundances did not predict which variant will dominate. Despite being less abundant at the initial point of incubation, ASV_a45d dominated over ASV_65f4 in half of the libraries after incubation with quercetin. However, when two fecal samples previously enriched in one or the other variant were combined, ASV_65f4 was always the strongest competitor and the dominant variant. Therefore, this evidence suggests that other processes besides initial relative abundances might be responsible for the dominance of the weaker competitor, ASV_a45d. For example, the explanation for this pattern of dominance might lay on the interactions with other species in the microbial community, thus species that might favor one or the other variant were searched.

In metataxonomic studies, a limiting factor in the analysis is that gut microbial communities are very variable at the species level among individuals, being more homogenous at the metabolic level (44), then shared species among libraries are scarcer. Despite of this, we were able to identify two genera that were associated with the dominance of ASV_65f4 or ASV_a45d. The relative abundance of the H_2S-producing genus, *Desulfovibrio*, was significantly higher when ASV_a45d dominated, indicating that ASV_65f4 might be affected by this species or its metabolites. A subsequent genomic comparative analysis revealed why hydrogen sulfide (H_2S) might affect this variant (see Chapter 3, 'Functional Profiling of Genomes from Flavonoid-Degrading Bacteria and Their Predictive Ecological Role in the Gastrointestinal Tract'). *Desulfovibrio* sp. is an acetate utilizing bacterium which produces hydrogen sulfide (45), a very toxic metabolite that bacteria detoxify (46). This might be converted to thiosulfate (47) and thiosulfate to tetrathionate (48). When tetrathionate becomes available, there are some bacteria that can use it as an electron donor for ethanolamine catabolism (49), as we show in Chapter 3, ASV_65f4 might be an ethanolamine-utilizing species, thus, in the presence of tetrathionate the enhanced competition for this substrate may affect ASV_65f4. Other explanations might be that hydrogen sulfide can inhibit the action of phosphodiesterases (50), enzymes needed for the conversion of phosphatidylethanolamine to ethanolamine or it may have a direct impact on ASV_65f4. Meanwhile, when ASV_65f4 dominated, the abundance of a succinate-utilizing bacterium, *Phascolarctobacterium,* was significantly higher. Two species of this genus are reported to only utilize succinate as a carbon source (51,52). Succinate is not a major fermentation product in human feces but saccharolytic bacteria which are abundant in the gastrointestinal tract can produce it and may be a substrate that bacteria can specialize on in order to coexist with bacteria that can readily utilize other more abundant carbon sources (51). The increase in abundance of *Phascolarctobacterium* sp. when ASV_65f4 dominated suggests that succinate became available under these conditions. Succinate is the product of fermentation of certain bacteria that use acetate as a carbon source and that under O_2 depletion accumulate succinate, they slowdown the tricarboxylic acid (TCA) cycle activity and may shift to an alternative route called glyoxylate shunt-based TCA cycle which uses fewer enzymes than the TCA cycle and generates glyoxylate and succinate (53,54).

Carbon sources are a major factor affecting the rate of flavonoid degradation and the dynamic operations of gut bacteria. We fed two diets to HMAM mice that varied largely in their content of plant polysaccharides. The diet low in fiber had corn starch, maltodextrin, and sucrose, while the diet high in fiber had ground wheat, ground corn, wheat middlings, dehulled soybean meal, and corn gluten meal. The diet high in fiber has a higher content of complex carbohydrates that resist digestion in the upper gut that can reach the colon and promote microbial fermentation (55). There are some studies that have shown the importance of dietary fiber for shaping the fate of flavonoid metabolism, for instance, mice fed resistant starch and FOS have higher levels of bacterial products of flavonoid metabolism (56,57).

Meanwhile, the abundance of *E. ramulus* in the gut has been shown to be positively influenced by the presence of dietary fiber (39). In our study, only the abundance of ASV_65f4 responded to the diet fed to the mice, this suggests that available carbon sources have the potential to differentially affect variants of *Flavonifractor*. Nevertheless, diet seemed to have no effect on whether ASV_65f4 or ASV_a45d dominated, pointing at the resilience of this pattern. The results of this study show that Flavonifractor-related variants are differentially affected by the host diet and the microbial communities associated with these variants exhibit different patterns of change when one or the other variant becomes enriched. Whether there is competition between Flavonifractor-related variants during flavonoid consumption warrants further investigation.

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Chapter 3. Functional Profiling of Genomes from Flavonoid-Degrading Bacteria and Their Predictive Ecological Role in the Gastrointestinal Tract

Abstract

Flavonoid research is focused on the health-promoting properties of these phenolic compounds. However, our understanding of gut-dwelling flavonoid-degrading bacteria is still limited and it is not known whether harboring certain bacterial species able to transform these compounds affects the overall metabolic outcomes associated with their consumption. In previous findings from our group, different variants of potential flavonoid-degrading bacteria were detected in a population of Wisconsin, USA. In order to predict the metabolic impact of harboring a specific population of flavonoid-degrading bacteria, we analyzed the functional profiles of different species of flavonoid-degraders, *Flavonifractor plautii* and *Eubacterium ramulus,* using a general annotation tool and one specialized on carbohydrate-active enzymes, that allows for reconstruction of complete pathways, and comparisons of orthologous gene clusters. This comparative genomic analysis enabled us to predict that there are groups of species with different carbohydrate utilization capablities, including galactose, galacturonate, and glucuronate. This is important since dietary compounds have been shown to impact distinctively the bioavailability of flavonoids. Additionally, we found one predicted glycoside hydrolase in *E. ramulus* that might have a domain with the ability to transfer hydroxyl ions between phenols, including the metabolic intermediate of flavonoid degradation, phloroglucinol. We also observed that a group of strains most related to *E. ramulus* might be important in the production of butyrate from protein sources. Other traits that may be important during opportunistic infections, such as ethanolamine utilization and presence of flagella, were enriched in a group of strains closely related to *F. plautii*. The genomic snapshot of these groups of flavonoid-degrading bacteria showed metabolic and structural differences (i.e. flagella) that might have different effects on the host and the gut microbiome.

Introduction

Flavonoids are 3-ring phenolic compounds produced by the secondary metabolism of plants. Their consumption in the human diet has been associated with health benefits on the immune, digestive, endocrine, nervous, and cardiovascular systems [1]–[6]. However, the intestinal microbiota has the capacity to transform these compounds in smaller phenolic compounds that may have different biological activity and bioavailability. Among the bacteria with a recognized flavonoid-degrading capability are *Flavonifractor plautii* and *Eubacterium ramulus.* Both of these gut bacteria are able to transform quercetin, one of the most abundant flavonoids in human diet, to phloroglucinol and 3,4-dihydroxyphenylacetic acid (DOPAC) [7], [8].

We recently showed that there are different variants of quercetin-degrading bacteria in human fecal sample libraries of a population of Wisconsin, USA. Two variants of each of these bacteria were present and became enriched after quercetin incubation (Chapter 2: “Gut-derived *Flavonifractor* species variants are differentially enriched during *in vitro* incubation with Quercetin”). *E. ramulus* variants shared 99.5% of identity in the variable region V4 of the 16S rRNA gene, meanwhile *Flavonifractor* variants were more distant, sharing only 96.4 % of identity in this same region. The closest relative for one of these

variants was *F. plautii* (100% identical), while the other variant was more closely related to *Flavonifractor* sp. strains An4 and An82 (98,6% identical), isolated by Medvecky and collaborators from chicken cecum and which have whole genome assembly projects [9]. Additionally, we observed metabolic differences between these two *Flavonifractor*-related variants.

In this study, we aimed to analyze the functional profile of the representative genomes of flavonoid-degraders, *F. plautii* YL31 and *E. ramulus* ATCC 29099, and their comparison to genomes of close relatives, including those most related to the *Flavonifractor*-variant enriched in fecal incubations with quercetin that were done in our previous work (*Flavonifractor* sp. strains An4 and An82). This analysis revealed metabolic and structural differences that were used to make ecological predictions that these flavonoid-degrading bacteria might present in their natural niche, the gastrointestinal tract.

Methods and Materials

Genomes used in this study. Twenty seven genome assemblies were downloaded from the NCBI genomes database on October 2019 (https://www.ncbi.nlm.nih.gov). A total of 5, 1, 1 and 20 genomes were downloaded for *E. ramulus, Eubacterium rectale* (non-quercetin degrader), *Eubacterium oxidoreducens* (quercetin degrader), and *Flavonifractor* spp., respectively. Accession numbers and information about completeness is presented in Table S3.1 (Appendix 3). In order to determine the species relationship of the genomes described as *E. ramulus* and *Flavonifractor* spp. the Average Nucleotide Identity (ANI) was calculated using the online tool JSpeciesWS (http://jspecies.ribohost.com/jspeciesws/) [10] which performs pairwise comparisons between two genomes calculating and indicating if a pair of genomes belongs to the same species and/or genus based on their percentage of identity. After this analysis, we selected all 5 genomes for *E. ramulus*. For *Flavonifractor* sp. we chose 8 genomes, 4 that represent the general features of the species *F. plautii* and 4 that represent the general features of the genus *Flavonifractor* (the 4 genomes belong to different species at a ANI percentage of 86-89%) and had the highest coverage in the ANI analysis (*Flavonifractor* sp. strains An4, An10, An82, and An306) (Fig. S3.1 and S3.2 in Appendix 3 show the phylogenetic relatedness of *F. plautii* and *Flavonifractor* sp. strains, respectively). Two genomes related to *E. ramulus*, UHGG_MGYG-HGUT-02278 and MGYG-HGUT-01456, did not have gene predictions, thus predictions were done using Prodigal (prokaryotic dynamic programming gene finding algorithm) [11].

Prediction of functional profiles. Annotation of functions was done using GhostKOALA (KEGG Orthology And Links Annotation, https://www.kegg.jp/ghostkoala/), an automatic annotation and mapping service using the database 'genus_prokaryotes' [12]. Other analyses that were performed were the prediction of signal peptides with SignalP-5.0 (http://www.cbs.dtu.dk/services/SignalP/) [13] and prediction of glycoside hydrolases with dbCAN2 meta server (automated Carbohydrate-active enzyme ANnotation, http://bcb.unl.edu/dbCAN2/) (predicted at least with 2 tools) [14]. Completeness of pathways was screened using the KEGG Mapper Reconstruction tool (https://www.genome.jp/kegg/tool/map_pathway.html).

Genome comparative analysis. Prediction of orthologous gene clusters was done using OrthoVenn2 (https://orthovenn2.bioinfotoolkits.net/home) [15]. Analysis for *Eubacterium* spp. and

Flavonifractor spp. genomes were run independently. We applied OrthoVenn2 clustering to identify gene clusters enriched in the groups most related and distantly related to *E. ramulus* ATCC 29099 or *F. plautii* YL31 in order to compare between groups. Proteins similar to flavonoid-degrading proteins were searched using the Blast tool in OrthoVenn2. For *E. ramulus*, Enoate Reductase (ERED) (AGS82961.1) and Phloretin hydrolase (AAQ12341.1), and for *Flavonifractor* spp. NADH oxidase (CUQ13575.1) and Phloretin hydrolase (OXE48401.1) were taken as reference proteins [16].

Evolutionary relationships of taxa. Evolutionary history was inferred using the UPGMA method [17]. Trees are drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Maximum Composite Likelihood method [18] and are in the units of the number of base substitutions per site. Evolutionary analyses were conducted in MEGA6 [19].

Results and Discussion

Representative genomes of *F. plautii* YL31 and *E. ramulus* ATCC 29099. Flavonoid-degrading bacteria influence host physiology transforming phenolic compounds with health benefits in smaller phenolic molecules that may have different biological activity and bioavailability compared to the parent compounds, but this type of bacteria are still under-characterized. This poses a limitation for the understanding of the interaction between gut microbiome, host, and diet. In the series of analyses that we performed, we first searched for the presence of complete KEGG modules, defined functional units of gene sets and reaction sets, in the representative genomes of *F. plautii* YL31 and *E. ramulus* ATCC 29099. Pathways for carbohydrate utilization, amino acid biosynthesis, secretory proteins and transport systems, vitamins biosynthesis, Short-Chain Fatty Acids (SCFAs) biosynthesis, antibiotic resistance, and oxygen tolerance are described below.

Carbohydrate metabolism. Both *F. plautii* YL31 and *E. ramulus* ATCC 29099 have the complete pathways for glycolysis and citrate cycle. Additionally, *E. ramulus* ATCC 29099 has the complete pathway for galactose degradation, this capacity is reported in the literature [20]. In the other hand, *F. plautii* YL31 has the pathways for the degradation of D-galacturonate and D-glucuronate. Galacturonic acid is the main monomer in pectin, while glucuronic acid is a glucose-derived sugar acid, which is linked via a glycosidic bond to other substances forming a glucuronide. Glucuronidation is a common mechanism of animal bodies for the detoxification of toxic substances, drugs or compounds that cannot be used as energy sources. Glucuronides can be hydrolyzed by bacterial beta-glucuronidases, then the aglycones are re-absorbed and enter the enterohepatic recirculation. This cycle contributes to prolonged exposure to certain compounds which can have health effects on the host. For example, when bacterial glucuronidases remove the glucuronic acid from carcinogens (e.g. dietary heterocyclic amines) this poses a health risk [21], while the same bacterial activity on glucuronidated flavonoids may help intestinal cells be exposed to aglycon flavonoids which can have a positive health effect [22]. *F. plautii* YL31 does not harbor any glycoside hydrolase families with beta-glucuronidase activity (GH1, GH2, GH30, GH79 or GH154) (Table 3.1). Thus, we predict that *F. plautii* YL31 only can use glucuronate as a carbon source when released by other bacteria. An analysis of other classes of glycoside hydrolases revealed that *E. ramulus* ATCC 29099 had 20 different classes of glycoside hydrolases (Table 3.2) while *F. plautii* YL31 only had 11 (Table 3.1),

which may indicate that *E. ramulus* ATCC 29099 has more versatility for the degradation of carbohydrates than *F. plautii* YL31. Interestingly, it has been reported that *F. plautii* strain I2 is only capable of degrading the flavonoid quercetin when it is in its aglycone form while *E. ramulus* strain wK1 is able to degrade the glycosides, as well [23].

Table 3. 1.

Comparison of predicted Glycoside Hydrolases for *Flavonifractor* spp.

Glycoside Hydrolase Class	YL31	2789STDY 5834932	ATCC 29863	An248	An4	An10	An82	An306
GH2	-	-	-	-	-	-	-	+
GH3	+P	+P	+P	+P	+	+	+P	+
GH4	+	+	+	+	-	-	-	-
GH13_4	-	-	-	-	+	-	+	-
GH13_9	+	+	+	+	+	+	+	+
GH13_11	-	-	-	-	+	-	+	-
GH13_20	-	-	-	-	+	+	+	+
GH13_31	-	-	-	-	+	-	+	+
GH13_39	+	+	+	+	+	+	+	+
GH18	+P	+P	+P	+P	+P	+	+P	+P
GH20	-	-	-	-	+P	-	-	-
GH25	+P	+P	+P	+P	+	+P	+	+
GH29	-	-	-	-	-	-	-	+
GH31	+P	+P	+P	+P	-	+	-	-
GH32	-	-	-	-	+	-	+	-
GH33	+P	+P	-	+P	-	-	+P	-
GH36	-	-	-	-	+	-	+	-
GH42	-	-	-	-	-	-	-	-
GH65	+	+	+	+	-	+	-	+
GH77	+	+	+	+	+	+	+	+
GH79	-	-	-	-	-	-	-	+
GH84	-	-	-	-	+P	-	-	-
GH112	-	-	-	-	-	-	-	+
GH125	+	+	+	+	-	+	-	-

+ GH predicted

+P GH predicted and has a signal peptide

- GH not predicted

Table 3. 2.

Comparison of predicted Glycoside Hydrolases for *Eubacterium* spp.

Glycoside Hydrolase Class	ATCC 29099	2789STDY5608891	MGYG01456	MGYG02278	Strain 21	*E. rectale*
GH2	+	+	+	+	+	+P
GH3	+P	+P	+P	+P	+P	+
GH13_9	+	+	+	+	+	+
GH13_14	-	-	-	+P	+P	+
GH13_18	+	+	+	+	+	+
GH13_20	+	+	+	+	+	+
GH13_31	+	+	+	+	+	+
GH13_39	+	+	+	+	+	-
GH18	+	+	+	+	+	+
GH20	+	+	+	+	+	-
GH23	+	+	+	-	-	+
GH30_5[a]	+P	+P	+P	+P	-	-
GH32	+	+	+	-	-	+
GH35	+	+	+	+	+	-
GH36	+	+	+	+	+	+
GH39	-	-	-	-	+	-
GH42	+	+	+	+	+	+
GH43_22	-	+P	-	-	-	-
GH43_24	-	-	-	+P	-	-
GH43_37	-	-	-	+P	-	-
GH51	+	+	+	+	-	+
GH73	-	+	-	+P	-	+P
GH77	+	+	+	+	+	+
GH78	-	-	+	-	-	-
GH101	+P	+P	+P	+P	-	-
GH112	+	+	+	+	+	+
GH136	+P	+P	+P	+P	+	-
GH1	-	-	-	-	-	+
GH5_2	-	-	-	-	-	+P
GH8	-	-	-	-	-	+
GH13_11	-	-	-	-	-	+
GH13_36	-	-	-	-	-	+P[b]
GH13_41	-	-	-	-	-	+P[b]
GH24	-	-	-	-	-	+
GH25	-	-	-	-	-	+P

GH31	-	-	-	-	-	+
GH43_10	-	-	-	-	-	+
GH43_12	-	-	-	-	-	+
GH53	-	-	-	-	-	+
GH94	-	-	-	-	-	+
GH133	-	-	-	-	-	+

+ GH predicted.

+P GH predicted and has a signal peptide.

- GH not predicted.

[a] Predicted Cna B domain protein in [*Clostridium*] *saccharolyticum* (Lachnospiraceae family) (Uniprot: D9R566).

[b] Predicted periplasmic amylase in *E. rectale* (Uniprot; GH13_36: C4ZC46 and GH13_41: C4ZGP6).

Note: *E. oxidoreducens* was excluded because it had few GH predictions.

Amino acid biosynthesis. The *de novo* microbial synthesis of amino acids represents a contribution to the host requirements for amino acids. For instance, Torrallardona and collaborators (2003) estimate that microbial lysine absorbed in the intestines contributes to 10 % of the requirement in pigs [24]. Another group has estimated this to be 5 to 9 % in healthy men under an adequate diet [25]. Both *F. plautii* YL31 and *E. ramulus* ATCC 29099 have complete pathways for the biosynthesis of essential amino acids histidine, lysine, arginine, valine, isoleucine, and leucine. Additionally, *F. plautii* YL31 has the complete pathway for the synthesis of serine and threonine and *E. ramulus* ATCC 29099 has the one for tryptophan. They also have the complete pathway for the synthesis of the conditionally essential amino acids, cysteine and proline.

Secretory proteins and transport systems. An analysis of signal peptides revealed non-cytosolic proteins (inner membrane, periplasmic, outer membrane/extracellular). In *E. ramulus* ATCC 29099, about 7.8 % of proteins had a secretory signal (270 out of 3461) while *F. plautii* YL31 had 8.7 % (312 out of 3592). Many of these proteins may be involved in the processing and/or transportation of dietary substrates, like carbohydrates, peptides, and amino acids. Among the non-cytosolic proteins with predicted function, *E. ramulus* ATCC 29099 had 9 predicted sugar transporters and 8 peptide/amino acid transport systems. Interestingly, *F. plautii* YL31 had a similar number of sugar transporters (8) but it had a much larger number of predicted peptide/amino acid transport systems, 25, which suggests that *F. plautii* YL31 may rely more on proteins for its nutrient requirements. In previous experiments, we have observed that *E. ramulus* ATCC 29099 grows well in minimal media supplemented with glucose [26] while *F. plautii* YL31 requires the addition of tryptone, yeast extract, meat extract, and lysine to the same media (it doesn't grow on glucose under laboratory conditions). Thus, this may indicate that the metabolism of *F. plautii* YL31 is favored toward the utilization of proteins, at least in *in vitro* cultures.

Vitamin biosynthesis. The human body cannot synthesize most vitamins, thus the gut microbiota is an important supplier of these vitamins. Both *F. plautii* YL31 and *E. ramulus* ATCC 29099 have the complete pathway for the biosynthesis of the complex vitamin cobalamin, the only vitamin exclusively

produced by microorganisms. Additionally, *E. ramulus* ATCC 29099 has also almost complete pathways (1 step missing) for the biosynthesis of thiamine, riboflavin, pantothenate, and tetrahydrofolate.

Short-Chain Fatty Acid (SCFA) biosynthesis. Both *F. plautii* YL31 and *E. ramulus* ATCC 29099 are known acetate and butyrate producers [20], [27]. As expected, our analysis revealed that both have the pathways acetyl-CoA fermentation to butanoate II and pyruvate fermentation to acetate I and IV. Butyrate or butanoate is essential for colon health, it is a major energy source for colonocytes, inhibits colon inflammation and carcinogenesis, and has an important role in regulating processes in the adipose tissue, skeletal muscle cells, liver, and pancreas [28]–[32].

Antibiotic resistance. Both *F. plautii* YL31 and *E. ramulus* ATCC 29099 have the complete pathways for vancomycin resistance, a predicted two-protein system that confers macrolide resistance to *Escherichia coli,* MacAB [33], and a deaminase that can inactivate the antibiotic blasticidin-S [34].

Oxygen tolerance. *F. plautii* YL31 and *E. ramulus* ATCC 29099 are strict anaerobes but may have some protection for transient oxygen exposure, as it has been observed for other strictly anaerobic bacteria [35], [36]. *F. plautii* YL31 has a predicted peroxidase and catalase (which consume H_2O_2) and superoxide dismutases (which consume O_2^-) (SOD1 and SOD2). This protection against reactive species may help *F. plautii* YL31 during an opportunistic infection, few infections by this bacterium have been reported [37], [38]. Meanwhile, *E. ramulus* ATCC 29099 only has a predicted 8-oxo-dGTP diphosphatase involved in the detoxification of oxidized GTP, a ROS-induced oxidation of guanine which is the most mutagenic base. It has been observed that anaerobic microorganisms lack some oxygen stress enzymes but they contain novel iron-containing proteins including hemerythrin-like proteins, desulfoferrodoxin, rubrerythrin, new types of rubredoxins, and a new enzyme termed superoxide reductase [39]. Both *F. plautii* YL31 and *E. ramulus* ATCC 29099 have predicted superoxide reductases and hemerythrins.

Comparison of the Genomes of *F. plautii* YL31 and *E. ramulus* ATCC 29099 to those from close relatives. After the analysis of the genomes of *E. ramulus* ATCC 29099 and *F. plautii* YL31, we performed comparisons with closed relatives. The Average Nucleotide Identity (ANI) analysis revealed which genomes were more closely related to these reference genomes, *E. ramulus* ATCC 29099 (Table S3.2 in Appendix 3) and *F. plautii* YL31 (Table S3.3 in Appendix 3). For *E. ramulus*, there were 2 genomes that had higher than 98.7% of identity with strain ATCC 29099 (the percentage for strains that belong to the same species is >95%), these were strains MGYG-HGUT-01456 and 2789STDY5608891. The other 2 genomes, MGYG-HGUT-02278 and strain 21, had only 89.2% of identity with *E. ramulus* ATCC 29099 but between them they shared 99% of identity. Thus, for subsequent analyses we considered these as two species clusters. For *Flavonifractor* spp., we also retrieved two group of genomes, one more closely related to the representative genome, *F. plautii* YL31 (*F. plautii* 2789STDY5834932, *F. plautii* ATCC 29863, and *F. plautii* An248) and another one more distant related to *F. plautii* YL31 (*Flavonifractor* sp. An4, *Flavonifractor* sp. An10, *Flavonifractor* sp. An82, and *Flavonifractor* sp. An306). This last group includes the two *Flavonifractor* sp. genomes (An4 and An82) that were most related to variant ASV_a45d from our previous study (Chapter 2, 'Gut-derived *Flavonifractor* species variants are differentially enriched during *in vitro* incubation with Quercetin'). So, for *Flavonifractor* spp. we also considered two groups of genomes, each

with 4 genomes. After selecting these groups of genomes, we compared the presence of different classes of predicted glycoside hydrolases, proteins involved in the degradation of flavonoids (enoate reductase and phloretin hydrolase), and enriched orthologous genes.

Glycoside hydrolases. All 5 strains of *E. ramulus* shared most of the glycoside hydrolase classes (Table 3.2). Few exceptions were GH23 and GH32 which were only present in the genomes most related to *E. ramulus* ATCC 29099. *E. rectale* shared less than half of the glycoside hydrolases classes with *E. ramulus* (16 out of 41). One prominent difference between *E. ramulus* strains and *E. rectale* was glycoside hydrolase class GH30_5 which was present in all *E. ramulus* strains but not in *E. rectale* (In *E. ramulus* ATCC 29099 corresponds to ERK42643.1: 312…763). In all cases, this glycoside hydrolase had a signal peptide and is a predicted Cna B-type domain protein with signal peptide (Uniprot: D9R566) in [*Clostridium*] *saccharolyticum* (Lachnospiraceae family). Interestingly, it is mentioned that other proteins contain domains with a similar fold, including the C-terminal domain of the beta subunit of a transhydroxylase, a molybdenum-containing enzyme from *Pelobacter acidigallici*. This enzyme catalyzes the conversion of pyrogallol to phloroglucinol (one of the metabolites from flavonoid degradation), and can transfer hydroxyl ions between phenols [40]. It will be interesting to evaluate if this protein has this activity in *E. ramulus*.

Another important observation was that the strains more closely related to *E. ramulus* ATCC 29099, all had predicted proteins in the GH13 class (subfamilies 9, 18, 20, 31, and 39) but none had a signal peptide. To this class belong enzymes with amylase activity involved in the degradation of starch, since starch is a large polysaccharide it might be partially degraded by proteins anchored in the membrane instead of transporting the whole molecule inside the cell. We have previously evidenced that *E. ramulus* ATCC 29099 is not able to use starch as a carbon source [26]. Meanwhile, *E. rectale* is a known starch degrader [41], in our analyses we observed 2 predicted proteins in the class GH13 (subfamilies 36 and 41) with signal peptide in the genome of *E. rectale,* these proteins may be responsible for *E. rectale* amylase activity. Similarly, *E. ramulus* MGYG02278 and *E. ramulus* strain 21 both had a predicted protein in the GH13 family (subfamily 14) with a signal peptide, it is possible then that these strains might be able to use starch, however, experimental evidence is not yet available.

In *Flavonifractor* spp., there were 12 glycoside hydrolase classes present in most of the strains (Table 3.1). Among these, 6 were present in all strains, 1 was present only in the group most closely related to *F. plautii* YL31, and 1 was present only in the most distant group (An4, An10, An82, and An306), the other vary according to the strain. This indicates that these strains might have different patterns of carbon metabolism which can affect the degradation of flavonoids since these compounds can be cometabolized with carbon sources [26], [42].

Enriched orthologous genes in <u>Eubacterium</u> spp. For *Eubacterium* spp. we did 3 analyses in which we looked for enriched orthologous genes in the following groups: a) *E. ramulus* ATCC 29099, *E. ramulus* 2789STDY5608891, and *E. ramulus* MGYG01456 (Table 3.3); b) *E. ramulus* 2789STDY5608891, *E. ramulus* ATCC29099, *E. ramulus* MGYG01456, *E. ramulus* 21, and *E. ramulus* MGYG02278 (Table 3.4); and c) *E. ramulus* strain 21, *E. ramulus* MGYG02278, *E. oxidoreducens, and E. rectale* (Table 3.5). In the first group (Table 3.3 and S3.4), the strains most closely related to *E. ramulus* ATCC29099 were significantly more

enriched in genes for L-lysine fermentation to acetate and butanoate. Metagenomic studies have revealed that the pathway for butyrate production from lysine has a high abundance in the human intestine [43]. The pathway from L-lysine to butanoyl-CoA was not present in *E. ramulus* strain 21, *E. ramulus* MGYG02278 or *E. rectale*. Thus, we predict that the presence of strains more closely related to *E. ramulus* ATCC 29099 is important for the generation of SCFAs from undigested proteins and amino acids in the colon. Other genes that were enriched in this group were involved in the degradation of rhamnose, however, this pathway was not complete and experimentally, *E. ramulus* ATCC 29099 does not use rhamnose as a carbon source [20].

Table 3. 3.

Gene clusters enriched in *E. ramulus* 2789STDY5608891/*E. ramulus* ATCC29099/*E. ramulus* MGYG01456.

Cluster	Count	Name	p-value
Cluster 1	4	L-lysine catabolic process to butyrate	3.27E-05
Cluster 2	2	rhamnose catabolic process	0.00157155
Cluster 3	2	pyridoxal phosphate biosynthetic process	0.00017873

When we analyzed what orthologous genes were enriched in all *E. ramulus* strains *vs E. oxidoreducens* and *E. rectale* (Table 3.4 and S3.5), we observed that genes for vitamin B1 (thiamine) binding and biosynthesis and tetrahydrofolate biosynthesis were enriched. As mentioned before, *E. ramulus* ATCC 29099 has almost complete pathways for the biosynthesis of these vitamins. Other genes that were enriched are involved in sporulation. The genus *Eubacterium* is known as asporogenous, this means that it harbors an incomplete set of genes for sporulation [44]. Although *E. ramulus* strains seem to have genes that *E. rectale* lack (*pspIIQ, spmA,* and *spmB*), it still does not have the complete set of genes.

At last, we looked for genes enriched in *E. ramulus* strain 21, *E. ramulus* MGYG02278, *E. oxidoreducens,* and *E. rectale* (Table 3.5 and S3.6) that were not enriched in the group most closely related to *E. ramulus* ATCC 29099. We found that genes for the biosynthesis of capsule and polysaccharides that formed the capsule were significantly enriched. There is no experimental evidence that points to the presence or lack of capsule in this group of bacteria, if they do produce it, the significance of this can be broad. Capsule-forming bacteria can influence host immunological responses, they might evade complement and/or antimicrobial peptides produced by the host's immune system and they can also exclude intestinal pathogens by inhibiting their attachment to host cells. Capsule may also improve bacterial fitness, for example, by providing protection from bacteriophages, and it can also affect other host-microbe and microbe-microbe interactions, as well as the formation of biofilms [45].

Table 3. 4.

Gene clusters enriched in *E. ramulus* 2789STDY5608891/*E. ramulus* ATCC29099/*E. ramulus* MGYG01456/*E. ramulus* 21/*E. ramulus* MGYG02278.

Cluster	Count	Name	p-value
Cluster 1	3	thiamine pyrophosphate binding	0.00021166
Cluster 2	6	thiamine diphosphate biosynthetic process	7.69E-05
Cluster 3	4	tetrahydrofolate biosynthetic process	0.00068406
Cluster 4	27	sporulation resulting in formation of a cellular spore	1.07E-12

Table 3. 5.

Gene clusters enriched in *E. ramulus* 21/*E. ramulus* MGYG02278/*E. oxidoreducens/E. rectale.*

Cluster	Count	Name	p-value
Cluster 1	3	polysaccharide biosynthetic process	5.45E-05
Cluster 2	2	capsule polysaccharide biosynthetic process	0.0005179

Enriched orthologous genes in Flavonifractor spp. For *Flavonifractor* spp., we analyzed orthologous genes enriched in the group more closely related to *F. plautii* YL31 (Table 3.6 and S3.7) and those enriched in the group more distantly related to *F. plautii* YL31 (Table 3.7 and S3.8). The *F. plautii* YL31-related group was enriched in several genes involved in ethanolamine utilization (Table 3.6 and S3.7). Ethanolamine can be used as carbon, nitrogen, and energy source, its catabolic process requires cobalamin (as mentioned before *F. plautii* YL31 has the complete biosynthesis pathway for this vitamin) and occurs in microcompartments called metabolosomes, which are protein-based organelle-like structures that protect the cell from the potential toxic effects of volatile intermediates; in the case of ethanolamine, acetaldehyde is produced. Ethanolamine is abundant in the gut thanks to the action of phosphodiesterases on the phosphatidylethanolamine on bacterial and mammalian cell membranes and could also originate from plant cells in the diet [46].

Table 3. 6.

Gene clusters enriched in *F. plautii* YL31/ *F. plautii* 2789STDY5834932/ *F. plautii* ATCC 29863/ *F. plautii* An248.

Cluster	Count	Name	p-value
Cluster 1	11	ethanolamine catabolic process	6.00E-07
Cluster 2	4	ornithine metabolic process	0.00028198
Cluster 3	3	polyhedral organelle	0.00255864
Cluster 4	5	bacterial-type flagellum-dependent swarming motility	2.33E-08
Cluster 5	5	chemotaxis	4.62E-07
Cluster 6	4	protein secretion	3.91E-06
Cluster 7	4	bacterial-type flagellum-dependent cell motility	1.14E-05

We noticed that both groups (closely and distantly related to *F. plautii* YL31) had ethanolamine utilization genes but *F. plautii* YL31-related group had these genes enriched, thus we reconstruct the operons for ethanolamine and 1,2-propanediol utilization (Fig. 3.1). Propanediol catabolic process also occurs in metabolosomes, produces a toxic volatile intermediate (propionaldehyde), requires cobalamin, and the pathway has homolog proteins to ethanolamine catabolic pathway that can be misannotated by automatic servers. The reconstruction of these operons revealed two ethanolamine operons (Eut operon 1 and 2) (Fig. 3.1) most similar to the reported Eut2 operon, which instead of the EutD phosphotransacetylase (PTAC) it encodes a homolog to the PduL PTAC, and in place of the EutR regulatory enzyme, it has a two-component regulatory system consisting of a signal transduction histidine kinase and a response regulator [47]. Eut operon 2 has more *eut* genes that encode for structural proteins for the microcompartment [48] than Eut operon 1 but both have the essential proteins for ethanolamine utilization, EutBC protein, which is an ethanolamine ammonia lyase that converts ethanolamine into acetaldehyde and ammonia, the reactivating enzyme EutA that acts on EutBC, the aldehyde dehydrogenase (AldDH) EutE for converting acetaldehyde to acetyl-CoA that enters the tricarboxylic acid cycle and other processes, and the PduL PTAC homolog for the formation of acetate. The *F. plautii* YL31-related group harbors Eut operon 1 and 2 while the group more distant related to *F. plautii* YL31 only has Eut operon 1 (Fig. 3.1). Eut operons 1 and 2 locate in different parts of the genome and their proteins are highly similar but not identical (data not shown).

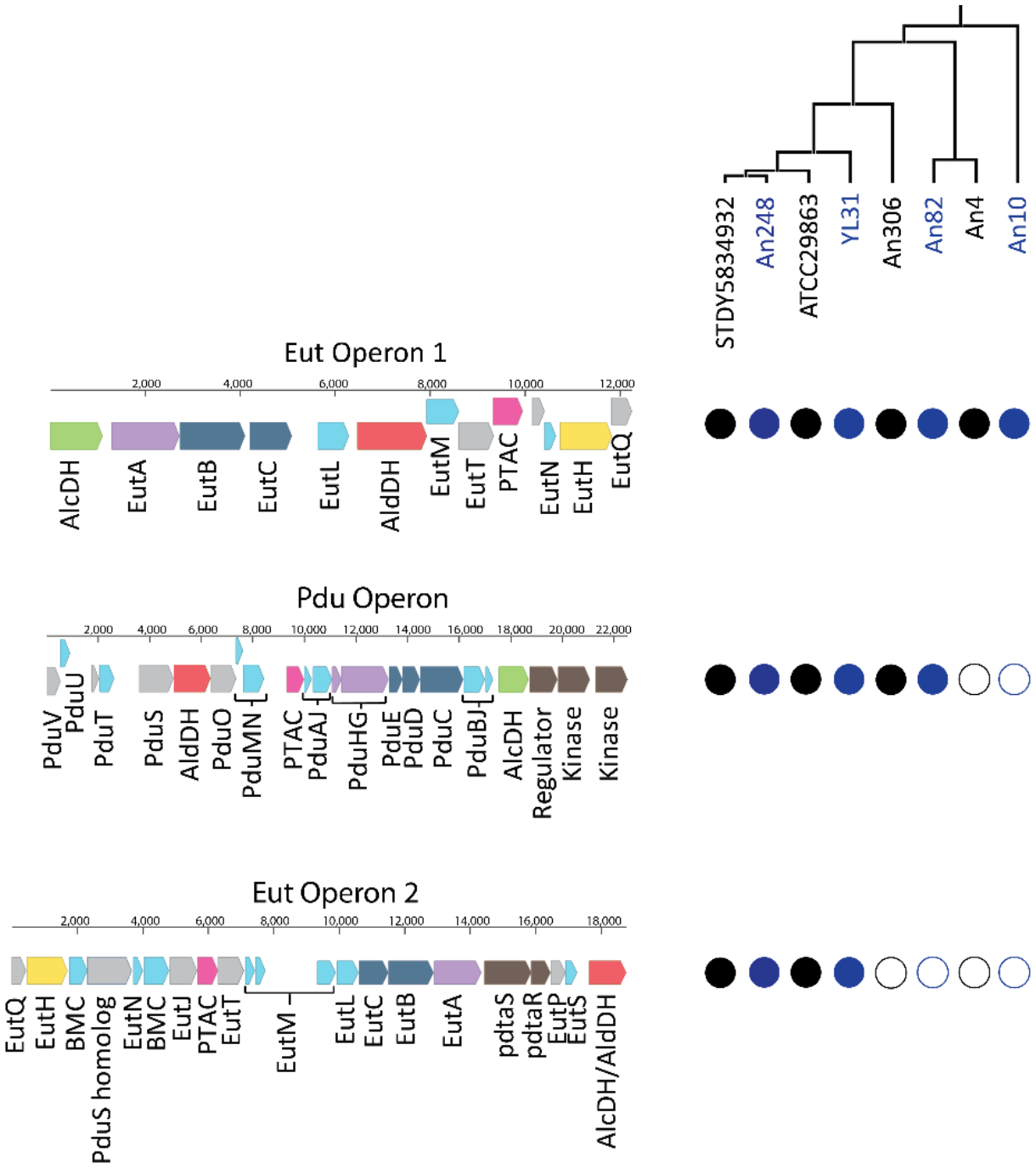

Figure 3. 1

Representative BMC Loci for *Flavonifractor* spp. Cartoon representation of Ethanolamine utilization operon (Eut operon 1 and 2) and 1,2-propanediol utilization operon (Pdu operon). Genes are drawn on *F. plautii* YL31 genome using Benchling [Biology Software] (2019). Eut operon 1 is 12,247 bp, Pdu operon is 22,527 bp, and Eut operon 2 is 18,769 bp. Abbreviations are as follows: AlcDH, Alcohol dehydrogenase; AldDH, Aldehyde dehydrogenase; PTAC, phosphotransacylase; BMC, bacterial microcompartment; pdtaS, two-component system, sensor histidine kinase; pdtaR, two-component system, response regulator.

Genes are color-coded according to their annotation: light blue, BMC-containing proteins; red, aldehyde dehydrogenase; green, alcohol dehydrogenase; solid pink, pduL-type phosphotransacylase; light purple, re-activating proteins; dark blue, signature enzymes (ethanolamine ammonia lyase subunits and propanediol dehydratase subunits); brown, regulatory element including two-component signaling elements; yellow, transporter; gray, other Eut or Pdu proteins. Circles show the presence (filled circle) or absence (white circle) of proteins in the strains depicted in the phylogenetic tree. The phylogenetic tree is as in Fig. 3.2. Table S3.9 (Appendix 3) contains all accession number for each protein in each strain.

Metabolosomes have different substrates and functions but their main function is dictated by the aldehyde-generating enzyme. In the case of ethanolamine and 1,2-propanediol, the presence of ethanolamine ammonia-lyase and 1,2-propanediol dehydratase, respectively, it is considered the signature enzymes that defines the substrate of the metabolosome [49]. Thus, we predict that the group with the two Eut operons it is more efficient in utilizing ethanolamine but we cannot discard that one of the operons might be also used for other processes, for example, in some cases class II aldolases were observed close to the operon as reported for other Eut operons [47]. The presence of ethanolamine catabolism in these strains might give an advantage under nutrient scarcity in the gastrointestinal tract since this compound is present in the membrane of epithelial intestinal cells which are constantly washed away in the mucus. It is possible that this pathway might be used during opportunistic infections.

Pdu operon was also found in members of both groups (Fig. 3.1). Some Pdu proteins were predicted using the automatic annotation server, however, some were missing an annotation; thus, we performed a similarity matrix between the possible proteins of *F. plautii* YL31 Pdu operon with the proteins encode in the Pdu operon of *Salmonella enterica* subsp. *enterica* serovar Typhimurium str. LT2, which has a well-established Pdu operon (Table S3.10 in Appendix 3). This process enabled us to reconstruct the Pdu operon in *F. plautii* YL31 genome (Fig. 3.1). This operon was found in all members of the *F. plautii* YL31-related group and only 2 members of the group more distantly related to *F. plautii* YL31 (*Flavonifractor* sp. strains An306 and An82). 1,2-propanediol catabolism is also important in the gastrointestinal tract since it is a microbial fermentation product that can be produced during the fermentation of rhamnose or fucose, sugars found in plant cell walls, bacterial capsules, and the glycoconjugates of eukaryotic cells (Badia, Ros, & Aguilar, 1985).

F. plautii YL31-related group was also enriched in the pathway for the degradation of L-ornithine (Table 3.6 and S3.7). This group can generate ornithine from the degradation of arginine and they have the complete pathway for the formation of acetyl-CoA from ornithine. Other enriched genes in this group encode proteins involved in the formation of flagella, flagellar proteins that interact with chemotaxis proteins, components of the flagellar motor that determine the direction of flagellar rotation, and the secretion of flagellar proteins (Table 3.6 and S3.7). The core set of flagellar genes [50] (26 genes) was identified in the 4 genomes belonging to the *F. plautii* YL31-related group, except for one of the genes that encode for a rod protein, FlgB, which was not found in *F. plautii* 2789STDY5834932. It has been reported that this genus can be motile or non-motile. Thus, we predict that *F. plautii* is one of the motile

species in this genus [27]. The breakdown of intestinal barrier has been associated with high levels of flagellin, a bacterial flagellar protein. In a healthy state, the host immunological defense downregulates the microbiome's production of flagella [51]. However, it is possible that this trait might be displayed during an opportunistic infection.

The group more distantly related to *F. plautii* YL31, to which strains An4, An10, An82, An306 belong to, was enriched in genes for galactose metabolism (Table 3.7 and S3.8). *F. plautii* YL31 and its close relatives do not possess the metabolic route for galactose degradation whereas strains An4, An10, An82, An100 do have the complete pathway. Thus, we propose that a strategy for the isolation of ASV_a45d from our previous study (Chapter 2) might be the use of galactose in the culture media. These strains might be important in dairy-rich diet since lactose is a disaccharide formed from one molecule of glucose plus one of galactose. Additionally, this group was enriched in riboflavin and Mo-molybdopterin cofactor biosynthetic processes but none of the strains have the complete pathways.

Flavonoid-degrading genes. Knowledge about the proteins involved in the degradation of flavonoids is still limited. In *E. ramulus*, enoate reductase (ERED) and phloretin hydrolase (Phy) have been experimentally confirmed. While in *F. plautii*, phloretin hydrolase (Phy) is predicted and the most probable homologue protein to enoate reductase is a NADH oxidase but there is no experimental evidence [16]. All *E. ramulus* strains harbored a protein highly similar to ERED and only *E. ramulus* strain 21 lacked a Phy protein (Fig. 3.2A and Table S3.11 and S3.12 in Appendix 3). For *Flavonifractor* spp., all strains closely related to *F. plautii* YL31 possess a protein very similar to the NADH oxidase and to the predicted Phy (Fig. 3.2B and Table S3.13 and S3.14 in Appendix 3). On the other hand, among the *Flavonifractor* strains that do not belong to the species *F. plautii,* all strains had a protein similar to Phy but only *Flavonifractor* sp. strains An82 and An306 had proteins highly similar to the NADH oxidase (Fig. 3.2B and Table S3.13 and S3.14 in Appendix 3). It is worth noting that predicted Phy proteins of strains An4, An10, An82, and An306 are 90-95% identical to the reference Phy (Table S3.14 in Appendix 3), meanwhile the predicted Phy for *E. oxidoreducens* is only 83% identical to the one from *E. ramulus* (Table S3.12 in Appendix 3). This suggests that *Flavonifractor* species might have a group of similar proteins involved in the degradation of flavonoids.

Table 3. 7.

Gene clusters enriched in *Flavonifractor* sp. An4/ *Flavonifractor* sp. An10/ *Flavonifractor* sp. An82/ *Flavonifractor* sp. An306.

Cluster	Count	Name	p-value
Cluster 1	4	Mo-molybdopterin cofactor biosynthetic process	8.54E-07
Cluster 2	3	riboflavin biosynthetic process	0.00044437
Cluster 3	2	galactose metabolic process	0.00249661

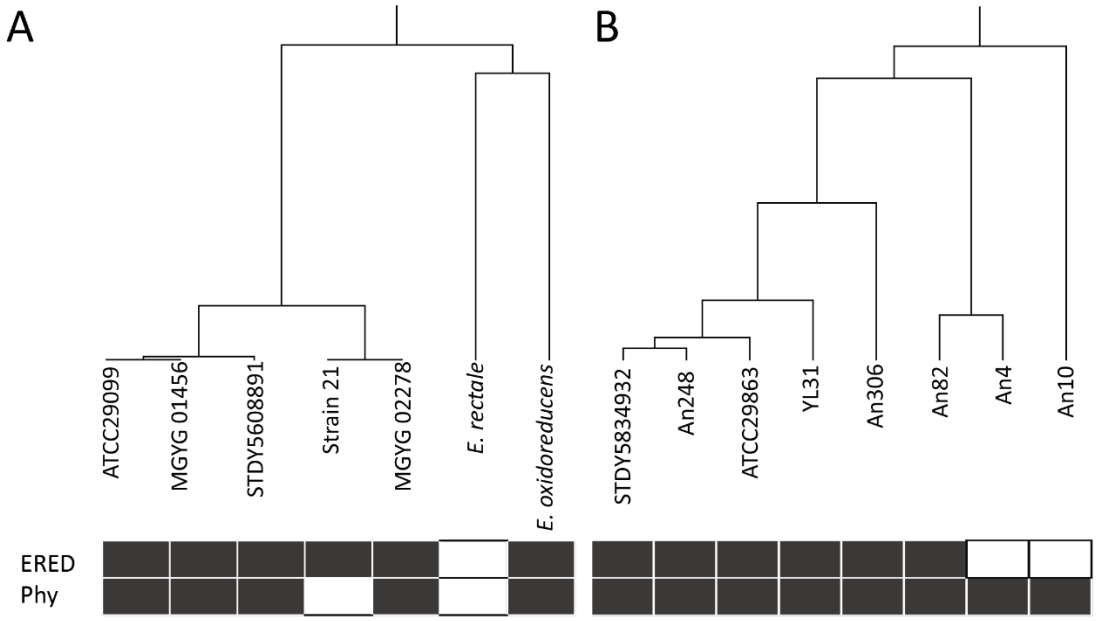

Figure 3. 2

Heatmap showing presence (gray) or absence (white) of proteins involved in flavonoid degradation in *Eubacterium* spp. (A) and *Flavonifractor* spp. (B) genomes. Optimal tree with the sum of branch length = 0.11524341 is shown for A and 0.05375762 for B. The analysis in A involved 7 nucleotide sequences: *E. ramulus* ATCC 29099 (ATCC29099), *E. ramulus* MGYG-HGUT-01456 (MGYG 01456), *E. ramulus* MGYG-HGUT-02278 (MGYG 02278), *E. ramulus* 2789STDY5608891 (STDY5608891)*, E. ramulus* strain 21 (Strain 21), *E. rectale* (*E.rectale*), and *E. oxidoreducens* (*E. oxidoreducens*). The analysis in B involved 8 nucleotide sequences: *F. plautii* 2789STDY5834932 (STDY5834932), *F. plautii* An248 (An248), *F. plautii* ATCC 29863 (ATCC29863), *F. plautii* YL31 (YL31), *Flavonifractor* sp. An306 (An306), *Flavonifractor* sp. An82 (An82), *Flavonifractor* sp. An4 (An4), and *Flavonifractor* sp. An10 (An10). All positions containing gaps and missing data were eliminated. There were a total of 1341 positions in the final dataset for A and 1008 positions in the final dataset for B. ERED, Enoate reductase; Phy, Phloretin hydrolase. Table S3.10-S3.13 (Appendix 3) show Blast results.

It must be said that strains An4 and An82 share an identical variable region V4 of the 16S rRNA gene but at the level of genome they only share 89% of identity, thus they belong to different species. In previous experiments in our laboratory, a sequence variant (ASV_a45d) identical to *Flavonifractor* sp. strains An82 and An4 in this variable region of the rRNA gene was enriched in *in vitro* fecal incubations with quercetin. Efforts to reconstruct the genome of ASV_a45d must take place in order to reveal which is the most closely related species. Meanwhile, the capacity of degrading quercetin of these strains must be confirmed in pure culture. If proven experimentally, this would mean that different species of the *Flavonifractor* genus are able to degrade flavonoids. And that they might have different metabolic and

structural traits, such as presence of flagella and use of carbon sources including host-derived compounds. Thus, they might have a different impact on the host and the gut microbiome.

Acknowledgments

We would like to thank the Doctorate program in Biosciences of La Sabana University and PhD scholarship 647 (Colciencias, Colombia) for supporting Gina Paola Rodriguez.

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General Discussion

In this study, we revealed aspects that are important for the microbial of ecology of quercetin-degrading bacteria, *F. plautii* and *E. ramulus*. To extend our understanding of the role of these bacteria in the complex interaction between host diet and gut microbiota, we first screened whether different dietary fibers have an impact on the quercetin-degrading capacity of *E. ramulus*. We observed that inulin, FOS, and arabinogalactan promoted the degradation of quercetin by this bacterium. However, in order to provide conditions more similar to the natural niche of this bacterium, we incorporated in our experiments another human gut symbiont, *B. thetaiotaomicron*, which it is a known versatile polysaccharide degrader but does not degrade quercetin. This simple model of gut microbiota revealed that non-quercetin degraders can promote the degradation of quercetin by *E. ramulus* in the presence of substrates that this bacterium is not able to use by its own (i.e. starch). As a consequence of this interaction, the production of butyrate, an important metabolite for colon health, was also enhanced. We therefore propose that the butyrogenic effect of starch [1] is partially mediated by the use of starch breakdown products by butyrate producers. This same effect that *B. thetaiotaomicron* had on the metabolism of *E. ramulus* was not observed with *F. plautii* (data not shown), indicating that flavonoid-degrading bacteria have different capacities of interacting with the gut microbiota and distinct nutrient requirements (at least under the laboratory conditions tested).

After these experiments with pure cultures and cocultures, we evaluated the degradation of quercetin by complex fecal communities in *in vitro* experiments. Our original objective was to observe if interindividual (fecal microbial communities from different subjects) differences in quercetin degradation exist. We did observe these differences (data not shown) but these were not consistent across different assays. An explanation might be that fecal matter individually sampled can have different number of bacterial cells per gram and/or concentration of carbon sources intrinsic to the feces. Nevertheless, these experiments showed another important insight about quercetin-degrading bacteria: the population that we sampled (Wisconsin, USA) harbors different variants closely related to known quercetin-degrading bacteria that showed distinct features. Under the conditions of our fecal incubation system, we found that some species from *Flavonifractor* spp. were more prevalent, thus we focused our analysis on them. There were two variants (named ASV_65f4 and ASV_a45d) that belong to different species of the genus *Flavonifractor* that presented a negative correlation in their relative abundances upon incubation with quercetin. One of the variants was closely related to the known quercetin-degrader, *F. plautii*, it had 100% identity with *F. plautii* in the variable region V4 of the rRNA gene. In order to continue studying these variants, we attempted to isolate them using the same media culture we use to grow *F. plautii* in pure culture. However, we could only retrieve the variant ASV_65f4 even from samples that became highly enriched with the variant ASV_a45d, which is more distantly related to *F. plautii*. This indicates that isolation attempts for variant ASV_a45d must use a different strategy. As we wanted to study the relation between both variants, we combined fecal matters that were previously enriched with one or the other variant. This approach shows that under these conditions, ASV_65f4 was a stronger competitor and indeed initial relative abundances of this variant were higher across all the 15 fecal samples that we studied. However, we propose that ASV_a45d might have an advantage over ASV_65f4 under certain

circumstances that might be given by the available carbon sources and/or interactions with the rest of the microbial community.

It has been widely described that the gut microbial community among different subjects have limited redundancy at the taxonomic level [2]. Additionally, some of our fecal samples were inoculated in HMAM mice that were subjected to an abrupt change in diet that impacted the taxonomic profile of the microbial community. In the other hand, we used media culture that lacked common sugars, this can restrict the enrichment of many bacteria. These factors can make difficult to establish the shared microbiota across experiments that correlated positively or negatively with the relative abundance of variants ASV_65f4 or ASV_a45d. Nevertheless, we observed that the genus *Desulfovibrio* was negatively correlated with ASV_65f4, which was the strongest competitor between the two *Flavonifractor*-related variants. *Desulfovibrio* sp. is an acetate-utilizing bacterium which produces hydrogen sulfide. This metabolite is very toxic and could impact ASV_65f4 directly. Another possibility is that when some bacteria detoxify hydrogen sulfide, they convert it to thiosulfate and from this tetrathionate can be generated. When tetrathionate becomes available, there are some microorganisms that can use it as an electron donor for ethanolamine catabolism. In our genomic comparison analysis, we revealed that the group of genomes most closely related to ASV_65f4 harbors the genetic potential for ethanolamine catabolism, this indicates that the presence of tetrathionate may increase the competition for this substrate which can affect ASV_65f4. Ethanolamine might be an important carbon source in our *in vitro* system as well as in the gastrointestinal tract since it can be formed from dead cell membranes.

Another important characteristic that we revealed in the group of genomes most closely related to ASV_65f4 was that they harbor a core set of flagellar genes while the group most related to ASV_a45d does not. This is an important difference since the presence of flagella and the utilization of ethanolamine (abundant in mammalian cells) can help during opportunistic infections, which have been reported for *F. plautii*. Meanwhile, for *E. ramulus*, we highlight the enriched genes for butyrogenesis from lysine in the group of genomes most closely related to *E. ramulus* ATCC 29099, this process is important in the gastrointestinal tract for the production of short-chain fatty acids from proteins and has been described in few species [3].

Overall, we showed aspects of the microbial ecology of known quercetin-degraders and related species among these the use of different substrates and interactions with the microbial community that are important for the understanding of the dynamic of quercetin degradation in the gastrointestinal tract.

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Conclusions and Future Research Perspectives

Gut microbiome research aims at understanding how the microbiota modulates the health of individuals considering lifestyles, diet and disease risk. Eventually, the field of microbial flavonoid degradation may lead to the characterization of biomarkers that will indicate the degree of the capacity of an individual's gut microbiota to degrade flavonoids with the goal of personalizing food intake based on the metabolic profile of the individual. It is possible that for some disease states (i.e. anxiety), the degradation of flavonoids will be desired while for other conditions a minimal degradation of the flavonoid might give a better outcome. The main conclusion drew from this study is that flavonoid-degrading bacteria cannot be understood in isolation but that these bacteria carry out complex interactions with abiotic and biotic components of their environment, including accompanying substrates of flavonoids and members of the microbial community. This means that there are factors that might influence the metabolic capability of these bacteria. Ultimately, the different interactions of flavonoid-degrading bacteria will affect the bioavailability and bioactivity of flavonoids, and these must be considered when using flavonoids as therapeutic compounds, for example, for people that are constantly exposed to high levels of oxidative stress (alcohol consumption, smoking and/or western diet) or that suffered from an inflammatory disease. In the future, it may be necessary to stratify subjects in clinical trials based on the flavonoid-degrading capacity of their gut microbiotas in order to understand the influence of the microbiome on the health outcome.

For future perspectives, it is important to evaluate fecal communities from other human populations in search for variants of quercetin-degrading bacteria and analyze if there are common variants across different populations. Attempts for the isolation of these variants must continue and we propose that in order to exclude the variant most closely related to *F. plautii*, galactose might be used as the only carbon source in the culture media. Through genome comparisons, it was shown that this substrate is not used by *F. plautii* but other strains of *Flavonifractor* sp. might use it. Upon isolation of different variants, their capacity for degrading flavonoids must be evaluated in pure culture. The rate of degradation of flavonoids might be tested using different dietary sources. Additionally, the utilization of ethanolamine for quercetin degradation by species of *Flavonifractor* is another topic that needs experimental evidence.

We also consider important to evaluate whether *E. ramulus*'s predicted GH30_5 protein has a transhydroxylase activity involved in the transfer of hydroxyl ions between phenols. This might reveal another important protein in flavonoid metabolism.

Appendix 1. Supplemental Material Chapter 1

Bacteroides thetaiotaomicron* Starch Utilization Promotes Quercetin Degradation and Butyrate Production by *Eubacterium ramulus

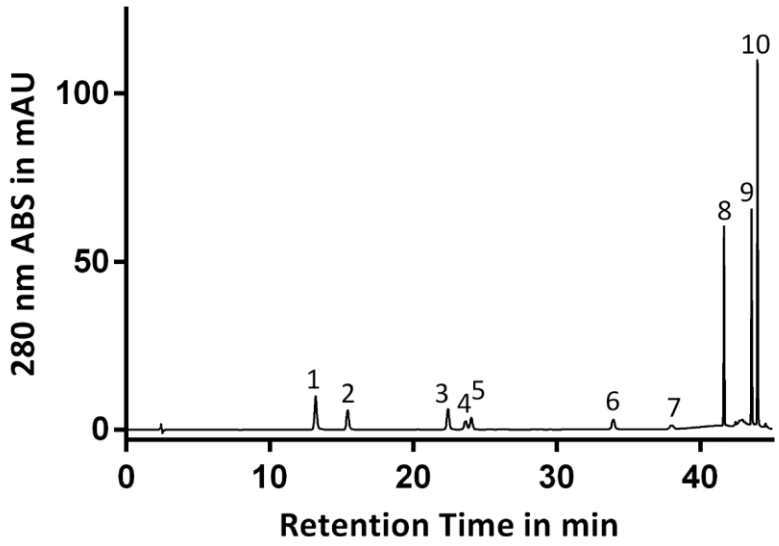


Figure S1.1. Chromatogram for calibration of standards. Reference compounds had the following retention times: 13.2 min for 3,4-dihydroxybenzoic acid (PCA, 1), 15.4 min for 3,4-dihydroxyphenylacetic acid (DOPAC, 2), 22.4 min for 3,4-dihydroxyphenylpropionic acid (3), 23.7 min for 3-hydroxybenzoic acid (4), 24.1 min for 3-hydroxyphenylacetic acid (5), 33.9 min for 3-(3-hydroxyphenyl)propionic acid + phenylacetic acid (6), 38.0 min for benzoic acid (7), 41.7 min for rutin (8), 43.6 min for quercetin (9), and 44.0 for genistein (10). Concentration of each analyte 100 µM.

Figure S1.2. Quercetin degradation assay inoculated with *E. ramulus* with different concentrations of glucose (0.1, 0.3, 0.6, and 0.9 mM) at 22 h of incubation. Degradation was monitored through the visualization of the yellow color of quercetin. No transformation of quercetin, yellow; transformation, transparent. Tubes correspond to representative results from 3 replicates.

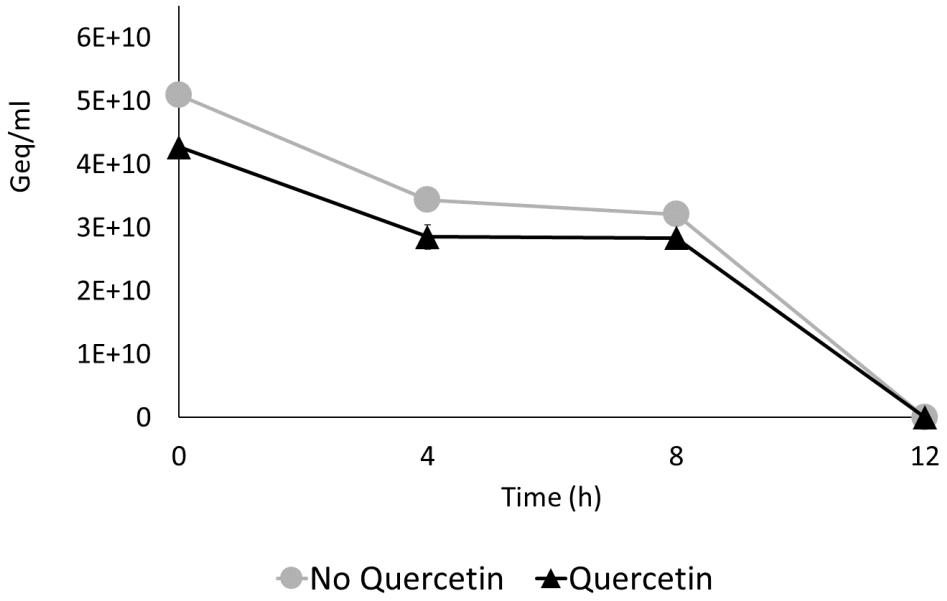


Figure S1.3. Lack of growth of *E. ramulus* in media supplemented with 1 % starch as carbon source with (black) and without Quercetin (gray). GEq, Genome equivalents.

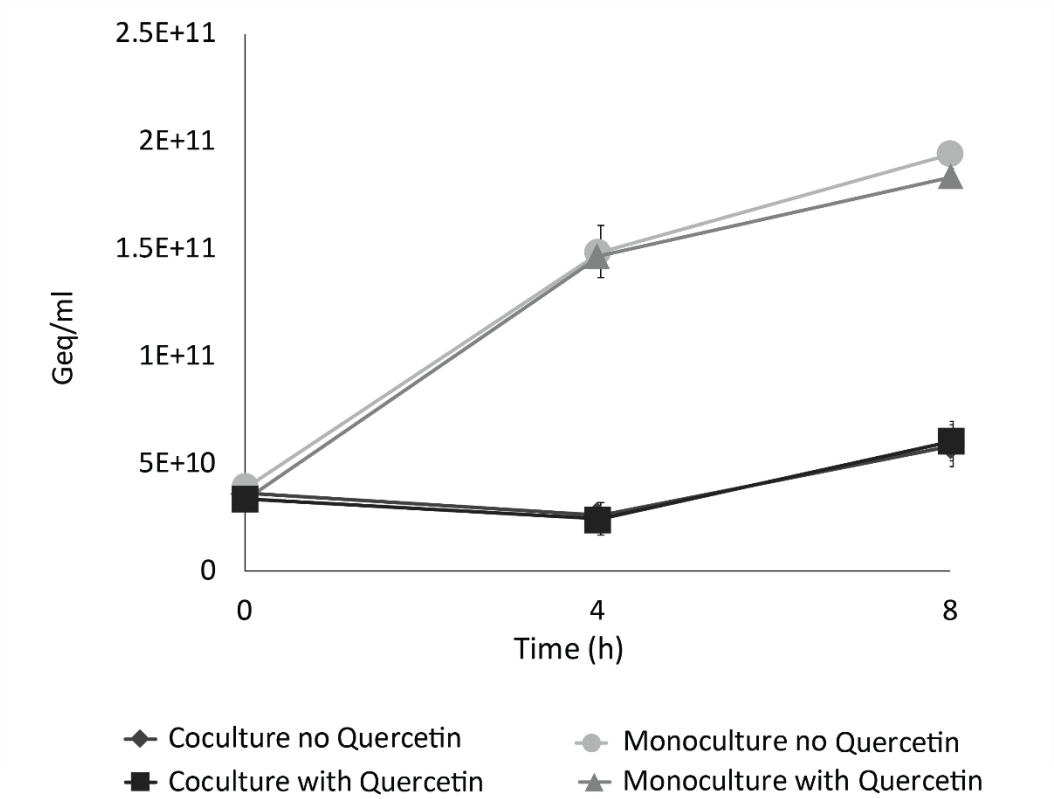


Figure S1.4. Growth of *E. ramulus* in monocultures and cocultures supplemented with 40 mM of glucose as carbon source with and without quercetin. GEq, Genome equivalents.

Table S1.1. HPLC reportable values for Quercetin and related compounds in monocultures and cocultures at 22 h.

Substrate	Culture	Q[q]	D[d]	C[c]	E[c]	F[f]	H[h]	I + J[i]	K[k]
No Carbon source	*B. thetaiotaomicron*	155.47	**<0.99**	<4.99	<0.99	<0.99	<0.99	<4.99	<4.99
No Carbon source	*B. thetaiotaomicron*	190.82	**<0.99**	<4.99	<0.99	<0.99	<0.99	<4.99	<4.99
No Carbon source	*B. thetaiotaomicron*	197.20	**<0.99**	<4.99	<0.99	<0.99	<0.99	<4.99	<4.99
Glucose	*B. thetaiotaomicron*	201.67	**3.93**	5.11	<0.99	<0.99	<0.99	<4.99	<4.99
Glucose	*B. thetaiotaomicron*	199.52	**4.13**	5.50	<0.99	<0.99	<0.99	<4.99	<4.99
Glucose	*B. thetaiotaomicron*	179.65	**4.10**	<4.99	<0.99	<0.99	<0.99	<4.99	<4.99
Starch	*B. thetaiotaomicron*	226.80	**3.63**	<4.99	<0.99	<0.99	<0.99	<4.99	<4.99
Starch	*B. thetaiotaomicron*	183.53	**3.60**	5.08	<0.99	<0.99	<0.99	<4.99	<4.99
Starch	*B. thetaiotaomicron*	192.92	**3.63**	5.03	<0.99	<0.99	<0.99	<4.99	<4.99
No Carbon source	*E.ramulus*	170.27	**26.25**	<4.99	<0.99	<0.99	<0.99	<4.99	<4.99
No Carbon source	*E.ramulus*	172.08	**22.29**	<4.99	<0.99	<0.99	<0.99	<4.99	<4.99
No Carbon source	*E.ramulus*	168.88	**20.73**	<4.99	<0.99	<0.99	<0.99	<4.99	<4.99
Glucose	*E.ramulus*	18.44	**113.76**	10.55	<0.99	<0.99	<0.99	<4.99	<4.99
Glucose	*E.ramulus*	2.98	**132.62**	9.81	<0.99	<0.99	<0.99	<4.99	<4.99
Glucose	*E.ramulus*	6.06	**131.26**	10.34	<0.99	<0.99	<0.99	<4.99	<4.99
Starch	*E.ramulus*	162.91	**47.63**	6.36	<0.99	<0.99	1.21	<4.99	<4.99
Starch	*E.ramulus*	203.09	**41.97**	5.67	<0.99	<0.99	<0.99	<4.99	<4.99
Starch	*E.ramulus*	173.60	**36.92**	<4.99	<0.99	<0.99	<0.99	<4.99	<4.99
No Carbon source	Coculture	171.92	**21.89**	<4.99	<0.99	<0.99	<0.99	<4.99	<4.99
No Carbon source	Coculture	165.69	**27.51**	<4.99	<0.99	<0.99	<0.99	<4.99	<4.99
No Carbon source	Coculture	166.26	**26.97**	<4.99	<0.99	<0.99	<0.99	<4.99	<4.99
Glucose	Coculture	25.56	**117.58**	12.21	<0.99	<0.99	<0.99	<4.99	<4.99
Glucose	Coculture	22.79	**117.77**	12.56	<0.99	<0.99	<0.99	<4.99	<4.99
Glucose	Coculture	18.70	**121.08**	11.93	<0.99	<0.99	<0.99	<4.99	<4.99
Starch	Coculture	34.33	**138.66**	6.42	<0.99	<0.99	<0.99	<4.99	<4.99
Starch	Coculture	39.18	**141.16**	6.05	<0.99	<0.99	<0.99	<4.99	<4.99
Starch	Coculture	25.34	**158.65**	6.37	<0.99	<0.99	<0.99	<4.99	<4.99

[q]Q, Quercetin; [d]D, 3,4-dihydroxyphenylacetic acid (DOPAC, in bold); [c]C, 3,4-dihydroxybenzoic acid (PCA); [e]E, 3,4-dihydroxyphenylpropionic acid; [f]F, 3-hydroxybenzoic acid; [h]H, 3-hydroxyphenylacetic acid; [i]I+J, 3-(3-hydroxyphenyl) propionic acid + phenylacetic acid; [k]K, benzoic acid. Concentration in µM. A number after the symbol "<" indicates less than the minimum detectable value.

Table S1.2. Change in Genome equivalents of *E. ramulus* and *B. thetaiotaomicron* in cultures with 1 % starch as carbon source (experiment independent from the one shown in Fig. 2) and no carbon source.

Experiment		Change in Geq/ml	
		No Carbon Source	Starch
Monoculture	*E. ramulus*	$-8.63\times10^{8} \pm 4.7\times10^{8}$ [a]	$-3.17E\times10^{9} \pm 5.3\times10^{9}$ [b]
	B. thetaiotaomicron	-	$2.07\times10^{11} \pm 1.8\times10^{10}$ [a]
Coculture	*E. ramulus*	-	$2.20E\times10^{10} \pm 2.6\times10^{9}$ [b]
	B. thetaiotaomicron	-	$2.78\times10^{11} \pm 1.5\times10^{10}$ [a]

GEq, Genome equivalents

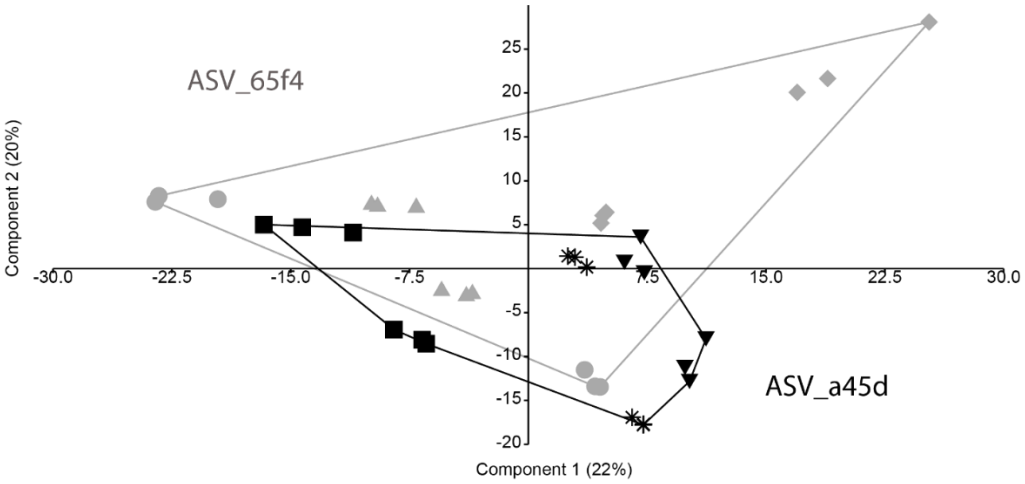
[a] Change in Geq/ml between time 0 h and 8 h.

[b] Change in Geq/ml between time 0 h and 12 h.

Appendix 2. Supplemental Material Chapter 2

Gut-derived *Flavonifractor* species variants are differentially enriched during *in vitro* incubation with Quercetin and persist in diets with different fiber contents

A



B

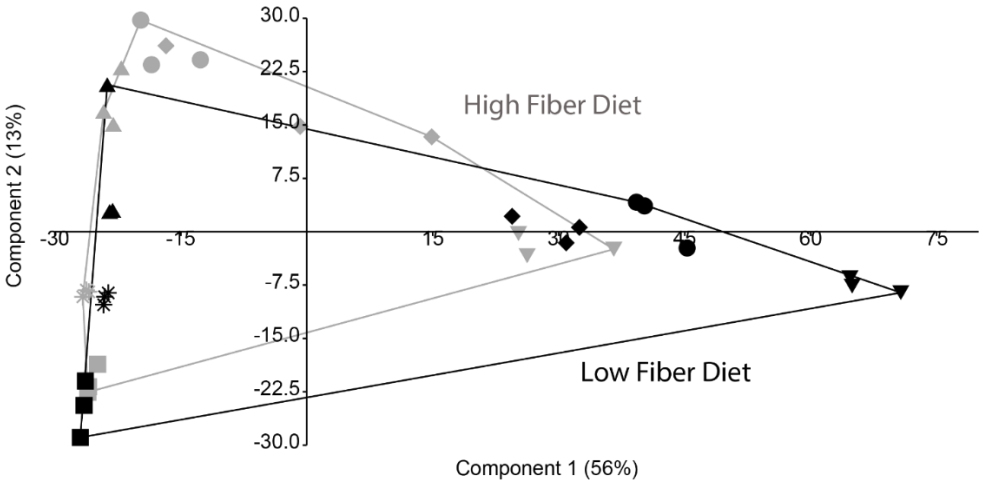
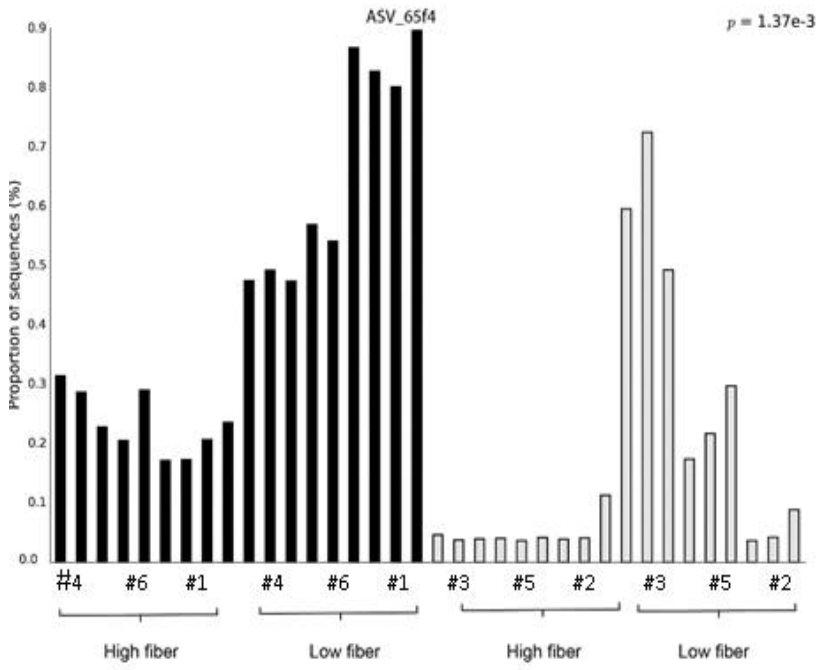


Figure S2.1. Principal component analysis (PCA) plot of the libraries from *in vitro* incubations with fecal samples from human microbiota-associated mice (HMAM) at 0 (A) and 7 days of incubation (B). In the top panel, libraries that were enriched in ASV_65f4 are shown in gray and libraries enriched in ASV_a45d are shown in black. In bottom panel, libraries from HMAM mice fed a high fiber diet are shown in gray and libraries from HMAM mice fed a low fiber diet in black. Each symbol represents a library (n=6, 3 replicates), different shapes represent libraries from a different subject: #1, star (∗); #2, diamond (♦); #3, dot (●); #4, inv. triangle (▼); #5, triangle (▲); and #6, square (■).

A



B

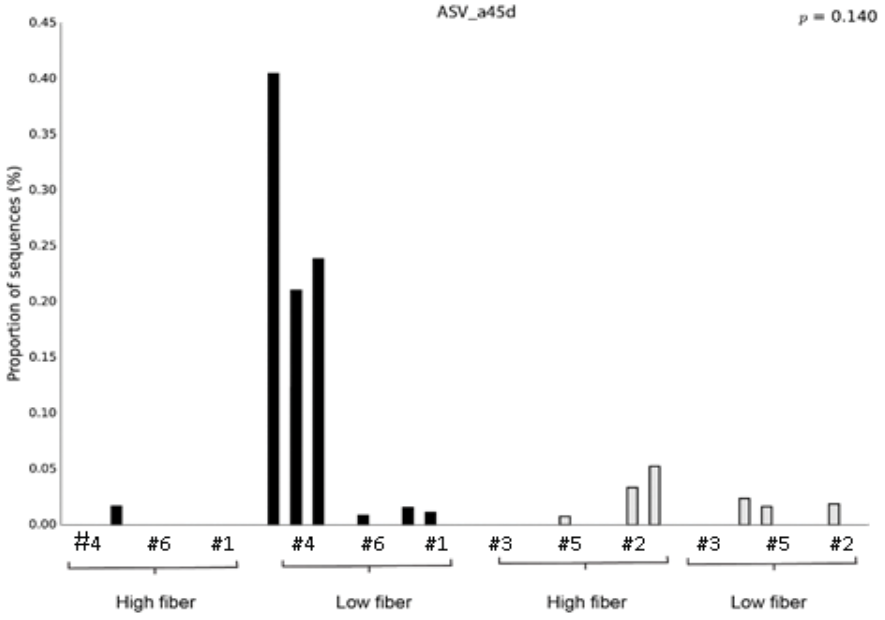


Figure S2.2. Initial relative abundances for ASV_65f4 (A) and ASV_a45d (B) in *in vitro* incubations with fecal samples from human microbiota-associated mice (HMAM) 0 days of incubation). Libraries that were enriched in ASV_65f4 are shown in gray and libraries enriched in ASV_a45d are shown in black.

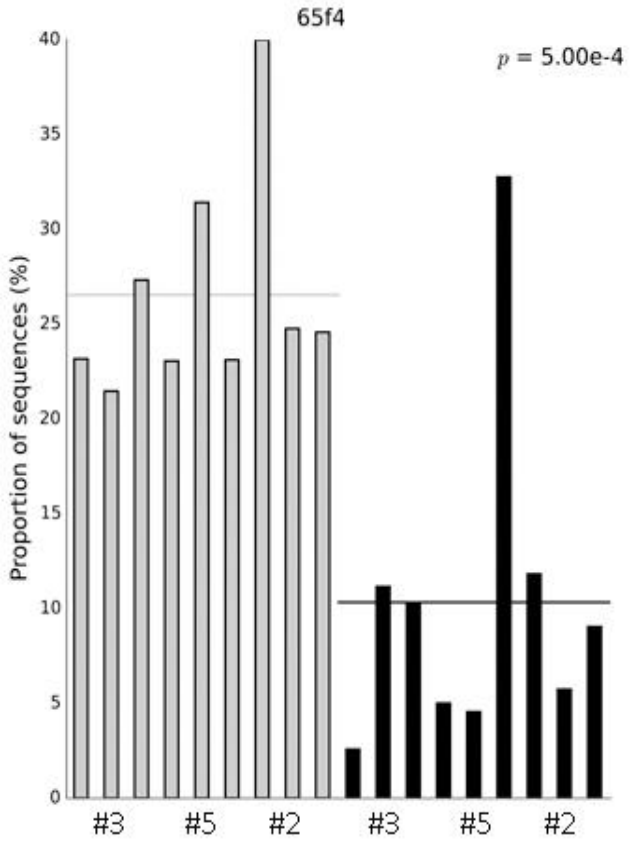
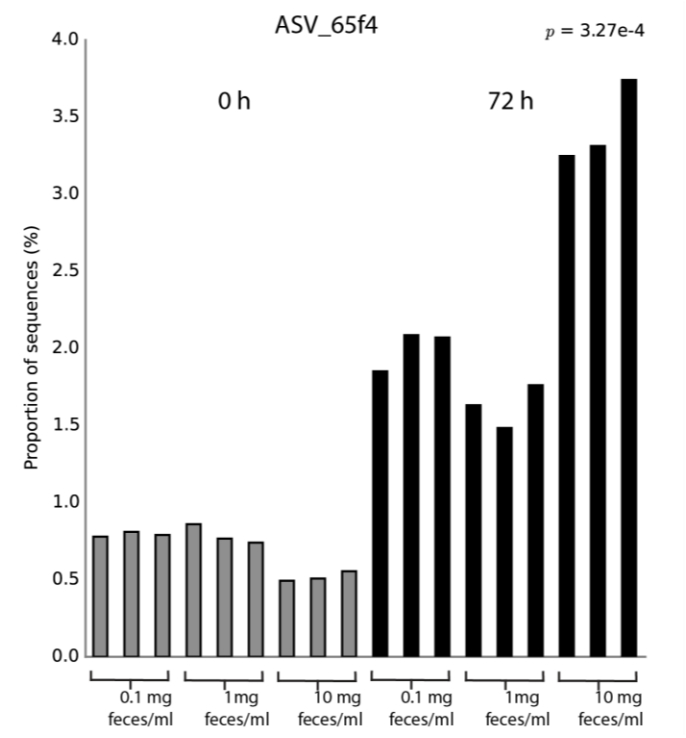


Figure S2.3. Relative abundances for ASV_65f4 libraries from *in vitro* incubations at 7th day of incubation with fecal samples from human microbiota-associated mice (HMAM) fed different diets. Libraries from HMAM mice fed a high fiber diet are shown in gray and libraries from HMAM mice fed a low fiber diet are shown in black. Libraries shown correspond only to those enriched in ASV_65f4 (subjects #2, #3, and #5).

A


ASV_65f4
p = 3.27e-4
0 h
72 h
Proportion of sequences (%)
4.0
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
0.1 mg feces/ml
1mg feces/ml
10 mg feces/ml
0.1 mg feces/ml
1mg feces/ml
10 mg feces/ml

B

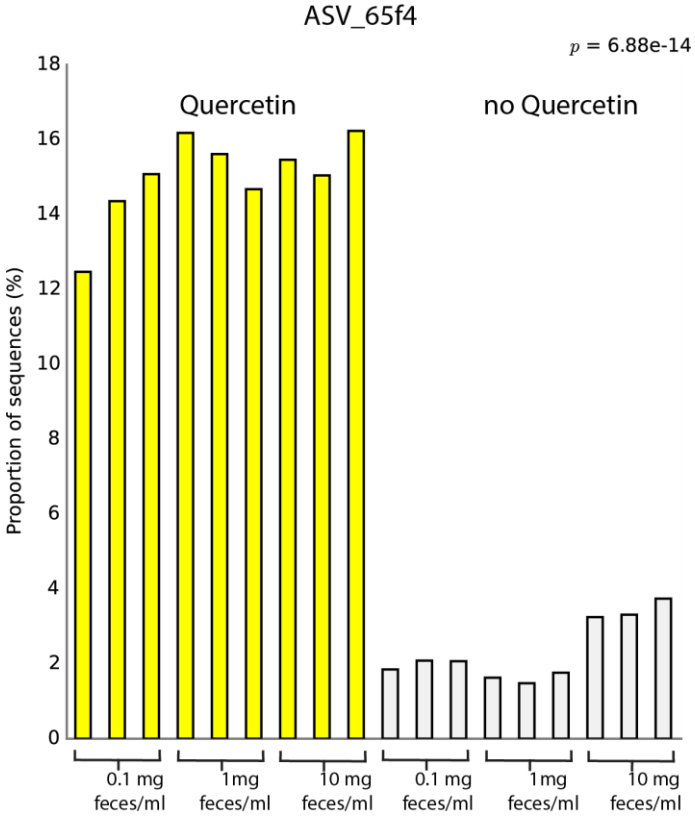

ASV_65f4
p = 6.88e-14
Quercetin
no Quercetin
Proportion of sequences (%)
18
16
14
12
10
8
6
4
2
0
0.1 mg feces/ml
1mg feces/ml
10 mg feces/ml
0.1 mg feces/ml
1mg feces/ml
10 mg feces/ml

Figure S2.4. Proportion of sequences (%) for ASV_65f4 in *in vitro* incubations with human fecal from subject #9. STAMP Bar plots for controls at 0 *vs* 72 h of incubation (A) and Quercetin treatment vs controls at 72 h (B).

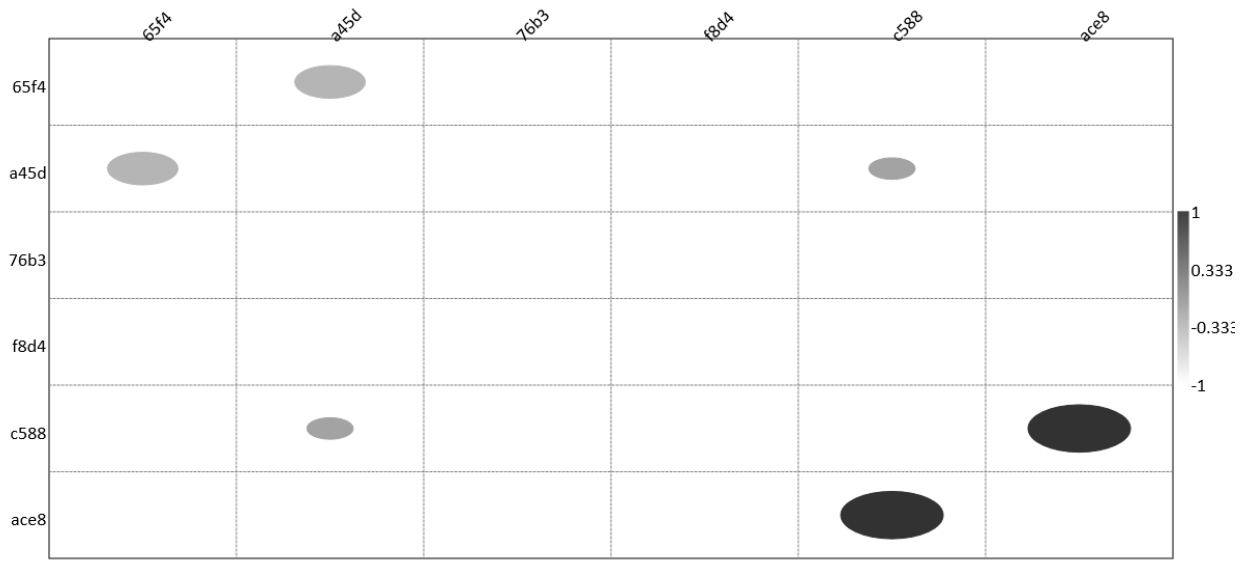


Figure S2.5. Univariate correlations between levels of six fecal taxa (ASVs) enriched in the presence of quercetin *in vitro*. Presence of an ellipse represents values below p=0.05. Spearman's rs and Bonferroni correction were applied. Dark gray is a positive correlation, light gray is a negative correlation. Only treatments with quercetin were analyzed (first and second incubation).

Table S2.1. Concentration (mM) in culture of Quercetin and metabolites related to flavonoid degradation for *in vitro* incubations with human fecal samples.

Subject	DOPAC	PCA	C	E	H	I + M	K	Quercetin
#1	**0.362**	<0.021	0.015	0.067	<0.004	<0.021	0.024	<0.004
#2	**0.385**	0.025	<0.004	<0.004	<0.004	<0.021	<0.021	<0.004
#3	**0.351**	0.025	<0.004	0.006	0.009	<0.021	<0.021	<0.004
#4	**0.385**	0.027	<0.004	<0.004	<0.004	<0.021	<0.021	<0.004
#5	**0.356**	0.024	<0.004	0.008	<0.004	<0.021	<0.021	<0.004
#6	**0.399**	0.025	<0.004	<0.004	<0.004	<0.021	<0.021	0.005
#8	**0.377**	<0.021	0.036	0.004	0.005	<0.021	<0.021	<0.004
#9	**0.325**	0.022	0.005	0.006	0.008	<0.021	<0.021	<0.004

3,4-dihydroxyphenylacetic acid (DOPAC)**,** 3,4-dihydroxybenzoic acid (PCA); 3,4-dihydroxyphenylpropionic acid (C); 3-hydroxybenzoic acid (E); 3-hydroxyphenylacetic acid (H); 3-(3-hydroxyphenyl) propionic acid (M) + phenylacetic acid (I); benzoic acid (K). A number after the symbol "<" indicates less than the minimum detectable value. Results from 2 replicates. Sample #7 was not measured. Samples were measured after completion of quercetin degradation (72 h).

Table S2.2. Distribution of Amplicon Sequence Variants (ASVs) significantly enriched in quercetin treatments *vs.* controls derived from *in vitro* incubations with human fecal samples.

	Human subject								
ASV	**#1**	**#2**	**#3**	**#4**	**#5**	**#6**	**#7**	**#8**	**#9**
65f4	+	+	+	+	+	+	+	+	+
a45d	Ns	Ns	-	+	-	+	+	-	Ns
76b3	-	+	-	Ns	-	-	Ns	-	Ns
f8d4	+	Ns	-	-	-	-	+	Ns	Ns
ace8	-	-	-	-	-	-	-	-	+
c588	-	Ns	-	Ns	Ns		-	-	+

+ $p < 0.05$

- not present

Ns $p > 0.05$

p-values calculated with STAMP (Parks et al., 2014).

Table S2.3. Estimates of Evolutionary Divergence between ASVs and Reference sequences. The number of base substitutions per site from between sequences are shown. Analyses were conducted using the Maximum Composite Likelihood model (Tamura et al., 2004). The analysis involved 44 nucleotide sequences. All positions containing gaps and missing data were eliminated. There were a total of 225 positions in the final dataset. Evolutionary analyses were conducted in MEGA6 (Tamura et al., 2013).

Organism or ASV	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31	32	33	34	35	36	37	38	39	40	41	42	43
1 65f46d8c10965a970fcc6949e8c793f6																																											
2 a45db01884f321cd22dcf0f2f1c20738	0.04																																										
3 76b37e21d00e3a7fe96be478db34f3fd	0.06	0.05																																									
4 f8d470aa418ce2d620ef4cd7323ae59d	0.07	0.06	0.03																																								
5 ace82a789a667f4a647ceeddf99ec8f4	0.16	0.18	0.17	0.18																																						
6 c588b62f0de4eb4d078a18ac941fad67	0.16	0.17	0.17	0.17	0.00																																					
7 Flavonifractor_plautii_Y18187	0.00	0.04	0.06	0.07	0.16	0.16																																				
8 Flavonifractor_plautii_EU874848	0.00	0.04	0.06	0.07	0.16	0.16	0.00																																			
9 Flavonifractor_plautii_AY730662	0.00	0.04	0.06	0.07	0.16	0.16	0.00	0.00																																		
10 Flavonifractor_AWSS01000025	0.00	0.04	0.06	0.07	0.16	0.16	0.00	0.00	0.00																																	
11 Flavonifractor_plautii_AGCK01000014	0.00	0.04	0.06	0.07	0.16	0.16	0.00	0.00	0.00	0.00																																
12 Flavonifractor_sp._An82_NFHG01000054.1:55-285	0.03	0.01	0.04	0.05	0.17	0.17	0.03	0.03	0.03	0.03	0.03																															
13 Flavonifractor_sp._An306_NFIQ01000164.1:558-787	0.03	0.02	0.05	0.06	0.18	0.18	0.03	0.03	0.03	0.03	0.03	0.02																														
14 Flavonifractor_sp._An4_NFIL01000023.1:55-285	0.03	0.01	0.04	0.05	0.17	0.17	0.03	0.03	0.03	0.03	0.03	0.00	0.02																													
15 Pseudoflavonifractor_capillosus_ATCC_29799_AY136666.1	0.04	0.04	0.07	0.08	0.17	0.17	0.04	0.04	0.04	0.04	0.04	0.04	0.05	0.04																													
16 Intestinimonas_FMGM01000011	0.07	0.06	0.03	0.00	0.18	0.17	0.07	0.07	0.07	0.07	0.07	0.05	0.06	0.05	0.08																													
17 Intestinimonas_HE974967	0.07	0.06	0.03	0.00	0.18	0.17	0.07	0.07	0.07	0.07	0.07	0.05	0.06	0.05	0.08	0.00																												
18 Intestinimonas_uncultured_KY285278	0.07	0.06	0.01	0.02	0.18	0.18	0.07	0.07	0.07	0.07	0.07	0.05	0.06	0.05	0.08	0.02	0.02																											
19 Intestinimonas_butyriciproducens_MJII01000001.3039866	0.07	0.06	0.03	0.00	0.18	0.17	0.07	0.07	0.07	0.07	0.07	0.05	0.06	0.05	0.08	0.00	0.00	0.02																										
20 Intestinimonas_butyriciproducens_KC311367	0.07	0.06	0.03	0.00	0.18	0.17	0.07	0.07	0.07	0.07	0.07	0.05	0.06	0.05	0.08	0.00	0.00	0.02	0.00																									
21 Intestinimonas_timonensis_LN870298	0.07	0.06	0.03	0.03	0.19	0.18	0.07	0.07	0.07	0.07	0.07	0.05	0.06	0.05	0.08	0.03	0.03	0.03	0.03	0.03																							
22 Eubacterium_ramulus_CYYA01000009	0.16	0.17	0.17	0.17	0.00	0.00	0.16	0.16	0.16	0.16	0.16	0.17	0.18	0.17	0.17	0.17	0.17	0.17	0.18	0.17	0.17	0.18																				
23 Eubacterium_ramulus_AJ011522	0.16	0.17	0.17	0.17	0.00	0.00	0.16	0.16	0.16	0.16	0.16	0.17	0.18	0.17	0.17	0.17	0.17	0.17	0.18	0.17	0.17	0.18	0.00																			
24 Eubacterium_ramulus_CYYA01000009(2)	0.16	0.17	0.17	0.17	0.00	0.00	0.16	0.16	0.16	0.16	0.16	0.17	0.18	0.17	0.17	0.17	0.17	0.17	0.18	0.17	0.17	0.18	0.00	0.00																		
25 Eubacterium_ramulus_LG085505	0.16	0.17	0.17	0.17	0.00	0.00	0.16	0.16	0.16	0.16	0.16	0.17	0.18	0.17	0.17	0.17	0.17	0.17	0.18	0.17	0.17	0.18	0.00	0.00	0.00																	
26 Eubacterium_ramulus_ATCC_29099_AWVJ01000187	0.16	0.17	0.17	0.17	0.00	0.00	0.16	0.16	0.16	0.16	0.16	0.17	0.18	0.17	0.17	0.17	0.17	0.17	0.18	0.17	0.17	0.18	0.00	0.00	0.00	0.00																
27 Eubacterium_oxidoreducens_FMXR01000001	0.18	0.20	0.18	0.17	0.07	0.07	0.18	0.18	0.18	0.18	0.18	0.19	0.20	0.19	0.19	0.17	0.17	0.17	0.17	0.17	0.17	0.17	0.07	0.07	0.07	0.07	0.07															
28 Eubacterium_rectale_AY169428	0.15	0.17	0.16	0.17	0.05	0.05	0.15	0.15	0.15	0.15	0.15	0.16	0.17	0.16	0.16	0.17	0.17	0.17	0.17	0.17	0.17	0.16	0.05	0.05	0.05	0.05	0.05	0.06														
29 Clostridium_butyricum_AB022592	0.24	0.26	0.26	0.25	0.20	0.19	0.24	0.24	0.24	0.24	0.24	0.25	0.26	0.25	0.23	0.25	0.25	0.25	0.25	0.25	0.26	0.19	0.19	0.19	0.19	0.19	0.19	0.19	0.19													
30 Eubacterium_multiforme_LV535406	0.23	0.26	0.26	0.26	0.19	0.19	0.23	0.23	0.23	0.23	0.23	0.25	0.25	0.25	0.21	0.26	0.26	0.26	0.26	0.26	0.26	0.19	0.19	0.19	0.19	0.19	0.18	0.18	0.08													
31 Eubacterium_barkeri_FNOU01000044	0.17	0.18	0.17	0.18	0.17	0.16	0.17	0.17	0.17	0.17	0.17	0.17	0.17	0.17	0.17	0.18	0.18	0.17	0.18	0.18	0.19	0.16	0.16	0.16	0.16	0.16	0.20	0.21	0.22	0.20												
32 Eubacterium_rangiferina_EU124830	0.14	0.16	0.16	0.16	0.06	0.06	0.14	0.14	0.14	0.14	0.14	0.16	0.16	0.16	0.16	0.16	0.16	0.17	0.16	0.16	0.17	0.06	0.06	0.06	0.06	0.06	0.06	0.06	0.19	0.19	0.17											
33 Eubacterium_callanderi_AM902700	0.21	0.21	0.20	0.20	0.15	0.15	0.21	0.21	0.21	0.21	0.21	0.21	0.21	0.21	0.20	0.20	0.20	0.20	0.20	0.20	0.20	0.15	0.15	0.15	0.15	0.15	0.16	0.18	0.23	0.20	0.05	0.17										
34 Eubacterium_pyruvativorans_AJ310135	0.30	0.32	0.32	0.32	0.25	0.25	0.30	0.30	0.30	0.30	0.30	0.31	0.30	0.31	0.28	0.32	0.32	0.33	0.32	0.32	0.34	0.25	0.25	0.25	0.25	0.25	0.26	0.25	0.21	0.21	0.24	0.25	0.25									
35 Eubacterium_oxidoreducens_AF202259	0.18	0.20	0.19	0.18	0.08	0.07	0.18	0.18	0.18	0.18	0.18	0.20	0.20	0.20	0.20	0.18	0.18	0.18	0.18	0.18	0.18	0.07	0.07	0.07	0.07	0.07	0.00	0.06	0.19	0.19	0.21	0.07	0.17	0.27								
36 Eubacterium_nitritogenes_AB018185	0.23	0.25	0.25	0.25	0.18	0.17	0.23	0.23	0.23	0.23	0.23	0.24	0.24	0.24	0.21	0.25	0.25	0.25	0.25	0.25	0.26	0.17	0.17	0.17	0.17	0.17	0.17	0.17	0.07	0.01	0.20	0.18	0.20	0.21	0.18							
37 Eubacterium_aggregans_AF073898	0.17	0.18	0.17	0.18	0.17	0.16	0.17	0.17	0.17	0.17	0.17	0.17	0.17	0.17	0.17	0.18	0.18	0.17	0.18	0.18	0.19	0.16	0.16	0.16	0.16	0.16	0.20	0.21	0.22	0.20	0.00	0.17	0.05	0.24	0.21	0.20						
38 Eubacterium_limosum_AB298909	0.21	0.21	0.20	0.20	0.15	0.15	0.21	0.21	0.21	0.21	0.21	0.21	0.21	0.21	0.20	0.20	0.20	0.20	0.20	0.20	0.20	0.15	0.15	0.15	0.15	0.15	0.16	0.18	0.23	0.20	0.05	0.17	0.00	0.25	0.17	0.20	0.05					
39 Eubacterium_plexicaudatum_AF157054	0.17	0.18	0.18	0.18	0.06	0.06	0.17	0.17	0.17	0.17	0.17	0.18	0.19	0.18	0.18	0.18	0.18	0.19	0.18	0.18	0.18	0.06	0.06	0.06	0.06	0.06	0.06	0.04	0.21	0.20	0.20	0.08	0.18	0.26	0.07	0.19	0.20	0.18				
40 Eubacterium_brachy_ATCC_33089_AXUD01000014	0.31	0.32	0.32	0.32	0.25	0.26	0.31	0.31	0.31	0.31	0.31	0.30	0.30	0.30	0.29	0.32	0.32	0.32	0.32	0.32	0.34	0.26	0.26	0.26	0.26	0.26	0.27	0.28	0.25	0.26	0.23	0.25	0.23	0.12	0.28	0.26	0.23	0.23	0.28			
41 Eubacterium_infirmum_AGWI01000033	0.29	0.30	0.30	0.31	0.25	0.24	0.29	0.29	0.29	0.29	0.29	0.30	0.28	0.30	0.26	0.31	0.31	0.31	0.31	0.31	0.31	0.24	0.24	0.24	0.24	0.24	0.25	0.25	0.24	0.20	0.24	0.24	0.23	0.09	0.26	0.20	0.24	0.23	0.25	0.11		
42 Eubacterium_nodatum_ATCC_33099_AZKM01000010	0.34	0.34	0.34	0.34	0.30	0.30	0.34	0.34	0.34	0.34	0.34	0.36	0.35	0.36	0.33	0.34	0.34	0.34	0.34	0.34	0.32	0.30	0.30	0.30	0.30	0.30	0.32	0.29	0.29	0.29	0.28	0.32	0.27	0.13	0.33	0.29	0.28	0.27	0.31	0.18	0.16	
43 Eubacterium_saphenum_ATCC_49989_ACON01000003	0.29	0.30	0.30	0.30	0.24	0.25	0.29	0.29	0.29	0.29	0.29	0.30	0.28	0.30	0.29	0.30	0.30	0.30	0.30	0.30	0.30	0.25	0.25	0.25	0.25	0.25	0.24	0.24	0.24	0.24	0.22	0.25	0.21	0.13	0.26	0.24	0.22	0.21	0.25	0.12	0.11	0.19
44 Bifidobacterium_adolescentis_AAXD02000034	1.19	1.24	1.34	1.32	1.13	1.13	1.19	1.19	1.19	1.19	1.19	1.25	1.20	1.25	1.23	1.32	1.32	1.39	1.32	1.32	1.36	1.13	1.13	1.13	1.13	1.13	1.10	1.17	1.19	1.18	1.23	1.16	1.25	1.12	1.10	1.15	1.23	1.25	1.17	1.16	1.13	1.28	1.156

Appendix 3. Supplemental material Chapter 3

Functional profiling of genomes from flavonoid-degrading bacteria and their predictive ecological role in the gastrointestinal tract

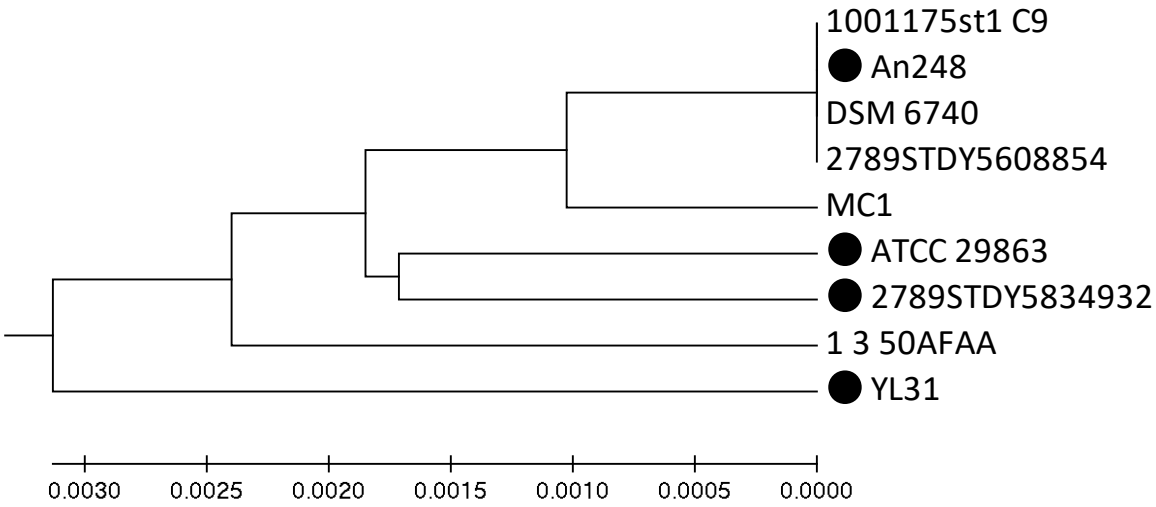


Figure S3.1. Evolutionary relationships of *F. plautii* strains. The evolutionary history was inferred using the UPGMA method. The optimal tree with the sum of branch length = 0.01324911 is shown. The evolutionary distances were computed using the Maximum Composite Likelihood method and are in the units of the number of base substitutions per site. The analysis involved 9 nucleotide sequences: *F. plautii* MC1, *F. plautii* An248*, F. plautii* 1001175st1_C9*, F. plautii* DSM 6740, *F. plautii* 2789STDY5608854, *F. plautii* 2789STDY5834932, *F. plautii* ATCC 29863, *F. plautii* 1_3_50AFAA, and *F. plautii* YL31. All positions containing gaps and missing data were eliminated. There were a total of 1465 positions in the final dataset corresponding to the 16S rRNA gene. Genomes selected for subsequent analyses are labeled with a black circle.

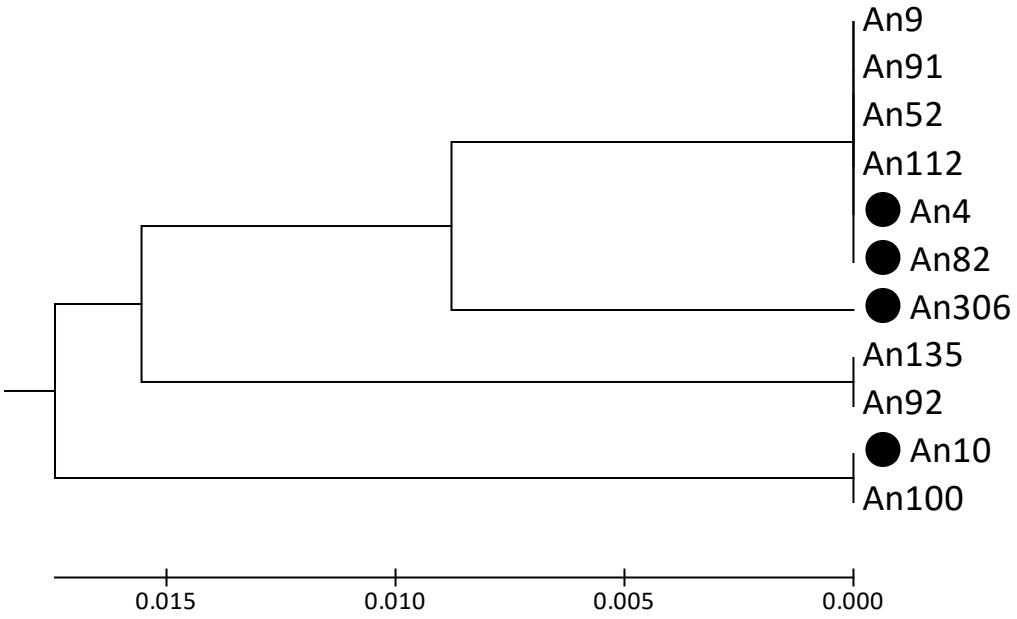


Figure S3.2. Evolutionary relationships of *Flavonifractor* sp. strains. The evolutionary history was inferred using the UPGMA method. The optimal tree with the sum of branch length = 0.05920312 is shown. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The analysis involved 11 nucleotide sequences: *Flavonifractor* sp. strains An4, An9, An10, An52, An82, An91, An92, An100, An112, An135, An306. All positions containing gaps and missing data were eliminated. There were a total of 230 positions in the final dataset. Genomes selected for subsequent analyses are labeled with a black circle.

Table S3.1. Genomes used in this study.

Organism name	Strain	Genome Id	Size (bp)	Assembly level	Genome representation
Flavonifractor plautii[a]	YL31	GCA_001688625.2	3,818,478	Complete Genome	full
Flavonifractor plautii	2789STDY5834932	GCA_001406055.1	4,115,051	Scaffold	full
Flavonifractor plautii	ATCC 29863	GCA_000239295.1	3,820,124	Scaffold	full
Flavonifractor plautii	DSM 6740	GCA_004345805.1	4,431,208	Scaffold	full
Flavonifractor plautii	An248	GCA_002159865.1	3,761,516	Contig	full
Flavonifractor plautii	MC1	GCA_901212615.1	3,923,577	Scaffold	full
Flavonifractor plautii	1001175st1_C9	GCA_005844565.1	4,011,075	Scaffold	full
Flavonifractor plautii	1_3_50AFAA	GCA_000760655.1	4,383,642	Scaffold	full
Flavonifractor plautii	2789STDY5608854	GCA_001404915.1	4,250,184	Scaffold	full
Flavonifractor sp.	An4	GCA_002161245.1	3,350,225	Contig	full
Flavonifractor sp.	An9	GCA_002161245.1	3,350,225	Contig	full
Flavonifractor sp.	An10	GCA_002161215.1	3,882,968	Contig	full
Flavonifractor sp.	An52	GCA_002159385.1	2,834,090	Contig	full
Flavonifractor sp.	An82	GCF_002159265.1	3,668,665	Contig	full
Flavonifractor sp.	An91	GCA_002159225.1	3,603,995	Contig	full
Flavonifractor sp.	An92	GCA_002159175.1	3,490,035	Contig	full
Flavonifractor sp.	An100	GCA_002161175.1	3,040,137	Contig	full
Flavonifractor sp.	An112	GCA_002161085.1	2,958,951	Contig	full
Flavonifractor sp.	An135	GCA_002160795.1	3,889,952	Contig	full
Flavonifractor sp.	An306	GCA_002159455.1	3,902,886	Contig	full
Eubacterium ramulus[a]	ATCC 29099	GCA_000469345.1	3,447,136	Scaffold	full
Eubacterium ramulus	2789STDY5608891	GCA_001406295.1	3,307,376	Scaffold	full
Eubacterium ramulus	21	GCA_003122485.1	3,487,636	Contig	full
Eubacterium ramulus	MGYG-HGUT-01456	GCA_902375155.1	3,447,136	Scaffold	full
Eubacterium ramulus	MGYG-HGUT-02278	GCA_902385375.1	3,656,239	Scaffold	full
Eubacterium oxidoreducens[a]	DSM 3217	GCA_900104415.1	2,912,287	Scaffold	full
Eubacterium rectale[a]	ATCC 33656	GCA_000020605.1	3,449,685	Complete Genome	full

[a] representative genome

Table S3.2. Average Nucleotide Identity (ANIm) and aligned percentage for *E. ramulus* genomes.

	MGYG-HGUT-02278	**MGYG-HGUT-01456**	**Strain 21**	**2789STDY5608891**	**ATCC 29099**
MGYG-HGUT-02278	*	89.22 [40.21]	99.05 [79.89]	89.04 [39.42]	89.22 [40.21]
MGYG-HGUT-01456	89.21 [41.98]	*	89.20 [40.53]	98.75 [80.31]	100.00 [99.70]
Strain 21	99.05 [82.87]	89.19 [40.09]	*	89.00 [39.88]	89.19 [40.09]
2789STDY5608891	89.04 [43.10]	98.75 [84.14]	89.01 [42.44]	*	98.75 [84.14]
ATCC 29099	89.21 [41.98]	100.00 [99.70]	89.20 [40.53]	98.75 [80.31]	*

Table S3.3. Average Nucleotide Identity (ANIm) and aligned percentage for 20 genomes belonging to *Flavonifractor* spp.

	Fp_1_3_50AFAA	Fp_2789STDY5834892	Fp_2789STDY5834932	Fp_100117 5st1_C9	Fp_An_248	Fp_ATCC_29863	Fp_DSM_6740	Fp_MC1	Fp_YL31	Fp_An_04	Fp_An_09	Fp_An_52	Fp_An_91	Fp_An_112	Fp_An_10	Fp_An_82	Fp_An_92	Fp_An_100	Fp_An_135	Fp_An_306
Fp_1_3_50AFAA	*	98.48 [81.09]	98.57 [76.17]	98.56 [78.29]	98.43 [74.34]	98.49 [75.71]	98.73 [83.41]	98.52 [76.06]	98.36 [75.24]	85.67 [19.57]	85.75 [21.08]	85.81 [19.25]	85.79 [20.65]	85.66 [19.94]	86.78 [38.64]	85.82 [23.13]	85.09 [14.23]	84.21 [5.54]	85.13 [14.93]	86.09 [25.45]
Fp_2789STDY5834892	98.49 [71.72]	*	98.34 [68.99]	98.88 [67.94]	98.37 [66.71]	98.47 [64.98]	98.87 [70.37]	98.27 [65.16]	98.32 [62.83]	85.78 [17.65]	85.74 [18.42]	85.86 [17.26]	86.14 [19.63]	85.76 [17.71]	87.25 [35.04]	86.21 [21.34]	85.63 [12.36]	85.30 [5.85]	85.45 [14.00]	86.33 [23.12]
Fp_2789STDY5834932	98.56 [80.27]	98.34 [82.31]	*	98.66 [79.31]	98.45 [79.26]	98.63 [77.23]	98.60 [78.89]	98.56 [77.97]	98.48 [78.20]	85.84 [20.72]	86.08 [22.15]	85.80 [20.65]	86.46 [22.16]	85.65 [21.03]	87.07 [41.22]	86.01 [24.70]	85.56 [15.82]	85.24 [5.84]	86.36 [17.58]	86.21 [27.55]
Fp_100117 5st1_C9	98.56 [84.33]	98.87 [82.89]	98.66 [81.33]	*	98.43 [76.63]	98.67 [82.74]	98.87 [84.25]	98.58 [76.83]	98.40 [77.28]	85.66 [21.13]	85.68 [22.50]	85.73 [21.29]	85.66 [22.48]	85.60 [22.22]	87.02 [41.59]	86.04 [25.38]	85.31 [15.87]	84.72 [6.10]	85.48 [16.33]	86.22 [28.26]
Fp_An_248	98.44 [84.52]	98.37 [85.57]	98.45 [86.14]	98.46 [81.03]	*	98.66 [81.58]	98.57 [83.97]	98.41 [82.91]	98.48 [84.75]	85.67 [22.44]	85.63 [23.44]	85.67 [22.62]	85.66 [23.63]	85.67 [23.08]	86.91 [43.72]	85.82 [26.37]	85.17 [16.23]	84.64 [5.73]	85.28 [17.32]	86.04 [28.99]
Fp_ATCC_29863	98.49 [84.95]	98.47 [82.90]	98.63 [82.72]	98.67 [86.45]	98.66 [80.44]	*	98.66 [84.60]	98.49 [79.64]	98.39 [80.87]	85.77 [22.20]	85.64 [22.65]	85.70 [22.00]	86.21 [24.16]	85.67 [22.97]	86.87 [42.51]	85.89 [26.18]	85.17 [15.95]	85.65 [6.98]	86.33 [18.09]	86.40 [29.42]
Fp_DSM_6740	98.72 [82.63]	98.88 [79.12]	98.61 [74.51]	98.87 [77.56]	98.54 [73.08]	98.67 [74.46]	*	98.64 [74.60]	98.45 [72.09]	85.64 [19.30]	85.63 [20.73]	85.95 [19.58]	85.65 [20.31]	85.59 [20.19]	87.10 [39.70]	86.15 [23.81]	85.24 [13.93]	84.90 [5.58]	85.35 [14.66]	86.21 [25.37]
Fp_MC1	98.54 [82.46]	98.27 [80.86]	98.57 [80.51]	98.58 [77.26]	98.42 [79.01]	98.49 [76.76]	98.64 [82.53]	*	98.30 [78.18]	85.81 [21.26]	86.08 [22.58]	85.93 [21.13]	86.46 [23.50]	85.66 [21.44]	86.95 [40.17]	86.06 [25.19]	85.16 [15.53]	84.53 [5.76]	86.15 [17.31]	86.33 [28.13]

Fp_YL31	98.36 [85.67]	98.31 [80.93]	98.48 [84.15]	98.41 [80.84]	98.48 [84.49]	98.38 [81.81]	98.45 [83.43]	98.30 [81.16]	*	85.61 [22.93]	85.73 [23.76]	85.78 [22.71]	85.59 [24.58]	85.61 [23.42]	86.84 [42.24]	85.83 [26.41]	85.19 [16.10]	85.06 [6.50]	85.17 [16.73]	85.92 [28.41]
Fp_An_04	85.68 [26.25]	85.79 [27.17]	85.84 [26.48]	85.66 [26.39]	85.67 [26.17]	85.78 [26.56]	85.65 [26.46]	85.82 [26.01]	85.61 [26.69]	*	97.62 [76.42]	96.71 [74.06]	96.90 [78.92]	96.73 [72.35]	86.16 [30.30]	89.36 [60.57]	85.78 [19.28]	86.70 [8.39]	86.34 [21.22]	86.89 [35.84]
Fp_An_09	85.75 [27.16]	85.75 [27.34]	86.08 [27.09]	85.69 [26.86]	85.63 [26.39]	85.64 [26.05]	85.63 [27.31]	86.09 [26.53]	85.73 [26.63]	97.62 [73.05]	*	96.62 [69.83]	96.77 [73.90]	96.79 [70.22]	87.18 [33.99]	89.41 [58.77]	85.98 [20.09]	87.02 [10.32]	86.64 [23.24]	86.68 [35.46]
Fp_An_52	85.81 [29.50]	85.86 [30.22]	85.79 [30.18]	85.72 [30.29]	85.67 [30.20]	85.70 [29.80]	85.95 [30.53]	85.94 [29.43]	85.77 [30.20]	96.71 [83.77]	96.61 [82.66]	*	97.03 [84.58]	97.17 [83.09]	86.17 [34.19]	89.11 [66.25]	85.77 [20.85]	85.52 [9.08]	85.93 [21.71]	86.63 [40.38]
Fp_An_91	85.79 [25.07]	86.14 [26.79]	86.46 [25.57]	85.66 [25.44]	85.66 [25.01]	86.21 [25.77]	85.65 [24.94]	86.45 [26.31]	85.58 [25.63]	96.90 [69.39]	96.77 [69.56]	97.03 [67.00]	*	97.05 [66.64]	86.44 [28.26]	89.12 [54.02]	85.99 [18.52]	88.37 [10.64]	87.44 [21.81]	87.02 [33.32]
Fp_An_112	85.66 [29.19]	85.76 [29.60]	85.65 [29.24]	85.59 [30.13]	85.67 [29.34]	85.67 [29.60]	85.60 [30.03]	85.66 [28.54]	85.61 [29.67]	96.73 [78.20]	96.78 [79.47]	97.17 [79.38]	97.05 [80.33]	*	86.19 [33.53]	89.00 [63.70]	85.83 [21.61]	86.50 [9.40]	85.87 [21.97]	86.84 [40.07]
Fp_An_10	86.78 [44.13]	87.23 [44.90]	87.05 [43.85]	87.01 [43.49]	86.90 [43.30]	86.86 [42.85]	87.09 [44.92]	86.95 [41.70]	86.83 [42.22]	86.16 [26.00]	87.18 [30.75]	86.17 [25.24]	86.44 [26.91]	86.20 [25.94]	*	87.11 [33.41]	85.65 [19.85]	87.36 [9.68]	85.93 [22.28]	87.44 [34.34]
Fp_An_82	85.83 [27.41]	86.20 [29.00]	86.02 [27.63]	86.04 [27.96]	85.82 [27.20]	85.89 [27.47]	86.16 [28.93]	86.06 [27.14]	85.83 [27.24]	89.36 [53.17]	89.42 [53.92]	89.12 [51.27]	89.11 [52.84]	89.00 [51.59]	87.11 [34.80]	*	86.57 [20.72]	87.70 [10.26]	87.41 [23.83]	87.13 [37.45]
Fp_An_92	85.09 [17.97]	85.62 [17.71]	85.54 [18.53]	85.31 [17.95]	85.17 [17.74]	85.17 [17.59]	85.23 [17.92]	85.16 [17.81]	85.19 [17.54]	85.76 [17.95]	85.98 [19.73]	85.75 [16.91]	85.97 [19.23]	85.81 [18.28]	85.65 [21.98]	86.57 [21.80]	*	86.03 [9.06]	98.91 [93.42]	86.14 [20.20]
Fp_An_100	84.23 [7.97]	85.28 [9.44]	85.26 [7.79]	84.73 [8.23]	84.66 [7.12]	85.67 [8.65]	84.91 [8.16]	84.55 [7.52]	85.08 [7.60]	86.70 [8.72]	87.02 [11.49]	85.52 [8.20]	88.37 [12.40]	86.51 [9.09]	87.36 [11.74]	87.70 [12.31]	86.03 [10.24]	*	87.39 [13.13]	87.85 [12.37]
Fp_An_135	85.13	85.45	86.35	85.48	85.28	86.33	85.36	86.14	85.16	86.33	86.64	85.91	87.43	85.86	85.93	87.40	98.91	87.39	*	86.79

	[16.58]	[17.79]	[18.48]	[16.63]	[16.85]	[17.86]	[16.73]	[17.73]	[16.29]	[17.61]	[20.36]	[15.97]	[20.03]	[16.88]	[21.71]	[22.40]	[84.35]	[10.71]	[20.63]
Fp_An_306	86.08 [29.02]	86.32 [30.13]	86.20 [29.07]	86.20 [29.52]	86.03 [28.40]	86.39 [29.60]	86.20 [29.23]	86.32 [29.32]	85.91 [27.71]	86.91 [29.80]	86.69 [30.52]	86.66 [29.21]	87.04 [31.18]	86.84 [30.40]	87.45 [33.66]	87.13 [35.29]	86.14 [18.51]	87.83 [10.12]	86.79 [21.67]	*

Coverage is given in brackets.

Table S3.4. Protein list for each orthologous gene clusters for the genomes of *E. ramulus* 2789STDY5608891, *E. ramulus* ATCC29099, and *E. ramulus* MGYG01456.

# cluster_name	protein_number	swiss_prot_id	go_annotation	protein_list
cluster2536	3	E3PRK0	GO:0019475; P:L-lysine catabolic process to acetate; IEA:UniProtKB-UniPathway	EramulusATCC|ERK46210.1;Eramulus2789STDY|CUN14552.1;EramulusMGYG01456|CABKSU010000112.1_56
cluster2628	3	Q9XBQ8	GO:0019475; P:L-lysine catabolic process to acetate; IEA:UniProtKB-UniPathway	EramulusATCC|ERK46208.1;Eramulus2789STDY|CUN14590.1;EramulusMGYG01456|CABKSU010000112.1_54
cluster2694	3	E3PRJ9	GO:0019475; P:L-lysine catabolic process to acetate; IEA:UniProtKB-UniPathway	EramulusATCC|ERK46209.1;Eramulus2789STDY|CUN14574.1;EramulusMGYG01456|CABKSU010000112.1_55
cluster2778	3	E3PRK1	GO:0019475; P:L-lysine catabolic process to acetate; IEA:UniProtKB-UniPathway	EramulusATCC|ERK46211.1;Eramulus2789STDY|CUN14537.1;EramulusMGYG01456|CABKSU010000112.1_57
cluster1258	6	E3PRK0	GO:0019475; P:L-lysine catabolic process to acetate; IEA:UniProtKB-UniPathway	EramulusMGYG02278|CABMEW010000002.1_59;Eoxidoreducens|SDB19416.1;Eramulus21|PWE86865.1;Eramulus2789STDY|CUM90605.1;EramulusMGYG01456|CABKSU010000018.1_53;EramulusATCC|ERK43036.1
cluster2730	3	C6DJR5	GO:0019299; P:rhamnose metabolic process; IEA:UniProtKB-UniRule	EramulusATCC|ERK41761.1;Eramulus2789STDY|CUN23050.1;EramulusMGYG01456|CABKSU010000033.1_13
cluster2803	3	Q65Q26	GO:0019301; P:rhamnose catabolic process; IEA:UniProtKB-UniPathway	EramulusATCC|ERK47447.1;Eramulus2789STDY|CUN23570.1;EramulusMGYG01456|CABKSU010000080.1_12

cluster2789	3	Q9KCM0	GO:0019301; P:rhamnose catabolic process; IEA:UniProtKB-UniPathway	EramulusATCC|ERK47449.1;Eramulus2789STDY|CUN23587.1;EramulusMGYG01456|CABKSU010000080.1_14
cluster2259	4	B8FZR3	GO:0042823; P:pyridoxal phosphate biosynthetic process; IEA:UniProtKB-UniRule	EramulusATCC|ERK46173.1;Eramulus2789STDY|CUN15211.1;EramulusMGYG01456|CABKSU010000112.1_21;Erectale|ACR76680.1
cluster2260	4	B8FZR4	GO:0042823; P:pyridoxal phosphate biosynthetic process; IEA:UniProtKB-UniRule	EramulusATCC|ERK46174.1;Eramulus2789STDY|CUN15191.1;EramulusMGYG01456|CABKSU010000112.1_22;Erectale|ACR76681.1

Table S3.5. Protein list for each orthologous gene clusters for the genomes of *E. ramulus* 2789STDY5608891, *E. ramulus* ATCC29099, *E. ramulus* MGYG01456, *E. ramulus* 21, and *E. ramulus* MGYG02278.

# cluster_name	protein_number	swiss_prot_id	go_annotation	protein_list
cluster1035	6	O26801	GO:0030976; F:thiamine pyrophosphate binding; IEA:InterPro	EramulusMGYG02278|CABMEW010000001.1_122;Eoxidoreducens|SDB28922.1;Eramulus21|PWE86535.1;Eramulus2789STDY|CUM93990.1;EramulusMGYG01456|CABKSU010000021.1_85;EramulusATCC|ERK42627.1
cluster1379	6	O26801	GO:0030976; F:thiamine pyrophosphate binding; IEA:InterPro	EramulusMGYG02278|CABMEW010000001.1_123;Eoxidoreducens|SDB28910.1;Eramulus21|PWE86536.1;Eramulus2789STDY|CUM94028.1;EramulusMGYG01456|CABKSU010000021.1_84;EramulusATCC|ERK42626.1
cluster1443	6	Q56317	GO:0030976; F:thiamine pyrophosphate binding; IEA:InterPro	EramulusMGYG02278|CABMEW010000002.1_153;Eoxidoreducens|SDB33464.1;Eramulus21|PWE87698.1;Eramulus2789STDY|CUM72979.1;EramulusMGYG01456|CABKSU010000006.1_37;EramulusATCC|ERK43358.1
cluster1092	6	Q9K9G5	GO:0009229; P:thiamine diphosphate biosynthetic process; IEA:UniProtKB-UniPathway	EramulusMGYG02278|CABMEW010000007.1_33;Eoxidoreducens|SDB27697.1;Eramulus21|PWE87606.1;Eramulus2789STDY|CUN14904.1;EramulusMGYG01456|CABKSU010000112.1_38;EramulusATCC|ERK46191.1
cluster1018	6	Q97LQ9	GO:0009229; P:thiamine diphosphate biosynthetic process; IEA:UniProtKB-UniPathway	EramulusMGYG02278|CABMEW010000009.1_82;Eramulus21|PWE86015.1;Eramulus2789STDY|CUM86707.1;EramulusMGYG01456|CABKSU010000057.1_74;EramulusATCC|ERK49886.1;Erectale|ACR74026.1
cluster1021	6	P61422	GO:0009229; P:thiamine diphosphate biosynthetic process; IEA:UniProtKB-UniPathway	EramulusMGYG02278|CABMEW010000001.1_199;Eramulus21|PWE86603.1;Eramulus2789STDY|CUN26534.1;EramulusMGYG01456|CABKSU010000063.1_69;EramulusATCC|ERK51838.1;Erectale|ACR76259.1
cluster1219	6	Q97EL4	GO:0009229; P:thiamine diphosphate biosynthetic process; IEA:UniProtKB-UniPathway	EramulusMGYG02278|CABMEW010000001.1_196;Eramulus21|PWE86600.1;Eramulus2789STDY|CUN26564.1;EramulusMGYG01456|CABKSU010000063.1_66;EramulusATCC|ERK51835.1;Erectale|ACR76261.1

cluster1308	6	A6LTL5	GO:0009229; P:thiamine diphosphate biosynthetic process; IEA:UniProtKB-UniPathway	EramulusMGYG02278|CABMEW010000009.1_80;Eramulus21|PWE86013.1;Eramulus2789STDY|CUM86750.1;EramulusMGYG01456|CABKSU010000057.1_72;EramulusATCC|ERK49884.1;Erectale|ACR74028.1
cluster1387	6	A0Q1U9	GO:0009229; P:thiamine diphosphate biosynthetic process; IEA:UniProtKB-UniPathway	EramulusMGYG02278|CABMEW010000001.1_202;Eramulus21|PWE86606.1;Eramulus2789STDY|CUN26517.1;EramulusMGYG01456|CABKSU010000063.1_71;EramulusATCC|ERK51842.1;Erectale|ACR76258.1
cluster1448	6	Q893R0	GO:0009229; P:thiamine diphosphate biosynthetic process; IEA:UniProtKB-UniPathway	EramulusMGYG02278|CABMEW010000001.1_197;Eramulus21|PWE86601.1;Eramulus2789STDY|CUN26552.1;EramulusMGYG01456|CABKSU010000063.1_67;EramulusATCC|ERK51836.1;Erectale|ACR76260.1
cluster1103	6	F4JYE9	GO:0046654; P:tetrahydrofolate biosynthetic process; IEA:UniProtKB-UniPathway	EramulusMGYG02278|CABMEW010000008.1_129;Eoxidoreducens|SDB27740.1;Eramulus21|PWE86909.1;Eramulus2789STDY|CUM88513.1;EramulusMGYG01456|CABKSU010000064.1_66;EramulusATCC|ERK49454.1
cluster1452	6	P59657	GO:0046654; P:tetrahydrofolate biosynthetic process; IEA:UniProtKB-UniPathway	EramulusMGYG02278|CABMEW010000008.1_132;Eoxidoreducens|SDB27717.1;Eramulus21|PWE86911.1;Eramulus2789STDY|CUM88575.1;EramulusMGYG01456|CABKSU010000064.1_63;EramulusATCC|ERK49451.1
cluster1512	6	B1L1S1	GO:0046654; P:tetrahydrofolate biosynthetic process; IEA:UniProtKB-UniRule	EramulusMGYG02278|CABMEW010000003.1_154;Eoxidoreducens|SDB03615.1;Eramulus21|PWE88095.1;Eramulus2789STDY|CUM71334.1;EramulusMGYG01456|CABKSU010000082.1_45;EramulusATCC|ERK47260.1
cluster1518	6	Q05621	GO:0046654; P:tetrahydrofolate biosynthetic process; IEA:UniProtKB-UniPathway	EramulusMGYG02278|CABMEW010000008.1_131;Eoxidoreducens|SDB27725.1;Eramulus21|PWE86910.1;Eramulus2789STDY|CUM88556.1;EramulusMGYG01456|CABKSU010000064.1_64;EramulusATCC|ERK49452.1
cluster1049	6	C0H450	GO:0030435; P:sporulation resulting in formation of a cellular spore; IEA:UniProtKB-KW	EramulusMGYG02278|CABMEW010000007.1_75;Eramulus21|PWE87646.1;Eramulus2789STDY|CUN04726.1;EramulusMGYG01456|CABKSU010000104.1_40;EramulusATCC|ERK46456.1;Erectale|ACR76162.1
cluster1058	6	P33658	GO:0030435; P:sporulation resulting in formation of a cellular spore; IEA:UniProtKB-KW	EramulusMGYG02278|CABMEW010000002.1_36;Eramulus21|PWE86849.1;Eramulus2789STDY|CUM90376.1;EramulusMGYG01456|CABKSU010000018.1_66;EramulusATCC|ERK42916.1;Erectale|ACR75980.1
cluster1071	6	Q8RB77	GO:0009847; P:spore germination; IEA:UniProtKB-UniRule	EramulusMGYG02278|CABMEW010000003.1_170;Eramulus21|PWE88042.1;Eramulus2789STDY|CUM77476.1;EramulusMGYG01456|CABKSU010000098.1_29;EramulusATCC|ERK46788.1;Erectale|ACR75363.1

cluster1087	6	P37558	GO:0030435; P:sporulation resulting in formation of a cellular spore; IEA:UniProtKB-KW	EramulusMGYG02278|CABMEW010000024.1_18;Eramulus21|PWE86103.1;Eramulus2789STDY|CUN21500.1;EramulusMGYG01456|CABKSU010000021.1_24;EramulusATCC|ERK42695.1;Erectale|ACR74321.1
cluster1090	6	B0K9G3	GO:0030435; P:sporulation resulting in formation of a cellular spore; IEA:UniProtKB-KW	EramulusMGYG02278|CABMEW010000008.1_33;Eramulus21|PWE86964.1;Eramulus2789STDY|CUN19136.1;EramulusMGYG01456|CABKSU010000122.1_35;EramulusATCC|ERK43707.1;Erectale|ACR76304.1
cluster1097	6	P40868	GO:0030435; P:sporulation resulting in formation of a cellular spore; IEA:UniProtKB-KW	EramulusMGYG02278|CABMEW010000008.1_50;Eramulus21|PWE86977.1;Eramulus2789STDY|CUN18942.1;EramulusMGYG01456|CABKSU010000122.1_23;EramulusATCC|ERK43768.1;Erectale|ACR74879.1
cluster1123	6	O34765	GO:0030435; P:sporulation resulting in formation of a cellular spore; IEA:UniProtKB-KW	EramulusMGYG02278|CABMEW010000004.1_48;Eramulus21|PWE87796.1;Eramulus2789STDY|CUM97308.1;EramulusMGYG01456|CABKSU010000038.1_42;EramulusATCC|ERK50468.1;Erectale|ACR75424.1
cluster1143	6	P24141	GO:0030435; P:sporulation resulting in formation of a cellular spore; IEA:UniProtKB-KW	EramulusMGYG02278|CABMEW010000023.1_14;Eramulus21|PWE86396.1;Eramulus2789STDY|CUM92553.1;EramulusMGYG01456|CABKSU010000009.1_19;EramulusATCC|ERK50833.1;Erectale|ACR74743.1
cluster1168	6	C0QYX7	GO:0030435; P:sporulation resulting in formation of a cellular spore; IEA:InterPro	EramulusMGYG02278|CABMEW010000002.1_140;Eramulus21|PWE87685.1;Eramulus2789STDY|CUM73190.1;EramulusMGYG01456|CABKSU010000006.1_9;EramulusATCC|ERK43329.1;Erectale|ACR75729.1
cluster1179	6	P06222	GO:0030435; P:sporulation resulting in formation of a cellular spore; IEA:UniProtKB-KW	EramulusMGYG02278|CABMEW010000002.1_33;Eramulus21|PWE86846.1;Eramulus2789STDY|CUM90327.1;EramulusMGYG01456|CABKSU010000018.1_69;EramulusATCC|ERK42919.1;Erectale|ACR75984.1
cluster1181	6	P40867	GO:0030435; P:sporulation resulting in formation of a cellular spore; IEA:UniProtKB-KW	EramulusMGYG02278|CABMEW010000008.1_49;Eramulus21|PWE86978.1;Eramulus2789STDY|CUN18958.1;EramulusMGYG01456|CABKSU010000122.1_24;EramulusATCC|ERK43769.1;Erectale|ACR74831.1
cluster1210	6	P49780	GO:0030435; P:sporulation resulting in formation of a cellular spore; IEA:UniProtKB-KW	EramulusMGYG02278|CABMEW010000002.1_77;Eramulus21|PWE86761.1;Eramulus2789STDY|CUM90965.1;EramulusMGYG01456|CABKSU010000018.1_35;EramulusATCC|ERK43016.1;Erectale|ACR75974.1
cluster1211	6	P39151	GO:0030435; P:sporulation resulting in formation of a cellular spore; IEA:UniProtKB-KW	EramulusMGYG02278|CABMEW010000017.1_17;Eramulus21|PWE85582.1;Eramulus2789STDY|CUN25808.1;EramulusMGYG01456|CABKSU010000016.1_9;EramulusATCC|ERK50672.1;Erectale|ACR74765.1

cluster1232	6	P62182	GO:0030435; P:sporulation resulting in formation of a cellular spore; IEA:UniProtKB-KW	EramulusMGYG02278|CABMEW010000018.1_60;Eramulus21|PWE86450.1;Eramulus2789STDY|CUM78110.1;EramulusMGYG01456|CABKSU010000098.1_11;EramulusATCC|ERK46768.1;Erectale|ACR75560.1
cluster1296	6	P35150	GO:0030435; P:sporulation resulting in formation of a cellular spore; IEA:UniProtKB-KW	EramulusMGYG02278|CABMEW010000004.1_207;Eramulus21|PWE86694.1;Eramulus2789STDY|CUM75745.1;EramulusMGYG01456|CABKSU010000091.1_14;EramulusATCC|ERK51326.1;Erectale|ACR75369.1
cluster1320	6	P15281	GO:0030435; P:sporulation resulting in formation of a cellular spore; IEA:UniProtKB-KW	EramulusMGYG02278|CABMEW010000001.1_326;Eramulus21|PWE85793.1;Eramulus2789STDY|CUN11272.1;EramulusMGYG01456|CABKSU010000113.1_4;EramulusATCC|ERK45983.1;Erectale|ACR74407.1
cluster1323	6	P49784	GO:0030435; P:sporulation resulting in formation of a cellular spore; IEA:UniProtKB-KW	EramulusMGYG02278|CABMEW010000002.1_81;Eramulus21|PWE86758.1;Eramulus2789STDY|CUM91043.1;EramulusMGYG01456|CABKSU010000018.1_31;EramulusATCC|ERK43012.1;Erectale|ACR75970.1
cluster1341	6	P49939	GO:0009847; P:spore germination; IEA:InterPro	EramulusMGYG02278|CABMEW010000017.1_14;Eramulus21|PWE85585.1;Eramulus2789STDY|CUN25779.1;EramulusMGYG01456|CABKSU010000016.1_6;EramulusATCC|ERK50669.1;Erectale|ACR74763.1
cluster1347	6	P49781	GO:0030435; P:sporulation resulting in formation of a cellular spore; IEA:UniProtKB-KW	EramulusMGYG02278|CABMEW010000002.1_78;Eramulus21|PWE86760.1;Eramulus2789STDY|CUM90990.1;EramulusMGYG01456|CABKSU010000018.1_34;EramulusATCC|ERK43015.1;Erectale|ACR75973.1
cluster1350	6	P17896	GO:0030435; P:sporulation resulting in formation of a cellular spore; IEA:UniProtKB-KW	EramulusMGYG02278|CABMEW010000003.1_118;Eramulus21|PWE88063.1;Eramulus2789STDY|CUM70758.1;EramulusMGYG01456|CABKSU010000082.1_72;EramulusATCC|ERK47288.1;Erectale|ACR75943.1
cluster1354	6	P21887	GO:0030435; P:sporulation resulting in formation of a cellular spore; IEA:UniProtKB-KW	EramulusMGYG02278|CABMEW010000012.1_40;Eramulus21|PWE86249.1;Eramulus2789STDY|CUN12557.1;EramulusMGYG01456|CABKSU010000004.1_7;EramulusATCC|ERK43473.1;Erectale|ACR74749.1
cluster1411	6	P37554	GO:0030435; P:sporulation resulting in formation of a cellular spore; IEA:UniProtKB-KW	EramulusMGYG02278|CABMEW010000010.1_27;Eramulus21|PWE85639.1;Eramulus2789STDY|CUM94881.1;EramulusMGYG01456|CABKSU010000021.1_40;EramulusATCC|ERK42713.1;Erectale|ACR76879.1
cluster1425	6	Q01367	GO:0030435; P:sporulation resulting in formation of a cellular spore; IEA:UniProtKB-KW	EramulusMGYG02278|CABMEW010000002.1_75;Eramulus21|PWE86763.1;Eramulus2789STDY|CUM90929.1;EramulusMGYG01456|CABKSU010000018.1_37;EramulusATCC|ERK43018.1;Erectale|ACR75976.1

cluster1446	6	Q00758	GO:0030435; P:sporulation resulting in formation of a cellular spore; IEA:UniProtKB-KW	EramulusMGYG02278|CABMEW010000011.1_26;Eramulus21|PWE85970.1;Eramulus2789STDY|CUM85205.1;EramulusMGYG01456|CABKSU010000025.1_86;EramulusATCC|ERK42356.1;Erectale|ACR75193.1
cluster1453	6	P24136	GO:0030435; P:sporulation resulting in formation of a cellular spore; IEA:UniProtKB-KW	EramulusMGYG02278|CABMEW010000023.1_16;Eramulus21|PWE86398.1;Eramulus2789STDY|CUM92503.1;EramulusMGYG01456|CABKSU010000009.1_21;EramulusATCC|ERK50835.1;Erectale|ACR74741.1
cluster1511	6	P06534	GO:0030435; P:sporulation resulting in formation of a cellular spore; IEA:UniProtKB-KW	EramulusMGYG02278|CABMEW010000003.1_109;Eramulus21|PWE88053.1;Eramulus2789STDY|CUM70574.1;EramulusMGYG01456|CABKSU010000082.1_80;EramulusATCC|ERK47297.1;Erectale|ACR75910.1
cluster1517	6	Q81SW4	GO:0030435; P:sporulation resulting in formation of a cellular spore; IDA:UniProtKB	EramulusMGYG02278|CABMEW010000002.1_68;Eramulus21|PWE86873.1;Eramulus2789STDY|CUM90795.1;EramulusMGYG01456|CABKSU010000018.1_44;EramulusATCC|ERK43026.1;Erectale|ACR75583.1
cluster1547	5	P26764	GO:0030435; P:sporulation resulting in formation of a cellular spore; IEA:UniProtKB-KW	EramulusMGYG02278|CABMEW010000008.1_34;Eramulus21|PWE86963.1;Eramulus2789STDY|CUN19119.1;EramulusMGYG01456|CABKSU010000122.1_34;EramulusATCC|ERK43706.1
cluster1559	5	P35157	GO:0030435; P:sporulation resulting in formation of a cellular spore; IEA:UniProtKB-KW	EramulusMGYG02278|CABMEW010000029.1_13;Eramulus21|PWE87422.1;Eramulus2789STDY|CUM82200.1;EramulusMGYG01456|CABKSU010000037.1_15;EramulusATCC|ERK41665.1
cluster1615	5	P35158	GO:0030435; P:sporulation resulting in formation of a cellular spore; IEA:UniProtKB-KW	EramulusMGYG02278|CABMEW010000029.1_19;Eramulus21|PWE87408.1;Eramulus2789STDY|CUM82178.1;EramulusMGYG01456|CABKSU010000037.1_16;EramulusATCC|ERK41666.1
cluster1649	5	P60495	GO:0030435; P:sporulation resulting in formation of a cellular spore; IEA:UniProtKB-KW	EramulusMGYG02278|CABMEW010000005.1_186;Eramulus21|PWE86926.1;Eramulus2789STDY|CUM88927.1;EramulusMGYG01456|CABKSU010000064.1_48;EramulusATCC|ERK49434.1
cluster1720	5	B1HVK6	GO:0030435; P:sporulation resulting in formation of a cellular spore; IEA:UniProtKB-UniRule	EramulusMGYG02278|CABMEW010000007.1_89;Eramulus21|PWE85503.1;Eramulus2789STDY|CUN04108.1;EramulusMGYG01456|CABKSU010000104.1_13;EramulusATCC|ERK46513.1
cluster1877	5	P71044	GO:0030435; P:sporulation resulting in formation of a cellular spore; IEA:UniProtKB-KW	EramulusMGYG02278|CABMEW010000002.1_101;Eramulus21|PWE87186.1;Eramulus2789STDY|CUN15166.1;EramulusMGYG01456|CABKSU010000112.1_26;EramulusATCC|ERK46178.1

clust er192 7	5	Q7W Y77	GO:0030435; P:sporulation resulting in formation of a cellular spore; IEA:UniProtKB-KW	EramulusMGYG02278|CABMEW010000005.1_187;Eramulus21|PWE86925.1;Era mulus2789STDY|CUM88907.1;EramulusMGYG01456|CABKSU010000064.1_49;E ramulusATCC|ERK49435.1

Table S3.6. Protein list for each orthologous gene clusters for the genomes of *E. oxidoreducens, E. rectale*, *E. ramulus* 21, and *E. ramulus* MGYG02278.

# cluster_name	protein_number	swiss_prot_id	go_annotation	protein_list
cluster1564	5	P39853	GO:0045227; P:capsule polysaccharide biosynthetic process; IEA:UniProtKB-UniPathway	EramulusMGYG02278|CABMEW010000009.1_19;Eoxidoreducens|SDB21266.1;Erectale|ACR76350.1;EramulusMGYG02278|CABMEW010000005.1_142;Eramulus21|PWE87843.1
cluster2123	4	Q0P8J8	GO:0045227; P:capsule polysaccharide biosynthetic process; IEA:UniProtKB-UniPathway	EramulusMGYG02278|CABMEW010000007.1_62;Eoxidoreducens|SDB17934.1;Eramulus21|PWE87642.1;Erectale|ACR76339.1
cluster1564	5	P39853	GO:0045227; P:capsule polysaccharide biosynthetic process; IEA:UniProtKB-UniPathway	EramulusMGYG02278|CABMEW010000009.1_19;Eoxidoreducens|SDB21266.1;Erectale|ACR76350.1;EramulusMGYG02278|CABMEW010000005.1_142;Eramulus21|PWE87843.1
cluster2123	4	Q0P8J8	GO:0045227; P:capsule polysaccharide biosynthetic process; IEA:UniProtKB-UniPathway	EramulusMGYG02278|CABMEW010000007.1_62;Eoxidoreducens|SDB17934.1;Eramulus21|PWE87642.1;Erectale|ACR76339.1
cluster381	7	P71057	GO:0000271; P:polysaccharide biosynthetic process; IEA:UniProtKB-KW	EramulusMGYG02278|CABMEW010000007.1_60;Eoxidoreducens|SDB06730.1;Eramulus21|PWE87643.1;Erectale|ACR76357.1;Eoxidoreducens|SDB06792.1;Eoxidoreducens|SDB06813.1;Eoxidoreducens|SDB30699.1
cluster2102	4	P71055	GO:0000271; P:polysaccharide biosynthetic process; IEA:UniProtKB-KW	EramulusMGYG02278|CABMEW010000007.1_64;Eoxidoreducens|SDB21844.1;Eramulus21|PWE87640.1;Erectale|ACR76356.1

cluster2117	4	P14184	GO:0009103; P:lipopolysaccharide biosynthetic process; IEA:UniProtKB-KW	EramulusMGYG02278|CABMEW010000007.1_42;Eramulus21|PWE87588.1;Erectale|ACR76380.1;Eoxidoreducens|SDB15620.1
cluster2190	4	Q58466	GO:0000271; P:polysaccharide biosynthetic process; IBA:GO_Central	EramulusMGYG02278|CABMEW010000009.1_15;Eramulus21|PWE87842.1;Erectale|ACR76349.1;Eoxidoreducens|SDB15544.1

Table S3.7. Protein list for each orthologous gene clusters for the genomes of *F. plautii* YL31, *F. plautii* 2789STDY5834932, *F. plautii* ATCC 29863, *F. plautii* An248.

# cluster_name	protein_number	swiss_prot_id	go_annotation	protein_list
cluster2635	4	Q892D0	GO:0046336; P:ethanolamine catabolic process; IEA:UniProtKB-UniPathway	2789STDY5834932|CUP71372.1;An248|OUO82145.1;YL31|ANU41982.1;ATCC_29863|EHM42371.1
cluster2683	4	Q9ZFV2	GO:0046336; P:ethanolamine catabolic process; IEA:UniProtKB-UniPathway	2789STDY5834932|CUP71298.1;An248|OUO82147.1;YL31|ANU41984.1;ATCC_29863|EHM42369.1
cluster2711	4	P0AEJ7	GO:0046336; P:ethanolamine catabolic process; IEA:UniProtKB-UniPathway	2789STDY5834932|CUP71331.1;An248|OUO82146.1;YL31|ANU41983.1;ATCC_29863|EHM42370.1
cluster2744	4	Q892D0	GO:0046336; P:ethanolamine catabolic process; IEA:UniProtKB-UniPathway	2789STDY5834932|CUQ41078.1;An248|OUO83463.1;YL31|ANU42383.1;ATCC_29863|EHM47953.1
cluster2792	4	P76552	GO:0046336; P:ethanolamine catabolic process; IEA:UniProtKB-UniPathway	2789STDY5834932|CUP71884.1;An248|OUO82132.1;YL31|ANU41970.1;ATCC_29863|EHM42337.1
cluster2801	4	P77277	GO:0046336; P:ethanolamine catabolic process; IEA:UniProtKB-UniPathway	2789STDY5834932|CUP71680.1;An248|OUO82137.1;YL31|ANU41975.1;ATCC_29863|EHM42332.1
cluster2809	4	Q9ZFV4	GO:0046336; P:ethanolamine catabolic process; IEA:UniProtKB-UniPathway	2789STDY5834932|CUP71597.1;An248|OUO82139.1;YL31|ANU41977.1;ATCC_29863|EHM42330.1
cluster2881	4	P76541	GO:0046336; P:ethanolamine catabolic process; IEA:UniProtKB-UniPathway	2789STDY5834932|CUP71414.1;An248|OUO82144.1;YL31|ANU41981.1;ATCC_29863|EHM42372.1
cluster2965	4	P0ABF5	GO:0046336; P:ethanolamine catabolic process; IEA:UniProtKB-UniPathway	2789STDY5834932|CUP71451.1;An248|OUO82143.1;YL31|ANU41980.1;ATCC_29863|EHM42373.1
cluster3013	4	P76552	GO:0046336; P:ethanolamine catabolic process; IEA:UniProtKB-UniPathway	2789STDY5834932|CUP57828.1;An248|OUO83125.1;YL31|ANU42625.1;ATCC_29863|EHM49955.1
cluster3062	4	P19264	GO:0046336; P:ethanolamine catabolic process; IBA:GO_Central	2789STDY5834932|CUQ41065.1;An248|OUO83464.1;YL31|ANU42382.1;ATCC_29863|EHM47952.1

cluster 2633	4	E3PY99	GO:0006591; P:ornithine metabolic process; TAS:UniProtKB	2789STDY5834932|CUQ29162.1;An248|OUO83539.1;YL31|ANU42311.1;ATCC_29863|EHM54568.1
cluster 2723	4	C1FW08	GO:0006591; P:ornithine metabolic process; IDA:UniProtKB	2789STDY5834932|CUQ29280.1;An248|OUO83532.1;YL31|ANU42318.1;ATCC_29863|EHM54561.1
cluster 2827	4	E3PY97	GO:0006591; P:ornithine metabolic process; IDA:UniProtKB	2789STDY5834932|CUQ29196.1;An248|OUO83537.1;YL31|ANU42313.1;ATCC_29863|EHM54566.1
cluster 2982	4	E3PY98	GO:0006591; P:ornithine metabolic process; IDA:UniProtKB	2789STDY5834932|CUQ29181.1;An248|OUO83538.1;YL31|ANU42312.1;ATCC_29863|EHM54567.1
cluster 2731	4	P0A1D2	GO:0031469; C:polyhedral organelle; IEA:InterPro	2789STDY5834932|CUP71147.1;An248|OUO82151.1;YL31|ANU41988.1;ATCC_29863|EHM42365.1
cluster 2871	4	P0A1D2	GO:0031469; C:polyhedral organelle; IEA:InterPro	2789STDY5834932|CUP99707.1;An248|OUO83195.1;YL31|ANU42686.1;ATCC_29863|EHM52290.1
cluster 2952	4	P0A1D2	GO:0031469; C:polyhedral organelle; IEA:InterPro	2789STDY5834932|CUP99735.1;An248|OUO83196.1;YL31|ANU42687.1;ATCC_29863|EHM52288.1
cluster 2244	5	P23446	GO:0071978; P:bacterial-type flagellum-dependent swarming motility; IBA:GO_Central	2789STDY5834932|CUP86983.1;An248|OUO84117.1;YL31|ANU40129.1;An306|OUO41293.1;ATCC_29863|EHM53550.1
cluster 2270	5	P39063	GO:0071978; P:bacterial-type flagellum-dependent swarming motility; IBA:GO_Central	2789STDY5834932|CUP87040.1;An248|OUO84119.1;YL31|ANU40131.1;An306|OUO41291.1;ATCC_29863|EHM53552.1
cluster 2305	5	P24501	GO:0071978; P:bacterial-type flagellum-dependent swarming motility; IBA:GO_Central	2789STDY5834932|CUP86639.1;An248|OUO84107.1;YL31|ANU40119.2;An306|OUO41301.1;ATCC_29863|EHM53539.1
cluster 2336	5	P23446	GO:0071978; P:bacterial-type flagellum-dependent swarming motility; IBA:GO_Central	2789STDY5834932|CUP87427.1;An248|OUO84131.1;YL31|ANU40143.1;An306|OUO41279.1;ATCC_29863|EHM53564.1
cluster 2463	5	P24500	GO:0071978; P:bacterial-type flagellum-dependent swarming motility; IBA:GO_Central	2789STDY5834932|CUP86599.1;An248|OUO84106.1;YL31|ANU40118.1;An306|OUO41302.1;ATCC_29863|EHM53538.1

cluster 2194	5	Q0AX B7	GO:0006935; P:chemotaxis; IEA:UniProtKB-UniRule	2789STDY5834932|CUP86231.1;An248|OUO84097.1;YL31|ANU40110.1;An306|OUO31958.1;ATCC_29863|EHM38114.1
cluster 2239	5	Q9X0 06	GO:0006935; P:chemotaxis; IEA:UniProtKB-KW	2789STDY5834932|CUP87493.1;An248|OUO84133.1;YL31|ANU40145.1;An306|OUO41277.1;ATCC_29863|EHM53566.1
cluster 2240	5	P2181 3	GO:0006935; P:chemotaxis; IEA:UniProtKB-KW	2789STDY5834932|CUP86340.1;An248|OUO84100.1;YL31|ANU40113.1;An306|OUO31960.1;ATCC_29863|EHM38117.1
cluster 2319	5	P2345 3	GO:0050918; P:positive chemotaxis; IMP:CACAO	2789STDY5834932|CUP87107.1;An248|OUO84121.1;YL31|ANU40133.2;An306|OUO41289.1;ATCC_29863|EHM53554.1
cluster 2350	5	Q0AY K9	GO:0006935; P:chemotaxis; IEA:UniProtKB-UniRule	2789STDY5834932|CUP87524.1;An248|OUO84134.1;YL31|ANU40146.1;An306|OUO41276.1;ATCC_29863|EHM53567.1
cluster 2437	5	Q9W Y63	GO:0006935; P:chemotaxis; IEA:UniProtKB-KW	2789STDY5834932|CUP86742.1;An248|OUO84110.1;YL31|ANU40122.1;An306|OUO41299.1;ATCC_29863|EHM53542.1
cluster 2210	5	O677 50	GO:0009306; P:protein secretion; IEA:InterPro	2789STDY5834932|CUP87233.1;An248|OUO84125.1;YL31|ANU40137.1;An306|OUO41285.1;ATCC_29863|EHM53558.1
cluster 2343	5	P3562 0	GO:0009306; P:protein secretion; IEA:InterPro	2789STDY5834932|CUP87365.1;An248|OUO84129.1;YL31|ANU40141.1;An306|OUO41281.1;ATCC_29863|EHM53562.1
cluster 2363	5	P3553 5	GO:0009306; P:protein secretion; IEA:InterPro	2789STDY5834932|CUP87266.1;An248|OUO84126.1;YL31|ANU40138.1;An306|OUO41284.1;ATCC_29863|EHM53559.1
cluster 2453	5	P3553 8	GO:0009306; P:protein secretion; IEA:InterPro	2789STDY5834932|CUP87336.1;An248|OUO84128.1;YL31|ANU40140.1;An306|OUO41282.1;ATCC_29863|EHM53561.1
cluster 1763	7	P8058 3	GO:0071973; P:bacterial-type flagellum-dependent cell motility; IEA:InterPro	2789STDY5834932|CUP86109.1;An248|OUO84093.1;YL31|ANU40106.1;An306|OUO41062.1;ATCC_29863|EHM38107.1;An306|OUO39651.1;An306|OUO31954.1
cluster 2130	5	P3981 0	GO:0071973; P:bacterial-type flagellum-dependent cell motility; IEA:InterPro	2789STDY5834932|CUQ42182.1;An248|OUO85163.1;YL31|ANU40099.1;An306|OUO42493.1;ATCC_29863|EHM43411.1
cluster 2207	5	A1SE Q0	GO:0071973; P:bacterial-type flagellum-dependent cell motility; IEA:InterPro	2789STDY5834932|CUP86675.1;An248|OUO84108.1;YL31|ANU40120.1;An306|OUO41312.1;ATCC_29863|EHM53540.1

cluster 2220	5	P24073	GO:1902021; P:regulation of bacterial-type flagellum-dependent cell motility; IMP:CACAO	2789STDY5834932|CUP87135.1;An248|OUO84122.1;YL31|ANU40134.1;An306|OUO41288.1;ATCC_29863|EHM53555.1
cluster 2486	5	Q8K9K4	GO:0071973; P:bacterial-type flagellum-dependent cell motility; IEA:InterPro	2789STDY5834932|CUP87460.1;An248|OUO84132.1;YL31|ANU40144.1;An306|OUO41278.1;ATCC_29863|EHM53565.1

Table S3.8. Protein list for each orthologous gene clusters for the genomes of *Flavonifractor* sp. An4, *Flavonifractor* sp. An9, *Flavonifractor* sp. An82, *Flavonifractor* sp. An306.

# cluster_ name	protein_ number	swiss_prot_id	go_annotation	protein_list
cluster2102	5	Q2RGL2	GO:0006777; P:Mo-molybdopterin cofactor biosynthetic process; IEA:UniProtKB-UniRule	An82|OUN22106.1;An4|OUO17000.1;An10|OUQ81968.1;An306|OUO42827.1;An306|OUO41792.1
cluster2541	5	P44902	GO:0006777; P:Mo-molybdopterin cofactor biosynthetic process; IEA:UniProtKB-KW	An4|OUO16999.1;An82|OUN22107.1;An10|OUQ81967.1;An306|OUO42828.1;An306|OUO41791.1
cluster3168	4	B6IQ15	GO:0006777; P:Mo-molybdopterin cofactor biosynthetic process; IEA:UniProtKB-UniRule	An4|OUO16998.1;An82|OUN22108.1;An10|OUQ81966.1;An306|OUO42829.1
cluster3210	4	Q8YY90	GO:0006777; P:Mo-molybdopterin cofactor biosynthetic process; IEA:UniProtKB-KW	An4|OUO16450.1;An82|OUN21730.1;An306|OUO41795.1;An10|OUQ83256.1
cluster3198	4	Q5WH08	GO:0009231; P:riboflavin biosynthetic process; IEA:UniProtKB-UniRule	An4|OUO17295.1;An82|OUN22005.1;An10|OUQ80892.1;An306|OUO44622.1
cluster3201	4	B2V4J4	GO:0009231; P:riboflavin biosynthetic process; IEA:UniProtKB-UniRule	An4|OUO17294.1;An82|OUN22004.1;An10|OUQ80891.1;An306|OUO44623.1
cluster3206	4	P50854	GO:0009231; P:riboflavin biosynthetic process; IEA:UniProtKB-UniPathway	An4|OUO17296.1;An82|OUN22006.1;An10|OUQ80893.1;An306|OUO44621.1
cluster3212	4	Q97EZ4	GO:0006012; P:galactose metabolic process; IEA:UniProtKB-UniRule	An4|OUO16988.1;An82|OUN20983.1;An10|OUQ83371.1;An306|OUO41913.1
cluster3213	4	A5VME2	GO:0006012; P:galactose metabolic process; IEA:UniProtKB-UniRule	An4|OUO16990.1;An82|OUN20981.1;An10|OUQ83373.1;An306|OUO41915.1

Table S3.9. Ids for Eut and Pdu proteins for *Flavonifractor* spp.

Protein	YL31	ATCC_29863	2789STDY5834932	An248	An306	An82	An10	An4
First Eut operon								
AlcDH	ANU39943.1	EHM41053.1	CUP45427.1	OUO85060.1	OUO39463.1	OUN22497.1	OUQ83249.1	OUO11851.1
EutA	ANU39944.1	EHM41052.1	CUP45465.1	OUO85061.1	OUO39462.1	OUN22498.1	OUQ83248.1	OUO11850.1
EutB	ANU39945.1	EHM41051.1	CUP45499.1	OUO85062.1	OUO39461.1	OUN22499.1	OUQ83247.1	OUO11849.1
EutC	ANU39946.1	EHM41050.1	CUP45537.1	OUO85063.1	OUO39460.1	OUN22500.1	OUQ83246.1	OUO11848.1
EutL	ANU39947.1	EHM41048.1	CUP45573.1	OUO85064.1	OUO39459.1	OUN22501.1	OUQ83245.1	OUO11846.1
AldDH	ANU39948.1	EHM41078.1	CUP45615.1	OUO85065.1	OUO39458.1	OUN22502.1	OUQ83244.1	OUO11845.1
EutM	CP015406.2: 490,436-491,117	EHM41077.1	CUP45688.1	NFJM01000001.1:255050-255725	OUO39492.1	OUN22646.1	OUQ83425.1	OUO11857.1
EutT	ANU39949.1	EHM41076.1	CUP45722.1	OUO85066.1	OUO39457.1	OUN22503.1	OUQ83243.1	OUO11844.1
PTAC	ANU39950.1	EHM41075.1	CUP45761.1	OUO85067.1	OUO39456.1	OUN22504.1	OUQ83242.1	OUO11843.1
36% identity with ethanolamine utilization protein	ANU39951.1	EHM41074.1	CUP45794.1	OUO85068.1	OUO39455.1	OUN22505.1	OUQ83241.1	OUO11842.1
EutN	ANU39952.1	EHM41073.1	CUP45828.1	OUO85069.1	OUO39454.1	OUN22506.1	OUQ83240.1	OUO11841.1

EutH	ANU39953.1	EHM41072.1	CUP45859.1	OUO85070.1	OUO39453.1	OUN22507.1	OUQ83239.1	OUO11840.1
EutQ	ANU39954.1	EHM41071.1	CUP45901.1	OUO85071.1	OUO39452.1	OUN22508.1	OUQ83238.1	OUO11839.1
Pdu Operon								
PduV	ANU41514.1	EHM40047.1	CUP30877.1	OUO82753.1	OUO37647.1	OUN20352.1		
PduU	ANU41515.2	EHM40046.1	CUP30904.1	OUO82944.1	OUO37656.1	OUN20407.1		
oxidoreductase	ANU41516.1	EHM40044.1	CUP30965.1	OUO82752.1	OUO37645.1	OUN20350.1		
PduT	ANU41517.1	EHM40043.1	CUP30990.1	OUO82751.1	OUO37644.1	OUN20349.1		
hypothetical protein	ANU41518.1	EHM40042.1	CUP31020.1	OUO82750.1	OUO37655.1	OUN20348.1		
PduS	ANU41519.1	EHM40041.1	CUP31053.1	OUO82749.1	OUO37643.1	OUN20347.1		
AldDH	ANU41520.1	EHM40040.1	CUP31081.1	OUO82748.1	OUO37642.1	OUN20346.1		
PduO	ANU41521.1	EHM40039.1	CUP31110.1	OUO82747.1	OUO37641.1	OUN20345.1		
PduN	ANU41522.1	EHM40038.1	CUP31136.1	OUO82746.1	OUO37640.1	OUN20344.1		
PduM	ANU41523.1	EHM40037.1	CUP31164.1	OUO82745.1	OUO37639.1	OUN20343.1		
PTAC	ANU41524.1	EHM40035.1	CUP31220.1	OUO82743.1	OUO37637.1	OUN20341.1		
PduA	ANU41525.1	EHM40034.1	CUP31258.1	OUO82742.1	OUO37636.1	OUN20340.1		

PduJ	ANU41526.1	EHM40033.1	CUP31293.1	OUO82741.1	OUO37635.1	OUN20339.1 (contig break)
PduH	ANU41527.1	EHM40032.1	CUP31326.1	OUO82740.1	OUO37634.1	OUN18837.1
PduG	ANU41528.1	EHM40031.1	CUP31355.1	OUO82739.1	OUO37633.1	OUN18838.1
PduE	ANU41529.1	EHM40030.1	CUP31392.1	OUO82738.1	OUO37632.1	OUN18839.1
PduD	ANU41530.1	EHM40029.1	CUP31436.1	OUO82737.1	OUO37631.1	OUN18840.1
PduC	ANU41531.1	EHM40028.1	CUP31491.1	OUO82736.1	OUO37630.1	OUN18841.1
PduB	ANU41532.1	EHM40027.1	CUP31544.1	OUO82735.1	OUO37629.1	OUN18842.1
PduJ	ANU41533.1	EHM40026.1	CUP31585.1	OUO82734.1	OUO37628.1	OUN18843.1
AlcDH	ANU41534.1	EHM40025.1	CUP31631.1	OUO82733.1	OUO37626.1	OUN18844.1
Regulator	ANU41535.1	EHM40024.1	CUP31669.1	OUO82732.1	OUO37625.1	OUN18845.1
Histidine kinase	ANU41536.1	EHM40023.1	CUP31727.1	OUO82731.1	OUO37624.1	OUN18846.1
Kinase	ANU41537.1	EHM40021.1	CUP31777.1	OUO82730.1	contig break	contig break
Second Eut operon						
EutQ	ANU41969.1	EHM42338.1	CUP71918.1	OUO82131.1		

EutH	ANU41970.1	EHM42337.1	CUP71884.1	OUO82132.1
BMC protein	ANU41971.1	EHM42336.1	CUP71851.1	OUO82133.1
PduS homolog, cobalamin reductase	ANU41972.1	EHM42335.1	CUP71805.1	OUO82134.1
EutN	ANU41973.1	EHM42334.1	CUP71761.1	OUO82135.1
36% identity with PduM	ANU41974.1	EHM42333.1	CUP71724.1	OUO82136.1
EutJ	ANU41975.1	EHM42332.1	CUP71680.1	OUO82137.1
PTAC	ANU41976.1	EHM42331.1	CUP71633.1	OUO82138.1
EutT	ANU41977.1	EHM42330.1	CUP71597.1	OUO82139.1
EutM	ANU41978.1	EHM42329.1	CUP71551.1	OUO82140.1
EutM	ANU41979.1	EHM42328.1	CUP71511.1	OUO82141.1
EutM	ANU41980.1	EHM42373.1	CUP71451.1	OUO82143.1
EutL	ANU41981.1	EHM42372.1	CUP71414.1	OUO82144.1
EutC	ANU41982.1	EHM42371.1	CUP71372.1	OUO82145.1
EutB	ANU41983.1	EHM42370.1	CUP71331.1	OUO82146.1
EutA	ANU41984.1	EHM42369.1	CUP71298.1	OUO82147.1

pdtaS; two-component system, sensor histidine kinase PdtaS [EC:2.7.13.3]	ANU41985.1	EHM42368.1	CUP71252.1	OUO82148.1
pdtaR; two-component system, response regulator PdtaR	ANU41986.1	EHM42367.1	CUP71219.1	OUO82149.1
EutP	ANU41977.1	EHM42366.1	CUP71185.1	OUO82150.1
EutS	ANU41988.1	EHM42355.1	CUP71147.1	OUO82151.1
AlcDH/AldDH	ANU41989.1	EHM42344.1	CUP71114.1	OUO82152.1

Table S3.10. Distance Matrix for Pdu Operon in *F. plautii* YL31 with the one in *Salmonella enterica* subsp. *enterica* serovar Typhimurium str. LT2.

***F. plautii* YL31**	***Salmonella enterica***	**Distance[a]**
ANU41514.1	NP 461001.1 PduV	1.124
ANU41515.2	NP 461000.1 PduU	0.431
ANU41517.1	NP 460999.1 PduT	0.856
ANU41519.1	NP 460998.1 PduS	0.856
ANU41520.1	NP 460996.1 PduP	0.431
ANU41521.1	NP 460995.1 PduO	0.744
ANU41522.1	NP 460994.1 PduN	0.744
ANU41524.1	NP 460992.1 PduL	0.470
ANU41525.1	NP 460983.1 PduA	0.255
ANU41526.1	NP 460990.1 PduJ	0.393
ANU41527.1	NP 460989.1 PduH	0.916
ANU41528.1	NP 460988.1 PduG	0.693
ANU41529.1	NP 460987.1 PduE	0.322
ANU41530.1	NP 460986.1 PduD	0.431
ANU41531.1	NP 460985.1 PduC	0.105
ANU41532.1	NP 460984.3 PduB	0.357
ANU41533.1	NP 460990.1 PduJ	0.223
ANU41534.1	NP 460997.1 PduQ	0.393

[a]Estimates of Evolutionary Divergence between Sequences, the number of amino acid substitutions per site from between sequences are shown. Analyses were conducted using the Poisson correction model (Zuckerkandl & Pauling, 1965). The analysis involved 42 amino acid sequences. All positions containing gaps and missing data were eliminated. There were a total of 40 positions in the final dataset. Evolutionary analyses were conducted in MEGA6.

Table S3.11. Blast results for Enoate Reductase (ERED) for *Eubacterium* spp.

Description	Score	eValue	Identities	Positives	Gaps
lcl|*E. ramulus* 2789STDY|CUN25748.1	1404	0	100	100	0
lcl|*E. ramulus* MGYG01456|CABKSU010000016.1_3	1399	0	99	99	0
lcl|*E. ramulus* ATCC|ERK50666.1	1399	0	99	99	0
lcl|*E. ramulus* MGYG02278|CABMEW010000007.1_31	1322	0	95	98	0
lcl|*E. ramulus* 21|PWE87608.1	1321	0	95	98	0
lcl|*E. oxidoreducens*|SDB03427.1	1118	0	79	88	0

Table S3.12. Blast results for Phloretin Hydrolase (Phy) for *Eubacterium* spp.

Description	Score	eValue	Identities	Positives	Gaps
lcl|*E. ramulus* ATCC|ERK50757.1	565	0	99	99	0
lcl|*E. ramulus* MGYG01456|CABKSU010000011.1_31	553	0	99	99	0
lcl|*E. ramulus* 2789STDY|CUN27723.1	550	0	99	99	0
lcl|*E. ramulus* MGYG02278|CABMEW010000016.1_10	520	0	91	96	0
lcl|*E. oxidoreducens*|SDB04098.1	403	5.00E-142	72	83	1

Table S3.13. Blast results for NADH oxidase for *Flavonifractor* spp.

Description	Score	eValue	Identities	Positives	Gaps
lcl|2789STDY5834932|CUQ36251.1	1353	0	99	99	0
lcl|ATCC_29863|EHM51844.1	1330	0	99	99	0
lcl|YL31|ANU42126.2	1328	0	99	99	0
lcl|An248|OUO81996.1	1300	0	98	98	0
lcl|An306|OUO41927.1	1259	0	92	97	0
lcl|An82|OUN23292.1	1251	0	93	96	0

Table S3.14. Blast results for Phloretin Hydrolase (Phy) for *Flavonifractor* spp.

Description	Score	eValue	Identities	Positives	Gaps
lcl|ATCC_29863|EHM54196.1	550	0	100	100	0
lcl|2789STDY5834932|CUQ29647.1	550	0	100	100	0
lcl|YL31|ANU42335.1	550	0	100	100	0
lcl|An248|OUO83512.1	547	0	99	99	0
lcl|An10|OUQ80369.1	507	0	91	95	0
lcl|An82|OUN23291.1	473	2.00E-169	85	91	0
lcl|An306|OUO41926.1	472	4.00E-169	86	90	0
lcl|An4|OUO11830.1	469	4.00E-168	83	90	0

Appendix 4. Advances in Gut Microbiome Research, Opening New Strategies to Cope with a Western Lifestyle

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Abbreviations: Body mass index (BMI), high-fat diet (HFAD), high-fructose diet (HFUD), hydrogen sulfide (H_2S), IL-1 receptor antagonist (IL-1ra), interleukin-10 (IL-10), interleukin-1β (IL-1β), interleukin-6 (IL-6), inflammatory bowel disease (IBD), irritable bowel syndrome (IBS), lipopolysaccharides (LPS), Nonalcoholic steatohepatitis (NASH), Nuclear Factor kappa beta (NF-κβ), short chain fatty acid (SCFA), soluble TNF-α receptor (sTNF-R), Toll like receptors (TLR), tumor necrosis factor-α (TNF-α), trimethylamine-N-oxide (TMAO), ω-3 polyunsaturated fatty acids (ω-3 PUFAs), ω-6 polyunsaturated fatty acids (ω-6 PUFA).

Abstract

The 'westernization' of global eating and lifestyle habits is associated with the growing rate of chronic diseases, mainly cardiovascular diseases, cancer, type 2 diabetes mellitus, and respiratory diseases. The primary prevention approach is to make nutritional and behavioral changes, however, there is another important determinant of our health that only recently has been considered and is the presence of beneficial microorganisms and their products in our gastrointestinal tract. Microorganisms living in our body can alter the fate of food, drugs, hormones, and xenobiotics, and recent studies point to the use of microorganisms that can counteract the harmful effects of certain compounds introduced or produced endogenously in our body. This review considers the effects of the western lifestyle on adiposity, glucose metabolism, oxidative markers and inflammation profile, emphasizes on the studies that have investigated bacterial strains and products of their metabolism that are beneficial under this lifestyle, and examines the screening strategies that recent studies are using to select the most promising probiotic isolates. In addition, we consider the relevance of studying the microbiota of metabolically healthy people under a western lifestyle for the understanding of the key components that delay the development of chronic diseases.

Introduction

Our modern societies have settled on large urban arrangements that have changed behavior and alimentary patterns. The establishment of new alimentary habits has been influenced by industrialization and technological advances which have minimized the time for preparing and consuming meals, reduced the cost of livestock, dairy, and sugar-sweetened products, vegetable oils, and flours, and increased the availability of these foods, especially, to low-income families[1]. Nowadays, the trend in nutritional epidemiology is the analysis of dietary patterns (i.e. through food-frequency questionnaires) to assess habits in food consumption. In this line, several studies have focused on identifying the main dietary factors that are common to the modern diet. For instance, Hu *et al.* (1998) identified a 'western dietary pattern' through factor analysis of dietary patterns among cohorts in the United States. The authors described this dietary pattern as a diet with a "higher intake of processed meat, red meat, butter, high-fat dairy products, eggs, and refined grains". Likewise, Slattery *et al.* (1999) identified a similar dietary pattern with "high levels of red meat, processed meat, fast food, refined grains, and sugar- containing foods, and low levels of vegetables (other than potatoes) and fruits, with the predominant fruit being canned fruit"[2], [3]. Importantly, the 'western diet' is no longer restricted to western societies, globalization and urbanization are increasing the worldwide exposition to this dietary pattern. For example, a Japanese study found a 'westernized Japanese pattern' associated with "high intakes of bread, meat, processed meat, fruit juice, coffee, black tea, soft drinks, sauces, mayonnaise, and dressing"[4]. Overall, the 'western diet' can be understood as a dietary pattern with a high intake of refined sugars, refined vegetable oils, and livestock products, and low intake of fresh fruits and vegetables[5].

Concerns with the modern alimentary pattern can be traced back to the scientific literature of 1939, when Weston Price published his findings on modern degeneration related to the modernized diet[6]. Currently, not only the modern dietary habits are of concern but also low-activity high-stress occupations, sedentarism, alcohol binge drinking, and smoking. These behavior and alimentary patterns will be defined from now on as the 'western lifestyle'[7]–[9]. This lifestyle is increasingly being associated with several conditions, including: obesity, alzheimer's disease[10], cardiovascular disease, type 2 diabetes mellitus[11], non-alcoholic fatty liver disease[12], hypertension[13], osteoporosis[14], autoimmunity[15], and cancer[16]. There are several risk factors for developing chronic diseases, including genetic, environmental, demographic, social and other factors that are not the scope of this review, instead the objective of this review is to relate the research made in the fields of microbiology, immunology, and nutrition to explain the role of gut microbiota as a risk factor of 'western lifestyle'-related chronic conditions, and then the strategies that are being developed to shift the gut microbiota from a risk factor toward a more protective state that helps ameliorate the effects of this lifestyle.

The Healthy Western Microbiota

The concept of the human microbiota, as first described by Joshua Lederberg, is defined as "the ecological community of commensal, symbiotic, and pathogenic microorganisms that literally share our body space"[17]. Major efforts are being made worldwide in order to understand the composition and functional states of the healthy gut microbiota. So far, projects like the Human Microbiome Project

Consortium among others[18]–[20] have found that the gut microbiome of healthy individuals varies significantly and only a few species from the dominant bacterial phyla have been consistently described, these are Firmicutes, Bacteroidetes and Actinobacteria, with Proteobacteria and Verrucomicrobia also present in lower abundance. Other studies also evidence that the microbiome of healthy and non-healthy states can be distinguished, as it is the case for ulcerative colitis, Crohn´s disease[20], chronic fatigue syndrome[21], rheumatoid arthritis[22], type I diabetes[23], and type II diabetes[24]; nevertheless, one study warns that the patient's treatment can exert changes in the microbiota[25].

Although not a single marker can be identified as representative of a healthy gut microbiome, a higher proportion of butyrate-producing and mucin-degrading bacteria has been mentioned in some studies[23], [26], [27]. Butyrate is a short chain fatty acid produced mainly by bacterial fermentation of non-digestible fiber in the colon, and a correct balance of a butyrate-producing microbiota may induce the synthesis of mucin in the gut epithelium thus maintaining gut integrity[23], [28]. Studies have shown that butyrate can enhance the assembly of tight junction proteins through regulation of AMP-activated protein kinase (AMPK), however the mechanism of AMPK activation is unknown[29]. Butyrate also has anti-inflammatory and anti-carcinogenesis effects, mainly by two mechanisms: activation of GPCRs (GPR41 and GPR43) and inhibiton of histone deacetylase (HDAC). Some of the effects of butyrate that have been observed are enhancement of the expression of certain pro-apoptotic genes in malignant cells and suppression of the pro-inflammatory pathway of Nuclear Factor kappa beta (NF-$\kappa\beta$)[30]. It is estimated that butyrate producers represent approximately 25% of all human faecal bacteria[31]. Meanwhile, bifidobacteria is another important group for colon health, they represent about <5% of the microbiota in adult subjects. In disease states like *Clostridium difficile* associated diarrhea, a 3 $\log_{10}$ reduction of this group of bacteria can occur[32]. Bifidobacteria contributes to colon health through the production of organic acids, like acetate and lactate, that are then used by butyrate-producing bacteria. Thus, a high abundance of butyrate-producers, mucin-degraders, and bifidobacteria could be an indicator of good health.

Another common feature in some studies is greater gut diversity in healthy states. In lean twins, a greater bacterial diversity has been observed compared to their obese twins[33], in patients with morbid obesity subjected to a gastric bypass an increased richness of gut microbiota was also observed after the surgery along with positive health outcomes[34], and another study analyzed the microbiota of non-colic and colic infants finding a higher microbiota diversity in non-colic infants during the first weeks after birth[35]. Hence, a high bacterial diversity can be another indicator of a healthy gut microbiota.

In terms of western diet, Yatsunenko *et al.* (2012) observed that American microbiomes were enriched with genes degrading simple sugars and amino acids[36]. As mentioned earlier, a western diet is characterized by a higher intake of processed meat and red meat, thus individuals following this diet may benefit from a Bacteroides-rich microbiota instead of a Prevotella-rich microbiota. This last type of microorganisms produces more trimethylamine from L-carnitine, a nutrient in red meat, which is then converted to pro-atherosclerotic trimethylamine-N-oxide (TMAO), increasing the risk of atherosclerosis. A study by Lozupone *et al*. (2014) evidenced that the immune dysfunction of HIV-infected individuals compromises their ability to select for bacteria that match their diet, thus HIV-positive individuals

following a western diet, instead of having a Bacteroides-rich microbiota have a Prevotella-rich microbiota, which is normally present in individuals consuming a plant-based diet, in consequence, these HIV-positive subjects have an increased incidence of several health risks, including cardiovascular disease[37]–[39]. Therefore, a Bacteroides-rich microbiota is of benefit under a western diet, as it has been associated with reduced cardiovascular risk.

In order to reveal the key components that make a healthy western microbiota, studies at the strain-level are needed. Evidence points that different strains have distinct effects, as it is the case with strains belonging to a genus enriched in people following a western diet, *Lactobacillus*[40]–[42]. For example, the administration of *Lactobacillus reuteri* ATCC PTA 4659 was associated with weight decrease in mice, whereas the administration of *L. reuteri* L6798 was associated with weight gain[43]. Differential effects have also been observed on the type of immunological response that the strain elicits, for example, *Lactobacillus salivarius* CECT5713 induced the anti-inflammatory cytokine IL-10, while *Lactobacillus fermentum* CECT5716 induced pro-inflammatory cytokines[44]. Studying the intestinal microbiota at the strain-level has been proved challenging due to the great variability at this taxonomic level among individuals and the lack of reliable, easy to use tools for accurate identification of bacteria at strain level, however, these studies indicate that the insights obtained at the phylum-level are limited and that the understanding of the functionality of strains can help delineate the boundaries of a healthy gut microbiota.

Metabolic healthy subjects under a western lifestyle

In order to better understand the healthy western microbiota, metabolic healthy individuals following a western lifestyle must be investigated. One potential group of people to be examined is metabolically healthy obeses. The prevalence of obesity in the United States has increased by 75% since 1980 along with the acquisition of a western diet, and is associated with an increased incidence of cardiovascular disease, type 2 diabetes mellitus, hypertension, stroke, dyslipidemia, osteoarthritis, and some cancers[45], [46]. However, ~10 to 30% of obese individuals are metabolically healthy and even have a lifelong health[47]. The physiological factors that characterized a metabolically healthy obese are decreased visceral and liver fat, number of macrophages in adipose tissue, mean adipocyte size, circulating C-reactive protein; while having an increase in serum adiponectin, and adipocyte insulin sensitivity[48]. The genetic background might play an important part in this scenario, as it has been observed that some ethnic groups at a higher body mass index (BMI) accumulate less liver fat, a factor that affects the metabolic outcome of the individual[49]. A study revealed that liver fat content is higher among Japanese than non-Hispanic whites despite a lower mean BMI, and the difference becomes more robust with a small increase in BMI; this might explain why obesity-related complications in Asians occur at a lower BMI[50].

In African Americans, high rates of fructose malabsorption have been associated with reduced liver fat[51]. African-Americans also appear to be more resistant to hypertriglyceridemia (high blood levels of triglycerides) associated with insulin resistance[52]. Geographical factors might be also involved; migrants from lower-to-higher chronic disease areas (i.e., Japaneses that migrate to the United States) acquire a higher risk of developing a chronic disease[53]. But even under a similar background, differences are observed. Naukkarinen *et al*. (2014) studied 16 Finnish pairs of identical twins in which one twin was

obese and the other lean, they found that despite all twin pairs being of the same age, had similar age of onset of obesity and weight difference, half of the obese co-twins were metabolically as healthy as their lean co-twins while the other half of the obese co-twins exhibited a typical response to obesity, this was increased insulin production and resistance, dyslipidaemia, fatty liver, and higher blood pressure; they also observed that the one factor that best predicted the metabolic outcome was the level of liver fat[49].

It is now recognized that the gut microbiota can influence liver fat in the host, thus the microbiota might be one of the factors modulating the individual susceptibility to chronic disease. A study that observed an association of microbiota and liver fat accumulation demonstrated that gut microbiota directly induced non-alcoholic fatty liver disease (NAFLD) in mice. The authors performed fecal transplantations from mice that developed, or not, liver steatosis (responders and non-responders, respectively) during a 16 week period of high-fat diet (HFAD) to receiver mice. The responder-receiver mice developed a higher level of liver steatosis and had higher levels of branched-chain fatty acids from bacterial amino acid fermentation than non-responder-receiver mice[54]. Similarly, nonalcoholic steatohepatitis (NASH) (severe hepatic steatosis and liver inflammation) patients had an increase in ester compounds and endogenous alcohol most likely produced from bacterial metabolism compared to patients with simple steatosis (fatty liver) and healthy volunteers. It is worth mentioning that healthy subjects and obese non-NASH patients had similar blood-ethanol concentrations[55], [56] (for a review see [57]), thus indicating that even under an obese state, non-NASH patients may harbor a microbiota whose functionality resembles the one on a healthy state. In addition, the administration of probiotics can exert a positive effect on liver fat accumulation, which will be mentioned later. One potential mechanism for liver fat accumulation is that bacteria can suppress the expression of a lipoprotein lipase inhibitor, the fasting-induced adipose factor (Fiaf), thus increasing the lipase activity in the gut and affecting the outflow of free fatty acids to the liver[58]. In order to advance our understanding of the factors influencing metabolic health during a western diet, it is important to explore the microbiome of metabolically healthy individuals following a western diet which stay healthy at an advanced age, such studies might reveal components of the microbiome that can counteract the accumulation of liver fat, protecting the host from further health outcomes.

The Western Lifestyle and Inflammation

Nowadays in the modern societies, an unbalanced diet, stress, and smoking can onset the inflammatory response daily, leading to a chronic low-grade systemic inflammation. Inflammation is the process through which the body limits pathogen invasion and controls tissue damage after injury. It is mediated by many soluble factors essential to signal immune cells to eliminate the aggressor and initiate tissue repair. Among these factors are secreted polypeptides called cytokines, which include tumor necrosis factor-α (TNF-α), interleukin-1β (IL-1β), interleukin-6 (IL-6), interleukin-10 (IL-10), IL-1 receptor antagonist (IL-1ra), and soluble TNF-α receptor (sTNF-R). TNF-α and IL-1β are pro-inflammatory cytokines, IL-6 has both anti- and pro-inflammatory properties, while IL-10, IL-1ra and sTNF-R are anti-inflammatory cytokines. In acute inflammation, the levels of cytokines rapidly increase several fold and decrease when the infection is controlled or the injury is healed. However, acute inflammation does not always subside, and can become a chronic low-grade inflammation characterized by a two- to three-fold increase in the

concentrations of cytokines and C-reactive protein, a molecule produced by the liver in response to inflammation[59].

One way the western lifestyle can cause inflammation is by increasing the number of compounds and microbial products with inflammatory capability (Fig. 4.1). Among these are: lipopolysaccharides (LPS) or endotoxins, D-lactate, acetaldehyde, hydrogen sulfide, toxic products of bacterial protein metabolism, and oxidative radicals, as described below. A role of antibiotics is also discussed.

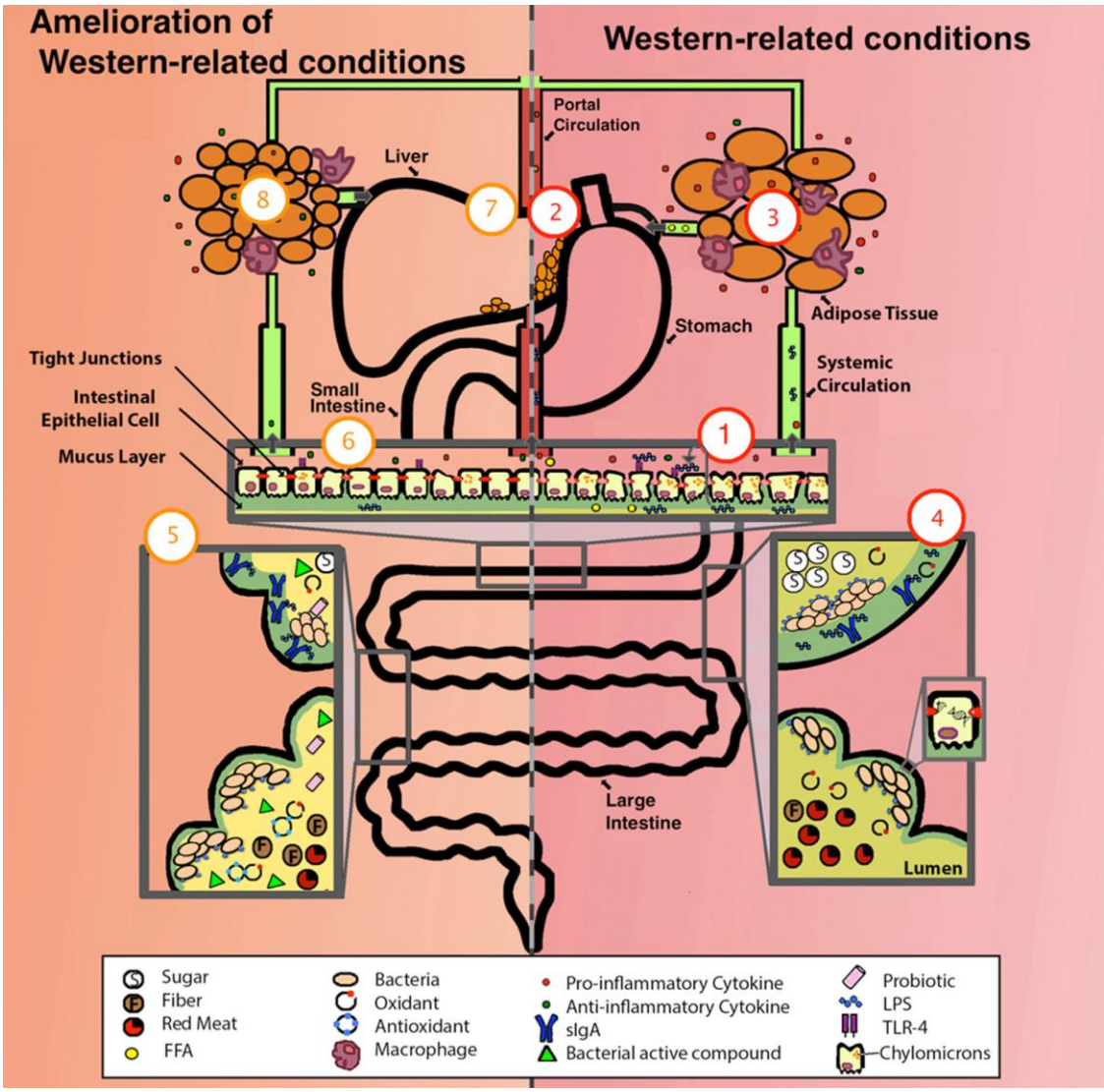

Figure 4.1. Comparison of a system suffering from western-related conditions (right, in red) and a system with amelioration of western-related conditions (left, in orange). One aspect of a western lifestyle is the higher intake of ω-6 PUFA (depicted as FFA), this enhances the formation of chylomicrons allowing the translocation of LPS, these then activate basolateral TLR which initiates a pro-inflammatory response, one overall consequence is the alteration of the gut epithelium and its permeability (depicted as deteriorated epithelium and compromised tight junctions), exacerbating inflammation by allowing the translocation of more LPS, pro-

inflammatory cytokines, FFA, among other luminal compounds (1). LPS/ pro-inflammatory cytokines/ FFA can enter portal and systemic circulation, one consequence is the alteration of fat metabolism, thus enhancing fat accumulation in liver (2), and in adipose tissue, adipocytes increase in size, FFA synthesis is enhanced (depicted as FFA in circulation), and an elevated pro-inflammatory state occurs (depicted as increased infiltration of macrophages and production of pro-inflammatory cytokines) (3). The western lifestyle includes higher intake of simple sugars and red-meat, lower intake of antioxidants (depicted as presence of oxidants), and sedentarism (depicted as low production of sIgA), some of the consequences are lower-capacity for antigen neutralization (depicted as LPS not bound to sIgA) and damage to the DNA of epithelial cells (depicted as DNA strand breakage) (4). For the amelioration of these conditions, a person can take different approaches, these include exercise (depicted as high production of sIgA), intake of dietary nutrients (i.e. polyphenols and ω-3 PUFAs) (depicted as antioxidants), probiotics, prebiotics, and SCFA (depicted as fiber, bacterial active compounds and probiotics) (5). Some of the effects of these approaches include the reestablishment of gut epithelium permeability and a decrease in LPS translocation, TLR activation, chylomicron formation, presence of LPS/cytokines/FFA in portal and systemic circulation (6), liver fat (7), adipocyte size, FFA synthesis, macrophage infiltration in adipose tissue (8), and an overall amelioration of the inflammatory state (depicted as a higher concentration of anti-inflammatory cytokines compared to pro-inflammatory cytokines [6 and 8]). FFA, free fatty acids. For more details see the text.

Lipopolysaccharides (LPS)

LPS are part of the outer membrane of Gram-negative bacteria and are one of the most important compounds that can induce a low-grade inflammation. LPS is bound by Toll like receptors (TLR) in cell surfaces, specifically TLR-4. Particularly in intestinal epithelial cells, these receptors mediate the inflammatory response triggering different mechanisms depending on its membrane location, apical or basolateral. Apical TLR are normally exposed to luminal antigens, including bacteria and their LPS, and their stimulation results in a homeostatic response and tolerance but not inflammation. In contrast, basolateral TLR are exposed to antigens only if these have crossed important epithelial barriers, and are potentially infectious. Therefore, basolateral TLR stimulation triggers the activation of the transcription factor NF-κβ, one of the most important mediators of the pro-inflammatory response[60]. Extra-luminal LPS can also reach the bloodstream, and subsequently, bind TLR on the surface of other cells, like blood vessel, muscle, joint, adipose, and hepatic Kupffer cells. Their activation affects processes like insulin signaling, adipose tissue differentiation, lipogenesis, and it has been suggested that the interaction LPS-adipocyte–macrophage can amplify the low-grade inflammation to the level of influencing metabolic disorders[61]. Thus, inflammation is a mechanism vital to set a prompt response to pathogens, however, LPS can onset a low-grade inflammatory response that may alter the metabolic status of the host by unknown molecular mechanisms[62]. LPS are being increasingly associated with a number of conditions summarized in Table 4.1.

Table 4.1. Conditions associated with high levels of plasmatic LPS.

Disease/Condition	Association with LPS
Depression and Neurodegenerative diseases	Peripheral inflammation can chronically activate brain microglia to produce elevated pro-inflammatory factors(Maes et al., 2013; L. Qin et al., 2007; Suffredini & Noveck, 2014).
Cardiovascular Disease and Atherosclerosis	Macrophages with a pro-inflammatory profile induced by TLR accumulate in blood vessel walls eventually forming a plaque(Caesar et al., 2010; Wiedermann, 1999).
Chronic Fatigue Syndrome	Serum levels of antibodies directed against LPS correlate to the level of fatigue(Maes & Leunis, 2008).
Cancer	LPS have been shown to increase the inflammatory activity of immune cells that generate oxidative radicals incrementing the chance of DNA damage in proliferating cells(Coussens & Werb, 2002), and they also increase the adhesiveness and metastatic capacity of cancer cells(Hsu et al., 2011).
Type 2 Diabetes Mellitus	LPS decreased insulin sensitivity in healthy subjects that had a reduced response to insulin 24h after a LPS infusion protocol(Mehta et al., 2010).
Obesity	LPS are identified as a triggering factor since a 4-week treatment of LPS in mice resulted in a similar whole-body, liver, and adipose tissue weight gain as in a HFAD(Cani et al., 2007).
Autism	The higher the level of LPS, the worse the social interaction of the patient(Emanuele et al., 2010).
Systemic Lupus Erythematosus disease	LPS increase systemic nucleosome release due to an enhancement of apoptosis and a decrease in the clearance of apoptotic cells(Licht, Van Bruggen, Oppers-Walgreen, Rijke, & Berden, 2001).
HIV-1	LPS lead to neurological dysfunctions since the increase of cytokine production affects the permeability of the blood-brain barrier allowing the trespassing of the virus into the brain(Dohgu & Banks, 2008).
Retinal pathologies	LPS are an underlying factor for their progression due to the sensitivity of the retinal pigment epithelium cells to inflammatory stress(Leung, Barnstable, & Tombran-Tink, 2009).
Autoimmune Joint Inflammation	An oral administration of LPS can exacerbate arthritis in animal models and antibiotics can suppress the recurrence of the disease(Yoshino, Sasatomi, Mori, & Sagai, 1999).

Several behaviors associated with the western lifestyle can affect the levels of plasmatic LPS. Among these are sedentarism, smoking, stress, and an unhealthy diet. Lira *et al*. (2010) showed that sedentary people had higher levels of plasmatic LPS than highly trained people at rest[63]. Pace *et al.* (2008) observed that cigarette smoke increased the expression of TLR4 and LPS binding[64]. Furthermore, it has been demonstrated that stress hormones stimulate the growth of LPS-containing bacteria such as *Yersinia enterocolitica* and *Escherichia coli*, and indeed, stress hormones achieved a 100,000-fold increase in viable *E. coli* in the cecum of mice within 24h and promote the synthesis of an autoinducer of bacterial growth[65]–[68]. Meanwhile, Cani *et al*. (2007) observed that a 4-week high-fat diet (HFAD) chronically increased plasma LPS concentration two to three times[69].

Among the factors that increase the abundance of plasmatic LPS, diet is the best studied. It is recognized that HFADs induce high levels of LPS in the blood through the stimulation of chylomicron (droplets of fat) formation in intestinal epithelial cells, this facilitates LPS transcellular transport across the gut epithelium and subsequently, LPS reach the bloodstream[70]. However, several investigations point that not every type of HFADs increases the concentration of plasmatic LPS. HFADs consisting of oils rich in ω-6 polyunsaturated fatty acids (ω-6 PUFA), like safflower oil, cause a markedly increase in the concentration of plasmatic LPS and pro-inflammatory cytokines compared to diets rich in coconut oil or fish oil, which instead are protective against a LPS challenge[71], [72]. Meanwhile, a high-fructose diet (HFUD) promotes a more pronounced increase in plasmatic LPS concentration than diets rich in glucose. The mechanism for this is unknown, but evidence suggests that HFUD effects are related to the gut microbiota, since observations that oral non-absorbable antibiotics (antibiotics that act locally in the gut) can prevent the increase of plasmatic LPS, while the knockout of the LPS receptor TLR-4 greatly decreases lipid peroxidation, expression of TNF-α, and accumulation of fat in the liver that occurs in fructose-fed mice[73]. In humans, HFUD is also associated with non-alcoholic fatty liver disease[74]′[75]. The distinct effects of different fats and sugars might explain some of the variability of diet response among studies.

Diet-dependent products of bacterial metabolism

Bacterial products of metabolism released in our gut depend heavily on diet, host secretions and digestive enzymes, local conditions of pH, oxygen and hydrogen, gut transit time, and the composition and activity of the microbiota, among other factors[76]. Undigested dietary residues that arrive to the large intestine are the main substrates of bacterial metabolism, along with diet-independent substrates like endogenous host secretions. Undigested carbohydrates are fermented mainly to short-chain fatty acids (SCFAs) (such as butyrate, acetate, and propionate) and gases (mainly carbon dioxide, hydrogen, and methane)[77]. However, an excessive consumption of carbohydrates can also increase the concentration of toxic compounds derived from microbial metabolism, as it is the case of D-lactate, which is produced during carbohydrate fermentation by D-lactic acid bacteria. This compound inhibits the transport of L-lactate and pyruvate, both essential for mitochondrial energy production[78]. Several conditions have been associated with high concentration of D-lactate, among these are chronic fatigue syndrome, diarrhea, short bowel syndrome, and diabetes[79]–[81]. Another toxic compound that has been associated with the excessive consumption of carbohydrates and alcoholic drinks is acetaldehyde. This

compound is produced by ethanol-oxidizing bacteria and yeast, and is formed during ethanol metabolism. When acetaldehyde is metabolized, oxidative radicals are generated, altering the permeability of the intestinal epithelium facilitating the translocations of luminal contents to the bloodstream[82]. Acetaldehyde is also a known carcinogenic compound[83], [84].

While carbohydrates are fermented in the proximal colon, amino acids are fermented in the distal colon and this results in branched-chain fatty acids and potentially toxic metabolites such as ammonia, phenols, indoles, amines, TMAO, and volatile sulfur compounds[85], some of which are associated with the increased incidence of colorectal cancer[86], [87] and atherosclerosis[88] in high-red meat diets, fresh or processed. In the case of ammonia, higher levels of this compound in the blood can enter the brain and cause conditions like hepatic encephalopathy. Ammonia is a concern in subjects with chronic diseases in the thyroid gland, kidneys, lungs, and liver, and it is been increasingly associated with diabetes, extreme obesity[89], and tumor promotion[86]. Interestingly, the evidence suggests that white meat (poultry and fish) do not have the same detrimental effects of red meat. A possible explanation is the higher content of dietary haem in red meat, which will provide a source of iron for some proteins that can form toxic nitrosating agents from nitric oxide under anaerobic conditions[90].

Another toxic compound of bacterial protein or carbohydrate metabolism is hydrogen sulfide (H_2S). This is produced by sulphate-reducing bacteria during the oxidation of a wide range of substrates found in the large intestine[91]. In western countries there is a high incidence of people with a sulphate-reducing bacteria, 50-70% compared to 10-20% of rural black Africans. The H_2S produced by this group of bacteria can cause DNA damage in susceptible subjects with genetic predisposition that compromises DNA repair, as it is observed in patients suffering from ulcerative colitis and colorectal cancer[92]. The concentration of these toxic compounds of bacterial protein and carbohydrate metabolism in the intestinal lumen might be the result of interplay between the microbiota capacity to produce them and the host capacity to clear them up; in addition, the metabolic effect of these compounds would depend on the host susceptibility.

Oxidative radicals

Oxidative radicals are normally produced in high concentrations during food digestion and are also generated during cigarette smoking. Ingestion of food with antioxidants can control the exposure to these compounds, consequently diets with low levels of antioxidants will not subside the constant oxidative stress that occur in the gut and lung epitheliums[93]–[95]. Overnutrition also increases the oxidative stress in the endoplasmatic reticulum, this activates a mediator of inflammation normally inactive in the hypothalamus, the kinase IKKβ, which regulates NF-$\kappa\beta$ through the phosphorylation of its inhibitor IκBα[96]. Oxidative stress also increases the activity of the PI 3-kinase and the myosin light chain kinase promoter that regulate the opening of the intestinal tight junction barrier. Thus oxidative stress mediates the enlargement of the spaces in the gut epithelium allowing the translocation of normally non-invasive bacteria or their toxic products and components, which will induce the activation of NF-κβ perpetuating a vicious cycle of NF-κβ activation and impairment of the tight junction barrier[97]′[98].

In summary, the NF-κβ pathway that mediates inflammation can be activated by several cellular stresses, including LPS and compounds that generate cellular damage, like D-lactate, acetaldehyde, and H_2S. Importantly, the activation of NF-κβ in parts of the body different from the gastrointestinal tract might eventually alter the permeability of the intestinal epithelium, facilitating the translocation of luminal materials, including LPS, which will exacerbate the low-grade inflammation state.

Antibiotics

The use of antibiotics in the modern era, including the extensive and inappropriate use in humans and animals, has changed the gut microbiota and this has diverse health implications. Broad-spectrum antibiotics can impact the gut microbiota causing a dysbiosis ("a pathological imbalance in a microbial ecological niche"[99]) which can alter the microbiota capacity to prevent the colonization and growth of pathogens and pathobionts with inflammatory capability. Two meta-analyses, one in >56,000 patients with *C. difficile* infection and the other in >7,000 inflammatory bowel disease (IBD) patients showed that antibiotics were a high risk factor for the development of these diseases[100], [101]. Depending on the class of antibiotic, the dosage, time of administration, and other antibiotic-independent factors, like genetic predisposition, sex, diet, physical activity, disease and environmental toxicants, antibiotics can exert effects on the weight (underweight and overweight states) and metabolic profile (pro-diabetic and anti-diabetic effect) of an individual (for a review see [102]). Antibiotic use carries other risks, like the dissemination of bacterial resistant genes and the alteration of the well established host-microbiota symbiosis through the eradication of important susceptible strains[103]. Recently, Moeller *et al.* (2016) demonstrated the cospeciation of certain symbiotic bacterial strains with hominids, including humans[104]. This unique set of symbionts might provide beneficial health effects to the host and could be under selective pressure by the modern use of antibiotics.

Approaches that Counteract the Western Lifestyle

The approaches that can effectively counteract the effects of the western lifestyle are the ones that mitigate the translocation of LPS, prevent toxic microbial metabolism, and modulate the pro-inflammatory response and oxidative stress. Among these approaches are: exercise, dietary compounds, probiotics, prebiotics, and short chain fatty acids (SCFAs) (Fig. 4.1 and Table 4.2), as described below.

Table 4.2. Summary of beneficial factors under a western lifestyle.

Disease/Condition	Association with LPS
Depression and Neurodegenerative diseases	Peripheral inflammation can chronically activate brain microglia to produce elevated pro-inflammatory factors (Maes et al., 2013; L. Qin et al., 2007; Suffredini & Noveck, 2014).
Cardiovascular Disease and Atherosclerosis	Macrophages with a pro-inflammatory profile induced by TLR accumulate in blood vessel walls eventually forming a plaque (Caesar et al., 2010; Wiedermann, 1999).
Chronic Fatigue Syndrome	Serum levels of antibodies directed against LPS correlate to the level of fatigue(Maes & Leunis, 2008).
Cancer	LPS have been shown to increase the inflammatory activity of immune cells that generate oxidative radicals incrementing the chance of DNA damage in proliferating cells(Coussens & Werb, 2002), and they also increase the adhesiveness and metastatic capacity of cancer cells(Hsu et al., 2011).
Type 2 Diabetes Mellitus	LPS decreased insulin sensitivity in healthy subjects that had a reduced response to insulin 24h after a LPS infusion protocol(Mehta et al., 2010).
Obesity	LPS are identified as a triggering factor since a 4-week treatment of LPS in mice resulted in a similar whole-body, liver, and adipose tissue weight gain as in a HFAD(Cani et al., 2007).
Autism	The higher the level of LPS, the worse the social interaction of the patient(Emanuele et al., 2010).
Systemic Lupus Erythematosus disease	LPS increase systemic nucleosome release due to an enhancement of apoptosis and a decrease in the clearance of apoptotic cells(Licht, Van Bruggen, Oppers-Walgreen, Rijke, & Berden, 2001).
HIV-1	LPS lead to neurological dysfunctions since the increase of cytokine production affects the permeability of the blood-brain barrier allowing the trespassing of the virus into the brain(Dohgu & Banks, 2008).
Retinal pathologies	LPS are an underlying factor for their progression due to the sensitivity of the retinal pigment epithelium cells to inflammatory stress(Leung, Barnstable, & Tombran-Tink, 2009).
Autoimmune Joint Inflammation	An oral administration of LPS can exacerbate arthritis in animal models and antibiotics can suppress the recurrence of the disease(Yoshino, Sasatomi, Mori, & Sagai, 1999).

Exercise

Regular moderate doses of physical activity can ameliorate the effect of an LPS insult. In addition, it has been shown that exercise: controls the levels of pro-inflammatory cytokines, is associated with less liver fat[105], [106], protects against insulin resistance[107], and increases the levels of SCFAs. The main SCFAs are butyrate, acetate and propionate, and these have anti-carcinogenic as well as anti-inflammatory properties and are essential for colon health[108]. Exercise can also modulate the microbiota, mice who exercised had lower intestinal and systemic bacterial loads than the group of sedentary mice, and had higher total and specific intestinal secretory immunoglobulin A (sIgA) which are the antibodies that control luminal antigens[109].

Dietary compounds

Dietary compounds can also be introduced to prevent the negative effects of a western lifestyle. Among these are: dietary polyphenols and ω-3 PUFAs. Polyphenols can be found in wine, cocoa, cranberry, grape, curcumin, propolis, coffee, and tea; they function as antioxidants[110], strengthen intestinal barrier function[111], prevent endotoxemia (presence of LPS in the blood), the loss of some beneficial bacterial strains, and the development of diabetes[112]–[114]. The other compounds are ω-3 PUFA, which are found in fish and olive oil, their addition to a high ω-6 PUFA diet can reverse some of the inflammatory effects of ω-6 PUFA, like immune cell infiltration and NF-κβ activation[115]. It is possible that the beneficial effects of some of these dietary compounds are exerted through the modulation of the microbiota. For example, the administration of cranberry extract and grape polyphenols is associated with an increased abundance of the beneficial genus *Akkermansia* even under a high sucrose and/or HFAD, while ω-3 PUFA have been shown to enrich *Lactobacillus* and *Bifidobacteria*[113], [115]–[117].

Probiotics

A probiotic can be defined as "a live microorganism that, when administered in adequate amounts, confers a health benefit on the host"[118]. Several recent studies have evaluated the benefits of probiotic supplementation in the absence of lifestyle changes[119]. One example is the study of Park *et al*. (2013) who observed that mice following a HFAD for 8 weeks and supplemented with *Lactobacillus curvatus* HY7601 and *Lactobacillus plantarum* KY1032 for another 10 weeks gained 38% less weight than the unsupplemented controls[120]. The same group also demonstrated that *L. curvatus* HY7601 and *L. plantarum* KY1032 at high (10^{10} cfu/d) or low dosage (10^{9} cfu/d) lowered plasma glucose, insulin, triglycerides, and oxidative stress levels in rodents fed a HFUD, while only at high doses lower liver mass and liver cholesterol were achieved[121].

The bacteria *Akkermansia muciniphila* and *Bacteroides uniformis* have also been evaluated under a HFAD. Everard *et al.* (2013) showed that *A. muciniphila* reduced plasma levels of LPS, adiposity, insulin resistance, body weight (without changing food intake), hyperglycemia, increased adipocyte differentiation and lipid oxidation. The supplementation of live cells of *A. muciniphila* also prevented the thinning of the mucus layer that occurred when mice were fed a HFAD[122]. Meanwhile, Cano *et al*. (2012)

showed that an oral administration of *B. uniformis* CECT 7771 significantly reduced total body weight gain, liver fat, levels of cholesterol and triglycerides. Furthermore, *B. uniformis* CECT 7771 improved glucose metabolism, insulin and leptin sensitivity, and immune function of macrophages and dendritic cells. The authors also measured the number of fat micelles per enterocyte as an indicator of intestinal lipid absorption which contributes to adiposity, and *B. uniformis* CECT 7771 also achieved a significant reduction in this aspect[123]. It was also demonstrated, in meat-fed rats, that *Lactobacillus acidophilus* strains NCFM and N-2 promoted a significantly lower production of free amines[124] and lowered significantly, in rats and subjects, the activity of cecal bacterial ß-glucuronidase, nitro-reductase, and azoreductase enzymes which are responsible for the generation of potential precarcinogenic compounds[125].

Studies have not only evaluated probiotic effects under a particular nutritional environment but have examined their effect on the treatment of alcohol-drinking and smoking induced diseases. Several studies have shown an improvement of alcohol-induced liver injury in mice and human subjects. For example, Kirpich *et al*. (2008) performed a pilot study evaluating the effect of a 5-day probiotic supplementation consisting of *Bifidobacterium bifidum* and *L. plantarum* 8PA3 on 66 alcoholic individuals, the subjects under the probiotic treatment had significantly lower alanine aminotransferase (ALT) and aspartate aminotransferase (AST) activity than those treated with standard therapy (abstinence plus vitamins)[126]. Then again, the same group demonstrated that a *Lactobacillus rhamnosus* GG supplementation during the last 2-weeks of a 8-week diet containing 5% alcohol significantly improved liver function and reduced alcohol-induced endotoxemia, and hepatic steatosis in mice[127]. Meanwhile, Naruszewicz *et al*. (2002) determined that the administration of *L. plantarum* 299v to heavy smokers for 6 weeks with no changes in lifestyle led to the significant reduction in systolic blood pressure, leptin, fibrinogen, IL-6, and monocytes adhesion to vein endothelial cells, thus reducing their risk of cardiovascular disease[128].

Prebiotics and SCFAs

Prebiotics are non-digestible fiber that promotes the growth of beneficial microorganisms in the gastrointestinal tract. Some examples are: inulin, fructooligosaccharides, resistant starch, pectin, among others. These are metabolized to SCFAs, mainly propionate, acetate and butyrate, which as mentioned earlier exert many beneficial health outcomes. SCFAs activate the SCFA receptor GPR43 that reduces insulin sensitivity in adipose tissue and hence its fat accumulation, thereby reducing the uptake, synthesis, and oxidation of toxic fatty acids in other tissues[129], [130]. They also increase proliferation and inhibit apoptosis of intestinal cells[131], hinders intestinal secretion of chylomicron into the circulation[132], and limits inflammation perhaps through inhibition of the NF-κβ pathway[133]. Galisteo *et al.* (2008) analyzed several studies that showed that prebiotics reduce all the abnormalities clustered in the metabolic syndrome, including: body weight gain, dyslipidemia, inflammation, hypertension, and insulin resistance[134].

One drawback of prebiotics is that they can cause intestinal tract discomfort in individuals with limited microbial capacity to ferment the prebiotic. Thus, novel approaches to deliver the benefits of

prebiotics have been developed. Chambers *et al*. (2014) designed an improved prebiotic compound linked to a SCFA, propionate, which exploits the benefits of prebiotics while reducing the amount to be administrated. SCFAs have many health benefits, but if they are supplemented orally, they will be absorbed in the upper part of the small intestine where their benefits are limited. In contrast, this new compound ensures the delivery of the SCFA directly to the colon and at the same time, reduces the patient complains about prebiotics (e.g. gas production and bloating). Only 10 grams of the inulin-propionate ester achieved a 2.5-fold increase in colonic propionate, this in consequence, prevented weight gain, abdominal adiposity, liver fat, and reduced insulin resistance significantly more than in the prebiotic-only control group[135], [136].

Probiotics and prebiotics are already been used clinically for the improvement of fatty liver[137], minimal hepatic encephalopathy[138], diabetes[139], abdominal adiposity[140], chronic fatigue syndrome[98], diarrhea, and *Clostridium difficile* disease[141], among others. Their supplementation is one alternative that is simple, safe, and that improves several health parameters simultaneously.

Mechanism-based Screening Strategies for the Identification of Beneficial Strains

There is great interest in developing commercial probiotic formulations that include new beneficial strains. Thus, several studies focus on different screening strategies to find promising strains that can favorably shape host pathways. These strains can act directly or indirectly on the cells of the immune system, epithelial cells, adipocytes, beta pancreatic cells, and can also control pathobionts. For instance, Cano *et al*. (2012) screened for the immunomodulation capabilities among different strains of *Bacteroides* spp., they carefully selected for a specific strain that had the lowest inflammatory potential on macrophages *in vitro*, specifically, low TNF-α and high IL-10 production[123]. Poutahidis *et al*. (2013) also demonstrated that *L. reuteri* protected the host from obesity through an immunomodulatory mechanism, specifically, *L. reuteri* had an effect on the IL-10-dependent function of CD4+ T cells. Interestingly, the researchers could replicate the phenotype of the probiotic-supplemented mice in naïve recipient rodents by transferring only the purified CD4+ T cells[40]. Meanwhile, Ito *et al*. (2003) screened the inhibitory activity of 49 lactic bacterial strains on lipid peroxidation *in vivo* and *in vitro*[142]. While Kullisaar *et al*. (2011) measured the capacity of *L. fermentum* ME-3 to reduce oxidative stress, blood triglyceride levels, and lipoprotein status postprandially (2h after a meal) in a randomized double-blind placebo-controlled study with 100 healthy subjects[143]. Lastly, Chung *et al.* (2016) screened for a FFAs-absorbing strain, *L. reuteri* JBD30 l, in a fecal sample of a healthy lean subject. The administration of this strain to experimental animals and human subjects under a clinical trial lowered the concentration of FFAs in the fluid of the small intestine thus increasing fecal fat excretion, the efficacy was comparable to the one obtained for orlistat, a FDA-approved pharmaceutical that also increases the content of fat in feces[144].

There are other reported mechanisms that can guide screening studies, among these are the increment in the expression of lectins against Gram-positive bacteria, e.g. *A. muciniphila* produces RegIIIγ[122]; the inhibition of T cell activation, e.g. *S. boulardii* produces a <3 kDa protein that has this effect[145]; production of phosphatases that can dephosphorylate LPS, as it has been observed also in *S. boulardii*[146]; upregulation of the expression of cytoprotective heat shock proteins that increase the

protection against oxidative damage and gut barrier loss in intestinal cells, e.g. *Bacillus subtilis* produces a quorum-sensing signal molecule, the competence- and sporulation-stimulating factor, which induces the heat shock protein Hsp27[147]; inhibition of the hydrogen peroxide-induced epithelial barrier disruption, e.g. *L. rhamnosus* GG produces two soluble proteins, p40 and p75, that control this aspect[148]; inhibition of NF-κβ pathway[149]; and enhancement of SCFA production[129], [130].

Conclusions

The western lifestyle causes the overproduction of inflammation signals and underprovides the means to block them, driving the body into a chronic low inflammation state. To avoid some negative consequences, people can introduce light exercise, simple dietary compounds, probiotics, prebiotics and/or SCFAs into their daily routine. Interestingly, several recent studies have proved that the effects of probiotics and prebiotics can even be exploited under a HFAD and smoking conditions, providing a way to extend the health of a person with a western lifestyle. The presence of probiotics in dairy products has made them well accepted and recognized by their health benefits on the gastrointestinal tract, and given that the clinical evidence points that they also have benefits on the lipid and glucose metabolism, gut permeability, mood, and immune system, it is foresighted that this field will keep introducing new probiotic strains to the market, perhaps specific formulations depending on the desired benefit. We proposed that for the advancement of this field, it is important to understand if there is a microbiological component that is extending the health of asymptomatic lean, overweight and obese people following a western diet, and the factors that increase the fitness of these strains in the western microbiome. Ultimately, considering that in western countries the most prevalent diseases are inflammatory in nature, it will be important that in the near future, inflammation markers would be routinely screened in the clinical setup and anti-inflammatory probiotics administered as an alternative preventive measure.

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Appendix 5. Research Products

Publications

Bacteroides thetaiotaomicron Starch Utilization Promotes Quercetin Degradation and Butyrate Production by *Eubacterium ramulus*. GP Rodriguez, MR Dorris, X Liu, BW Bolling, A Acosta-Gonzalez, F Rey. Frontiers in microbiology 10, 1145.

Advances in gut microbiome research, opening new strategies to cope with a western lifestyle. GP Rodriguez-Castaño, A Caro-Quintero, A Reyes, F Lizcano. Frontiers in genetics 7, 224.

Research Visit

Wisconsin University, Department of Bacteriology. Madison, WI, USA. July 1st-December 1st, 2017.

Poster Presentations

7th Conference on Beneficial Microbes. Madison, WI, USA. July 8th-11th, 2018.

Bogota Microbial Meeting, BOMM. Bogota, Colombia. August 9th-10th, 2018.

Oral Presentation

Bogota Microbial Meeting, BOMM. Bogota, Colombia. July 25th, 2019.