INTERACTIONS BETWEEN DIETARY FLAVONOID APIGENIN AND HUMAN

GUT MICROBIOTA *IN VITRO*

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ABSTRACT OF THE DISSERTATION

Interactions between Dietary Flavonoid Apigenin and Human Gut Microbiota *in Vitro*

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Apigenin is a monomeric flavonoid widely distributed in plants and is found in human diet. It has low toxicity and multiple reported beneficial bioactivities. Apigenin reaches the colon region intact and interacts with the human gut microbiota. Published reviews on apigenin focused its pharmacokinetics, cancer chemoprevention potentials or interactions with drugs, but no review is available on the antimicrobial effects. There is little research on the mutual effects between apigenin and gut bacteria. Therefore, there exists a knowledge gap. The objective of the dissertation is to study the interactions between dietary flavonoids and human gut microbiota *in vitro*, at both single strain and community levels. To achieve this objective, three sub-objectives were established: to gain an overall understanding of the effects of apigenin on microbes on both a single species and a gut microbiota community; to study the effects of apigenin on the growth of *Enterococcus caccae* SS-1777 as a single strain and on a gut bacteria community *in vitro*,

respectively; to study the degradation of apigenin by *Enterococcus caccae* SS-1777 and a human gut microbiota *in vitro*, respectively.

The effect of apigenin on the single gut bacteria strains, *Bacteroides galacturonicus*, *Bifidobacterium catenulatum*, *Lactobacillus rhamnosus* GG, and *Enterococcus caccae,* was examined by measuring their anaerobic growth profiles. The effect of apigenin on a gut microbiota community was studied by culturing fecal inoculum under *in vitro* conditions simulating the human ascending colon. 16S rRNA gene sequencing and GC-MS analysis quantified changes in the community structure. Single molecule RNA sequencing was used to reveal the response of *Enterococcus caccae* to apigenin. The degradation of apigenin in *E. caccae* culture and in human gut microbiota culture *in vitro* was measured by treating the cultures and quantifying the concentration of apigenin and its potential degradation products with UPLC-ESI-MS/MS.

Results show that there were two-way interactions between apigenin and human gut bacteria on both single strain and community levels. *Enterococcus caccae* was effectively inhibited by apigenin when cultured alone; however, genus *Enterococcus* was enhanced when tested in a community setting. Single molecule RNA sequencing found that *Enterococcus caccae* responded to apigenin by up-regulating genes involved in DNA repair, stress response, cell wall/membrane synthesis, and protein folding. Apigenin did not have a major effect on the structure and SCFAs production of the human gut microbiota *in vitro*. In *Enterococcus caccae* culture, apigenin concentration decreased with time, but not to zero and no targeted degradation product was detected. In the community, apigenin was fully degraded and 3-(4-hydroxyphenyl)propionic acid was the main end product. Apigenin was stable in sterile media. Such interactions provide more

information on how the human gut microbiota would respond to dietary apigenin, the fate of apigenin in bacterial culture, and the potential mechanisms of the health benefits of apigenin.

Table of Contents

List of Tables

List of Illustrations

Chapter 1 Introduction

It is universally acknowledged, that plants, in particular vegetables and fruits, are an important part in a healthy diet. Other than providing essential nutrients like protein, carbohydrates, vitamins, etc., plants contain phytochemicals that have various biological activities. One major group of phytochemicals is flavonoid. Flavonoids are widely distributed in nature in different forms (Sharma, Kaur, Katnoria, & Nagpal, 2017), but the core chemical structure of flavonoids is a 15-carbon skeleton consisted of two phenyl rings (A and B) and a heterocyclic ring (C) ("flavonoid (CHEBI:47916)," n.d.). Flavonoids are often found in nature as glycosides, as their glycosides are more soluble and more stable than the aglycone forms (Rothwell, Knaze, & Zamora-Ros, 2017). These glycosides can withstand food processing and cooking, survive the digestion and absorption in the upper gastrointestinal (GI) tract and finally, most of the oral intake reach the colon region without being broken down (Cardona, Andrés-Lacueva, Tulipani, Tinahones, & Queipo-Ortuño, 2013).

It has long been known that the human GI tract is colonized by bacteria, especially in the colon where the microbial density could be as high as 10^{11}-10^{12} cfu/mL (O'Hara & Shanahan, 2006). This microbial collection has protective, structural, and metabolic functions and are closely relevant to human health, yet, the mechanisms of its functions and influencing factors are only beginning to be studied and revealed (O'Hara & Shanahan, 2006).

As the flavonoids can reach the colon region intact and the colon is inhabited by the gut microbiota, flavonoids and the bacteria will interact with each other upon contact. This interaction is in two ways. Flavonoids may exert some effects on the gut bacteria. Some flavonoids have been found to be able to inhibit microbial growth (Cushnie & Lamb, 2005). Cell physiology may also be altered at a molecular level besides the phenotypical changes in growth profile. A bacterial community's structure and function may also be changed as its different members respond to flavonoids differently. In the opposite direction, as flavonoids modulate bacteria growth, the bacteria can use flavonoids as substrates, taking up the compound, degrading it, and generating other metabolites (Blaut & Clavel, 2007; Schoefer et al., 2003).

The objective of the projects presented in this dissertation was to study the interactions between dietary flavonoids and human gut microbiota *in vitro* as the information on such interaction is very limited. We started with screening several flavonoids by testing the growth modulation effect on some commensal bacteria. Based on the screening results, we focused our research on one dietary flavone, apigenin, and its interactions with commensal bacteria *Enterococcus caccae* SS-1777 as well as the human gut microbiota *in vitro* respectively. Three sub-objectives were developed to guide the completion of the projects.

- Sub-objective 1: To gain an overall understanding of the effects of apigenin on microbes on both a single species and a gut microbiota community.

- Sub-objective 2: To study the effects of apigenin on the growth of *Enterococcus caccae* SS-1777 as a single strain and on a gut bacteria community *in vitro*, respectively.

- Sub-objective 3: To study the degradation of apigenin by *Enterococcus caccae* SS-1777 and a human gut microbiota *in vitro*, respectively.

The following three chapters will each address one of the sub-objectives in the order they are listed.

Chapter 2 Antimicrobial Effects of Apigenin and Its Interactions with Human Gut

Microbiota

2.1 Introduction

Flavonoids belong to a type of phytochemicals called polyphenols, which are secondary metabolites produced by plants (Sharma et al., 2017). By producing flavonoids, plants possess a defense mechanism to protect against UV-B, ward off microbial infection and herbivory (Harborne & Williams, 2000). "The Handbook of Natural Flavonoids" published in 1999 contains information of 6467 known flavonoid structures, with formulae, references and information on biological activities (Harborne Baxter, H., 1999). Among the over 6000 different flavonoids, quercetin, kaempferol, myricetin, apigenin, and luteolin are the five most common plant flavonoids (Tang, Chen, Huang, & Li, 2017a). Apigenin, 4',5,7-trihydroxy-flavone, is one of the predominant monomeric flavonoids found in the diet (Figure 2.1) (Bhagwat, Haytowitz, & Holden, 2011). Based on the chemical structure of its backbone, apigenin is a flavone, one of the subclasses of flavonoids. Apigenin has gained attention among researchers partly due to its low toxicity and multiple beneficial bioactivities. The largest number of published reviews on apigenin focus on its effects on various cancers (Ali, Rahul, Naz, Jyoti, & Siddique, 2017; Michael G.L. Hertog, Hollman, Katan, Daan, & Kromhout, 1993; Lefort & Blay, 2013; Madunić, Madunić, Gajski, Popić, & Garaj-Vrhovac, 2018; Nabavi, Habtemariam, Daglia, & Nabavi, 2015; Patel, Shukla, & Gupta, 2007; Rothwell et al., 2017; Sak, 2017; Shukla & Gupta, 2010a; Tang et al., 2017a). The reviews included findings using eukaryotic cells, animal models or epidemiological studies, covering the

pharmacokinetics, cancer chemoprevention, drug interactions, but no review is available on the antimicrobial effects of apigenin although there are many individual research papers on bacterial inhibitory activities from natural flavonoids. This review aims to fill the gap by providing an overview of reported results from antimicrobial tests using apigenin. Another interesting aspect of the effects of apigenin on bacteria is its interactions with gut microbes. Apigenin has a low solubility (Lakshmanan et al., 2015; Li, Robinson, & Birt, 1997) and a low bioavailability (Tang et al., 2017a), thus may come into contact with the colon microbiota and be metabolized into smaller and more bioavailable molecules (Cardona et al., 2013). Apigenin may also affect the composition and functionality of gut microbiota. With apigenin and the gut microbiota potentially impact one another, there may exist interactions between them. Therefore, the dietary sources of apigenin, certain relevant chemical and biological properties, ADME (absorption, distribution, metabolism, and excretion) of apigenin, the antimicrobial effects of apigenin and its interactions with gut microbiota will be presented and discussed in this review.
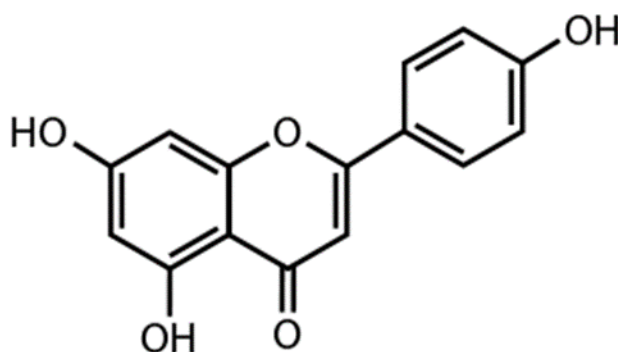
Figure 2.1 Apigenin (4',5,7-trihydroxy-flavone)

2.2 Apigenin: dietary source and daily intake levels

Before discussion of effects of dietary apigenin on gut bacteria, it is necessary to look at the dietary intake level of apigenin. The distribution of apigenin in the plant kingdom is wide, as it has been found in many vegetables, herbs, and fruits (Bhagwat, Haytowitz, & Holden, 2015). Fresh parsley, vine spinach, celery seed spices, green celery heart, Chinese celery and dried oregano are dietary sources of high apigenin content (Bhagwat et al., 2011). Other plants in which apigenin has been identified include red and white sorghum, rutabagas, oranges, kumquats, onions, wheat sprouts, tea, and cilantro (Bhagwat et al., 2011; W. J. Lee, Chen, Wang, Lin, & Tseng, 2008; Patel et al., 2007). Dried parsley spices has a particularly high level of apigenin (4503.50 mg / 100 g) that far exceeds any other vegetables or herbs (Bhagwat et al., 2011). Chamomile tea, high in apigenin content (840 mg / 100 g), is one of the most common source of apigenin intake from a single ingredient (Mckay & Blumberg, 2006). In nature, apigenin is typically found in a glycosylated form, with the tricyclic core structure linked to a sugar moiety through hydroxyl groups (O-glycosides) or directly to carbon (C-glycosides). The common apigenin glycosides are apigenin-7-apioglucoside (apiin), apigenin-7-O-glucoside (apigentrin), apigenin-8-C-glucoside (vitexin), apigenin-6-C-glucoside (isovitexin), apigenin-7-O-neohesperidoside (rhoifolin), and apigenin 6-C-glucoside 8-C-arabinoside (schaftoside) (Lefort & Blay, 2013; Simirgiotis, Schmeda-Hirschmann, Bórquez, & Kennelly, 2013; Tang et al., 2017a).

Since apigenin is widely spread in food items, and a diet high in flavonoids has been reported with many beneficial health effects, an estimation of the daily intake of

apigenin could be useful in the correct interpretation of the relationship between health outcomes and apigenin. Though dietary intake values have been obtained for some individual flavonoids (Probst, Guan, & Kent, 2018), when it comes to the flavone, apigenin, relevant research is very limited (Janssen, 1997; Mullie, Clarys, Deriemaeker, & Hebbelinck, 2007). Higher estimation of daily dietary intake of flavonoids is 1 g as glycosides or 650 mg as aglycones (Kuhnau, 1976), while another study estimated an average of only 23 mg/day in adults in the Netherlands (Michael G.L. Hertog et al., 1993). In a study around 1990, the mean flavanol and flavone intake among US health professionals was approximately 20 - 22 mg per day (Sampson, Rimm, Hollman, de Vries, & Katan, 2002). In the aforementioned study in Netherland in 1988, most of the average daily intake was attributed to quercetin, about 16 mg/day, and the actual daily intake of apigenin was only about 0.69 mg/day (Michael G.L. Hertog et al., 1993). Another study on the Dutch diet estimated that the average daily intake of apigenin is about 1 mg in Dutch diet (Janssen, 1997). A similar number, 1.5 ± 4.9 mg/day (range 0 - 30.3), was reported in a group of female Flemish dietitians (Mullie et al., 2007). A more recent paper provided information of the mean intake of flavonoid compounds in adults in the European Union by country, region and overall (Vogiatzoglou et al., 2015). The European average of apigenin intake is 3 ± 1 mg/day using the food consumption data from the European Food Safety Authority (EFSA) and the FLAVIOLA Food Composition Database. The daily intake level of apigenin is 4.23 mg/day in China (Cao, Zhang, Chen, & Zhao, 2017), while among middle-aged and older women in the US, it is 0.13 – 1.35 mg/d (L. Wang et al., 2009). This intake value among US women is similar to the reported 0.2-1.3 mg/day among 66,940 married women in an US nurses' health study

from 1984 through 2002 (Gates et al., 2007). Mean daily apigenin intake of major dietary sources of flavones in adult Australians was measured to be 0.45 mg/day (Somerset & Johannot, 2008).

The above information indicates that, as a phytochemical present in multiple food sources, quantification of dietary intake for apigenin is difficult and has large variation (Probst et al., 2018). One primary method for dietary intake level estimation relies on dietary assessment, which includes a diet history interview or food frequency questionnaires, and compares this against a food composition database (Probst et al., 2018). Accordingly, daily intake various with diet, depending on time and geographical location. The reliability of the information collected from subjects, as well as the inclusiveness, extensiveness (Probst et al., 2018), and demographic specificity of the database can all impact the accuracy of intake levels obtained. Other than the inaccuracy in the data, more information is available on the daily amount of total or combined groups of flavonoids than a single flavonoid of interest (Probst et al., 2018).

2.2 Chemical properties: solubility and stability

Understanding of the solubility and the stability of apigenin is the basis of any experiment that looks into the antimicrobial effects of apigenin in aqueous solutions. Flavonoids are often found in nature as glycosides and phenolic acids, as esters in the aqueous environment of the plant cell vacuole (Rothwell et al., 2017). Apigenin is the aglycone form and it is present in plants naturally as several apigenin glycosides as mentioned in the previous section. Those glycoside conjugates, primarily as apigenin-7-*O*-glucoside, and acylated derivatives are more water soluble than apigenin (Shukla &

Gupta, 2010b) and their structures have a major impact on their absorption and bioavailability, with the best bioavailability occurring when apigenin is bound to β-glycosides (Patel et al., 2007).

Apigenin is practically insoluble in highly polar solvents such as water (0.00135 mg/mL), and nonpolar solvents such as silicon fluid (0.0728 mg/mL) and safflower oil (0.0317 mg/mL) (Lakshmanan et al., 2015; Li et al., 1997). Other reports on apigenin's solubility ranges from 0.001 to 1.63 mg/mL (Nabavi et al., 2015). Apigenin is insoluble in water, yet, it is freely soluble in dimethylsulfoxide (DMSO) (Ali, Rahul, Naz, et al., 2017). One source estimated the solubility to be more than 100 mg/mL (Li et al., 1997), while another showed that the approximate solubility of apigenin in ethanol, DMSO, and dimethyl formamide (DMF) purged with inert gas to be 0.3, 15, and 25 mg/mL ("Apigenin (CAS 520-36-5) | Cayman Chemical," n.d.). Flavonoids are also better soluble in methanol than in water (Biesaga, 2011). As a result, organic solvents like DMSO (M. Wang et al., 2017) and Tween 80 (Zhang, Liu, Huang, Gao, & Qian, 2012) are used to dissolve apigenin prior to their addition to an aqueous solution to increase solubility. Different carriers such as ethosomes (Shen, Zhang, Wang, Xu, & Feng, 2014), polymeric micelles of Pluronic P123 and Solutol HS 15 (Zhai et al., 2013), and carbon nanopowder (Pforte, Hempel, & Jacobasch, 1999), or self-microemulsifying delivery system (L. Zhao, Zhang, Meng, Wang, & Zhai, 2013) are also developed and tested to enhance efficacy of apigenin. Taking into consideration its high permeability, apigenin is categorized as a Class II drug according to Biopharmaceutics Classification System (BCS), whose characteristics are low solubility and high permeability (Telange et al., 2017; Zhang et al., 2012).

Pure apigenin is generally regarded as unstable for long term storage at room temperature, and thus requires storage at -20°C (Patel et al., 2007). For use in experiments, it is recommended that fresh solutions be made as needed ("Apigenin (CAS 520-36-5) | Cayman Chemical," n.d.). Some researchers have looked into the stability of apigenin under various conditions. After heated reflux in water for 30 min, maceration for 24 hours or microwave irradiation for 5 min under 500W, 93-95% of apigenin was recovered for each condition (Biesaga, 2011). However, ultrasonic extraction caused the product to be degraded with only an 86% recovery rate (Biesaga, 2011). Another research showed that, in rat plasma, apigenin were stable under three conditions: 24 hours under room temperature; at least 4 weeks when kept frozen at -20°C and after at least three freeze-thaw cycles (Duan et al., 2011; Shi et al., 2011).

The decomposition for flavonoids depends on the number of substituents in the flavonoid molecule. Hydroxyl groups promote degradation of flavonoids, whereas sugar moiety and methoxyl groups protect flavonoids from degradation during microwave and ultrasonic assisted extraction (Biesaga, 2011). Therefore, the glucosides, other than having a higher solubility than the aglycone, are also chemically more stable, which may be why they have enhanced biological activities (Gurung, Kim, Oh, & Sohng, 2013).

2.3 Safety: mutagenicity and hemolysis tests

Safety is an important aspect of dietary components. In general, dietary plants containing flavonoids have not been associated with negative health impact, but rather considered as beneficial. Evidences support that a flavonoid-rich diet is inversely associated with cancer risk (Bosetti et al., 2005; Heederik, Kromhout, Burema, Biersteker,

& Kromhout, 1990; Michaël G.L. Hertog, Feskens, Hollman, Katan, & Kromhout, 1994; Hoensch, Groh, Edler, & Kirch, 2008; Knekt et al., 1997; Rossi et al., 2008). Therefore, consumption of flavonoids has been assumed as safe. One of the favorable characteristics of apigenin is its low toxicity (Ali, Rahul, Naz, et al., 2017; Patel et al., 2007; T. Way, Kao, & Lin, 2004; Xu & Lee, 2001).

Results of an Ames test, one of the oldest methods employed for *in vitro* testing of carcinogenicity using *Salmonella* strains (Ali, Rahul, Falaq Naz, Jyoti, & Siddique, 2017), showed that apigenin was not mutagenic or toxic when tested alone (Birt, Walker, Tibbels, & Bresnick, 1986). Not only was apigenin not mutagenic (Czeczot et al., 1990), it protected against multiple genotoxic agents, such as sodium azide, 9- amino acridine (Ali, Rahul, Falaq Naz, et al., 2017; Gulluce et al., 2015). Apigenin prevented the reverted mutations as a result of sodium azide exposure using histidine auxotroph *Salmonella typhimurium* (TA100) strain and the hindrance percent of apigenin was 98.17% (Hashemi, Long, Entezari, Nafisi, & Nowroozii, 2009). Apigenin 7-O-glucoside inhibited sodium azide mutagenicity in *Salmonella typhimurium* TA1535 at 0.4 μM with an inhibition rate of 27.2% and 9-aminoacridine in *S. typhimurium* TA1537 at 0.2 μM with an inhibition rate of 91.1%. In the yeast deletion assay using mutagens ethyl methanesulfonate and acridine, the inhibition rates were from 4% to 57.7% (Gulluce et al., 2015).

Apart from mutagenesis, hemolytic activity is another measurement of safety. Hemolytic tests showed that the percent hemolysis of apigenin was below permissible limit of 5% after 30 min treatment. Being hemocompatible indicates that apigenin is safe for intravenous application, and suggests that apigenin is non-toxic in mammalian

systems (Banerjee, Banerjee, Das, & Mandal, 2015). Apigenin was able to significantly attenuate the hemolytic activity of the purified Pneumolysin, the pore-forming toxin secreted by *S. pneumoniae*, in a concentration-dependent manner (Song et al., 2016).

As can be concluded from the Ames tests and hemolytic tests, apigenin is not mutagenic or hemolytic.

Toxicity is dependent on the amount. Taking apigenin glucosides in daily diets can hardly reach the therapeutic doses used in clinical trial (Tang et al., 2017a) and have not been reported to be harmful. However, as consumers intentional increase apigenin intake from dietary supplements or pharmaceutical sources, safety concerns may arise with a higher level of apigenin exposure.

2.4 Pharmacokinetics: absorption, distribution, metabolism and excretion

The relevance of pharmacokinetics of apigenin in this review is that it determines how much apigenin taken in orally is available to the human gut microbiota. A summary of ADME and drug interactions of apigenin can be found in the review by Tang *et al* (Tang, Chen, Huang, & Li, 2017b). In this section, information is provided to show that oral bioavailability of apigenin is poor; it is either excreted unabsorbed in the urine or feces or rapidly metabolized after absorption (Tang et al., 2017a).

2.4.1 Absorption

About 5-10% of total polyphenol intake, mostly monomers and dimers, may be absorbed in the small intestine (Cardona et al., 2013). Gastrointestinal tract plays a significant role in the metabolism and conjugation of apigenin before entry into the

systemic circulation and the liver (Y. Liu & Hu, 2002). In a perfused rat intestinal model, aglycone apigenin were rapidly absorbed (Y. Liu & Hu, 2002). Apigenin can be transported by both passive and active carrier-mediated saturable mechanism in the duodenum and jejunum, and passive transport mechanisms in the ileum and colon, primarily (Zhang et al., 2012). Oral administration of apigenin resulted in with a peak concentration (C_{max}) 1.33 ± 0.24 µg/mL and area under the curve (AUC_{0-t}) of 11.76 ± 1.52 µg · hour/mL in rats, which were very low blood apigenin levels (Ding et al., 2014). Contradicting conclusions have been drawn regarding the rate of absorption of apigenin. One study using rats concluded that apigenin has a slow absorption because after a single oral administration of radiolabeled apigenin, radioactivity appeared in the blood 24 hours later (Angéline Gradolatto et al., 2005). Another concluded the opposite, as the plasma concentrations apigenin reached peak level at 3.9 hours after dosing (T. Chen, Li, Lu, Jiang, & Su, 2007a).

2.4.2 Distribution

Multiple studies have been performed to measure the distribution and excretion of apigenin *in vivo*. Following an IV bolus injection of apigenin at 20 mg/kg, the mean value of systemic clearance was 6.12 ± 0.79 L/h/kg (Wan et al., 2007). In the previously mentioned study with rats, after a single oral administration of radiolabeled apigenin, the elimination half-time was high with a value of 91.8 hours (Angéline Gradolatto et al., 2005). The distribution volume was 259 ml, and the plasmatic clearance was 1.95 ml/h (Angéline Gradolatto et al., 2005). After 10 days, 1.2% of radioactivity was recovered in the blood, 0.4% in the kidneys, 9.4% in the intestine, 1.2% in the liver, and 24.8% in the rest of the body (Angéline Gradolatto et

al., 2005). Another study showed that steady-state levels of apigenin in mice plasma, hepatic and small intestinal, after ingestion of 0.2% flavones with their diet (including 1.1 mmol/kg apigenin) for a week, were 0.09 ± 0.08 nmol/mL, 1.5 ± 1.0 nmol/g, and 86 ± 47 nmol/g, respectively (Cai, Boocock, Steward, & Gescher, 2007).This is in the similar range to the other study. Apigenin distributes well into the tissues (Y. Liu & Hu, 2002).

2.4.3 Metabolism

The absorbed apigenin may go through extensive Phase I and Phase II metabolism (Cardona et al., 2013). In the rat liver, metabolism of apigenin was found to involve Phase I Enzymes in the presence of NADPH (nicotinamide adenine dinucleotide phosphate), P450 (cytochrome P450 enzymes), or FMO (flavin-containing monooxygenase) (Tang et al., 2017a). Phase II biotransformation of apigenin involves both enteric and enterohepatic cycling (T. Chen et al., 2007a). The conjugation reactions glucuronidation and sulfation are the essential phase II metabolic pathways of apigenin (Tang et al., 2017a). In both rat and human, apigenin has been reported to produce major metabolites of glucuronidated and sulfated conjugates (J. Chen, Lin, & Hu, 2003). The major hepatic metabolite of apigenin is luteolin (Angéline Gradolatto et al., 2005). The absorbed apigenin in blood circulation and tissues is in the form of glucuronide, sulfate conjugates or luteolin (Tang et al., 2017a). There were three β-monoglucuronides that appeared after glucuronidation reaction, while only one product appeared after sulfation reaction (Tang et al., 2017a). In human hepatic cell line, Hep G2, as well as intestinal cell line, Caco-2, apigenin induced phase II detoxifying enzyme UDP-glucuronosyltransferase UGT1A1 (Walle & Walle, 2002)(Švehlíková et al.,

2004). In addition, glucuronidation reactions also occur in the intestine, and intestinal disposition may be more important than hepatic disposition in the first-pass metabolism of apigenin (J. Chen et al., 2003).

2.4.4 Excretion

Excretion of apigenin after oral intake, especially through feces, is a good indication of the phenomenon that dietary apigenin are available for metabolism by the gut microbiota. In the aforementioned experiment using rats, after a single oral administration of radiolabeled apigenin, 51.0% of radioactivity was recovered in urine, 12.0% in feces within 10 days. In the same research, it was discovered that sex and age of the rats affected apigenin conjugates eliminated via the urinary route (Angéline Gradolatto et al., 2005). This research also concluded that apigenin has a slow metabolism and a slow elimination phase. Thus, a possible accumulation of this flavonoid in the body (Angéline Gradolatto et al., 2005).

The other study that suggested that apigenin has a quick absorption and a slow elimination phase also hypothesized a possible accumulation of apigenin. In this study, 28.6% and 16.6% of the apigenin was recovered in feces and in urine, respectively, summing up to a total recovery rate of 45.2% (T. Chen et al., 2007a). The cumulative apigenin excreted in the bile was 6.34% of the dose (T. Chen et al., 2007a).

In a human intervention study, a basic diet was supplemented with parsley, providing 3.73 - 4.49 mg apigenin/ Megajoule for one week, and the excretion of apigenin was measured (Nielsen et al., 1999). The experimental design differs from the animal experiments as it used human as subjects and plant as the dietary source of apigenin instead of pure compound. The results differ from the animal experiments,

too. The average urinary excretion of apigenin was significantly higher during intervention with parsley (20.7 - 5727.3 µg/24 hours) than during the basic diet (0 - 1571.7 µg/24 hours). No difference between males and females was observed in the mean excretion of apigenin. The amount of apigenin excreted ended abruptly after parsley supplementation was halted. Therefore, urinary excretion and clearance of apigenin is quick, with the excretion half-life estimated to be approximately 12 hours.

It is worth mentioning again that apigenin exists in plants as glycosides naturally. Glycosides of flavonoids may be absorbed from the small intestine by a mechanism involving the glucose transport pathway (Gee, DuPont, Rhodes, & Johnson, 1998). Epithelial β-glucosidase-mediated deglycosylation is a critical step in the absorption and metabolism of dietary flavonoid glycosides. It is hypothesized that apigenin glucosides can be hydrolyzed into apigenin by cytosolic β-glucosidase (CBG) and lactase-phlorizin hydrolase (LPH), enzymes produced by the liver or intestinal cells, or the gut microflora (Manach, Scalbert, Morand, Rémésy, & Jiménez, 2004; Németh et al., 2003; Tang et al., 2017a). LPH has been shown to hydrolyze flavonoid glycosides and is proposed to be brush-border enzymes (Y. Liu & Hu, 2002), while CBG functions inside cells (Manach et al., 2004). One study presented a time-profile of distribution of flavonoids in the rat stomach lumen and wall, small intestine lumen and wall, cecum lumen and wall after gavaging with purified flavonoid extract from illuminated parsley (Pforte et al., 1999). It was found that glycosides transported to the cecum were mostly absorbed or deglycosylated rapidly as the cecum luminal contents flavonoids contained only aglycones. Therefore, *in vitro* simulation of the impact of apigenin in the colon should use the aglycone form, apigenin.

2.5 Usage as herbal medicine or functional food

Pharmacological potential of apigenin may be reflected by the use of plants containing it as herbal medicine or functional food in different cultures. Plants containing apigenin, along with other flavonoids, have been used to battle diseases in many cultures. Apigenin has been identified as an active ingredient in *Scutellaria barbata* D. Don (Lamiaceae) (Sato et al., 2000), *Castanea sativa* Mill. (Fagaceae) (Basile et al., 2000a), *Portulaca oleracea* L. (Nayaka, Londonkar, Umesh, & Tukappa, 2014), *Marrubium globosum* ssp. *Libanoticum* (Rigano et al., 2007), *Combretum erythrophyllum* (Combretaceae) (Martini, Katerere, & Eloff, 2004), *Aquilegia oxysepala* (Yu, Yi, & Liang, 2007) and propolis (Koru et al., 2007), among which most are traditional herbal or alternative medicines. Chamomile tea, which is extremely rich in apigenin, has been used as a folk medicine for relieving indigestion or gastritis (Patel et al., 2007). Chamomile is also used in mouth rinse, skin care products and vapor inhalant to reduce inflammation (Patel et al., 2007). There are two types of chamomile: German chamomile and Roman chamomile, with the former more commonly used as a dietary supplement (Vogiatzoglou et al., 2015). Some preliminary studies suggest that a chamomile dietary supplement might be helpful for generalized anxiety disorder. Researchers conducted a randomized, double-blind, placebo-controlled trial to test the effects of chamomile extract in patients diagnosed with mild to moderate GAD. Compared with placebo, chamomile was associated with a clinically meaningful and statistically significant greater reduction in mean Hamilton Anxiety Rating (HAM-A) scores (Amsterdam et al., 2009). Although the

researchers suggest that other chamomile species, preparations, and formulations might produce different results (Amsterdam et al., 2009).

As for the safety of chamomile, there have been reports of allergic reactions as well as interactions between chamomile and cyclosporine and warfarin, which can cause serious consequences (Vogiatzoglou et al., 2015).

2.6 Pharmacological activities of apigenin

As one of the five major flavonoids in plants, apigenin has been extensively studied for its biological functions. Several reviews are available on the bioactivities of apigenin focusing on different aspects including its health functionality (Ali, Rahul, Falaq Naz, et al., 2017) and cancer chemoprevention potential (Lefort & Blay, 2013; Patel et al., 2007; Shukla & Gupta, 2010a). These reviews captured the large body of research on pharmacological activities of apigenin and provided expert opinions on this subject.

Research on apigenin first began in the 1960s, and it was proposed to be chemo preventative in the 1980s (Birt et al., 1986; Lefort & Blay, 2013; Patel et al., 2007). Recently, apigenin has received much attention because it has low intrinsic toxicity (Tang, Chen, Huang, & Li, 2017c; T.-D. Way, Kao, & Lin, 2004) and it exerts differential effects on normal versus cancer cell growth, survival, or apoptosis in several different types of cells (Czeczot et al., 1990; Lefort & Blay, 2013; Patel et al., 2007).

The reported biological functions of apigenin include anti-oxidant, anti-mutagenic, anti-carcinogenic, anti-inflammatory, anti-proliferative, and anti-progression (Patel et al., 2007). Apigenin is a moderate antioxidant compound because the double bond at the 2,3 carbon makes the structure more reactive, despite the absence of the hydroxyl group at

position 3 and a catechol structure in the B-ring (Sichel, Corsaro, Scalia, Di Bilio, & Bonomo, 1991; Tripoli, Guardia, Giammanco, Majo, & Giammanco, 2007). Apigenin can act as an anti-inflammatory agent. It inhibits the production of proinflammatory cytokines IL-1β, IL-8, and TNF in lipopolysaccharide-stimulated human monocytes and mouse macrophages (Nicholas et al., 2007). It also inhibited TNF-induced NF-κB transcriptional activation in NIH/3T3 cells as well as acute carrageenan-induced paw edema in mice (Funakoshi-Tago, Nakamura, Tago, Mashino, & Kasahara, 2011). Apigenin exhibited anti-inflammatory effect on murine microglia cell line by reducing the production of nitric oxide and prostaglandin E_2 and was found to be protective against ischemia in neuronal cells (Ha et al., 2008). Anti-inflammatory activity of apigenin has also being reported in lipopolysaccharide-induced inflammation in acute lung injury (J. Wang et al., 2014). A DNA protective capacity of apigenin was reported against free radicals generated by H_2O_2, resp. Fe^{2+} (Romanová, Vachálková, Cipák, Ovesná, & Rauko, 2001).

The anti-mutagenic effect has been reported in *in vitro* cell models, *in vivo* experiments, and AMES test using bacterial models showing that apigenin could prevent, inhibit or reverse chemically induced genotoxicity (Birt, Mitchell, Gold, Pour, & Pinch, n.d.; M.-L. Kuo, Lee, & Lin, 1992; Patel et al., 2007; Van Dross, Xue, Knudson, & Pelling, 2003). The anti-carcinogenic effect of apigenin has been widely reported. Apigenin has been found to be protective against multiple types of cancer including breast cancer (W. J. Lee et al., 2008), cervical cancer, colon cancer, leukemia (Ruela-de-Sousa et al., 2010), lung cancer, prostate cancer (Shukla & Gupta, 2008), skin cancer, thyroid cancer, endometrial cancer, neuroblastoma, and adrenocortical cancer (Patel et al.,

2007; Shukla & Gupta, 2010b). Derivatized compound based on apigenin displayed higher antiproliferative activity in human lung, cervical, hepatocellular liver and breast cancer cell lines than apigenin itself (R. Liu et al., 2013).

Apigenin showed synergistic effects with antitumor drugs such as paclitaxel, 5-fluorouracil, PLX4032 and N-(4-hydroxyphenyl) retinamide by enhancing their bioavailability or efficacy (Tang et al., 2017c). Apigenin may also serve as a dietary supplement along with small molecule inhibitors to improve radioiodine therapeutic efficacy on invasive tumor margins and thus minimizing future metastasis (Lakshmanan et al., 2015).

2.7 Antimicrobial effects of apigenin and mechanism

The antimicrobial effects of dietary flavonoids have been studied extensively. Although there are many publications reporting findings on apigenin, there is no review that summarizes the findings. The following part aims to include available information in publications and to make meaningful conclusions.

2.7.1 Antibacterial activities

The antibacterial potential of apigenin has been tested against many bacteria species and various strains within the same species. Broth microdilution and agar dilution are the most popular methods in which the minimal inhibitory concentrations (MICs) are determined as the lowest concentration of treatment that showed no growth after incubation (Özçelik, Murat, & Orhan, 2011) (Dong et al., 2013) (Mamadalieva et al., 2011) (Cushnie, Hamilton, & Lamb, 2003b). However, results obtained from the two methods may not necessarily coincide (D. Wu et al., 2008). It has been suggested this

might result from the different solubilities of the tested compound in liquid and the agar gel culture media, and the variability in MIC judgment criteria (Cushnie & Lamb, 2005). Results of the antibacterial and antifungal tests of apigenin measured by its MICs are summarized in Table 2.1. Due to the limited amount of apigenin that could dissolve in the media, apigenin is considered not active if the MIC is above 128 µg/mL in this review. MIC values are strain-specific, making it difficult to summarize the effects of apigenin in a shorter and more general way.

Apigenin could not inhibit the growth of *S. aureus* (8325-4, ATCC 29213, wood 46, and BAA-1717) (Dong et al., 2013). Despite the reported lack of activity against *S. aureus*, apigenin was found to remarkably decrease the production of α-hemolysin at low concentrations in a concentration-dependent manner (Dong et al., 2013). Alpha-hemolysin is a pore-forming cytotoxin that is secreted by most *S. aureus* strains, essential for the pathogenesis of *S. aureus* pneumonia (Dong et al., 2013). Apigenin protected A549 cells from α-hemolysin-mediated injury in the adenocarcinomic human alveolar basal epithelial cells (A549 cells) and *S. aureus* co-culture system (Dong et al., 2013), as well as alleviated injury of the lung tissue and decreased cytokine levels in the bronchoalveolar lavage fluid in the mouse model of *S. aureus pneumonia* (Dong et al., 2013). Therefore, the protective effect apigenin did not come from a reduction in bacterial quantity, but more likely an altered cell physiology. When apigenin was applied with LysGH15, the lysin derived from phage GH15 with high efficiency and a broad lytic spectrum against MRSA, a synergism was observed using a mouse *S. aureus* pneumonia model (Xia et al., 2015).

Reverse antibiotics (RA) are chemicals that are ineffective against antibiotic-susceptible bacteria but active against the relevant antibiotic-resistant bacteria (Hiramatsu et al., 2012). RAs can help put a stop in the accumulation of antibiotic resistance by bacteria, for that treatment with RA kills the bacteria that has acquired the antibiotic resistance and leaves only the bacteria that possess the original phenotype resistant to RA and susceptible to that antibiotic (Hiramatsu et al., 2012). Apigenin has RA activities against quinolone-resistant *Staphylococcus aureus* (Morimoto, Baba, Sasaki, & Hiramatsu, 2015). The minimum inhibitory concentrations (MICs) of apigenin against quinolone-resistant *Staphylococcus aureus* strain Mu50 and quinolone-susceptible *S. aureus* FDA 209P are 4 mg/L and more than 128 mg/L (Morimoto et al., 2015). Apigenin was also found to reverse bacterial resistance to cephalosporin ceftazidime in *Enterobacter cloacae* (Eumkeb & Chukrathok, 2013). The MIC of apigenin in ceftazidime-resistant *Enterobacter cloacae* (CREC) was higher than 512 µg/mL, which indicated no inhibitive effects (Eumkeb & Chukrathok, 2013). Ceftazidime applied in combination with apigenin showed a synergistic effect with a fractional inhibitory concentration index smaller than 0.01 (Eumkeb & Chukrathok, 2013). The 5,7- OH group of A ring and one 4'- OH group of the B ring in apigenin were found important in reversing antimicrobial resistance (Eumkeb & Chukrathok, 2013). The significantly enhanced the activities of ceftazidime by apigenin may have been the results of peptidoglycan synthesis inhibition, certain β-lactamase enzymes inhibition, and alteration of outer membrane and cytoplasmic membrane permeabilization (Eumkeb & Chukrathok, 2013).

Contrasting results have been observed on apigenin's effect on *Helicobacter pylori in vitro*. While one study saw no inhibitory effect against thirteen randomly selected clinical strains of *H. pylori* from antral biopsies and a reference strain *H. pylori* ATCC 43504 (Konstantinopoulou, Karioti, Skaltsas, & Skaltsa, 2003), apigenin showed moderate antibacterial activity in another study, with a MIC of 25 µg/mL against both *H. pylori* SS1 and *H. pylori* ATCC 43504 (D. Wu et al., 2008). An *in vivo* experiment using Mongolian gerbils, apigenin treatments (30–60 mg/kg body weight/day) effectively decreased *H. pylori*-induced atrophic gastritis and N'-methyl-N'-nitro-N-nitroso-guanidine (MNNG)-induced dysplasia/gastric cancer rates (C. H. Kuo et al., 2014). The dose of 60 mg apigenin/kg bodyweight/day significantly decreased *H. pylori* colonization and *H. pylori*-induced histological changes of neutrophil and monocyte infiltrations and atrophic gastritis (C. H. Kuo et al., 2014).

Streptococcus mutans is the main pathogen responsible for the development of dental caries in humans. The organism synthesizes glucans during adhesive interactions with tooth surface and other oral bacteria. Apigenin was found capable of inhibiting water insoluble glucans synthesis and reducing incidence of dental caries with minimal effects on the viability of oral flora populations *in vivo* when applied topically (H Koo et al., 2005; Hyun Koo et al., 2003).

Apigenin C-glucoside's antibacterial activity was reported to be weak (Taiwo & Igbeneghu, 2014). It was hypothesized that glycosylation causes a reduction in lipophilicity and consequently diminishes the ability to penetrate bacterial membrane (Taiwo & Igbeneghu, 2014). Some derivatives of apigenin showed increased antibacterial activity against *Staphylococcus aureus*, *Bacillus subtilis*, *Escherichia coli*, and

Pseudomonas aeruginosa, especially 7-[3-(Morpholin-4-yl)propoxy]-5-hydroxy-2-(4-hydroxyphenyl)-4H-chromen-4-one (R. Liu et al., 2013). Given the prevalence of antibiotic resistance, apigenin or its derivatives could be a candidate as a new antibiotic or as a dietary supplement to enhance performance of antibiotics.

There are many other studies that tested apigenin-containing plant extracts' antimicrobial effects. Plant extracts could have several other bioactive components, thus further research is needed to accurately attribute the cause of antimicrobial effect. Those studies are not included in this review.

Some insight is available on the mechanism of the antibacterial activity of apigenin. It has been previously indicated that apigenin affects nucleic acids, type II fatty acids and D-Alanine: D-Alanine ligase in bacteria (M. Wang et al., 2017; D. Wu et al., 2008). In a research where apigenin inhibited *Staphylococcus aureus*, mode of action was compared to other antibiotics with known mechanism by clustering the treatments based on intracellular metabolites. Apigenin was clustered with rifampicin and norfloxacin which target RNA polymerase, and gyrase and topoisomerase IV, respectively (Yu et al., 2007), which indicates that the target of apigenin could be nucleic acid (Sato et al., 2000). The target DNA gyrase was also reported in one other study (Ohemeng, Schwender, Fu, & Barrett, 1993). Both the D-Alanine: D-Alanine ligase and type II fatty acid synthetic pathway are involved in cell membrane/wall synthesis. It was hypothesized that cell membrane and cell wall synthesis is likely a major target of apigenin in *Enterococcus caccae* (M. Wang et al., 2017). Apigenin was found to also induce stress response genes and protein chaperone genes expression (M. Wang et al., 2017).

Although the methods employed in determining the inhibitory effects are different, nearly all studies focused on pathogenic bacteria and cultured the bacteria under aerobic conditions. Since dietary apigenin will enter colon, it is worthwhile to study the effects of apigenin on commensal gut bacteria under anaerobic culture conditions.

Table 2.1 The antibacterial and antifungal effects of apigenin measured by its minimum inhibitory concentration (MIC). ATCC, American type culture collection; RSKK, Refik Saydam Central Hygiene Institute.

Strain	MIC µg/mL	Method	Reference
Antibacterial activity			
E. coli ATCC 35218	4	Broth microdilution with no growth under the microscope	(Özçelik et al., 2011)
E. coli clinical isolate ESβL+	128		
P. aeruginosa ATCC 10145	2		
P. aeruginosa clinical isolate	32		
P. mirabilis ATCC 7002	4		
P. mirabilis clinical isolate ESβL+	128		
K. pneumoniae RSKK 574	8		
K. pneumoniae clinical isolate ESβL+	128		
A. baumannii RSKK 02026	2		
A. baumannii clinical isolate	64		
S. aureus ATCC 25923	16		
S. aureus clinical isolate MRSA	> 128		
E. faecalis ATCC 29212	8		
E. faecalis clinical isolate	128		
B. subtilis ATCC 6633	8		
B. subtilis clinical isolate	16		
20 *S. aureus* strains MRSA and MSSA	3.9-15.6	Disc-diffusion test	(Sato et al., 2000)
S. pyogenes ATCC 12344	0.5 mM	Broth microdilution with no visual turbidity and no growth on agar after incubation	(Mamadalieva et al., 2011)
MRSA NTCC 10442	Not active		
E. coli ATCC 25922	0.5 mM		
P. aeruginosa ATCC 27853	Not active		
S. aureus 25923	400	Broth microdilution	(Lucarini et al., 2015)
S. pyogenes 19615	50		
E. faecalis 19433	> 400		
E. coli 14948	>400		
S. Choleraesuis 10708	>400		

Organism	MIC	Method	Reference
P. aeruginosa 27853	400		
34 *S. aureus* strains	> 4000	Agar dilution	(Su, Ma, Wen, Wang, & Zhang, 2014)
S. aureus ATCC 13709	no inhibition	Broth dilution with no visible growth	(Basile et al., 2000b)
P. vulgaris ATCC 12454	no inhibition		
K. pneumoniae ATCC 10031	128		
E. cloacae ATCC 10699	64		
P. aeruginosa ATCC 27853	64		
E. coli ATCC 11229	128		
E. aerogens ATCC 13048	64		
S. aureus NCTC 10788, NCTC 6571, NCTC 11940, ATCC 33591, NCTC 11561, NCIMB 9968	Low activity	Agar dilution with strong, moderate, weak or absent growth	(Cushnie et al., 2003b)
E. faecalis NCIMB 775, NCTC 12201, NCTC 12203	Not active		
E. faecium NCTC 7171, NCTC 12202, NCTC 12204	Not active		
E. coli NCTC 12241, NCTC 11954, NCTC 11560	Not active		
P. aeruginosa NCTC 10662, NCTC 8506, NCTC 8203	Not active		
E. coli JM 109	200	Agar dilution	(D. Wu et al., 2008)
E. coli 25922	> 200		
H. pylori SS1	25		
H. pylori ATCC 43504	25		
S. aureus ATCC 6538	Not active	Broth microdilution with no growth under the microscope	(Konstanti nopoulou et al., 2003)
B. cereus clinical isolate	Not active		
M. flavus ATCC 10240	Not active		
P. aeruginosa ATCC 27853	Not active		
H. pylori ATCC 43504 and clinical isolates	Not active		
P. mirabilis clinical isolate	Not active		
E. coli ATCC 35218	Not active		
S. aureus ATCC 29213, ATCC 10832, BAA 1717, 8325-4, DU 1090	> 1024	Broth microdilution	(Dong et al., 2013)
ceftazidime-resistant *Enterobacter cloacae*	> 512	Broth microdilution	(Eumkeb & Chukratho k, 2013)
S. aureus strain Mu50	4	Broth microdilution	(Morimoto et al., 2015)
S. aureus FDA 209P	> 128		
Antifungal activity			
C. albicans ATCC 90028	Not active	Broth microdilution with no visual turbidity and no growth on agar after incubation	(Mamadali eva et al., 2011)
C. glabrata ATCC MYA2950	0.25 mM		
C. albicans ATCC 10231	8	Broth microdilution	(Özçelik

C. parapsilosis ATCC 22019	16	with no growth under the microscope	et al., 2011)

2.7.2 Antiviral activity

Apigenin has been reported to be able to inhibit multiple viruses, including enterovirus 71, herpes simplex virus HSV-1 and HSV-2, hepatitis C virus, influenza virus, foot-and-mouth disease virus, and African swine fever virus, but not coxsackievirus A16. The details are described as follows.

One of the major causative agents for hand, foot and mouth disease (HFMD), enterovirus 71 (EV71), is a member of genus *Enterovirus* in Picornaviridae family (Lv et al., 2014). Research found that apigenin inhibited EV71-mediated cytopathogenic effect and EV71 replication (Lv et al., 2014). Viral polyprotein expression, EV71-induced cell apoptosis, intracellular reactive oxygen species (ROS) generation and cytokines up-regulation were inhibited (Lv et al., 2014). Apigenin could interfere with viral internal ribosome entry site (IRES) activity and inhibit EV71-induced c-Jun N-terminal kinase (JNK) activation which is critical for viral replication (Lv et al., 2014). Another research tested methanol extract of *Paulownia tomentosa* flower for antiviral activity against enterovirus 71 (EV71) and coxsackievirus A16 (CAV16) (Ji et al., 2015). CAV16 is another one of the predominant etiologic agents of hand, foot, and mouth disease (Ji et al., 2015). The extract had no effect against CAV16 infection, but apigenin was again identified as an active component to inhibit EV71 (Ji et al., 2015). The EC_{50} value, inhibitory concentration of compound that produces 50% inhibition of virus-induced cytopathic effects, for apigenin to block EV71 infection was 11.0 µM, with a selectivity index (SI) of approximately 9.3 (Ji et al., 2015). Similar compounds like naringenin and quercetin, were not active against EV71 infection (Ji et al., 2015).

In another study, apigenin, as a bioactive compound in *Ocimum basilicum*, also known as sweet basil, showed a broad spectrum of antiviral activity (Chiang, Ng, Cheng, Chiang, & Lin, 2005). The highest activities were against Herpes simplex virus HSV-2 (EC_{50} = 9.7 mg/L; SI = 6.2), adenovirus ADV-3 (EC_{50} = 11.1 mg/L; selectivity index SI = 5.4), hepatitis B surface antigen (EC_{50} = 7.1 mg/L; SI = 2.3) and hepatitis B e antigen (EC_{50} = 12.8 mg/L; SI = 1.3). Apigenin inhibited HSV-1 in Madin-Darby canine kidney (MDBK) cells within the concentrations of 0.4–1.6 µg/ml (Özçelik et al., 2011).

Apigenin was found to be able to inhibit Hepatitis C virus (HCV) replication *in vitro* (Shibata et al., 2014). Apigenin decreased the expression levels of mature microRNA miR122, a liver-specific miRNA that positively regulates HCV replication, through inhibition of TRBP (transactivating response RNA-binding protein) phosphorylation without significantly affecting cell growth (Shibata et al., 2014). Therefore, apigenin intake, either through regular diet or supplements, may be beneficial for chronically infected patients (Shibata et al., 2014).

Apigenin showed significant anti-influenza virus activities with IC_{50} of 1.34 µg/mL in MDBK cells (Q. Wu et al., 2010).

Animal disease related viruses were also found to be inhibited by apigenin. Foot-and-mouth disease (FMD) is a highly contagious and clinically acute viral disease of domestic and wild cloven-hoofed animals worldwide caused by FMD virus (FMDV) (Qian et al., 2015). FMDV belongs to the *Aphthovirus* genus of the Picornaviridae family (Qian et al., 2015). Apigenin inhibited FMDV infection at the viral post-entry stage with no extracellular virucidal activity (Qian et al., 2015).

Similar to EV71, apigenin interfered with the translational activity of FMDV by internal ribosome entry site (Qian et al., 2015).

African swine fever virus (ASFV) causes serious diseases in domestic pigs. A dose-dependent anti-ASFV effect of apigenin was reported *in vitro* (Hakobyan et al., n.d.). Apigenin was highly effective at the early stages of infection and reduced the ASFV yield by more than 99.99 % when it was added at 1 hour post infection (Hakobyan et al., n.d.). Apigenin inhibited ASFV-specific protein synthesis and viral factory formation. Continuous apigenin treatment prevented cytopathic effect in ASFV-infected cells (Hakobyan et al., n.d.).

2.7.3 Antifungal activity

C. albicans ATCC 10231 and *C. parapsilosis* ATCC 22019 were reported to be inhibited by apigenin with MIC of 8 and 16 µg/mL, respectively (Özçelik et al., 2011). Apigenin could be used as an antifungal agent in the clinical treatment of dermatophytosis (Singh, Kumar, & Joshi, 2014). Mice were experimentally induced to develop dermatophytosis with *Trichophyton mentagrophytes* and lesions were treated with two concentrations of apigenin ointment, 2.5 mg/g and 5mg/g. Apigenin exhibited similar effect as the reference drug Terbinafine (Singh et al., 2014). Applied at 5 mg/g for both apigenin and Terbinafine, complete recovery from the infection was recorded on the 12th day in both groups. With 2.5 mg/g ointment, the infection was cured on the 16th day (Singh et al., 2014). A recent paper reported that apigenin induced cell shrinkage in *Candida albicans*, altering the cell membrane potential and causing leakage of intracellular components (H. Lee, Woo, & Lee, 2018).

2.7.4 Antiparasitic activity

Leishmaniasis is a disease that affects more than 12 million people worldwide with around 2 million new cases each year. It is caused by the protozoa parasite *Leishmania amazonesis* (Alvar et al., 2012). Apigenin treatment for 24 hours resulted in concentration-dependent inhibition of cellular proliferation with IC_{50} equals 23.7 μM and increased reactive oxygen species (ROS) generation. These effects exerted by apigenin were protected by glutathione and N-acetyl-L-cysteine, but not oxidized glutathione. Other mechanisms of the negative effects of apigenin on *Leishmania amazonesis* include extensive swelling in parasite mitochondria, altered mitochondrial membrane potential, rupture of the trans-Golgi network, and cytoplasmic vacuolization (Fonseca-Silva, Canto-Cavalheiro, Menna-Barreto, & Almeida-Amaral, 2015).

2.8 Apigenin and human gut microbiota

As shown earlier in the ADME section of the review, part of the apigenin intake is excreted in the feces and apigenin aglycone was detected in cecum luminal content, thus the bacterial community in colon is exposed to dietary apigenin. Once apigenin enters the colon, it becomes the substrate of the pool of various enzymes produced by the gut microbiota. Human gut microbiota has been found to harbor enzymes that could degrade apigenin.

Some, not all, commensal gut bacteria are capable of degrading apigenin on their own. *Bacteroides distasonis* was found to be capable of converting apigenin-7-glucoside to apigenin, but it was not the case with *E. coli* (Hanske, Loh, Sczesny, Blaut, & Braune, 2009). *Eubacterium ramulus*, a strictly anaerobic human gut bacteria, is able to metabolize apigenin, as well as quercetin, naringenin, daidzein and genistein (Blaut &

Clavel, 2007). It possesses a phloretin-hydrolase able to break the phloretin C-C bond (Schoefer, Braune, & Blaut, 2004). Another anaerobic bacterium isolated from human feces, *Clostridium orbiscindens*, was found to degrade apigenin to 3-(4-hydroxyphenyl)propionic acid with phloretin and naringenin as the two intermediates (Schoefer et al., 2003). This degradation product 3-(4-hydroxyphenyl)propionic acid, also known as desaminotyrosine (DAT), is beneficial during influenza by triggering type I interferon signaling and in turn augmenting antiviral responses by phagocytic cells; therefore preventing inflammation and severe disease (Steed et al., 2017). It is possible that the anti-inflammatory activity and protective effects on lung tissues mentioned in the previous sections are mediated by microbial metabolites from apigenin as well (Dong et al., 2013; J. Wang et al., 2014). Degradation of apigenin and its glycosides most likely involves multiple bacteria, with complementary and overlapping functionalities.

Some information is available on how the human gut microbiota metabolize apigenin glycosides using *in vitro* experiments or animal models. Hanske *et al* performed a research demonstrating that the bioavailability of apigenin-7-glucoside (A7G) is influenced by human intestinal microbiota using rats and *in vitro* culture of human gut micorbiota in test tubes (Hanske et al., 2009). The *in vitro* culture experiment was carried out by incubating 10 mL culture of human gut microbiota and apigenin-7-glucoside mixture in airtight tubes for 24 hours at 37°C (Hanske et al., 2009). Results showed that human fecal suspensions converted A7G completely within 5 hours of incubation (Hanske et al., 2009). A7G concentration remained largely stable without the presence of bacteria (Hanske et al., 2009). Apigenin and naringenin were transiently formed as intermediate metabolites from A7G (Hanske et al., 2009). The end products of A7G

microbial degradation were 3-(4-hydroxyphenyl)propionic acid (4-HPPA) and trace amounts of 3-(3-hydroxyphenyl)propionic acid (3-HPPA) (Hanske et al., 2009). In rat models, germ-free rats excreted via urine A7G, apigenin, and luteolin uniformly within 48 hours after application of A7G, both in free and conjugated forms (Hanske et al., 2009). Additional metabolites were excreted by human-microbiota associated (HMA) rats in urine: naringenin, phloretin, 3-(3,4-dihydroxyphenyl)propionic acid (3,4-DHPPA), 4-HPPA, 4-hydroxycinnamic acid (4-HCA), and 3-HPPA, with A7G, apigenin, luteolin, naringenin, phloretin, 4-HPPA, and 4-HCA in both their free and conjugated forms and only free forms of 3,4-DHPPA and 3-HPPA (Hanske et al., 2009). A7G, apigenin, and luteolin, mainly as conjugates, were observed in germ-free rats' fecal excretion, while they were excreted at a considerably lower level in HMA rats (Hanske et al., 2009). This study strongly support that the gut microbiota is involved in the metabolic fate of apigenin after consumption.

Another research using an *in vitro* system to study the fermentation of apigenin showed that the number of metabolites formed is donor dependent with 3-(4-hydroxyphenyl)propionic acid, 3-hydroxyphenyl-acetic acid, and 3-phenylpropionic acid being the common metabolites detected with microbiota samples from all three different donors (Vollmer et al., 2018). This study also found that fermentation rates of *C*-glycosides are slower compared to the rates of *O*-glycosides.

While microbiota facilitate degradation of apigenin, apigenin and its metabolites may also modify the structure and function of gut microbiota considering its effects on bacteria. The effect on the modulation of the gut ecology is still poorly understood (Cardona et al., 2013). Throughout the study by Hanske *et al*, the similarity of the

intestinal microbiota composition of rats was determined by PCR-coupled denaturing gradient gel electrophoresis and was reported to be ranged from 63.3 to 75.8%. However, no detailed information was available on compositional changes of the community although there seemed to be a change. The *in vitro* experiment was carried in test tubes without pH control and a freshly inoculated microbiota. It would be more ideal to perform the *in vitro* incubation of apigenin and gut bacteria in a chemostat and establish a stable community. In the aforementioned research that used samples from three different donors, no significant changes in the microbiota composition and short chain fatty acid levels as products of carbohydrate fermentation were not detected between incubations with different phenolic compounds (Vollmer et al., 2018). In another short term *in vitro* fermentation experiment using human gut microbiota and apigenin, apigenin slightly enhanced overall microbial growth and microbial diversity compared to the control and some statistically different changes between the control and apigenin treated groups at the phylum level were observed (M. Wang et al., 2017). However, those differences may not be big enough to make a practical significance.

2.9 Conclusion

Apigenin has been shown to possess antibacterial, antiviral, antifungal, and antibacterial activities. The antimicrobial effects of apigenin is strain specific and limited by its solubility. Still, it could be a good starting point to investigate what the antimicrobial structure in apigenin is, or how to use it to complement or enhance effectiveness of antibiotics. Broth microdilution and agar dilution are the most popular methods to determine the minimal inhibitory concentrations, however, due to system

differences, there are discrepancies between the values obtained from the two methods. There seems to be no consensus on which method is better in general.

Nearly all studies looking at the antibacterial effects of apigenin used pathogenic bacteria and cultured the bacteria under aerobic conditions. Some published research looked at other similar dietary phytochemicals (Duda-Chodak, 2012; Firrman et al., 2016a, 2018). The effects of apigenin on commensal gut bacteria under anaerobic conditions is a new territory to explore.

Initial information on the interactions between apigenin and the human gut microbiota is available. It is now known that a few gut bacteria are able to degrade apigenin into smaller molecules, some of which are better absorbed than apigenin and are more biologically active. Inconclusive results have been reported on how apigenin affects the structure of the gut microbiota, but likely the effect would be mild due to the dietary intake level of apigenin and the complex ecological relations within the gut microbiota. We also know that the rate of such interaction depends on the form of apigenin and gut microbiota composition (Vollmer et al., 2018). Also because of the complexity of human gut microbiota, there are still much to investigate. Our knowledge of how apigenin affects human gut microbiota and how it in turn modulates human health is still very limited. It is yet to be discovered what other gut bacteria can metabolize apigenin and what are the metabolic products. There might be potential cross-feeding phenomenon in apigenin degradation, as the metabolites from one bacterium could be the substrate for another. The impact of dietary apigenin on human health through gut microbiota can be explored through metabolomics as the smaller molecules produced by the gut microbiota may be more relevant than the structure of the gut microbiota itself. Host characteristics

such as gender, age, and disease status may also play a role in affecting the interactions between apigenin and the gut microbiota.

Chapter 3 Apigenin Impacts the Growth of the Gut Microbiota and Alters the Gene Expression of *Enterococcus*

3.1 Introduction

Flavonoids are a type of polyphenol found in fruits, beverages, and vegetables. The average daily intake of flavonoids can reach more than 600 milligrams in a healthy diet (Augustin Scalbert & Williamson, 2000)(Pan, Chiou, Chen, & Ho, 2015). Flavonoids are categorized into six classes based on their structure: flavones, flavonols, flavanones, flavan-3-ols, anthocyanidins, and isoflavones. Flavonoids exist in a glycosylated form naturally (Gee et al., 1998). After deglycosylation, glycosides lose attached sugar molecules and become corresponding aglycones.

Processing and cooking foods rarely cause deglycosylation (Gee et al., 1998). After ingestion, foods go through digestion and absorption in our gastrointestinal tract. Due to the nature of their structure, flavonoids are resistant to acid hydrolysis in the stomach and degradation in the small intestine. Although a limited amount of absorption occurs in the small intestine, it is estimated that 90-95% of flavonoids reach the colon unaltered and unabsorbed (A Scalbert & Williamson, 2000). Human GI tract is inhabited by microorganisms. This collection of microorganisms is called the microbiota. Once the flavonoids enter the colon region, they become available to gut bacteria.

The relationship between flavonoids and gut microbiota is a two-way interaction. Human gut microbiota is involved in the metabolism and absorption of flavonoids. Upon contact with gut microbiota, flavonoids go through extensive degradation: glycosidic linkages are cleaved and glycosides quickly become aglycones, and the polymer structure

is broken down into smaller, low-molecular-weight metabolites, the heterocyclic backbone can also be cleaved (Cardona et al., 2013). On the other hand, dietary flavonoids and the phenolic metabolites produced in the degradation can in turn modify the composition of gut microbiota, alter bacterial cell physiology and change metabolic profile of the bacterial community (Cardona et al., 2013). The integrated effects from this two-way interaction can contribute to host health and may provide insight into mechanisms behind benefits of consuming flavonoids. However, study of the two sides of this interaction is rather unbalanced, most research on this subject has looked only at the degradation of flavonoids by the gut microbiota, while the effect of flavonoids on the ecology of the microbiota remains largely unclear (Cardona et al., 2013).

Six dietary flavonoids, apigenin, kaempferol, hesperitin, luteolin, myricetin, and tangeritin, were chosen for this study. They come from three classes. They have been identified in many edible plants and have been demonstrated to possess a wide range of pharmacological activities, including chemo-preventative, anti-inflammatory, and anti-oxidant capabilities. Only completed analysis and discussion from apigenin treated group will be presented here.

3.2 Technical Background

3.2.1 Apigenin

The polyphenol apigenin (Figure 3.1) is one of the major flavonoids found in many fruits, vegetables, and herbs. Celery and parsley in particular provide high levels of apigenin, approximately 19 and 215 mg per 100 g, respectively (Bhagwat et al., 2011), but it is also found in rutabagas, oranges, onions, wheat sprouts, tea, cilantro, and chamomile (W. J. Lee et al., 2008). Daily intake varies with diet and geographical location, making it difficult to calculate an average intake for a large population. One study in The Netherlands determined the average daily intake of major flavones, such as apigenin, to be about 16 mg per day (Gee et al., 1998), whereas the average daily intake of apigenin was about 1 mg in a Dutch diet (Janssen, 1997) and 1.5 ± 4.9 mg/day (range 0–30.3) in a group of female Flemish dietitians (Mullie et al., 2007).
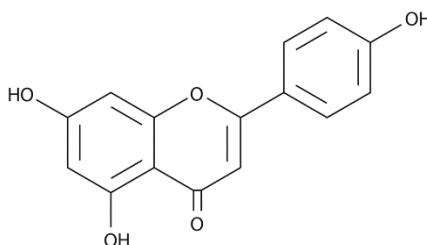
Figure 3.1 The chemical structure of apigenin (5,7-dihydroxy-2-(4-hydroxyphenyl)-4H-1-benzopyran-4-one).

Research has found that a diet rich in the flavonoid apigenin has multiple beneficial properties. Apigenin has been identified as an active ingredient in the traditional, antibacterial, herbal medicines *Scutellaria barbata D*. Don (Lamiaceae) (Sato et al., 2000), *Castanea sativa* Mill. (Fagaceae) (Basile et al., 2000a), *Portulaca oleracea*

L. (Nayaka et al., 2014), *Marrubium globosum* ssp. *Libanoticum* (Rigano et al., 2007), *Combretum erythrophyllum* (Combretaceae) (Martini et al., 2004), and *Aquilegia oxysepala* (Yu et al., 2007). These herbal medicines have been used in treating multiple symptoms including coughing (Basile et al., 2000a), digestive and biliary complaints (Rigano et al., 2007), abdominal pains and venereal diseases (Martini et al., 2004), gynopathy, irregular menstruation and metrorrhagia (Yu et al., 2007) in different cultures. Studies using eukaryotic cells have found apigenin to be protective against multiple types of cancer (W. J. Lee et al., 2008), including colon cancer, and therefore apigenin is considered a promising plant-derived chemopreventive agent (Patel et al., 2007). In eukaryotic cells, apigenin exhibits anti-inflammatory properties (Nicholas et al., 2007) and is considered an antioxidant, protecting DNA against free radicals generated by H2O2 or Fe2+ (Romanová et al., 2001).

Apigenin is a flavonoid, which is a type of plant polyphenol. It is present in food and ingested as its glycoside conjugates, primarily as apigenin-7-O-glucoside, and acylated derivatives (Shukla & Gupta, 2010b). These forms are water soluble, and their structures have a major impact on their absorption and bioavailability, with apigenin bound to β-glycosides having the best bioavailability (Patel et al., 2007). About 5–10% of total polyphenol intake, mostly monomers and dimers, are absorbed in the small intestine (Cardona et al., 2013). Since only a small portion is absorbed, it is estimated that 90–95% of total polyphenols ingested reach the colon region intact (Cardona et al., 2013). One study done on rats detected that 28.6% of ingested apigenin was excreted in feces within 72 h after oral administration (T. Chen et al., 2007a). In another study in rat, 12.0% and 9.4% of the single oral administration of radiolabeled apigenin was recovered in feces

and in the intestine, respectively, within 10 days (Angeline Gradolatto et al., 2005). These results demonstrate that a substantial amount of apigenin passes through the small intestine and is available to interact with the gut microbiota (T. Chen et al., 2007a; Angeline Gradolatto et al., 2005).

3.2.2 Gut microbiota and flavonoids

The human colon gut microbiota has been found to harbor enzymes that could degrade apigenin (Hanske et al., 2009; Schoefer et al., 2003). In germ-free mice that consumed apigenin, apigenin and its conjugates were found to be in urine and feces, while in human microbiota-associated mice, additional degradation products and their conjugates were detected (Hanske et al., 2009). Bacterial cell extracts from *Eubacterium ramulus* and *Bacteroides distasonis* were found to be capable of converting apigenin-7-glucoside to apigenin, however, this was not the case with *Escherichia coli* (Migula) (Hanske et al., 2009). Another anaerobic bacterium isolated from human feces, *Clostridium orbiscindens*, was found to degrade apigenin to 3-(4-hydroxyphenyl)propionic acid with phloretin and naringenin as intermediates (Schoefer et al., 2003). Therefore, it seems that the ability of bacteria to modify apigenin is strain specific, and not universal. In the context of the gut microbiota, degradation of apigenin and its glycosides most likely involves multiple bacteria, with complementary and overlapping functionalities.

3.2.3 Short chain fatty acids

Colonic microbial fermentation of food substrates produces short chain fatty acids (SCFA), including acetate, propionate, butyrate, isobutyrate, 2-methylpropionate, valerate, isovalerate, hexanoate (Nicholson et al., 2012). These SCFAs are readily

absorbed, and the primary products are acetate, propionate, and butyrate (Macfarlane & Macfarlane, 2003)(Wong, de Souza, Kendall, Emam, & Jenkins, 2006). The majority of SCFA in the gut are derived from bacterial breakdown of complex carbohydrates, specifically resistant starches and dietary fiber in the proximal bowel, but digestion of proteins and peptides contribute to SCFA production as food residues pass through the bowel as well (Macfarlane & Macfarlane, 2003)(Wong et al., 2006). Substrate availability in the human adult colon is about 20–60 g carbohydrates and 5–20 g protein per day (Tremaroli & Bäckhed, 2012). The rates, amounts and types of SCFA produced are determined by substrate availability, bacterial composition of the microbiota and intestinal transit time (Macfarlane & Macfarlane, 2003)(Wong et al., 2006). An example is that fermentation of dietary fructans increases when gnotobiotic mice are co-colonized with *Bacteroides thetaiotaomicron* and *Methanobrevibacter smithii* compared to *Bacteroides thetaiotaomicron* alone. *B. thetaiotaomicron* produces more acetate and formate, and *M. smithii* uses formate for methanogenesis. In the case of co-colonization, carbohydrate fermentation efficiency and energy absorption are enhanced, resulting in an increased adiposity (Samuel & Gordon, 2006). Microbiota composition differs between obese and lean population, as well as SCFAs production. One study reported that overweight or obese subjects had higher fecal acetate, propionate, butyrate, valerate and total short chain fatty acid concentrations than the lean group and that fecal total SCFA and F:B ratio are correlated (Fernandes, Su, Rahat-Rozenbloom, Wolever, & Comelli, 2014). Bacteria closely related to SCFAs are the Clostridial clusters IV and XIVa of Firmicutes, including species of *Eubacterium*, *Roseburia*, *Faecalibacterium*, and *Coprococcus* (Nicholson et al., 2012). Bacterial hydrogen metabolism also affects the

way in which SCFA are made. Inorganic electron acceptors (nitrate, sulfate) affect bacterial hydrogen metabolism, favoring the formation of more oxidized SCFA such as acetate, and leading to a decrease of more reduced fatty acids, such as butyrate (Macfarlane & Macfarlane, 2003).

SCFAs exhibit many potential biological functions. In the colon, where they are produced, they decrease colonic pH, inhibit the growth of pathogens, contribute to gut motility and wound healing (Tremaroli & Bäckhed, 2012), stimulate water and sodium absorption, provide energy to the colonic epithelial cells, as well as affecting colorectal cancer. As they are readily absorbed, they participate in cholesterol synthesis; and play a role in inflammation, obesity, insulin resistance and type 2 diabetes (Nicholson et al., 2012).

Butyrate is almost completely consumed by the colonic epithelium, and it is a major source of energy for colonocytes (Guarner & Malagelada, 2003). About 70% to 90% of butyrate is metabolized by the colonocyte (Smith et al., 2013). Butyrate promotes cell differentiation, cell-cycle arrest and apoptosis of transformed colonocytes. It also inhibits the enzyme, histone deacetylase, and decreases the transformation of primary to secondary bile acids as a result of colonic acidification. Sodium butyrate exerts an antiproliferative activity on many cells types, showing preventive effects on colon cancer and adenoma development (Smith et al., 2013). Butyrate also stimulates immunogenicity of cancer cells (Smith et al., 2013).

Acetate enters the peripheral circulation to be metabolized by peripheral tissues, particularly muscle (Guarner & Malagelada, 2003). Propionate is largely taken up by the liver and is a gluconeogenerator (Wong et al., 2006)(Guarner & Malagelada, 2003).

Acetate and propionate work antagonistically in cholesterol synthesis. Acetate has been shown to increase cholesterol synthesis, while propionate has been shown to inhibit cholesterol synthesis. Therefore, decreasing the acetate: propionate ratio may reduce serum lipids and potentially cardiovascular disease risk (Wong et al., 2006). Acetate and propionate might modulate glucose metabolism and lower glycemic responses to oral glucose or standard meal (Guarner & Malagelada, 2003).

3.3 Objectives

Previous reports have found that apigenin can also affect the growth of some bacteria, inhibiting the growth of species such as *Klebsiella pneumoniae*, *Enterobacter cloacae*, *Pseudomonas aeruginosa*, *Escherichia coli* (Migula), *Salmonella typhimurium*, *Vibrio harveyi*, *Proteus mirabilis*, and *Enterobacter aerogens*, but not, or only mildly inhibiting, *Staphylococcus aureus*, *Escherichia coli* (MG 1655), *Bacillus subtilis* or *Proteus vulgaris* (Basile et al., 2000a; Cushnie, Hamilton, & Lamb, 2003a; Nayaka et al., 2014; Ulanowska, Tkaczyk, Konopa, & Węgrzyn, 2006). Although the methods employed in determining the inhibitory effects are different, nearly all studies looking at the effect of apigenin on bacterial growth have focused on pathogenic bacteria that are cultured under aerobic conditions. While these results prove that apigenin can functionally act on the growth of some bacteria, the commensal strains of the gut microbiota were not tested.

The objective of this paper was to study how apigenin, a dietary flavone with various pharmacological activities, impacts the growth and metabolism of the gut microbiota. Initially, a reductionist model was adopted to determine if apigenin would have an effect on the growth of representative gut microbial strains *Bacteroides*

galacturonicus, *Bifidobacterium catenulatum*, *Lactobacillus rhamnosus* GG, and *Enterococcus caccae* by analyzing their anaerobic growth profiles for 24 h. Results of this initial study indicated that apigenin had a varying effect on the growth of the different types of bacteria. Based on these results, whether or not apigenin would have an effect on a gut microbiota community structure, or short chain fatty acid production, was examined by culturing a fecal inoculum in the presence of apigenin under *in vitro* anaerobic growth conditions simulating the human ascending colon region. These results indicated that apigenin may have a unique interaction with *Enterococcus*. In order to better understand this interaction at the molecular level, single molecule RNA sequencing was performed on *Enterococcus caccae*, with and without apigenin, to produce a full transcriptome profile for gene expression. Comparison of these profiles revealed the effect of apigenin on *Enterococcus caccae* at genotypic level. Taken together, these results provide a detailed analysis on the effect of apigenin on the gut microbiota community, and in particular *Enterococcus caccae*.

3.4 Methods & Materials

3.4.1 Media preparation

Difco^{TM} Lactobacilli MRS broth (Becton, Dickinson and Company, (Franklin Lakes, NJ, USA) was made by resuspending 55.00 g of MRS powder in a total volume of 1 L deionized, distilled water. The solution was heated with agitation until the powder was completely dissolved.

Bacteroides galacturonicus medium was made using the following ingredients: Sodium polygalacturonate 4.00 g, trypticase peptone 5.00 g (Becton Dickinson), yeast

extract 2.50 g, MgSO$_4$ × 7 H$_2$O 2.50 g, CaCl$_2$ × 2 H$_2$O 0.15 g, FeSO$_4$ × 7 H$_2$O 20.00 mg, (NH$_4$)$_2$SO$_4$ 1.40 g, L-cysteine 1.00 g, resazurine stock solution 1.5 mL, NaHCO$_3$ 40 mL 5% (w/v) solution, in a final volume of 1 L deionized, distilled water. Final pH was adjusted to 7.1 using 10M NaOH or 37% HCl.

Tryticase Soy Yeast Extract Medium for *Enterococcus caccae* was made with the following ingredients: 30.00 g trypticase soy broth (Becton Dickinson), and 3.00 g yeast extract, in a final volume of 1 L deionized, distilled water. Final pH was adjusted to 7.0–7.2 using 10 M NaOH or 37% HCl.

Bifidobacterium catenulatum medium was made with the following ingredients: Casein peptone tryptic digest 10.00 g (Becton Dickinson), yeast extract 5.00 g, meat extract 5.00 g, Bacto Soytone 5.00 g (Becton Dickinson), glucose 10.00 g, K$_2$HPO$_4$ 2.00 g, MgSO$_4$ × 7 H$_2$O 0.20 g, Tween80 1.00 mL, NaCl 5.00 g, cysteine-HCl × H$_2$O 0.50 g, resazurin stock solution 1 mL, salt solution 40 mL in a final volume of 1 L deionized, distilled water. Cysteine-HCl × H$_2$O was added after the medium had been boiled and cooled. Final pH was adjusted to 6.8 using 10 M NaOH or 37% HCl. Resazurin stock solution was made by dissolving 0.1 g resazurin in 100 mL deionized, distilled H$_2$O. Salt solution was made by dissolving the following in 1 L deionized, distilled water: CaCl$_2$ × 2 H$_2$O 0.25 g, MgSO$_4$ × 7 H$_2$O 0.50 g, K$_2$HPO$_4$ 1.00 g, KH$_2$PO$_4$ 1.00 g, NaHCO$_3$ 10.00 g, NaCl 2.00 g, then filter sterilized.

Basal medium (Rechner et al., 2004) was made fresh before use with the following ingredients: Peptone 2 g (Becton Dickinson), yeast extract 2 g, L-cysteine 0.5 g, NaCl 0.1 g, K$_2$HPO$_4$ 40 mg, KH$_2$PO$_4$ 40 mg, MgSO$_4$ × 7H$_2$O 10 mg, CaCl$_2$ × 2H$_2$O 6.7 mg, Tween80 2 mL, resazurine stock solution 1.5 mL (resazurine stock solution:

resazurine 0.1 g, deionized, distilled water 100 mL), with a final volume of 1 L deionized, distilled water. The final pH was adjusted to 5.8 using 10M NaOH or 37% HCl.

All broths were autoclaved at 121 °C for 30 min. Before being transferred into a Bactron anaerobic chamber to cool overnight, the broth was boiled under negative pressure using nitrogen gas for 10 min to remove any oxygen from the solution. All above anaerobic broths were made fresh every two to three weeks and stored at room temperature under oxygen free conditions after cooling in the anaerobic chamber. All ingredients were purchased from Sigma unless otherwise labeled.

3.4.2 Apigenin stock solutions

Apigenin ($\geq$98%, Item No.10010275) was purchased from Cayman Chemical Company (Ann Arbor, MI, USA) and was stored at -20 °C prior to use. For the single strain bacteria growth tests, stock solutions were made at 500 times the final concentration in an Eppendorf tube with DMSO (Sigma, St. Louis, MO, USA) before being transferred into the anaerobic chamber. Each tube was vortexed to ensure homogenization. Stock solutions were diluted with media at a 1:9 volume ratio and a final volume of 100 µL of each diluted solution was injected into a sealed Hungate tube (Chemglass, Vineland, NJ, USA) containing 5 mL of media. Final concentrations of apigenin tested were 5, 12.5, 25, 50, and 100 µg/mL.

For use in the batch culture experiment, apigenin was made into stock solutions 500 times the final concentration in DMSO to increase solubility before being transferred into the anaerobic chamber, as described above. The stock was then diluted with basal

medium at 1:1 ratio in the anaerobic chamber to avoid corrosion of pure DMSO on plastics. Final concentration for apigenin in each bioreactor was 100 µg/mL.

3.4.3 Single strain bacteria preparation

Lactobacillus rhamnosus GG (LGG, 53103)) was purchased from ATCC (Manassas, VA, USA). The other four strains were ordered from the company Deutsche SammLung von Mikroorganismen und Zellkulturen GmbH (DSMZ, Braunschweig, Germany): freeze dried ampoule of 3978, type strain; *Bacteroides galacturonicus* N6, freeze dried ampoule of 19829, type strain; *Enterococcus caccae* SS-1777(*E. caccae*), freeze dried ampoule of 16992, type strain, *Bifidobacterium catenulatum* B669 (*B. catenulatum*). Each bacterial strain was recovered from frozen aliquots in strain specific broths (described above) and grown overnight in the anaerobic chamber at 37 °C sequentially at least twice prior to use in order to ensure recovery from freezing.

3.4.4 Growth curve measurement of single strain bacteria

All steps of microbial culture were performed using a Bactron anaerobic chamber to ensure oxygen free conditions. For the single strain experiments, anaerobic broth was aliquoted into Hungate tubes prior to starting an experiment, 5 mL per tube. The tubes were sealed with a rubber septum (Chemglass) and screw cap lid (Chemglass), and stored at room temperature in the anaerobic chamber until needed. Each Hungate tube containing 5 mL pre-aliquoted broth was injected with 100 µL of the corresponding diluted stock solution of apigenin with a 1 mL needle and a 25-gauge syringe.

The cultures of bacteria grown overnight were diluted in their specific broth to 0.5 McFarland units above background. Each 5 mL Hungate tube containing the appropriate anaerobic broth and the desired concentration of apigenin was injected with 100 µL of

this culture using a 1 mL syringe and a 25-gauge needle. Each Hungate tube was briefly vortexed after injection to ensure proper distribution and the McFarland units for each culture were determined using a DEN-1 densitometer (Grant Instruments, Cambridge, UK). After adding apigenin and the bacteria, the McFarland units were determined as the time 0 read. The cultures were then placed into the anaerobic incubator set to 37 °C. Each culture was removed temporarily from the incubator at 4, 8, 12, and 24 h post inoculation, briefly vortexed, and the McFarland units measured using a densitometer. For each strain at each concentration of apigenin, six Hungate tubes of broth were used. Three tubes were designated as a broth control, consisting of broth containing the desired concentration of apigenin only. The other three tubes were designated as the experimental group, consisting of broth containing apigenin which were also inoculated with bacteria. The sets with DMSO only, with 0 μg/mL of apigenin, were designated as the control group.

3.4.5 Figures and statistics for single strain bacteria growth curve

For the growth of a single bacterial strain, the McFarland readings from each group were adjusted by subtracting the broth control read from the experimental read. The adjusted numbers were plotted in a growth curve as McFarland units above broth-only over time. For each time point, a 2-tailed, unpaired homoscedastic Student's t-test was run to determine if the difference between the control and the experimental groups was statistically significant, $p < 0.05$.

3.4.6 Inoculum preparation for batch culture experiment

In order to prepare a fecal inoculum to use in the batch culture experiment, 630 mL of basal medium was sterilely transferred into a bioreactor after the empty vessel had

been flushed sterilely with nitrogen for 10 min. The transferred medium was then sparged with nitrogen for 20 min at 37 °C to achieve anaerobic conditions. Resazurin color indicator was used to confirm anaerobic conditions. The temperature was maintained at 37 °C with constant stirring, and the pH was computer controlled, and set to 5.8 ± 0.1 using 0.5 M HCl and 0.5 M NaOH.

After the removal of oxygen from the basal media, 70 mL of defrosted human gut microbiota preparation (OpenBiome; Somerville, MA, USA) was added into the bioreactor, mixed three times with a syringe and allowed to grow for 16 h overnight. The donor of the gut microbiota preparation was a healthy female between 25–45 years of age, with a normal BMI, who had been following a non-vegetarian American diet, and had been antibiotic free for at least six months. The preparation was stored at - 80 °C and thawed at 37 °C on the day of use. After complete thawing and thorough mixing, half of the preparation was used in the first replicate of batch fermentation experiment. The other half was frozen at - 80 °C immediately after aliquoting and was used in the second replicate of the batch fermentation experiment.

3.4.7 Batch culture fermentation

Batch culture was performed in a series of bioreactors using SHIME®, Simulator of the human intestinal microbial ecosystem (Ghent University-ProDigest, Ghent, Belgium). SHIME® was originally designed to simulate the complete GI tract, but for this experiment it was set up to simulate only the ascending colon. The glass bioreactors have double jackets allowing heating water to circulate across all the vessels, maintaining 37 °C. The lids were assembled with two head space flush ports, one media sparging port, one sample port for removing medium during the experiment, acid and base ports, and a

pH probe. The entire system was sterilized before use. pH probes were sterilized with 75% ethanol and the rest were autoclaved at 121 °C for 15 min.

Prior to the start of the experiment, 540 mL of basal medium was sterilely transferred into each bioreactor using the same method as described above. Apigenin stock solution was made as described above and added to the vessel using a syringe. One vessel received DMSO only was deemed as the control group. After adding the basal medium and the apigenin, 3 mL of sample was harvested and tested for OD_{600} in a G10S UV-Vis spectrophotometer (Thermo Fisher, Waltham, MA, USA).

At time 0 h, 60 mL of inoculum prepared overnight was added into each bioreactor. Nitrogen was sparged through each vessel for 10 min to ensure anaerobic condition. Growth condition was maintained for 48 h at 37 °C with constant stirring and pH controlled at 5.8 ± 0.1. Samples were harvested at 4, 8, 12, 24, and 48 h post inoculation and OD600 values were measured. Another set of samples were centrifuged at 5000 g for 10 min and the supernatants were then filtered with 0.20 Micron PES filters (Corning, Corning, NY, USA). Both the pellets and filtered supernatant were immediately stored at - 80 °C until further analysis.

3.4.8 DNA extraction

A DNA extraction was performed on harvested samples using a bead beater-CTAB extraction method. For each sample, 500 µL CTAB buffer and 500 µL of phenol-chloroform-isoamyl alcohol (PCI) were added to a pellet spun down from the collected samples. After vortexing, all liquid was transferred to a bead-beating tube, prefilled tube kit, with Triple-Pure™ High Impact Zirconium Beads of diameter 0.1 mm (Benchmark Scientific, Sayerville, NJ, USA). Bead beating tubes were placed in a BeadBug™

microtube homogenizer (Benchmark Scientific) for two, 20-s bead-beating rounds, with a 20 s interval in between. Tubes were centrifuged at 3000 g for 5 min. 300 µL of the supernatant were transferred to a new 1.5 mL Eppendorf tube. Five hundred µL of CTAB buffer were added to the original bead-beating tube. Tubes were homogenized again with two rounds of 20-s bead-beatings and a 20 s interval in between. Tubes were centrifuged at 3000 g for 5 min. 300 µL of the supernatant were transferred and combined with the previous 300 µL. Six hundred µL of Chloroform-isoamyl alcohol (CI) were added into the 1.5 mL Genemate tube, inverted and spun for 10 s with a minifuge (VWR, Radnor, PA, USA). Five hundred µL of the upper aqueous phase were transferred to a new 2 mL Eppendorf tube. One thousand µL PEG-6000 precipitation solution were added and incubated at room temperature for 2 h. Tubes were then centrifuged for 10 min at 18,200 g. Supernatant was removed and the pellet was washed with 1 mL ice cold 70% ethanol. Tubes were centrifuged again for 10 min at 18,200 g. Supernatant was removed and the tubes were air-dried for 30 min under laminar flow in a Biosafety Cabinet (Labconco, Kansas City, MO, USA). Samples were then eluded with 75 µL of PCR grade water (Roche, Brandford, CT, USA).

CTAB buffer was made by dissolving 4.20 g K_2HPO_4 and 4.091 g NaCl in 200 mL deionized, distilled water. The solution was autoclaved at 120 °C for 30 min. The day before use, 10 g of Hexadecyltrimethylammonium bromide (CTAB) was added to the autoclaved solution and heated to 60 °C with agitation until the particles were completely dissolved. PEG-6000 precipitation solution was made by completely dissolving 300 g of polyethylene glycol 6000 (Alfa Aesar, Haverhill, MA) and 93.5 g NaCl in 1000 mL

deionized, distilled water. The solution was autoclaved at 120 °C for 30 min and later stored at room temperature.

3.4.9 DNA sequencing

Barcoded PCR primers annealing to the V1-V2 region of the 16S rRNA gene was used for library generation using the primer sequences 27F (AGAGTTTGATCCTGGCTCAG) and 338R (TGCTGCCTCCCGTAGGAGT) (McKenna et al., 2008; G. D. Wu et al., 2010). PCR reactions containing 50 nanograms of DNA and 10 pM of each primer was carried out in quadruplicate using high fidelity Accuprime Taq (Invitrogen, Carlsbad, CA, USA). The resulting 16S rDNA amplicons were purified using a 1:1 volume of Agencourt AmPure XP beads (Beckman-Coulter, Brea, CA, USA), quantified using PicoGreen, pooled in equal amounts, and sequenced on the Illumina MiSeq (San Diego, CA, USA) using 2×250 bp chemistry. Extraction blanks and DNA free water were subjected to the same amplification and purification procedure to allow for empirical assessment of environmental and reagent contamination. Positive controls, consisting of eight artificial 16S gene fragments synthesized in gene blocks and combined in known abundances, were also be included (D. Kim et al., 2017).

3.4.10 Bioinformatics processing

Sequence data was processed using QIIME version 1.9 (D. Kim et al., 2017). Read pairs were joined to form a complete V1-V2 amplicon sequence if they had a minimum overlap of 35 base pairs and maximum overlap difference of 15%. Then they were quality filtered with a minimum quality threshold of Q20. Operational Taxonomic Units (OTUs) were selected by clustering reads at 97% sequence similarity (Edgar, 2010). Taxonomic assignments were generated by comparison to the Greengenes reference

database (McDonald et al., 2012), using the consensus method implemented in QIIME. A phylogenetic tree was inferred from the out data using FastTree (Price, Dehal, & Arkin, 2010). For each time point, a 2-tailed, unpaired homoscedastic Student's t-test was run to determine if the percentage difference of a given OUT, genus, order, or phylum between the control and the experimental groups was statistically significant, $p < 0.05$.

3.4.11 RNA extraction

Enterococcus caccae cultured with DMSO only and 25 µg/mL of apigenin were harvested at 8 h post inoculation. Two samples were collected from each group, centrifuged at 5000 g for 5 min at 4 °C to pellet the cells, and the supernatant discarded. RNA from these samples was purified using an Ambion PureLink® RNA Mini Kit (Cat no. 12183018A, ThermoFisher, Waltham, MA, USA).

3.4.12 RNA sequencing: rRNA removal, cDNA Synthesis, Poly A tailing and Blocking

Ribosomal RNA depletion of the bacterial total RNA was performed using the Illumina Bacterial Ribo-Zero kit (#MRZMB126). Magnetic beads were prepared by vortexing and aliquoting 225 µL per reaction into RNase-free tubes, placing on magnetic stand, and removing supernatant. Beads were washed twice with RNase free water, followed by addition of 65 µL of Magnetic Bead Resuspension solution and 1 µL of RiboGuard RNase Inhibitor.

RNA was treated with rRNA Removal solution by combining ~1 µg of total RNA with RNase-free water, 4 µL of Ribo-zero rRNA Reaction Buffer, and 8 µL Ribo-zero Removal Solution to a total of 40 µL, and incubating at room temperature for 5 minutes.

The probe-hybridized RNA solution was then transferred to the magnetic beads and the solution was mixed, incubated at room temperature for 5 minutes and subsequently at 50°C for 5 minutes. Tubes were removed from the heat and placed on the magnetic stand. Supernatant containing 90 µL of rRNA depleted sample was removed and placed on ice until purified.

Ethanol precipitation was performed by adjusting the volume of sample to 180 µL with water, followed by addition of 18 µL of 3M sodium acetate, 2 µL of glycogen (10 mg/ml), and 600 µL of 100% ethanol. This solution was gently vortexed and placed at 80°C for overnight. After approximately 16 hours, the solution was centrifuged at 10,000 g for 30 minutes and supernatant was carefully removed. The pellet was washed twice with a 70% ethanol solution, air dried, and dissolved in RNase free water. Concentration was measured using the nanodrop.

First strand cDNA synthesis was carried out by initially incubating 7 µL of sample plus water (~100 ng RNA) at 95°C for 5 minutes and chilling on ice for 2 minutes. While on ice, 5 µL of 50 ng/µL random hexamers and 1 µL of dNTP mix were added to the RNA and the mixture was incubated at 65°C for 5 minutes. While on ice, 4 µL of 5x buffer and 1 µL of 0.1 M DTT were mixed in to the solution and incubated at 15°C for 20 minutes. Finally, 1 µL of RNase Inhibitor and 1.5 µL of Superscript III Reverse Transcriptase (Invitrogen #18080-044) were added. The following program was run on the thermal cycler: 25°C for 10 minutes, 40°C for 40 minutes, 55°C for 50 minutes, and 85°C for 5 minutes. Upon completion, 1 µL each of RNase H and RNase If was used to digest the RNA strand at 37°C for 30 minutes. EdgeBio Performa DTR Gel filtration cartridges (#42453) were used to purify the cDNA.

3' Poly A tailing was initiated by addition of 4 µL of 10x TdT buffer and 4 µL of 2.5 mM $CoCl_2$ and water to 9 µL of cDNA in a final volume of 33 µL, denatured at 95 °C for 5 minutes, and snap cooled. Then 2 µL of 1 mM dATP and 0.5 µL of 20 U/µL Terminal Transferase (NEB #M0315) were added and the solution was incubated for 10 minutes at 37 °C. Finally, 2 µL of 1 mM ddATP was spiked into the reaction and it was incubated at 37 °C for ½ hour, and 70 °C for 10 minutes to inactivate the enzyme.

3.4.13 RNA sequencing analysis

The *Enterococcous caceae* gene sequences were downloaded from the National Center for Biotechnology Information (NCBI) under the accession number NZ_KB946335. Then UCLUST (Edgar, 2010) with default settings and an identity of 90% was used to cluster all the reads from the 4 helicos samples against reference genes from the *E. caceae*. Gene expression was measured by reads per kilobase of mapped reads (RPKM (Mortazavi, Williams, McCue, Schaeffer, & Wold, 2008)), where the number of reads mapped to the gene was normalized to the length of the genes and the total number of mapped reads to all the genes. Because each sample contains two replicates, differentially expressed genes were identified using the Fisher's exact test implemented in the edgeR (Robinson, McCarthy, & Smyth, 2010) package with replicates. This method fits a negative binomial distribution by estimating the sample dispersion from the replicates and identifies differentially expressed genes that show statistical significance in fold change.

3.4.14 Short chain fatty acid quantification by GC-MS

Samples were harvested from each bioreactor at 4, 24, and 48 h post inoculation. Samples were centrifuged (Sorvall Legend XTR, ThermoFisher, Waltham, MA, USA) at

5000 rpm for 10 min. The supernatant was drawn into a syringe and sterile filtered using a 0.22 PES (Corning) syringe filter and transferred new 5 mL vials. The fluid was then stored in - 80 °C freezer until analysis.

To begin SCFA quantification, the samples were thawed at 40 °C for 30 min. The total SCFAs were extracted from the media with diethyl ether for GCMS (QP2010 Ultra, Shimadzu Scientific, Columbia, MD, USA) analysis. The GC-MS method involved injecting 1 µL of sample into the 260 °C injection port. Using a split ratio of 1:20 and a flow rate of 1.00 mL/min of helium, the sample was deposited on the Stabilwax-DA 30 m × 0.25 mm column (Restek Corporation, Bellefonte, PA, USA) which was held at 125 °C for 1 min and then ramped to 170 °C at 30 °C/min, then to 220 °C at 10 °C/min and then to 250 °C at 50 °C/min where the temperature was held for 3 min. The interface temperature between the GC and MS was held at 250 °C and the ion source temperature was 220 °C.

For each time point, a 2-tailed, unpaired homoscedastic Student's t-test was run to determine if the concentration difference between the control and the experimental groups was statistically significant, $p < 0.05$.

3.5 Results and Discussion

3.5.1 The effect of apigenin on single strain bacterial growth

The effect of apigenin on growth of *Lactobacillus rhamnosus* GG (*L. rhamnosus GG*), *Bacteroides galacturonicus (B. galacturonicus)*, *Enterococcus caccae* (*E. caccae),* *and Bifidobacterium catenulatum (B. catenulatum*) was examined by comparing the growth profile of bacteria treated with increasing concentrations of apigenin to the control group containing no apigenin. Such comparison revealed differences in growth over time, through lag, log, and stationary phases. Single strains of commensal gut bacteria were selected and cultured independently because this allowed comparison between different strains, and the effect of apigenin would not be masked by complex background activities that would be present in a community.

In general, apigenin had a minimal effect on growth of *L. rhamnosus GG.* The lowest dose of 25 µg/mL apigenin had no effect on growth at any time point (Figure 3.2 A). The only significant change in growth was observed as a slight inhibition by 50 µg/mL of apigenin at 8 and 12 hours post inoculation (Figure 3.2 A). The McFarland units (MU) for the control at 8 and 12 hours were 1.867 ± 0.058 and 9.067 ± 0.021 respectively, whereas the MU for the apigenin treated group (50 µg/mL) were 1.600 ± 0.058 and 8.400 ± 0.289, respectively. The MU readings at 8 and 12 hours with 100 µg/mL of apigenin had larger variability, thus was not statistically significant.

Growth of *B. galacturonicus* was inhibited by the presence of apigenin in a dose dependent manner at both 12 and 24 hours post inoculation (Figure 3.2 B). At 12 hours, the MU readings for *B. galacturonicus* treated with apigenin were 2.333 ± 0.058, 2.100 ± 0.100,
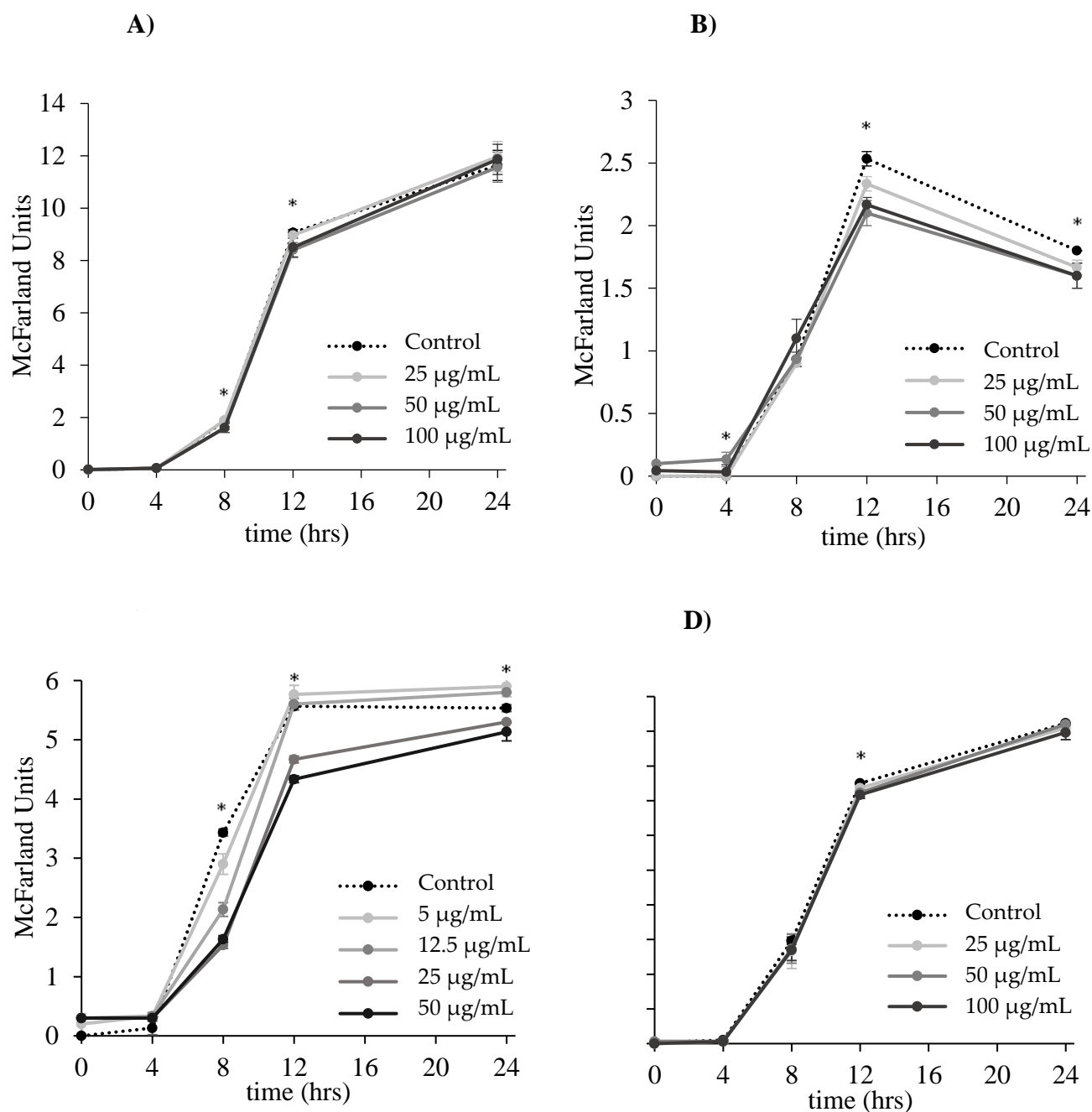
Figure 3.2 The effect of apigenin on the growth of single gut bacterial strains. Bacteria were inoculated in strain-specific media containing apigenin. McFarland unit values were determined using a densitometer at 0, 4, 8, 12, and 24 h post inoculation. The dotted line represents the control group in which bacteria grew with no apigenin added. The * mark indicates at least one experimental group was statistically significant from the control at that time point ($p < 0.05$). The 24 h growth curve with increasing concentrations of

apigenin for (**A**) *Lactobacillus rhamnosus* GG; (**B**) *Bacteroides galacturonicus*; (**C**) *Enterococcus caccae*; (**D**) *Bifidobacterium catenulatum.*

2.167 ± 0.058 for 25 µg/mL, 50 µg/mL, and 100 µg/mL apigenin respectively, compared to the control which had an MU reading of 2.533 ± 0.058. At 24 hours post inoculation these reading had decreased, with MU levels of 1.667 ± 0.058, 1.600 ± 0.026, 1.600 ± 0.026 for 25 µg/mL, 50 µg/mL, and 100 µg/mL apigenin respectively, compared to the control which had an MU reading of 1.800 ± 0.000.

The addition of apigenin had an interesting, and somewhat dichotomous effect on growth of *E. caccae* (Figure 3.2 C). At 8 hours post inoculation, there was a significant, and dose dependent inhibition of growth in which the control group had an MU of 3.433 ± 0.057 and the treatment groups had MUs of 2.900 ± 0.173, 2.133 ± 0.115, 1.533 ± 0.058, 1.633 ± 0.058 for concentrations of 5 µg/mL, 12.5 µg/mL, 25 µg/mL, and 50 µg/mL apigenin respectively (Figure 3.2 C). In fact, 25 µg/mL of apigenin was able to inhibit the growth of *E. caccae* to 52.9% of control at 8 hours and 83.8% at 12 hours. Interestingly, at 12 hours post inoculation, only the 25 and 50 µg/mL doses inhibited growth, while the 5 and 12.5 µg/mL doses had either no effect, or a slight enhancement of growth. At 24 hours post inoculation, the 25 and 50 µg/mL doses still inhibited growth, while the 5 and 12.5 µg/mL had slight enhancement of growth.

Growth of *B. catenulatum* was only minimally affected by the presence of apigenin. This can be observed in the growth curves, which illustrate no real changes in growth regardless of the dose of apigenin (Figure 3.2 D). Yet, it should be noted that at 12 hours post inoculation there was a statistically significant inhibition of growth by 50 µg/mL and 100 µg/mL of apigenin (Figure 3.2 D). However, this difference was nominal; the control

at 12 hours had an MU of 7.500 ± 0.000, and the 50 µg/mL and 25 µg/mL treated groups

had MUs of 7.233 ± 0.115 and 7.167 ± 0.100 respectively.

Apigenin is a flavonoid widely consumed in the diet, yet little is known regarding

its ability to alter the gut microbiota. Using a reductionist model, the effect of apigenin on

the growth of single gut commensal bacteria was first analyzed (Figure 3.2). These

results demonstrated that apigenin is able to influence growth of some gut microbes, but

that this effect is species dependent. The results of this experiment clearly demonstrated

that growth of *L. rhamnosus GG* and *B.catenulatum* were not truly affected by apigenin.

This is not surprising, considering that *L. rhamnosus GG* is specifically used as a

probiotic because it is unaffected by harsh conditions such as acid and bile (Koskenniemi

et al., 2011) and *B. catenulatum* is unaffected by other polyphenols, such as quercetin

(Firrman et al., 2016b). It is possible that *L. rhamnosus GG* and *B. catenulatum* may

respond to apigenin at a higher concentration, however, the low solubility of apigenin in

media limits the concentration to no more than 100 µg/mL.

Apigenin effectively inhibited growth of both *E. caccae* and *B. galacturonicus.*

Among the four strains, *B. galacturonicus* is the only Gram-negative strain. However, the

observed inhibitory effect is not considered to be determined by Gram stain status. This

was concluded based on the observation that both G+ and G- strains were inhibited (*E.*

caccae and *B. galacturonicus*, respectively), however, not all G+ strains were inhibited.

The effect of apigenin on *E. caccae* specifically revealed a divergent pattern

between the treatment groups. For the 5 and 12.5 µg/mL apigenin groups, inhibition was

observed during early exponential phase. Yet, the rate of cell density increase was similar

between the control and apigenin treated groups after 8 hours, with a potential enhancement of growth due to the addition of apigenin (Figure 3.2 C). However, for the 25 and 50 µg/mL apigenin groups, growth was significantly inhibited after 4 hours post inoculation (Figure 3.2 C). A possible reason for this difference is that the lower concentrations of apigenin were able to inhibit *E. caccae* growth prior to 12 hours just enough so that there were more nutrients left in the media, and less metabolic waste, allowing for growth to surpass the control group at 12 and 24 hours post inoculation. These results indicate that *E. caccae* is able to either degrade apigenin into byproducts that are no longer inhibitory, or overcome this inhibition through genetic regulation.

Species of the *Enterococcus* genus have been identified in human and animal gastro-intestinal (GI) tracts, as well as in the guts of insects, traditional fermented food and dairy products, and in various extra enteric environments including soil, sediments, beach sand, aquatic and terrestrial vegetation and water(rivers, streams, and creeks) (Byappanahalli, Nevers, Korajkic, Staley, & Harwood, 2012; Lebreton, Willems, & Gilmore, 2014). *Enterococcus* is considered a commensal opportunist and emerged in the 1970s as some of the leading causes of multidrug-resistant, hospital-acquired infections (Lebreton et al., 2014). Considering that *Enterococci* have been known to cause multidrug-resistant infections, and how close *E. caccae* is related to the most abundant *Enterococcus* species in human gut, the inhibitory effect of apigenin provides an opportunity for developing new antibiotics.

The results of the single strain testing revealed that the ability of apigenin to influence growth of gut commensal bacteria was individualistic. Some species were affected and others were not, and among those affected the growth profiles were modified

in different ways. Therefore, whether or not apigenin would have an effect on a complete gut community was questioned. Since a community has hundreds of species that are able to interact, a reductionist model cannot be used to determine a community effect. To examine the effect of apigenin on a complete community, bioreactors were set to simulate the ascending colon region only using a batch culture model. The ascending colon region was chosen because it is where the apigenin in foods first interacts with the gut bacteria; the other colon regions would be interacting with a lower concentration of apigenin or its degradation products as it has been passed from previous regions. It should be noted that the effects of apigenin could very possibly vary across different regions or microenvironments of the colon.

3.5.2 The effect of apigenin on a human gut microbial community *in vitro*

The effect of apigenin on a human gut bacteria community was determined using batch culture fermentation. For this experiment, two bioreactors containing basal media were inoculated with the same human fecal material and run in parallel, with one bioreactor serving as a control (no apigenin) and the other as apigenin treated (100 µg/mL). Samples were harvested at 4, 8, 12, 24 and 48 hours post inoculation to measure culture density, microbial composition, and SCFAs production.

Readings of culture density found that apigenin promoted overall growth of the microbial community *in vitro*, with a significant enhancement of growth observed for 8, 12, and, 24 hours post inoculation (Figure 3.3). The OD_{600nm} for the control group at 8, 12, 24, and 48 hours were 0.573 ± 0.003, 0.557 ± 0.002, 0.365 ± 0.002, 0.358 ± 0.031 respectively; and for the apigenin-treated group were 0.593 ± 0.002, 0.603 ± 0.004, 0.412 ± 0.002, and 0.395 ± 0.001 respectively. Analysis of the alpha diversity for both

communities using the Shannon index found that microbial diversity increased with fermenting time, with the apigenin treated group having a statistically significantly higher Shannon index at 8, 24, and 48 hours post inoculation (Figure 3.3).
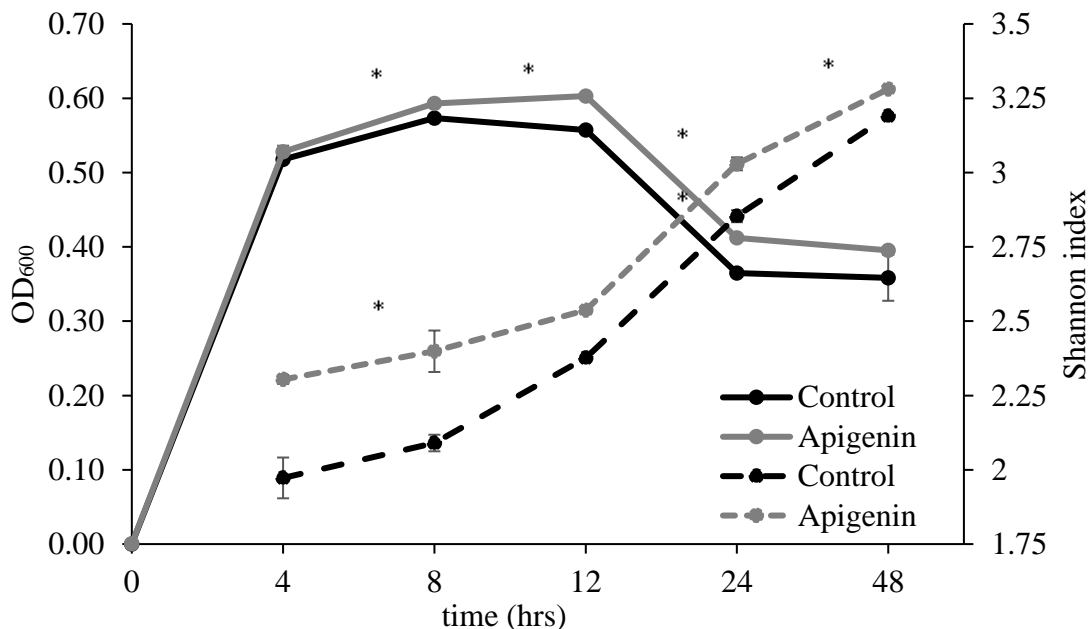
Figure 3.3 Culture density and alpha diversity over time. The culture density for the control and apigenin (100 µg/mL) treated groups based on the OD_{600} reading over time is represented using the left axis. The alpha diversity based on the Shannon index is represented using the right axis. The * mark indicates at least one experimental group was statistically significant from the control at that time point ($p < 0.05$).

The community composition over time for each group was determined using 16S rRNA sequencing to produce a community profile based on OTUs that were more than 0.1% of the community for at least one time point (Figure 3.4). In both the control and apigenin treated groups, there were fewer dominant OTUs at 4 and 8 hours post inoculation. *Clostridium* species and Peptostreptococcaceae family were the most abundant taxa present, totaling 76.67% and 55.57% for control and apigenin groups

respectively. By 24 and 48 hours post inoculation the microbiota culture composition resembled that of a more of a mature community. At 48 hours, 20 and 16 more OTUs grew to more than 0.1% compared to 4 hours post inoculation for control and apigenin treated group, and the initially dominant *Clostridium* species and Peptostreptococcaceae family were reduced to 1.9% and 2.07% in control and apigenin groups respectively. At 48 hours, genera *Enterococcus*, *Lactococcus*, and *Bacteroides* were the most abundant OTUs for both the control and apigenin groups, contributing to 53.62% of the control community and 55.55% the apigenin-treated community.
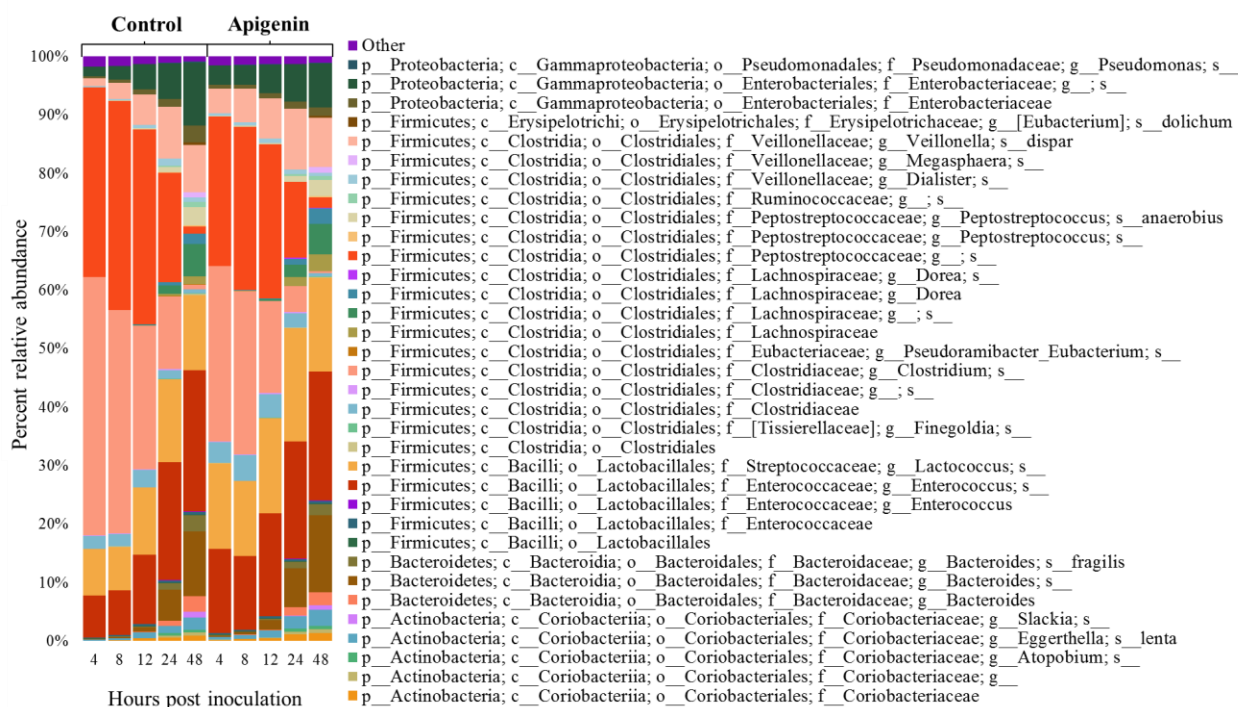
Figure 3.4 Gut bacterial community composition changes over 48 hours. Community composition was determined based on relative abundance. OTUs that were more than 0.1% in at least one time point are presented. Diversity of the community increased over time for both the control and apigenin (100 µg/mL) treated cultures.

The addition of apigenin differentially effected the four major phyla in human gut microbiota, which are Firmicutes, Bacteroidetes, Proteobacteria, and Actinobacteria (Figure 3.5). For both the control and apigenin treated groups, the percentage of Firmicutes decreased with time, but remained the most dominant phylum over the course of 48 hours (Figure 3.5 A). At 4 hours, Firmicutes consisted of $97.36 \pm 0.06\%$ of the control community and $94.74 \pm 0.25\%$ in the apigenin-treated community. At this early time point, apigenin significantly reduced the percentage of Firmicutes, but had no effect at any other time points.

For Bacteroidetes, the percentages in both groups increased with time (Figure 3.5 B). At 4 hours and 12 hours post inoculation, apigenin significantly increased the percentage of Bacteroidetes, from $0.14 \pm 0.01\%$ and $1.07 \pm 0.12\%$ in the control to $0.28 \pm 0.03\%$ and $1.95 \pm 0.10\%$ in apigenin-treated groups (Figure 3.5 B). As a result of the decrease in Firmicutes percentage and the increase in Bacteroidetes percentage, the Firmicutes/Bacteroidetes ratio was decreased with apigenin treatment. At 4 hours, the F/B ratio was 695.4 in control and 338.5 in apigenin-treated group; the 12 hours, the ratios were 86.0 and 46.2, respectively.

The percent of phylum Proteobacteria increased over time for both the control and apigenin treated groups (Figure 3.5 C). The addition of apigenin resulted in an enhancement of Proteobacteria at 4 hours post inoculation, with the control having $1.99 \pm 0.05\%$ Proteobacteria and the apigenin-treatment group having $4.00 \pm 0.10\%$ Proteobacteria (Figure 3.5 C). At 8, 12, and 24 hours post inoculation the amount of Proteobacteria was similar for both groups. Conversely, at 48 hours, Proteobacteria

percentage in apigenin-treated group, 9.14 ± 1.09%, was significantly lower than the
control, 13.95 ± 0.93% (Figure 3.5 C).

A)

B)

C)

D)
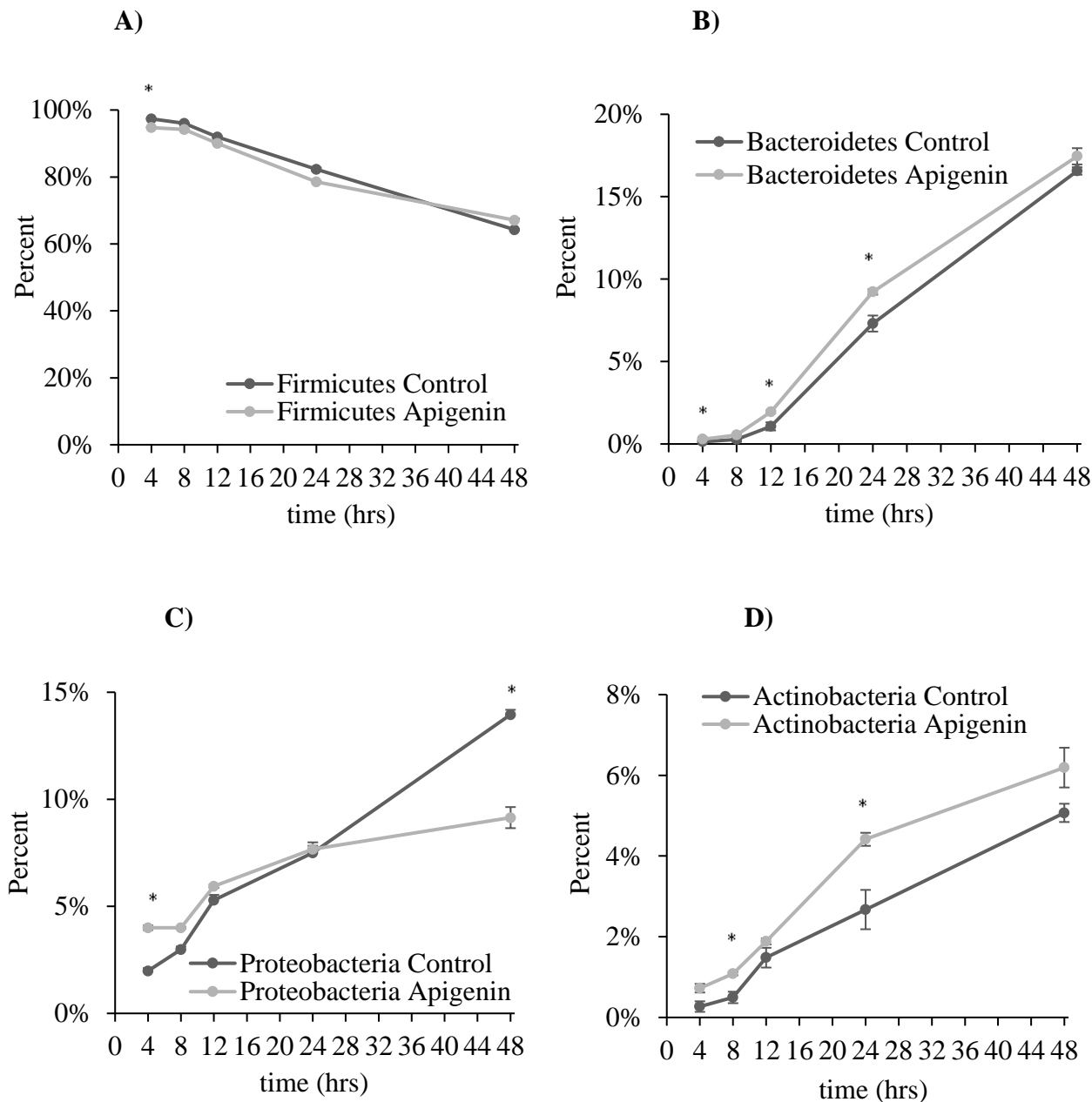
Figure 3.5 Phylum composition in control and apigenin groups. Bioreactors
containing basal media were inoculated with human fecal homogenate, and
samples harvested at 4, 8, 12, 24, 48 h post inoculation. Microbial composition
of each sample was determined by 16S rRNA sequencing, and percentages of

the four major phyla for both the control and apigenin-treated groups were compared at each time point. The * mark indicates the experimental group was statistically significant from the control at that time point ($p < 0.05$). (**A**) Firmicutes; (**B**) Bacteroidetes; (**C**) Proteobacteria; (**D**) Actinobacteria.

For both the control and apigenin groups, the percentage of phylum Actinobacteria increased over time (Figure 3.5 D). At 8 hours and 24 hours, Actinobacteria percentages were significantly higher in the apigenin treated group (Figure 3.5 D). The control group had $0.50 \pm 0.14\%$ and $2.67 \pm 0.49\%$ of Actinobacteria for the two time points respectively, while apigenin-treated group had $1.09 \pm 0.04\%$ and $4.41 \pm 0.16\%$. While the difference between the groups at 48 hours post inoculation is not statistically significant, there is a definite enhancement in growth for the apigenin treated group, $5.07 \pm 0.23\%$ in control group and $6.19 \pm 0.49\%$ in apigenin-treated group.

Results from testing the effect of apigenin on *E. caccae* under axenic conditions demonstrated that the addition of apigenin influenced strain growth (Figure 3.2 C). Therefore, whether or not this occurred in the community setting was evaluated. Since 16S rRNA sequencing of the V1-V2 region is unable to distinguish between species of *Enterococcus*, the effect of apigenin on the Lactobacillales order and *Enterococcus* genus was examined (Figure 3.6). Analysis of the growth trend revealed that proportions of order Lactobacillales were significantly enhanced at 8, 12, and 24 hours post inoculation for the apigenin treated group (Figure 3.6 A). Looking at the genus level, the addition of apigenin resulted in a significant increase for *Enterococcus* at 4 and 8 hours post inoculation (Figure 3.6 B). At 4 hours, the control proportion was $7.18 \pm 1.61\%$ for *Enterococcus* genus, and the apigenin-treated proportion was $14.38 \pm 0.05\%$. At 8 hours,

the proportions were 7.61 ± 1.04% for control; 12.61 ± 0.83% for the apigenin treated group.

The growth trends were almost identical between the Lactobacillales order and *Enterococcus* genus. This is because the Lactobacillales order was largely composed of *Enterococcus* in this experiment. This effect of apigenin was different from when *E. caccae* was cultured alone. While the single strain growth was inhibited by apigenin effectively, apigenin helped *Enterococcus* genus grew to a higher percentage in a community setting.

A)

B)
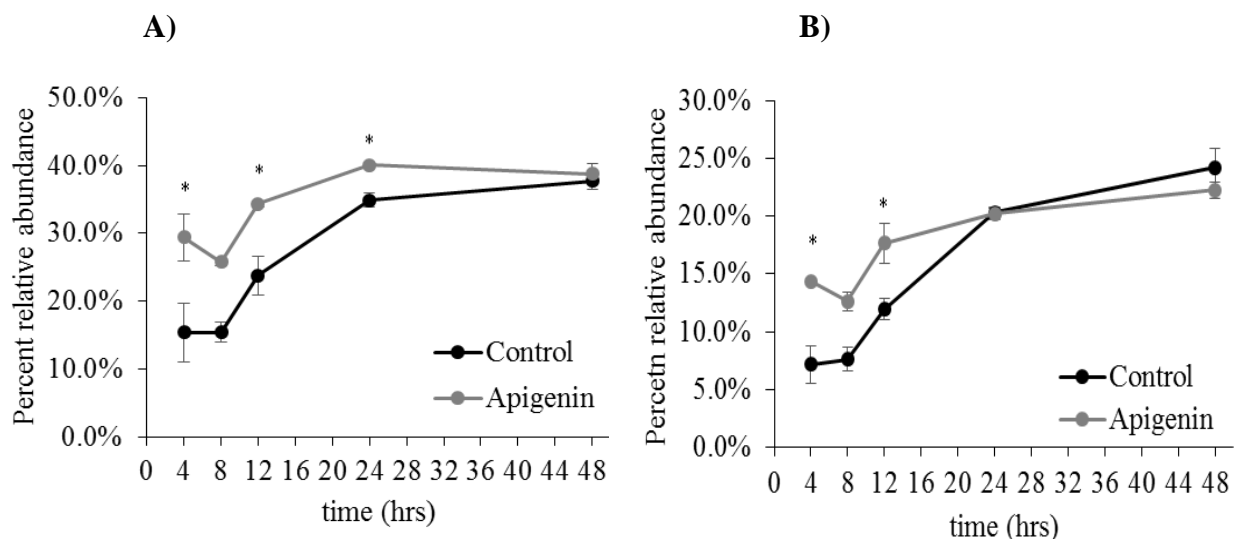
Figure 3.6 Apigenin enhances growth of *Enterococcus* in a community. The percent relative abundance for order Lactobacillus and genus *Enterococcus* was determined based on 16S rRNA sequencing. The * mark indicates that the experimental group (100 µg/mL apigenin) was statistically significant from the control at that time point, according to a 2-tailed, Student's *t*-test ($p < 0.05$). (A) Lactobacillales order; (B) *Enterococcus* genus.

In batch culture of the microbiota, it was observed that in both the control and apigenin groups, microbial diversity increased with time, represented by an increasing number of OTUs of higher abundance over the course of 48 hours. This is not surprising since after inoculation the bacteria were able to enter into an exponential growth phase,

allowing the community to mature and species that were below detection level in the fecal sample to develop. The amount of growth for both the control and apigenin was similar, with enhancement by apigenin, as determined by optical density and alpha diversity using the Shannon index. These observations are important because they indicate that apigenin was not able to affect the ability for the gut microbiota to grow and mature.

There were some statistically different changes between the control and apigenin treated groups at the phylum level. However, other than for Proteobacteria at 48 hours, those differences were not as drastic as the inhibitory effect observed on *Enterococcus caccae*. This is not entirely surprising since phyla contain a large number of OTUs which make them more robust than a single strain and the effect of apigenin may occur at an intra-phylum level. However, the small changes noted did result in a change to the Firmicutes/Bacteroidetes ratio, which was lower in the apigenin treated microbiota. Since a higher Firmicutes/Bacteroidetes ratio has been associated with obesity (Ley, Turnbaugh, Klein, & Gordon, 2006), lowering the F/B ratio indicates that apigenin has the potential to shift the microbiota away from the one associated with obesity.

Interestingly, apigenin did not inhibit growth of *Enterococci* in the community. On the contrary, growth of genus *Enterococci* was enhanced with apigenin at 4 and 8 hours post inoculation. This is opposite to what happened when apigenin was tested with *E. caccae* alone. One possible explanation is that apigenin was degraded or taken up by other species in the community, and as a result *Enterococci* were exposed to less apigenin. Other bacteria more susceptible to apigenin were inhibited and thus opened up more niche for *Enterococci*. It is also possible that the *Enterococci* in this fecal sample

were not sensitive to apigenin. Determination if this is the case would require identification of the *Enterococcus* species and examination of their growth in the presence of apigenin.

3.5.3 Apigenin influences short chain fatty acids production in a community setting

The most abundant short chain fatty acids detected in the culture were acetate, propionate, and butyrate. Other SCFAs, including 2-methyl-propionate, 2-methyl-butyrate, pentanoic acid, 2-methyl-pentanoic acid, 4-methyl-pentanoic acid were also detected, but only at low or trace concentrations. The amount of acetate, propionate, and butyrate produced all increased with time for both the control and apigenin treated groups (Figure 3.7). Acetate production was significantly higher in the samples containing apigenin at 48 hours post inoculation, 17.546 ± 0.597 mmol/L, compared to the control, 15.539 ± 0.772 mmol/L (Figure 3.7 A). Propionate concentration was significantly higher in the apigenin-treated samples at 24 and 48 hours post inoculation (Figure 3.7 B). The control group concentrations were 3.221 ± 0.055 mmol/L, 3.579 ± 0.120 mmol/L, and apigenin-treated concentrations were 3.413 ± 0.108 mmol/L and 4.171 ± 0.121 mmol/L for 24 and 48 hours, respectively. Apigenin-treated gut microbiota produced more butyrate for all three time points tested, with the control, in the order of 4, 24, and 48 hours, producing 0.318 ± 0.015 mmol/L, 0.658 ± 0.014 mmol/L, and 1.097 ± 0.045 mmol/L and apigenin-treated group producing 0.381 ± 0.021 mmol/L, 0.803 ± 0.022 mmol/L, and 1.287 ± 0.027 mmol/L of butyrate (Figure 3.7 C).

The gut microbiota community not only resides in the colon, but is a functional part of the environment. Fermentation of substrates by the gut microbiota produces short

chain fatty acids, which are absorbed by the colonic cells. Therefore, the effect of apigenin on the gut microbiota may not be limited to community structure, but could also result in changes to the production of SCFAs. In this case, it is not just about an effect on who is there, but what they are doing, and what they are producing.

The results of this study are in accordance with previous findings in that the most abundant SCFAs produced were acetate, followed by propionate, and butyrate (Dorsten et al., 2012). In this study, the production of all three major SCFAs were enhanced by apigenin. SCFAs participate in the regulation of mucin secretion. Goblet-cell-specific Mucin 2 (MUC-2) is the most prominent mucin secreted by intestinal epithelial cells. It has been demonstrated that butyrate and propionate both induce an increase in MUC-2 mRNA levels (Burger-van Paassen et al., 2009). It is known that prostaglandins (PG) enhance mucin secretion and are key players in mucoprotection. SCFA can differentially regulate PG production, promoting the production of more potent Prostaglandin E1 (PGE1) over PGE2, thus stimulating MUC-2 expression in intestinal epithelial cells (Willemsen, Koetsier, van Deventer, & van Tol, 2003). Apigenin, by enhancing the production of butyrate and propionate, may stimulate mucin production. This would be protective for gut lining and mucin-dependent gut bacteria, e.g. *Akkermansia muciniphila*. This effect is yet to be tested *in vivo*.

Butyrate, which was significantly higher in apigenin-treated samples at all three time points, is almost completely consumed by the colonic epithelium, and it is a major source of energy for colonocytes (Guarner & Malagelada, 2003). It has been estimated that 70% to 90% of butyrate is metabolized by the colonocyte (Smith et al., 2013), where it promotes cell differentiation, cell-cycle arrest and apoptosis of transformed

colonocytes. Sodium butyrate exerts an antiproliferative activity on many cells types, showing preventive effects on colon cancer and adenoma development (Smith et al., 2013). Butyrate also stimulates immunogenicity of cancer cells (Smith et al., 2013). A diet high in apigenin may be contributing to health by increasing the amount of butyrate produced by the microbiota.
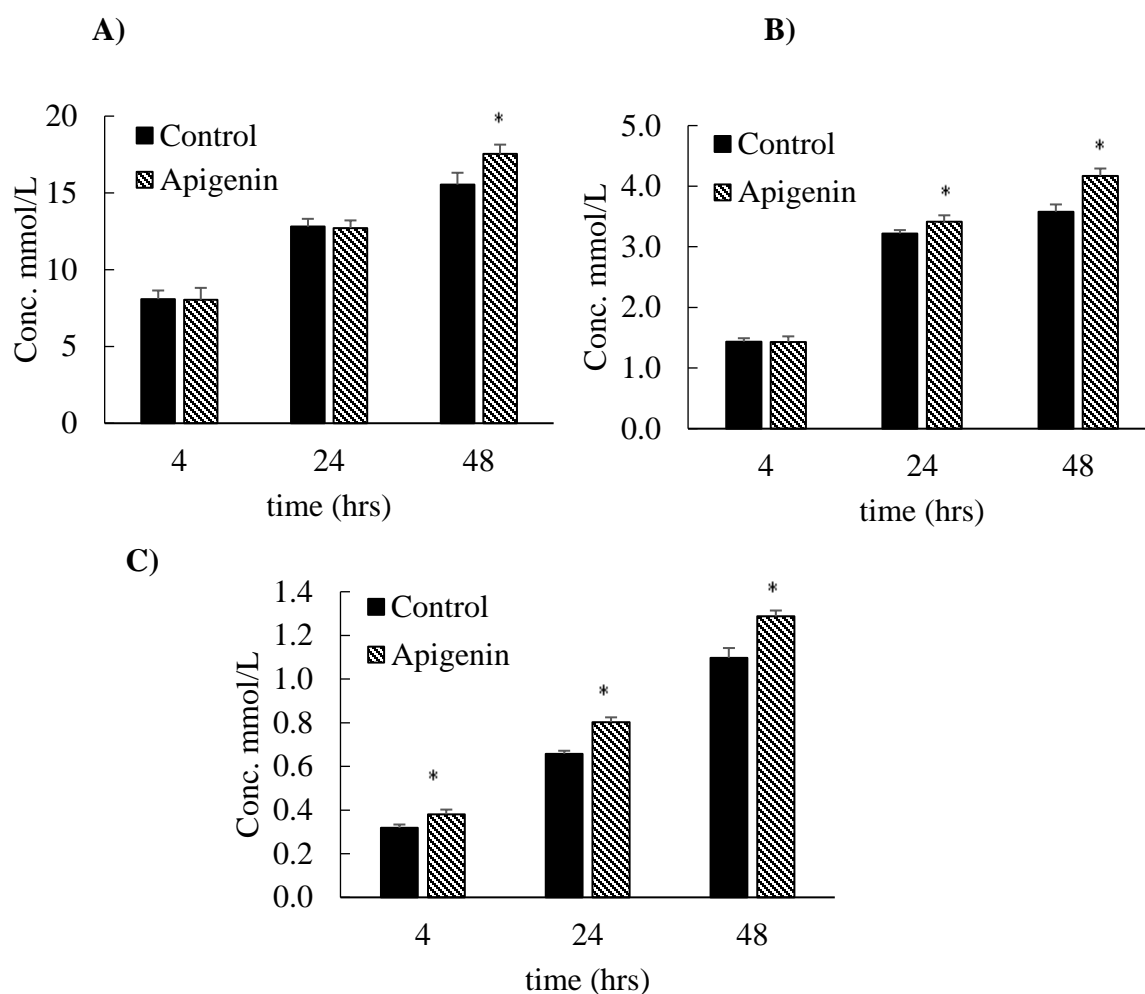
Figure 3.7 Major short chain fatty acid production in control and apigenin group at 4, 24, and 48 hours post inoculation. mmol/L is millimolar per liter. Amounts of the short chain fatty acids acetate, propionate, and butyrate were measured using a GC/MS. The * mark indicates the experimental group was statistically significant from the control at that time point ($p < 0.05$). (**A**) Acetate; (**B**) Propionate; (**C**) Butyrate.

3.5.4 Apigenin induces changes to the genetic expression profile of *Enterococcus caccae*

The gene expression profiles of *E. caccae* grown with and without the presence of 25 µg/mL apigenin were assembled and compared. A total of 764 individual genes were identified; 141 of these had expression levels significantly different from the control ($p < 0.05$). Among the 141 genes, 97 were up-regulated, and 44 were down-regulated. For brevity, only genes with a greater than 1.5-fold change were considered in the discussion. Setting the threshold at a fold change of 1.5, 60 genes were up-regulated and 30 were down regulated. Their fold change, number, and brief description of function can be found in Table 3.1. Of the up-regulated genes, 17 were hypothetical proteins, and 18 of the down-regulated genes were hypothetical proteins.

The results of single strain and community studies demonstrated that apigenin had a divergent effect on growth of *E. caccae,* inhibiting growth in a single strain setting and enhancing growth in a community setting. In order to elucidate how apigenin and *E. caccae* may interact at the genetic level, resulting in a change in growth status, RNA expression was evaluated. Among all the 764 genes identified, 290 were described as hypothetical proteins, including the most upregulated and the most downregulated ones, thus their identities and functions are unknown. Setting the threshold at a fold change of 1.5 times among those are not hypothetical proteins, 43 genes were up-regulated and 12 were down regulated.

Table 3.2 RNA expression profiles

    A) *Enterococcus caccae* genes upregulated in response to apigenin

Gene	Description	Fold ▲	Description of function
UC7_RS11 115	hypothetical protein	7.0	Unknown
UC7_RS15590	hypothetical protein	5.9	Unknown
UC7_RS11110	glycerophosphoryl diester phosphodiesterase	2.9	Phospholipid metabolism
UC7_RS11955	tyrosine recombinase XerC	2.8	DNA dimer resolution
UC7_RS11545	hypothetical protein	2.7	Unknown
UC7_RS16330	acyl carrier protein	2.6	Fatty acid biosynthesis
UC7_RS16340	MarR family transcriptional regulator	2.5	Stress response
UC7_RS13900	glycine cleavage system protein H	2.4	Glycine degradation
UC7_RS11945	ATP-dependent protease ATPase subunit HslU	2.3	Protein quality control
UC7_RS16335	3-oxoacyl-ACP synthase III	2.3	Type II fatty acid synthesis
UC7_RS13125	hypothetical protein	2.2	Unknown
UC7_RS11950	ATP-dependent protease subunit HslV	2.2	Protein quality control
UC7_RS14535	chaperonin	2.2	Protein folding
UC7_RS11290	phosphoglycerol transferase	2.1	Lipoteichoic acid biosynthesis
UC7_RS15845	hypothetical protein	2.1	Unknown
UC7_RS13905	spx/MgsR family transcriptional regulator	2.1	Stress response
UC7_RS11940	GTP-sensing transcriptional pleiotropic repressor CodY	2.1	Transcriptional regulation
UC7_RS13910	cell cycle protein FtsW	2.1	Cell division
UC7_RS12345	CBS domain-containing protein	2.1	metabolism
UC7_RS13095	N5-carboxyaminoimidazole ribonucleotide mutase	2.1	Purine synthesis
UC7_RS15130	acyltransferase	2.1	Cell wall peptidoglycan synthesis
UC7_RS12825	phenazine biosynthesis protein PhzF	2.0	Phenazine biosynthesis
UC7_RS13565	hypothetical protein	2.0	Unknown
UC7_RS15200	excinuclease ABC subunit B	2.0	DNA repair
UC7_RS13270	neutral zinc metallopeptidase	1.9	Extracellular metabolism
UC7_RS14315	magnesium-transporting ATPase	1.9	Magnesium transportation

UC7_RS13560	diaminopimelate dehydrogenase	1.8	Lysine biosynthesis
UC7_RS11285	hypothetical protein	1.8	Unknown
UC7_RS12620	undecaprenyl pyrophosphate synthase	1.8	Peptidoglycan biosynthesis
UC7_RS15260	hypothetical protein	1.7	Unknown
UC7_RS16325	enoyl-ACP reductase II	1.7	Fatty acid biosynthesis
UC7_RS12170	hypothetical protein	1.7	Unknown
UC7_RS11935	aldolase 1 epimerase LacX	1.7	Carbohydrate metabolism
UC7_RS11340	cold-shock protein	1.7	Stress response
UC7_RS15195	UvrABC system protein A	1.7	DNA repair
UC7_RS13110	hypothetical protein	1.7	Unknown
UC7_RS13815	hypothetical protein	1.7	Unknown
UC7_RS13990	hypothetical protein	1.7	Unknown
UC7_RS13115	N-acetylmuramoyl-L-alanine amidase	1.7	Cell separation
UC7_RS16345	hypothetical protein	1.7	Unknown
UC7_RS12340	2_3_4_5-tetrahydropyridine-2_6-dicarboxylate N-acetyltransferase	1.7	Lysine biosynthesis
UC7_RS13365	NrdR family transcriptional regulator	1.7	DNA synthesis and repair
UC7_RS14530	molecular chaperone GroEL	1.7	Protein folding
UC7_RS14300	GNAT family acetyltransferase	1.6	Transcriptional regulation
UC7_RS16190	penicillin-binding protein 2B	1.6	Cell wall synthesis
UC7_RS13090	phosphoribosylaminoimidazole carboxylase ATPase subunit	1.6	Purine and pyrimidine ribonucleotide biosynthesis
UC7_RS15255	hypothetical protein	1.6	Unknown
UC7_RS13120	hypothetical protein	1.6	Unknown
UC7_RS13335	chaperone protein ClpB	1.6	Protein folding
UC7_RS14685	DNA-binding response regulator	1.6	Transcriptional regulation
UC7_RS11985	S26 family signal peptidase	1.6	Protein maturation
UC7_RS12190	elongation factor Tu	1.6	Protein synthesis
UC7_RS15900	D-alanine--poly(phosphoribitol) ligase subunit 2	1.6	Cell wall/membrane metabolism
UC7_RS16320	malonyl CoA-ACP transacylase	1.5	Fatty acid and

Gene	Description	Fold	Description of function
			polyketide synthesis
UC7_RS13160	penicillin-binding protein	1.5	Cell wall synthesis
UC7_RS15715	50S ribosomal protein L10	1.5	Protein translation
UC7_RS15600	phosphocarrier protein HPr	1.5	Carbohydrate phosphorylation
UC7_RS13265	hypothetical protein	1.5	Unknown
UC7_RS15250	hypothetical protein	1.5	Unknown
UC7_RS12410	signal peptidase I	1.5	Protein maturation

B) *Enterococcus caccae* **genes downregulated in response to apigenin**

Gene	Description	Fold ▼	Description of function
UC7_RS16160	hypothetical protein	2.4	Unknown
UC7_RS16570	hypothetical protein	2.2	Unknown
UC7_RS14780	hypothetical protein	2.0	Unknown
UC7_RS13490	Lsa family ABC-F type ribosomal protection protein	2.0	Ribosomal protection
UC7_RS14785	hypothetical protein	1.9	Unknown
UC7_RS11535	hypothetical protein	1.9	Unknown
UC7_RS15700	general stress protein GlsB	1.9	Stress response
UC7_RS14775	hypothetical protein	1.8	Unknown
UC7_RS15645	hypothetical protein	1.8	Unknown
UC7_RS16575	hypothetical protein	1.8	Unknown
UC7_RS16225	WxL domain surface protein	1.7	Surface protein binding
UC7_RS13920	hypothetical protein	1.7	Unknown
UC7_RS15555	MocD family protein	1.7	Catabolism of mannopine
UC7_RS13450	hypothetical protein	1.7	Unknown
UC7_RS16165	hypothetical protein	1.7	Unknown
UC7_RS13865	SufS family cysteine desulfurase	1.6	Fe-S protein biosynthesis
UC7_RS13860	iron-sulfur cluster assembly scaffold protein	1.6	Iron-sulfur cluster formation
UC7_RS14795	deoxycytidine triphosphate deaminase	1.6	Nucleotide

			metabolism
UC7_RS13855	FeS assembly protein SufB	1.6	Iron-sulfur cluster formation
UC7_RS13455	hypothetical protein	1.6	Unknown
UC7_RS11040	hypothetical protein	1.6	Unknown
UC7_RS12130	hypothetical protein	1.6	Unknown
UC7_RS13465	hypothetical protein	1.5	Unknown
UC7_RS12125	hypothetical protein	1.5	Unknown
UC7_RS14270	ATP synthase subunit C	1.5	ATP synthesis
UC7_RS16230	hypothetical protein	1.5	Unknown
UC7_RS16220	hypothetical protein	1.5	Unknown
UC7_RS11885	cardiolipin synthase	1.5	Phospholipid biosynthesis
UC7_RS14805	ATP-grasp domain-containing protein	1.5	metabolism
UC7_RS15830	thioredoxin-disulfide reductase	1.5	Redox regulation

It has been previously indicated that apigenin effects nucleic acids, type II fatty acids and D-alanine:D-alanine ligase in bacteria. Related genes could be upregulated by apigenin as the bacteria compensates for the inhibition exerted on the end products, e.g. enzymes. The results of the gene expression for this experiment demonstrated that Tyrosine recombinase XerC, which is responsible for chromosome dimer resolution during DNA replication (Le Bourgeois et al., 2007), was upregulated by 2.8 fold; excinuclease ABC subunit B, NrdR family transcriptional regulator, and UvrABC system protein A, which are involved in DNA repair were upregulated by 2, 1.7, and 1.7 fold respectively (Crowley et al., 2006; Grinberg et al., 2006; Sambir et al., 2011; Truglio, Croteau, Houten, & Kisker, 2006), which supports the findings that nucleic acids are one of the targets of apigenin. Results also showed that genes for an acyl carrier protein and 3-oxoacyl-ACP synthase III, two components involved in Type II fatty acid synthesis (Bi, Zhu, Wang, & Cronan, 2014; Okuo Nishida, Akihiko Kawaguchi, 1986; Park et al., 2016;

Taguchi et al., 2006), were upregulated by 2.6 and 2.3 fold, respectively, which indicates that apigenin alters the type II fatty acid biosynthesis. D-alanine-poly(phosphoribitol) ligase subunit 2, which participates in cell wall and cell membrane metabolism (Kumariya, Sood, Rajput, & Garsa, 2015) was also regulated by 1.6 fold, which could be a result of the inhibitory effect of apigenin targeting D-alanine ligase.

Cell membrane/wall synthesis is likely a major target of apigenin in *Enterococcus caccae*. The aforementioned targets: D-alanine:D-alanine ligase and Type II fatty acid synthetic pathway are both involved in cell membrane/wall synthesis. The Type II fatty acid synthetic pathway is the principal route for membrane phospholipid acyl chains production (White, Zheng, Zhang, & Rock, 2005). Several other genes related to cell membrane or cell wall metabolism were also found to be upregulated, including: glycerophosphoryl diester phosphodiesterase, upregulated 2.9 fold, is involved in phospholipid metabolism (Arias et al., 2011; Tran et al., 2013); phosphoglyceroltransferase, upregulated 2.1 fold, is involved in lipoteichoic acid biosynthesis (Mohamadzadeh et al., n.d.; Morath, Stadelmaier, Geyer, Schmidt, & Hartung, 2002); acyltransferase, upregulated 2.1 fold, is involved in cell wall peptidoglycan synthesis (Goffin & Ghuysen, 2002; Lyu et al., 2014); penicillin-binding protein and penicillin-binding protein 2B, upregulated 1.5 and 1.6 folds respectively, are involved in cell wall synthesis (Daniel, Harry, & Errington, 2000; Sauvage et al., 2008); N-acetylmuramoyl-L-alanine amidase, upregulated 1.7 fold, participates in cell separation (Layec, Decaris, & Leblond-Bourget, 2008); undecaprenyl pyrophosphate synthase, upregulated 1.8 fold, participates in peptidoglycan biosynthesis (Y. H. Lee & Helmann, 2013).

Several general stress response genes were also found to be upregulated due to apigenin treatment. The MarR family transcriptional regulator was upregulated 2.5 fold compared to the control (Perera & Grove, 2010), spx/MgsR family transcriptional regulator was upregulated by 2.1 fold (Zuber, 2009, 2013), and the cold-shock protein was upregulated by 1.7 fold. This is a sign that the cell was turning on responsive protection mechanism to overcome damages caused by apigenin. As a result, fewer energy and resources were used for cell growth and division.

Protein chaperones facilitate correct folding of proteins and help with refold of misfolded ones (Y. E. Kim, Hipp, Bracher, Hayer-Hartl, & Ulrich Hartl, 2013). Several protein chaperones and elements involved in controlling protein quality were upregulated: chaperonin by 2.2 folds; molecular chaperone GroEL by 1.7 folds, chaperone protein ClpB by 1.6 folds, and ATP-dependent protease ATPase subunit HslU by 2.3 folds, and ATP-dependent protease subunit HslV by 2.2 folds. The latter two are involved in protein quality control (Alcántara & Zúniga, 2012). This could indicate an increase in misfolding of proteins in the cell as a result of apigenin treatment.

Among the genes that were down regulated, two participate in Iron-sulfur cluster formation and one is an element in Iron-sulfur biosynthesis: FeS assembly protein SufB by 1.6 folds; iron-sulfur cluster assembly scaffold protein by 1.6 folds; and SufS family cysteine desulfurase by 1.6 folds. Fe-S cluster is regulated under homeostatic control, meaning that it is regulated according to cellular requirement (Andrews, Robinson, & Rodríguez-Quiñones, 2003). A decrease in expression of related genes could be the result or the cause of slower growth rate in apigenin-treated cells, as iron is an important metal ion for bacterial growth.

3.6 Conclusion

The results of testing using a reductionist model demonstrated that this effect varied with species. Among the species tested, the most effective inhibition by apigenin was observed on *E. caccae*. Analysis of RNA expression indicated that apigenin affects *E. caccae* cell wall/membrane synthesis and increased the incidence of protein misfolding. *B. galacturonicus* growth was also inhibited by apigenin, while *B. catenulatum* and *L. rhamnosus* GG were not affected. In the bacterial community cultured in vitro, apigenin promoted overall growth and diversity, lowered Firmicutes to Bacteroidetes ratio, and promoted production of short chain fatty acids, including butyrate, which is associated with health. Taken together, these observations may explain the health benefits of an apigenin-rich diet. In order to look at apigenin on the microbiota at different regions, a continuous feeding model could be established using SHIME® to look at the effect on all colon regions. The continuous feeding model will also provide the possibility to look at the long-term effect of apigenin.

Chapter 4 Degradation of Apigenin by *Enterococcus caccae* and Human Gut

Microbiota *in Vitro*

4.1 Introduction

Flavonoids are a class of plant secondary metabolite that are widely distributed in nature (Sharma et al., 2017). The chemical structure of flavonoids is characterized with a 15-carbon skeleton consisted of two phenyl rings (A and B) and a heterocyclic ring (C), abbreviated as C6-C3-C6 ("flavonoid (CHEBI:47916)," n.d.). The variation of the heterocyclic ring (C) structure leads to further categorization of flavonoids into subclasses. Formation of oligomers or polymers, as well as substitution, contribute to the complexity of each flavonoid (Vollmer et al., 2018). The natural forms of flavonoids are often conjugated with sugars or organic acids (Vollmer et al., 2018).

Apigenin, 4',5,7-trihydroxy-flavone (Figure 4.1), is a monomeric flavonoid found in the diet (Bhagwat et al., 2011). Dried parsley spices and chamomile tea are the two dietary items with the highest concentration of apigenin (Bhagwat et al., 2011; Mckay & Blumberg, 2006). Nevertheless, apigenin is widely distributed in the plant kingdom as it is found in many vegetables, herbs, and fruits (Bhagwat et al., 2015). Other than parsley and chamomile, the list includes vine spinach, celery seed spices, green celery heart, Chinese celery, dried oregano, red and white sorghum, rutabagas, oranges, kumquats, onions, wheat sprouts, tea, and cilantro (Bhagwat et al., 2011; W. J. Lee et al., 2008; Patel et al., 2007). Just as flavonoids exist in plants naturally as glycosides, apigenin is no exception. Apigenin is typically found in a glycosylated form, with the tricyclic core

structure linked to a sugar moiety through hydroxyl groups forming O-glycosides, or directly to carbon forming C-glycosides (Tang et al., 2017a). The common apigenin glycosides are apigenin-7-apioglucoside (apiin), apigenin-7-O-glucoside (apigentrin), apigenin-8-C-glucoside (vitexin), apigenin-6-C-glucoside (isovitexin), apigenin-7-O-neohesperidoside (rhoifolin), and apigenin 6-C-glucoside 8-C-arabinoside (schaftoside) (Lefort & Blay, 2013; Simirgiotis et al., 2013; Tang et al., 2017a). Several studies have been conducted to estimate the daily intake of apigenin and the results varied over a wide range (0.13 – 4.23 mg/day) because quantification of dietary intake for apigenin is difficult and various with diet, demographic, and age, depending on time and geographical location (Cao et al., 2017; Gates et al., 2007; Michael G.L. Hertog et al., 1993; Janssen, 1997; Mullie et al., 2007; Somerset & Johannot, 2008; Vogiatzoglou et al., 2015; L. Wang et al., 2009).
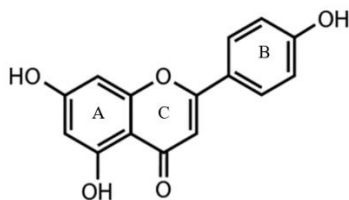
Figure 4.1 Chemical structure of apigenin, 4',5,7-trihydroxy-flavone.

Flavonoids have been widely studied and are associated with many therapeutic potentials (Agrawal, 2011). A search for herbal medicines with apigenin identified as the or one of the active ingredients resulted in a long list including *Scutellaria barbata* (Sato et al., 2000), *Castanea sativa* (Basile et al., 2000a), *Portulaca oleracea* (Nayaka et al., 2014), *Marrubium globosum* (Rigano et al., 2007), *Combretum erythrophyllum* (Martini et al., 2004), *Aquilegia oxysepala* (Yu et al., 2007) and propolis (Koru et al.,

2007). Chamomile tea, which is extremely rich in apigenin, has been used as a folk medicine for relieving indigestion or gastritis (Patel et al., 2007).

Research show that pure apigenin compound possesses pharmacological activities, including being anti-inflammatory, anti-carcinogenic, anti-toxicant (Ali, Rahul, Falaq Naz, et al., 2017), anti-oxidant (Sichel et al., 1991; Tripoli et al., 2007), chemo preventative (Lefort & Blay, 2013; Patel et al., 2007; Shukla & Gupta, 2010a), and is helpful for rheumatoid arthritis, autoimmune disorders, Parkinson's disease, Alzheimer's disease, and various type of cancers (Ali, Rahul, Falaq Naz, et al., 2017).

Ingested dietary apigenin, mostly in its glycosylated forms, then goes through digestion and absorption in the gastrointestinal tract. For total polyphenols, it is estimated that 90–95% of total ingested amount reach the colon region intact (Cardona et al., 2013). Detection and quantification of fecal apigenin in rats after oral intake of apigenin proves that the colon region is exposed to dietary apigenin (T. Chen, Li, Lu, Jiang, & Su, 2007b; Angeline Gradolatto et al., 2005). Therefore, interactions between dietary apigenin and the gut microbiota are very likely with ingested apigenin being broken down by the collection of different microbes into compounds of different structure and bioactivity and the gut microbiota composition and function being modified by apigenin and its breakdown products. Nevertheless, information is limited on the interactions.

It is not uncommon to study the antibacterial effects of flavonoids (Cushnie & Lamb, 2005). Based on the review on the antimicrobial effects of apigenin (manuscript in preparation), most investigation was interested in the inhibitory effects on pathogens under aerobic growth conditions. Previous research done in our lab has looked into the

effects of apigenin on several commensal gut bacteria strains (M. Wang et al., 2017). Our results showed that apigenin effectively inhibited the growth of *Enterococcus caccae* SS-1777 during the early exponential phase as well as altering the strain's genetical expression by up-regulating genes involved in DNA repair, stress response, cell wall synthesis, and protein folding. As the inhibitory effect on the rate of binary fission was not observed later in the growth curve, one hypothesis was that the single dose of apigenin administered at the time of inoculation degraded with time and microbial growth.

Gut bacteria have various levels of capability to degrade apigenin or the glycosides. While *Bacteroides distasonis* was found to be capable of converting apigenin-7-glucoside to apigenin, *E. coli* was not found to be capable of the same function (Hanske et al., 2009). *Eubacterium ramulus* is able to degrade of number of flavonoids including apigenin, quercetin, naringenin, daidzein and genistein (Blaut & Clavel, 2007) because it possesses a phloretin-hydrolase which can cleave the phloretin C-C bond (Schoefer et al., 2004). Another anaerobic bacterium isolated from human feces, *Clostridium orbiscindens*, degrades apigenin to phloretin and naringenin as intermediates, then to 3-(4-hydroxyphenyl)propionic acid as a final product (Schoefer et al., 2003). This degradation product 3-(4-Hydroxyphenyl)propionic acid, also known as desaminotyrosine (DAT) can offer protective effects during influenza (Steed et al., 2017). This is an example of gut microbiota mediated bioactivity in which the end product after microbial fermentation offers additional functions than the original dietary compound. Given the diversity of gut bacterial composition, it is reasonable to think that there are other gut bacteria that can degrade apigenin and may produce similar or different forms of intermediates and end

products. These intermediates and end products may be utilized by other bacteria as well. Researches that looked into metabolism of apigenin by a gut microbial community will be presented in the following paragraphs.

Those studies either used an *in vitro* model or a rat model. Results from *in vitro* models showed that human fecal suspensions converted apigenin *O*-glycoside (Hanske et al., 2009) or apigenin *C*-glycoside (Vollmer et al., 2018) to several smaller compounds, including those mentioned earlier. In the degradation of apigenin glycosides, apigenin and naringenin were transiently formed as intermediate metabolites and the end products include 3-(4-hydroxyphenyl)propionic acid (4HPPA), 3-hydroxyphenyl-acetic acid, 3-phenylpropionic acid, and trace amounts of 3-(3-hydroxyphenyl)propionic acid (3HPPA) (Hanske et al., 2009). Although samples from different donors would ferment at different rates and yield different metabolite profiles (Vollmer et al., 2018). Without the presence of bacteria, apigenin glycosides concentration remained largely stable (Hanske et al., 2009).

In the animal model, germ-free rats excreted apigenin glycoside, apigenin, and luteolin after oral intake of the glycoside (Hanske et al., 2009). In human-microbiota associated (HMA) rats, additional metabolites were detected in the urine: naringenin, phloretin, 3-(3,4-dihydroxyphenyl)propionic acid (3,4DHPPA), 4HPPA, 4-hydroxycinnamic acid (4HCA), and 3HPPA (Hanske et al., 2009). The glycoside, apigenin, and luteolin were found in germ-free rats' feces, but only at a much lower level in the fecal material of HMA rats (Hanske et al., 2009). These findings clearly indicate that the gut microbiota is involved in conversion of dietary apigenin.

The other side of the interactions between dietary apigenin and the gut microbiota that apigenin and its metabolites can also modify the structure and function of gut microbiota considering its effects on bacteria. Evidence for this modification capability is limited (Cardona et al., 2013). In one of the studies mentioned above, the intestinal microbiota composition of rats was determined by PCR-coupled denaturing gradient gel electrophoresis (Hanske et al., 2009). The similarity of the compositions over the course of the experiment, including before and after apigenin glycoside treatment, was from 63.3 to 75.8%, which were not significantly different between time points (Hanske et al., 2009). Previous *in vitro* experiment done by our lab showed by 16S rRNA gene sequencing that genus *Enterococcus* was enhanced in a community by apigenin although it was effectively inhibited by apigenin on its own (M. Wang et al., 2017). There were some changes between the control and apigenin treated groups at the phylum level, but the differences were too small to confidently assign any practical significance to them (M. Wang et al., 2017).

The main objective of this research is to study the degradation of apigenin in *Enterococcus caccae* SS-1777 (*E. caccae*) culture and in human gut microbiota culture *in vitro* to help explain why the inhibitory effect of apigenin on *Enterococcus caccae* was limited to mid-exponential phase and to also provide insight on the degradation of apigenin aglycone by a gut bacteria community. At the same time, the microbial community structure and its function as in short chain fatty acids production will be evaluated by 16S rRNA gene sequencing and GC-MS, respectively.

4.2 Materials and Methods

4.2.1 Degradation of apigenin in *E. caccae* culture

Single strain bacteria preparation

Freeze-dried ampoule of 19829, Type strain; *Enterococcus caccae* SS-1777 (*E. caccae*) was ordered from the company Deutsche SammLung von Mikroorganismen und Zellkulturen GmbH (DSMZ) in Germany. The bacterial strain was recovered from frozen aliquots in Tryticase Soy Yeast Extract medium and grown overnight in the anaerobic chamber at 37 °C sequentially at least twice prior to use in order to ensure recovery from freezing.

Media preparation

Tryticase soy yeast extract medium for *Enterococcus caccae* was made by adding 30.00 g Trypticase Soy broth (Becton Dickinson), and 3.00 g Yeast extract, in deionized distilled water with a final volume of 1 L. Final pH was adjusted to 7.0 - 7.2 using 10M NaOH or 37% HCl. The broth was autoclaved at 120 °C for 30 minutes. Before being transferred into a Bactron anaerobic chamber to cool overnight, the broth was boiled under negative pressure using nitrogen gas for 10 minutes to remove any oxygen from the solution. Then the bottles were stored at room temperature in the anaerobic chamber until needed.

Apigenin stock solution

Apigenin (≥ 98%, Item No.10010275) was purchased from Cayman Chemical Company (Michigan, USA) and was stored at -20 °C prior to use. For the single strain bacteria growth tests, stock solutions were made at 500 times the final concentration in a 2mL microcentrifuge tube (GeneMate, BioExpress, VWR International, Radnor,

Pennsylvania) with DMSO before being transferred into the anaerobic chamber. Each tube was vortexed to ensure homogenization. A volume of 1 mL of apigenin stock solution was injected into a sealed Hungate bottle (Chemglass, Vineland, New Jersey, USA) containing the correct amount of trypticase soy yeast extract medium. For the volumes of the medium, please see the next section. The concentration of apigenin tested was 100 µg/mL. All chemicals were purchased from Sigma unless otherwise labeled.

Growth curve measurement of *E. caccae* and sample collection

Each replicate of the *E. caccae* growth curve measurement and degradation of apigenin experiment included three groups: the apigenin broth control, consisting of broth containing the 100 µg/mL of apigenin only; the bacteria control group containing DMSO inoculated with bacteria, with 0 µg/mL of apigenin; the experimental group, consisting of broth containing apigenin which were also inoculated with bacteria. Tryticase soy yeast extract broth was aliquoted into glass Hungate bottles (Chemglass, Vineland, New Jersey, USA) prior to starting an experiment, 489 mLs per bottle for apigenin-bacteria group and bacteria only group, 499 mLs per bottle for apigenin only group. Each of the Hungate bottles for the experimental group and broth control group pre-aliquoted broth was added with 1 mL of the corresponding diluted stock solution of apigenin using a pipettor. The bottle for bacterial control group was added with 1 mL of pure DMSO. The bottles were sealed with a rubber stopper (Chemglass, Vineland, New Jersey, USA) and aluminum seal (Chemglass, Vineland, New Jersey, USA).

The cultures of *E. caccae* grown overnight were diluted in trypticase soy yeast extract broth to 0.5 McFarland units over the broth only read with the help of a DEN-1

densitometer (Grant Instruments, Cambridge, UK). Each 490 mLs Hungate bottle containing the appropriate anaerobic broth and the desired concentration of apigenin was injected with 10 mLs of this culture using a 10 mL syringe and a 25-gauge needle. Each Hungate bottle was shaken after injection to ensure proper distribution. These initial steps of single strain microbial culture were performed using a Bactron anaerobic chamber to ensure oxygen free conditions. The bottles were then taken out of the anaerobic chamber. Because of the stoppers and seals, the anaerobic conditions inside the bottles were maintained. Immediately, 5 mLs of samples were taken from each bottle and the bottles were then placed into an incubator set to 37° C, shaken at 200 rpm. Duplicates of OD_{600} reading were taken with a G10S UV-Vis spectrophotometer (Thermo Fisher, Waltham, MA, USA) as the time 0 read. Each OD_{600} was taken with 1 mL of sample. The rest of the samples, 3 mLs from each group, were spun for 10 min at 5000 g. The supernatants were filtered and frozen individually at - 80 °C immediately for future UPLC-MS analysis. Each bottle was removed temporarily from the incubator at 0.5, 1, 2, 4, 6, 8, 12, and 24 hours post inoculation. The samples at each time point were processed in the same way as the time 0 samples. This experiment was replicated for three times.

Figures and statistics for single strain bacteria growth curve:

For the growth of *E. caccae*, the OD_{600} readings from each group were adjusted by first subtracting the broth control read from the experimental read, and then adjusted by subtracting the time 0 value from all time points. The adjusted numbers were plotted in a growth curve as OD_{600} over time. For each time point a 2-tailed, Student's t-test was

run to determine if the difference between the control and the experimental groups was statistically significant.

4.2.2 Degradation of apigenin by human gut microbiota culture *in vitro*

Apigenin stock solution

For use in the batch culture experiment, apigenin was made into stock solutions 1200 times the final concentration in DMSO. The stock was then diluted with sterile deionized distilled water at 1:1 ratio to avoid corrosion of pure DMSO on plastics. After application of apigenin treatment, the concentration for apigenin in the treated bioreactor was 100 µg/mL, while the controlled group received the same amount of DMSO and sterile deionized distilled water mixture.

Media preparation

Modified MShime® medium was made from ProDigest MShime® feed (ProDigest, Gent, Belgium) with additional 2 g/L type II mucin from porcine stomach and 0.5 g/L of Oxgall dehydrated fresh bile (Difco™ Becton Dickinson). The ProDigest MShime medium itself contains 1.2 g of arabinogalactan, 2 g of pectin, 0.5 g of xylan, 0.4 g of glucose, 3 g of yeast extract, 1 g of special peptone, 2 g of mucin, 0.5 g of L-cysteine-HCl, and 4 g of starch, per liter. The broth was autoclaved at 120 °C for 30 minutes. The modified MShime® medium was made fresh before use.

Inoculum preparation

Frozen human gut microbiota preparation (OpenBiome; MA, USA) was obtained commercially. The donor of the gut microbiota preparation was a healthy female between 25 - 45 years of age, with a normal BMI, who had been following a non-vegetarian

American diet, and had been antibiotic free for at least six months. The preparation was stored at - 80 °C and thawed at 37 °C on the day of use.

Bioreactors set-up

Batch culture was performed with a BioFlo® 320 next-generation bioprocess control station using two bioreactors (Eppendorf, Hamburg, Germany) in parallel. Bioreactors were made of glass with double jackets allowing heating water to circulate across all the vessels, maintaining 37 °C. The lids for the bioreactors had a propeller, individual ports for acid, base, waste, sample, nitrogen, pH probe, dissolved oxygen probe, temperature probe, as well as an exhaust. Except for the pH probes, the entire system was sterilized before use by autoclaving at 121 °C for 15 min with 250 mLs Dulbecco's Phosphate-Buffered Saline with calcium and magnesium (DPBS) (Thermo Fisher, Waltham, MA, USA) to protect the dissolved oxygen probe. The pumps for transferring liquids as well as the pH and dissolved oxygen probes were calibrated following instructions from the manual provided by the manufacturer of the bioprocess control station.

Prior to the start of the experiment, the autoclaved bioreactors were allowed to cool and flushed with 1 L autoclaved sterile deionized distilled water twice using sterile transfer technique to remove the DPBS. Then, 1200 mLs of autoclaved modified MShime® medium was sterilely transferred into each bioreactor. The pH probes were sterilized with 75 % ethanol and assembled onto the lids after air-dry.

The *in vitro* culture system was initialized to bring the media to a condition that was suitable for inoculation. At the end of the initialization, the system temperature was

maintained at 37 °C; the dissolved oxygen level was kept at 0 % by constant flushing of nitrogen; the propeller was mixing the bacterial culture media at 100 RPM; and the pH was kept within 5.7 – 5.9. After the system has reached this status, it was monitored for another 1 - 2 hours to ensure that the pH and anaerobic conditions were properly maintained.

Inoculation and stabilization of the human gut microbiota using bioreactors

Before inoculate the system, 60 mLs of media was removed from each bioreactor so that the total volume stayed the same. Then 60 mLs of the defrosted human gut microbiota preparation was added into each bioreactor at the same time. Each bioreactor content was mixed for three times with a syringe to ensure that all of the inoculum went into the media and had been properly mixed. Then the newly inoculated cultures were allowed to grow for 12 - 16 hours overnight with proper temperature, pH, agitation, and anaerobic condition, but without feeding or waste removal.

After the first 12 - 16 hours, samples were collected from each bioreactor and the feeding cycles were turned on. Every 8 hours, the waste pump would run at 80 mL/min for 3 mins to return the volume in each bioreactor to 1000 mL, then 200 mL of fresh modified MShime® medium was added. The total retention time is 48 hours. The system was allowed to stabilized for three weeks and samples were collected once per day right before the next feeding cycle started. For two days before applying treatments, more samples were collected from each unit at 0.5, 1, 2, 4, 6, 7.25 hours after completion of feeding. Samples were centrifuged at $5000 \times g$ for 10 min and the supernatants were then

filtered with 0.20 Micron PES filters (Corning, Corning, NY, USA). Both the pellets and filtered supernatant were immediately stored at - 80 °C until further analysis.

Experiments were replicated twice and in the third replication, the stabilization period was shortened to 13 days as evidence from the first two replicates showed that the bacterial communities were stable after 10 days.

Apigenin treatment and sample collection

Apigenin treatment was applied on the 22nd day after inoculation. Before applying the treatment, samples were harvested from reach bioreactors to serve as the baseline. Additional samples were also harvested to for non-microbial control groups as well as for calibration curve development. Apigenin stock solution was made as described above. As soon as the feeding process was completed, the stock solution was added to the vessel using a syringe, and was mixed for five times to ensure complete delivery. The vessel that received DMSO only was deemed as the control group. The two vessels were no longer fed fresh modified MShime® medium after the treatment nor was waste removed from the vessels, but all other conditions remained the same as in the stabilization period. Immediately after administration of the treatments, a set of samples deemed as time 0 were collected in different aliquots that summed to 26 mLs and were centrifuged at 5000 $\times$ g for 10 min and the supernatants were then filtered with 0.20 Micron PES filters (Corning, Corning, NY, USA). Both the pellets and filtered supernatant were immediately stored at - 80 °C until further analysis. Additional sets of samples were collected with the same method at time 0.25, 0.5, 1, 2, 4, 6, 8, 12 and 24 hours post treatment.

Two additional sets of degradation experiments that did not involve bacteria were conducted. Freshly made autoclaved modified MShime® medium were aliquoted into two Hungate bottles, therefore deemed as the media groups. The final volume was 50 mL per bottle and apigenin stock solution was added to one of the bottles to achieve the final concentration of 100 µg/mL. The other one received the same amount of DMSO to serve as the control bottle. The second set used the additional samples collected before the bioreactors were treated with apigenin. Those additional samples were centrifuged at $5000 \times g$ for 10 min and the supernatants were then filtered with 0.20 Micron PES filters upon harvest and then they were immediately stored at - 80 °C. On the day of the degradation experiment, samples were taken out and thawed at 37 °C. Then, the samples were sterile filtered into two Hungate bottles, 50 mL each. These bottles were designated as the supernatant group. One of them received apigenin stock solution to achieve the final concentration of 100 µg/mL. The other one received the same amount of DMSO to serve as the control bottle. Samples were collected for these two non-microbial groups at time 0, 0.25, 0.5, 1, 2, 4, 6, 8, 12 and 24 hours post treatment and filtered with 0.20 Micron PES filters then immediately stored at - 80 °C until further analysis. Samples taken from unfermented media groups were not filtered because the matrix would not go through the filter without clogging it.

DNA extraction and sequencing

DNA extraction and sequencing was performed following the method described in Chapter 3 (M. Wang et al., 2017).

Short Chain Fatty Acid Quantification by GC-MS

The method used to quantify GC-MS was described in Chapter 3 (M. Wang et al., 2017).

4.2.3 Detection of apigenin and its degradation products

Chemicals

Apigenin (A, 4',5,7-trihydroxy flavone), naringenin (N, 4',5,7-trihydroxy flavanone), luteolin (L, 3',4',5,7-tetrahydroxyflavone), phloretin (P, 2,6-dihydroxy-4-methoxyacetophenone), Phloroglucinol (PG, 1,3,5-trihydroxybenzene) were purchased from Cayman Chemical Company (Ann Arbor, MI, USA). Eriodictyol (E, 3',4',5,7-tetrahydroxy flavanone), 3-(3-hydroxyphenyl)propionic acid (3HPPA), 3-(4-hydroxyphenyl)propionic acid (4HPPA, desaminotyrosine), 3,4-dihydroxyphenylacetic acid (3,4DHPA), 4-hydroxyphenylacetic acid (4HPAA), 4-hydroxycinnamic acid (4HCA), 4-hydroxybenzoic acid (4HBA), 3,4-dihydroxyhydrocinnamic acid (3,4DHCA) were purchased from Sigma-Aldrich (St. Louis, MO, USA). All chemicals were stored at −20 °C prior to use. The names, abbreviations, and structures of the potential microbial metabolites from apigenin included in this study are shown in Table 4.1 and Figure 4.2.

Table 4.1 Names and abbreviations of the compounds targeted in UPLC-ESI-MS/MS analysis.

Index	Compound	Synonym	Abbr.
1	Apigenin	4',5,7-Trihydroxy flavone	A
2	Naringenin	4',5,7-Trihydroxy flavanone	N
3	Luteolin	3',4',5,7-Tetrahydroxyflavone	L
4	Phloretin	2,6-Dihydroxy-4-methoxyacetophenone	P
5	Phloroglucinol	1,3,5-Trihydroxybenzene/1,3,5-benzenetriol	PG
6	Eriodictyol	3',4',5,7-Tetrahydroxy flavanone	E
7	3-(3-Hydroxyphenyl)propionic acid	3-(3-Hydroxyphenyl)propanoic acid	3HPPA
8	3-(4-Hydroxyphenyl)propionic acid	Phloretic acid / desaminotyrosine	4HPPA

9	3,4-Dihydroxyphenylacetic acid	Homoprotocatechuic acid	3,4DHPA
10	4-Hydroxyphenylacetic acid	p-Hydroxyphenylacetic acid	4HPAA
11	4-Hydroxycinnamic acid	p-Coumaric acid	4HCA
12	4-Hydroxybenzoic acid	p-Hydroxybenzoic acid	4HBA
13	3,4-Dihydroxyhydrocinnamic acid	Hydrocaffeic acid	3,4DHCA

a) Naringenin   b) Luteolin   c) Phloretin   d) Phloroglucinol

e) Eriodictyol   f) 3-(3-Hydroxyphenyl) propionic acid   g) 3-(4-Hydroxyphenyl) propionic acid   h) 3,4-Dihydroxyphenylacetic acid

i) 4-Hydroxyphenylacetic acid   j) 4-Hydroxycinnamic acid   k) 4-Hydroxybenzoic acid   l) 3,4-Dihydroxyhydrocinnamic acid
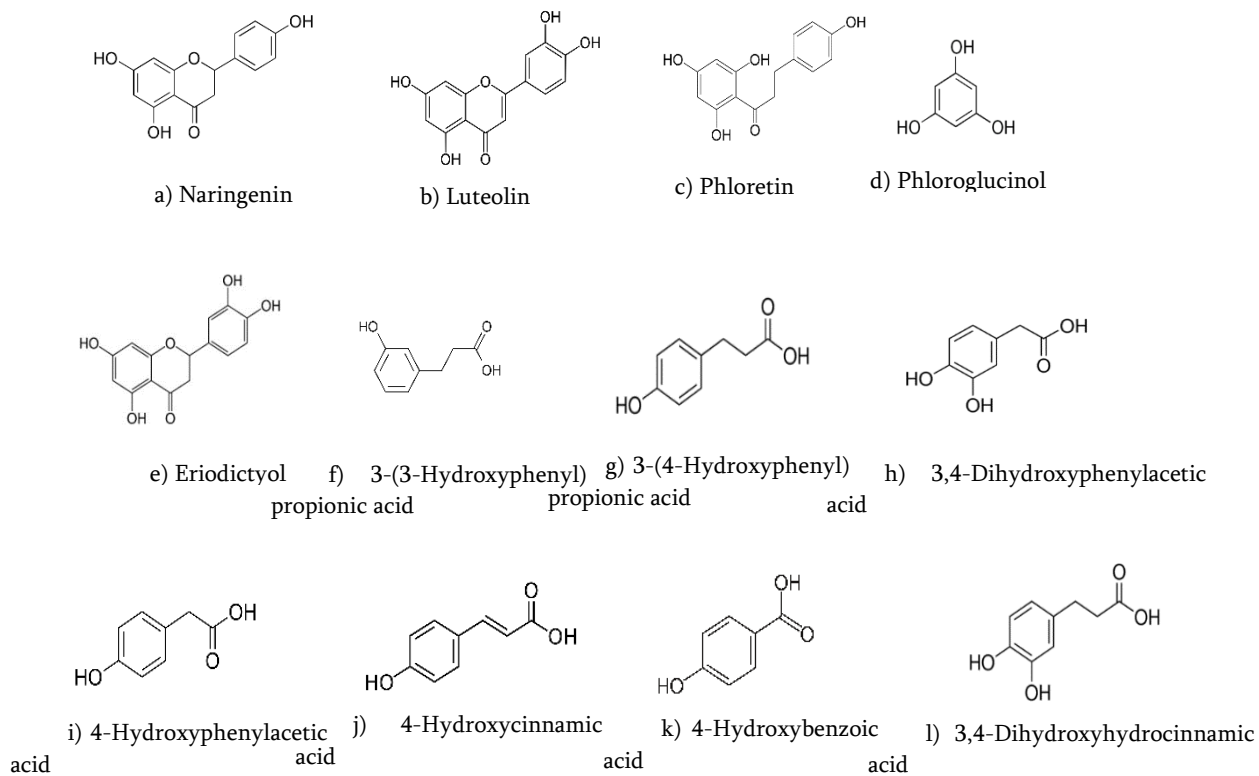
Figure 4.2 Structures of the potential apigenin metabolites targeted in UPLC-ESI-MS/MS analysis.

Sample extraction

The extraction method is modified based on the method described by Zhao, *et al* (2016) (M. Zhao et al., 2016). Previously mentioned supernatant samples stored at -80 °C were thawed at 40 °C for the minimal amount of time. Fully thawed sample was mixed thoroughly by inverting the tube five times. Then, 120 µLs of sample were transferred to a 2 mL microcentrifuge tube (GeneMate, BioExpress, VWR International, Radnor,

Pennsylvania). For extraction, 390 µLs of methanol were added to the same tube and thoroughly mixed on the hula hoop (Lifetechnologies, ThermoFisher Scientific, Waltham, MA) for 2 minutes. After mixing, mixtures were centrifuged (Centrisart, Sartorius, Bohemia, NY) at 3000 rpm for 3 minutes. The whole organic liquid phase was transferred to a new centrifuge tube and evaporated to dryness without heat in a Savant SPD111V SpeedVac^{TM} Concentrator (ThermoFisher Scientific, Waltham, MA, USA) connected to a Savant UVS400 universal vacuum system (ThermoFisher Scientific, Waltham, MA, USA) for two hours. Samples were reconstituted with 120 µLs of methanol and vortexed for 10 seconds or longer as needed. Finally, samples were centrifuged at 14,000 rpm for 10 minutes and 80 µLs of the supernatant were transferred into a vial for UPLC-ESI-MS/MS analysis.

UPLC-ESI-MS/MS analysis

UPLC analysis of apigenin and its potential degradation products were performed using a Waters Acquity H class. Separation of the target compounds were carried out using BEH C18 column (2.1 mm x 100 mm, 1.7 µm) equipped with a Waters VanGuard precolumn (Waters, Milford, MA, USA). The chromatography separation temperature was 35 °C. The mobile phase used was 0.1 % formic acid in water (A) and 0.1 % formic acid in acetonitrile (B). The gradient elution used is as such: 10 % B progressed linearly over 0 - 5.5 min to 75 % B, then over 5.5 - 6 min progressed to 100 % and 100 % B was maintained for 4 min from 6 - 10min, then percentage of solution B was decreased linearly to 10 % from 10 - 12.5 min and held for 1.5 min until the run was completed at 14 min. The flow rate was set to 200 µL/min, and the injection volume was 2 µL.

For detecting apigenin and its potential metabolites, targeted mass analyses were performed in negative ion mode on a Xevo TQ-S micro Triple Quadrupole Mass Spectrometry (Waters, Milford, MA, USA). The following mass spectrometer settings were used: capillary voltage = 3000 V, desolvation temperature = 500 °C, source gas flow desolvation = 1000 L/hour, source gas flow cone = 20 L/hour, analyzer collision energy = 20 volts, source temperature = 150 °C, collision gas used was argon.

For each substance, the time of detection was predetermined based on separation results obtained from UPLC-PDA analysis and the time frames can be found in Table 4.2. Information for characteristic daughter ions, collision voltage and cone voltage settings in multiple reaction monitoring (MRM) for targeted compounds can be found in Table 4.3. For quantification, an external matrix calibration was used. The matrix consisted of the unfiltered fermented medium taken from the bioreactors and processed in the same way as the samples after being spiked with the targeted compounds at desired concentrations. Concentrations included in the calibration curve were 0.25, 0.50, 0.75, 2.5, 5.0, 7.5, 25, 50, 75, 100, 120 µg/mL. Calibration curves for all tested compounds were of second order and excluded the origin. Coefficients for each term, axis transformation, coefficient of determination information for each calibration curve can be found in Table 4.4.

Table 4.2 Detection time window of targeted compounds.

Compound	Beginning Time (min)	End Time (min)
Phloroglucinol	0.00	2.49
4HBA	3.00	5.00
4HPAA	3.50	6.00
4HCA	4.50	6.00
4HPP	4.50	6.00
3HPP	4.50	6.49
3,4DHPA	0.00	14.00
3,4DHCA	3.00	5.50

Apigenin	6.00	8.00
Naringenin	6.00	8.00
Phloretin	6.00	8.00
Luteolin	5.50	7.00
Eriodictyol	5.50	7.00

Table 4.3 Information of daughter ions, collision voltage and cone voltage settings in multiple reaction monitoring for targeted compounds.

Compound names	Parent (m/z)	Daughter (m/z)	Cone (V)	Collision (V)
Phloroglucinol	125.1274	56.9743	2	16
		96.8844	2	12
4HBA	136.9998	64.9863	32	24
		74.953	32	26
		93.5702	32	24
4HPAA	150.9516	78.9799	12	16
		92.9159	12	20
		107.554	12	16
4HCA	163.0154	92.966	2	26
		93.5463	2	26
		119.449	2	26
4HPP	164.9673	58.9891	34	12
		92.9682	34	10
		121.5323	34	16
3HPP	164.9673	92.9699	38	18
		105.9851	38	22
		121.5973	38	12
3,4DHPA	167.0104	94.9973	36	20
		104.9556	36	16
		123.7603	36	14
3,4DHCA	180.8984	58.9241	44	16
		108.9143	44	14
		121.081	44	16
Apigenin	269.0209	116.9886	66	36
		148.6857	66	26
		151.6069	66	26
Naringenin	271.0366	106.9633	52	28
		118.9447	52	28
		150.9615	52	16
Phloretin	273.116	80.9949	50	32
		122.9796	50	22
		167.0364	50	14
Luteolin	284.952	106.9669	62	30
		133.0011	62	32
		150.965	62	24
Eriodictyol	287.0315	106.9648	46	24
		134.9566	46	38
		150.9645	46	14

Table 4.4 Calibration curves for each targeted compound.

Compound name	Coefficient of determination R^2	Coefficient for term x^2	Coefficient for term x	Y axis intercept	Axis transformation
3HPP	0.998574	-100.647	32551.2	352696	None
4HBA	0.997673	-0.00489263	0.943608	7.87508	Ln
4HCA	0.998545	-0.0319595	1.0478	9.75714	Ln
4HPAA	0.999213	-3.52332	1413.81	74593	None
4HPP	0.999449	-7.93222	5014.21	3812.64	None
34DHCA	0.998749	-46.6625	28115.9	52793.3	None
34DHPA	0.998512	0.193837	116.984	-170.997	None
Apigenin	0.982208	-0.0358249	1.11374	7.62863	Ln
Eriodictyol	0.991184	-539.35	170062	-347638	None
Luteolin	0.99533	0.0172786	0.934545	8.91328	Ln
Naringenin	0.988821	-226.378	99379	-299189	None
Phloretin	0.982098	338.346	142665	-594677	None
Phloroglucinol	0.998741	-2.84417	1882.07	11414.8	None

4.3 Results

4.3.1 While apigenin inhibited *E. caccae* growth, its concentration declined in the culture.

Three groups were used in the experiment to determine the decline in apigenin concentration in trypticase soy yeast extract broth and construction of growth profiles: the apigenin broth control with 100 µg/mL of apigenin; the bacteria control group containing DMSO inoculated with bacteria and no apigenin; the experimental group, containing apigenin and bacteria.

The growth profiles of *E. caccae* treated with 100 µg/mL of apigenin and the control group that received the same amount of stock solution solvent were constructed (Figure 4.3 a) with duplicated OD_{600} readings taken at 0, 0.5, 1, 2, 4, 6, 8, 12, and 24 hours post inoculation. The cell density in the apigenin treated group was significantly lower than the control group at 6, 8, 12, and 24 hours. At those time points, the control group had

OD_{600} readings of 0.362 ± 0.001, 0.768 ± 0.003, 0.844 ± 0.009, 0.857 ± 0.001, respectively, while the apigenin treated group had readings of 0.234 ± 0.004, 0.59 ± 0.001, 0.763 ± 0.001, 0.765 ± 0.001. Therefore, for those time points, cell density in apigenin treated groups were 64.73 %, 76.82 %, 90.46 %, and 89.32 % of the control group and the differences between OD_{600} readings were statistically significant ($p < 0.05$).
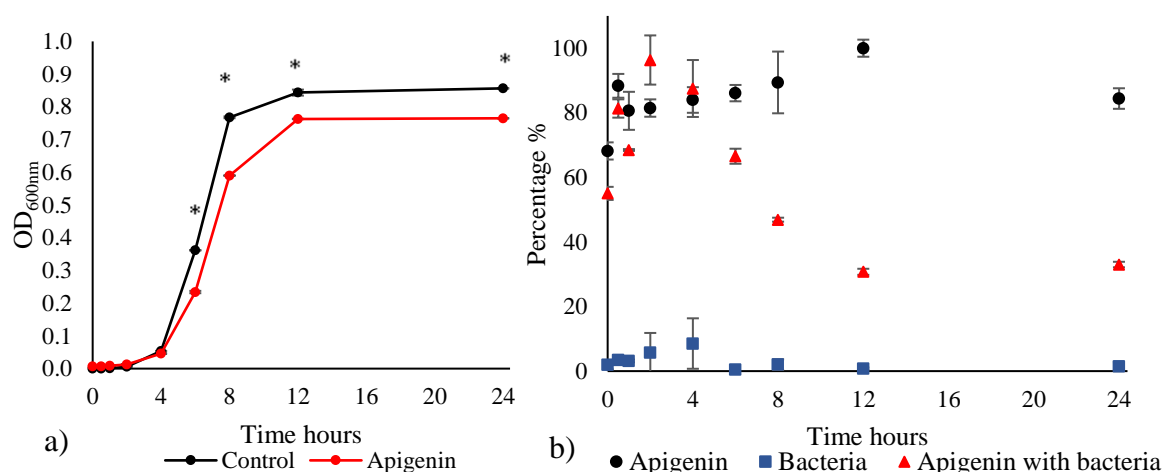

Figure 4.3 Concentration of apigenin and microbial growth in *E. caccae* culture. a) Growth of *E. caccae* with and without the presence of 100 µg/mL apigenin measured by optical density at 600 nm. Error bars indicate standard deviation. b) Concentration of apigenin measured by UPLC-ESI-MS/MS in trypticase soy yeast extract medium with and without *E. caccae*. The initial concentration in apigenin groups were 100 µg/mL. A bacteria only group served as the blank. Measurements were taken at 0, 0.5, 1, 2, 4, 6, 8, 12, 24 hours post inoculation. * indicates P -value < 0.05 in unpaired homoscedastic Student t-test.

The concentration of apigenin and its potential degradation products were determined using targeted UPLC-ESI-MS/MS. Relative concentration, the percentage against the highest concentration detected, was found to be the best way to showcase the disappearance of apigenin in the broth and to produce comparable results across replicates. The highest concentration of apigenin (40.67 ± 0.20 µg/mL) was detected in the apigenin broth control group at 12 hours, therefore considered 100 %. For the apigenin broth control group, the relative concentrations and the standard deviation of apigenin at 0, 0.5, 1, 2, 4, 6, 8, 12, 24 hours post addition of the single dose of apigenin

were 68.18 ± 2.66 %, 88.35 ± 3.69 %, 80.60 ± 5.89 %, 81.46 ± 2.69 %, 83.96 ± 3.97 %,

86.09 ± 2.53 %, 89.39 ± 9.56 %, 100 ± 2.65 %, 84.41 ± 3.18 %, respectively. For the

same time points in the apigenin treated experimental group with bacteria, the relative

concentrations were 55.04 ± 2.02 %, 81.33 ± 2.84 %, 68.47 ± 0.28 %, 96.34 ± 7.62 %,

87.52 ± 8.82 %, 66.54 ± 2.33 %, 46.89 ± 0.61 %, 30.74 ± 0.94 %, 33.00 ± 0.85 %. In the

bacteria control group where no apigenin was added, the detected relative concentrations

were 1.91 ± 1.38 %, 3.49 ± 0.33 %, 3.16 ± 0.41 %, 5.77 ± 6.05 %, 8.54 ± 7.81 %, 0.50 ±

0.00 %, 2.01 ± 1.64 %, 0.72 ± 0.39 %, 1.45 ± 1.30 %. The trends (Figure 4.3b) showed

that after a brief increase from 0 to 0.5 hours, apigenin concentration remained stable

with no obvious sign of degradation in sterile broth kept at 37 °C for 24 hours; there was

no sign of inherent apigenin from the broth itself, although there was a slight upward shift

at around 4 hours; detected apigenin concentration increased in the initial two hours, then

gradually decreased until 12 hours post inoculation; the concentration at 24 hours was

similar to the one at 12 hours. At 24 hours, only 34.25% of apigenin were left compared

to the highest concentration detected in the apigenin experimental group. None of the

targeted potential degradation products was detected.

4.3.2 The effect of apigenin on a human gut microbial community *in vitro*

The effect of apigenin on a human gut bacterial community was determined using

bioreactors through inoculation, stabilization, and treatment phases. Two bioreactors

were inoculated with the same human fecal material and run in parallel. After

stabilization, one bioreactor was treated with a single dose of 100 µg/mL apigenin and

the other one was designated as control, receiving no apigenin. Samples were harvested

over the whole three phases to measure the effect of apigenin on the human gut microbial community as reflected in microbial composition and SCFAs production.

The majority of the community after stabilization was Clostridia, accounting for more than 75 % in most samples. The control unit and the experimental unit showed similar trends (Figure 4.4 a,b) in microbial composition after inoculation and during stabilization, although the exact percentage of each class varied slightly between the two groups. Due to this variation, composition during the stabilization period was used to compare with the composition after the bioreactors had received either DMSO (control group) or apigenin stock solution (experimental group) to determine the effect of DMSO and apigenin on the composition of the human gut microbiota *in vitro* (Figure 4.4 c,d). Microbial composition showed gentle fluctuation during each feeding cycles and, in both control group and experimental group, there were more Clostridia and less Bacilli on day 14 compared to day 12 and day 13. Neither DMSO nor apigenin had distinctive effects on the microbial composition of the communities cultured *in vitro*.

Short chain fatty acids (SCFAs) concentration were determined with GC-MS. The most abundant short chain fatty acids in the culture were acetate, butyrate, and propionate. Several other SCFAs, namely 2-methylpropionate, 2-methylbutyrate, pentanoic acid, 2-methylpentanoic acid and 4-methylpentanoic acid were also detected. As they were only in low or trace concentrations, detailed information on them are not included here. Due to the slight variation of microbial composition, short chain fatty acid production from two feeding cycles, on two days and one day before the day of treatment respectively, was compared with that in the feeding cycle when treatment was applied. Percentage was

used to account for the different amount of acid and base added to the two systems as well. The most abundant short chain fatty acid was acetic acid. In the order of two days before the treatment, one day before the treatment, and treatment day, the percentages of acetic acid (Figure 4.5 a) at 45 min before feeding ended were 65.47 ± 0.68 %, 67.28 ± 1.78 %, 66.26 ± 4.57%, when the feeding ended, the percentages were 68.82 ± 2.50 %, 67.49 ± 1.29 %, 69.53 ± 2.13 %; at 30 min after feeding/treatment, the percentages were 67.22 ± 1.40 %, 68.52 ± 5.04 %, 69.32 ± 3.25 %; at 1 hour after feeding/treatment, the percentages were 69.17 ± 0.50 %, 68.43 ± 2.34 %, 67.97 ± 1.54 %; at 2 hours after feeding/treatment, the percentages were 67.40 ± 3.31 %, 66.54 ± 13.04 %, 66.79 ± 0.14 %; at 4 hours after feeding/treatment, the percentages were 66.64 ± 1.47 %, 67.96 ± 3.07 %, 67.08 ± 1.22 %; at 6 hours after feeding/treatment, the percentages were 64.35 ± 0.21 %, 66.42 ± 13.17 %, 66.05 ± 1.17 %; at 7.25 hours after feeding/treatment, the percentages were 64.68 ± 3.85 %, 65.68 ± 0.74 %, 67.55 ± 1.37 %.

For butyric acid (Figure 4.5 b), the second most abundant SCFA detected in this experiment, in the same order as the results for acetic acid was presented, the percentages at 45 min before feeding ended were 24.21 ± 0.19 %, 21.99 ± 0.54 %, 23.85 ± 0.97 %; when the feeding ended, the percentages were 21.66 ± 1.08 %, 22.08 ± 0.61 %, 21.27 ± 0.46 %; at 30 min after feeding/treatment, the percentages were 22.90 ± 0.13 %, 21.32 ± 0.19 %, 22.04 ± 0.42 %; at 1 hour after feeding/treatment, the percentages were 21.56 ± 0.49 %, 21.23 ± 0.32 %, 22.04 ± 0.42 %; at 2 hours after feeding/treatment, the percentages were 22.48 ± 0.41 %, 22.69 ± 2.41 %, 23.67 ± 0.07 %; at 4 hours after feeding/treatment, the percentages were 22.95 ± 0.62 %, 21.88 ± 0.78 %, 23.50 ± 0.09 %;

at 6 hours after feeding/treatment, the percentages were 24.64 ± 0.37 %, 22.39 ± 2.08 %, 24.48 ± 0.16 %; at 7.25 hours after feeding/treatment, the percentages were 24.38 ± 0.70 %, 23.31 ± 0.63 %, 23.40 ± 0.30 %.

For propionic acid (Figure 4.5 c), the percentages at 45 min before feeding ended were 10.32 ± 0.09 %, 10.73 ± 0.15 %, 9.88 ± 0.46 %; when the feeding ended, the percentages were 9.52 ± 0.22 %, 10.44 ± 0.36 %, 9.20 ± 0.25%; at 30 min after feeding/treatment, the percentages were 9.88 ± 0.09 %, 10.16 ± 0.23 %, 8.64 ± 0.23 %; at 1 hour after feeding/treatment, the percentages were 9.27 ± 0.17 %, 10.34 ± 0.21 %, 9.40 ± 0.27 %; at 2 hours after feeding/treatment, the percentages were 10.12 ± 0.13 %, 10.78 ± 0.90 %, 9.54 ± 0.05 %; at 4 hours after feeding/treatment, the percentages were 10.40 ± 0.21 %, 10.16 ± 0.23 %, 9.42 ± 0.11 %; at 6 hours after feeding/treatment, the percentages were 11.01 ± 0.10 %, 11.18 ± 1.06 %, 9.47 ± 0.13 %; at 7.25 hours after feeding/treatment, the percentages were 10.94 ± 0.39 %, 11.01 ± 0.27 %, 9.05 ± 0.17 %. Using the criteria that only if in unpaired homoscedastic Student t-test, baseline 1 and 2 have the same mean, but treatment group is significantly different from both baseline means, there was no significant difference expect for the decrease of propionic acid percentage after apigenin treatment at 0.5, 4, and 7.25 hours post treatment. At 6 hours, the difference is very close to be considered as significantly different.

4.3.3 Apigenin degraded in human gut microbiota culture *in vitro* and 4HPPA was the main end product

In the present study, the degradation of apigenin were determined in several independent different conditions *in vitro*: in the human gut microbiota, sterile fresh

medium, and sterile fermented medium by measuring the concentrations of apigenin and its potential degradation products (Figure 4.6). In each condition, there was a treated group (100 µg/mL) and a control group (DMSO).

In the human gut microbiota culture, there was no apigenin in the system prior to the treatment (Figure 4.6 a). The concentration of apigenin in the media increased from when it was added to 2 hours later. After reaching peak concentration at two hours, apigenin disappeared rapidly, losing more than 60 % over the next two hours. The decrease trend continued until 8 hours after administration of apigenin when there was close to no apigenin left in the media. As the apigenin concentration started dropping, the concentration of 4HPPA started to increase. As there was no 4HPPA inherently in the media nor was it produced by an untreated gut microbiota, the only source of 4HPPA was the breaking down of apigenin. When apigenin concentration was decreasing rapidly, the rate of increase of 4HPPA concentration was also fast. When apigenin concentration was slowly going down, the increase in 4HPPA also slowed done. The conversion from apigenin to 4HPPA did not occur at a 1:1 ratio. For a total decrease in apigenin concentration from 100% to 1.43%, 4HPPA concentration increased by only an equivalent of 27.43%, about 1/3. From 12 hours to 24 hours, when there was no apigenin in the system, the concentration of 4HPPA decreased as it may have been converted into other compounds. Setting the time apigenin treatment was applied as time 0, the relative apigenin concentration in the treated bioreactor at time 45 min prior to 0, 0, 0.25, 0.5, 1, 2, 4, 6, 8, 12, 24 hours post treatment were 0.00 ± 0.00 %, 53.97 ± 1.11 %, 78.62 ± 0.70 %, 89.21 ± 1.87 %, 74.78 ± 1.53 %, 100.00 ± 0.49 %, 36.84 ± 1.63 %, 13.29 ± 0.27 %, 1.43

± 0.13 %, 0.00 ± 0.00 %, 0.00 ± 0.00 %; relative apigenin concentrations in the control

group were all zero after adjusting for negative values; 4HPPA in the treated group were

not detected until 4 hours post treatment and the relative concentrations for the following

time points were 4.79 ± 0.37 %, 18.04 ± 0.36 %, 21.88 ± 0.68%, 27.04 ± 0.70 %, 15.94 ±

2.22 %. 4HPPA concentration was below the limit of detection in the control group that

the equipment and software failed to pick it up. Similar trends were observed in all three

replications. In one out of the three replications, phloroglucinol was picked up in the

treatment group but not the control group, and its relative concentrations at 6, 8, 12, 24

hours post treatment were 12.23 ± 1.22 %, 14.33 ± 0.83 %, 18.06 ± 0.47 %, and 5.82 ±

2.08 %. The other targeted compounds were not detected or quantified by UPLC-ESI-

MS/MS.

Apigenin measurement remained stable in fresh modified MShime® medium

incubated at 37 °C in darkness over 24 hours with some inherent fluctuations, while no

apigenin was detected in the control group (Figure 4.6b). The relative concentration of

apigenin in fresh modified MShime® medium incubated at 37 °C were 99.74 ± 2.59 %,

100.00 ± 1.18 %, 93.29 ± 7.14 %, 96.29 ± 4.45 %, 99.70 ± 2.39 %, 98.36 ± 2.86 %, 99.89

± 1.91 %, 99.53 ± 1.68 %, 97.98 ± 0.83 %, 94.63 ± 0.25 % for 0, 0.25, 0.5, 1, 2, 4, 6, 8,

12, 24 hours post addition of apigenin respectively. The corresponding relative

concentrations in control group (DMSO) were 0.03 ± 0.10%, 0.01 ± 0.03 %, 0.00 ±

0.00 %, 0.03 ± 0.04 %, 0.06 ± 0.10 %, 0.04 ± 0.08 %, 0.00 ± 0.00 %, 0.05 ± 0.06 %, 0.04

± 0.09 %, 0.02 ± 0.10 %. No targeted potential metabolic products were detected, which

was expected as the concentration of apigenin stayed about the same level throughout the 24 hours.

After addition of apigenin to sterile filtered bioreactor supernatant, its concentration briefly increased in the first 15min, then decreased to about 4% of the peak concentration in the next 15min. For the 23.5 hours after that, the apigenin concentrations remained at the low level, with a slight downward trend, from 3.96% to 2.05% (Figure 4.6c). No apigenin was detected in the control group (DMSO). The relative concentration of apigenin in sterile bioreactor supernatant incubated at 37 °C were 87.84 ± 1.47 %, 100.00 ± 1.87 %, 3.96 ± 0.02 %, 3.42 ± 0.03 %, 2.51 ± 0.02 %, 2.11 ± 0.03 %, 2.92 ± 0.05 %, 2.58 ± 0.02 %, 2.02 ± 0.09 %, 2.05 ± 0.02 % for 0, 0.25, 0.5, 1, 2, 4, 6, 8, 12, 24 hours post addition of apigenin respectively. The corresponding relative concentrations in control group (DMSO) were 0.00 ± 0.00 %, 0.00 ± 0.00 %, 0.00 ± 0.00 %, 0.00 ± 0.00 %, 0.00 ± 0.00 %, 0.00 ± 0.00 %, 0.00 ± 0.00 %, 0.00 ± 0.00 %, 0.00 ± 0.00 %, 0.02 ± 0.05 %. No targeted potential metabolic products were detected, although apigenin concentration very rapidly decreased.
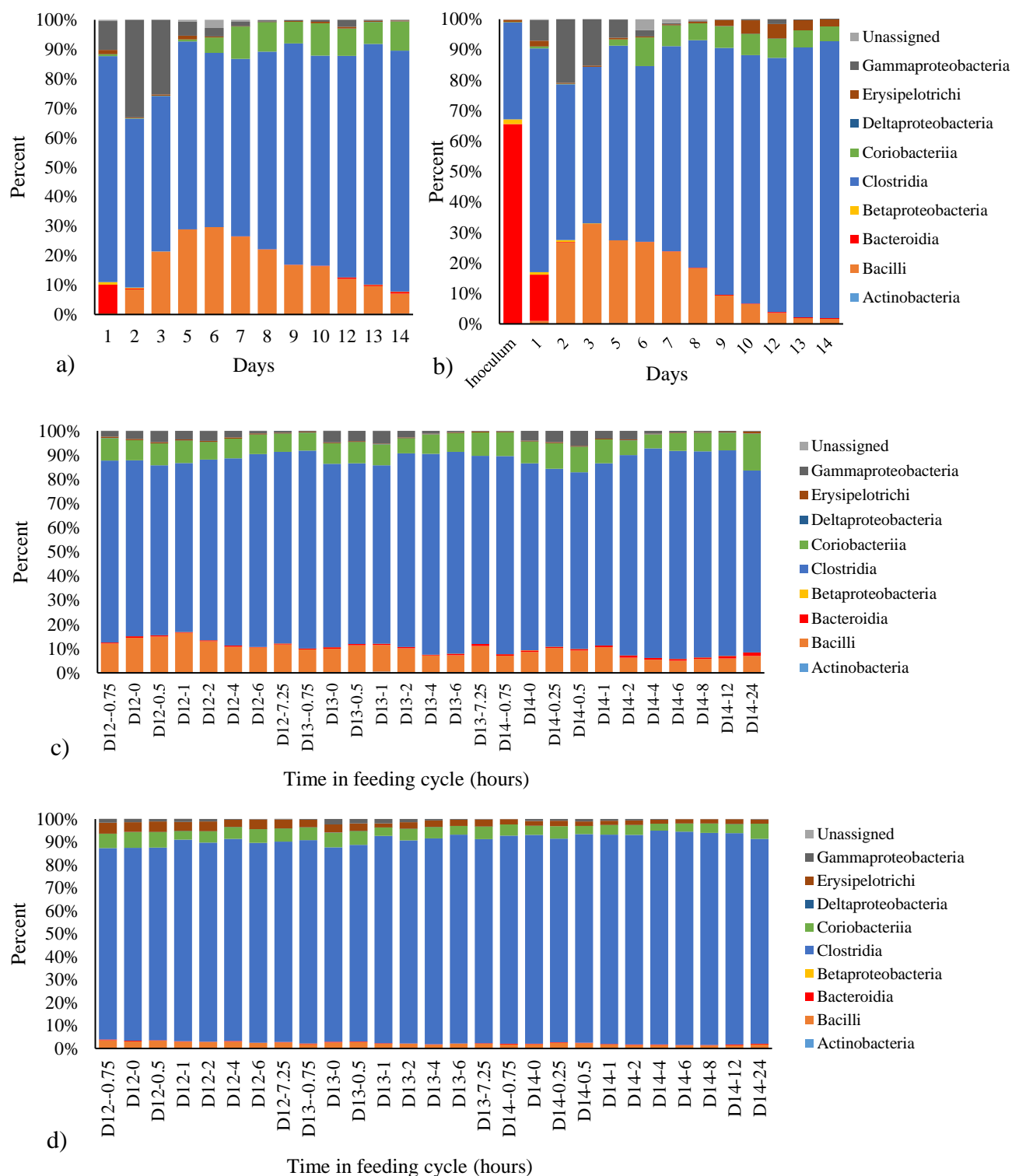
Figure 4.4 Human gut microbial community composition at class level. a) The control group bioreactor composition at 15 min before a feeding cycle started. b) The inoculum and experimental group (100 µg/mL apigenin) bioreactor composition at 15 min before a feeding cycle started over 14 days. c) The control group bioreactor composition in three consecutive days, the treatment day and two days before. d) The experimental group bioreactor composition in three consecutive days, the treatment day and two days prior. Community composition was determined based on relative abundance of OTUs determined by Illumina sequencing grouped at the class level.
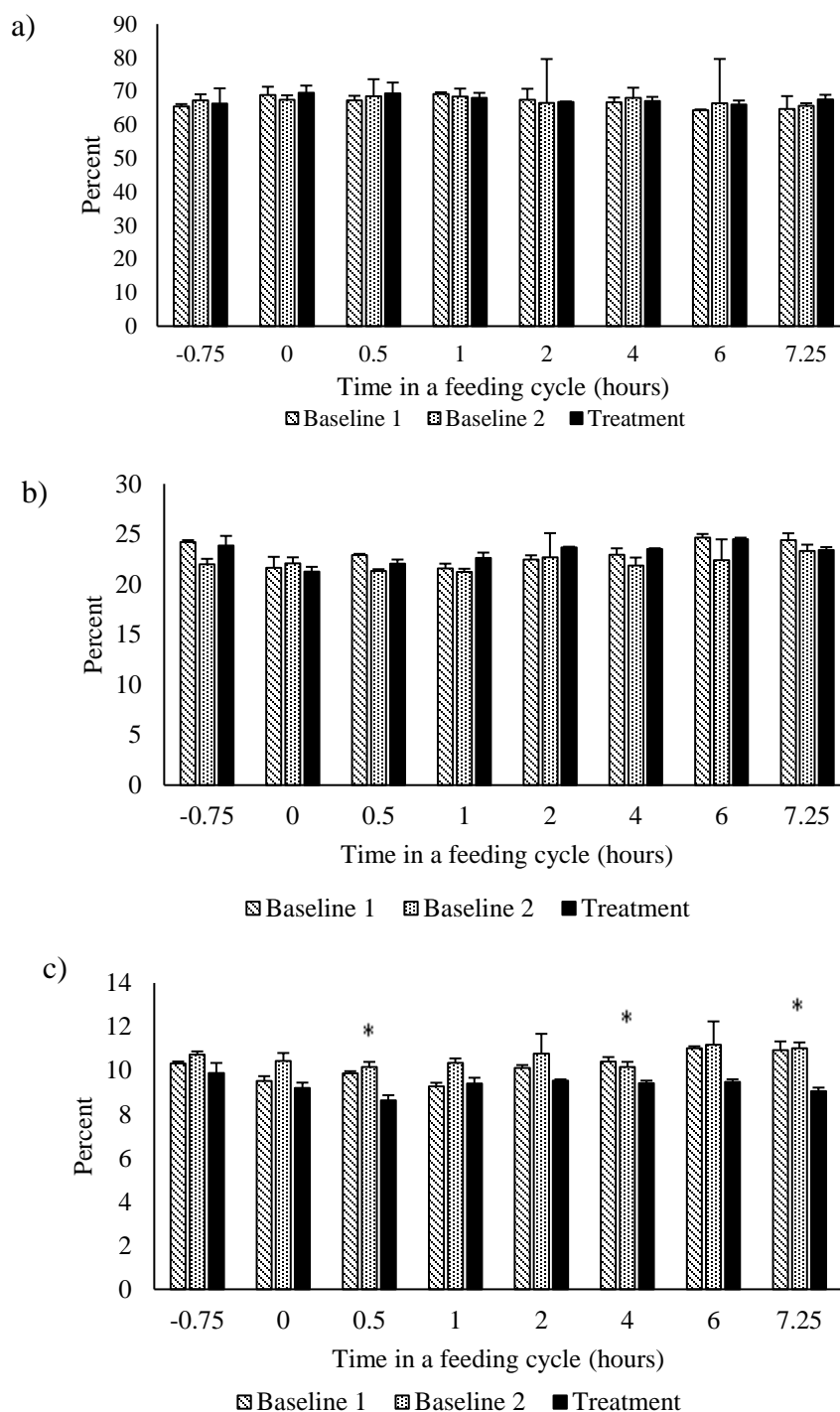
Figure 4.5 Percentage of three major short chain fatty acid production by the gut microbial community over one feeding cycle in three consecutive days, the treatment day and two days prior. Amounts of the short chain fatty acids acetate, propionate, and butyrate were measured using a GC/MS and divided against the total amount of short chain fatty acids quantified for the percentages. a) Acetate; b) Butyrate; c) Propionate. * indicates that using unpaired homoscedastic Student t-test, baseline 1 and 2 have the same mean, but treatment group is significantly different from both baseline means.

Figure 4.6 The concentrations of apigenin and its potential degradation products the degradation of apigenin in several independent different conditions *in vitro*. a) in the human gut microbiota, b) sterile fresh medium, c) sterile fermented medium. In each condition, there was a control group (DMSO) and a treated group (100 µg/mL).

4.4 Discussion

4.4.1 Chemicals and concentration

The aglycone form of apigenin was used as the treatment instead of the more prevalent natural existing glycosides because of two considerations. First, 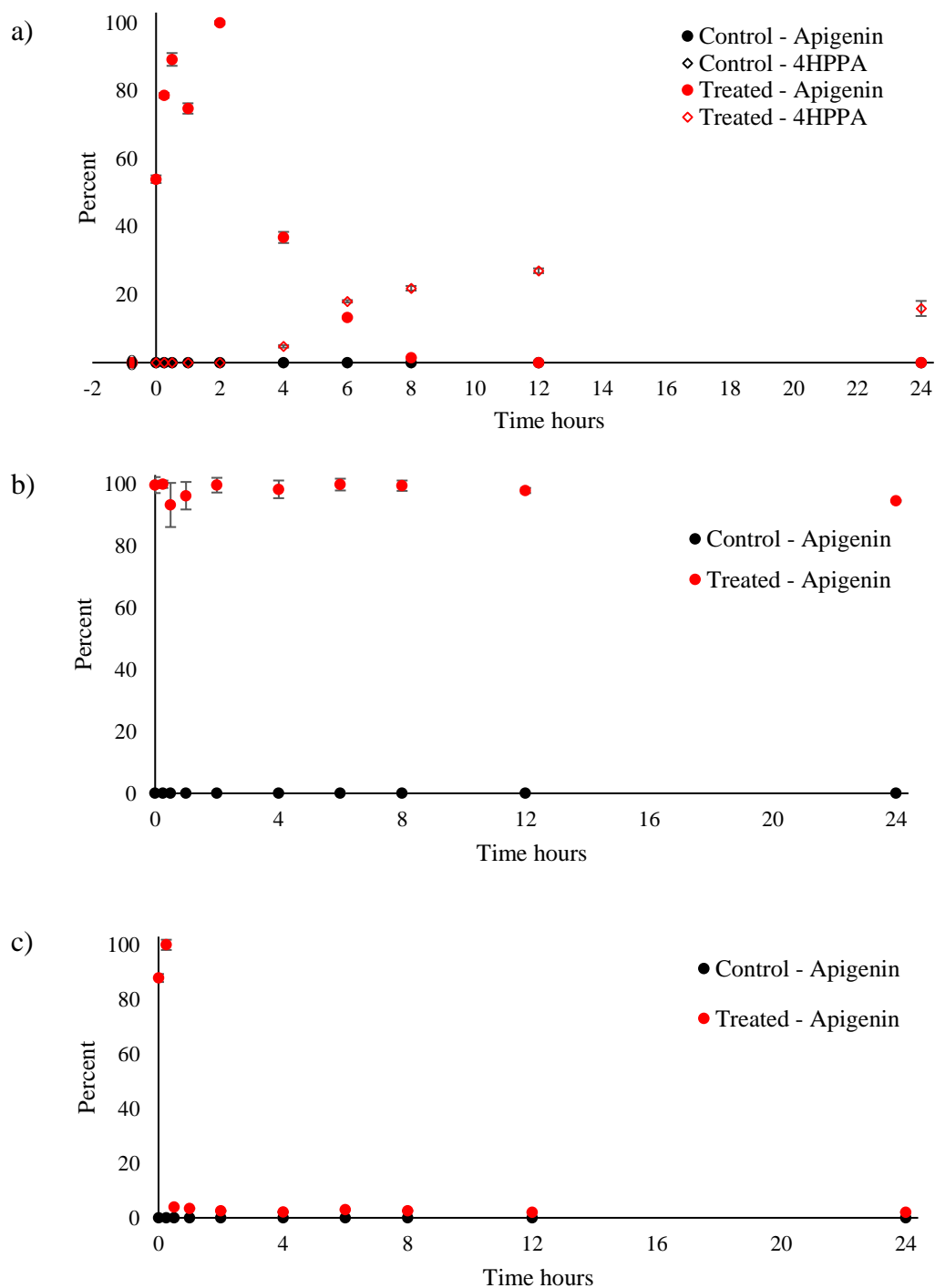not all bacteria possess the ability to cleave the sugar molecule from the apigenin backbone structure. There has not been any report on the ability of the *E. caccae* strain is able to perform such task. To study how *E. caccae* degrades apigenin, using the glycosides may prevent the degradation from being initiated. Second, as in rats gavaged with purified flavonoid extract from parsley, it was found that the cecum luminal contents contained only aglycone form of this flavonoid (Pforte et al., 1999). It was suggested that glycosides transported to the cecum were mostly absorbed or deglycosylated rapidly (Pforte et al., 1999). Therefore, in this present study, the *in vitro* simulation of the impact of apigenin in the colon used the aglycone form. The concentration used was 100 µg/mL based on previous research (M. Wang et al., 2017). By keeping it at the same level as the concentration tested on an *in vitro* gut microbiota community, results were more comparable. This concentration did not surpass the solubility of apigenin in media, but was also designed to be high so that any effects on bacteria or the degradation phenomenon would be distinct to observe. Flavonoids in general have limited solubility in water, therefore DMSO was used as the organic solvent to facilitate apigenin dissolve into the aqueous media. Based on the results in *E. caccae* growth and gut microbial community structure as well as results from other experiments performed in our lab, the concentration of DMSO used did not cause any interference to the effects we were observing in this study.

4.4.2 Degradation of apigenin in *E. caccae*

The effects of apigenin on the growth of *E. caccae* from this experiment were in line with previous findings despite of the different types of equipment to measure cell density (M. Wang et al., 2017), a densitometer that measures turbidity verses a spectrometer that measures absorbance of light.

In both the group where apigenin was added to sterile media and the group where apigenin was added to *E. caccae* culture, apigenin concentration increased during the first half an hour. Considering that all solid particles were removed during UPLC sample processing, the detected concentration only captured the dissolved apigenin in the system, but would not have included undissolved apigenin, apigenin entrapped by insoluble organic matrix in the media, or apigenin attached to or contained within the bacteria. This initial increase in apigenin concentration could reflect the slow dissolving of apigenin into the solution, but also could be from the rising temperature of media as sitting in the incubator for an extended period of time allowed to fully reach 37 °C. Apigenin concentration remained largely unchanged with no obvious sign of degradation in sterile trypticase soy yeast extract broth kept at 37 °C for 24 hours. This result is similar to reports of apigenin being stable after maceration for 24 hours (Biesaga, 2011) and 24 hours under room temperature in rat plasma (Duan et al., 2011; Shi et al., 2011)

This shows that although most flavonoids are considered unstable and should be stored refrigerated or frozen, apigenin may be more stable than cautiously expected. In the apigenin treated bacteria group, a similar initial increase in concentration was observed from 0 to 2 hours. Then the detected concentration gradually decreased until 12

hours post inoculation; the concentration at 24 hours was similar to the one at 12 hours, with only 34.25 % the peak concentration. Unexpectedly, none of the targeted potential degradation products was detected. This could be because that the list of potential degradation products was not complete and *E. caccae* metabolized apigenin into something else. Combining information with the *E. caccae* growth curve, there is another possible explanation. *E. caccae* entered exponential phase close to 4 hours post inoculation and cell number increased until around 12 hours post inoculation. This happened concurrently as detected concentrations of apigenin in the media kept dropping. During the stationary phase of *E. caccae* growth, cell number remained stable as the rate of cells dying is about the same as the rate of new cells forming; apigenin concentration at 24 hours was close to the one at 12 hours, but not zero. Taking all the information, the decrease of apigenin concentration was more likely caused by up-take of live *E. caccae* cells rather than degradation. It has been hypothesized that glycosylation causes a reduction in lipophilicity and consequently diminishes the ability to penetrate bacterial membrane and therefore renders the apigenin glycosides less effective to inhibit microbial growth (Taiwo & Igbeneghu, 2014). Penetrating the cell membrane might be a critical step for apigenin to exert its effects on bacteria.

After *E. caccae* had taken up the apigenin into itself, it did not metabolize apigenin or it did not release the metabolic products. It is not clear how the bacteria take up apigenin, whether it was inside the cell or attached to the surface. However, this can help explain the range-limited dose-dependent inhibitory effects of apigenin on *E. caccae* reported earlier (M. Wang et al., 2017). As the number of cells in the media was limited, there was a finite maximum amount of apigenin that could be taken up by *E. caccae* and

exert its antimicrobial effects, within the dose-dependent range of concentration, the higher apigenin treatment, the more apigenin would be taken up by the cells; beyond the dose-dependent range, any additional apigenin would remain free in the media and be of no effect on bacteria. This potential explanation needs to be validated by further experiments that measure the amount of apigenin in the media and in or on the bacterial cells. Previous single molecule RNA sequencing of apigenin treated *E. caccae* at 8 hours post inoculation indicated that cell membrane and cell wall synthesis is likely a major target of apigenin in *Enterococcus caccae* (M. Wang et al., 2017). Many of the up-regulated genes were involved in cell membrane/wall synthesis, such as the D-Alanine: D-Alanine ligase and Type II fatty acid synthetic pathway. These genes could have been upregulated to compensate for the inhibition exerted on the end products like enzymes or cell wall/membrane, which could possibly reflect apigenin attaching to or penetrating the cell wall/membrane.

In most of the samples from the control group in which no apigenin was added, no or very low signals of apigenin were detected, although artifact from changes in media composition due to incubation at 37 °C might have caused a slight upward shift at around 4 hours.

4.4.3 Culturing the human gut microbiota *in vitro*

The bioreactors were set up to simulate the condition in the proximal, or ascending, colon. The proximal colon was chosen because dietary apigenin would be of the highest concentration in this part of the colon than anywhere downstream. Interactions in the transverse and descending colon may involve mainly degradation

products from the ascending colon instead of intact apigenin. Therefore, pH of the *in vitro* culture systems was set at 5.8 with a dead band of 0.1 as the pH in human colon regions is about 5.7 in the caecum, and gradually increases to pH 6.7 in the rectum (Fallingborg, 1999). The media used in the present study was modified based on what was used in another *in vitro* simulation study (L. S. Liu et al., 2018) that successfully stabilized a human gut microbiota similar to the inoculum composition. However, due the difference in system components, mainly without a stomach region where the MShime® feed can be digested with pancreatic juice with the presence of bile salt, the modified MShime® feed selected for Clostridia, causing the community composition to shift away from that of the inoculum. To determine the suitability of a culture media, long term experiment that extend to stabilization phase is needed, as the results showed that during the first few days after inoculation, the favorable effect on Clostridia was not obvious comparing to after the community composition had stabilized.

Previous experiments in our lab showed that a three-week stabilization period was sufficient, hence the apigenin treatment was applied on the 22nd in this study. As results later showed that the community composition stabilized at around two weeks post inoculation.

4.4.4 Interactions between apigenin and the human gut microbiota *in vitro*

The effects of apigenin on the human gut microbiota were investigated from two aspects, the structure as determined by 16S rDNA sequencing and the function as reflected in SCFAs production. Apigenin did not have a major effect on the microbiota structure. This conclusion is in line with a couple previous publications

(Vollmer et al., 2018; M. Wang et al., 2017). The gut microbial community has a complex composition and many components to interact with apigenin, therefore the effect on each single strain might be minimum. It could also be that the bacteria in the stabilized community are in a phase similar to the stationary phase and would not be as sensitive to apigenin as the *E. caccae* tested as a single strain where apigenin was added at the same time of inoculation. As for short chain fatty acid production, propionic acid percentage in total SCFAs seemed to be inhibited by apigenin treatment, while acetic acid and butyric acid product was not really altered. Percentage was used here to account for the potential concentration differences between samples due to different amount of acid and base added, or subtle uncontrollable differences in system volume due to inherent variation of pump speed.

The effects of gut microbiota on apigenin can be seen by comparing the concentrations of apigenin in the treated bioreactor, sterile fresh media, and the sterile supernatant taken from gut microbial culture over 24 hours. There was no apigenin in the media and the bacteria community itself was not able to generate apigenin. After apigenin was added to the bioreactor, the concentration of apigenin in the media increased until reaching peak concentration at 2 hours. This could be because that not all apigenin dissolved initially and more apigenin went into the media later and was available to be quantified in the samples. Temperature could not be used to explain the increase as the system was kept at 37 °C at all times. However, another explanation could be that apigenin was trapped in the media matrix and was released slowly. The apigenin treatment was applied when the system had just finished

receiving fresh media, therefore the media matrix was rich in nutrients such as polysaccharides and large proteins. Some of the apigenin may be attached to those large biopolymers and thus was filtered out during sample processing. As the bacteria utilized the nutrients and broke them down, apigenin was released into the media and the concentration in the aqueous phase increased. After the 2 - hour time point, apigenin disappeared rapidly, losing more than 60 % of its peak concentration over the next two hours. Such trend continued until 8 hours after administration of apigenin, albeit at a lower rate. As the apigenin concentration started dropping, 4HPPA started to show in the system. As there was no 4HPPA inherently in the media nor was it produced by an untreated gut microbiota, it had to be from apigenin. There was a negative correlation between apigenin concentration and 4HPPA concentration. The conversion from apigenin to 4HPPA was not one to one, but rather, close to, three to one. It has been reported by several other papers that 4HPPA is one of the main microbial degradation products from apigenin (Hanske et al., 2009; Schoefer et al., 2003; Vollmer et al., 2018) with potential health benefits in fighting influenza (Steed et al., 2017). In one of the replications, phloroglucinol was also found to be a degradation product occurring later than 4HPPA. The degradation of apigenin, its rate and types of metabolites produced, is highly dependent on the microbiota community itself. As one research reported that fecal samples from three different donors generated very different degradation profiles in the *in vitro* degradation of apigenin glycosides (Vollmer et al., 2018). It may be the characteristic of the fecal sample we used that only 4HPPA was detected in all three replicates for the bacteria capable of degrading apigenin and produce certain smaller compounds might have been missing

or have been present at a low abundance. Some bacteria in the community might take up apigenin into the cells like we hypothesized with *E. caccae* and did not produce smaller metabolites. This can help to understand why apigenin was not converted to 4HPPA at a 1:1 ratio. Detection of apigenin in complex microbial media can be a challenge occurring not only in the present study. In the study by Vollmer *et al* (Vollmer et al., 2018), the concentration of apigenin treatment applied was 200 μM, but the highest detected concentrations in three independent experiments were 106 ± 16.0 μM, 22.9 ± 14.5 μM, 25.3 ± 6.55 μM. In the present study, the highest detected concentrations following addition of 100 μg/mL of apigenin in three independent replicates were 23.83 ± 0.17 μg/mL, 95.90 ± 1.18 μg/mL, and 40.67 ± 0.20 μg/mL. Despite the different extraction methods and the external matrix calibration curve adopted for apigenin and its metabolites, total recovery was not consistently achieved in either study. Similar to the findings in our work, Vollmer *et al* reported 4HPPA as the main end product of apigenin, however, the time to generate this product varied with samples from different donors. Since the fecal samples were from the same donor, this time frame is more consistent in the present study. Vollmer *et al* reported additional degradation products, phenylacetic acid and 3-phenylpropionic acid, occurring at 24 and 48 hours post apigenin treatment with some, but not all, fecal samples. Compared with results obtained from *in vitro* simulations, the major hepatic metabolite of apigenin is luteolin (Angéline Gradolatto et al., 2005) and *in vivo* experiment using human microbiota-associated rats (Hanske et al., 2009) reported many more metabolites including: naringenin, eriodyctiol, phloretin, 3-(3,4-dihydroxyphenyl) propionic acid, 3-(4-hydroxyphenyl)propionic acid, 3-(3-

hydroxyphenyl)propionic acid, and 4-hydroxycinnamic acid, none of which was detected in the present study. Such difference shows that the host and gut microbiota both contribute to the metabolism of apigenin. Metabolites produced by germ free animals or gut bacteria individually are much simpler than the combined effects. And some of the health benefits of plant flavonoids may be mediated through the smaller end metabolic products.

Similar to the sterile media group in the single strain experiments, apigenin measurement remained stable in fresh modified MShime® medium incubated at 37 °C in darkness over 24 hours.

Some unexpected results were seen with the addition of apigenin to sterile filtered bioreactor supernatant. Apigenin concentration briefly increased in the first 15 minutes, then decreased to about 4 % of the peak concentration in the next 15 minutes. The concentration decreased from 3.96 % to 2.05 % over the next 23.5 hours. The sudden drastic drop of concentration in fermented media without the presence of bacteria is curious as a similar pattern was not seen in the group with bacteria. As the fermented supernatant was filter sterilized, there was no bacteria and nothing larger than 0.20 Micron in the solution. Why would the absence of bacteria and large particles from the media lead to a more rapid degradation? One possible explanation is that apigenin can be degraded by some soluble contents in the media, potentially enzymes released from bacterial cells, however, large particles like the polysaccharides and proteins mentioned earlier protects apigenin by entrapping it and preventing it from becoming substrates for enzymes. Without the protection from a

complex matrix, apigenin were exposed to the efficient degradation of enzymes. However, one drawback of this explanation is that no degradation product was detected. When researching the effects of flavonoids and gut bacteria on each other, the focus tends to be on the flavonoids and the bacteria when there is a third component in the system, the media, containing nutrients, salts, wastes, *etc*., and is constantly mediating all the reactions and interacting with the other two components. Food is a complex matrix and dietary apigenin is always mixed with or attached to food components when it is consumed. It would be of interest to develop *in vitro* models that simulate the release of dietary flavonoids from food matrix and integrate them into the available microbiol ecology simulators.

To further investigate the mechanism and more precise fate of apigenin in both single strain and bacterial community culture, more aggressive separation techniques and isotope labeled apigenin could help understand the distribution of apigenin in the cells and the media matrix, as well as the pathways of generating various metabolic products.

4.5 Conclusion

Based on the results from single strain and community experiments, apigenin inhibited the growth of commensal gut bacteria *E. caccae* during early exponential phase, but it did not have a major effect on the structure and SCFAs production of the human gut microbiota *in vitro*. In *E. caccae* culture, apigenin concentration decreased as the cell number increased and no targeted degradation product were detected. In the human gut microbiota *in vitro* culture, apigenin was fully degraded and 3-(4-

hydroxyphenyl)propionic acid was the main end product among the list of compounds tested. Apigenin was stable in fresh sterile media. The interactions between apigenin and gut microbiota are, in fact, interactions between apigenin, gut microbiota, and the media.

Chapter 5 Conclusion

The objective of the dissertation is to provide an overall review of studies on antimicrobial effects of apigenin and what has been reported on apigenin and human gut microbiota, to study the interactions between dietary flavonoids and human gut microbiota *in vitro*, at both single strain and community levels. There were three sub-objectives to this dissertation, the findings for each sub-objective are summarized as follows.

- Sub-objective 1: To gain an overall understanding of the effects of apigenin on microbes on both a single species and a gut microbiota community.

Based on literature review, apigenin has been shown to possess antibacterial, antiviral, antifungal, and antibacterial activities. The antimicrobial effects of apigenin is strain specific and limited by its solubility. Broth microdilution and agar dilution are the most popular methods to determine the minimal inhibitory concentrations, however, due to system differences, there are discrepancies between the values obtained from the two methods. There seems to be no consensus on which method is better in general.

Reports on the effects of apigenin on commensal gut bacteria under anaerobic conditions is much fewer compared to studies using pathogenic bacteria and aerobic conditions.

Initial information on the interactions between apigenin and the human gut microbiota is available. It is now known that a few gut bacteria are able to degrade apigenin into smaller molecules, some of which are better absorbed than apigenin and are more biologically active. Inconclusive results have been reported on how apigenin affects the structure of the gut microbiota, but likely the effect would be mild due to the dietary

intake level of apigenin and the complex ecological relations within the gut microbiota. We also know that the rate of such interaction depends on the form of apigenin and gut microbiota composition. Our knowledge of how human gut microbiota mediates the health impact of apigenin is limited.

- Sub-objective 2: To study the effects of apigenin on the growth of *Enterococcus caccae* SS-1777 as a single strain and a gut bacteria community *in vitro*, respectively.

  Among the species tested, the most effective inhibition by apigenin was observed on *E. caccae*. Analysis of RNA expression indicated that apigenin affects *E. caccae* cell wall/membrane synthesis and increased the incidence of protein misfolding. *B. galacturonicus* growth was also inhibited by apigenin, while *B. catenulatum* and *L. rhamnosus* GG were not affected. In the bacterial community cultured for 48 hours *in vitro*, apigenin promoted overall growth and diversity.

- Sub-objective 3: To study the degradation of apigenin by *Enterococcus caccae* SS-1777 and a human gut microbiota *in vitro*, respectively.

  Apigenin did not have a major effect on the structure and SCFAs production of the human gut microbiota *in vitro*. In *E. caccae* culture, apigenin concentration decreased as the cell number increased and no targeted degradation product were detected. There were still apigenin in the media at the end of 24 hours. In the human gut microbiota *in vitro* culture, apigenin was fully degraded and 3-(4-hydroxyphenyl)propionic acid was the main end product detected. Apigenin did not degraded when incubated in fresh sterile media.

This dissertation provided the first overall review of studies on antimicrobial effects of apigenin and what has been reported on apigenin and human gut microbiota. The effects of apigenin on *E. caccae* from both phenotypic and genotypic aspects were described and the level of apigenin was measured to show interaction between apigenin and a single gut bacterial strain. The effects of apigenin on both a freshly inoculated and a stabilized gut microbiota community *in vitro* were documented and the degradation of apigenin in the stabilized gut microbiota community was observed with generation of end product of reported bioactivity.

Chapter 6 Recommendations and future work

In future studies, it is worthwhile to keep in mind that the interactions between apigenin and gut microbiota are, in fact, interactions between apigenin, gut microbiota, and the media. We should consider interactions between the three system components when designing the experiments, not only choosing the best media for the bacteria, but also consider its compatibility with the treatment interested, and save samples that can serve as the proper control group.

To determine how much bias and what type will be caused by a culture media, evaluation experiment should not end before a stabilized community has been formed.

It seemed that, in order to inhibit *E. caccae* growth, apigenin treatment might have to be applied at inoculation. To form a thorough understanding of the mechanism of apigenin's effect on *E. caccae*, additional single molecule sequencing may be performed on treated samples from time points earlier than 8 hours, the one tested in this dissertation. To answer why no apigenin degradation product was detected in *E. caccae* culture, more studies are needed to track the distribution of apigenin. Separating bacterial cell components, separating the solids, hydrocolloids, and aqueous phase in the media matrix and measure apigenin concentrations in each of them, perhaps, with the help of isotope labeled apigenin, might overcome challenges posed by the complex bacterial culture media.

Acknowledgment of Previous Publication

The third chapter of the dissertation is composed of an independent article already published. The previously published material used represents the original work of the student. Citation information for the paper is the follows:

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The second and the fourth chapters of the dissertation are intended for future publications.

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