Human Gut Microbiome and Quercetin

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The existing evidence suggests that gut microbiota is capable of flavonoid biotransformation to generate bioactive metabolites including 2,4,6-trihydroxybenzoic acid (2,4,6-THBA), 3,4-dihydroxybenzoic acid (3,4-DHBA), and 3,4-dihydroxyphenylacetic acid (DOPAC).

Keywords: chemoprevention ; colorectal cancer ; flavonoids ; quercetin ; gut microbiome ; bioactive metabolites ; probiotics ; gut health

1. Introduction

In the past, clinical and epidemiological studies largely focused on the ability of flavonols to prevent cancer. Of these flavonols, quercetin has been extensively studied not only for its anticancer effects, but also for its anti-inflammatory, antithrombotic, anti-neurodegenerative, anti-infectious and immunomodulatory activities [1][2]. Quercetin is one of the most abundant flavonoids in the diet and can be found in many foods including onions, apples, grapes, berries, citrus fruits, tea, cherries and broccoli. It has been estimated that humans consume 10–100 mg of quercetin every day on average through their diet [1]. The reported health benefits are predicted to occur through quercetin's (a) interaction with cellular receptors, (b) modification of signal transduction pathways, (c) antioxidant properties and (d) apoptosis regulation, to name a few [3][4][5].

Quercetin, however, is not freely present as an aglycone in most of these food sources. It is usually conjugated to a sugar moiety (forming glycosides) that confers water solubility and chemical stability to these compounds [6]. The sugar moiety attached to quercetin is usually glucose or rhamnose, but it could also be galactose, arabinose, xylose or other sugars [7]. Quercetin glycosides are poorly absorbed from the intestines, and hence these linkages are cleaved by enzymes present either in the small intestine or the colon to facilitate absorption [8][9]. As the enzymes produced by the human body are sometimes incapable of cleaving the glycosidic linkages, microbial enzymes produced in the gut have been implicated in these deconjugation reactions. For example, studies have suggested that the hydrolysis of rutin (quercetin-3-O-rutinoside; from onions, berries, etc.) to quercetin occurs through the action of gut microbiota [10][11]. Studies highlighting the importance of gut microflora in human health are now emerging, wherein the microbiota is considered as a separate organ in itself [12][13][14][15].

Numerous studies have implicated the dysregulation of the microbial ecology in the intestines to be a cause of colorectal cancers (CRC). These studies have also indicated that diet is a major factor governing dysbiosis wherein regular intake of fruits and vegetables seems to favorably shift the ecology of gut microbes to a cohort that can prevent the occurrence of CRC [16][17][18][19][20][21]. Increasingly, studies are also implicating a beneficial role of microbial metabolites in human health, especially in cancer [22][23][24]. Taken together, these studies are indicative of the importance of gut microbes and their flavonoid catabolites in influencing human health. Hence, identifying the mechanisms involved in flavonoid catabolism and the various microbial enzymes involved in this process would aid in understanding and establishing the key contributors to the generation of beneficial metabolites.

Studies conducted thus far have identified quercetin-2,3-dioxygenase (quercetinase) as the first enzyme in *Bacillus spp.* that is required for the metabolism of quercetin [25]. Cleavage of quercetin by this enzyme followed by the action of gut esterases on the intermediary product (2-(3,4-dihydroxybenzoyloxy)-4,6-dihydroxybenzoate) can yield 2,4,6-trihydroxybenzoic acid (2,4,6-THBA) and 3,4-dihydroxybenzoic acid (3,4-DHBA) which are shown to have antiproliferative properties in cancel cell lines. Other studies have also demonstrated that alternative pathways may also exist for quercetin degradation, generating 3,4-dihydroxyphenylacetic acid (DOPAC) through biotransformation of this flavonoid by other bacteria [23][26]. The generation of DOPAC from quercetin is predicted to occur through the involvement of phloretin hydrolase [27]. A recent study also suggested the involvement of ene-reductases in the biotransformation of flavones and flavonols [28]. Apart from these enzymes, other enzymes known to be involved in flavonoid metabolism include chalcone

isomerase and enoate reductase from *Eubacterium ramulus* [29] as well as peroxidases, dehydrogenases, demethylases and tyrosinases from a variety of bacteria [30].

2. Current Insights on Human Gut Microbiome and Quercetin

The flavonoid quercetin has been shown to have health benefits against numerous disorders including inflammation, hypertension, obesity and atherosclerosis [31] and in the prevention of many types of cancers [3][17]. Increasing evidence in the literature suggests that the metabolites of quercetin may be responsible for these observed health benefits. Three observations that support the role of quercetin metabolites in human health include (1) pH-dependent degradation of quercetin in the intestines (in the basic environment), (2) low absorption of quercetin in the intestines resulting in low bioavailability and (3) the biotransforming capability of resident gut microflora. In this regard, the bacteria responsible for the biotransformation of flavonoids such as quercetin are poorly understood. Therefore, we performed screening of the gut microbiota culture collection developed by our group to identify the species responsible for quercetin biotransformation. Our screening yielded five bacteria capable of degrading quercetin. These include *B. glycinifermentans*, *F. plautii*, *B. eggerthii*, *O. scatoligenes* and *E. eligens*. The results presented in this study also demonstrate the ability of select human gut bacteria to generate the metabolites 2,4,6-THBA, 3,4-DHBA and DOPAC. These results now tie in well with our previously published reports where we demonstrated the ability of some of these hydroxybenzoic acid metabolites (2,4,6-THBA, 3,4-DHBA and 3,4,5-THBA) to inhibit cancer cell growth [24]. This report also constitutes the first demonstration of quercetin degradation by *B. glycinifermentans, B. eggerthii, O. scatoligenes* and *E. eligens*. All the five species identified in this study have been demonstrated to be present in human fecal content, suggesting that these species in the gut are capable of biotransforming quercetin. Our demonstration of the ability of *F. plautii* and *Lactobacillus* species (positive control) to degrade quercetin is also consistent with the previously published reports [23][26][32][33].

Although *F. plautii* was previously reported to degrade quercetin to generate DOPAC, the research described in this report for the first time identified the ability of *B. glycinifermentans* to biotransform quercetin to generate 2,4,6-THBA and 3,4-DHBA. *B. glycinifermentans*, which was reported to be part of human fecal content by Ghimire et al. [34], was initially characterized as being present in fermented soybean paste, hence the name *B. glycinifermentans* [35]. Interestingly, based on the analysis of its complete genome, this bacterium was suggested for use as a probiotic for livestock to enhance immune stimulation, enzyme production and pathogen inhibition [36]. The link between our observation that it is capable of biotransforming quercetin to generate 2,4,6-THBA and 3,4-DHBA and its suggested use as a probiotic in the previous report [36] makes this bacterial strain an interesting candidate for the maintenance of human gut health.

The detection of DOPAC as a metabolite of quercetin generated by *F. plautii* in this study confirms the previous reports in literature [32]. In this study, we did not detect the presence of 2,4,6-THBA and 3,4-DHBA in the spent medium from *F. plautii*. Although the reason for this is currently unclear, it is possible that the amounts of the other metabolites (2,4,6-THBA and 3,4-DHBA) generated from this bacterium might have been below the detection levels of the HPLC technique employed. Similarly, the lack of detection of DOPAC in the spent medium from *B. glycinifermentans* may be attributed to its low levels in the samples. It is to be noted that TBLASTN analysis revealed the presence of homologs of quercetinase, phloretin hydrolase and pirin-like protein in both *B. glycinifermentasns* and *F. plautii*. Consistent with this, both species exhibited the presence of transcripts for quercetinase and phloretin hydrolase; however, the abundance of pirin-like protein implicated in quercetin degradation appears to be differentially regulated with a more than two-fold increase in *B. glycinifermentans* and a more than two-fold decrease in *F. plautii*. This differential regulation may account for the differences in the metabolites produced as detected by HPLC. Alternatively, it is also possible that the quercetin degradation pathway utilized by *F. plautii* is radically different from that of *B. glycinifermentans* and hence generates different metabolites. This is supported by the observation that the spent culture supernatant from *B. glycinifermentans* had quercetin-degrading enzyme activity while the culture supernatant of *F. plautii* did not. This observation also suggests that the quercetin-degrading enzymes in *B. glycinifermentans* and *F. plautii* are likely to be differentially localized (secreted vs. membrane-bound/intracellular). Furthermore, the degradation kinetics demonstrates that *F. plautii* completely degrades quercetin within 12 h of incubation, whereas *B. glycinifermentans* requires around 48 h for complete quercetin degradation. This, along with the minimal enzyme activity detected in the cell lysate, suggests that the degradation of quercetin by *F. plautii* may require the presence of live bacterial cells. A recent study by Yang et al. demonstrated the involvement of ene-reductase, chalcone isomerase, enoate reductase and phloretin hydrolase in the generation of metabolites from flavones and flavonols [28]. Hence, the lack of quercetin degradation in the culture supernatant and minimal activity in the pellet observed in our study may have also been related to the requirement of four different enzymes for the generation of DOPAC from quercetin. Alternatively, it is also possible that the enzymes involved in *F. plautii* are sensitive to the experimental conditions or require other cofactors when assayed in vitro, which may not be the

case for *B. glycinifermentans*. The observation that pirin-like protein, which was previously reported to have quercetin-2,3-dioxygenase activity [37], was differentially expressed in *B. glycinifermentans* and *F. plautii* may provide a link to the differences observed in the generation of metabolites between the two species of bacteria. Hence, further research should shed light on the specific pathways utilized by these bacteria for quercetin biotransformation.

The importance of 2,4,6-THBA and 3,4-DHBA in the inhibition of cancer cell growth was well-established by our group previously [24], and in the present study, we demonstrated that *F. plautii* is able to produce DOPAC at concentrations sufficient to produce an antiproliferative effect on cancer cells when tested ex vivo. It is to be noted that DOPAC has also been shown to inhibit cancer cell proliferation by other investigators in various cancer cell types [22][26][38]; this is believed to occur through its antioxidant properties. The demonstration of the ability of the bacterial culture supernatant of *F. plautii* grown in the presence of quercetin to inhibit colony formation is, to our knowledge, the first report showing the direct effect of bacteria-generated metabolites on cancer cell growth and, therefore, is a very significant finding. We observed that while 1 mL supernatant from the *F. plautii* culture was sufficient to inhibit colony formation in the HCT-116 cells, 1 mL culture supernatant from the *B. glycinifermentans* was insufficient to exert a similar inhibitory effect. Quantification of the metabolites indicated that the amount of 3,4-DHBA generated in our experiments from *B. glycinifermentans* was lower (40.66 µM) than that required for effective inhibition (250 µM) [24]. As the addition of >1 mL bacterial culture supernatant to 10 mL cell culture medium may affect osmolarity of the medium and, in turn, cancer cell growth in the culture, the effect of larger volumes of bacterial supernatants on colony formation was not tested. As an alternative strategy, we performed concentration of the supernatants through rota-evaporation at 65 °C under vacuum conditions. Addition of the 5× and 10× concentrated samples dissolved in the cell culture medium failed to inhibit cancer cell growth (data not shown) for the supernatant obtained from the *F. plautii* culture. We believe that this could be related to the instability of DOPAC during the process of concentration; for example, temperature may affect the stability of DOPAC. Therefore, alternative methods of concentration need to be explored to demonstrate the effectiveness of bacterial supernatants where metabolites are generated at lower concentrations (such as for *B. glycinifermentans*) against cancer cell growth.

While the human gut is known to harbor 300–500 species of bacteria [39][40], this study investigated the potential of only 94 bacterial species to degrade quercetin. Therefore, additional screening is required to establish the contribution of other bacterial strains to CRC prevention as well. However, it is interesting to note that of the 94 strains screened, only five exhibited the ability to degrade quercetin. This suggests that the flavonoid-biotransforming ability may be narrowly restricted to only a few species of bacteria, highlighting the importance of these bacteria in the prevention of CRC. While the focus of our study was on quercetin, it is to be noted that the diet also contains other flavonoid members (such as anthocyanins, epigallocatechin gallate, catechins, cyanidin-3-glucoside, etc.) that may be biotransformed by the gut microflora [41]. For example, DOPAC, 2,4,6-THBA and 3,4-DHBA have been reported to be metabolites produced upon green and black tea consumption [22][42] while 2,4,6-THBA and 3,4-DHBA have been demonstrated to be generated upon the consumption of the anthocyanin, cyanidin-3-glucoside [9]. Additionally, phenolic acids have also been shown to be produced from the intestinal degradation of fibers by colonic bacteria [43].

In this study, the bacterial strains were grown individually to screen for their ability to degrade quercetin, but it is still unknown how quercetin may be degraded in the presence of other bacteria, in cocultures. It is known that some bacterial species influence the growth of others; diet is also suggested to contribute to this selection [41][44]. Therefore, additional studies are required to establish how diet influences the growth of these species and overall degradation of quercetin in vivo. It will be interesting to explore the metabolism of quercetin by other species of bacteria, enzymes involved in this process and characterize the metabolites generated individually or in a community setting. Additional studies are also required to test the bacterial culture supernatants containing these metabolites for their ability to inhibit cancer cell proliferation.

3. Conclusions

The research described in this report identified five species of bacteria capable of degrading quercetin to give different bioactive metabolites, some of which have been previously characterized to have antiproliferative effects against cancer cells. This study also established clear differences between two bacterial species (*B. glycinifermentans* and *F. plautii*) in terms of their ability to degrade quercetin; in addition, it also showed the generation of different metabolites. We also demonstrated for the first time the inhibitory effect of the bacterial culture supernatant from *F. plautii* against cancer cell growth, paving the way for similar studies with other bacterial culture supernatants. We believe that bacteria-mediated biotransformation of flavonoids and generation of bioactive metabolites are important contributors to colorectal cancer prevention observed in flavonoid-rich diets.

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