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BIOTRANSFORMATION OF RESVERATROL AND ITS IMPLICATIONS IN BIOLOGICAL ACTIVITIES IN THE COLON

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BIOTRANSFORMATION OF RESVERATROL AND ITS IMPLICATIONS IN BIOLOGICAL ACTIVITIES IN THE COLON

A Dissertation Presented

by

FANG LI

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DEDICATION

I dedicate this thesis to

my beloved families and friends

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I would like to express my sincere gratitude to my advisor, Dr. Hang Xiao, for providing me an opportunity to fulfill my dream in his group. With his continuous support, his kind guidance, his motivation and insight on science, I was trained and prepared to be a mature researcher. Without his help and guidance, I cannot complete this work. I treasure the time and experience in Xiao's group. Also, I would like to thank my committee members, Dr. Guodong Zhang and Dr. Richard J. Wood, for their kindly guidance, valuable time, feedbacks and recommendations that improve the quality of this dissertation.

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ABSTRACT

BIOTRANSFORMATION OF RESVERATROL AND ITS IMPLICATIONS IN BIOLOGICAL ACTIVITIES IN THE COLON

SEPTEMBER 2018

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Resveratrol (RES) is a natural polyphenol compound with a wide range of health-promoting activities, including protective effects against colon cancer and renal disease. However, the premise of these benefits has been dampened since RES shows a poor oral bioavailability due to its rapid and extensive biotransformation after oral consumption. The paradox (low bioavailability but high bioactivity) warrants further investigations to determine the contribution of RES metabolites to the health benefits associated with RES. We identified 11 metabolites of RES in mice with high-resolution HPLC-MS/MS, then quantified two major metabolites - dihydro-resveratrol (DHR) and lunularin (LUN). To further understand the chemopreventative effects of RES metabolites in the kidney and colon, the inhibitory effects of RES, DHR, LUN, and their combinations at the

concentrations equivalent to those found in mouse tissues were determined in corresponding cell models. Our results demonstrated that DHR and LUN exhibited much stronger anti-inflammatory, anti-clonogenic and anti-proliferative effects than did RES at physiologically relevant levels. Moreover, the combination of RES, DHR and LUN produced the strongest inhibitory effects, while the contribution of RES was marginal. DHR and LUN might be the main force that responsible for the chemopreventive effects attributed to RES.

Accumulating evidence indicated that gut microbiota plays important roles in the pathogenesis of colitis, and microbiota composition could be modulated by dietary components. Therefore, ameliorating colitis-associated bacterial dysbiosis by dietary components may be a unique strategy to improve gut health. Herein, we determined the effects of resveratrol on gut microbiota and their implications in anti-colonic inflammation in mice with colitis induced by dextran sodium sulfate (DSS). Our results reinforce the protective effects of resveratrol in intestinal inflammation by alleviating the body weight loss, reducing the disease activity index, attenuating tissue damage and down-regulating the expression of pro-inflammatory cytokines such as IL-2, GM-CSF, IL-1β, IL-6 and TNF-α in the colon of DSS-treated mice. Moreover, dietary resveratrol restored the microbial richness and evenness in DSS-treated mice. Specifically, at the genus level resveratrol effectively reduced the richness of *Akkermansia, Dorea, Sutterella* and *Bilophila* and increased the abundance of *Bifidobacterium* in colitic mice. Furthermore, a

Pearson's correlation analysis indicated that resveratrol suppressed pro-inflammatory cytokines were strongly correlated with gut microbiota composition. Overall, our results suggested that dietary resveratrol attenuated inflammation perhaps by modulating the microbial composition.

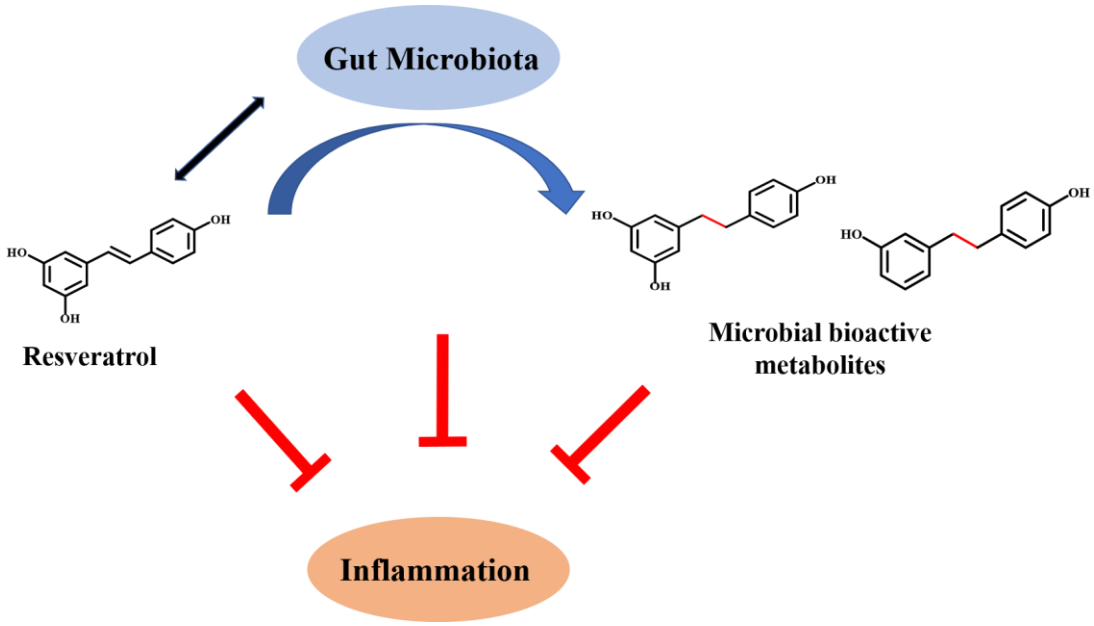

Fig. 1 Graphic abstract

The necessary role of gut microbiota in the biotransformation of RES was evidenced by antibiotic treated mice. DHR, LUN, and their conjugates were completely absent in the antibiotic treated mice. Moreover, we found that in colitic mice LUN and its conjugates were extinguished, which may associate with the altered gut microbiota composition and structure. Most interestingly, we found that RES metabolites at the concentrations equivalent to that observed in the colonic tissues in colitic mice exhibited significantly stronger chemopreventative effects than that observed in the healthy mice.

These results suggested that absence of LUN may resulted in stronger biological activities of RES. Overall, our results provided a solid scientific basis for understanding the chemopreventive mechanisms of RES from the perspective of biotransformation and gut microbiota and are of great value for future research on RES in prevention and treatment of colonic diseases in humans.

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CHAPTER 1

INTRODUCTION

Resveratrol (*trans*-3, 5, 4'- trihydroxystilbene, RES), a stilbenoid, abundant in grapes, wines, peanuts and mulberries. Its health beneficial bioactivities have been recognized and studied widely. Accumulating evidence demonstrated the superior pharmacological properties of RES such anti-cancer [1], anti-diabetes [2], anti-oxidant [3] and cardiovascular protective effects [4]. However, RES is subjected to extensive biotransformation mediated by drug-metabolizing enzymes, which may produce metabolites with different bioactivities in comparison with the parent compound [5, 6]. Therefore, it is critical to establish the detailed information of the biotransformation and tissue distribution of RES to better understand its mechanism of action. On the other hand, metabolites may achieve higher concentrations than their parent compound at certain tissues [7]. Therefore, the metabolites rather than the consumed components, may mainly responsible for the biological effects at site of function. Regarding these concerns, one of our current thesis aims is to identify and quantify metabolites of RES present in mouse tissue, gastrointestinal tract (GIT) and biological fluids. And the potential chemopreventive effects of RES metabolites were further investigated in corresponding cell-based models.

Over 1 million residents in the USA are estimated to be suffering from inflammatory bowel disease (IBD) [8]. And one of the most important and devastating complications of

long-term IBD is colorectal cancer development [9]. Phytochemicals enriched diet have been explored as a prophylactic tool to moderate the disease course [10]. Consistent results showed that RES could down-regulated inflammatory biomarkers, reduced oxidative stress and attenuated clinical symptoms in experimental murine colitis models [1, 3, 11]. However, the contribution of its metabolites in this process is not clear. Moreover, the implication of gut microbiota dysbiosis on the pathogenesis of IBD has been highlighted recently [12]. High accumulation of RES and its metabolites in cecum and colon suggesting the dual interactions existing between gut microbiota and RES. Therefore, it is of great significance to explore the mechanisms of anti-colitis effects of RES from the gut microbial and metabolic perspectives.

The microbial biotransformation of dietary polyphenols, including ring fission, reduction, demethylation, hydrolyzation glycosides, and dihydroxylation, has been well described [13, 14]. On the one hand, significantly interindividual differences exist in gut microbial composition, which may lead to different microbial transformation patterns of polyphenols. As in the case of production of either equol or *O*-demethylangolensin and dihydrodaidzein from daidzein [15]. Given the complex metabolism of other polyphenols by gut microbiota, we speculated the pronounced interindividual different routes of resveratrol biotransformation by the human gut microbiota. On the other hand, gut microbiota composition could be dramatically altered in various diseases [16]. It is crucial to systematically discuss the effects of altered gut microbiota on the biotransformation of

resveratrol and its important implications in biological activities of RES.

Our ***long-term goal*** is to the set up a database of bioavailability and bioactivities of diet-based resveratrol administration in different diseases models and elucidate its implication on various chemopreventive effects of RES. To reach this goal, our ***main objective*** of this dissertation is to elucidate the biotransformation of resveratrol and its implication on gut inflammation in DSS-induced colitis model. Our ***central hypothesis*** is that resveratrol is extensively metabolized *in vivo*, and its metabolites are at least partially responsible for the chemopreventive effects that attributed to RES. Dietary resveratrol will restore the microbial dysbiosis in the colitic mice. In turn, its metabolic profile will be changed in colitic mice. As a result, the biological activities of resveratrol will be different in colitic mice.

We will test our central hypothesis and achieve our objective by completing the following ***specific aims***:

Specific Aim 1: Identification and quantification of resveratrol metabolites in mice tissue after sustained oral consumption of RES. Mice will receive 0.5% (w/w) of RES for four weeks. RES metabolites will be identified with Orbitrap HPLC-MS/MS in the urine and fecal samples. The concentrations of RES metabolites will be quantified with HPLC-MS in the organs/tissues, serum, bile, and gastrointestinal tract.

Specific Aim 2: Evaluate the chemopreventive effects of RES metabolites at

physiological levels in cell-based model. The *in vitro* anti-inflammatory efficacy of RES metabolites will be determined in lipopolysaccharide (LPS)-stimulated RAW 264.7 macrophages. The in vitro growth inhibitory effects of RES metabolites on human renal carcinoma cell lines and colon cancer cell lines will be determined by using cell viability assay and colony formation assay.

Specific Aim 3: Evaluate the effects of dietary resveratrol on gut microbiota and its implication on gut inflammation in DSS-induced colitic mice. Dextran sulfate sodium (DSS)-induced colitis mice model will be used to assess the anti-inflammatory efficacy of RES *in vivo*. Histological evaluation will be performed to assess the colon morphological damage. ELISA assay will be used to determine the expression of inflammatory cytokines. Gut microbiota composition will be analyzed with 16S-RNA based sequencing technique.

Specific Aim 4: Determine the role of gut microbiota in biotransformation of RES and its implication in the anti-inflammation in the colon. Broad spectrum antibiotic will be used to wash out the gut microbiota in mice. The metabolites of RES will be identified and quantified with HPLC-MS in antibiotic treated mice. 300 mg of resveratrol capsules will be assigned to eleven healthy volunteers every day for three days. The metabolites in the urine samples will be quantified with HPLC-MS. We expect to observe pronounced interindividual differences in RES biotransformation induced by gut microbiota. The metabolites of RES in the cecum, colon, bile, liver, kidney, and serum in healthy mice and DSS-treated mice will be analyzed and compared with HPLC-MS. We expect to observe

distinguished metabolism patterns of RES in healthy mice *vs* DSS-treated mice. The *in vitro* anti-inflammatory efficacy of RES metabolites will be determined in lipopolysaccharide (LPS)-stimulated RAW 264.7 macrophages. The in vitro growth inhibitory effects of RES metabolites on human colon cancer cell lines will be determined by using cell viability assay.

CHAPTER 2

LITERATURE REVIEW

2.1 Introduction of biotransformation

2.1.1 Biotransformation

Since the chemopreventive agents in food are subjected to digestion, understanding of metabolic fate of bioactive food components is crucial in elucidation the efficacy of these compounds. Dietary bioactive compounds are regarded as xenobiotics by human body. Therefore, as a self-defense, biotransformation will take actions to chemically modify dietary bioactive compounds and eliminate them. Biotransformation can be defined as production of chemical changes on compounds that are not their natural substrates by using the biological systems [17]. In general, biotransformation reactions are divided into two categories known as phase I and phase II metabolism. Phase I reactions including oxidation, reduction and hydrolytic reaction, are mediated by enzymes such as CYP family and amidases [18]. In phase II reactions, small polar molecules, like sulfate and glucuronides, conjugate with appropriate functional groups of substrates. Glucuronosyltransferase, sulfotransferase and N-acetyltransferase are three most common enzymes that mediate phase II metabolism [19]. Although the process of biotransformation on one hand produces active metabolites from some dietary compounds [7]. On the other hand, it reduces the plasma concentrations of dietary compounds [5]. Therefore,

elucidating the biotransformation of bioactive compounds is essential to better define the bioavailability of dietary bioactive compounds and further evaluate their biological activity.

2.1.2 Implication of biotransformation in bioavailability

Bioavailability theoretically defined as the fraction of the administered dose that reaches the general circulation unchanged [20]. It often characterized by the absorption, tissue distribution, metabolism and elimination of the dietary bioactive compounds.

Different bioactive compounds show different rates of absorption. Biotransformation can be a speed-limit step. Some of flavonoids naturally occurred as glycosides in plants, which means glycoside moieties are attached to the flavonoids backbone. Glucosides need to undergo deglycosylation prior to be absorbed [21], which is carried out by intracellular cytoplasmic β-glucosidase [22]. For example, quercetin-3-*O*-rutinoside is deglycosylated by rhamnosidases and β-glucosidase before absorbing by epithelial cells [23]. However, recent evidence suggests that anthocyanins, e.g. cyanidin-3-glucoside, are rapidly and efficiently absorbed in the small intestine intact without deglycosylation [24].

The biotransformation also plays an important role in the disposition of dietary compounds, especially in the enteroenteric and enterohepatic circulation. Small intestine and liver are the crucial tissues responsible for various biotransformation leading to different conjugated forms of dietary bioactive compounds. However, kidney and other tissue also involved in the metabolism of dietary bioactive compounds. Take anthocyanins

as an example (Fig. 2) [20]. After ingestion, only native anthocyanins are detected in stomach. Once arriving at small intestine, extensive glucuronidation, sulphoation and methylation of anthocyanins occurs. After absorption and intestinal biotransformation, hepatic metabolism of anthocyanins takes place. Through enterohepatic recirculation, part of the resultant anthocyanins can be recycled back to the small intestine through bile excretion. As a result, anthocyanins metabolites (methylated and glucurono-conjugated derivatives) are identified in various organs including bladder, prostate, testes, heart, adipose tissue, liver, eyes, cerebellum and kidney as shown in Fig.2. The rest of anthocyanins will pass from the small intestine to the large intestine where the gut microbiota further bio-transform anthocyanins and their conjugates.

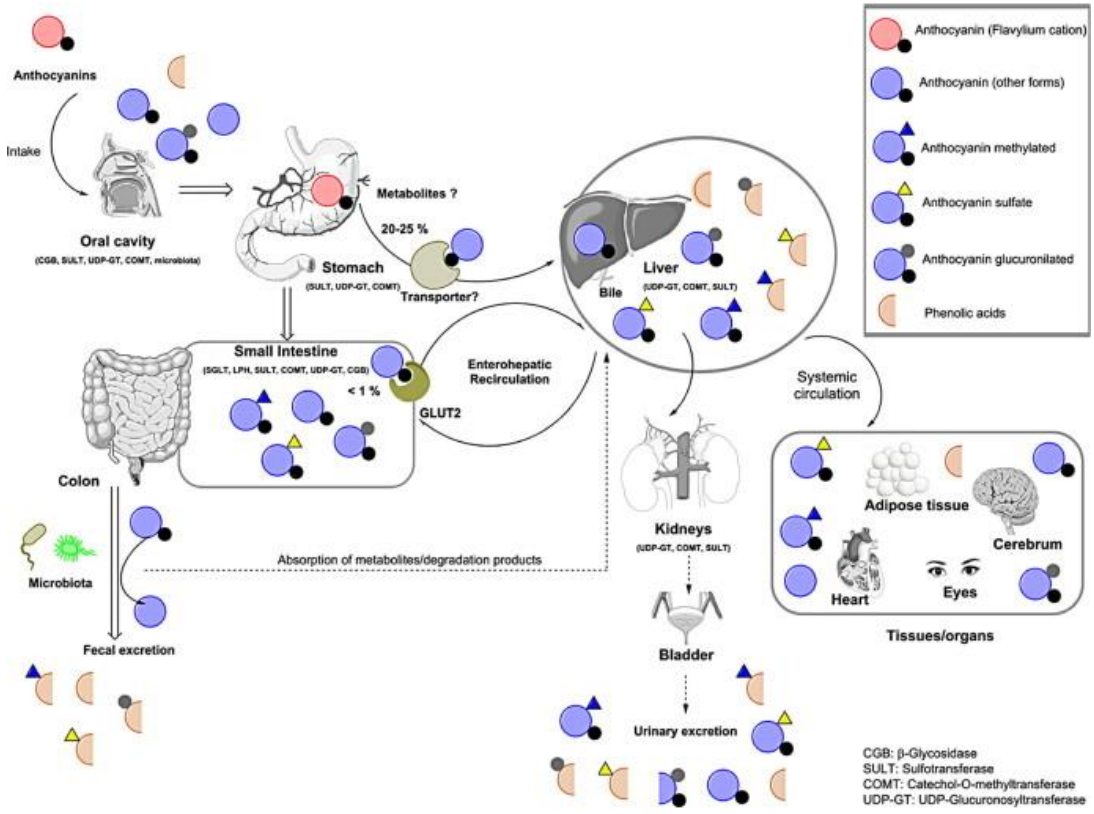


Fig. 2 Hypothetic pathways of anthocyanins absorption, distribution, metabolism and excretion based on current information. Adopted from reference [17].

2.1.3 Implication of biotransformation on bioactivity

Biotransformation of dietary bioactive compounds affects properties such as mass, charge and hydrophobicity, which may influence their ability to cross biological membranes. It is also likely impacting their half-life in plasma and rates of excretion, further influences their bioactivities. Quercetin is one of the most extensively studied polyphenols. It has been shown that some conjugates of quercetin possess significant anti-oxidative, anti-inflammatory and anti-angiogenic properties [25, 26]. Conjugates derivate from dietary polyphenols often have weakened bioactivities compared to the parent compounds, and this seems to be the case for RES based on previous studies [6]. While, Wu et al. demonstrated that metabolites of nobiletin showed much stronger anti-carcinoma and anti-inflammatory effects than nobiletin itself [27]. Therefore, it is important to determine the impacts of biotransformation on biological activities of dietary bioactive compounds.

2.2 Inflammatory bowel disease

2.2.1 Introduction of inflammatory bowel disease

Inflammatory bowel disease (IBD), including relapsing-remitting inflammatory

disorders Crohn's disease and ulcerative colitis, is a chronic inflammatory disease of gastrointestinal tract [28]. Over 1 million residents in the USA are estimated to be suffering from IBD [8]. One of the most important and devastating complications of long-term IBD is colorectal cancer development [9]. The precise etiology of IBD remains unknown, the most accepted hypothesis to date is that aberrant and continuing immune response against the commensal gut microbiota in genetically susceptible hosts [28, 29]. Fig. 3 summarized

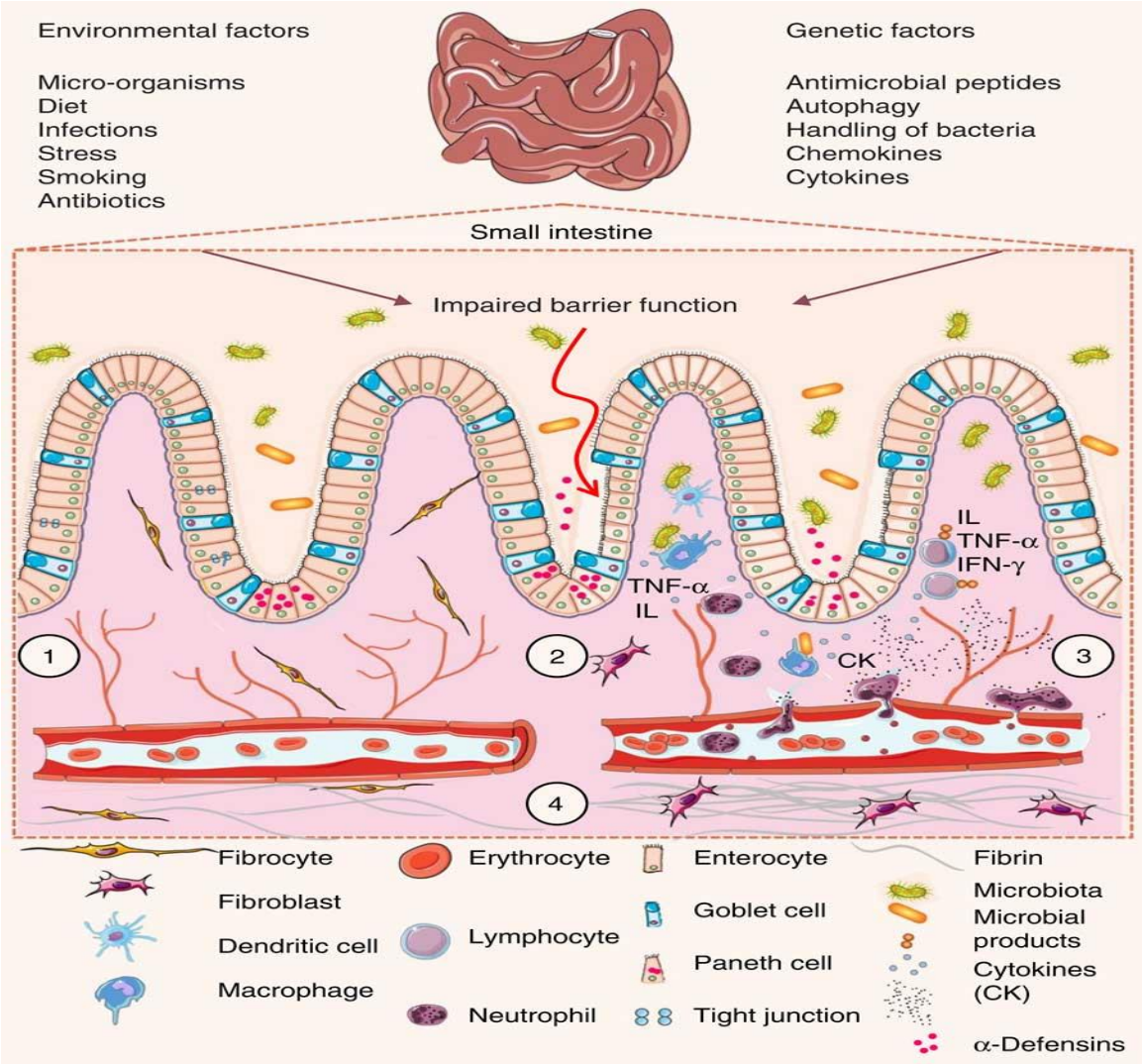


Fig. 3 Pathophysiology of inflammatory bowel disease. Adopted from reference [30].

The proposed pathophysiology of IBD. Tight junction is disrupted due to genetic or environmental factors, which causes increased the change of uptake of commensal bacteria and microbial products. Immune cells including macrophages, dendritic cells lead to immune system activation and cytokines production. If acute inflammation cannot be resolved by immune systems, chronic intestinal inflammation develops [30].

2.2.2 Preventive effects of phytochemicals on inflammatory bowel disease

IBD conduct a growing health concern due to increasing incidence worldwide. The current notion on pathogenesis of IBD is the involvement of diet, gut microbiome and epigenetics. Among these factors, diet plays an important role in modulating the gut microbiota and influencing epigenetic changes [10]. Therefore, improving diet quality could be applied as a prophylactic tool to moderate the disease course. Consequently, many phytochemicals enriched diet have been explored as preventive treatments recently [31]. Previous studies indicated that high prevalence of IBD in northern Europe, United Kingdom and North America may associated with their typical diet [32]. Paik et al demonstrated that high-fat diet exacerbated IBD in Mdr1a-/- male mice [33]. While, dietary polyphenols such as curcumin, quercetin, ellagic acid and resveratrol possess both protective and therapeutic effects in the management of IBD via enhancing antioxidant defense and suppressing inflammatory pathways [34]. Galvez et al. indicated that rutin can

alleviate the inflammation and mucosa lesion by modulation the levels of internleukin-1 and tumor necrosis factor alpha [35]. Green tea polyphenols are widely studying for their strong anti-oxidative effects. Several studies demonstrated the preventive effects of green tea polyphenols on IBD [36].

2.3 Gut microbiota

2.3.1 Introduction of gut microbiota

The collection of bacteria, archaea, and eukarya living in the gastrointestinal tract (GIT) is termed as the "gut microbiota" [37]. The number of bacterial cells inhabiting in the GIT accounted ~10 times more than the number of human cells [37]. Gut microbiota exerts a marked influence on the health of host by metabolizing nutrients, harvesting energy, regulating immune system, and protecting intestinal epithelium [38]. Alteration of gut microbiota composition (dysbiosis) is associated with the pathogenesis of many disease.

Advanced sequencing techniques including 16S rRNA based bacterial sequencing and whole-genome shotgun metagenomics allowed researches to survey the breath of the gut microbiota. 16s rRNA is a popular approach, since 16S ribosomal RNA gene is present in all bacteria and archaea, which allows species to be easily identified [39]. Whole-genome shotgun metagenomics is more reliable than 16S rRNA based sequencing techniques due to its higher resolution [39]. MetaHit and the Human Microbiome Project have provided

the comprehensive data of human gut microbiota [40] 2172 species have been identified from human samples, which are classified into 12 phyla, of which 93.5% belonged to *Proteobacteria, Firmicutes, Actinobacteria and Bacteroidetes* [40].

Gut microbiota begin to develop since from birth. In the early stage, the microbiota is in low diversity and dominated by Actinobacteria and Proteobacteria[41]. In the first year of life, the microbial diversity increases, and the microbiota composition is distinct from microbial profile in the adult [42]. By around 2.5 years old, the microbial diversity, function, and composition are similar with adults [41]. Recently, cohort studies indicate that gut microbial composition shifted, function compromised, and diversity decreased in elder populations [43].

2.3.2 Diet shapes the gut microbiota

Diet can rapidly and reproducibly alter gut microbiota [44]. Food components (e.g. fiber, polysaccharides, polyphenols), which are non-digestible to human enzymes, are subjected to the intestinal microbial metabolism.

Dietary patterns, such as Western diets or plant-based diet, are strongly associated with distinct gut microbiota compositions [44]. Previous studies observed that *Clostridum cluster XIVa, Bcteroides/Prevotella, Bacteroides thetaiotaomicron*, and *Faecalibacterum prausnitzii* were enriched in vegetarians compared with omnivores [45]. Moreover, another comprehensive research reported that irrespective of age, the least microbial diversity was

observed in adults of Americans *vs* Venezuela and Malawi [46].

Specific foods, such as whole grains, fruits, and vegetables, can partially get rid of the metabolizing in the upper GIT, reach to the large intestine, and interact with gut microbiota. Martinez et al. demonstrated that whole grains interventions led to an increased microbial diversity, as well as a rise in the Firmicutes/Bacteroidetes ratio [47]. A placebo-controlled crossover study revealed that wild blueberry drink enriched the gut microbial diverstity and increased the amount of *Bifidobacterium spp* [48]. After consumption of red wine that abundant with polyphenols, the relative counts of *Bifidobacterium, Enterococcus, and Eggerthella lenta* were increased [49].

It is obvious that diet has an important influence on the structure and composition of gut microbiota. Furthermore, the end products of bacterial metabolism, especially vitamins and short-chain fatty acids (SCFA), are vital for human health.

2.3.3 Microbial biotransformation of dietary polyphenols

It has been estimated that only 5-10% of the total intake polyphenol can be absorbed in the small intestine. The remaining 90-95% of intake polyphenol may accumulate in the large intestinal lumen, where they are subjected to the enzymatic activities of gut microbial community [50]. Currently, it is estimated that 500-1000 different microbial species inhabit the gastrointestinal tract, reaching the highest concentrations in the colon (up to 10^{12} cells per gram of feces) [50]. Thus, the encountering of gut microbiota and polyphenols can lead

to the development of two-way mutual reactions. In one direction, polyphenols are bio-transformed into their metabolites by gut microbiota that may results in the changed biological activities. Bacterial metabolites are different from those that can be generated by transferase enzymes because bacterial processes occur under anaerobic conditions and are based mainly on reactions of reduction and/or hydrolysis.

Accordingly, luteolin is metabolized by colon microbiota (*C. orbiscindens, Enterococcus avium*), breaking down its C-ring towards to phloretin chalcone, 3-(3,4-dihydroxyphenyl) - propionic acid and 4-hydroxycinnamic acid, which are absorbed and excreted by urine [51]. Ellagic acid is largely metabolized by the gut microbiota, giving rise to urolithin A and its analog known as urolithin B [52]. However, it is noteworthy that there is a large interindividual variation in colonic microbiota composition, which may cause variations in the timing, quantity, and types of metabolites produced in large intestine.

In another way, polyphenols modulate the composition of gut microbial community mostly by enhancing the probiotics and inhibiting the pathogenic bacteria. The interactions between dietary bioactive compounds and gut microbiota may impact on human host health. Several polyphenols have been recognized as potential prebiotics that can enrich the beneficial bacteria in gut. It has been reported that grape seed extract resulted in the dramatically increasing of *Lactobacillus*, *Lachnospiraceae* and *Ruminococcaceae* [53]. It has been demonstrated that dietary supplemented-curcumin increased the abundance of *Lactobacillales*, and decreased *Coriobacterales* order in AMO/Il10 model [54].

Pterostilbene, an analog of resveratrol, modified intestinal bacteria composition toward to a healthier microbial profile in obesity rats [55]. Overall, two-way, reciprocal interactions of gut microbiota and phenolic compounds have an important impact on the bioavailability of phenolic compounds and human health.

2.3.4 The role of gut microbiota in inflammatory bowel disease

The gut microbiota exerts a marked influence on the host during homeostasis and disease. The possible implication of gut microbiota in the pathogenesis of IBD has been highlighted recently. Multiple lines of evidence suggested the important role of gut microbiota in the progress of IBD. Firstly, Metagenomic and 16S RNA based sequencing studies demonstrated reduced diversity and altered composition of gut microbiota in patients with IBD compared with healthy subjects [56-58]. Secondly, germ-free animals were protected against colitis development in DSS-treated mice [12]. Thirdly, IL-10-deficient mice, a murine model of IBD, did not develop colitis under germ-free conditions [59]. All these results suggested that microbial factors might directly contribute to the development of colonic inflammation and adverse metabolic consequences.

The gut microbiota has become an important target in the treatment of IBD, and therapy has focused on correcting intestinal microbial imbalance. The gut microbiota, including symbiotic, probiotic, and pathogenic microorganisms, plays an important role in human health. Its balance can be destroyed by the introduction of invasive pathogens,

activation of immune cells, and production of pro-inflammatory cytokines [60]. Meantime, interaction of dietary compounds and gut microbiota have been shown in great part to affect the immune response and the inflammatory status [61]. Unravelling the complex interactions underlying diet, IBD and gut microbiota will have implications for the development of novel therapies.

2.4 Resveratrol

2.4.1 Introduction of resveratrol

Natural phytochemicals presenting in our diet have seen a wide range of acceptability for the prevention and treatment of diseases. Resveratrol (3,4',5-trihydroxystilbene) is a polyphenolic compound, presenting itself in both *trans*- and *cis*- isomeric forms naturally. It is found abundantly in red wine, grapes, peanuts and Japanese knotweed. The concentrations of resveratrol in grapes varies from 0.16 to 3.54 μg/g depends the varieties [62]. The concentrations of resveratrol in various types of wines ranged from 0.1 to 14.3 mg/L [62].

The use of resveratrol as a health-promoting dietary supplement is rapidly increasing in market due to its numerous benefits including anti-oxidant, anti-cancer, anti-aging, anti-inflammatory and the prevention of cardiovascular disease [63]. Furthermore, resveratrol plays an effective role against various pathways such as, apoptosis, inflammation, oxidative damage and angiogenesis [63]. The high efficiency of resveratrol might be owing

to the three hydroxyl groups in its structure as shown in Fig. 4. Recent clinical trials proved that resveratrol is well tolerated and pharmacologically safe at doses up to 5 g/day [64].

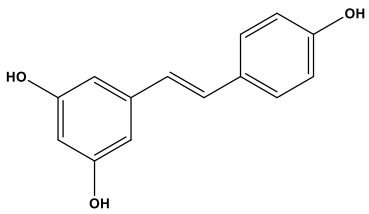


Fig. 4 Chemical structure of trans-resveratrol

2.4.2 Current studies on biotransformation of resveratrol

Low solubility of resveratrol in water and organic solvents (log *P* = 3.10), determined by its chemical structure, impacts its absorption *in vivo*. Studies on metabolic conversion and tissue distribution of RES are continuously undertaken [5, 65-70]. Fig. 5 summarized the absorption and metabolism of resveratrol *in vivo*. the Studies convincingly demonstrated that RES was highly absorbed but rapidly and extensively metabolized, predominantly into glucuronides and sulfates derivatives, and only trace amount of RES could be detected in the plasma [5, 65-68]. The maximum plasma peak concentration of unmetabolized resveratrol was observed at 30-90 mins after oral consumption [5]. The Appearance of the second peak at 6h after resveratrol intake indicated the enteric recirculation and reabsorption of resveratrol [5]. Twelve conjugated metabolites of resveratrol were identified, including resveratrol-3-O-sulfate, resveratrol-3-O-glucuronide, resveratrol-4-O-glucuronide, resveratrol diglucuronide, resveratrol-sulfoglucuronide,

resveratrol trisulfate, and resveratrol disulfate [66]. By using ^{14}C-labeled resveratrol, dihydro-resveratrol (DHR) was firstly identified as a microbial metabolite of

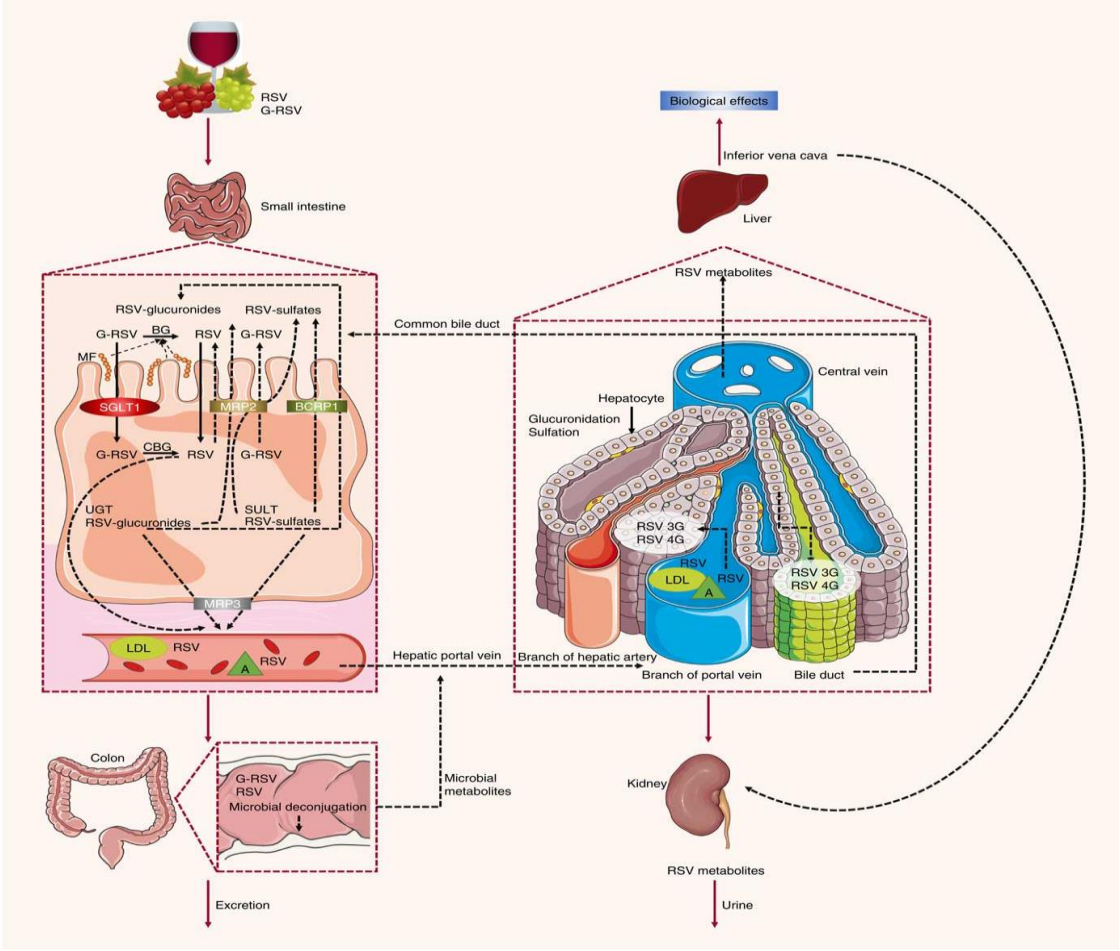


Fig. 5 Absorption and metabolism of resveratrol. RSV stood for resveratrol, G-RSV stood for glycoside resveratrol, CBG stood for cytosolic glucosidase, BG stood for beta-glycosidases, UGT stood for uridine-5'-diphosphate-glucuronosyltransferase, SULT stood for beta-glycosidase, UGT stood for uridine-5'-diphosphage-glucuronosyltransferase, SULT stood for sulfotransferase, MRP2 stood for multidrug resistance protein, BCRP1 stood for breast cancer resistance protein 1. Figure is adopted from reference [30].

resveratrol [5]. Later, the DHR derived metabolites were identified as well, such as DHR diglucuronide, DHR sulfoglucuronide, DHR trisulfate, DHR-3-O-glucuronide, DHR sulfate, and DHR disulfate [71], which together with its conjugates (sulfates and glucuronides) accounted for as much as 50% of an oral RES dose [72]. With the in analytical techniques lunularin (LUN) and 3,4'-dihydroxy-trans-stilbenes have been recognized as microbial metabolites of RES in rats and humans [73-76]. Nevertheless, their concentrations in tissues/plasma and their potential contribution to the pharmacological efficacy of RES have not been probed yet.

2.4.3 Biological effects of resveratrol

Tons of researches suggests the low toxicity and high bioactivity of resveratrol. Many studies have shown that resveratrol possess anti-oxidant, anti-cancer, anti-aging, anti-inflammatory and the prevention of cardiovascular disease [63]. It is worth mentioning that the effects of resveratrol heavily depending on its doses. At low concentrations (5 to 10 μM), resveratrol increased various cancer cells proliferation, while at higher concentrations (higher than 15 μM) itinduced apoptosis [62]. It also has been reported that in cardiovascular diseases, at low dose (5 to 10 μM) resveratrol functioned as anti-oxidant. While at higher concentrations, it acts as prooxidant [77]. Consequently, resveratrol possesses biphasic properties over low to high concentration, which is a critical point when design it as a chemopreventative agent.

Resveratrol has been reported to suppress proliferation of various tumor cells including myeloid, breast, lung, liver, prostate, colon and stomach [78]. Moreover, resveratrol induces apoptosis through various pathways: cell cycle arrest, silent information regulator-mediated pathways, and caspase-8-dependent pathways [79]. Besides, resveratrol exhibits neuroprotective activity by enhancing glutathione and decreasing malondialdehyde levels [80]. Lee et al, demonstrated that resveratrol considerably attenuates dopamine-induced cell death in neuroblastoma cells by activating the antiapoptotic factor Bcl-2 and inhibiting caspase-3 [81].

2.4.4 Biological effects of resveratrol metabolites

Nonetheless, these health benefits elicited by RES have been questioned, since it is rapidly and extensively metabolized upon digestion, resulting in low plasma concentrations [72]. This raises the impetus sparking the scientific inquiry that whether sufficient levels of RES can be attained at the proposed function sites and whether RES metabolites might contribute to the beneficial effects associated with the parent compound.

Therefore, researches diverted attentions to the chemopreventive effects of RES-sulfates and RES-glucuronides [6, 82-85]. It has been reported that relative to RES, its glucuronide and sulfate metabolites showed comparable or some degree of activities in anti-oxidation [6], anti-inflammation [84, 85], and cytotoxicity against SW480, SW620 and SMMC-7721 cancer cell lines [6, 82]. Aires et al. reported that the mixture of RES-3-

O-sulfate, RES-3-*O*-gucuronide, and RES-4'-*O*-glucuronide at the human plasma concentrations exhibited synergistic inhibition effects on proliferation of colon cancer cells [82]. Patel and Brown fed mice with RES-3-*O*-sulfate and RES-4'-*O*-sulfate proved that sulfate metabolites served as an intracellular pool in the body from which RES could be regenerated locally, and further demonstrated that RES mono-sulfates could inhibit the growth of HT-29 cancer cells at clinically relevant concentrations [83]. Only limited reports focused on DHR's biological activities, especially at physiological levels. Lu et al. [6] reported that DHR exhibited comparable activity compared to RES in inhibiting NO production and liver cancer cells proliferation. Tang et al. accentuated that DHR showed comparable inhibition effects with RES on pancreatic oxidative damage [86]. Along with the discovering of new metabolites, it is important to reveal the contributions of resveratrol metabolites on its biological activities.

2.4.5 Effects of resveratrol on inflammatory bowel diseases

Consistent results convinced the anti-inflammatory attribute of resveratrol. Very recently a randomized, double-blind, placebo-controlled pilot study demonstrated that 6 weeks supplementation with 500 mg of resveratrol significantly reduced the levels of TNF-α and hs-CRP in the plasma [87]. The mechanisms underlying anti-inflammation of resveratrol are explained from following perspectives: down-regulation of inflammatory MAPK, and NF-κB pathways, reduction of COX-2, modification the expression of cytokines, diminish the leukocytes and alleviation of the clinical symptoms [88]. However,

the involvement of gut microbiota in this progress has never been reported.

CHAPTER 3

BIOTRANSFORMATION OF RESVERATROL IN THE GASTROINTESTINAL TRACT DICTATES ITS BIOLOGICAL ACTIVITIES

3.1 Introduction

Resveratrol (*trans*-3, 5, 4'- trihydroxystilbene, RES), a phytochemical abundant in grapes, wines, peanuts and mulberries, has attracted much scientific attentions because of its potentially beneficial effects on numerous disorders, *i.e.* colon cancers [1], cardiovascular disease [4], diabetes [2], neurodegenerative diseases [89], and renal carcinoma [90]. Nonetheless, these health benefits elicited by RES have been questioned, since it is rapidly and extensively metabolized upon digestion, resulting in low plasma concentrations [72]. This raises the impetus sparking the scientific inquiry that whether sufficient levels of RES can be attained at the proposed function sites and whether RES metabolites might contribute to the beneficial effects associated with the parent compound.

Regarding these concerns, studies on metabolic conversion and tissue distribution of RES are continuously undertaken [5, 65-70]. Studies convincingly demonstrated that RES was highly absorbed but rapidly and extensively metabolized, predominantly into glucuronides and sulfates derivatives, and only trace amount of RES could be detected in

the plasma [5, 65-68]. Therefore, researches diverted attentions to the chemopreventive effects of RES-sulfates and RES-glucuronides [6, 82-85]. It has been reported that relative to RES, its glucuronide and sulfate metabolites showed comparable or some degree of activities in anti-oxidation [6], anti-inflammation [84, 85], and cytotoxicity against SW480, SW620 and SMMC-7721 cancer cell lines [6, 82]. Aires et al. reported that the mixture of RES-3-*O*-sulfate, RES-3-*O*-gucuronide, and RES-4'-*O*-glucuronide at the human plasma concentrations exhibited synergistic inhibition effects on proliferation of colon cancer cells [82]. Patel and Brown fed mice with RES-3-*O*-sulfate and RES-4'-*O*-sulfate proved that sulfate metabolites served as an intracellular pool in the body from which RES could be regenerated locally, and further demonstrated that RES mono-sulfates could inhibit the growth of HT-29 cancer cells at clinically relevant concentrations [83]. These findings partially explained the RES paradox.

With the advances in analytical techniques, dihydro-resveratrol (DHR) was identified as a microbial metabolite of RES in mice and humans [5, 65, 66, 91, 92], which together with its conjugates (sulfates and glucuronides) accounted for as much as 50% of an oral RES dose [72]. However, only limited reports focused on DHR's biological activities, especially at physiological levels. Lu et al. [6] reported that DHR exhibited comparable activity compared to RES in inhibiting NO production and liver cancer cells proliferation. Tang et al. accentuated that DHR showed comparable inhibition effects with RES on

pancreatic oxidative damage [86]. Very recently, lunularin (LUN) and 3,4'-dihydroxy-trans-stilbenes have been recognized as microbial metabolites of RES in rats and humans [73-76]. Nevertheless, their concentrations in tissues/plasma and their potential contribution to the pharmacological efficacy of RES have not been probed yet.

Regarding these research preceding, the purpose of this study was to identify and quantify metabolites present in mouse tissues and biological fluid with Orbitrap Fusion high-resolution HPLC-MS/MS after sustained oral consumption of RES. Based on the concentrations observed in mouse kidney and colon, we further investigate the anti-inflammatory, anti-clonogenic, and anti-proliferative efficacy of RES metabolites alone or in combination in renal and colon cancer cells. Our findings provided important scientific support to the notion that RES's biological activities could be compensated by its metabolites.

3.2 Materials and methods

3.2.1 Materials

RES (>99% purity) was purchased from Quality Phytochemicals (Edison, NJ, USA). Pinostilbene (PIN) (>98% purity) and DHR (>98% purity) were obtained from Yuanye Bio-Technology Co., Ltd (Shanghai, China). LUN (>98% purity) was purchased from Aikon Biopharma LLC (Nanjing, China). Sulfatase (type H-1, from Helix pomatia,

containing sulfatase and β-glucuronidase) was obtained from Sigma-Aldrich (St. Louis, MO, USA). Acetonitrile (ACN), methanol (MeOH), acetic acid and ethyl acetate were purchased from Fisher Scientific (Fairlawn, NJ, USA). All these solvents are HPLC grade.

3.2.2 Animas, diets and experimental design

The Institutional Animal Care and Use Committee, University of Massachusetts-Amherst approved all animal experiments performed. Twenty male CD-1 mice (5-week old) were obtained from Charles River Laboratory (Wilmington, MA, USA). After one week of diet acclimation, ten mice were randomly chosen to receive standard AIN93G diet containing 0.05% (w/w) RES, while the other ten remaining on standard diet. Urine and feces were collected with metabolic cages. All mice were sacrificed with CO_2 asphyxiation after four weeks. Heart, liver, spleen, lung, kidney, brain, stomach, small intestine (transversely cut equally into four parts), cecum, colon and bile were collected and stored at -80°C for further analysis. Blood samples were centrifuged at 3 000g for 15 mins at 4°C to collect serum.

3.2.3 Sample preparation

Serum, bile and urine samples were extracted according to Menet et al. [93]. Briefly, aliquots of samples were vortex-mixed with ten volume of acidified (2.5% acetic acid) ACN and stood at ice for 20 min to precipitate the protein. After centrifugation (14000 rpm,

10min, 4°C), the supernatant was evaporated to dryness using a Speed Vac Concentrator (Savant Thermo Fisher Scientific Inc., Agawam, MA, USA). Tissue samples were prepared according to Juan et al. with modifications [67]. Briefly, aliquots of tissues were homogenized with 10 volume of MeOH/water/acetic acid (80:20:2.5) solution using a Bead Ruptor Homogenizer (Omni International, Kennesaw, GA, USA). The homogenates were then centrifuged at 14000 rpm for 5 min. Specially for kidney samples, the homogenate was sonicated for 20 min before centrifugation. The residues were extracted one more time and combined methanolic layers were evaporated to dryness under vacuum. All sulfated and glucuronide metabolites were measured by enzymatic hydrolysis of the processed samples with β-glucuronidase and sulfatase as reference described [74]. The internal standard PIN (5 μmol/L) was routinely used in all the samples. The dried extractions were reconstituted in 50 μL 50% MeOH for further analysis.

3.2.4 Orbitrap Fusion high resolution HPLC-MS/MS and HPLC-MS analysis

Identification of RES metabolites was performed in an Ultimate 3000 RSLC HPLC (Thermo Scientific, USA) coupled with Orbitrap Fusion High-Resolution Mass Spectrometer (Thermo Scientific, Waltham, MA, USA). The metabolites were eluted with a Zorbax SB-Aq C18 column (Agilent Technologies, Santa Clara, CA, USA) at flow rate of 0.6 mL/min. Mobile phase A was 5% acetonitrile/water, mobile phase B was 100% acetonitrile. Gradient elution started at 15% solvent B, linear gradient from 15 to 70%

solvent B over 18 min, held at 70% B for 3 min, then followed by washing and reconditioning the column. The Mass-spectra conditions were optimized at negative electrospray ionization mode, as follows: ion spray voltage 3.5 kv, ion transfer tube temperature 325°C, vaporizer temperature 275°C, sheath gas 15 Arb, aux gas 6 Arb, Orbitrap resolution 120K, and collision energy 30%. Data acquisition and processing were accomplished using Xcalibur V4.1 (Thermo Scientific).

The concentration of RES and its metabolites were quantified by using the Shimadzu Model 2020 HPLC-MS (Shimadzu, Kyoto, Japan). The conditions of chromatography and Mass-Spectra were same with Orbitrap Fusion HPLC-MS/MS, except the sample injection volume was 20 μL rather than 5 μL. The data was processed with Labsolutions Software (Shimadzu).

3.2.5 *In vitro* fermentation

Mouse fecal samples were collected from cecum and colon and placed into anaerobic chamber (A35 anaerobic workstation, Whitley, USA) immediately. Aliquots of fecal samples were suspended in gifu anaerobic broth (Hemedia). Small intestine digesta were collected from RES-fed mice and incubated with fecal suspension under anoxic conditions for 24 h. Digesta was defined as the complex aqueous suspensions of undigested matters

and solubilized nutrients in GIT lumen [94]. 1 mL of samples were collected and extracted with ethyl acetate for further HPLC-MS analysis.

3.2.6 Cell viability assay, colony formation assay, and nitric oxide assay

We adopted various cell models to investigate the multiple biological activities of RES and its metabolites, including anti-proliferation of cancer cells, anti-inflammation, anti-oxidant and anti-colony formation. Mouse TLR-4 (mTLR-4) cell was purchased from InvivoGen (San Diego, CA, USA). All other cells were purchased from American Type Cell Collection (ATCC, Manassa, VA, USA). HT-29, HCT-116, A498 and 786-O were applied to MTT and colony formation assays as described previously to explore the anti-proliferatory effects [95-97]. Nitric oxide assay (anti-inflammation) were performed according to Guo et al. in RAW264.7 macrophage model [95, 96]. mTLR-4 cells are designed to specifically study that whether toll-like receptor 4 (TLR-4) mediated NF-κB pathway involved. This experiment was conducted following manufacture's HEK-Blue protocol.

3.2.7 Statistical analysis

All data were expression as Mean ± SD. The statistical significance was assessed by one-way ANOVA with post hoc Tukey HSD test. P value < 0.05 was considered as statistically significant.

3.3 Results and discussion

3.3.1 Identification of RES metabolites

To investigate the biological properties of resveratrol metabolites, the very first step should be the identification of metabolites. By using high-resolution HPLC-MS/MS, RES metabolites were characterized in urine and fecal samples. Blank urine and fecal samples were injected to discard confounding ions not related to RES-derived metabolites (results not shown). Eleven metabolites were detected in urine and fecal samples including two microbial metabolites-DHR and LUN, three RES conjugates (RES-sulfate, RES-glucuronide, and RES-sulfoglucuronide), four DHR conjugates (DHR-sulfate, DHR-glucuronide, DHR-diglucuronides, and DHR-sulfate-glucuronide) and two LUN conjugates (LUN-sulfate and LUN-glucuronide), as shown in Table 1 and Fig. 6. In agreement with previous publications, the main RES metabolites were RES-sulfates, RES-glucuronides, and RES-sulfoglucuronide as well as the microbiota-derived DHR and its corresponding sulfate, glucuronide, diglucuronides, and sulfoglucuronide conjugates [5, 65-67, 91, 92]. LUN was identified as a microbial metabolite of RES in urine and fecal samples recently [74, 76]. Importantly, for the first time, our results revealed that LUN conjugates were important metabolites derived from resveratrol.

Table 1 List of resveratrol metabolites identified with HPLC-MS/MS

#	Metabolites	Retention time (min)	m/z [M-H]	MS/MS fragment
1	Resveratrol (RES)	13.67	227.0708	227.0702, 185.079, 143.0493
2	RES-sulfate	9.75/9.96	307.027	307.0269, 227.0702, 185.0595
3	RES-glucuronide	8.45/6.48	403.1029	403.1026, 227.0703, 113.0236, 175.0293
4	RES-sulfoglucuronide	6.86	483.0597	307.027, 227.0715, 113.0223
5	Dihydro-resveratrol (DHR)	13.42	229.0865	229.0837, 123.0442, 81.0339
6	DHR-sulfate	9.65	309.0433	309.0424, 229.0395, 123.0443
7	DHR-glucuronide	8.55/8.60	405.1186	405.1161, 229.0865, 113.0235
8	DHR-diglucuronides	6.43	581.1506	405.1160, 229.0875, 175.0238, 113.0236
9	DHR-sulfoglucuronide	7.01	485.0754	485.0757,405.152, 309.0425, 113.0237
10	Lunularin (LUN)	17.14	213.0916	213.0912, 106.0415, 107.0494
11	LUN-sulfate	10.35/10.56	293.0484	293.0476, 213.0910, 79.9567, 107.0494
12	LUN-glucuronide	9.85	389.1236	389.1267, 213.0910, 113.0235

The spectra of metabolites were shown in Supplementary Fig. S1. RES, DHR and LUN were fully identified by direct comparison with the authentic standards. The rest of metabolites were tentatively identified regarding to their pseudo molecular ions and daughter ions within 5ppm measurement error. For example, RES glucuronide showed a $[M-H]^-$ ion at *m/z* 403.1029, which further fragmented to form a product ion of *m/z* 227.0703 that corresponded to RES itself after the loss of fragment at 113.0236 (Fig. S1). This results was consistent with previous report [91]. The exact positions of these conjugations in the resveratrol molecule were not established.

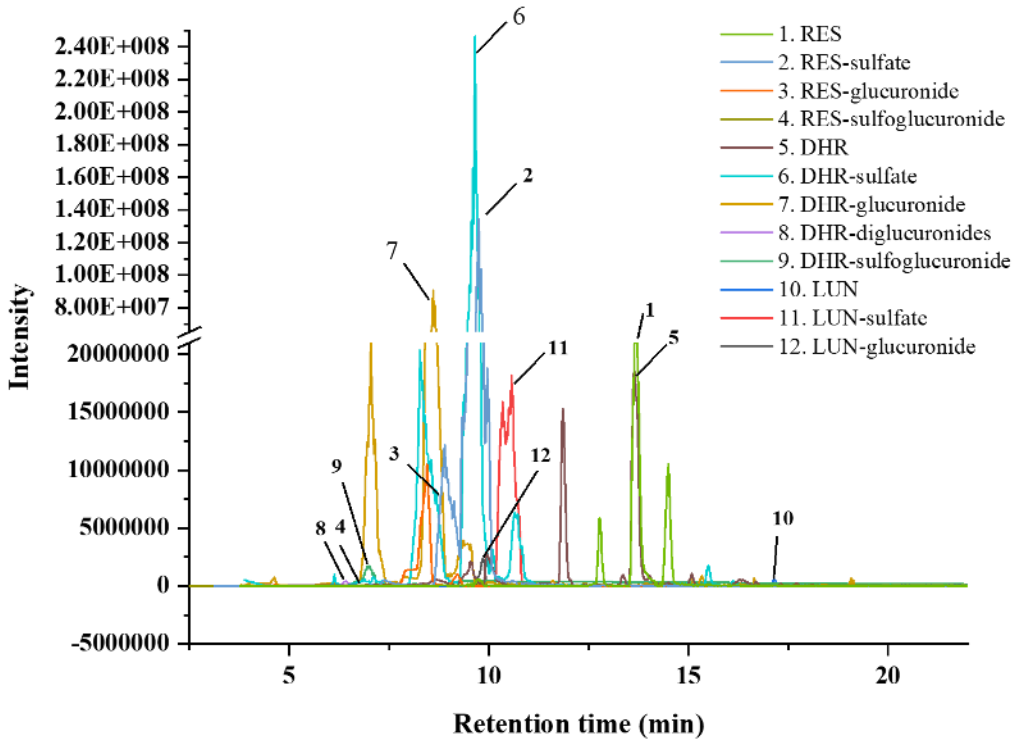


Fig. 6 Chromatogram of eleven RES metabolites identified in urine and fecal samples after sustained oral consumption of RES.

3.3.2 Distribution of RES and its metabolites in tissues and biological fluids

We further quantified the levels of RES and its metabolites in tissues/organs, gastrointestinal tract (GIT) and biological fluids. Due to the paucity of available standards for sulfate and glucuronide conjugates, their concentrations were assessed by enzymatic hydrolysis as the whole, presenting as RES-conjugates, DHR-conjugates and LUN-conjugates, respectively. We noticed that not even trace amount of unmetabolized RES was detected in bile, liver, kidney and serum (Fig. 7A-7D), which implied that RES underwent

extensive metabolism after oral consumption. More interestingly, DHR, LUN and their conjugates were much more abundant than RES-conjugates (Fig. 7A-7D). RES and its conjugates together were presented as RES-M and the same applied to DHR and LUN. As shown in Fig. 7, DHR-M and LUN-M were 5.3- and 4.6- folds higher in bile, 1.2- and 4.8-folds higher in serum, 10.3- and 3.4- folds higher in liver and 2.9- and 3.1-folds higher in kidney than RES-M, respectively. Above results suggested that besides RES-sulfate, RES-glucuronide and RES-sulfoglucuronide that were reported widely before, RES was more profoundly metabolized into DHR, LUN and their conjugates after oral consumption. Higher amounts of DHR-M than RES-M were reported before [72], while our current study emphasized the high abundance of LUN-M in tissues for the first time. Considering the absence of RES in tissues and limited bioactivity of RES conjugates, this was very useful information since it indicated that DHR-M and LUN-M might play more important roles than RES-M after oral administration of RES, due to their much greater abundance than RES-M in tissues.

Moreover, high concentration of RES (179.1 μmol/L) and its conjugates (145.3 μmol/L) were detected in urine (Fig. 7E). This result suggested that higher amount of RES-M was excreted compared with DHR-M and LUN-M through urine, which further supported previous results.

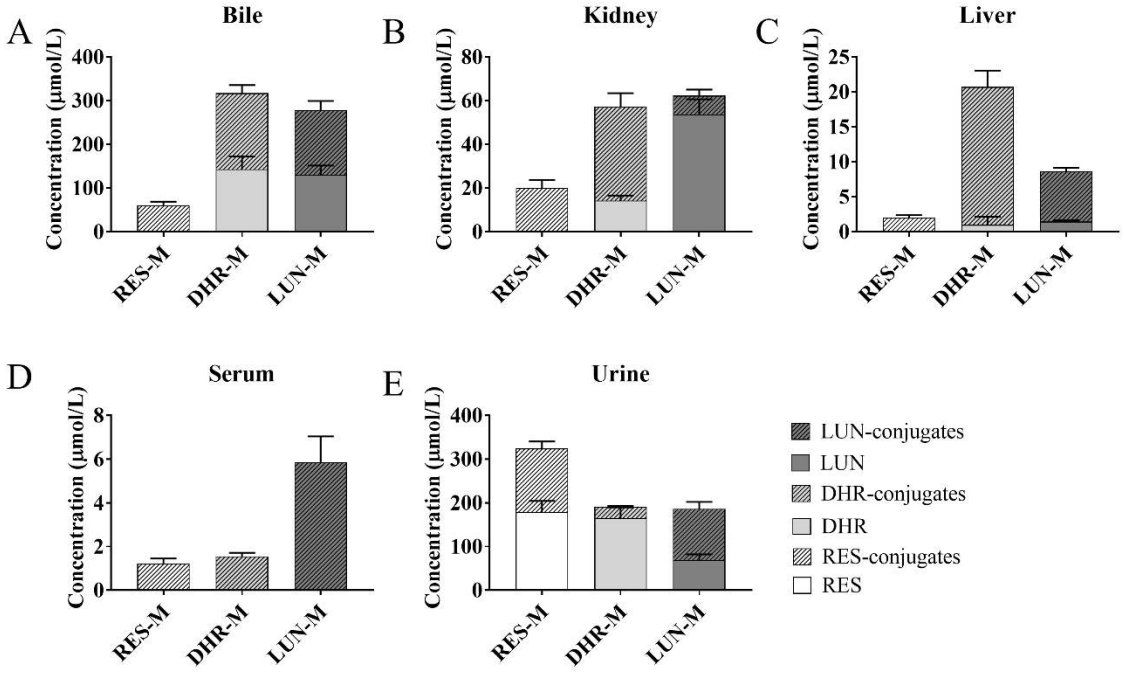


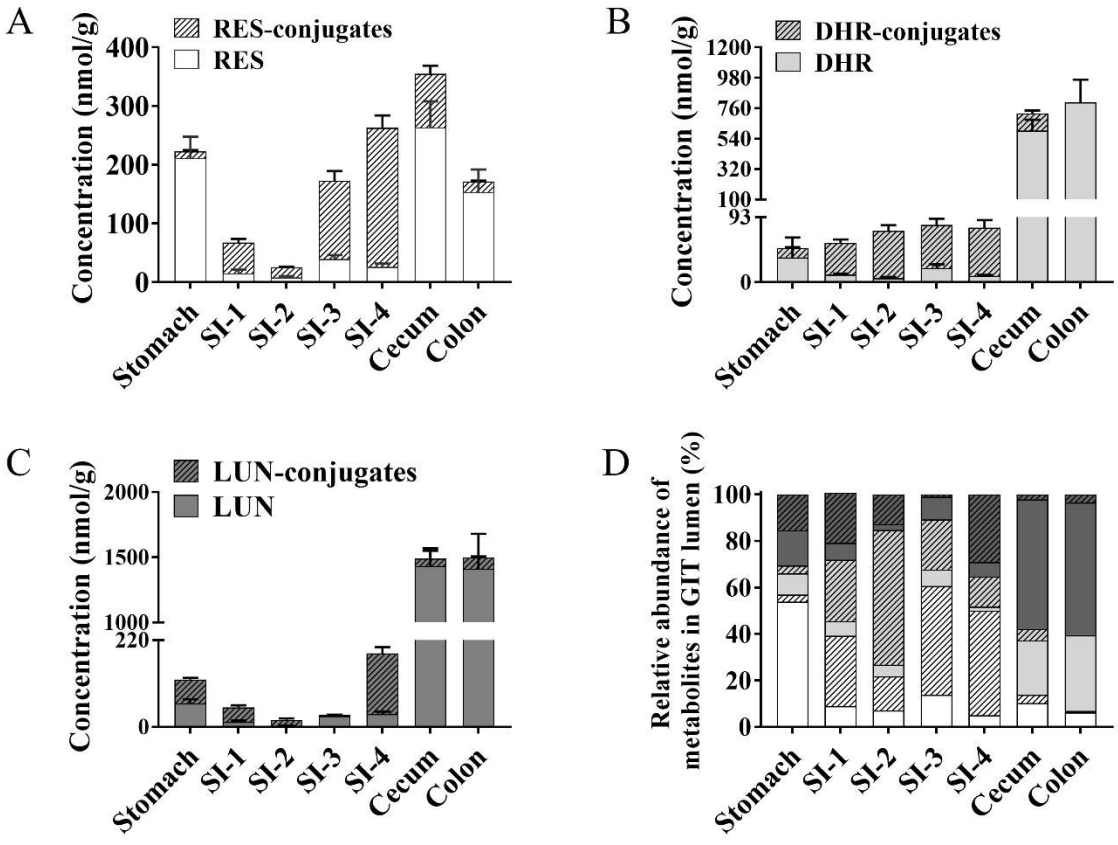
Fig. 7 Distribution of RES, DHR, LUN and their conjugates in bile (A), kidney (B), liver (C), serum (D) and urine (E). Data presented as mean ± SD (n=8).

3.3.3 Distribution of RES and its metabolites in GIT

For better understanding the metabolic pathways of RES after oral consumption, we also examined the accumulation of RES metabolites in both the digesta and the gastrointestinal (GI) tissues. Small intestine (SI) was equally cut into four segments labelled as SI-1, 2, 3, and 4, referring to as the duodenum, jejunum, proximal ileum and distal ileum in human.

Considerable amount of unmetabolized RES were detected in the stomach digesta, as well as the occurrence of lesser extent of RES-conjugates, DHR, LUN, DHR-conjugates,

and LUN-conjugates (Fig. 8A). Conjugates in stomach digesta could attribute to the metabolizing ability of gastric cells [98]. The presence of DHR and its conjugates in the stomach digesta has been reported before by Azorin-Ortuno et al [66], which was tentatively explained by the presence of microbial groups in the stomach and SI involved in the formation of DHR. This may also explain the appearance of LUN and its conjugates in the stomach lumen. While, mice eat their feces, which contained large volumes of DHR and LUN, could be another potential reason.



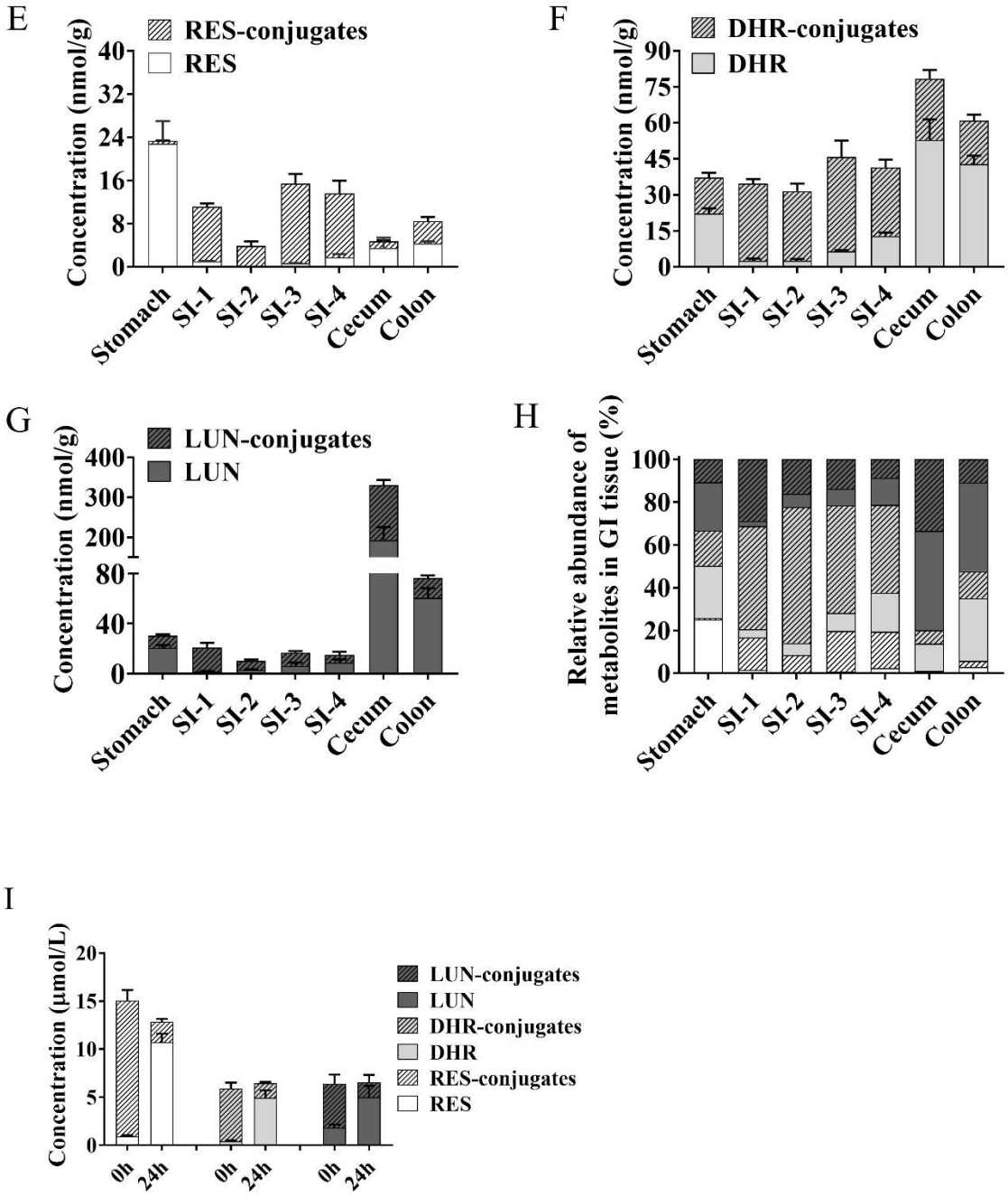


Fig. 8 Levels of RES metabolites in gastrointestinal digesta and tissues. (A-C) Concentration of RES, DHR, LUN and their corresponding conjugates in digesta. (D) Relative abundance of RES metabolites in digesta. (E-G) Concentration of RES, DHR,

LUN and their corresponding conjugates in GIT tissues. (H) Relative abundance of RES metabolites in GIT tissues. (I) Concentration of RES, DHR, LUN and their conjugates before and after anaerobic fermentation with mouse fecal bacteria. Data presented as mean ± SD (n=8).

Free-formed RES, DHR and LUN dominated (78.06%) in the stomach digesta. While once the digesta arriving at the SI, the concentration of free-formed metabolites showed a dramatic decline, meanwhile their conjugates significantly increased to 80.81% of total RES metabolites (average of SI-1 to SI-4) (Fig. 8A-8D). The abundance of conjugates in the SI digesta could due to several contributions together: catalyzed by transferase enzymes that rich in SI enterocytes, an active efflux of conjugates to the lumen content, and biliary secretion of the enterohepatic circulation [5, 66, 99]

However, when digesta reached the cecum and colon, conjugated metabolites including those from DHR and LUN dramatically dropped down below 10.6% of the total metabolites, while their corresponding parent compounds rocketed up (Fig. 8A-8D). Similar situation have been reported in previous study [66]. We also noticed that the RES-M dropped down from 47.97% of the total metabolites in the SI digesta (average) to 10.46% in the large intestine inner content (average of the cecum and colon). While the abundance of DHR-M and LUN-M increased at the meantime. (Fig. 8A-8D). Given the richness of gut microbiota in the cecum and colon, we hypothesized that gut microbiota plays a role in

the de-conjugation process and the production of DHR and LUN in the large intestine. To examine our hypothesis, we incubated SI digesta, which was collected from RES-fed mice, with mice-derived gut microbiota and determined the levels of free- and conjugated-metabolites before and after the fermentation. We successfully observed that after 24h anaerobic incubation, majority of the conjugates were converted back to their parent compounds (Fig. 8I). We also found that the total amount of RES and its conjugates decreased by 3.20 μmol/L after fermentation, while DHR-M increased by 1.12 μmol/L and LUN-M increased by 0.16 μmol/L (Fig. 8I). Though this result did not strictly consistent with our *in vivo* data, it still highly suggested the involvement of gut microbiota in the biotransformation of RES to DHR and LUN. Bode et al. successfully identified *Slackia equolifaciens* and *Adlercreutzia equolifaciens* as two DHR producers from human stools [74]. The LUN producers, however, still had not been identified. The low increased-proportion of DHR-M and LUN-M might due to the limited *in vitro* fermentation conditions so that the bacteria composition was different with that *in vivo*. The relative high concentrations of DHR and LUN in the bile, could be considered because of the reabsorption from the large intestine [100] (Fig. 7A).

The distribution of RES and its metabolites in the GI tissues had similar pattern with GIT digesta, as shown in Fig. 8E-8H, excepting the lower concentrations and lesser percentage of RES and its conjugates. The luxuriant enzymes in epithelial cells, such as

cytochromes P450, UDP-glucuronosyltransferases, sulfotransferases, could be an explanation of this observation [19]. Based on above discussion, the proposed metabolic pathway of resveratrol was summarized in Fig. 9.

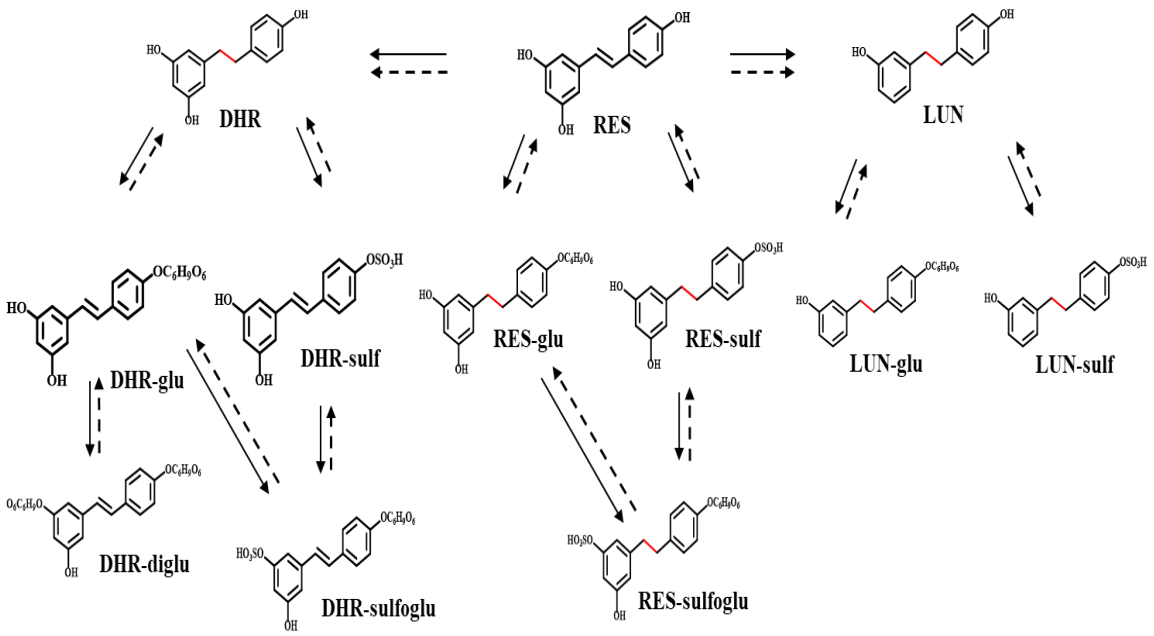


Fig. 9 Proposed metabolic pathway of RES. Solid arrows indicated metabolism conducted by various transferases. Dotted arrows indicated the involvement of gut microbiota.

3.3.4 Metabolites of RES showed stronger chemopreventive effects than RES at physiological concentrations

There are compelling evidences that RES exerts multiply chemopreventive properties against various diseases. Nevertheless, these health benefits remained controversial due to the low oral bioavailability and fast metabolism of RES [5, 72]. Consequently, the potential

bioactivity of RES metabolites is an important question to solve to better understand RES's mechanisms of action. Conjugates derivate from dietary polyphenols often have weakened bioactivities compared to the parent compounds, and this seems to be the case for RES based on previous studies [6, 101]. Therefore, we focused on the chemopreventive effects of two free-formed phase I metabolites (DHR and LUN) of RES in our study. RES exhibited colon and renal protective effects and high levels of DHR and LUN were detected in kidney and colon [1, 90]. Thus, the chemopreventative effects of DHR and LUN were examined in renal and colonic cell lines. To establish the protective effects of RES and its metabolites in a physiologically relevant manner, we deliberately used the concentrations measured in tissues to determine their bioactivities.

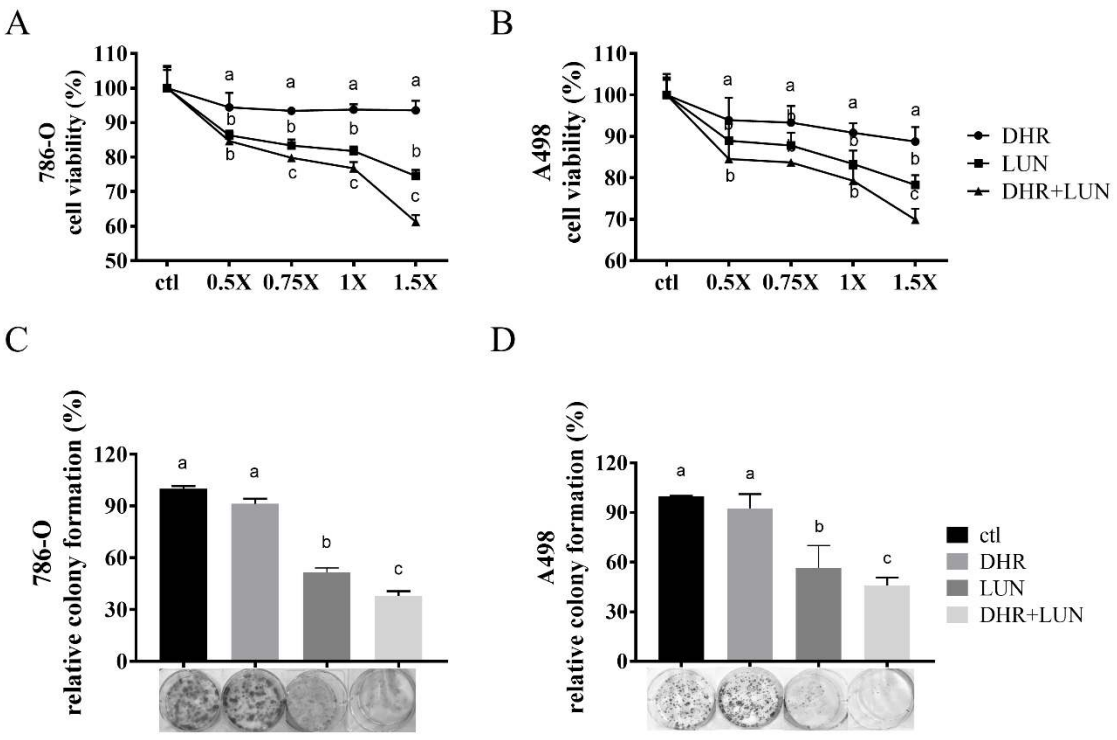


Fig. 10 Renal protective effects of DHR and LUN at renal relevant concentrations. Growth inhibitory effects of DHR and LUN in 786-O (A) and A498 (B) renal carcinoma cells. DHR and LUN potently inhibited colony formation of 786-O (C) and A498 (D) cells. Data presented as mean ± SD (n=3). Different letters indicate statistical differences ($P<0.05$).

Regarding the zero concentration of RES in kidney, the renal protective effects of RES inevitably drove attentions to its metabolites [90]. A498, a "classical" human renal carcinoma cell line, is widely used as a model of clear cell renal cell carcinoma (ccRCC) [102]. 786-O, with the phenotype of ccRCC, is primary cell line that most commonly used in renal carcinoma focused researches [102]. These two cell lines were adopted to evaluate the anti-proliferatory and anti-clonogenic effects of DHR and LUN at renal relevant concentrations. Four levels of DHR, LUN and DHR+LUN were used at 0.5, 0.75, 1 and 1.5×. Concentration at 1× was equivalent to the concentrations (DHR: 14.3 nmol/g; LUN: 53.6 nmol/g) found in kidney tissues. Concentrations at 0.5, 0.75 and 1.5× were half, three quarters and one and half times of the concentrations of RES metabolites at 1×. As shown in Fig. 10, DHR and LUN inhibited the growth of both 786-O and A498 cells in a dose-dependent manner. LUN showed stronger inhibitory effects than DHR in both 786-O and A498 cells at all tested concentrations ($P<0.05$). LUN caused 15.6, 16.5, 18.2 and 25.4% of inhibitions on 786-O cells at 0.5, 0.75, 1 and 1.5×, respectively. The combination of DHR and LUN produced stronger inhibitory effects, i.e., at concentration of 1×, treatment of DHR+LUN caused 23.2% death of 786-O cells. Similar results were also observed in

A498 cells. A498 and 786-O cells were also subjected to anti-colony formation assay at the 1× concentration. The formed-colonies were scanned and counted as shown in Fig. 10C, 7D. LUN significantly inhibited the clonogenic formation of A498 and 786-O cells by 43.38 and 48.44%, respectively (P<0.01). Combination of DHR and LUN exhibited stronger inhibitory effects than LUN alone (P<0.05). As shown in Fig. 10C-10D, DHR+LUN suppressed the colony formation by 54.15 and 62.03% in A498 and 786-O cells, respectively. These results suggested that renal protective effects of RES might highly relevant to its metabolites (DHR and LUN) in the kidney.

In order to establish the anti-colitis and anti-colon cancer effects of DHR and LUN in a physiologically relevant manner, we determined their inhibitory effects on two widely used human colon cancer cell lines (HCT-116 and HT-29) at the concentrations found in the mouse colonic tissues. Treatment of 1× stood for concentrations measured in the colonic tissue, that was, 4.3 μmol/L of RES, 42.8 μmol/L of DHR and 60.5 μmol/L of LUN. DHR showed a tendency to suppress the proliferation of HCT-116 cells, although it did not achieve a statistical significance. While, LUN profoundly and dose-dependently inhibited the multiplication of HCT-116 cells since concentration of 0.75× compared to RES (P<0.01). Markedly, DHR+LUN together showed stronger inhibitory effects than DHR and LUN alone, i.e., at 1.0×， compared to 8.2 and 12.7% of inhibition induced by DHR and LUN, DHR+LUN inhibited cell growth by 24.8% (P<0.05) (Fig. 11A).

Interestingly, combination of all three compounds (RES+DHR+LUN) exerted the strongest inhibition, however, it was not statistically greater than that produced by combination of two metabolites (DHR+LUN). This finding further demonstrated the meager contribution of RES itself to its *in vivo* protective effects against colon cancer. It is noteworthy that at the concentration of 1.5×, DHR+LUN+RES showed no significant inhibition on the growth of normal human colon CCD-18Co cells (Supplementary Fig. S2). This result indicated that cytotoxic effects of RES, DHR and LUN were cancer cell specific.

In addition, HT-29 cells were subjected to different treatments at 1×. After 12 days of incubation, the colonies formed were photographed and quantified as shown in Fig. 11B. The numbers of colonies formed followed the order of RES > DHR > LUN > DHR+LUN > RES+DHR+LUN. Compared to 5.0% of suppression caused by RES alone, treatments of DHR, LUN, DHR+LUN and RES+DHR+LUN restricted the clonogenic survival of HT-29 cells by 11.3, 35.2, 45.0 and 56.7%, respectively (P<0.05) (Fig. 11B). These results indicated that RES metabolites might play more vital role in promoting the reproductive death of colon cancer cells than RES itself in the colonic tissue after oral consumption of RES.

Besides the anti-proliferative and anti-clonogenic effects, RES metabolites also exerted stronger anti-inflammatory ability than RES at the colonic concentrations (Fig. 11C. LPS-stimulated RAW 264.7 macrophages were used to determine the anti-inflammatory

potency of DHR and LUN. LUN behaved a dose-dependent inhibition on LPS-induced NO production (an important inflammatory mediator) by 14.2, 21.6, 28.3 and 38.2% at 0.5, 0.75, 1 and 1.5×, respectively (P<0.01). Overall, DHR did not produce a significant anti-inflammatory ability, which was consistent with previous study [6]. The combined treatments of DHR and LUN caused a significant decrease on production of NO compared with LUN alone (P<0.01), i.e., 19.1, 26.8, 36.9 and 46.2% of NO inhibition throughout the concentration range. Most interestingly, the combination of all three compounds (RES, DHR and LUN) did not produce stronger inhibitory effects in comparison with the combination of DHR and LUN (Fig. 11C).

To obtain further understanding of the anti-inflammatory signaling pathway, we employed mTLR-4 cells to exam the involvement of NF-κB pathway. Stimulation of mTLR-4 cells with a bacterial toxin LPS activated NF-κB and activator protein 1 (AP-1), which induces the production of secreted embryonic alkaline phosphatase (SEAP). As shown in Fig. 8D, single treatment of DHR and LUN at a concentration range of 0.75× to 1.5×, caused a significant dose-dependent inhibition on SEAP production compared to RES alone (P<0.01). Furthermore, cotreatments with serial concentrations of DHR+LUN resulted in suppression of SEAP production by 25.4, 40.6, 51.5 and 75.6% at 0.5, 0.75, 1 and 1.5×, respectively. The involvement of RES strengthened the inhibitory effects of DHR+LUN on SEAP expression, but not statistically significant at lower concentrations

(0.5 and 0.75×) (Fig. 11D). However, further analyses were required to clarify the specific molecular mechanisms of anti-inflammatory effects of RES metabolites. Nonetheless, it should be noted that RES exhibited stronger inhibitory effects on these cancer cell lines [6] and NO production than DHR and LUN at the same concentration, however, this dose range of RES is not achievable in our *in vivo* feeding study (Supplementary Fig. S3). Overall, our results demonstrated that colonic metabolites of RES played more important roles than RES itself in inhibiting cancer cell growth and inflammation.

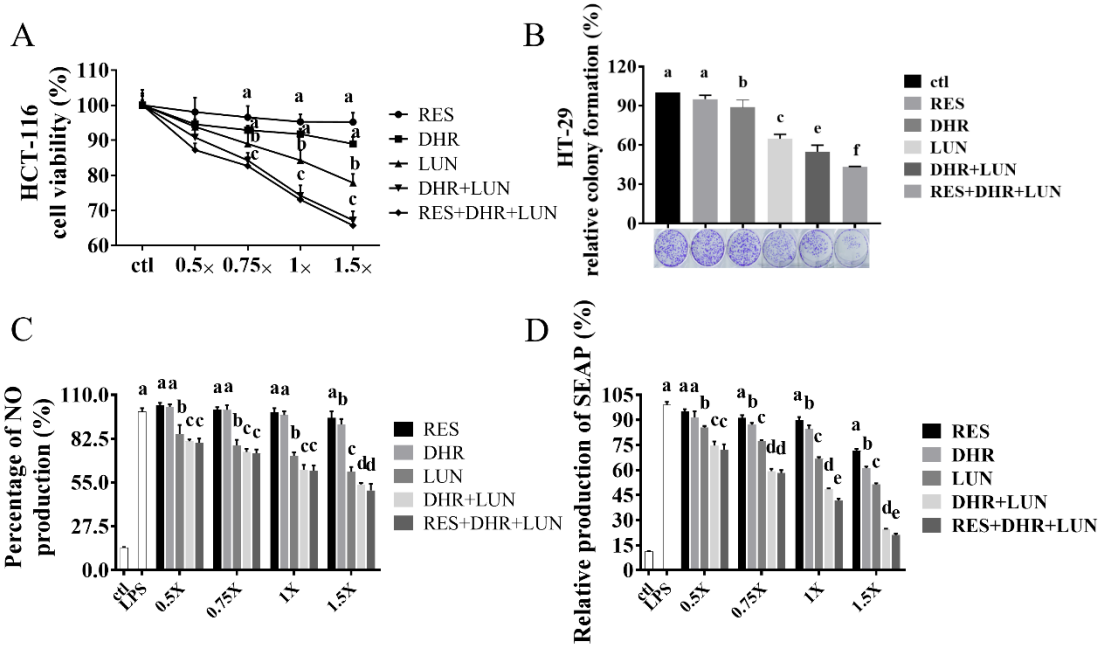


Fig. 11 Chemopreventive effects of RES, DHR and LUN at colonic tissue levels. (A) Growth inhibitory effects of RES, DHR and LUN on HCT-116 cancer cell line. (B) Anti-clonogenic effects of RES, DHR and LUN on HT-29 cancer cell line. (C) Percentage of

inhibition on NO production by RES, DHR, LUN and their combination in LPS-stimulated RAW 264.7 cells. (D) Inhibitory effects of RES, DHR, LUN and their combination on LPS-induced SEAP production. Data presented as mean ± SD (n=3). Different letters indicate statistical differences ($P<0.05$).

3.4 Conclusion

The present study for the first time systemically determined the biotransformation of RES in mice, with focuses on its metabolic fate in the GIT. Our results demonstrated that DHR, LUN and their corresponding conjugates were dominated metabolites of RES after sustained oral consumption of RES, rather than RES-sulfates and RES-glucuronides. More importantly, we found that DHR, LUN and their combination exerted much stronger chemo-preventive and anti-inflammatory effects in the renal and colonic tissues, at their concentrations achieved in these tissues, suggesting that DHR and LUN may greatly contribute to the chemopreventive properties elicited by RES in the kidney and colon. Overall, our findings provided a solid scientific basis for understanding the chemopreventive mechanisms of RES from the perspective of biotransformation, and are of great value for future research on RES in prevention and treatment of renal and colonic diseases in humans

CHAPTER 4

ANTI-INFLAMMATORY EFFECTS OF RESVERTROL ASSOCIATED WITH THE ALTERATION OF GUT MICROBIOTA

4.1 Introduction

Inflammatory bowel disease (IBD), including relapsing-remitting inflammatory disorders Crohn's disease and ulcerative colitis, is a chronic inflammatory disease of gastrointestinal tract [28]. Over 1 million residents in the USA are estimated to be suffering from IBD [8]. One of the most important and devastating complications of long-term IBD is colorectal cancer development [9]. The precise etiology of IBD remains unknown, the most accepted hypothesis to date is that aberrant and continuing immune response against the commensal gut microbiota in genetically susceptible hosts [28, 29]. The possible implication of gut microbiota in the pathogenesis of IBD has been highlighted recently. Multiple lines of evidence suggested the important role of gut microbiota in the progress of IBD. Firstly, Metagenomic and 16S RNA based sequencing studies demonstrated reduced diversity and altered composition of gut microbiota in patients with IBD compared with healthy subjects [56-58]. Secondly, germ-free animals were protected against colitis development in DSS-treated mice [12]. Thirdly, IL-10-deficient mice, a murine model of IBD, did not develop colitis under germ-free conditions [59]. All these results suggested

that microbial factors might directly contribute to the development of colonic inflammation and adverse metabolic consequences.

The gut microbiota has become an important target in the treatment of IBD, and therapy has focused on correcting intestinal microbial imbalance. The gut microbiota, including symbiotic, probiotic, and pathogenic microorganisms, plays an important role in human health. Its balance can be destroyed by the introduction of invasive pathogens, activation of immune cells, and production of pro-inflammatory cytokines [60]. Diet has an influence in shaping the development and composition of the gut microbiota and many phytochemicals enriched diet have been explored as preventive treatments of IBD recently [31].

Resveratrol (3,5,4'-trihydroxy-trans-stilbene) is a natural polyphenol abundant in grapes, wines and peanuts. Consistent results showed that resveratrol could down-regulated inflammatory biomarkers, reduced oxidative stress and attenuated clinical symptoms in experimental murine colitis models [1, 3, 103]. A recent human study claimed that 6-weeks supplementation of resveratrol could improve clinical colitis [87]. Moreover, high accumulation of resveratrol in the gastrointestinal tract (65.1%) was reported, suggesting it could arrive intact at the colon, where it may interact with micro flora [71]. In the view of direct contact and dual interaction existing between resveratrol and gut microbiota, possible composition modifications in gut microbiota might be expected as a result of

consumption of resveratrol, which ultimately contribute to its anti-inflammation property.

Given the complex relationship among diet, IBD and gut microbiota. The purpose of this study was to elucidate whether supplementation of trans-resveratrol could counteract gut microbiota dysbiosis produced by DSS-induced colitis and whether these changes might be correlated with anti-colitis effects of resveratrol.

4.2 Materials and Methods

4.2.1 Animals and experimental design

Institutional Animal Care and Use Committee of University of Massachusetts Amherst approved the protocol for the animal experiment. 40 male wild-type CD-1 mice (6-8 weeks old) were obtained from Charles River Laboratories (Wilmington, MA, USA). Animals were randomly assigned to four groups and housed in groups of five mice per cage in a temperature-controlled environment (22±2 °C) with 65 % relative humidity and fixed 12 h light/dark cycle. After one-week diet acclimation, four groups were distributed as follows: the control group (RES-, DSS-; n=10), which was fed with standard chow (AIN93G diet); the resveratrol group (RES+, DSS-; n=10), which was fed with the standard diet supplemented with 0.025% (w/w) resveratrol (>99% purity) (Quality Phytochemicals, NJ, USA); the DSS group (RES-, DSS+; n=10), which received the standard diet and 1.5% DSS water (wt/v, dextran sulfate sodium salt) (International Lab, Chicago, IL, USA); and

the resveratrol-DSS group (DSS+, RES+; n=10), which received resveratrol (0.025% w/w) supplemented diet and 1.5 % DSS drinking water (wt/v). 1.5% DSS was administered in the drinking tap sterilized water ad libitum for 4 days followed by 7 days of pure water for recovery, and this cycle was repeated four times. Body weight and disease activity index (DAI) were monitored at every last day of recovery cycles and DSS cycles. At the end of the fourth cycle of DSS treatment, all mice were sacrificed with CO_2 asphyxiation. The liver, spleen and kidney were removed and weighted. After measurement of weight and length, the entire colon was cut into two pieces longitudinally. Half of the colon was fixed in 10% buffered formalin (pH=7.4) for further histopathological analysis. The other half of the colon was stored at -80°C for ELISA analysis. The feces collected from colon were stored at -80°C for further sequencing analysis.

4.2.2 DAI and histological assessment

DAI was determined by scoring extent of rectal bleeding (Score: 0, none; 1, hemoccult positive; 2, blood; 3, gross bleeding), stool consistency (Score: 0, normal; 1, soft but form; 2, soft; 3, diarrhea) and weight loss (Score: 0, none; 1, 1~5%; 2, 5~10%; 3, 10~20%; 4, >20%) [104]. The final macroscopic score for each animal is the sum of each individual score.

Formalin fixed colon tissues was processed for paraffin embedding, sectioning (5 μm),

and haematoxylin and eosin (H&E) staining as previously described [7, 105, 106]. Three parameters were graded: surface epithelial loss, crypt destruction and inflammatory cell infiltration into the mucosa based on the established criteria [107]. A score of 0-4 was assigned to each parameter (maximum macroscopic score = 12).

4.2.3 Enzyme-linked immunosorbent assay (ELISA)

Colonic mucosa was scraped and homogenized in MSD Tris lysis buffer (Meso Scale Discovery, Rockville, MD, USA) contained with 1% protease inhibitor cocktail (Boston Bioproducts, Ashland, MA, USA). Supernatants were collected by centrifuging at 14000 rpm for 20min at 4°C, then loaded in sandwich enzyme-linked immunosorbent assay kits (Meso Scale Discovery, Rockville, MD, USA) to determine the concentrations of cytokines according to the manufacturer's instructions.

4.2.4 16S rRNA analysis and Illumina Mi-Seq sequencing

Fresh fecal samples were collected from colon and immediately frozen at −80°C for future analyses. Bacterial DNA was extracted from feces by using the PowerFecal DNA isolation kit (MoBio Laboratories, Inc., Carlsbad, CA), following supplier's protocols. The DNA was quantified by the NanoDrop spectrophotometer (Thermo Scientific, Waltham, MA, USA) and normalized to 20 ng/μL. 16s rRNA genes were amplified with PCR by using Forward primer

5'TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGCCTACGGGNGGCWGCAG and Reverse Primer = 5'GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGGACTACHVGGGTATCTAATCC as described by Klindworth et al. [108]. Amplicons of interest was verified by gel electrophoresis. If required, low yields were further amplified. PCR products were then purified with AMPure XP beads (Beckman Coulter, Danvers MA, US) to remove primer dimers and other small mispriming products. Dual indices and Illumina sequencing adapters were attached by PCR using the Nextera XT Index Kit (Illumina, San Diego, CA, US). The quantity and size of PCR products were determined and verified by using Qubit dsDNA BR Assay kit (Life technology, Carlsbad, CA, US) and DNA analysis ScreenTape Assay on Tape Station 2200 (Agilent Technologies, Santa Clara, CA, US). Final PCR products were pooled in equimolar concentration and denatured by NaOH. After combining the amplicon library and PhiX control, the samples were loaded onto the 600-cycle MiSeq Reagent kit v3 cartridge and sequenced on an Illumina MiSeq platform (Illumina Inc, San Diego, CA, USA).

4.2.5 Statistical analysis

Raw Illumina Miseq sequence data were processed with Quantitative Insights Into Microbial Ecology (QIIME) software pipeline v1.9.1[109]. Generated operational taxonomic units (OTUs) were further subjected to alpha- and beta-diversity and principal

coordinate analysis (PCoA) analyses using QIIME. Cladogram and Linear discriminant analysis (LDA) characterized by LEfSe tool [110]. The LEfSe analysis was performed using an alpha value of 0.01 for both the factorial Kruskal-Wallis rank sum test and pairwise Wilcoxon test and a threshold of 2.0 for the LDA. The approach used was an all-against-all multi-class analysis. Correlation analyses were performed with SPSS 17.0 software (Chicago, IL, USA). Data were presented as the mean ± standard error of the mean (SEM). The statistical significance of differences among groups was performed using one-way ANOVA followed by Tukey-Kramer multiple comparison post hoc analysis. The differences between groups were considered significant at $P< 0.05$.

4.3 Results

4.3.1 Dietary resveratrol attenuated colitis symptoms

Cyclic administration of DSS in drinking water resulted in the establishment of chronic colitis and the development of colorectal dysplasia in mice [111]. Body weight was monitored at the end of every DSS and recovery cycles, as an indicator of colitis severity. Lower percentage of body weight gain was observed since the end of the first DSS cycle. With increased DSS-treatment cycles, the percentage of body weight gain in DSS group was getting lower and lower than control group and statistically significant at the fourth DSS cycle ($P < 0.05$), while dietary resveratrol significantly counteracted this trend in DSS-

treated mice (Fig. 12A). The final body weight was 48.98 ± 5.06 g for control group, and significantly decreased to 36.92 ± 3.05 g for DSS group ($P < 0.01$). Whilst, dietary resveratrol supplementation ameliorated the body weight loss by elevating the final body weight to 44.78 ± 1.61 g in DSS-treated mice ($P < 0.01$) (Fig. 12B). DAI exhibits features of fecal consistency, haematochezia and body weight loss, and is used to evaluate inflammation severity in mice with colitis [104]. The DAI score was as high as 7.9 ± 1.9 in DSS treated group, whereas it was markedly attenuated by 1.8-fold with resveratrol treatment ($P< 0.05$) (Fig. 12C). Colon weight/length ratio is another index that correlated with the progression of colorectal inflammation. As shown in Fig.12D, compared to the DSS group, dietary supplement of resveratrol rescued the inflammation-associated elevation of colon weight/length ratio ($P < 0.05$). Furthermore, remarkable increased in the ratio of spleen to final body weight were observed in DSS-treated mice compared with control mice. Dietary resveratrol significantly decreased this spleen to final body weight ratio to control level ($P < 0.05$) (Fig. 12E). These results suggested that dietary resveratrol offered protective effects against DSS-induced colitis.

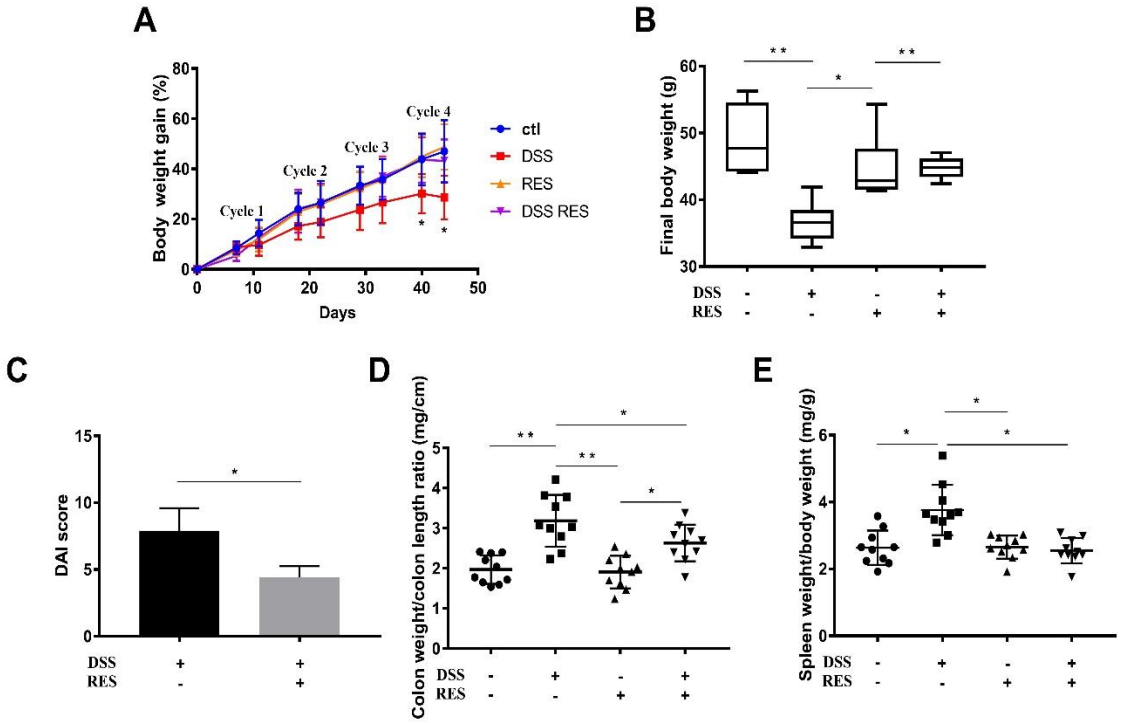
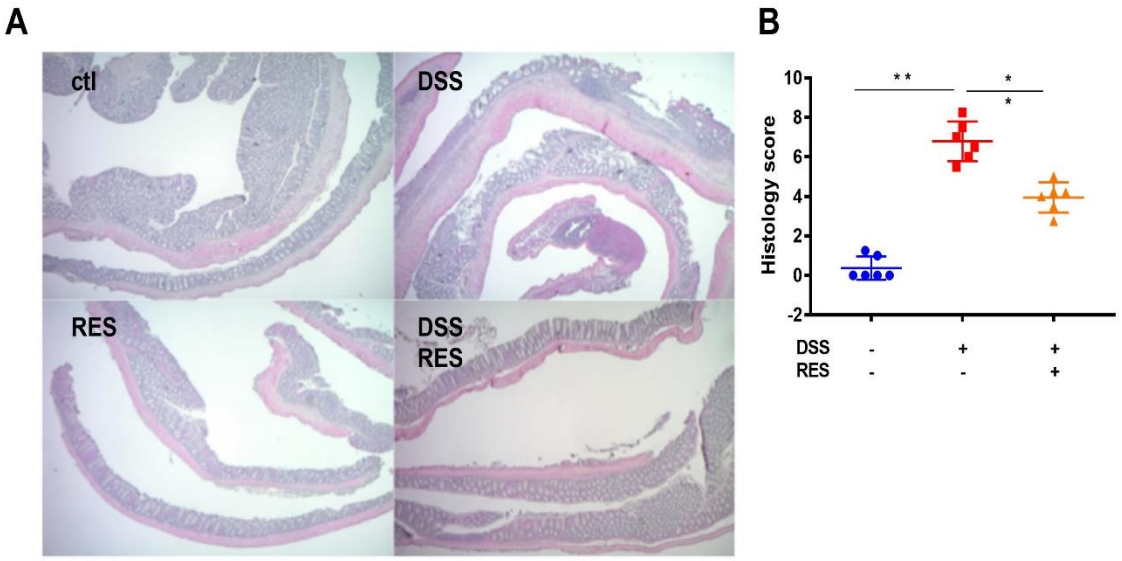


Fig. 12 Dietary resveratrol attenuated colitis symptoms. (A) Percentage of body weight gain during entire experiment; (B) Final body weight; (C) Disease activity index (DAI); (D) Colon weight to colon length ratio; (E) Spleen weight to body weight ratio. Data are mean ± SD (n=10). Significant differences are indicated: *, $P<0.05$, **, $P<0.01$.

4.3.2 Dietary resveratrol suppressed DSS-induced colitis by improving colonic tissue damage and reducing the colonic levels of pro-inflammatory cytokines

Histological H&E staining of the colonic segments from DSS group showed severe inflammatory lesions, supported by the significant distortion of crypts structure, infiltration of inflammatory cell, edema of submucosa, and formation of dysplasia (Fig. 13A).

Resveratrol supplementation significantly mitigated morphological alteration associated with DSS treatment, thus protected colonic structure. In majority of areas, the epithelium and mucosal architecture remained intact (Fig. 13A). Damage of colonic mucosa assessed by overall histological score was 1.7-fold higher ($P < 0.01$) in the DSS group compared to the resveratrol-DSS group (Fig. 13B).



Cytokines play a vital role in the pathogenesis of IBD. The imbalance between anti-inflammatory and pro-inflammatory cytokines results in colonic diseases [112]. The expression levels of granulocyte-macrophage colony-stimulating factor (GM-CSF), interferon gamma (IFN-γ), Interleukin 10 (IL-10), Interleukin 2 (IL-2), Interleukin 1 beta (IL-1β), Interleukin 6 (IL-6), keratinocyte chemoattractant/human growth-regulated oncogene (KC/GRO), and tumor necrosis factor alpha (TNF-α) in colon mucosa were

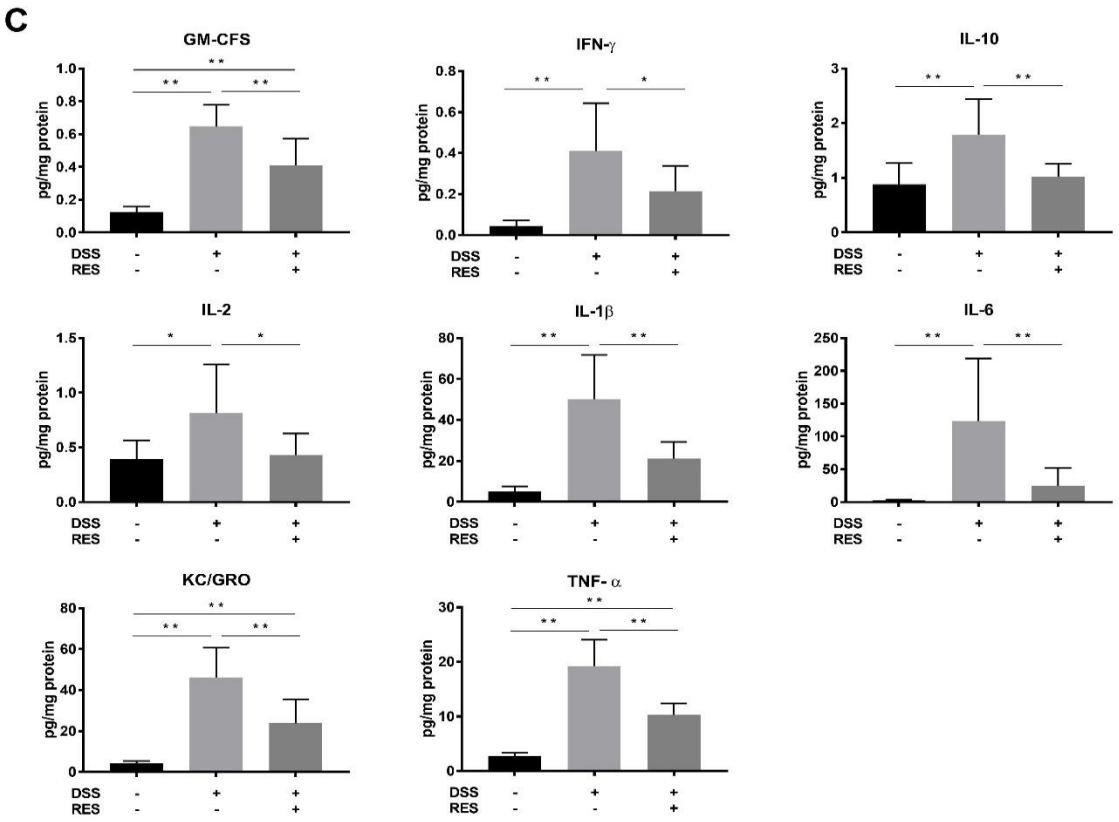


Fig. 13 Dietary resveratrol improved tissue damage and suppressed the overexpression of inflammatory cytokines. (A) Representative colon tissue images of H&E-stained colorectum sections (60X magnification); (B) Histology score; (C) Concentration of inflammatory cytokines (GM-CFS, IFN-γ, IL-10, IL-2, IL-1β, IL-6, KC/GRO, and TNF-α) in colonic mucosa. Data are mean ± SD (n= 6-8). Significant differences are indicated: *, P<0.05; **, P<0.01.

increased to 5.2, 8.7, 2.0, 2.1, 10.0, 47.9, 10.9 and 7.0-fold over control levels, respectively, in mice consumed 1.5% DSS drinking water ($P < 0.05$) (Fig. 13C). While consumption of resveratrol markedly reduced their colonic expression by 44.2%, 48.5%, 43.2%, 46.8%,

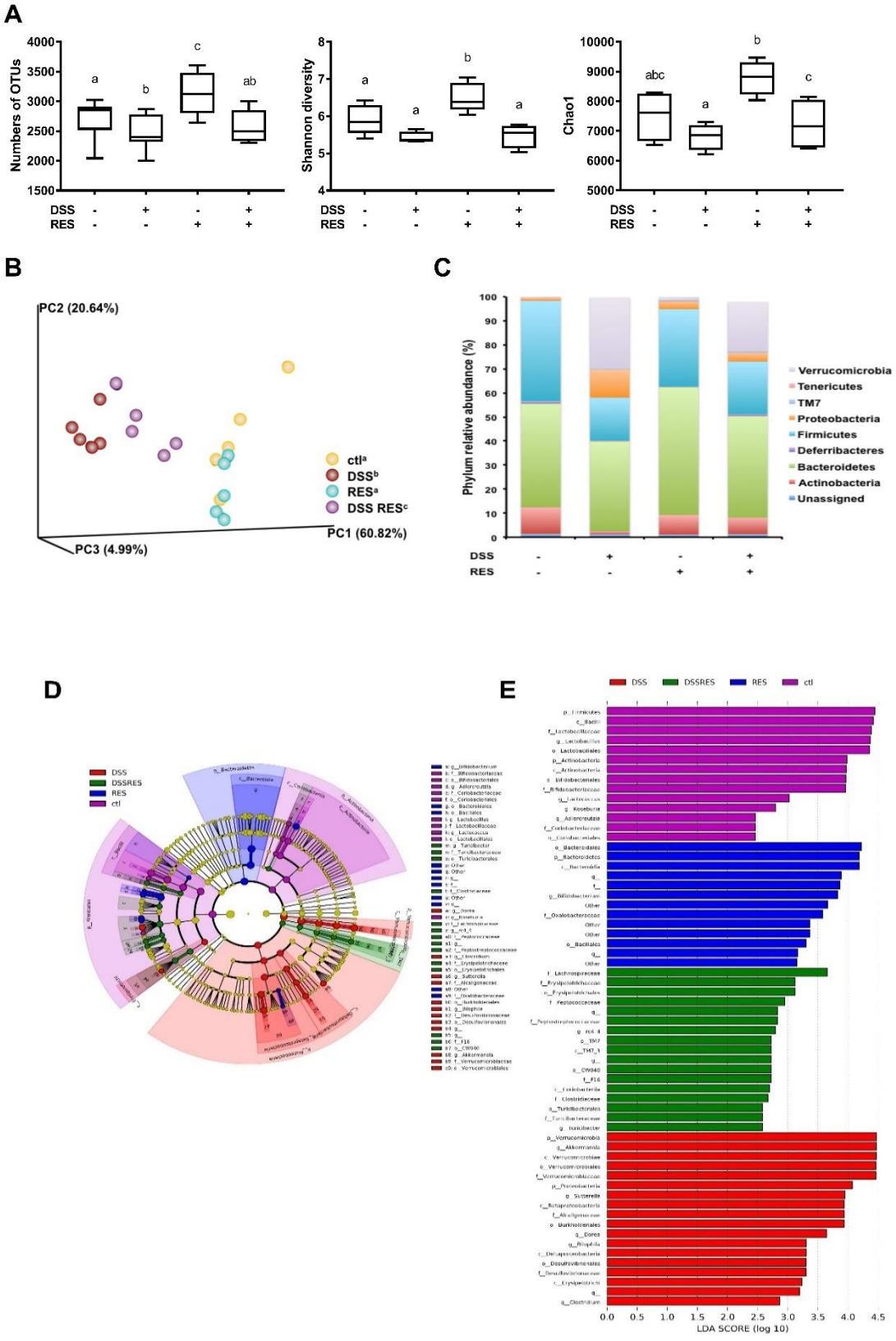
58.0%, 79.9%， 48.5%, and 46.5%, respectively ($P < 0.05$) (Fig. 13C). Taken pathological changes together, these results suggested the effective capacity of dietary resveratrol to mitigate the severity of DSS-induced colitis by down-regulating the level of colonic pro-inflammatory cytokines.

4.3.3 Resveratrol partially rescued gut microbiota dysbiosis induced by DSS

Ilumina Mi-Seq sequencing of the 16S rRNA gene of fecal samples analyzed effects of dietary resveratrol on gut microbiota structure and composition. Expressive changes observed in the microbial ecology (Fig. 14). The terms of OTUs mean a cluster of related (no less than 97% similarity) 16S rRNA sequences [113]. Numbers of OTUs, Shannon diversity, Chao1, and phylogenetic distance (PD) whole tree are common indexes of α-diversity that indicate the depth of sequence coverage and community diversity within samples [114]. As shown in Fig. 14A, dietary resveratrol significantly enriched the bacterial diversity versus control group ($P < 0.05$), while DSS treatment prevented the enrichment of microbiota caused by resveratrol ($P < 0.05$). Bacterial α-diversity was marginally decreased in DSS-treated group compared with control group ($P < 0.05$) for Numbers of OTUs), suggesting similar richness and evenness of community species between these two groups (Fig. 14A). There were no significant differences of PD whole tree among groups (Data not shown). Additionally, the structure of fecal microbiota was further assessed by β-diversity, which generated based on the number of shared species

between samples [27]. The weighted β-diversity (weighted UniFrac distances) analysis suggested that DSS treatment dramatically shifted the fecal microbiota, supported by a significant distance between control and DSS group ($P = 0.009$). While dietary resveratrol dragged the plot back toward the control group and clearly distinguished with DSS group ($P = 0.008$) (Fig. 14B), which implied that administration of resveratrol partially blunted the dysbiosis caused by DSS. The presence of overlapping clusters between control and resveratrol group illustrated the relative similarity of microbial profiles between these two groups (Fig. 14B).

To profile the specific changes in the gut microbiota, we analyzed the relative abundance of the predominant taxa identified from sequencing in all groups (Fig 14C-14F). At the phylum level, there were no significant differences existed between control and resveratrol group, except the up-regulation of *Bacteroidetes* in resveratrol group ($P < 0.05$) (Fig. 14C-14E). This result was consistent with previous studies [115]. DSS treatment significantly altered the bacterial structure by contributing the expansion of


A
Numbers of OTUs
Shannon diversity
Chao1
DSS
RES
B
PC2 (20.64%)
PC3 (4.99%)
PC1 (60.82%)
ctl
DSS
RES
DSS RES
C
Phylum relative abundance (%)
Verrucomicrobia
Tenericutes
TM7
Proteobacteria
Firmicutes
Deferribacteres
Bacteroidetes
Actinobacteria
Unassigned
D
E
DSS
DSSRES
RES
ctl
LDA SCORE (log 10)

Verrucomicrobia and *Proteobacteria* and contraction of *Actinobacteria* and *Firmcutes* *versus* control group ($P < 0.05$). However, consumption of resveratrol partially reversed this dysbiosis by down-regulation the abundance of *Verrucomicrobia* from 29.7% to 20.8% and *Proteobacteria* from 11.7% to 3.6% and up-regulation the abundance of *Actinobacteria* from 1.2% to 6.9% ($P < 0.05$) (Fig. 14C-14E). At the genus level, the aberrant dominance of *Akkermansia* (29.7% of sequences) were observed in DSS group, while it accounted no more than 1.5% of whole composition in control and resveratrol group. In contrast, dietary resveratrol significantly inhibited the excessively proliferation of *Akkermansia* (18.8%) in DSS-treated mice ($P < 0.05$) (Fig. 14D-14F). Similarly, resveratrol supplementation significantly inhibited the abnormal bloom of *Boliphila, Sutterella* and *Dorea* in DSS-fed mice ($P < 0.05$) (Fig. 14D-14F). We also noticed that the consumption of resveratrol significantly lifted the *Bifidobacterium* counts to 8.0% compared to 5.7% in control group ($P < 0.05$). Meanwhile, its relative abundance dropped down to 1.2% in DSS group and recovered to control level (6.7%) in DSS-resveratrol group ($P < 0.05$) (Fig. 14D-14F). These results indicated that resveratrol treatment restored the gut microbiota communities to compositions resembling those of the healthy control group.

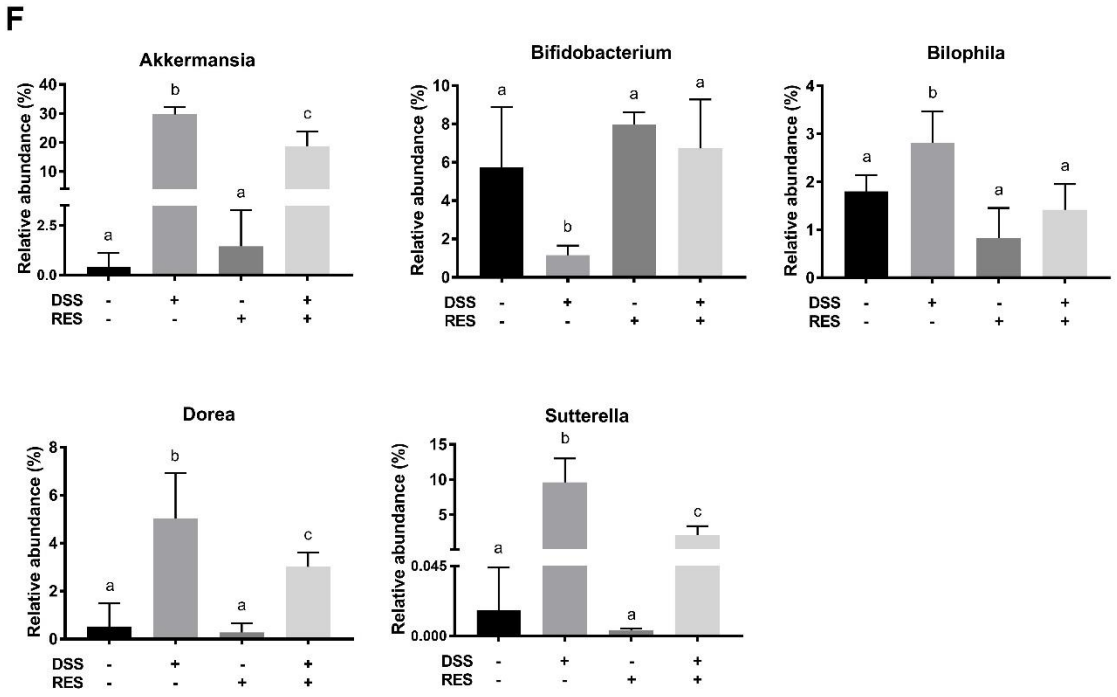


Fig. 14 Dietary resveratrol partially rescued gut microbiota dysbiosis in colitic mice. (A) α-diversity of gut microbiota with different indices - numbers of OTUs, Shannon index and Chao1 ($P < 0.05$); (B) PCoA plots based on weighted UniFrac distances representing changes of gut microbial community among different groups ($P<0.01$); (C) Gut microbiota composition at the phylum level; (E) Cladogram generated from default LEfSe analysis showing the most differentially abundant taxa enriched in microbiota among different groups ($\alpha < 0.1$ for factorial Kruskal-Wallis test); (F) LDA scores of the differentially abundant taxa (with LDA score >2 and and significance of $\alpha < 0.1$, determined by Kruskal-Wallis test); (G) Relative abundance of gut microbiota at the genus level ($P < 0.05$). Data are mean ± SD (n=5). Different letters represent statistically differences.

4.3.4 Alterations in gut microbiota correlated with aberrant cytokine expression

To determine whether alteration of the gut microbiota could be associated with abnormal expression of inflammatory cytokines, we examined the correlation between gut microbiota phylotypes and colonic cytokines (Fig. 15). As a result, consistent positive linkages were detected between potentially pathogenic organisms enriched in DSS group and pro-inflammatory cytokines. For example, at the phylum level *Proteobacteria* were significantly positively correlated with GM-CSF, IL-1β, IL-2, IL-6, KC/GRO and TNF-α ($p < 0.05$). Similarly, the abundance of genera *Boliphila* and *Sutterella* who belong to *Proteobacteria* positively correlated with pro-inflammatory cytokines, including IL-1β, IL-6 and IFN-γ ($p < 0.05$). We also observed that health promoting gut microbiota were negatively correlated with pro-inflammatory cytokines and positively correlated with anti-inflammatory cytokines. Specifically, *Bifidobacterium* was highly negatively correlated with IL-1β, IL-6, KC/GRO and TNF-α ($P < 0.05$), and positively correlated with IL-4. Same to *Lactobacillus*, negative correlations were observed with pro-inflammatory cytokines (GM-CSF, IFN-γ, IL-1β, KC/GRO, and TNF-α) ($P < 0.05$). These data suggested that mutation in bacterial composition might influence changes in the expression of inflammatory cytokines in colon.

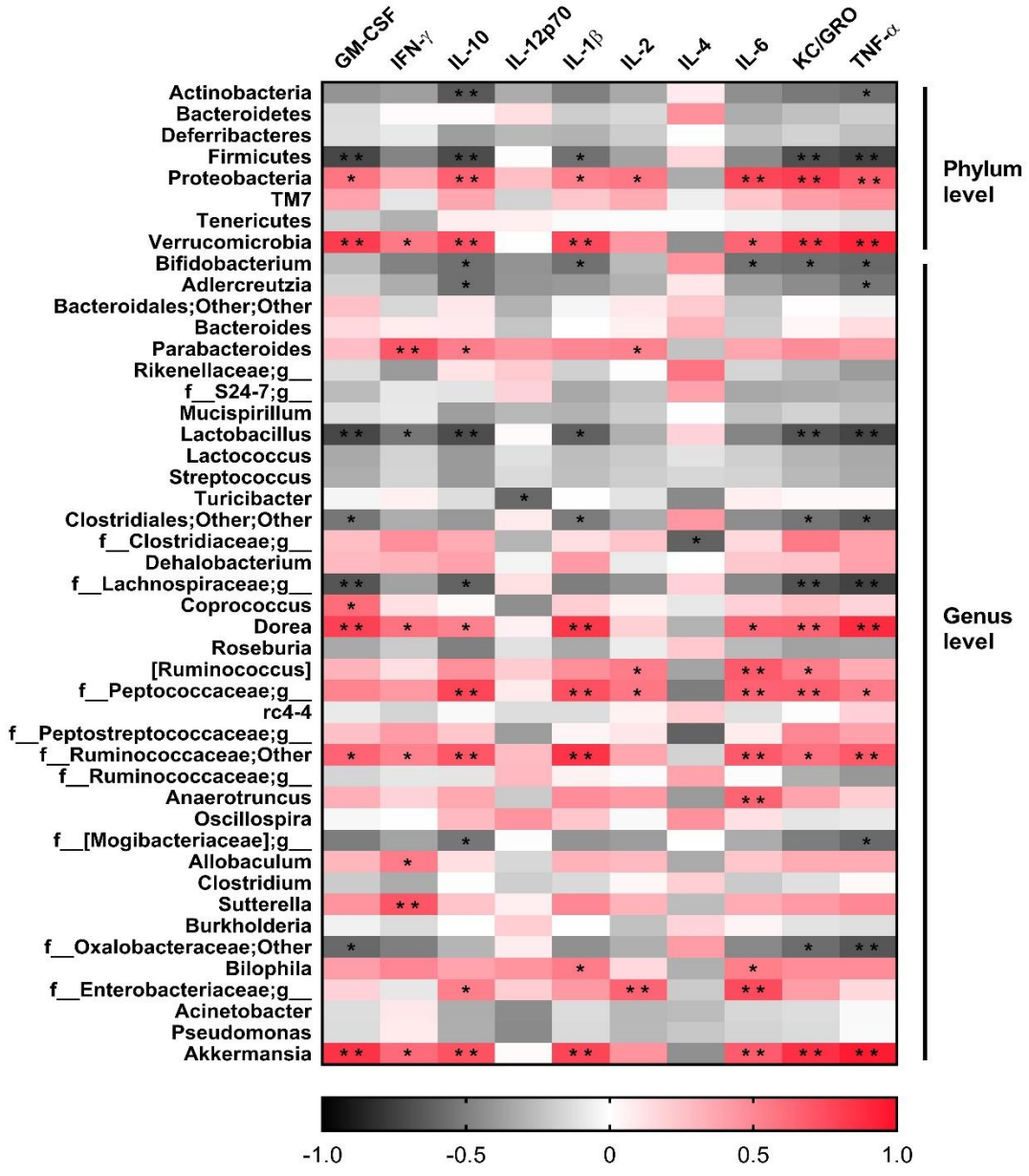


Fig. 15 Heatmap of correlation between gut microbiota and inflammatory cytokines. Positive correlation is in red. Negative corelation is in black. Significant differences are indicated: *, P<0.05; * *, P<0.01.

4.4 Discussion

IBD conduct a growing health concern due to increasing incidence worldwide. The current notion on pathogenesis of IBD is the involvement of diet, gut microbiome and epigentics. Among these factors, diet plays an important role in modulating the gut microbiota and influencing epigenetic changes [10]. Therefore, improving diet quality could be applied as a prophylactic tool to moderate the disease course. Consequently, many phytochemicals enriched diet have been explored as preventive treatments recently [31]. The protective effects of resveratrol against acute or chronic colitis in different models were demonstrated in previous studies [3, 103, 116, 117]. However, the mechanism of anti-inflammation effects of resveratrol, especially the role of gut microbiota has not been illustrated yet. DSS (a chemical agent) induces colitis by disrupting the epithelial barrier and increasing colonic permeability, which is critical for the IBD onset. This impairment of gut barrier allows for gut-derived bacteria, disrupts balance of probiotics and pathogens, and leads to increased interactions between microbiota and immune system [118]. A complex interplay exists among diet, IBD, intestinal epithelium and microbial communities in the maintenance of gastrointestinal homeostasis. Therefore, it is of great significance to further explore the mechanisms of anti-colitis effects of resveratrol from the gut microbial perspective. One previous research studied the impacts of dietary resveratrol on several gut bacteria in acute colitis model by traditional counting method [117]. Now 16S rRNA-based sequencing allows fully identifying and profiling the structure and composition of gut microbiota in our study.

In this research, we investigated the interplay between dietary resveratrol, gut microbiota and chronic colitis in the *in vivo* model. The resveratrol dose assayed is equivalent to 2 mg/kg/day in humans approximately, which is conveniently achievable through dietary supplementation. We found that consumed achievable dose of resveratrol significantly inhibited colitis by alleviating the loss of body weight, DAI, colon shortening, tissue injury and inflammatory cytokine changes induced by DSS treatment. These results agree with former researches [3, 116, 117], which suggested that resveratrol exerts an anti-inflammatory action in DSS-induced colitis and is capable of preventing DSS induced colitis.

Cytokines play a crucial role in driving and mediating intestinal inflammation. The severity of IBD is associated with up-regulation of pro-inflammatory cytokines and down-regulation of anti-inflammatory cytokines. Hence rebalance levels of these cytokines are potential therapies to alleviate IBD [112]. As found in ELISA analysis, seven pro-inflammatory cytokines (GM-CSF, IFN-γ, IL-1β, IL-2, IL-6, KC/GRO, and TNF-α) were overrepresented in DSS-treated group as shown in Fig. 10C. TNF-α is one of the most effective proinflammatory cytokines in IBD that can directly disrupted epithelial barrier and inducted apoptosis in epithelial cells [119]. IFN-γ is an indispensable proinflammatory cytokine in the initiation of DSS colitis with pleiotropic functions including stimulation of T and natural killer (NK) cells and modulation chemokines [120]. IL-1β signals are

required for the development of severe inflammation in both T cell-mediated and T cell-independent colitis [121]. Over expression of IL-6 can activate the anti-apoptotic genes Bcl-xl, leading to the expansion of T-cell, which further induce the chronic intestinal inflammation [122]. In line with previous studies, dietary resveratrol significantly mitigated the overexpression of TNF-α, IFN-γ, IL-1β and IL-6 in DSS-treated mice (Fig. 13C) [1, 103, 123]. The precise role of GM-CSF in the pathogenesis of IBD is contradictory now. It is believed that production of GM-CSF is related to a delayed neutrophil apoptosis, which contributes to the tissue injury in IBD and an increased secretion of GM-CSF has been found in mucosal lesions of mouse model and IBD patients [124, 125]. In contrast, some studies indicate that GM-CSF plays a protective role in intestinal infection and benefits in overcoming bacteria invasion [27]. Here we for the first-time report that dietary resveratrol down-regulated the enhanced GM-CSF production in DSS-induced colitis mice model (Fig. 13C). KC/GRO is a CXC chemokine also known as chemokine (C-X-C motif) ligand 1 (CXCL1). During inflammation, it is involved in neutrophil activation and recruitment [126]. It has been reported that the level of CXCL1 in serum is highly correlated with the grade of disease in the IBD patients [126]. We firstly indicate here that dietary resveratrol inhibited DSS-induced colitis through CXCL1-mediated pathways (Fig. 13C). IL-2 is discovered as a key cytokine that supports the proliferation and differentiation of regulatory T cells. Subsequently, aberrant expression of IL-2 promotes the imbalance between effector T cells and regulatory T cells, which leads to multi-organ autoimmune

disease and inflammation [127]. We firstly revealed the expanded expression of IL-2 in DSS-induced colitis mice model. More meaningful our result showed that dietary resveratrol down-regulated IL-2 to the level of control group in DSS-treated mice (Fig. 13C). In addition, we also evaluated the expression levels of two anti-inflammatory cytokines (IL-4 and IL-10) in colonic mucosa. IL-10 exerts its anti-inflammatory property by inhibiting pro-inflammatory cytokines such as IL-1, IL-6, IL-12 and TNF-α as well as chemokines [128]. In our study the expression changes of IL-10 showed similar pattern with other pro-inflammatory cytokines - augmented in DSS group and cut down in DSS-resveratrol group (Fig. 13C). This result is contradictory with previous study [129]. We speculated the possible cause behind this finding is stressful reaction to extremely severe inflammation (overexpress of IL-10 to overcome expansion of seven proinflammatory cytokines) [130]. Concentration of IL-4 monitored as well. No significant differences existed among groups (data not shown). Above results suggested the potent anti-inflammatory effects of dietary resveratrol via inhibition of pro-inflammatory mediator's production.

The impact of dietary resveratrol on relative abundance of the predominant taxa in all samples was evaluated based on the following criteria: phylum, class, order, family, and genus. In accordance with previous research, the dominant bacterial phyla in healthy adults are *Firmicutes*, *Bacteroidetes*, and *Actinobacteria*, normally with lower abundances of

Verrucomicrobia and *Proteobacteria* [131]. Consistently, we found that *Firmicutes*, *Bacteroidetes* and *Actinobacteria* accounted more than 95% of the whole gut microbial composition in control group. While in DSS group, these three phyla only occupied 56.5%. At the meantime, *Proteobacteria* and *Verrucomicrobia* increased to 41.4% of the gut microbial composition in DSS group (Fig. 14C). At the genus level, a lifted proliferation of *Lactobacillus* and *Bifidobacterium* was observed in resveratrol group, which suggested that dietary resveratrol could promote the enrichment of probiotics (Fig. 14D-14F). In fact, several studies have demonstrated a protective effect of *Lactobacillus* and *Bifidobacterium* in DSS-induced colitis, impacting multiply parameters like proinflammatory cytokines and oxidative damage [132]. More meaningfully, we found that resveratrol successfully restored the frequencies of *Bifidobacterim* to control level in DSS-treated mice (Fig. 14D-14F). From other side, dietary resveratrol also improved the dysbiosis of some potential pathogens induced by the colitis, such as *Akkermansia*, *Biliphila* and *Sutterella* (Fig. 14D-14F). *Akkermansia* is a Gram-negative mucin-degrading bacterium and the only member of *Verrucomicrobia* phylum identified to date [133]. Seregin *et al.* concluded that *Akkermansia* acted as a pathobiont to promote colitis in a genetically susceptible host under the regulation of nod-like receptor 6 (NLRP6) [134]. Moreover, the enrichment of *Akkermansia* has been reported in faeces of mice with DSS-induced colitis and patients suffering from colorectal cancer [135, 136]. *Biliphila* belonging to phylum of *Proteobacteria* is sulfate-reducing bacteria associated with IBD and appendicitis [137].

Sutterella Wadsworthensis (belonging to genus of *Sutterella* and phylum of *Proteobacteria*) has been suggested could induce inflammation cascades by occupy the mucus layer [138]. Our results revealed that dietary resveratrol strikingly reversed these unfavorable changes in DSS-treated mice (Fig. 14D-14F). We also observed significantly differences of *Dorea* (belonging to phylum of *Firmicutes*)) richness among different groups (Fig. 14F). Limited researches on *Dorea* have been published. One clinical study indicated that *Dorea* represented lower level in IBD patients, suggesting *Dorea* may act as a probiotic [139]. To our best knowledge, this is the first time demonstrate that dietary resveratrol alleviated microbial community perturbations by selectively restoring the proliferation of probiotics and blunting the expansion of potential pathogens in DSS-induced colitis mice model. These findings could suggest a possible prebiotic effect of resveratrol on the microbiota, which may influence the host metabolism. Nevertheless, the mechanism of how resveratrol affects the gut microbiota remains an important field of future research.

To better understanding the relationship between dietary resveratrol, inflammatory cytokines and gut microbiota, we analyzed the correlation between cytokines and gut microbiota. As observed in Fig. 15, pro-inflammatory cytokines (GM-CSF, IFN-γ, IL-1β, IL-2, IL-6, KC/GRO, and TNF-α) were significantly positively correlated with potential pathogenic gut microbiome (*Akkermansia*, *Bilophila, Parabacteroides*, *Ruminococcus*, and *Sutterella*) and negatively correlated with probiotics (*Bifidobacterium*, *Lactobacillus* and

Roseburia). Positive correlation between TNF-α with family *Ruminococcaceas*e and genus *Parabacteroides*, and negative correlation with family *Rikenellaceae*, family *Lachnospiracease* and genus *Lactobacillus* were reported in previous studies [140, 141], suggesting a crucial role of gut microbiota in TNF-α production [142]. The protective effect of *Lactobacillus* and *Bifidobacterium* in DSS-induced colitis by modulating proinflammatory cytokines has been discussed [132]. Accordingly, we demonstrated their positive correlation with anti-inflammatory cytokine IL-4 and negative correlation with potential pathogens (Fig. 15). Here we for the first time systematically report the complex correlation between inflammation-associated cytokines and gut microbiota abundance in DSS-induced colitis model. These results offer a clue to further investigate the causality between these two events and illustrate the pathogenesis of IBD.

In conclusion, our results reinforced the important role of dietary resveratrol (2 mg/kg/day) as a multitargeted anti-inflammatory compound. The effects of resveratrol on DSS-induced colitis and the gut microbiota were clearly revealed, and the complex correlation between inflammatory cytokines and gut microbiota was systematically displayed. However, whether the anti-inflammatory effects observed are due exclusively to the direct resveratrol action or it is also mediated by the gut microbiota deserves further investigation.

CHAPTER 5

EFFECTS OF ALTERED GUT MICROBIOTA ON BIOTRANSFORMATION OF RESVERATROL

5.1 Introduction

Resveratrol is a phytochemical that can be found in variety of foods and plants, such as grapes, peanuts, pistachios, berries, and Japanese knotweed [143]. Resveratrol is marketed by many nutritional supplement companies due to its wide range of health benefits functions. Resveratrol decreased trimethylamine-N-Oxide levels and increased hepatic bile acid neosynthesis, thus potentially attenuated atherosclerosis [115]. Fat storage was lowered by resveratrol in obesity mice [144]. Resveratrol also exhibited comparable beneficial effects with calorie restriction diet on extending the lifespan of mice and *Caenorhabditis elegans* [145, 146]. In addition, resveratrol may act as an anti-inflammatory and anti-cancer agent [82].

Though vast amount of data has become available, the biological effects of resveratrol and their underlying mechanism are not fully understood yet. This may partially due to the knowledge gap of the metabolic fate of resveratrol. Fully elucidating he metabolic route of resveratrol is crucial, because the derived metabolites may have significantly different chemical, which will lead to different biological characters. Numerous studies have

provided consistent information about the conjugation of resveratrol via intestinal and hepatic metabolism [5, 70, 93]. Our recent studies emphasized the critical role of microbial metabolites of resveratrol: dihydro-resveratrol (DHR) and lunularin (LUN), not only because of their high concentration, but also their stronger chemopreventive effects on anti-inflammation and anti-proliferation (unpublished data) at achieved tissue levels. These innovative results driven our attentions from hepatic and small intestinal to microbial biotransformation of resveratrol.

The microbial biotransformation of dietary polyphenols, including ring fission, reduction, demethylation, hydrolyzation glycosides, and dihydroxylation, has been well described [13, 14]. On the one hand, significantly interindividual differences exist in gut microbial composition, which may lead to different microbial transformation patterns of polyphenols. As in the case of production of either equol or *O*-demethylangolensin and dihydrodaidzein from daidzein [15]. On the other hand, gut microbiota composition could be dramatically altered in various diseases [16]. Previous study indicated that gut microbiota diversity and balance between pathogens and probiotic were significantly disturbed in DSS-induced colitic mice (unpublished data).

Given these complex situations, the first step of our current study was to prove that gut microbiota was necessary in the production of DHR and LUN by using antibiotic treated mice. Then a human study was performed to elucidate the different interindividual

routes of resveratrol transformation by gut microbiota. Lastly, we identified, quantified, and compared the distribution of DHR and LUN in DSS-treated colitic mice and healthy mice, which had vital implications in biological activities of resveratrol. Overall, our current study for the first time systematically discussed the effects of altered gut microbiota on the biotransformation of resveratrol and its important implications in biological activities of resveratrol, which should be taken into account during investigation of health-promoting effects of this polyphenol.

5.2 Materials and Methods

5.2.1 Materials

RES (>99% purity) was purchased from Quality Phytochemicals (Edison, NJ, USA). Pinostilbene (PIN) (>98% purity) and DHR (>98% purity) were obtained from Yuanye Bio-Technology Co., Ltd (Shanghai, China). LUN (>98% purity) was purchased from Aikon Biopharma LLC (Nanjing, China). Sulfatase (type H-1, from Helix pomatia, containing sulfatase and β-glucuronidase) was obtained from Sigma-Aldrich (St. Louis, MO, USA). Acetonitrile (ACN), methanol (MeOH), acetic acid, and ethyl acetate were purchased from Fisher Scientific (Fairlawn, NJ, USA). All these solvents are HPLC grade.

5.2.2 Animals models, diets and treatments

The Institutional Animal Care and Use Committee, University of Massachusetts-Amherst approved all animal experiments performed. Ten male CD-1 mice (6-week old) were obtained from Charles River Laboratory (Wilmington, MA, USA). Mice were housed individually in a cage and cage bedding was changed two times per day. After one week of diet acclimation, all mice receive resveratrol enriched diet, which contained 0.025% (w/w) RES in standard AIN93G diet for five days. From day 6, all mice continuously received resveratrol enriched diet but drink antibiotic water (AB), which contained broad-spectrum antibiotics (ampicillin and neomycin) [147]. At the day 10, all mice were sacrificed with CO_2 asphyxiation. The urine and fecal samples were collected everyday with Labsand (Braintree, MA) for further HPLC-MS analysis.

Twenty male CD-1 mice (6-week old) were obtained from Charles River Laboratory (Wilmington, MA, USA). After one week of diet acclimation, mice were randomly divided into two groups. Ten mice in RES group fed standard AIN93G diet containing 0.025% (w/w) of RES and normal drinking water. The other ten mice in DSS RES group fed standard AIN93G diet enriched with 0.025% (w/w) of RES and received 1.5% DSS water (wt/v, dextran sulfate sodium salt) (International Lab, Chicago, IL). 1.5% DSS was administered in the drinking tap sterilized water ad libitum for 4 days followed by 7 days of pure water for recovery, and this cycle was repeated four times. At the end of the fourth cycle of DSS treatment, all mice were sacrificed with CO_2 asphyxiation. Heart, liver, spleen,

lung, kidney, brain, stomach, small intestine (transversely cut equally into four parts), cecum, colon and bile were collected and stored at -80°C for further analysis. Blood samples were centrifuged at 3 000g for 15 mins at 4°C to collect serum.

5.2.3 Sample preparation

Serum, bile and urine samples were extracted according to Menet et al. [93]. Briefly, aliquots of samples were vortex-mixed with ten volume of acidified (2.5% acetic acid) ACN and stood at ice for 20 min to precipitate the protein. After centrifugation (14000 rpm, 10min, 4°C), the supernatant was evaporated to dryness using a Speed Vac Concentrator (Savant Thermo Fisher Scientific Inc., Agawam, MA). Tissue and fecal samples were prepared according to Juan et al. with modifications [67]. Briefly, aliquots of tissues were homogenized with 10 volume of MeOH/water/acetic acid (80:20:2.5) solution using a Bead Ruptor Homogenizer (Omni International, Kennesaw, GA). The homogenates were then centrifuged at 14000 rpm for 5 min. Specially for kidney samples, the homogenate was sonicated for 20 min before centrifugation. The residues were extracted one more time and combined methanolic layers were evaporated to dryness under vacuum. All sulfated and glucuronide metabolites were measured by enzymatic hydrolysis of the processed samples with β-glucuronidase and sulfatase as reference described [74]. The internal standard PIN (5 μmol/L) was routinely used in all the samples. The dried extractions were reconstituted in 50 μL 50% MeOH for further analysis.

5.2.4 Sample analysis with HPLC-MS

The concentration of RES and its metabolites were quantified by using the Shimadzu Model 2020 HPLC-MS (Shimadzu, Kyoto). The metabolites were eluted with a Zorbax SB-Aq C18 column (Agilent Technologies, Santa Clara, CA, USA) at flow rate of 0.6 mL/min. Mobile phase A was 5% acetonitrile/water, mobile phase B was 100% acetonitrile. Gradient elution started at 15% solvent B, linear gradient from 15 to 70% solvent B over 18 min, held at 70% B for 3 min, then followed by washing and reconditioning the column. The Mass-spectra conditions were optimized at negative electrospray ionization mode, as follows: Heat block temperature 400°C, desolvation line temperature 250°C, interface voltage -3.5 kv, and drying gas flow 15 L/min. Data acquisition and processing were accomplished using Labsolutions Software (Shimadzu).

5.2.5 Cell viability assay, colony formation assay, nitric oxide assay and anti-oxidant assay

We adopted various cell models to investigate the multiple biological activities of RES and its metabolites at achieved colon tissue levels, including anti-proliferation of cancer cells, anti-inflammation, anti-oxidant and anti-colony formation. All cells were purchased from American Type Cell Collection (ATCC, Manassa, VA, USA). HT-29 and HCT-116 were applied to MTT and colony formation assays as described previously to explore the anti-proliferative effects [95-97]. Nitric oxide assay (anti-inflammation) were performed

according to Guo et al. in RAW264.7 macrophage model [95, 96]. Caco-2 cells based anti-oxidant assay consulted with previous reference [148].

5.2.6 Statistical analysis

All data were expression as Mean ± standard deviation (SD). The statistical significance was assessed by one-way ANOVA with post hoc Tukey HSD test. P value < 0.05 was considered as statistically significant.

5.3 Results and discussion

5.3.1 DHR, LUN and their conjugates absent in antibiotic treated mice

Walle et al identified DHR as a major metabolite of resveratrol, that was likely produced by the intestinal microbiota for the first time [5]. Later, *Slackia equolifaciens*, *Adlercreutzia equolifaciens*, and *Eggerthella lenta* ATCC 43055 were identified as DHR producers [74, 75]. LUN was identified as a microbial metabolite of resveratrol in an *in vitro* fermentation study by Bode el al firstly, though the responsible producer was not identified [74]. These results indicated that the production of DHR and LUN was associated with gut microbiota, but still could not exclude the potential involvement of enzymes abundant in liver, kidney and upper gastrointestinal tract, like CYP450 [149]. Especially, DHR, LUN and their conjugates were detected in the stomach content and tissue in mice (unpublished data). Therefore, broad-spectrum antibiotic was used to wash

out the gut microbiota in mice in our study. The metabolites of resveratrol were compared before and after antibiotic treatments. Previous studies demonstrated that antibiotics treatments significantly decreased diversity, richness and evenness of the fecal microbiota [150]. Ampicillin and Neomycin were chosen due to their poor absorption in small intestine, which resulted in better target to the commensal microbes [151].

As shown in Fig. 16A, 16C, all mice could metabolize RES to DHR, while only three mice could produce DHR before antibiotic treatment. LUN was a minor metabolite in mice #2, which only occupied for 4.59% of the total metabolites. While DHR and its conjugates account 84.75% of the metabolites in #2 mice (Fig. 16C). In mice #6 and #8, LUN and its conjugates accounted over 50% of the metabolites in the urine. These results could attribute to the interindividual differences in gut microbiota. In other five mice, RES-conjugates, DHR, and DHR-conjugates were major metabolites of RES. Unmetabolized RES taken up to 17.9% of the total derivates (Fig. 16C). These results were consistent with our previous studies.

Strikingly, DHR, LUN and their conjugates were totally disappeared after five days antibiotic treatment as shown in Fig. 16B. These results for the first time fully demonstrated that DHR and LUN are strictly microbial metabolites of resveratrol. The appearance of DHR, LUN and their conjugates in the upper GIT and tissues were due to the reabsorption of DHR and LUN in large intestine. These findings provided fundamental information in

the metabolic pathway of resveratrol after oral consumption in human.

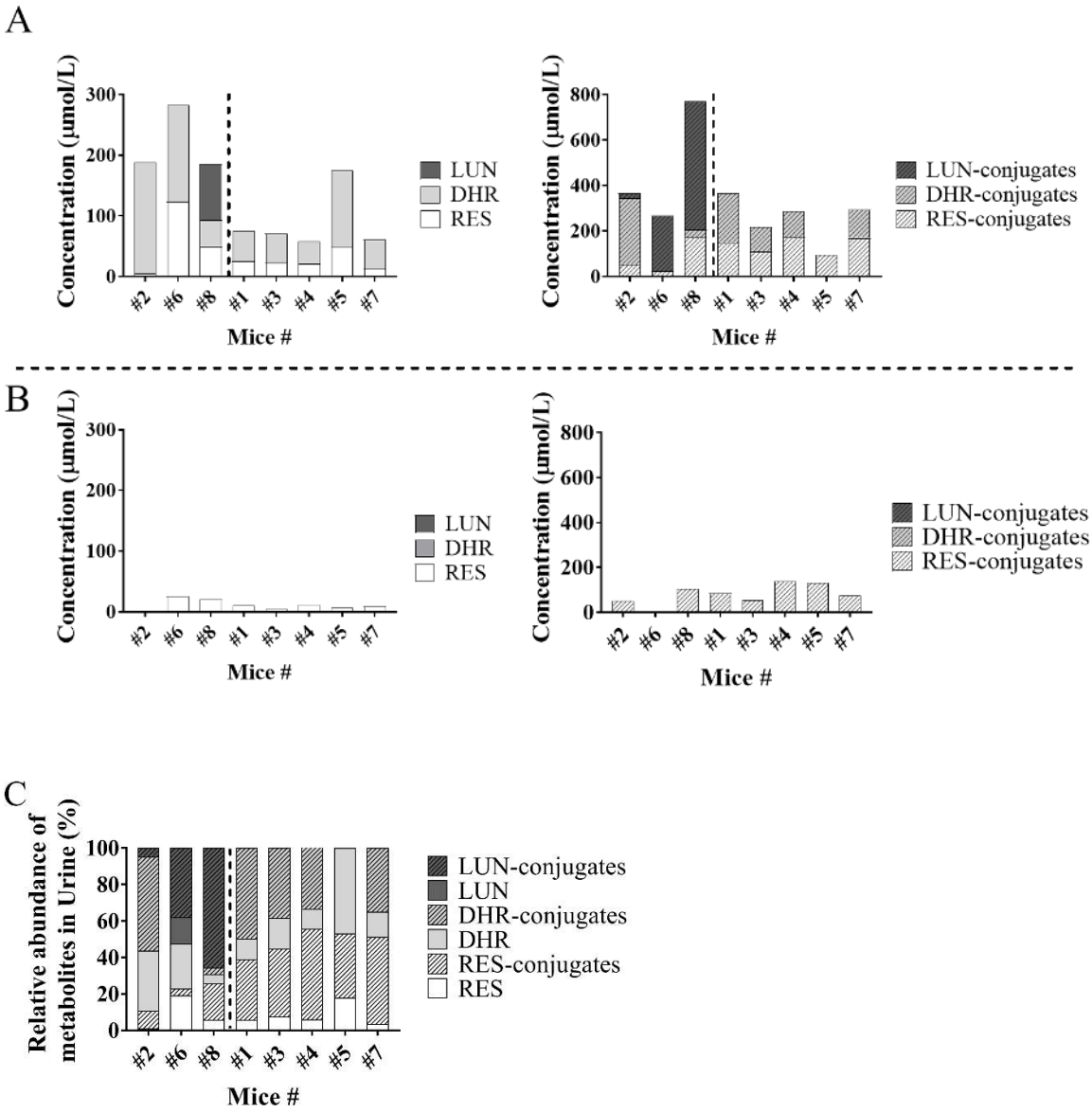


Fig. 16 Metabolites of resveratrol before and after antibiotic treatment in the urine. (A) Metabolites of resveratrol in the urine before antibiotic treatment. (B) Metabolites of resveratrol in the urine after antibiotic treatment. (C) Relative abundance of resveratrol metabolites in the urine before antibiotic treatment.

5.3.2 Biotransformation of RES in DSS-induced colitis mice

We had observed that the anti-inflammatory and anti-proliferative effects of DHR and LUN were significantly stronger than RES at the observed colon tissues levels (unpublished data). Dramatically gut microbiota alteration was also announced in our previous research (unpublished data). Taken all these observations together, we hypothesized that altered gut microbiota in DSS-induced colitis mice would change the biotransformation of RES in large intestine and further impact its biological activities.

The concentrations of RES, DHR, LUN and their conjugates in the colon and cecum tissues were compared between heathy mice and DSS-treated colitis mice (Fig. 17). Most strikingly, LUN and its conjugates were totally deracinated in the cecum and colon tissues in the DSS-treated mice (Fig. 17). It was also noteworthy that the concentrations of RES were 34.39 and 6.49 nmol/g, respectively in the cecum and colon tissues in the DSS-treated mice, which were much higher than those in the healthy mice (cecum: 4.32 nmol/g; colon:1.32 nmol/g). We speculated that the disappeared LUN and increased RES in the large intestine in colitic mice may resulted in different biological effects of RES at the site of function. The conjugated RES and DHR in the cecum were higher in colitic mice compared with healthy mice, which indicated the weaker deconjugation capacity of gut microbiota in the cecum in the colitic mice (Fig. 17).

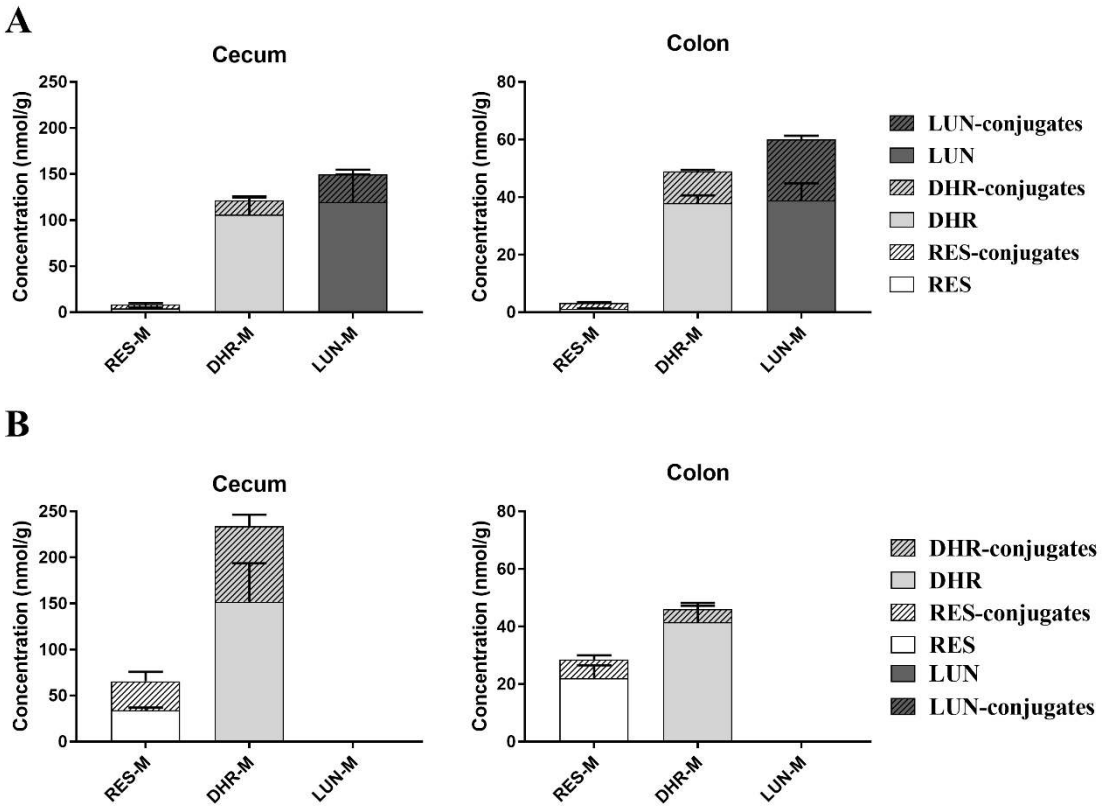


Fig. 17 Concentration of RES metabolites in the cecum and colon tissues in the healthy mice (A) and colitic mice (B).

To further confirm the distinguished metabolism patterns in colitic mice *vs* healthy mice. The distribution of RES metabolites in the bile, liver, kidney, and serum were analyzed. Generally, LUN and its conjugation remained unshown in the colitic mice (Fig. 18), which further supported that LUN was produced by gut microbiota. The concentrations of all metabolites were lower in colitic mice compared with healthy mice, which may due to the lower food intake and/or impaired gastrointestinal absorption

function in colitic mice [152, 153].

DHR and LUN were reabsorbed from large intestine and concentrated in the bile. We consistently observed the absence of LUN and its conjugates in the bile in colitic mice (Fig. 18). In the bile, the concentration of RES-M was as high as 335.23 μmol/L, which was much higher than DHR-M (165.80 μmol/L) and LUN-M (174.66 μmol/L) in healthy mice. Among these, quiet amount of unmetabolized RES (10.23 μmol/L), DHR (49.69 μmol/L), and LUN (52.07 μmol/L) were observed in healthy mice (Fig. 20). While, in the colitic mice RES-M was apparently lower than DHR-M, which may due to the lower food intake and/or impaired small and large intestinal function in colitic mice [152, 153]. The concentrated DHR and LUN further entered in to the enterohepatic circulation and underwent extensive phase II metabolism.

In the serum, no free formed metabolites were detected in both colitic mice and healthy mice. Patel and Brown fed mice with RES-3-*O*-sulfate and RES-4'-*O*-sulfate proved that conjugated metabolites served as an intracellular pool in the body from which RES could be regenerated locally, this could be the same case for DHR and LUN [83]. Thus, in the liver and kidney, free formed DHR and LUN were observed. Especially, in the kidney free-formed metabolites accounted 67.36% of the total metabolites in healthy mice. Our previous study indicated that at the achieved tissue levels, free formed DHR and LUN exhibited stronger anti-cancer effects than RES alone in the renal carcinoma cell lines.

The lower concentration of free-formed RES and DHR in the kidney in colitic mice may compromised these heathy benefits.

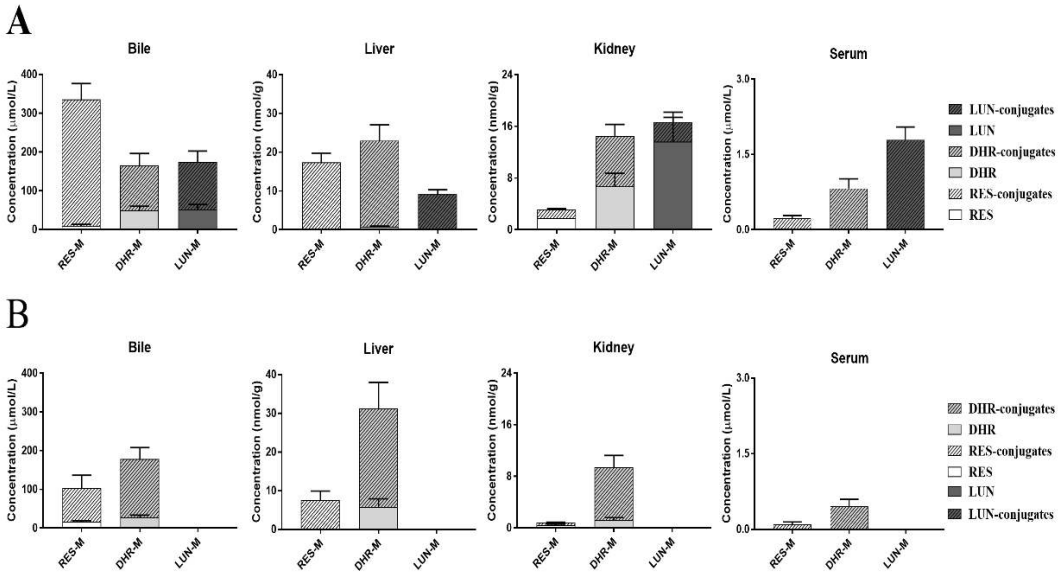


Fig. 18 The distribution of RES metabolites in the bile, liver, kidney, and serum in the colitic mice vs healthy mice.

5.3.4 Stronger chemopreventative effects of RES metabolites at concentrations equivalent to that found in the colon tissues in the colitic mice *vs* healthy mice

we found that DHR, LUN and their combination exerted much stronger chemo-preventive and anti-inflammatory effects in the colonic tissues, at their concentrations achieved in the tissues, suggesting that DHR and LUN may greatly contribute to the chemopreventive properties elicited by RES in the colon. Meanwhile, above data indicated that LUN and its metabolites were disappeared in the DSS-treated mice

due to the altered gut microbiota. These preliminary data guaranteed the further investigations on chemopreventative effects of RES metabolites at the tissue relevant levels in the colitic mice. To establish the protective effects of RES and its metabolites in a physiologically relevant manner, we deliberately used the concentrations measured in the colonic tissues to determine their bioactivities.

To establish the anti-colitis and anti-colon cancer effects of DHR and LUN in a physiologically relevant manner, we determined their inhibitory effects on two widely used human colon cancer cell lines (HCT-116 and HT-29) at the concentrations found in the mouse colonic tissues. Treatment of 1× stood for concentrations measured in the colonic tissue, that was combinations of 1.32 nmol/g of RES, 37.96 nmol/g of DHR and 38.89 nmol/g of LUN in the healthy mice or 22.07 nmol/g of RES and 41.53 nmol/g of DHR in the DSS-treated mice (Fig. 19A). Generally, RES metabolites at the concentrations equal to that found in the colitic mice exhibited stronger anti-inflammation and anti-proliferation abilities than that found in the healthy mice (Fig. 19 B-D), which explained the anti-colitis effects of resveratrol supplementation in DSS-induced colitis mice (unpublished data).

LPS-stimulated RAW 264.7 macrophages were used to determine the anti-inflammatory potency of RES metabolites. RES metabolites at concentrations equivalent to that in the healthy mice showed a tendency to suppress the production of NO (an important inflammatory mediator). While, the inhibitory effects of RES metabolites at

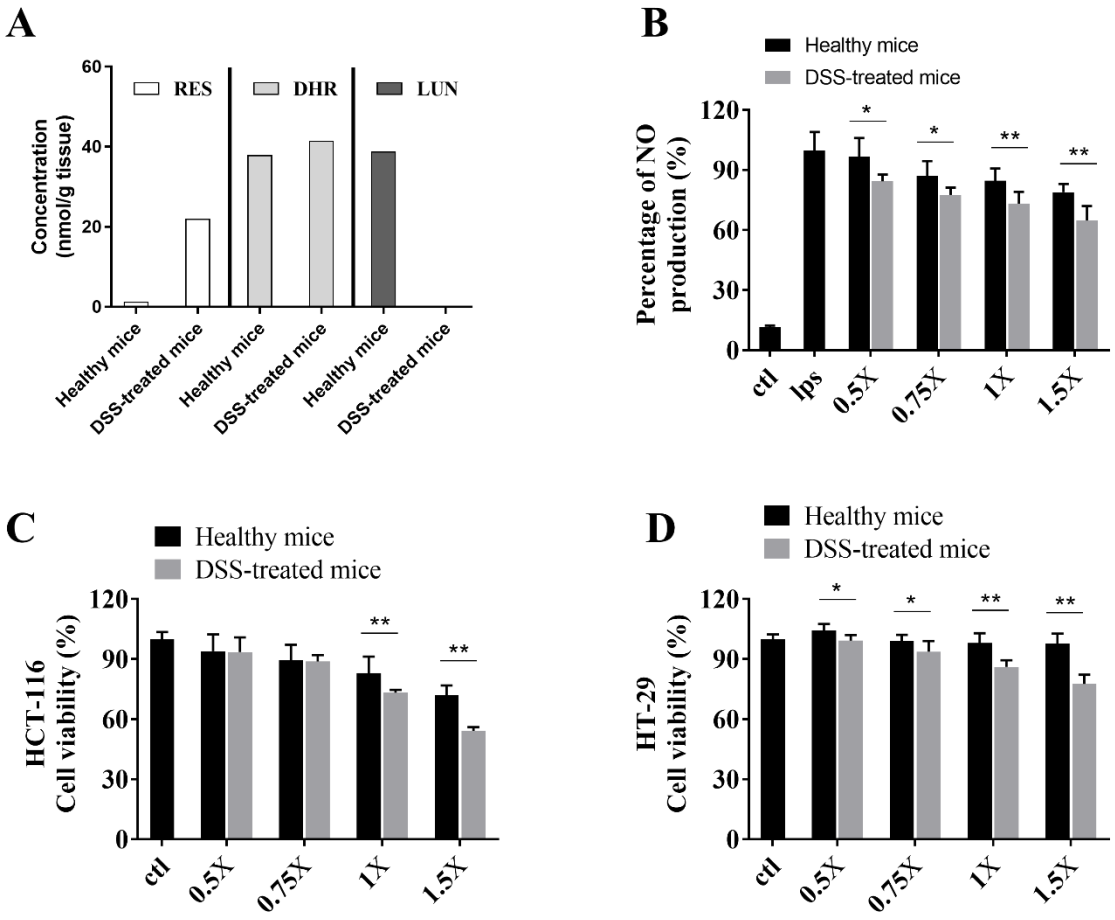


Fig. 19 Chemopreventative effects of RES metabolites in the DSS-treated mice vs healthy mice. (A) Concentrations of RES metabolites in the colonic tissue in healthy mice vs colitic mice. (B) Percentage of inhibition on NO production by RES metabolites in LPS-stimulated RAW 264.7 cells. (C) Growth inhibitory effects of RES metabolites on HCT-116 cancer cell line. (D) Anti-proliferative effects of RES metabolites on HT-29 cancer cell line. Data presented as mean ± SD (n=3). Statistical differences were indicated as: *, $P <$

0 .05; **, $P < 0.01$.

concentrations relevant to that in the colitic mice increased by 12.2, 9.59, 11.44, and 14.08% at 0.5, 0.75, 1 and 1.5×, respectively, compared with healthy mice ($P < 0.05$) (Fig. 19).

In addition, HCT-116 and HT-29 cell lines were subjected to a series of treatments. Generally, HCT-116 cell line was more vulnerable than HT-29 upon the treatments of RES metabolites. For example, RES metabolites at the healthy mice relevant levels of 1× suppressed 16.95 and 1.86 % of the growth of HCT-116 cell lines *vs* HT-29 cell lines (Fig. 19C, 19D). While at the concentrations equal to that found in tDSS-treated mice in the colonic tissue, 26.80 and 13.83% of HCT-116 and HT-29 cells were suppressed (Fig. 19C, 19D). The stronger chemopreventative effects of RES metabolites at concentrations relevant to that found in the colitic mice than that observed in healthy mice may due to the higher concentration of RES in the colitic mice. Our previous study demonstrated that RES exhibited stronger chemopreventative effects than DHR and LUN at the same concentrations (supplementary figures Fig. S3). These results provided a vital scientific basis for understanding the chemopreventative mechanisms of RES from the perspective of biotransformation of RES by gut microbiota.

5.4 Conclusion

In current study, we systematically and deeply discussed the complex relations

among gut microbiota, RES biotransformation and biological activities of RES. The study for the first time fully demonstrated that gut microbiota mediated metabolism was the only way to bio-transformed RES to DHR and LUN *in vivo*. Our results further indicated the important role of gut microbiota in the pronounced interindividual variations in biotransformation of RES. RES in LUN non-producers may have higher bioavailability due to the lower urine excretion. We also firstly reported that in the colitic mice LUN and its conjugates were extinct due to the altered gut microbiota compositions and structures. More meaningfully, we found that RES metabolites at the concentrations equivalent to that observed in the colonic tissues in colitic mice exhibited significantly stronger chemopreventative effects than that observed in the healthy mice. These results suggested that absence of LUN may resulted in stronger biological activities of RES. Overall, our finding provided comprehensive scientific knowledge on the interactions between gut microbiota and RES metabolism, and its implications in the biological activities of RES.

CHAPTER 6

CONCLUDING REMARKS

Over 1 million residents in the USA are estimated to be suffering from inflammatory bowel disease, which include Crohn's disease and ulcerative colitis. One of the most important and devastating complications of long-term IBD is colorectal cancer development. Epidemiological evidences suggested that diet enriched with fruits and vegetables were strongly associated with lower cancer occurrence. Resveratrol has attracted much scientific attentions because of its potentially beneficial effects on numerous disorders, including colon inflammation and colon cancer.

The present dissertation for the first time systemically determined the biotransformation of RES in mice, with focuses on its metabolic fate in the GIT. Our results demonstrated that DHR, LUN and their corresponding conjugates were dominated metabolites of RES after sustained oral consumption of RES, rather than RES-sulfates and RES-glucuronides. More importantly, we found that DHR, LUN and their combination exerted much stronger chemo-preventive and anti-inflammatory effects in the renal and colonic tissues, at their concentrations achieved in these tissues, suggesting that DHR and LUN may greatly contribute to the chemopreventive properties elicited by RES in the kidney and colon.

Accumulating evidence indicated that gut microbiota plays important roles in the pathogenesis of colitis, and microbiota composition could be modulated by dietary components. Herein, we determined the effects of resveratrol on gut microbiota and their implication in anti-colonic inflammation in mice with colitis induced by dextran sodium sulfate (DSS). Our results reinforce the protective effects of resveratrol in intestinal inflammation by alleviating the body weight loss, reducing the disease activity index, attenuating tissue damage and modulating inflammatory cytokines. Moreover, dietary resveratrol restored the microbial richness and evenness in DSS-treated mice.

The complex relations among gut microbiota, RES biotransformation and biological activities of RES were deeply discussed. Our results demonstrated that gut microbiota mediated metabolism was the only way to bio-transformed RES to DHR and LUN *in vivo*. Gut microbiota played an important role in the pronounced interindividual variations in biotransformation of RES. RES in LUN non-producers may have higher bioavailability due to the lower urine excretion. We also firstly reported here that in the colitic mice LUN and its conjugates were extinct due to the altered gut microbiota compositions and structures. More meaningfully, we found that RES metabolites at the concentrations equivalent to that observed in the colonic tissues in colitic mice exhibited significantly stronger chemopreventative effects than that observed in the healthy mice. These results suggested that absence of LUN may resulted in stronger biological activities

of RES. Overall, the findings presented in this dissertation provided a solid scientific basis for understanding the chemopreventive mechanisms of RES from the perspective of biotransformation and are of great value for future research on RES in prevention and treatment of colonic diseases in humans.

APPENDIX

THE FIGURES

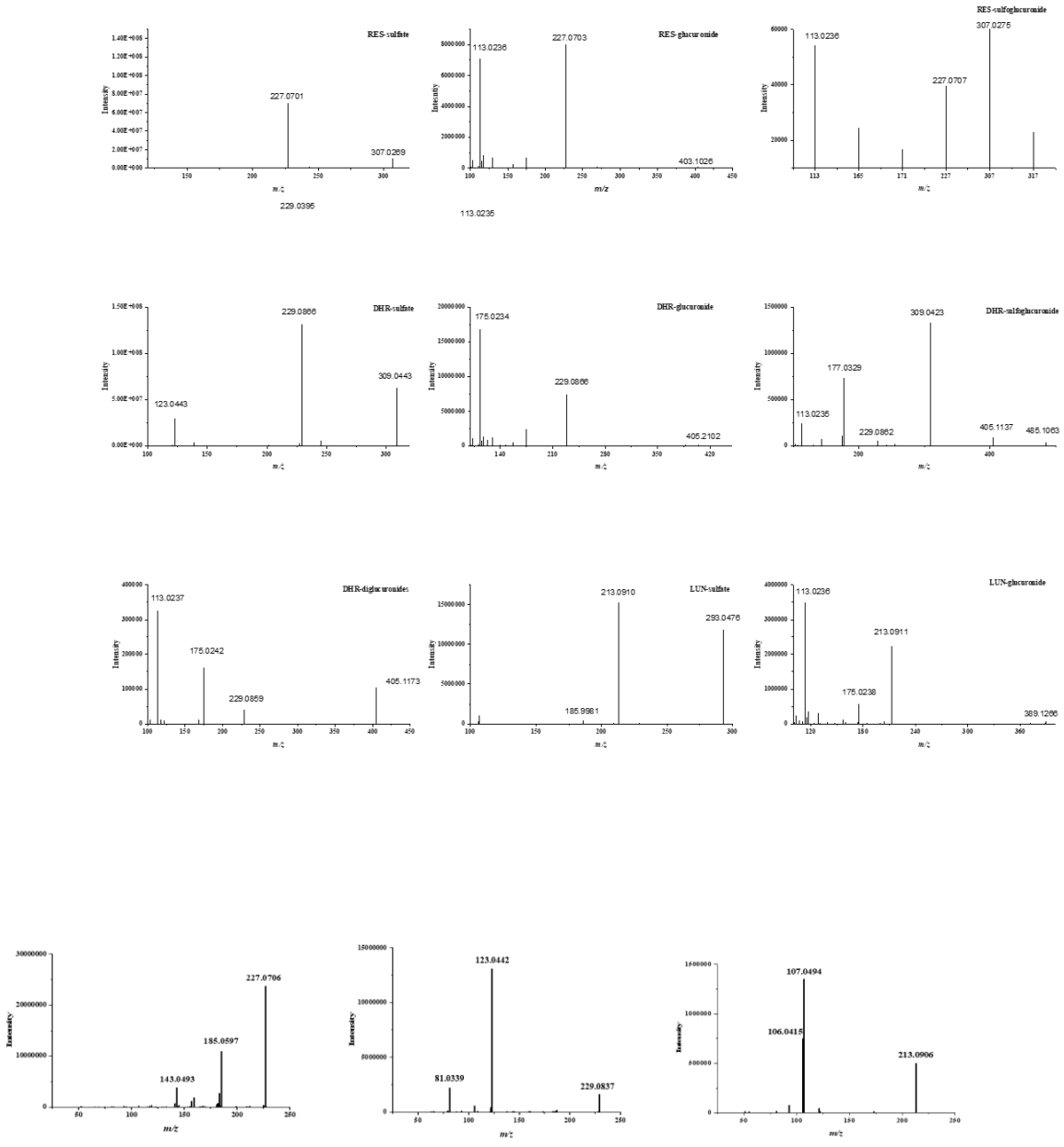


Fig. 20 Mass spectra of RES metabolites at negative mode.

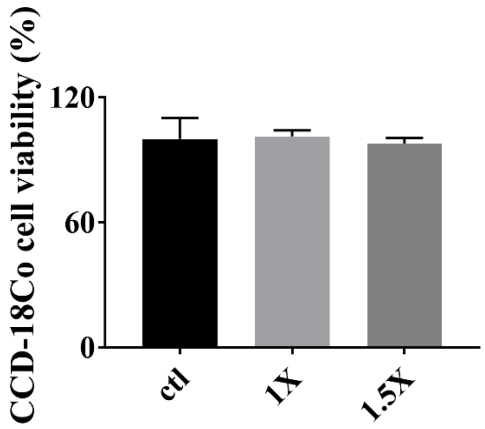


Fig. 21 Anti-proliferative effects of RES, DHR and LUN on normal human colon CCD-18Co cell lines.

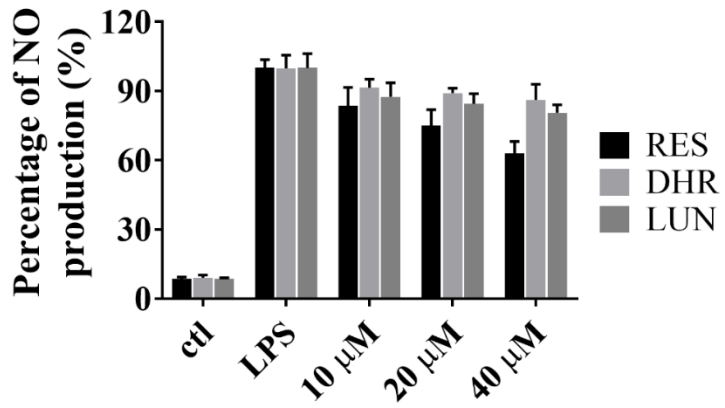


Fig. 22 Percentage of inhibition on NO production by RES, DHR, LUN in LPS-stimulated RAW 264.7 cells at concentrations of 10 μM, 20 μM, and 40 μM.

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