ANTHOCYANINS IN CHRONIC INFLAMMATORY BOWEL DISEASE IN RATS

A Thesis submitted by

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Abstract

Inflammatory Bowel Disease (IBD), including Crohn's disease and ulcerative colitis, is an uncontrolled inflammation of the gastrointestinal tract with unclear aetiology affecting over 85,000 Australians and 5 million globally. IBD produces symptoms such as weight loss, bloody diarrhoea and severe abdominal pain. Multiple factors are involved in IBD pathogenesis including genetic predisposition, gut microbial imbalance, mucosal barrier malfunction, immune system dysfunction and environmental factors.

Current drug therapy for IBD includes sulphasalazine, aminosalicylates, corticosteroids, tumour necrosis factor blockers, immunomodulators (azathioprine-mercaptopurine/methotrexate), anti-integrins alpha4beta2 (vedolizumab), anti-interleukin-12 (ustekinumab), and small molecules including janus kinase inhibitor (tofacitinib). As nutrition can influence some of the causative factors, dietary modulation in IBD patients may be a preventive and therapeutic approach. In particular, anthocyanins such as cyanidin 3-glucoside (C3G) present in common foods such as Queen Garnet plum and purple carrots and pelargonidin 3-glucoside (P3G) in strawberry have anti-inflammatory activities that may be effective in IBD. The aim of my thesis is to understand whether these anthocyanin-containing functional foods improve the structure and function of the gastrointestinal tract, and improve gut bacteria in a rat model of chronic IBD.

The first objective of my thesis was to mimic chronic human IBD in rats. I developed a model of reversible chronic IBD in young male Wistar rats using 0.5% dextran sodium sulphate (DSS) in drinking water for 12 weeks. DSS induced IBD with increased diarrhoea, haematochezia (passage of fresh blood through the anus, usually with stools), infiltration of inflammatory cells in ileum and colon, depletion of mucosal epithelial layer including villi, crypts, goblet cells, and gut microbiota imbalance with increased Proteobacteria phylum and decreased commensal bacteria. IBD symptoms were reversed with replacement of 0.5% DSS with water or the standard IBD drug treatment, sulphasalazine (300 mg/kg body weight/day) for the final 6 weeks.

The second objective was to investigate the effects of functional foods to attenuate IBD in the rat model. For the last 6 weeks of the protocol, either Queen

Garnet plum juice, purple carrot juice or pure C3G at 8mg/kg/day, or strawberry powder at 8mg P3G/kg/day was added in the food to the rats fed with either 0% or 0.5% DSS water. Queen Garnet plum, purple carrot and C3G improved IBD symptoms with reduced diarrhoea and haematochezia. The ileum and colon showed reduced infiltration of inflammatory cells and increased villi length and crypt depth. C3G improved the gut bacteria homeostasis. Similarly, P3G-containing strawberry mitigated the signs of IBD with reduced stool bleeding and diarrhoea, improved ileum and colon structure, suppressed infiltration of inflammatory cells and restored goblet cells. C3G and P3G were as effective as the standard drug sulphasalazine in this model suggesting their promising role in human IBD therapy.

My PhD thesis concludes that functional foods containing anthocyanins may be an alternate or complementary treatment for IBD patients owing to their anti-inflammatory activity, and the ability to balance gut bacteria. Further mechanistic studies and clinical trials are warranted for their inclusion in complementary therapy of human IBD.

Keywords

Inflammatory bowel disease; gut bacteria; dextran sodium sulphate; anthocyanins; Queen Garnet plum; purple carrot; strawberry.

Certification of Thesis

This Thesis is the work of **Naga Koteswara Rao Ghattamaneni,** except where otherwise acknowledged, with the majority of the authorship of the papers presented as a Thesis by Publication undertaken by the Student. The work is original and has not previously been submitted for any other award, except where acknowledged.

Principal Supervisor: Professor Lindsay Brown

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Student and supervisors' signatures of endorsement are held at the University.

Statement of Authorship

Chapter 2. Nutraceuticals in rodent models as potential treatments for human Inflammatory Bowel Disease. Naga KR Ghattamaneni, Sunil K Panchal, Lindsay Brown

NKRG was responsible for 60% of conception and design of this chapter, 60% of analysis and interpretation of literature, 70% of drafting and writing and 20% of final important editorial input; SKP was responsible for 10% of conception and design, 10% of analysis and interpretation of literature, 10% of drafting and writing and 30% of final important editorial input; LB was responsible for 30% of conception and design, 30% of analysis and interpretation of literature, 20% of drafting and writing and 50% of final important editorial input.

Chapter 3. An improved rat model for chronic inflammatory bowel disease. Naga KR Ghattamaneni, Sunil K Panchal, Lindsay Brown

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Chapter 4. Cyanidin 3-glucoside attenuates inflammatory bowel disease in rats. Naga KR Ghattamaneni, Sunil K Panchal, Lindsay Brown

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Chapter 5. Pelargonidin 3-glucoside from strawberry improves chronic inflammatory bowel disease in rats. Naga KR Ghattamaneni, Sunil K Panchal, Lindsay Brown

NKRG was responsible for 50% of conception and design, 80% collection of data, 60% of analysis and interpretation of data, 55% of drafting and writing and 20% of final important editorial input; SKP was responsible for 25% of conception and design, 20% collection of data, 20% of analysis and interpretation of data, 20% of drafting and writing and 35% of final important editorial input; LB was responsible for 25% of conception and design, 20% of analysis and interpretation of data, 25% of drafting and writing and 45% of final important editorial input.

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Chapter 1. Introduction

Introduction

Overview of IBD

Inflammatory bowel disease (IBD) is defined as an uncontrolled chronic inflammation of the gastrointestinal tract (1). IBD is widespread across the world and there has been a steady increase in its incidence (2, 3). Over the last 50 years, IBD has become a common disease from an unusual one (2). There has been a steady increase in the incidence of IBD in Western countries and now it is also increasing in previously low incidence areas of Europe and Asia (3). It is more common in industrialised regions, possibly due to the rise in adopting a Westernised lifestyle indicating strong environmental influences on its development (4, 5). As of 2018, more than 85,000 Australians have IBD and 1 in 250 Australians are affected with IBD (6). There is a possibility of more than 100,000 IBD patients in Australia by 2022 (6), which is supported by increasing hospitalisations over the years (7). The prevalence of IBD is higher in developed countries than in developing countries and it is predominantly seen in higher socio-economic groups (3).

IBD produces symptoms such as weight loss, diarrhoea with blood and severe abdominal pain. The initial diagnosis for IBD relies on laboratory tests for stool and blood examination. Further examination may involve imaging and endoscopy methods (3). The precise aetiology and pathology of IBD is yet to be clarified however it is hypothesised that IBD could be due to genetic, microbial, environmental and host immune factors that interact in a complex manner (8).

Possible molecular players in IBD

In IBD, there is an imbalance between immune tolerance and activated defence against intestinal microbiota and this can lead to aberrant and excessive immunological responses (9). Higher permeability of the intestine is observed in IBD due to breakdown of epithelial tight junctions and this leads to imbalance between gut microbiota and the immune system (10). There is also an enhanced production of pro-inflammatory cytokines and chemokines and increased expression of adhesion molecules (11). The pro-inflammatory cytokines interleukin 1-beta and tumour necrosis factor (TNF) can trigger the release of free radicals. TNF can also initiate inflammation. The TNF signalling pathway controlled by nuclear factor kappa-light-chain-enhancer of activated B cells (NF-Kb) is responsible for the expression of

adhesion molecules that are key in leukocyte recruitment to the inflamed intestine (12). In IBD patients, leukocytes infiltrate the inflamed bowel wall, generating large amounts of reactive oxygen species (ROS) that cause intestinal tissue damage (13). Excessive production of ROS or reduced removal of ROS can lead to IBD (14).

Treatments for IBD

IBD has no medical cure so it requires lifetime healthcare. Treatment options for treating IBD include corticosteroids (15), aminosalicylates (16), antibiotics (17), immunosuppressants (18) and biologics (19). To minimise the adverse effects of drugs, new methods of drug delivery are being investigated such as artificial cell microencapsulation (20). Surgery is available in severe cases to remove the extremely affected parts of the intestine (21).

Chronic drug administration increases the risk of adverse effects in IBD patients (22). Biologics treatment and surgery are expensive for IBD patients (23). An alternative to the drug therapy alone are the dietary interventions which may be a valuable complementary therapy along with conventional drugs with lower dose for chronic treatment of IBD (24).

Diet as cause and treatment in IBD

Diet as an environmental factor is of great importance in IBD patients as it can modulate the disease determinants (12, 25). Diet can influence the intestinal microbiota, affect intestinal permeability, improve immune function and alter gene expression of gut cells (26). Diet has a prominent role in defining the microbiota in the intestine, possibly transforming commensal microflora to pathogenic microflora thereby inducing IBD, as nearly 57% of intestine microflora changes are due to diet whereas genetics causes nearly 12% (27).

The incidence of IBD is increased by adopting the Westernised lifestyle and diet. Epidemiological studies showed that high consumption of sugar and fast food, and low consumption of fruits and vegetables, increased the risk of IBD (23, 28). Due to methodological limitations in these epidemiological studies, the role of specific dietary components in IBD development is suggestive but inconclusive (26, 29, 30).

Nutraceuticals or functional foods are defined as foods or food components that improve physiological processes that lead to disease. Foods rich in antioxidants prevent ROS accumulation and have been proposed as treatments for IBD. Curcumin, a phytochemical in turmeric showed anti-inflammatory activity when tested on colonic mucosal biopsies from active IBD patients (31). Therefore, curcumin may be useful in treating IBD patients. An increased intake of omega-3 fatty acids improved the symptoms of IBD patients whereas diet rich in omega-6 fatty acids and inadequate in omega-3 fatty acids can worsen the symptoms (32). Foods containing fibre such as *Plantago ovata* seeds and germinated barley foodstuff are beneficial for patients with severe IBD (33). Thus, nutraceuticals can be a major player in IBD therapy as a therapeutic intervention to suppress active IBD (30).

Animal models of human IBD

It is a general experimental approach to investigate the efficacy of an intervention in an animal model before proceeding to studies in diseased humans. Thus, if a nutraceutical has shown clear promising results as a positive intervention in an animal model of IBD that mimics the human disease, the efficacy of this intervention can then be tested in IBD patients. It is easier to perform controlled experiments in animals with a standardised diet and the intestines of the animals can be investigated with ease, unlike humans. The animal model is used since metabolic responses by the body to pathophysiological changes following an inflamed gut involve different organ systems that cannot be represented in non-animal or *in vitro* studies. Cell culture studies only help us to understand about one cell type and not the complex interactions between the tissues in the body. Whole animal studies are the only way in which we can indicate the therapeutic potential of treating IBD with natural anti-inflammatory agents preceding studies in humans with IBD. For these studies, we need to use a reliable whole animal model.

There are many models for studying IBD pathogenesis and treatment, usually in mice and sometimes in rats. IBD can be induced chemically, genetically or by germ-free gut environment (34). The most commonly used chemically induced model is with administration of dextran sodium sulphate (DSS). DSS is administered in the drinking water to the animals, causing both inflammation in the gastrointestinal tract by disruption of the intestinal epithelial membrane and an imbalance of the gut microflora

which mimics human IBD (34). It is hypothesised that the toxic effects of DSS lead to loss of gastrointestinal tract epithelial membrane. This loss leads to movement of gut microbiota from the intestinal lumen to the intestinal crypts and causes inflammation due to excess innate and adaptive immune reactions (34). Thus, a pathological scenario of IBD is established which mimics the human IBD condition (35). DSS induces imbalance of gut microbiota in the mice with a decreased population of *Lactobacillus* and an increased population of *Akkermansia* and *Desulfovibrio* (36) and mimics human gut microbacterial changes in IBD. Therefore, this model is extensively used to investigate the efficacy and mechanisms of action of drugs and nutraceuticals in IBD.

There are limitations with the existing animal models of IBD. Most of the studies with DSS are acute IBD models with DSS administration for around 7 days only using high concentrations of 1.5-10% DSS (37-39). The concentration of DSS and the duration of DSS administration determine the severity of the symptoms (40). The high concentration of DSS induces an acute inflammatory response in the intestine within a few days. However, human IBD is a chronic condition that gradually aggravates and can last for decades with many relapses. Therefore, it is essential to characterise interventions in a chronic DSS animal model for the treatment of IBD. An appropriate DSS concentration for an appropriate time needs to be chosen to produce marked but relatively constant symptoms for a prolonged period without causing severe disease or death of the animal. Many of the studies have used mice as the animal model but a rat model may be more suitable owing to the larger size of the rat with more blood and tissue sample for analysis. A model with these characteristics can then be used to investigate interventions with functional foods. Sulphasalazine, the standard drug treatment for treating IBD, can be used as a positive control in the development of the chronic IBD rat model and the interventions in treating DSS-induced IBD (41).

DSS-induced IBD and diet

The acute DSS model has been widely used to study dietary interventions with anti-inflammatory and anti-oxidant activity. Antioxidants such as green tea polyphenols when administered to DSS-induced IBD mice ameliorated IBD symptoms (42). Green tea is a good source of nutraceuticals such as the catechins that can effectively promote intestinal health by reducing inflammation. Diet supplemented

with peracetylated (-)-epigallocatechin-3-gallate (AcEGCG) improved the symptoms of DSS-induced colitis in mice (37). A dietary combination of quercitrin and olive oil supplemented with fish oil containing eicosapentaenoic acid and docosahexaenoic acid which are omega-3 polyunsaturated fatty acids fed to rats with DSS-induced IBD ameliorated the intestinal inflammation (43). Ellagic acid found in pomegranate fruit reduced intestinal inflammation in acute and chronic models of DSS-induced IBD in mice (38). These studies indicate that DSS-induced IBD animal model is a good choice to test the efficacy of functional foods to treat IBD in humans.

Possible treatments for IBD

Purple foods are rich in natural anthocyanins, including cyanidin 3-O-β-D-glucoside (C3G), that are potential therapeutic agents in IBD due to their antioxidant and anti-inflammatory actions especially in intestinal cells, and also by modulation of the gut microbiota (44, 45). Anthocyanins extracted from blueberries showed protective effects on trinitrobenzene sulfonic acid (TNBS)-induced IBD model of mice (46). Our research group showed that Queen Garnet plum juice given to diet-induced obese rats at 8mgC3G/kg bw/day reversed the symptoms of metabolic syndrome including hepatic and cardiac inflammation (47). Our previous studies on purple carrots (*Daucus carota* subsp *sativus*), another food source of C3G, reported improved structural and functional changes of the heart and liver due to its anti-inflammatory action in diet-induced metabolic syndrome rats as a model of chronic low-grade inflammation (48). Strawberry (*Fragaria* × *ananassa*) contains pelargonidin 3-glucoside (P3G), another important anthocyanin present in many fruits (49, 50). P3G has anti-inflammatory and radical scavenging properties observed in human whole blood cell cultures and in carrageenan induced pleurisy mouse model (51, 52). Therefore, these foods rich in C3G or P3G may be potential treatments for IBD and it is a good approach to test them in an appropriate DSS-induced chronic IBD rat model.

Aim

To determine the changes in the structure and function of the gastrointestinal tract in inflammatory bowel disease (IBD) following treatment with anti-inflammatory compounds from food.

Objectives

- The study will develop a chronic model of inflammatory bowel disease (IBD) that mimics human IBD in young male Wistar rats by administration of dextran sodium sulphate (DSS); and
- This chronic IBD rat model will be studied to assess various functional foods as possible interventions to reverse the structural and functional changes of the inflamed gastrointestinal tract.

Hypotheses

- That low chronic dosage of DSS in rats will mimic the symptoms of human IBD; and
- That functional foods will reverse the gastrointestinal tract symptoms in IBD.

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Chapter 2. Nutraceuticals in rodent models as potential treatments for human Inflammatory Bowel Disease

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Review

Nutraceuticals in rodent models as potential treatments for human Inflammatory Bowel Disease

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ABSTRACT

Inflammatory Bowel Disease (IBD) is characterized by chronic inflammation of all or part of the digestive tract. Nutraceuticals include bioactive compounds such as polyphenols with anti-inflammatory activities, thus these products have the potential to treat chronic inflammatory diseases. We have emphasized the role of nutraceuticals in ameliorating the symptoms of IBD in rodent models of human IBD through modulation of key pathogenic mechanisms including dysbiosis, oxidative stress, increased inflammatory cytokines, immune system dysregulation, and inflammatory cell signaling pathways. Nutraceuticals have an important role in IBD patients as a preventive approach to extend remission phases and as a therapeutic intervention to suppress active IBD. Further clinical trials on nutraceuticals with positive results in rodent models are warranted.

1. Introduction

Inflammatory bowel disease (IBD) is an umbrella term for uncontrolled inflammation of the mucosa in the gastrointestinal tract that primarily includes two chronic disorders, ulcerative colitis and Crohn's disease, in humans [1]. Ulcerative colitis mainly involves the colon, commonly the distal end and rectum, affecting the inner lining of the gut or mucosa. Crohn's disease involves any part of the gastrointestinal tract with the terminal ileum and colon most commonly affected through the gut wall including mucosa and submucosa [2,3]. The symptoms of IBD are similar in humans and rodent models, although there are differences in behavioral and dietary patterns such as coprophagy, and the cause of IBD in animal models. Thus, animal models cannot fully mimic human disease but we can understand the development of the disease which strengthens the use of rodent models to test treatments [4,5]. Human and rodent gut microbiota composition are different and definition of their gut microbiota may help to develop a closely-related rodent model of human IBD [6]. Genetic factors are being discovered to link with human IBD and novel genetic mice models are being developed to study the mechanism of IBD, despite differences in human and mice genetics that may cause differences in intestinal physiology [6]. Potential treatments include nutraceuticals, defined as extracts of foods that help in preventing or treating diseases or disorders [7]. Rodent models of human IBD treated with nutraceuticals as prevention or reversal treatments either acutely for 1–3 weeks or chronically for 8–18 weeks showed attenuation of disease symptoms [8,9]. This review emphasizes that nutraceuticals have potential roles in the therapy of IBD in humans, often suggested by preclinical studies in rodent models of IBD. The papers were selected based on relevant keywords such as IBD; Crohn's disease; ulcerative colitis; inflammation; gut microbiota; cytokines; phytochemicals; polyphenols; nutraceuticals; probiotics; prebiotics; animal model; dextran sodium sulfate (DSS); and 2,4,6-trinitrobenzenesulfonic acid (TNBS) in the search engine PubMed from 1979 to February 2018. References cited by key studies and reviews were also checked.

2. Epidemiology

The number of patients with IBD is constantly increasing as IBD is now a global disease although it is more prevalent in developed westernized countries such as western Europe, Canada, USA, Australia, and New Zealand than in developing areas including countries in Asia, Africa, and South America [10,11]. The prevalence of IBD is highest in Europe followed by North America. In Europe, the highest prevalence of ulcerative colitis was reported as 505 per 100,000 in Norway with Crohn's disease highest in Germany with 322 per 100,000; in North America, ulcerative colitis was 286 per 100,000 in the USA; Crohn's disease was 319 per 100,000 in Canada [12]. The highest prevalence of IBD in Africa was reported in Algeria in both ulcerative colitis at 19 per 100,000 and Crohn's disease at 11 per 100,000 [12]. In South America, the prevalence of ulcerative colitis was 41 per 100,000 in Puerto Rico and Crohn's disease was 44 per 100,000 in Barbados; in Asia, Lebanon

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was highest in both Crohn's disease at 53.1 per 100,000 and ulcerative colitis at 106 per 100,000 [12]. Since the Second World War, there has been a steady increase in the incidence of ulcerative colitis in Western countries and now it is increasing in previously low incidence areas of Europe and Asia [13,14]. The prevalence of Crohn's disease is higher in developed countries than in developing countries and it is predominantly seen in higher socio-economic classes [15]. Since the turn of the 21st century, the incidence of IBD has stabilized or reduced in developed countries while there has been a rapid increase in newly industrialized developing countries, but its prevalence is still higher in developed westernized countries [11]. This may have widespread consequences with the prediction for 2027 that millions of mostly younger IBD patients in the newly industrialized countries will be ready to enter the work-force if the increase in IBD cases continues at the current pace [11]. The difference in prevalence of IBD between developed and developing nations has been explained by the "hygiene hypothesis" which states that the people living in the developed countries are less exposed to infections or unhygienic conditions so they are prone to lose protective organisms that promote immune response, but putative pathogens are increased, leading to chronic immune diseases in these individuals [15]. However, an increased risk of ulcerative colitis in a north Indian population with poor hygiene does not support this "hygiene hypothesis" [16].

IBD produces symptoms such as weight loss, diarrhea with blood loss, and severe abdominal pain that can markedly increase morbidity [17]. Patients are diagnosed by fecal examinations, colonoscopy, gastroscopy, and blood tests, and often with imaging modalities such as computer tomography, magnetic resonance imaging, and capsule endoscopy [15].

The precise etiology of IBD is still unclear. IBD is more common in industrialized regions, indicating that there are strong environmental influences [17,18]. A single causative agent or mechanism cannot explain all aspects of IBD. The chronic intestinal inflammation in IBD could result from microbial factors, genetic factors, mucosal barrier malfunction, immune system dysfunction, and environmental factors, all of which are interlinked [3,19–24]. IBD has no medical cure and requires medication that extends the time in remission which improves quality of life [25]. Many rodent models have been used to test medications that may improve symptoms and extend remission in human IBD. However, these models do not completely reproduce the causes or symptoms of human IBD. As an example, TNBS/DNBS-induced colitis in rodents follows rectal administration of TNBS/DNBS and ethanol of varying concentrations which results in Th1 inflammation and ulceration of colon and rectum similar to Crohn's disease patients [26,27]. However, depending on the species or strains of rodents, TNBS can affect Th2 inflammation that is an ulcerative colitis model [27]. Orally given DSS and rectally administered acetic acid models affect the colon predominantly and produce ulcerative colitis symptoms [28,29], whereas genetic models of IL-10 knockout and HLA-B27 develop spontaneous colitis similar to human IBD [29].

3. Current drug treatment options for IBD in humans

Treatment options for IBD include corticosteroids such as budesonide and prednisolone [30,31], aminosalicylates including sulfasalazine as a pro-drug of 5-aminosalicylate [32–34], antibiotics such as metronidazole [35,36], immunosuppressants including azathioprine and mercaptopurine [37–40], and antibodies such as infliximab and adalimumab referred to as biologics [41–48]. Some of the recent drugs for IBD treatment include further biologics, including ustekinumab [49] and tofacitinib [50,51]; the anti-TNF (tumor necrosis factor) antibody, golimumab [52]; and the humanized monoclonal antibody, vedolizumab [53,54]. Another option is budesonide MMX as a novel oral formulation of budesonide using Multi-Matrix System (MMX®, Cosmo Pharmaceuticals, Milan) technology which extends the drug release in the colon [55]. New methods of drug delivery to minimize the adverse effects of drugs are being investigated such as artificial cell microencapsulation that allows the drug to cross the low pH of the stomach for controlled time delivery in the colon [56]. Surgery may be necessary in severe cases to remove the extremely affected parts of the intestine in Crohn's disease [57]. Nearly 70% of Crohn's disease patients and 30% of ulcerative colitis patients undergo surgery when medications are not effective in disease control [15]. Personalized medicine is patient-specific medication that aims to optimize the efficacy of the treatment with reduced adverse drug effects and at a lower cost [25], so getting the right medicine to the right patient at the right time. Infliximab, a TNF antagonist drug, was found to have better efficacy for Crohn's disease patients with specific characteristics such as young age, Crohn's colitis, and increased CRP concentrations [58,59]. Home-based screening is a rapid, simple, and cost-effective method used by the patient at home to test stool calprotectin with a smartphone and IB*Doc* software [60]. The test gave similar results to ELISA calprotectin assay with reduced cost of traveling to the clinic, reduced burden on the clinic resources and patient-friendly sampling techniques [60]. Telemedicine is the application of information and communication technology to interact with the patient without direct contact [61]. A study in Dartmouth-Hitchcock Medical Center showed that telemedicine is a low-cost method which improved the quality of life with reduced hospital visits and increased office visits by the IBD patients [62]. Internet-based patient management tools are another approach to improve patient adherence to treatment and keep track of any changes in the symptoms of IBD [63]. A Danish study showed improved adherence of Crohn's disease patients to infliximab treatment through web-based patient management [64].

Drug therapy of IBD requires chronic administration of drugs that are not effective in all patients and may increase the risk of adverse effects [57,65–67]. Treatment with biologics is very expensive, as is surgery [68]. Surgical treatment of IBD can lead to malabsorption of nutrients [57]. Therefore, dietary interventions with nutraceuticals may be one of the complementary methods of chronic treatment for IBD, if they can improve the remission phase when given with conventional drugs and possibly reduce the adverse effects from these conventional drugs [69]. Different animal models, mostly in rodents such as mice and rats, are used for *in vivo* testing of nutraceuticals as treatments for IBD as detailed in Table 1.

4. Modulation of gut microbiota by nutraceuticals

Any imbalance in the local distribution, metabolic function, or qualitative and quantitative changes of the microbiota leads to a state known as dysbiois, which is associated with chronic diseases including IBD [76–79]. It is yet to be proven that dysbiosis has a direct causal link with IBD [76].

Prebiotics are food components or supplements which selectively stimulate the growth or activity, or both, of one or many microbes of a genus or species in the gut resulting in improved health of the host [80]. Prebiotics are mostly complex carbohydrates derived from fruits, vegetables, and grains which are not metabolized in the stomach or small intestine but are fermented in the colon leading to improved metabolic activity of the gut microflora [81]. Lactulose displayed its prebiotic properties on TNBS colitis rat model by promoting the growth of Lactobacilli and Bifidobacteria, reducing the production of colonic pro-inflammatory markers including TNF and leukotriene B_4 (LTB_4), and inhibition of iNOS expression [82]. In mice with DSS-induced colitis, oral administration of inulin attenuated gut inflammation and increased Lactobacilli counts [83]. Prebiotics were also tested on transgenic models such as spontaneous colitis HLA-B27 rats in which fructo-oligosaccharides, unlike inulin, increased the counts of cecal and fecal Bifidobacteria, and also increased the cecal Bacteroides counts. However, fructo-oligosaccharides did not change the diversity of Bifidobacteria [75]. The increased Bifidobacteria correlated negatively with chronic intestinal inflammation in HLA-B27 colitis rats [75]. Bifidobacteria and Bacteroides reduced intestinal inflammation in HLA-B27

Table 1
Rodent models of IBD and the research outcomes after treatment with nutraceuticals.

Animal model	Treatment	Research outcome	Remarks
TNBS colitis rat (female Wistar) n = 10	Rosmarinic acid, apigenin, and luteolin [70]. (10 and 25 mg/kg of *L. dentata* and *L. stoechas* extracts, treatment from day 1–7, preventive)	Lowered the degree of mucosal ulceration and leukocyte infiltration, goblet cells regenerated partially, decreased colonic MPO activity, increased GSH content, downregulated colonic iNOS expression, decreased colonic expression of the pro-inflammatory cytokines IL-1β and IL-6, MCP-1, and ICAM-1 and increased TFF-3 and MUC-3.	Rosmarinic acid, apigenin, and luteolin from *L. dentata* and *L. stoechas* led to repair of intestinal epithelial barrier and down-regulated the immune response.
TNBS colitis rat (male Wistar) n = 6	(-)-Hydroxycitric acid [71]. (0.5 and 1 g/kg of *G. cambogia* extract (51.2% (-)-hydroxycitric acid), treatment from day 1–6, preventive)	Improved Reduced colonic macroscopic damage, reduced MPO activity, COX-2, iNOS, PGE_2, and IL-1β colonic concentrations, prevented DNA damage.	(−)-Hydroxycitric acid in *Garcinia cambogia* extract reduced colon injury through its anti-inflammatory activity.
DSS IBD mice (female C57BL/6) n = 6	Curcumin polymer [8]. (50 mg/kg of curcumin, treatment from day 1–7, preventive)	Less weight loss, severe diarrhea reduced, colon length increased, epithelium and crypt architecture restored, reduced neutrophil invasion, decreased colonic MPO activity and MDA content, IL-6 and TNF production reduced in colonic tissue.	Solubility and partition coefficient of curcumin increased, alleviated symptoms of IBD.
(female Balb/c mice) n = 10	Anthocyanins [9]. (1 & 10% anthocyanin extract (60–70% anthocyanins), treatment from 2 weeks prior to DSS administration, 3 weeks (acute) and 10 weeks (chronic), preventive)	Improved the colon tissue lining, increased colon length, lowered production of IFN-γ, TNF, and IL-6 from mesenteric lymph node cells.	Anthocyanins from bilberry had anti-inflammatory effect on colon and reduced acute and chronic colitis.
DNBS colitis rat (male Sprague–Dawley) n = 6	Punicalagin [72]. (4 mg/kg of punicalagin, treatment from 10 days before colitis and one week after colitis induction, preventive)	Stool characteristics improved, colon mucosal damage and severity of inflammation reduced, lowered MDA concentrations and MPO activity, decreased NO concentrations and increased SOD activity, mRNA levels of TNF, IL-18, IL-1β, and NF-κβ reduced.	Punicalagin from pomegranate juice was effective in ameliorating IBD symptoms.
Acetic acid colitis rat (Male Wistar) n = 12	Amentoflavone [73]. (10 mg/kg of amentoflavone, treatment from 5 days before colitis and one day after colitis induction, preventive)	Inhibited colonic ulceration, normal epithelium and mucosa with cryptitis absent, reduced MPO activity, TNF, IL-1β, IL-6; normalized colonic tissue concentrations of GSH, SOD activity; inhibited NF-κB signaling pathway.	Amentoflavone from *Biophytum sensitivum* was as effective as sulfasalazine in treating IBD symptoms.
IL-10 KO spontaneous colitis mice n = 7	6-Gingerol and 6-shoagol [74]. (0.3 mg/rat of GDNPs 2, treatment for 18 weeks, preventive)	Increased colon length, decreased spleen weight, reduced colonic MPO activity, reduced mucosal inflammation, decreased expression of colonic pro-inflammatory cytokines TNF and IL-1β.	Nanoparticles with 6-gingerol and 6-shoagol targeted inflamed colon and showed anti-inflammatory activity.
HLA-B27 spontaneous colitis rat n = 12	Fructo-oligosaccharide and inulin [75]. (8 g/kg of inulin and fructo-oligosaccharide, treatment from 4 to 16 weeks, preventive)	Reduced cecal and colonic inflammation, reduced colonic IL-1β expression, increased cecal and fecal Bifidobacteria, decreased Clostridium cluster XI, decreased Enterobacteriaceae and Clostridium difficile toxin B, inflammatory score correlated with gut microbiota analysis.	Fructo-oligosaccharide and inulin reduced chronic intestinal inflammation by modulation of gut microbiota.

TNBS, 2,4,6-Trinitrobenzenesulfonic acid; MPO, Myeloperoxidase; GSH, Glutathione; iNOS, Inducible nitric oxide synthase; IL-1β, Interleukin 1 beta; IL-6, Interleukin 6; MCP-1, Monocyte chemoattractant protein 1; ICAM-1, Intercellular adhesion molecule 1; TFF-3, Trefoil factor 3; MUC-3, Mucin 3; TNF, Tumor necrosis factor; COX-2, Cyclooxygenase 2; PGE_2, Prostaglandin E2; NF-κB, Nuclear factor-κB; DSS, Dextran sodium sulfate, MDA, Malondialdehyde; IFN-γ, Interferon gamma; DNBS, Dinitrobenzene sulfonic acid; NO, Nitric oxide; SOD, Superoxide dismutase; IL-18, Interleukin 18; IL-10 KO, Interleukin-10-/- knockout; GDNP, ginger-derived nanoparticles.

rats, IL-10 KO mice, and children as well [84–86]. Chitosan oligosaccharide, a biodegradation product of the dietary fiber chitosan, was effective in treating the acute and chronic models of DSS-induced IBD in mice. Chitosan oligosaccharide decreased apoptosis of the intestinal epithelial cells and inhibited NF-κB signaling, thus it could lower intestinal inflammation [87].

The role of prebiotics is yet to be well established clinically in IBD patients compared to animal studies, so many clinical trials are in progress. The reduction of *C. difficile* overgrowth by fructo-oligosaccharides and inulin supports their therapeutic use as ulcerative colitis patients suffer from *C. difficile* toxin that causes dysbioisis [88,89]. Human studies with fructo-oligosaccharides resulted in increased fecal and mucosal Bifidobacteria content in Crohn's disease patients and decreased disease activity [90]. In a clinical trial on active ulcerative colitis, patients were randomized to 2 groups and were either given oligofructose-enriched inulin (12 g/d) or placebo for 2 weeks together with 3 g/d of mesalazine [62]. The oligofructose-enriched inulin test group had reduced disease activity and lower amounts of the inflammatory marker, fecal calprotectin, compared to placebo group, suggesting that this prebiotic along with mesalazine can ameliorate gut inflammation [91]. Prebiotics such as inulin, resistant starch, and β-glucan could not maintain remission in active Crohn's disease patients even when formulated as Synbiotic 2000 [92]. However, the drawbacks of the study were its small size of which many did not have inflammatory state of the disease, and a low daily dose of synbiotic. The low fermentable, oligo-, di-, mono-saccharides, and polyol (FODMAP) diet, which includes prebiotics such as inulin, is being studied as a dietary intervention to treat IBD patients with some studies showing positive results, but more clinical trials are warranted [93–95].

Since ulcerative colitis and Crohn's disease patients have dysbiosis, modulation of gut microbiota by intake of probiotics or prebiotics may be a strategy to restore the gut homeostasis [96–98]. However, it is still in the experimental stage, and more investigations on prebiotics and probiotics are being conducted in rodents and humans to find new treatments for IBD. Diet supplemented with dietary fiber from *Plantago ovata* seeds that breaks down to butyrate in colon controlled the re-appearance of severity of the disease in ulcerative colitis patients [99]. DSS-induced IBD rats given fiber of germinated barley in their food

showed reduced diarrhea and mucosal damage with an increased repair process of the damaged colonic mucosa structure, thus ameliorating IBD [100]. Further, due to the positive effect of fiber in germinated barley foodstuff, this food also reduced the clinical activity of ulcerative colitis and prolonged the remission time of ulcerative colitis in humans; as a prebiotic, germinated barley increased butyrate concentrations in intestinal lumen that effectively triggered the growth of commensal bacteria [101,102]. In Crohn's disease patients, a fiber-rich, unrefined-carbohydrate diet was associated with reduced hospital visits and surgical treatments, thus improving prognosis of the disease and prolonging the relapse time [103].

Gut microbial homeostasis may be improved by other interventions, including the use of probiotics, synbiotics, and fecal microbiota transplantation. Probiotics such as VSL#3 and I3.1 are mixtures of live microbes that can improve gut homeostasis and health [104] by decreasing enteric pathogens, promoting commensal and symbiotic microbiota by interacting with the gut microflora and regulating innate immunity [81,105,106]. Most probiotics contain Lactobacillus and Bifidobacteria species [107,108]. Synbiotics, the combination of probiotics and prebiotics, were tested for their efficacy in treating IBD in spontaneous colitis rats [109] and in ulcerative colitis patients [110]. Fecal microbial transplantation reverses dysbiosis by introducing stool of healthy person to IBD patients thereby transferring the commensal gut microbiota into the gut of the IBD patient and inhibiting the disease [111–113]. Due to complexity of the relationship between the host and gut microbiota, more research is needed to define the role of microbiota in human and animal studies to improve our understanding in the pathogenesis of IBD.

5. Activation of antioxidant defense

Increased production of reactive oxygen species leads to oxidative stress causing oxidative modification of macromolecules and finally tissue damage [114]. In IBD patients, leukocytes infiltrate the inflamed bowel wall, generating increased reactive oxygen species that causes intestinal tissue damage [115]. Excessive production of reactive oxygen species or reduced removal of reactive oxygen species by antioxidants can lead to IBD [116]. Many enzymes such as myeloperoxidase, nitric oxide synthases (NOS), and cyclooxygenases (COXs) play vital roles in endogenous reactive oxygen species generation [117,118]. However, the antioxidant cellular system normalizes the increased oxidative state with many intracellular enzymatic antioxidants, including superoxide dismutase (SOD), glutathione peroxidase, catalase as well as nonenzymatic glutathione [119]. Studies on butyrate usually obtained from dietary fiber showed reduced reactive oxygen species concentrations thus inhibiting intestinal inflammation [120]. Punicalagin from pomegranate juice was effective in increasing anti-oxidant status in the colon tissue by increasing SOD [121].

Polyphenols from concentrated apple extract, predominantly chlorogenic acid, when given to rats with acetic acid-induced colitis, downregulated iNOS and upregulated copper and zinc superoxide dismutase (CuZnSOD) [122]. Many biologically active food components such as (−)-hydroxycitric acid, curcumin, punicalagin, amentoflavone, 6-gingerol, and 6-shoagol decreased colitis in animal models by downregulation of myeloperoxidase, iNOS, COX-2, and MDA, upregulation of antioxidant enzymes such as catalase, and reduced glutathione concentrations [8,71–74].

Antioxidants such as green tea polyphenols given to DSS-induced IBD mice prevented the reduction of colon length, improved blood concentrations of reduced glutathione, and lowered TNF and serum amyloid A concentrations [123]. Green tea is a good source of nutraceuticals such as catechins that can promote intestinal health by reducing inflammation. Anti-oxidant rich foods can mitigate the free radical-derived inflammatory conditions in the intestine and improve the symptoms of IBD [124,125].

6. Modulation of anti-inflammatory activity

In IBD patients, there is an increased production of pro-inflammatory cytokines including TNF, INF-γ, IL-6, IL-1β, and chemokines, and increased expression of adhesion molecules [126]. IL-10 is an immune regulator in intestinal mucosa and prevents the rise of pro-inflammatory agents [127]. The pro-inflammatory cytokines IL-1β and TNF can trigger the release of free radicals such as NO which aggravates the inflammation cascade [128]. The TNF signaling pathway controlled by NF-κB is responsible for the expression of adhesion molecules such as vascular cell adhesion molecule 1 (VCAM-1) and intercellular adhesion molecule 1 (ICAM-1) in the endothelium [129]. These adhesion molecules are major determinants of leukocyte recruitment to the inflamed intestine [23]. Anthocyanins prevented inflammation in colitis mice and improved the colon by lowering the concentrations of the pro-inflammatory cytokines, IFN-γ, TNF, and IL-6 [9]. In ulcerative colitis patients, these anthocyanins reduced the colonic tissue expression of IFN-γ R2, the signal transducing part of the IFN-γ receptor, thereby inhibiting the inflammatory activity of IFN-γ in the colon [130], and increased the serum concentrations of tissue protective cytokines IL-22 and IL-10. However, in the same study, only patients in remission had decreased serum concentrations of pro-inflammatory cytokines TNF and MCP-1. These studies indicate that anthocyanins from fruits including bilberry are potential therapeutic interventions for IBD.

Curcumin, a phytochemical in turmeric with anti-inflammatory activity, was tested on colonic mucosal biopsies and myofibroblasts cultured *ex vivo* from patients with active IBD. Curcumin-treated biopsies increased IL-10 production, decreased p38 MAPK (mitogen-activated protein kinases activation), and IL-1β production while curcumin-treated myofibroblasts inhibited matrix metalloproteinase-3 (MMP-3) expression [131]. Therefore, curcumin may be useful in treating IBD patients.

In rats with DSS-induced IBD, a dietary combination of quercitrin and olive oil supplemented with fish oil containing eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) ameliorated the intestinal inflammation [132]. The inflammation was suppressed through various mechanisms by the antioxidant activity and also by inhibition of the production of TNF and IL-1β by quercitrin. Fish oil also caused inhibition of TNF and LTB_4 production along with its antioxidant activity. A nested case-control study within a European prospective cohort study reported that higher intake of linoleic acid, an omega-6 polyunsaturated fatty acid (PUFA), increased the risk of ulcerative colitis [133]. However, an increased intake of omega-3 PUFA such as EPA or DHA lowered gut mucosal disease activity and histological scores improved in ulcerative colitis patients [134], indicating that a diet rich in omega-6 PUFA and inadequate in omega-3 PUFA can activate intestinal pro-inflammatory reactions.

Aloe has anti-inflammatory properties and its components aloe, aloin, and aloe-gel were tested individually in DSS-induced IBD mice [135]. These aloe components reduced the colonic mucosal TNF and IL-1β concentrations and decreased the plasma concentrations of LTB_4 and TNF. Thus, aloe could effectively reduce the intestinal inflammation in IBD mice [135].

Inflammatory cytokines greatly exacerbate the mucosal barrier damage and some of the nutraceuticals mentioned in Table 1 lowered concentrations of pro-inflammatory cytokines such as ICAM-1, IFN-γ, TNF, IL-6, and increased mucosal protective proteins in animal models of colitis. Diets rich in anti-inflammatory foods can mitigate the inflammatory insult in IBD patients and improve their treatment outcomes.

7. Modulation of immune system dysregulation

Dysregulation of the immune system is a common feature in IBD pathogenesis and the role of innate and adaptive immune cells in IBD has been reviewed [136]. Foods that can regulate the immune cells such

as T cells and B cells can improve the condition of IBD patients. There is an imbalance of regulatory and effector cells in active IBD, with effector T cells (Th_1, Th_2) increased over regulatory T cells (Th_3, Treg) [137,138]. IL-23 can trigger chronic intestinal inflammation mediated through innate or adaptive immune mechanisms and elicits IL-17-dependent autoimmunity [139–141]. IL-17 activates the NF-κB and MAPK signaling pathways, and upregulation of inflammatory molecules such as IL-6, IL-8, and MCP-1 [142]. Dysfunctional dendritic cells can cause disruption of intestinal mucosal barrier by activating inflammatory cells, including primed T cells to secrete pro-inflammatory cytokines [143]. This causes imbalance between the pro-inflammatory and anti-inflammatory responses leading to IBD.

In DSS-induced colitis mice, TCRαβ cells are essential for their anti-inflammatory action, along with IL-10, B cells, and γδ T cells, to induce the protective action of apple polyphenols [144]. Curcumin ameliorated colitis in TNBS colitis mice by inactivating the dendritic cells because of inhibition of the phosphorylation of the three members (JAK2, STAT3, and STAT6), thus modulating the JAK/STAT/SOCS signaling pathway [145]. Epigallocatechin-3-gallate (EGCG) and DHA inhibited the production of IL-17 and TNF in the cell line Kit 225, similar to Th_{17} cells [146]. The conjugate of DHA and 5-HT, docosahexaenoyl serotonin (DHA-5-HT), a gut-specific endogenously produced mediator, modulated the IL-17/Th_{17} signaling response by inhibiting Th_{17} pro-inflammatory mediators, IL-17 and CCL-20 [147]. Kaempferol, a flavonoid with anti-inflammatory and immune-modulatory activities, ameliorated colitis in DSS-treated mice by decreasing inflammatory mediators and improved repair of damaged intestinal epithelial layer [148]. This suggests that modulation of immunoregulatory activity can be a major target for therapy with nutraceuticals to treat IBD.

8. Modulation of cell signaling pathways

Major cell signaling pathways affected in IBD are the MAPK, Janus kinase-signal transducer and activator of transcription (JAK/STAT), NF-κB signaling, and nuclear factor erythroid 2-related factor 2 (Nrf2) pathways. MAPK cascades are divided into three subgroups: extracellular signal-regulated kinase (ERK), c-jun N-terminal kinase (JNK), and p38 which are implicated in IBD [149]. Activation of NF-κB signaling pathway causes release of pro-inflammatory cytokines TNF and IL-1 β in IBD [2]. Nrf2 is a master regulator in activation of transcription of anti-inflammatory and anti-oxidant proteins such as NAD(P)H:quinone oxidoreductase 1 (NQO1), glutathione-S-transferase, glutathione peroxidase (GPX2), thioredoxin and heme oxygenase-1 (HO-1) [150]. Diet supplemented with peracetylated (−)-EGCG improved the symptoms of DSS-induced colitis in mice [151]. The positive effect of peracetylated EGCG against colitis correlated with higher expression of HO-1 which is activated through signaling of ERK1/2 and acetylation of Nrf2, thereby abating DSS-induced colitis [125].

Curcumin can modulate more than one of these pathways to inhibit the production of pro-inflammatory cytokines. Curcumin-treated biopsies of IBD children and adults decreased p38 MAPK activation [131]. In TNBS colitis mice, curcumin modulated JAK/STAT/SOCS signaling pathway and repaired the gut tissue [145]. Curcumin reduced colonic inflammation by inhibition of pro-inflammatory pathways, including the NF-κB and MAPK pathways, in multidrug resistance gene-deficient ($Mdr1a^{-/-}$) IBD mice [152]. Further, curcumin treatment of ($Mdr1a^{-/-}$) IBD mice inhibited key pro-inflammatory transcription factors such as ERK and PI3 K complex [153].

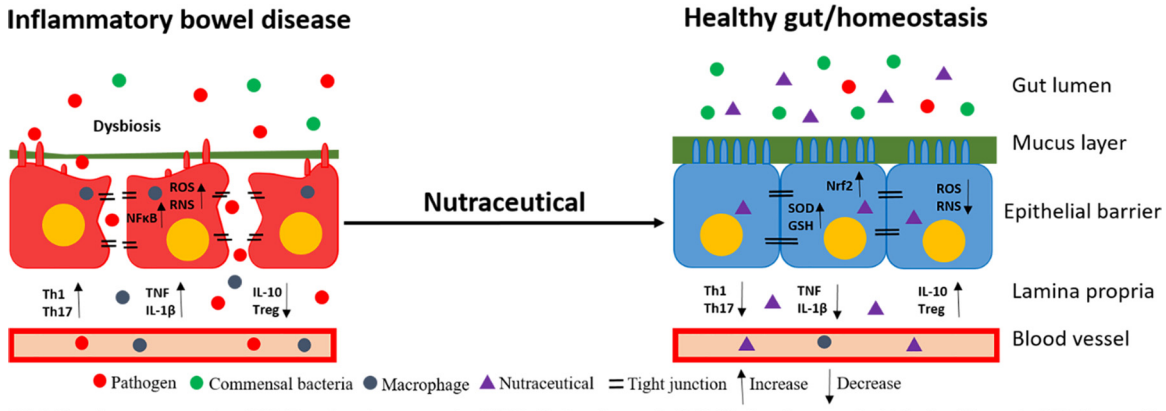
Polyphenols found in Marie Ménard apples reduced colitis when fed to HLA-B27 transgenic rats by downregulating the pathways of prostaglandin synthesis, MAPK signaling, and TNF and NF-κB pathways [154]. Zingerone and punicalagin also ameliorated IBD by inhibition of the NF-κB pathway [72,155]. Ellagic acid found in pomegranate fruit has been studied in acute and chronic models of DSS-induced IBD in mice where it reduced intestinal inflammation thereby decreasing the severity of the disease by down-regulating COX-2 and inducible nitric oxide synthase (iNOS) and blocking the signaling pathways, NF-κB, Signal transducer and activator of transcription 3 (STAT3), and p38 MAPK [156]. The anti-inflammatory ellagitannins from pomegranate reversed the gastrointestinal changes in DSS-induced IBD rats [157].

Broccoli and pak choi containing glucosinolates up-regulated the expression of Nrf2 target genes such as Nqo1 and GPx2 in azoxymethane (AOM)/DSS induced colitis-associated cancer in mice and inhibited colon inflammation and carcinogenesis [158]. In general, Nrf2 is inactively bound to Kelch-like ECH-associated protein 1 (Keap 1). Nrf2 is activated by either Keap1 cystein thiol modification or phosphorylation of Nrf2 by upstream kinases such as ERK, JNK, and P38 MAPK. Further, Nrf2 is translocated into the nucleus, where it binds to antioxidant response elements located in the promoter region of genes that activate transcription of phase-2 detoxifying enzymes and anti-oxidant proteins [150]. Some of the polyphenols found in *Perilla frutescens* have shown anti-inflammatory activity [159]. Luteolin effectively inhibited TNF, IL-1β, IL-6, and IL-17A in monocytes on LPS stimulation; apigenin decreased secretion of IL17A and increased IL-10 whereas rosmarinic acid had no effect on TNF, IL-6, and IL-17A but inhibited IL-1β secretion and increased Treg population [160]. However, rosmarinic acid inhibited the expression of IL-1β, IL-6, and IL-22 in colonic tissues of DSS-induced colitis in mice and suppressed COX-2 and iNOS through inhibition of NF-κB and STAT3 signaling pathways [159]. This suggests that, if *Perilla frutescens* extract was given as a treatment, the positive effects of the individual polyphenols would help in reducing colitis. In a recent study, *Perilla frutescens* extract suppressed DSS-induced colitis is mice by inhibiting NF-κB and STAT3 signaling pathways and activating Nrf2 signaling pathway thereby increasing HO-1 levels [161].

IL-8 is released by macrophages during intestinal inflammation and recruits neutrophils thereby increasing the inflammation at the tissue site [162,163]. Treatment of CCD841CoN human normal colon epithelial cells with *Perilla frutescens* extract after TNF-α insult reduced the expression of inflammatory CXCR2, a receptor of IL-8 [161]. Further, probiotics such as VSL#3 prebiotic formula inhibited DSS-induced colitis in rats by decreasing the colonic tissue expression of TNF, COX-2, NF- κB, pAkt, and IL-6, and increasing IL-10. This suggests that the treatment acts by suppressing the PI3 K/Akt and NF-κB inflammatory pathways [105]. Amentoflavone also reduced colitis by inhibiting the activation and translocation of NF-κB inflammatory pathway [73]. Modulation of cell signaling pathways, especially the NF-κB pathway, is the key in regulating gut homeostasis by nutraceuticals. The NF-κB pathway linked to the production of TNF is suppressed by many nutraceuticals, thereby improving the intestinal homeostasis and ameliorating the inflammation. These studies suggest that foods as a whole or enriched extracts of active ingredients rather than individual active compounds play a vital role in regulating IBD pathogenesis through modulation of gut microbiota, anti-oxidant, anti-inflammatory, immunomodulatory, and cell-signaling pathways as shown in Fig. 1 [75,122,123,131,156]. These mechanisms may work together to maintain a healthy gastrointestinal tract in IBD patients. However, the potential limitations of translating results obtained in rodent models to human IBD patients should be noted, including calculating a suitable dose and length of intervention, mimicking disease severity and periods of remission, differences in gastrointestinal anatomy and physiology between rats and humans, and the possibility of providing long-term follow-up and care of the human patients.

9. Conclusions

Nutraceuticals have an important role in IBD patients as a preventive approach to extend remission phases and also as a therapeutic intervention to suppress active IBD [164]. Active nutraceuticals may work as a low-cost complementary approach with minimal adverse effects to ameliorate the signs of IBD as effectively as current



ROS, Reactive oxygen species; RNS, Reactive nitrogen species; NFκB, Nuclear factor-κB; Nrf2, Nuclear factor erythroid 2-related factor 2; SOD, Superoxide dismutase; GSH, Glutathione; Th1, T helper cell type 1; Th17, T helper cell type 17; TNF, Tumor necrosis factor; IL-1β, Interleukin-1 beta; IL-10, Interleukin-10; Treg, Regulatory T cell;

Fig. 1. Inflammatory bowel disease (IBD) is characterized by various factors including dysbiosis, loss of mucus and epithelial barrier along with tight junctions that lead to increased intestinal permeability, increase in free radicals that lead to oxidative stress, increased activation of NF-κB cell signaling pathway and expression of pro-inflammatory cytokines TNF, IL-1β. There is also increased activation and number of innate and active immune cells such as macrophages, Th1 and Th2 cells and decreased Treg cells and IL-10. Nutraceuticals can modulate the factors implicated in IBD and help achieve gut homeostasis.

therapeutic options. More studies especially clinical trials are necessary to ascertain the limits of the usefulness of nutraceuticals as therapies in IBD.

Conflicts of interest

There are no conflicts of interest.

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Chapter 3. An improved rat model for chronic inflammatory bowel disease

An improved rat model for chronic inflammatory bowel disease

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Abstract

Background: Inflammatory bowel disease (IBD), an uncontrolled inflammation of the gastrointestinal tract, can be induced in rodents with dextran sodium sulfate (DSS). DSS causes inflammation in the gastrointestinal tract by disruption of the intestinal epithelial barrier.

Methods: This project developed a model of chronic IBD in young male Wistar rats testing responses to DSS (0%, 0.25%, 0.5%, or 1% in drinking water) for six weeks and further with 0.5% DSS for twelve weeks.

Results: Gastrointestinal tract changes were observed as diarrhea and bloody stools. Extra-intestinal parameters including oral glucose tolerance test, systolic blood pressure, bone mineral content, fat and lean mass, and left ventricular stiffness were measured. Rats given 0.5% DSS for twelve weeks showed increased, chronic, and sustained gastrointestinal changes with bloody diarrhea and anal bleeding, with a small loss of body weight. Histological examination showed marked infiltration of inflammatory cells throughout the gastrointestinal tract with crypt distortion. Gut bacteria diversity profiling data analyzed from fecal samples showed increases in Proteobacteria phylum. The replacement of DSS with water or sulfasalazine treatment for the final six weeks reversed these symptoms. No changes were observed in extra-intestinal parameters, so DSS-induced inflammation and cellular damage was limited to the gastrointestinal tract. Thus, chronic 0.5% DSS produces selective gastrointestinal changes mimicking chronic IBD in humans and is reversible.

Conclusion: This study provides an improved model using a lower dose of DSS for longer duration to mimic the chronic nature of gastrointestinal inflammation in human IBD. This model will be useful to test proposed interventions to treat human IBD.

Keywords: inflammatory bowel disease, rats, dextran sodium sulfate, sulfasalazine

INTRODUCTION

Inflammatory bowel disease (IBD) in humans, primarily Crohn's disease and ulcerative colitis, is an uncontrolled chronic inflammation of the mucosa in the gastrointestinal tract that gradually worsens and can last for decades with many relapses (1). There has been a steady increase in IBD incidence in Western and developed countries, with increasing incidence in Asia (2). IBD is more common in industrialized regions, possibly due to the increase in adopting a Westernized lifestyle, suggesting strong environmental influences on its development (3). IBD produces symptoms such as weight loss, bloody diarrhea, and severe abdominal pain (4). Microbial and genetic factors, mucosal barrier malfunction, immune/inflammatory system dysfunction and environmental factors contribute to the development of chronic intestinal inflammation (3). The gut bacterial compostion varies in IBD patients from healthy people due to thinning of mucosal barrier caused by inflammation (5-9).

Treatments for human IBD are developed from interventions in rodent models, induced either chemically or genetically (10). The most commonly used method in rodents is by oral treatment with dextran sodium sulfate (DSS) in the drinking water (10). DSS has been usually given at concentrations of 2% and greater in the drinking water for 5-7 days to induce acute IBD, and to lesser extent used at 1-2.5% for 10-28 days to induce chronic IBD in mice and rats (11-14). However, these models do not present a stable disease state, and interventions are usually for prevention rather than reversal, so different to clinical treatment in humans. IBD treatment options have been widely available for many years with sulfasalazine approved in 1950 in the USA and on the WHO List of Essential Medicines for treating intestinal inflammation including Crohn's disease and ulcerative colitis (15). In the USA and Canada, mesalamine (mesalazine or 5-aminosalicylic acid) as the active metabolite of sulfasalazine is one of the most prescribed drugs for IBD (16).

The aim of this study was to develop a stable chronic model that mimics human IBD and can be used to test interventions for reversal. We have characterized treatment with different DSS doses for 6 weeks, then with 0.5% DSS for 12 weeks in rats, then determining whether reversal of inflammation in the intestine is possible by replacing 0.5% DSS with normal water for the final six weeks. We also determined whether DSS-induced changes were restricted to the intestine by measuring cardiovascular, liver, and metabolic parameters. We then analyzed the responses to chronic sulfasalazine treatment as a reversal protocol in this IBD rat model. This is the first study to analyze whether chronic low-dose DSS induced stable IBD in rats with similar changes as in humans that can be reversed by an intervention used in humans for IBD.

MATERIALS AND METHODS

Diets and measurements in living rats

All experiments were approved by the University of Southern Queensland Animal Ethics Committee under the guidelines of the National Health and Medical Research Council of Australia. Male Wistar rats (8-9 weeks old, weighing 338 ± 1 g, n = 96) were purchased from the Animal Resource Centre, Perth. In the first study, rats were randomly divided into four experimental groups (n=10) and were fed with standard laboratory chow diet. Group one received normal drinking water. Groups two, three, and four received 0.25%, 0.5%, or 1% DSS (molecular weight: 36,000-50,000 Da, MP Biomedicals) in drinking water, respectively, for six weeks. In the second study, the rats were randomly divided into two experimental groups (n=12) and were fed with standard laboratory chow diet. One group was administered 0.5% DSS in drinking water for twelve weeks and the other group was administered 0.5% DSS in drinking water for the first six weeks and normal water for the next six weeks. In the third study, the rats were randomly divided into four groups (n = 8). Two groups were fed with only powdered food either with normal water (C) or 0.5% DSS water (D) for twelve weeks. The

remaining two groups received powdered food and normal water (CS) or 0.5% DSS water (DS) for twelve weeks together with powdered food containing sulfasalazine (4.6 g/kg food) for the final six weeks of the twelve week protocol. All rats were provided free access to food and water and were individually housed in temperature-controlled (20 ± 2°C), 12-hour light-dark conditions. Rats were monitored daily for body weight, and food and water intakes.

Rat stool scores were measured every day to assess the onset and progress of IBD. The stool consistency score was defined as 0-formed, 1-mild-soft, 2-very soft, and 3-watery soft (diarrhea). The stool bleeding score was taken as follows 0-normal color, 1-brown color, 2-reddish color, and 3-bloody red (17). Instead of disease activity index, it was more informative to show changes in individual components such as stool consistency and stool bleeding.

Dual-energy X-ray absorptiometric measurements were performed at the end of the protocol using a Norland XR36 DXA instrument (Norland Corp, Fort Atkinson, WI) under anesthesia with Zoletil (tiletamine 10 mg/kg, zolazepam 10 mg/kg, i.p.) and Ilium Xylazil (xylazine 6 mg/kg, i.p.). Scans were analyzed using the manufacturer's recommended software for use in laboratory animals (Small Subject Analysis Software, version 2.5.3/1.3.1; Norland Corp) (18). The precision error of lean mass for replicate measurements, with repositioning, was 3.2%.

For measurement of gastrointestinal permeability, rats were deprived of food for 4 hours, then gavaged with 2 mL of the probe solution containing 0.5g/mL sucrose, 0.04g/mL mannitol, 0.06g/mL lactulose, and 0.03g/mL sucralose (Sigma-Aldrich Australia, Sydney, NSW, Australia) (19). For the next 3 hours, rats were deprived of food and water. The urine was collected in 100µL of a 10% thymol in isopropanol solution at regular intervals for 24 hours.

Systolic blood pressure was measured non-invasively under anesthesia with Zoletil (tiletamine 10 mg/kg, zolazepam 10 mg/kg; i.p.). The measurements were recorded using physiological pressure transducers and Chart software on a MacLab system (ADI Instruments) (20). Abdominal circumference was measured at the same time with a measuring tape.

For oral glucose tolerance testing, rats were food-deprived for 12 hours (20). Basal blood glucose concentrations were measured following the food deprivation period using Medisense Precision Q.I.D. glucose meter in blood taken from the tail vein. The rats were given 2g/kg body weight via 40% glucose solution by oral gavage. Tail vein blood samples were then taken after 30, 60, 90, and 120 minutes and blood glucose concentrations were measured (20).

Two hours before euthanasia, rats in the third study were deprived of food. At the time of food deprivation, rats were administered 0.1mL charcoal solution/10g bodyweight of a 10% charcoal solution in 5% gum arabic by oral gavage. At euthanasia, the furthermost point the charcoal had moved from the pyloric sphincter was determined. The upper gastrointestinal tract motility was estimated as a percentage of the travelled distance to the total length from the pyloric sphincter to the ileocecal junction (21).

Measurements after euthanasia

Euthanasia was induced by i.p. injection of pentobarbitone sodium (Lethabarb®, 100mg/kg; Virbac, Peakhurst, NSW, Australia). Heparin (200 IU; Sigma-Aldrich Australia) was administered into the right femoral vein. The abdomen was then opened and blood (~5 mL) was withdrawn from the abdominal aorta and collected into heparinized tubes. Blood was centrifuged at 5000*g* for 15 minutes to obtain plasma. Plasma was stored at -20°C for further characterization. Hearts were then removed from rats for isolated Langendorff heart preparation. A latex balloon catheter was inserted into the left ventricle of the isolated heart connected to Capto SP844 MLT844 physiological pressure transducer and Chart software on

a MacLab system (ADI Instruments, Sydney, NSW, Australia). Isovolumetric function of the isolated heart was measured to calculate diastolic stiffness constant (κ, dimensionless) (20).

The small intestine and large intestine were separated and their lengths were measured. A small portion of the distal ileum and distal colon were separated for histological examination. The distal ileum and distal colon (~1.5 cm) were separated and placed in Tyrode's buffer. The lumen was washed with Tyrode's buffer and the tissue was placed in an organ bath chamber filled with Tyrode's buffer bubbled with 95% O_2–5% CO_2, maintained at 35°C and allowed to stabilize at a resting tension of approximately 10mN. Concentration-response (contraction) curves were recorded with acetylcholine (Sigma-Aldrich Australia) using pressure transducers and Chart software on a MacLab system (ADI Instruments).

After the heart perfusion, the left ventricle along with septum and the right ventricle were separated and weighed. Kidney, spleen, and liver were collected, blotted dry, and weighed from each rat. Abdominal fat pads were removed as retroperitoneal, epididymal, and omental fat pads and weighed. All organ weights were normalized to the tibial length at the time of organ isolation (expressed as mg/mm) (20).

Plasma lipid concentrations and activities of plasma enzymes were determined using kits and controls supplied by Olympus using an Olympus analyser (AU 400, Tokyo, Japan) (20).

The isolated portions of ileum and colon for histology were fixed in 10% neutral buffered formalin for three days. Thereafter, the tissues were dehydrated and embedded in paraffin wax. Thin 5µm sections were cut from the paraffin embedded tissues. The cut sections of the tissues were stained with hematoxylin and eosin stains and were observed by using 20× objective lens of an Olympus BX51 microscope (Olympus, Melville, NY, USA) to determine the infiltration of inflammatory cells and damage to the intestinal tissue (20).

Analysis of the sugars present in the urine samples was performed at the Central Analytical Research Facility of the Queensland University of Technology, Gardens Point, Brisbane. Myo-inositol (Sigma-Aldrich Australia) was added to the urine samples as the internal standard (1 mg/mL). Calibration curves of the four sugars (mannitol, sucrose, lactulose, and sucralose; stock solutions, 1mg/mL) were undertaken from 0.075 to 1 mg/mL with myo-inositol (1mg/mL). 50µL of urine sample was added to 450µL of cold methanol and vortexed followed by centrifugation for 5 minutes at 14000*g* and 5°C. 3µL of the supernatant was aliquoted in a glass insert tube. All samples were vacuum-concentrated to dryness for 30 minutes before derivatization of the samples in two steps (22). In the first step, the samples underwent oximation under incubation with 20µL of 30mg/mL Meox (methoxyamine + pyridine) (Sigma-Aldrich, Sydney, Australia) for 2 hours at 37°C and 500 rpm in a thermomixer (Eppendorf, Melbourne, Australia). The second step was silylation of the samples by incubation with 40µL of N,O-bis(trimethylsilyl)trifluoroacetamide (BSTFA) + 1% trichloromethylsilane (TMCS) silylation reagent (Thermo Scientific, Scoresby, Australia) for 40 minutes under similar conditions. The glass insert tubes with the samples were transferred to auto-sampler GC vials.

Sugar analysis was performed using GCMS Shimadzu TQ8040 (Shimadzu, Sydney, Australia). Samples (1µL) were injected in split ratio (2:1) in the injection port at 250°C, and the sugars were separated on the SH-Rxi-5Sil-MS column (30m × 0.25mm × 0.25µm film thickness) with the column flow of 0.76 mL/minute, and helium carrier gas flow of 4.15 mL/minute. The initial oven temperature was set at 200°C for 1 minute and then increased at 10°C/minute to 250°C, 1°C/minute to 260°C, 3°C/minute to 275°C and held for 2 minutes, 15°C/minute to 300°C and held for 1 minute. The total run was 25.67 minutes. The MS detector ion source temperature was 250°C and the interface temperature was 280°C. The samples were

detected in Selected Ion Monitoring method. Cumulative percent recovery of each sugar and intestine permeability were calculated (23-25).

The fecal samples of the rats were collected from the colon at euthanasia and stored at -80 °C for analysis of gut microbiota diversity profiling. Microbiota profiling was performed at the Austrlaian Genomic Research Facility, Adelaide, Australia (26). The PCR amplification of the gDNA was undertaken with primers specific to V3–V4 region of 16S rRNA gene. The primers used were F341 (5′-CCTAYGGGRBGCASCAG-3′) and R806 (5′-GGACTACNNGGGTATCTAAT-3′). R statistical software was used according to the developer's instructions to generate heatmaps for relative abundance of bacterial phyla and species. Venn diagrams were prepared for observed taxonomical units (OTUs) indicating richness of species, in not less than 50% of the samples of each group using the online plotting tool Venny 2.1.0 (http://bioinfogp.cnb.csic.es/tools/venny/index.html). Complex heatmaps of the bacterial phyla and species greater than 1% in abundance were generated by R software using R/bioconductor package (27).

Statistics

All data are expressed as mean ± standard error of the mean (SEM). Results from control, 0.25% DSS, 0.5% DSS, and 1% DSS were analyzed for variance by using one-way ANOVA. The data were tested with Neumann-Keuls multiple comparison *post hoc* test and $P<0.05$ was considered as significant. The results of 0.5% DSS for 6 and 12 weeks were compared by using unpaired Student's *t* test. The results from the sulfasalazine study were analyzed for variance by using two-way ANOVA followed by Neumann-Keuls multiple comparison *post hoc* test. The statistical analyses were run by using GraphPad Prism version 6 for Windows (GraphPad Software, La Jolla, CA, USA).

RESULTS

DSS dose-dependent changes over 6 weeks

DSS produced dose- and time-dependent increases in stool consistency and stool bleeding scores (Fig. 1A and 1B). Control rats maintained normal stool texture and color whereas 1% DSS rats had the most marked changes with watery bloody stool, with occasional anal bleeding. DSS treatment had no effect on small intestinal length but there was a dose-dependent reduction of colon length in DSS groups (Table 1). Histological examination of the colon and ileum showed dose-dependent predicted changes in the epithelial membrane and crypt architecture of control, 0.25%, 0.5%, and 1% DSS rats (Fig. 2). In the colon and ileum of 0.5% DSS and 1% DSS groups, there was marked loss of epithelial layer and branched crypts, crypt and mucosal atrophy, reduction of villi length and crypt height along with inflammatory cell infiltration (Fig. 2C, 2D, 2G and 2H). 0.25% DSS rats had only minimal inflammation (Fig. 2B and 2F) while the controls had healthy tissue (Fig. 2A and 2E). Fig. 3 shows the fecal bacteria taxonomic diversity in rats treated with 0% DSS or 0.5% DSS for 6 weeks, 0.5% DSS for 12 weeks and replacement of 0.5% DSS with water at 6 weeks. This figure highlights the mean abundances of the major bacterial phyla, Venn diagram of operational taxonomic units and relative abundances of species among the groups. The gut bacteria analyzed from fecal samples showed that 0.5% DSS treatment for 6 weeks decreased *Bifidobacterium pseudolongum* of phylum Actinobacteria and increased order Clostridiales, *Ruminococcus gnavus* and genus *Oscillospira* which belong to phylum Firmicutes compared to control (0% DSS) rats (Fig. 3D).

DSS rats had higher food intake and energy intake than control rats (Table S1). However, DSS produced dose-dependent decreases in the feed conversion efficiency which is reflected as the reduced body weight gain, and final body weight in 1% DSS rats (Tables 1 and S1; Fig. 1C). 0.5% DSS for 6 weeks caused no body weight change because DSS caused localized effects in the intestine causing inflammation without causing changes in food

absorption, mimicking low grade chronic inflammation. DSS rats showed no differences in metabolic parameters with the exception of increased blood glucose area under the curve and higher total cholesterol in 0.5% DSS rats (Tables 1 and S1). Only 0.5% DSS rats had small increases in systolic blood pressure (Table S1). The heart function and organ weights were unchanged except for the increased spleen weight in 1% DSS rats which is uncommon in IBD and not related to inflammation (Tables 1 and S1). Plasma alanine transaminase activity remained unchanged with DSS treatment (Table 1). As 1% DSS produced severe inflammation, a reduced dose of 0.5% DSS was chosen with an extended protocol of 12 weeks to examine whether this dose produced reversible, stable, and moderate chronic IBD in rats.

DSS dose-dependent changes over 12 weeks

The stool consistency and bleeding scores showed similar changes after 6 or 12 weeks of treatment with 0.5% DSS (Fig. S1A and S1B). 0.5% DSS reduced the colon length without changing small intestinal length (Table S2). The ileum and colon of rats with 0.5% DSS for 12 weeks had marked mucosal inflammation, epithelial membrane loss, cryptitis, forked crypts, and crypt abscesses (Fig. S3A, S3C, and S3E-H). The increase from 6 weeks to 12 weeks with 0.5% DSS increased the inflammation of ileum and colon. The increase from 6 weeks to 12 weeks with 0.5% DSS gave similar species distribution of gut bacteria in fecal samples except for the decreased counts of *Lactobacillus* genus and the increased counts of *Streptococcus* genus, both belonging to phylum Firmicutes (Fig. 3E).

The total fat mass remained unchanged and other metabolic parameters are mentioned in Table S2. The cardiovascular parameters, organ weights, and plasma liver enzyme activities were similar after 6 or 12 weeks DSS intervention except for a decrease in liver weight (Table S2).

Return to normalcy with replacement of DSS by water

The stool consistency and bleeding scores improved with replacement of 0.5% DSS with water for the final 6 weeks (Fig. S2C and S2D). Replacement of 0.5% DSS for the final 6 weeks prevented the shortening of colon but there was no change in small intestinal length (Table S3). The inflammation of ileum and colon was reversed with replacement of 0.5% DSS with water for 6 weeks (Fig. S3B and S3D). Replacement of 0.5% DSS with water did not alter the maximal force of contraction (mN) to acetylcholine in isolated ileum and colon preparations (ileum:D=31.6 ± 8.6, D+W=13.4 ± 7.3; colon:D=78.2 ± 11.6, D+W=57.9 ± 18.9).

The intestinal permeability test was performed for the two groups in the 12-week study, the 0.5% DSS group and the replacement of 0.5% DSS with water group. Cumulative percent recoveries of sucrose (gastroduodenal permeability marker), lactulose, mannitol, and sucralose (colon permeability marker) at 3, 6, 9, 21, and 24 h were similar in both groups (Fig. S4). For all four sugars, the 24-h % excretion of the oral dose was similar in both groups. The lactulose/mannitol ratio (small intestine permeability marker), sucralose/lactulose ratio, and sucralose/mannitol ratio were similar in both the groups indicating no change in the whole gut permeability (Fig. S5).

The mean abundances of the phyla Actinobacteria and Bacteriodetes decreased while phylum Firmicutes increased in the fecal samples of rats treated with DSS for 6 and 12 weeks (Fig. 3A and 4). Replacement of 0.5% DSS with water at 6 weeks reduced Proteobacteria compared to DSS rats for 12 weeks (Fig. 3B). Replacement of 0.5% DSS with water at 6 weeks decreased the populations of Firmicutes phylum comprising of *Ruminococcus gnavus* of Lachnospiraceae family and also Ruminococcaceae family including *Oscillospira* genus (Fig. 3F). After 12 weeks, DSS decreased the number of species compared to control rats while water replacement of DSS increased the number of species (Fig. 3C). There was no change in Shannon diversity index, an indicator of alpha diversity (C6=2.1±0.1, D6=2.3±0.1, D12=2.4±0.1, D+W=1.9±0.3). Heatmaps of relative abundances of bacterial phyla and species

with dendograms showed variability among the groups of the rats with control and the reversal DSS groups as one cluster while 0.5% DSS for weeks 6 and 12 groups as one cluster indicating there was a shift of gut bacteria composition back to normal after replacement of 0.5% DSS with water at 6 weeks including the family S24-7, *Bifidobacterium pseudolongum* (Fig. 4 and 5).

Replacement of 0.5% DSS with water at 6 weeks did not alter food, water, energy intakes, or feed efficiency at 12 weeks; however, during the first four weeks, there was lower body weight gain even though both the groups had similar diet (Table S3; Fig. S2A and S2B). Rats fed with 0.5% DSS water for 12 weeks had lesser omental fat, higher total fat mass, and bone mineral content, with no change in blood glucose area under the curve (Table S3). The plasma concentrations of non-esterified fatty acids and total cholesterol remained unchanged but there was a decrease in triglycerides concentrations with the replacement of 0.5% DSS with water at 6 weeks (Table S3). Replacement of 0.5% DSS with water at 6 weeks did not change cardiovascular parameters, liver parameters, or organ weights except that the left ventricular diastolic stiffness constant was higher (Table S3).

Sulfasalazine on 0.5% DSS-induced IBD

The doses of sulfasalazine based on food intake measurements were 346 ± 23 mg/kg in CS rats and 350 ± 36 mg/kg in DS rats. The stool consistency and stool bleeding scores of CS rats were minimal and similar to C rats (Fig. 6A and 6B). Sulfasalazine improved stool consistency and stool bleeding scores in DS rats (Fig. 6A and 6B). The small intestinal length increased in CS rats compared to C rats (Table 2). However, colon length and gastric transit remained unchanged in CS group (Table 2). The small intestinal length increased in DS rats compared to D rats (Table 2) but colon length and gastric transit remained unchanged (Table 2). The histology of ileum and colon showed healthy mucosal layer in CS rats as in C rats (Fig. 7A, 7B, 7E, and 7F). Sulfasalazine improved the ileum of DS rats with increased villi length

and decreased inflammation compared to D rats (Fig. 7C and 7D), while the colon of DS rats showed higher crypt numbers, increased crypt heights, and improved epithelial membranes compared to D rats (Fig. 7G and 7H). The isolated ileum and colon preparations of CS rats gave similar maximal contractile responses (mN) to acetylcholine when compared to C rats (ileum: C=27.3 ± 4.9, CS=28.9 ± 5.4; colon: C=64.9 ± 8.8, CS=63.9 ± 15.9). The isolated ileum preparations of DS rats gave higher maximal contractile responses (mN) to acetylcholine than the D rats whereas the colon preparations showed no change among the groups (ileum:D=19.9 ± 4.1, DS=44.5 ± 6.1; colon: D=68.2 ± 7.8, DS=61.8 ± 11.7). The cumulative percent recovery of sucrose, mannitol, and sucralose at 3, 6, 9, 21, and 24 h, and whole gut permeability indicator sucralose/mannitol were unchanged with sulfasalazine treatment (Fig. S6A-S6D).

Mean abundances of the major phyla differed among the groups (Fig. 8A). The heatmaps for the bacterial phyla showed C and DS rats clustered together whereas D and DS rats clustered together for species abundance (Fig. 9 & 10). Phylum Proteobacteria increased with DSS and reversed to normal with sulfasalazine (Fig. 8B). The observed OTUs were lower in CS and DS rats (Fig. 8C) and there was no difference in Shannon diversity index among the C, CS, D, and DS rats (C=2.3±0.1, CS=2.2±0.1, D=2.4±0.1, DS=2.5±0.1). *Streptococcus* genus was increased with DSS and normalized with sulfasalazine (Fig. 8D). Cluster 2 species of families S24-7, Lachnospiraceae, order Clostridiales and genus *Lactobacuillus* were most abundant among the four groups (Fig. 10).

CS rats had decreased final body weight but no difference in body weight gain, food intake, water intake, energy intake, or feed efficiency compared to C and D rats (Table S4). Sulfasalazine resulted in DS rats with lower final body weight but with no difference in body weight gain, food intake, water intake, energy intake, or feed efficiency compared to D rats (Table S4). CS rats had decreased abdominal fat pads compared to the C rats, whereas omental fat decreased in D and CS rats (Table 2 and S4), which was unexpected change with no logical

explanation. DS rats had decreased retroperitoneal fat pads compared to D rats, whereas abdominal fat pads, epididymal fat, and omental fat were similar to D rats (Table S4). There were no changes in blood glucose concentrations or organ weights in CS rats (Table 2 and S4). Blood glucose concentrations and organ weights remained unchanged in DS rats (Table 2 and S4).

DISCUSSION

Our initial finding was that 0.5% DSS for 6 or 12 weeks produced stable, moderate, and chronic symptoms based on the stool characteristics and histological examination. We then showed that replacement of 0.5% DSS with water for the final 6 weeks of the 12 weeks protocol normalized the stool characteristics and the ileum and colon tissue damage. Extra-intestinal effects on glucose tolerance, blood pressure, cardiac and liver function, body weight, and bone mineral parameters were minimal. The intestinal permeability was not affected by this 0.5% DSS protocol for up to 12 weeks. However, there were changes in gut microbiota in the 0.5% DSS rats after 12 weeks with decreased counts of *Lactobacillus sp* and increased counts of *Streptococcus sp* and Proteobacteria phylum. Thus, 0.5% DSS in the drinking water for 12 weeks was the optimal dose in male Wistar rats to reflect human inflammatory bowel disease. To further validate our model, we examined the responses to sulfasalazine treatment in the 0.5% DSS rats for the final 6 weeks of the 12 week protocol. Sulfasalazine normalized the stool characteristics, repaired the gut epithelial membrane with reduced inflammation and improved the gut bacterial profile. Our results suggest that chronic dosage with 0.5% DSS in rats mimics human IBD, both in symptoms and management. This model should allow relevant results to be obtained for new treatments before translation studies in humans with IBD.

The major limitation of our study is that, while DSS is the causative agent in this model, the cause of the human disease is unknown and therefore not mimicked in this study. Further, we have characterized functional and structural changes, but molecular changes were not

362 investigated. In addition, a longer protocol in rats could produce cardiovascular changes as
363 found in humans with IBD including an increased risk of cardiovascular disease and endothelial
364 dysfunction (28, 29) and other extra-intestinal changes due to chronic systemic inflammation
365 following increases in intestinal permeability.

366 Many rodent models have been used to study the pathogenesis and treatment of IBD of
367 which chemically-induced IBD with DSS is quite common. The concentration and the duration
368 of DSS administration determine the severity of the symptoms (30). Most DSS models are
369 acute with administration of 2-5% DSS ranging from 5 days to 7 weeks in healthy rodents to
370 produce a rapidly worsening disease state; further, these studies tested prevention of IBD by
371 dietary interventions with anti-inflammatory and anti-oxidant activity (12-14, 31-33). As these
372 studies investigated preventive therapy for a short period, this may not be relevant to IBD
373 patients who have existing chronic disease which requires reversal therapy. Treatment with
374 0.5% DSS in this study produced a relatively stable gastrointestinal disease state that can test
375 possible reversal interventions such as functional foods or new drugs. As an example, we
376 treated rats with sulfasalazine for the final 6 weeks as this drug is widely used in human IBD
377 patients. Thus, 0.5% DSS for 12 weeks produces a chronic IBD model in male rats with clinical,
378 physiological, morphological, histological, and dysbiosis symptoms similar to chronic human
379 IBD that can be treated with sulfasalazine.

380 DSS causes intestinal inflammation extending from the rectum towards the distal colon
381 and further to the rest of the colon (34). There was colon repair and re-epithelization with
382 squamous epithelium due to replacement of 0.5% DSS in drinking water with normal water as
383 observed in other studies in mice (35). Though the exact mechanism of action of DSS-induced
384 inflammation is unclear, DSS causes loss of epithelial barrier integrity and leads to disruption
385 of epithelial membrane which is further aggravated by apoptosis and reduced rate of cell
386 renewal thereby leading to reduced colon length (36). This compromised epithelial barrier in

DSS rats is consistent with the changes in gut structure and function indicated by stool characteristics, gut histology, gut motility and gut bacteria in our study. DSS decreased the stool consistency causing diarrhea due to inefficient absorption of water and electrolytes (37). The severely inflamed intestinal wall can be further damaged by internal fissures, fistulae and increased microvasculature which leads to gastrointestinal bleeding causing bleeding stools, even anal bleeding at times (38). This leads to movement of gut microbiota from the intestinal lumen to the intestinal crypts and causes inflammation due to excess innate and adaptive immune reactions and these symptoms in rats mimic human IBD (39).

Increased intestinal permeability is observed in IBD patients due to the disruption of tight junctions in the intestinal epithelial membrane, and there is a leakage of LPS or its binding protein into the systemic circulation triggering the immune system to secrete inflammatory cytokines leading to systemic inflammation (40). The intestinal permeability was not increased in our model although the stool characteristics and histology indicate that intestinal architecture is compromised to some extent, but not sufficient to increase the permeability of large molecules such as LPS suggesting that there is no systemic inflammatory damage after 12 weeks' treatment with DSS. Although most IBD patients show increased intestinal permeability at 10 years after the onset of IBD, there is increased epithelial cell turnover and tight junctions in the chronic recovery stage as observed in Crohn's disease patients in remission or DSS-induced IBD rats after replacing DSS with water (41, 42). The increased intestinal permeability could be a cause or effect of inflammation and therefore depend on the severity and extent of inflammation (41, 43). It is not clear whether increased intestinal permeability precedes intestinal inflammation or is the consequence of inflammation (44). Decreased intestinal motility is observed in severely inflamed intestine of IBD patients and also animal models (45) but was not observed after 12 weeks of 0.5% DSS, suggesting that this change occurs later in the disease progression. Decreased intestinal circular smooth muscle

412 contractility is due to dampened activity of L-type Ca^{2+} channels caused by gut inflammation;
413 sulfasalazine, an inhibitor of inflammatory NF-κB, improved colonic contraction (46), as
414 observed in our study.

415 In the normal human gut, Firmicutes and Bacteroidetes are the most abundant phyla
416 followed by phyla Actinobacteria and Proteobacteria; however, during dysbiosis which is seen
417 in IBD patients, there are decreases in Firmicutes and increases in Proteobacteria and
418 Actinobacteria phyla (47), as observed in our study in rats. *B. pseudolongum* with gut protective
419 effects was the dominant bifidobacterial population in the healthy adult human intestinal
420 biopsies and was reduced in IBD patients and 0.5% DSS treated rats (48, 49), as observed in
421 our study. *Lactobaciluus* genus, a commensal bacteria that maintains the gut mucosal
422 homeostasis, was underrepresented in Crohn's disease and ulcerative colitis patients (50),
423 which was similar to our observation in 0.5% DSS rats after 12 weeks. Decline of Lactobacillus
424 may impair the gut barrier leading to invasion of bacteria and inflammation. Consistent with
425 our results, *Streptococcus* spp was increased among Iranian IBD patients in active and
426 remission stage (51). The mucolytic bacteria *Ruminococcus gnavus* predominated in Crohn's
427 disease and ulcerative colitis patients compared to healthy individuals (52). This was reflected
428 in our study with an increase in 0.5% DSS rats and decrease after replacing 0.5% DSS with
429 water suggesting that there was a reversal in the gut dysbiosis. Overall, our model showed
430 changes in the gut bacteria similar to the human IBD dysbiosis condition that could be reversed
431 with sulfasalazine.

432 Extra-intestinal parameters were only changed to a minor extent in our IBD model but
433 obesity could aggravate IBD (53). Hyperplasia of fat around inflamed intestine in Crohn's
434 disease may allow colonization and translocation of intestinal bacteria but we found reduced
435 omental fat in DSS rats (54). This topic needs to be explored more as few have studied the link
436 between visceral adipose tissue and IBD. The plasma lipid inflammatory markers were normal

in IBD children unlike in IBD adults (55). Unlike in our model, IBD patients after a prolonged period of disease may be prone to osteopenia and osteoporosis that depends on age, sex, BMI, and largely corticosteroid therapy (56). Even though it seems to be controversial whether IBD leads to cardiovascular complications, a recent study concluded that IBD patients did not show any changes in cardiovascular parameters such as systolic blood pressure and diabetes (57) and our rats did not show endovascular complications. Abnormality of liver functional tests was found to be transient in IBD patients and return to normal (58), similar to our study. Absence of extra-intestinal abnormalities in our model indicates that there is no systemic inflammation which could occur after a much longer period of disease progression.

In conclusion, we have presented an improved rat model for chronic IBD with relatively stable disease parameters to allow studies on reversal of symptoms, with no changes in extra-intestinal parameters, reversal after removal of the inflammatory stimulus and therapeutic effectiveness of the standard drug treatment of sulfasalazine. Thus, this model could be used to test compounds that may reverse IBD symptoms which could then be further tested in human clinical trials. Positive trials may eventually lead to an enhanced life-style for IBD patients.

Author contributions

N.K.R.G., S.K.P., and L.B. developed the original study aims. N.K.R.G. conducted the experiments. N.K.R.G., S.K.P., and L.B. analyzed and interpreted the data; N.K.R.G., S.K.P., and L.B. prepared manuscript drafts and contributed to the final version. L.B. has been the corresponding author throughout the writing process. All authors read and approved the final manuscript.

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Table 1. *Metabolic, cardiovascular, hepatic, and gastrointestinal parameters in rats treated with dextran sodium sulfate (DSS) (0%, 0.25%, 0.5%, or 1%) for 6 weeks.*

Variables	***0% DSS***	***0.25% DSS***	***0.5% DSS***	***1% DSS***
Final body weight, g	491 ± 6^{ab}	503 ± 8^{a}	505 ± 8^{a}	470 ± 8^{b}
Abdominal fat pads, mg/mm tibial length	575 ± 36	636 ± 51	704 ± 68	536 ± 40
Total fat mass, g	137 ± 8	158 ± 18	152 ± 16	111 ± 11
Total lean mass, g	332 ± 10	318 ± 10	308 ± 16	325 ± 12
Fasting blood glucose 0 minutes, mmol/L	4.3 ± 0.2	4.3 ± 0.2	4.3 ± 0.2	4.9 ± 0.3
Area under the curve, mmol/L×minutes	721 ± 15^{b}	860 ± 29^{a}	810 ± 25^{a}	804 ± 30^{a}
Plasma non-esterified fatty acids, mmol/L	2.8 ± 0.3	2.6 ± 0.2	3.3 ± 0.4	3.1 ± 0.2
Plasma triglycerides, mmol/L	0.8 ± 0.2	0.7 ± 0.1	1.2 ± 0.2	1.0 ± 0.1
Plasma total cholesterol, mmol/L	1.1 ± 0.1^{b}	1.2 ± 0.1^{b}	1.6 ± 0.1^{a}	1.4 ± 0.1^{ab}
Left ventricular diastolic stiffness constant (κ)	26.8 ± 1.8	25.7 ± 1.3	22.8 ± 2.1	26.7 ± 1.5
Plasma alanine transaminase activity, U/L	26.1 ± 6.2	20.5 ± 2.6	19.1 ± 1.9	22.1 ± 2.2
Small intestinal length, cm	124 ± 1	114 ± 6	126 ± 3	124 ± 2
Colon length, cm	24.4 ± 0.5^{a}	19.7 ± 0.6^{b}	20.2 ± 0.5^{b}	18.1 ± 0.6^{c}

All values are mean ± SEM, n = 6-10. Mean values within a row with a different superscript are significantly different, $P<0.05$.

Table 2. *Metabolic and gastrointestinal parameters in rats treated with sulfasalazine*

Variables	***C***	***CS***	***D***	***DS***	***P-Value***		
					DSS	***Sulfasalazine***	***Interaction***
Fasting blood glucose, mmol/L	4.2 ± 0.1	4.7 ± 0.1	4.5 ± 0.1	4.4 ± 0.2	1.00	0.14	0.031
Area under the curve, mmol/L×minutes	727 ± 18	725 ± 21	740 ± 9	767 ± 31	0.2	0.56	0.50
Abdominal fat pads, mg/mm	897 ± 71^{a}	567 ± 72^{b}	775 ± 44^{ab}	675 ± 66^{ab}	0.64	0.0008	0.037
Small intestine length, cm	114 ± 3^{b}	129 ± 2^{a}	120 ± 2^{ab}	127 ± 4^{a}	0.49	0.0007	0.18
Colon length, cm	22.6 ± 1.4^{a}	22.9 ± 0.3^{a}	18.9 ± 0.9^{b}	18.4 ± 1.0^{b}	0.003	0.029	0.33
Gastrointestinal transit, %	76.8 ± 5.2	86.5 ± 3.5	86.6 ± 3.2	87.6 ± 4.4	0.22	0.23	0.32

All values are mean ± SEM, n = 6-8. Mean values within a row with a different superscript are significantly different, $P<0.05$. C, control (0% DSS); D, 0.5% DSS; CS, C + sulfasalazine; DS, D + sulfasalazine; DSS, dextran sodium sulfate; CS and DS rats were treated with sulfasalazine for the last 6 weeks of the 12 week protocol.

Figure legends

Fig. 1. Effect of dextran sodium sulfate on stool consistency (A), stool bleeding (B), and body weight (C) in rats. Values are mean ± SEM, n =10. Endpoint means without a common letter differ, $P < 0.05$. Stool consistency score, 0-formed, 1-mild-soft, 2-very soft, 3-watery soft (diarrhea). Stool bleeding score, 0-normal color, 1-brown color, 2-reddish color, 3-bloody red.

Fig. 2. Effect of dextran sodium sulfate on inflammation in the intestine. Hematoxylin and eosin staining of ileum and distal colon showing infiltration of inflammatory cells "in" (C, D, G, and H), epithelial disruption "ed" (C, D, G, and H), crypt distortion "cd" (C, D, G, and H), branched crypt "bc" (H), and mucosal atrophy "ma" (C, D, G, and H) (×20). Ileum of rats treated with dextran sodium sulfate at 0% (A), 0.25% (B), 0.5% (C), and 1% (D). Colon of rats treated with dextran sodium sulfate at 0% (E), 0.25% (F), 0.5% (G), and 1% (H).

Fig. 3. Taxonomic diversity of fecal microbiota in rats treated with 0% DSS (C), 0.5% DSS for 6 weeks (D6), 0.5% DSS for 12 weeks (D12), and 0.5% DSS for 6 weeks and water for next 6 weeks (D+W), n=6/group. (A) Mean abundances of the major bacterial phyla as percentage of the total population among the four groups of rats, (B) relative abundance of Proteobacteria phylum, (C) Venn diagram of observed taxonomic units (OTUs), relative abundances of species significantly different among C and D6 rats (D), D6 and D12 rats (E), D12 and D+W rats (F). DSS, dextran sodium sulfate.

Fig. 4. Heatmap of bacterial phyla obtained from fecal microbiota in rats treated with 0% DSS (C), 0.5% DSS for 6 weeks (D6), 0.5% DSS for 12 weeks (D12), and 0.5% DSS for 6 weeks and water for next 6 weeks (DW), n=6/group. The sample groups are on the horizontal axis and the bacterial phyla are on the vertical axis. Dark red- highest value, dark blue- lowest value. DSS, dextran sodium sulfate.

Fig. 5. Heatmap of bacterial species obtained from fecal microbiota in rats treated with 0% DSS (C), 0.5% DSS for 6 weeks (D6), 0.5% DSS for 12 weeks (D12), and 0.5% DSS for 6 weeks and water for next 6 weeks (DW), n=6/group. The sample groups are on the horizontal axis and the bacterial species are on the vertical axis. Dark red- highest value, dark blue- lowest value. DSS, dextran sodium sulfate.

Fig. 6. Effect of sulfasalazine on stool consistency (A) and stool bleeding (B) in rats given 0.5% DSS water (DS) or normal water (CS) in comparison to control (C) and 0.5% DSS (D) rats for twelve weeks. Values are mean ± SEM, n = 6-8/group. Endpoint means with a different alphabet differ, $P < 0.05$. DSS, dextran sodium sulfate. Stool consistency score, 0-formed, 1-mild-soft, 2-very soft, 3-watery soft (diarrhea). Stool bleeding score, 0-normal color, 1-brown color, 2-reddish color, 3-bloody red.

Fig. 7. Effect of sulfasalazine on inflammation in the intestine. Hematoxylin and eosin staining of ileum and distal colon showing infiltration of inflammatory cells “in” (B and F), epithelial disruption “ed” (B and F), crypt distortion “cd” (B and F), cryptitis “cy” (F) and mucosal atrophy “ma” (B and F) (×20). Ileum of rats treated with normal water, 0.5% DSS water for 12 weeks (A and B), and ileum of rats treated with normal water and 0.5% DSS water for 12 weeks and sulfasalazine in the food for last 6 weeks (C and D). Colon of rats treated with normal water and 0.5% DSS water for 12 weeks (E and F) and colon of rats treated with normal water and 0.5% DSS water for 12 weeks and sulfasalazine in the food for last 6 weeks (G and H). DSS, dextran sodium sulfate. Intestinal inflammation shows signs of Crohn’s disease in ileum with cryptitis, mucosal atrophy and also signs of ulcerative colitis in colon with the crypt distortion and mucosal atrophy.

Fig. 8. Taxonomic diversity of fecal microbiota in rats given sulfasalazine with 0.5% DSS water (DS) or normal water (CS) in comparison to control (C) and 0.5% DSS (D) rats for twelve weeks. (A) Mean abundances of the major bacterial phyla as percentage of the total population

among the four groups of rats, (B) relative abundance of Proteobacteria phylum, (C) Venn diagram of observed taxonomical units (OTUs) and (D) Relative abundances of species.

Fig. 9. Heatmap of bacterial phyla obtained from fecal microbiota in rats given sulfasalazine with 0.5% DSS water (DS) or normal water (CS) in comparison to control (C) and 0.5% DSS (D) rats for twelve weeks, n=6. The sample groups are on the horizontal axis and the bacterial phyla are on the vertical axis. Dark red- highest value, dark blue- lowest value.

Fig. 10. Heatmap of bacterial species obtained from fecal microbiota in rats given sulfasalazine with 0.5% DSS water (DS) or normal water (CS) in comparison to control (C) and 0.5% DSS (D) rats for twelve weeks, n=6. The sample groups are on the horizontal axis and the bacterial species are on the vertical axis. Dark red- highest value, dark blue- lowest value.

Figure 1

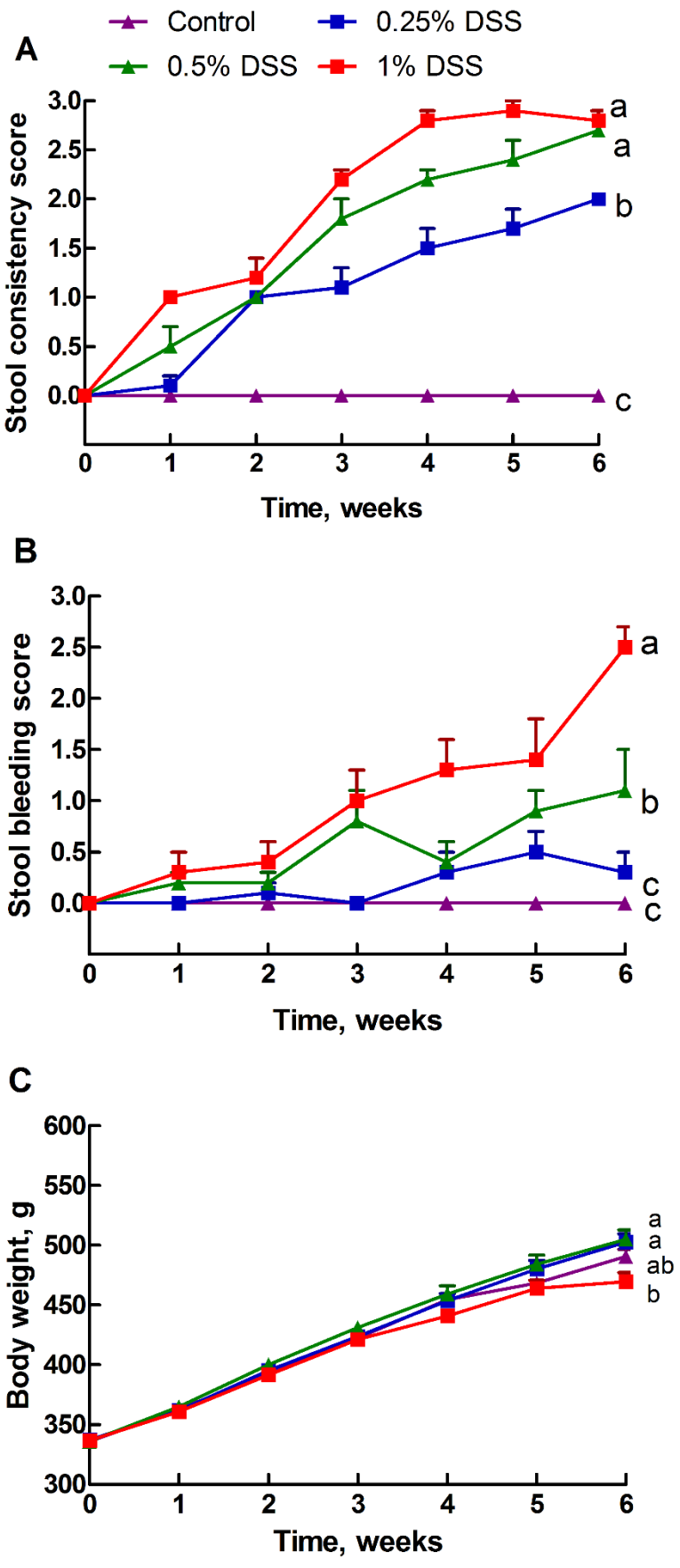
A
Control
0.25% DSS
0.5% DSS
1% DSS
Stool consistency score
3.0
2.5
2.0
1.5
1.0
0.5
0.0
0
1
2
3
4
5
6
Time, weeks
a
a
b
c
B
Stool bleeding score
3.0
2.5
2.0
1.5
1.0
0.5
0.0
0
1
2
3
4
5
6
Time, weeks
a
b
c
c
C
Body weight, g
600
550
500
450
400
350
300
0
1
2
3
4
5
6
Time, weeks
a
a
ab
b

Figure 2

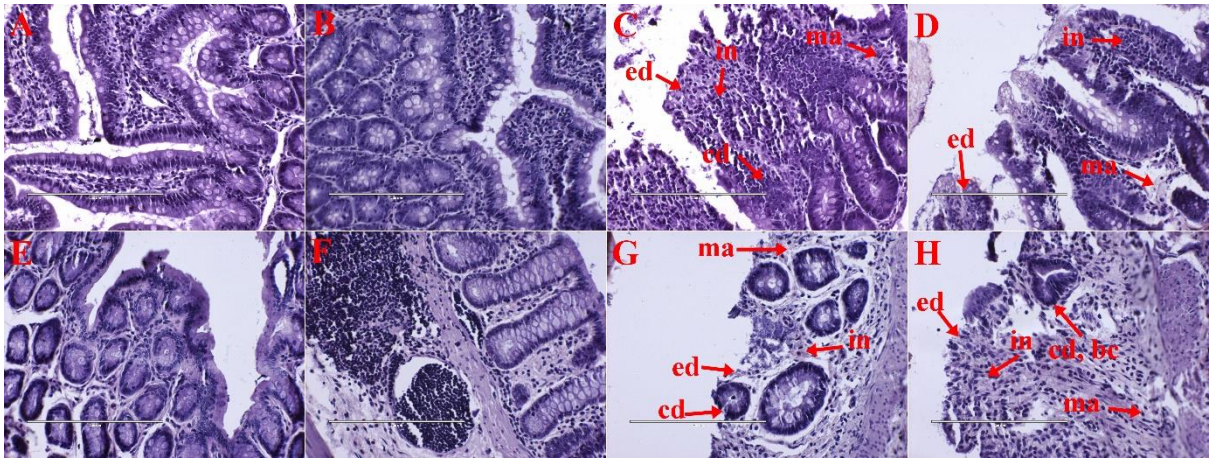

Figure 3

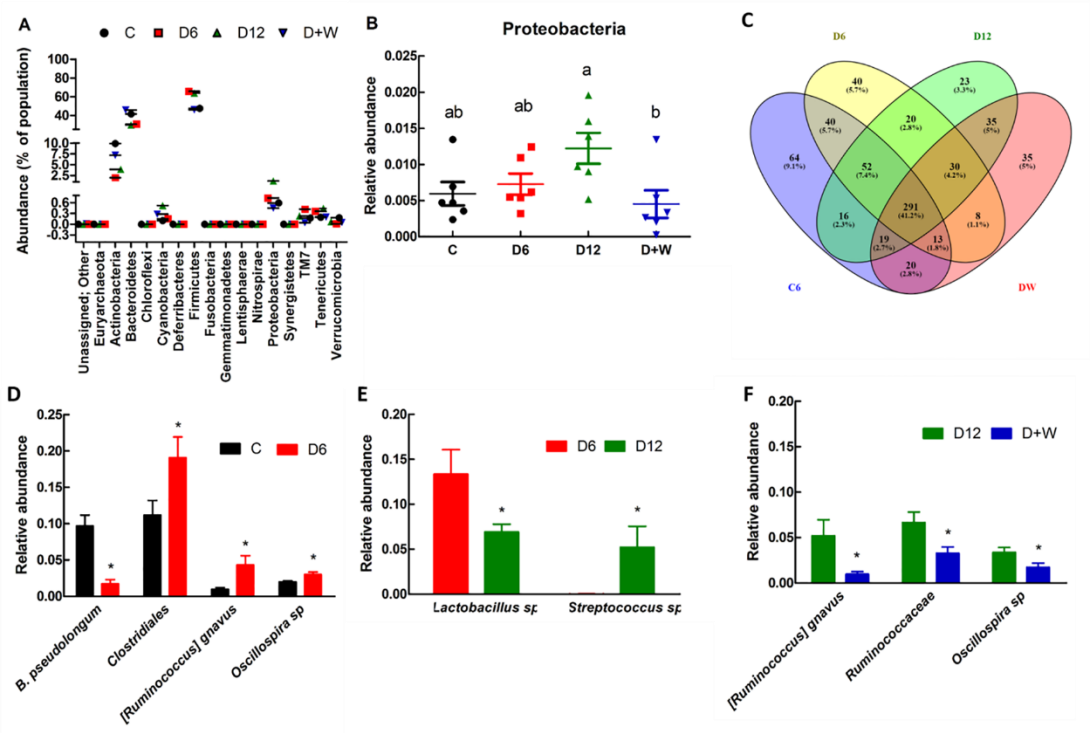

Figure 4

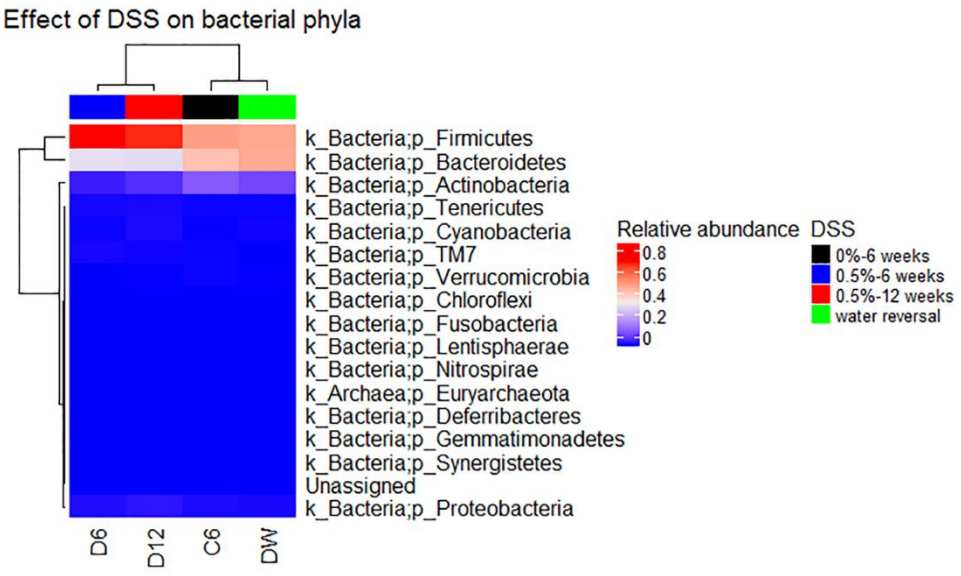

Figure 5

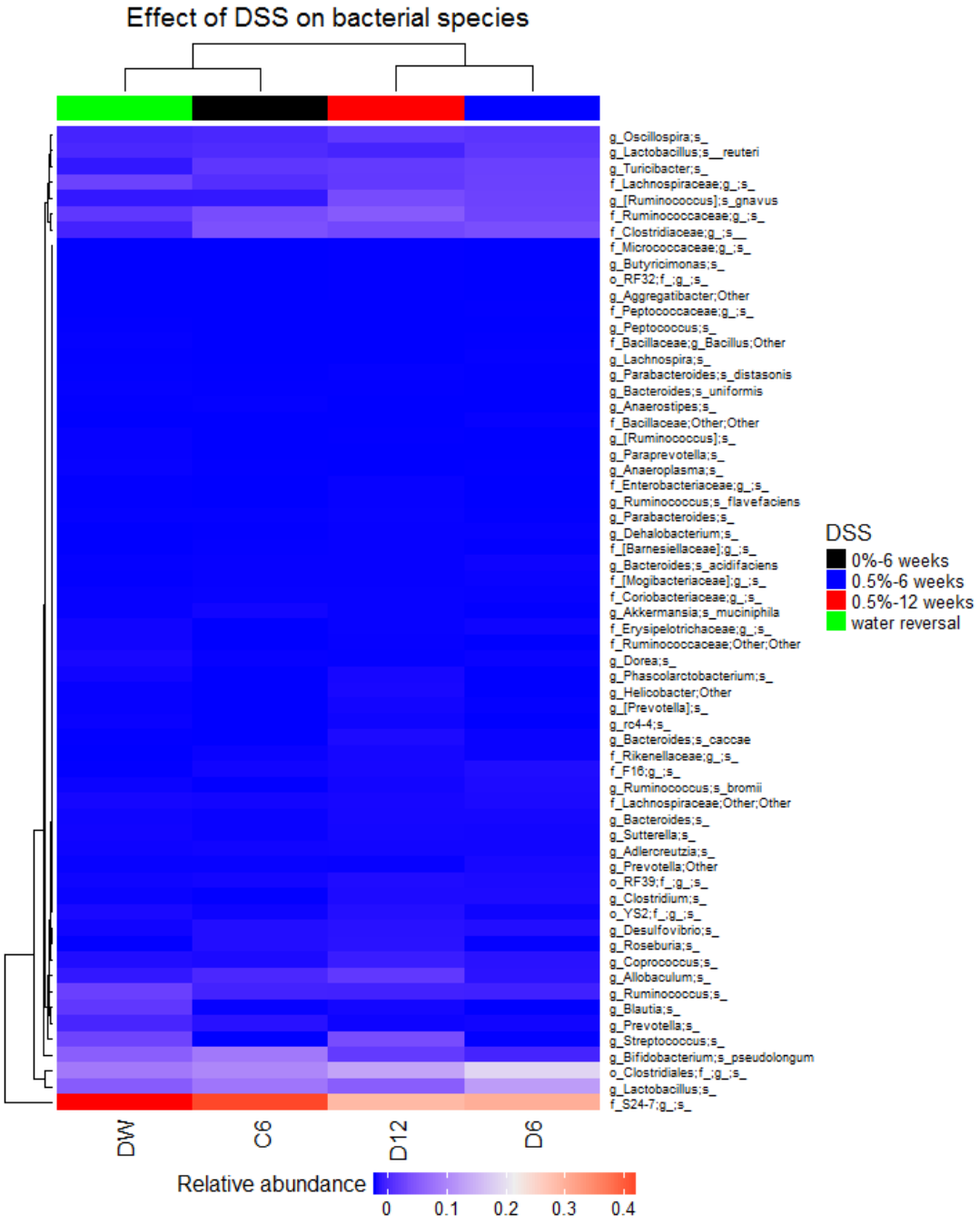
Effect of DSS on bacterial species
g_Oscillospira;s_
g_Lactobacillus;s__reuteri
g_Turicibacter;s_
f_Lachnospiraceae;g_;s_
g_[Ruminococcus];s_gnavus
f_Ruminococcaceae;g_;s_
f_Clostridiaceae;g_;s__
f_Micrococcaceae;g_;s_
g_Butyricimonas;s_
o_RF32;f_;g_;s_
g_Aggregatibacter;Other
f_Peptococcaceae;g_;s_
g_Peptococcus;s_
f_Bacillaceae;g_Bacillus;Other
g_Lachnospira;s_
g_Parabacteroides;s_distasonis
g_Bacteroides;s_uniformis
g_Anaerostipes;s_
f_Bacillaceae;Other;Other
g_[Ruminococcus];s_
g_Paraprevotella;s_
g_Anaeroplasma;s_
f_Enterobacteriaceae;g_;s_
g_Ruminococcus;s_flavefaciens
g_Parabacteroides;s_
g_Dehalobacterium;s_
f_[Barnesiellaceae];g_;s_
g_Bacteroides;s_acidifaciens
f_[Mogibacteriaceae];g_;s_
f_Coriobacteriaceae;g_;s_
g_Akkermansia;s_muciniphila
f_Erysipelotrichaceae;g_;s_
f_Ruminococcaceae;Other;Other
g_Dorea;s_
g_Phascolarctobacterium;s_
g_Helicobacter;Other
g_[Prevotella];s_
g_rc4-4;s_
g_Bacteroides;s_caccae
f_Rikenellaceae;g_;s_
f_F16;g_;s_
g_Ruminococcus;s_bromii
f_Lachnospiraceae;Other;Other
g_Bacteroides;s_
g_Sutterella;s_
g_Adlercreutzia;s_
g_Prevotella;Other
o_RF39;f_;g_;s_
g_Clostridium;s_
o_YS2;f_;g_;s_
g_Desulfovibrio;s_
g_Roseburia;s_
g_Coprococcus;s_
g_Allobaculum;s_
g_Ruminococcus;s_
g_Blautia;s_
g_Prevotella;s_
g_Streptococcus;s_
g_Bifidobacterium;s_pseudolongum
o_Clostridiales;f_;g_;s_
g_Lactobacillus;s_
f_S24-7;g_;s_
DW
C6
D12
D6
Relative abundance
0
0.1
0.2
0.3
0.4
DSS
0%-6 weeks
0.5%-6 weeks
0.5%-12 weeks
water reversal

Figure 6

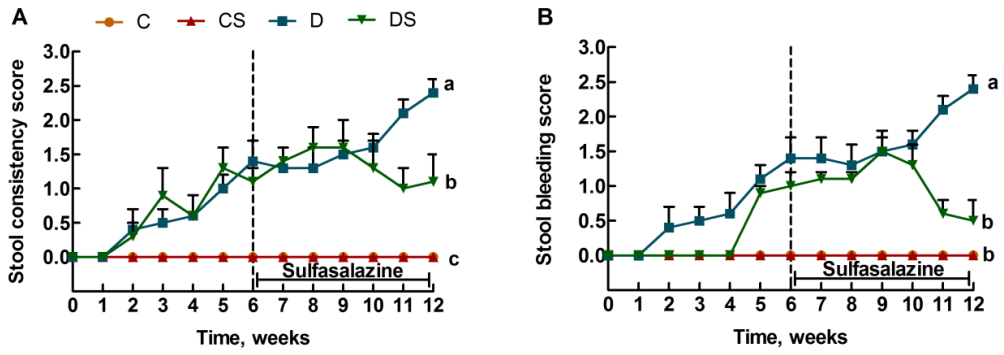

Figure 7

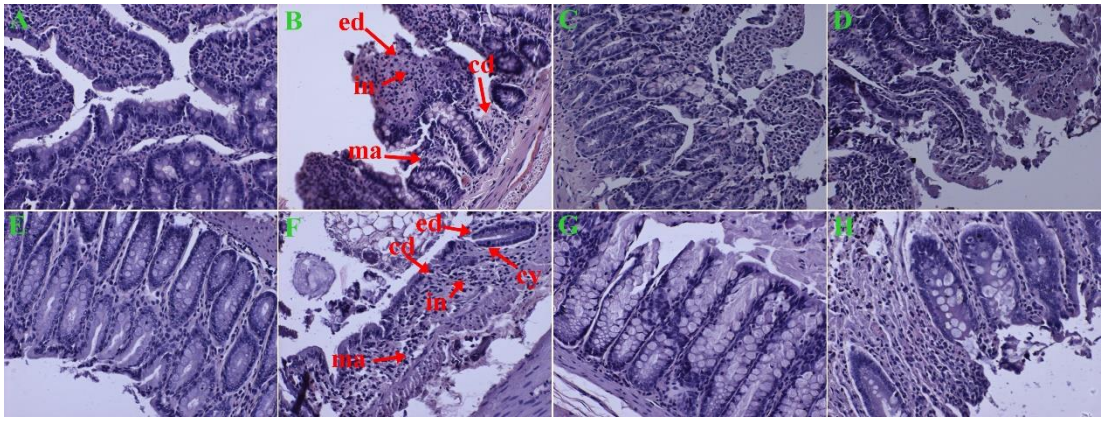

Figure 8

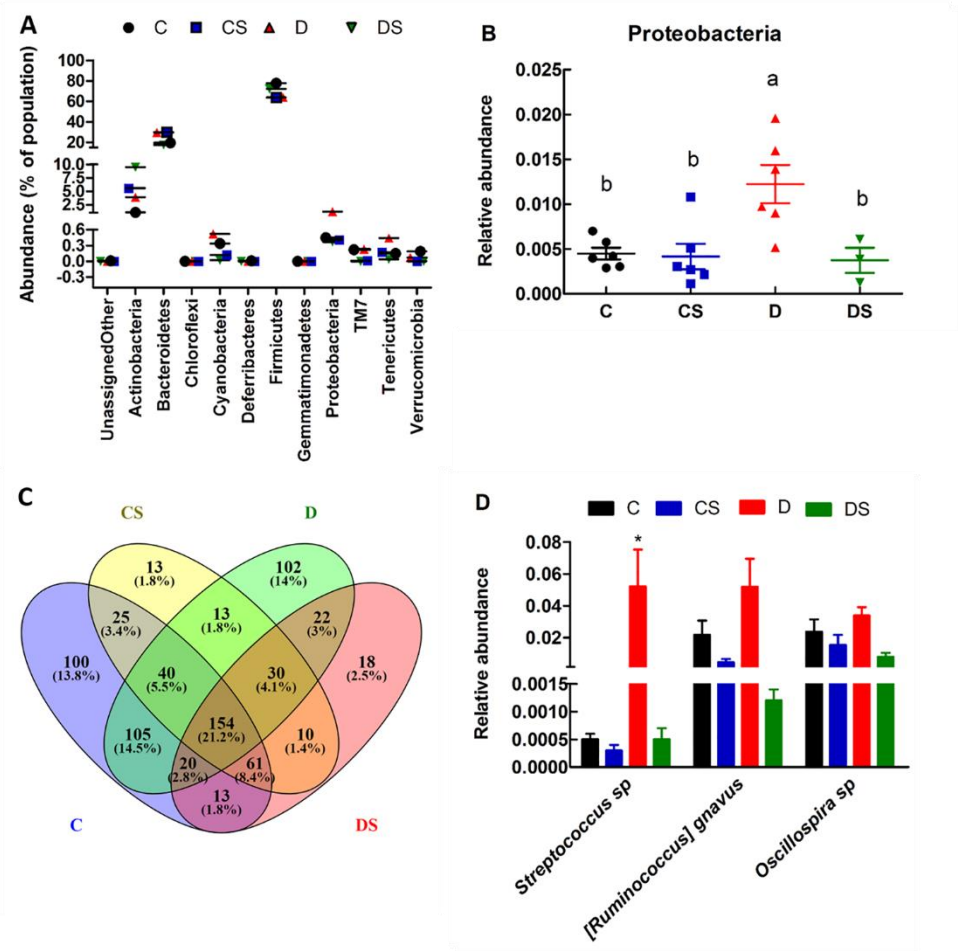

Figure 9

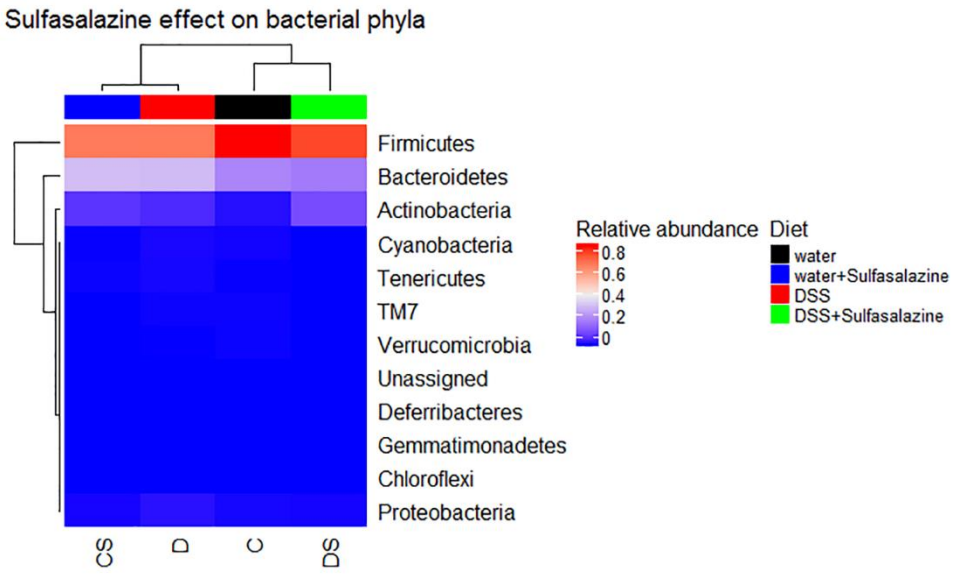

Figure 10

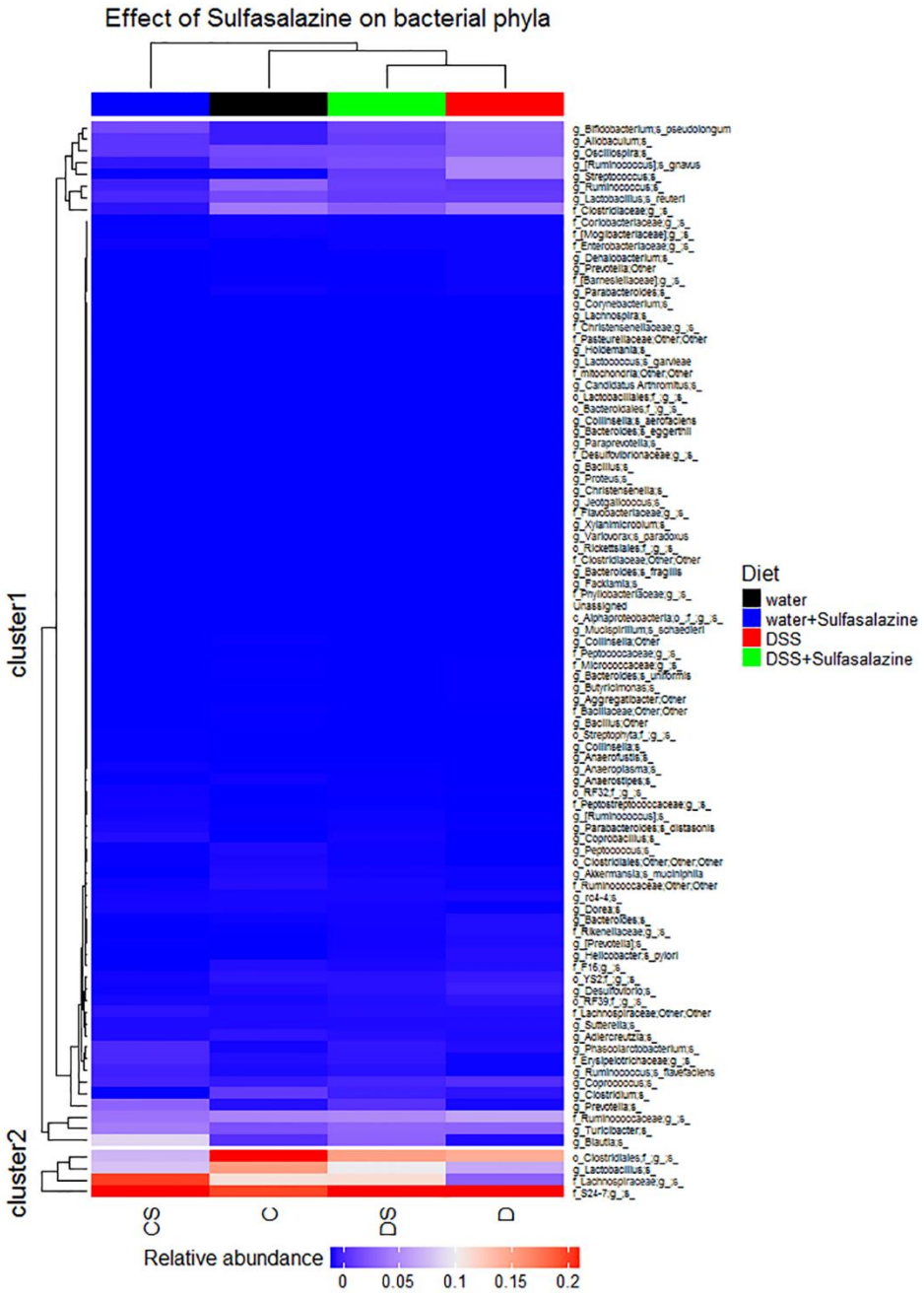
Effect of Sulfasalazine on bacterial phyla
cluster1
cluster2
CS
C
DS
D
Relative abundance
0
0.05
0.1
0.15
0.2
Diet
water
water+Sulfasalazine
DSS
DSS+Sulfasalazine

Table S1. *Dietary, metabolic, and organ weight parameters in rats treated with DSS (0%, 0.25%, 0.5%, or 1%) for 6 weeks*

Variables	***0% DSS***	***0.25% DSS***	***0.5% DSS***	***1% DSS***
Initial body weight, g	336 ± 1	337 ± 1	338 ± 2	337 ± 1
Final body weight, g	491 ± 6^{ab}	503 ± 8^{a}	505 ± 8^{a}	470 ± 8^{b}
Body weight gain, g	155 ± 6^{a}	166 ± 7^{a}	168 ± 8^{a}	133 ± 7^{b}
Food intake, g/d	31.9 ± 0.4^{b}	39.5 ± 1^{a}	39.5 ± 1.3^{a}	38.2 ± 1^{a}
Water intake, g/d	49.1 ± 3.2^{b}	53.7 ± 2.8^{ab}	64.1 ± 4.0^{a}	62.1 ± 4.8^{ab}
Energy intake, kJ/d	440 ± 5^{b}	544 ± 14^{a}	545 ± 18^{a}	528 ± 13^{a}
Feed efficiency, g/kJ	0.35 ± 0.01^{a}	0.31 ± 0.01^{a}	0.31 ± 0.02^{a}	0.25 ± 0.01^{b}
Abdominal circumference, cm	21.2 ± 0.2	21.1 ± 0.2	21.8 ± 0.4	21.3 ± 0.1
Retroperitoneal fat, mg/mm tibial length	229 ± 15	263 ± 25	293 ± 34	206 ± 16
Epididymal fat, mg/mm tibial length	157 ± 18	177 ± 19	205 ± 18	156 ± 14
Omental fat, mg/mm tibial length	190 ± 11	196 ± 14	206 ± 18	175 ± 12
Bone mineral density, g/cm²	0.168 ± 0.003	0.172 ± 0.003	0.165 ± 0.003	0.165 ± 0.003
Bone mineral content, g	12.2 ± 0.3	12.9 ± 0.6	12.4 ± 0.5	11.6 ± 0.3
Blood glucose 30 minutes, mmol/L	6.6 ± 0.2	7.7 ± 0.3	7.5 ± 0.3	7.2 ± 0.5
Blood glucose 120 minutes, mmol/L	5.5 ± 0.2	6.1 ± 0.3	6.1 ± 0.3	5.6 ± 0.2
Systolic blood pressure, mmHg	124 ± 3^{b}	126 ± 1^{b}	136 ± 3^{a}	133 ± 2^{ab}
LV + Septum wet weight, mg/mm tibial length	22.0 ± 0.8	22.7 ± 1.1	20.5 ± 3.5	21.9 ± 0.5
RV wet weight, mg/mm tibial length	4.75 ± 0.31	4.78 ± 0.35	4.62 ± 0.17	4.12 ± 0.14
Liver wet weight, mg/mm tibial length	352 ± 17	357 ± 22	390 ± 11	369 ± 8
Kidney wet weight, mg/mm tibial length	65.9 ± 2.3	65.5 ± 2.5	70.1 ± 1.8	68.9 ± 1.3
Spleen wet weight, mg/mm tibial length	23.9 ± 0.8^{b}	23.8 ± 1.0^{b}	25.0 ±1.1^{b}	30.0 ± 2.1^{a}

All values are mean ± SEM, n = 6-10. Mean values within a row with a different superscript are significantly different, $P<0.05$. DSS, dextran sodium sulfate; LV, left ventricle; RV, right ventricle.

Table S2. *Dietary, metabolic, cardiovascular, and liver parameters in rats treated with 0.5% DSS for either 6 weeks or 12 weeks*

Variables	***0.5% DSS for 6 weeks***	***0.5% DSS for 12 weeks***
Initial body weight, g	338 ± 2	340 ± 1
Body weight at 6 weeks, g	505 ± 8	503 ± 5
Body weight at 12 weeks, g	-	572 ± 9
Abdominal circumference, cm	21.8 ± 0.4	23.4 ± 0.3*
Abdominal fat pads, mg/mm tibial length	704 ± 69	513 ± 29*
Retroperitoneal fat, mg/mm tibial length	293 ± 34	225 ± 17
Epididymal fat, mg/mm tibial length	205 ± 18	142 ± 8*
Omental fat, mg/mm tibial length	206 ± 18	146 ± 11*
Total fat mass, g	152 ± 16	152 ± 8
Total lean mass, g	308 ± 16	394 ± 10*
Bone mineral density, g/cm²	0.165 ± 0.003	0.180 ± 0.003*
Bone mineral content, g	12.4 ± 0.5	14.6 ± 0.3*
Fasting blood glucose, mmol/L	4.3 ± 0.2	4.1 ± 0.1
Blood glucose at 30 minutes, mmol/L	7.5 ± 0.3	5.8 ± 0.2*
Blood glucose 120 minutes, mmol/L	6.1 ± 0.3	4.8 ± 0.2*
Area under the curve, mmol/L×minutes	810 ± 25	671 ± 20*
Plasma non-esterified fatty acids, mmol/L	3.3 ± 0.4	3.2 ± 0.2
Plasma triglycerides, mmol/L	1.2 ± 0.2	1.2 ± 0.1
Plasma total cholesterol, mmol/L	1.6 ± 0.1	1.7 ± 0.2
Systolic blood pressure, mmHg	136 ± 3	125 ± 1*
LV diastolic stiffness constant (κ)	22.8 ± 2.1	22.5 ± 1.0
LV + Septum wet weight, mg/mm tibial length	20.5 ± 3.5	24.2 ± 1.4
RV wet weight, mg/mm tibial length	4.62 ± 0.17	4.49 ± 0.30
Liver wet weight, mg/mm tibial length	390 ± 11	349 ± 11*
Plasma alanine transaminase activity, U/L	19.1 ± 1.9	24.0 ± 2.4
Kidney wet weight, mg/mm tibial length	70.1 ± 1.8	66.5 ± 2.1
Spleen wet weight, mg/mm tibial length	25.0 ± 1.1	24.4 ± 0.6
Small intestine length, cm	126 ± 3	120 ± 3
Colon length, cm	20.2 ± 0.5	17.3 ± 0.7*

All values are mean ± SEM, n = 6-10. Mean values within a row with a different superscript are significantly different, $P<0.05$. DSS, dextran sodium sulfate; LV, left ventricle; RV, right ventricle.

Table S3. *Dietary, metabolic, cardiovascular, liver, and gastrointestinal parameters in rats treated with 0.5% DSS or 0.5% DSS + water for 12 weeks*

Variables	***0.5% DSS for 12 weeks***	***DSS + water***
Body weight gain, g	233 ± 10	171 ± 8*
Feed efficiency, g/kJ	0.46 ± 0.02	0.34 ± 0.01*
Abdominal circumference, cm	23.4 ± 0.3	22.4 ± 0.3*
Abdominal fat pads, mg/mm tibial length	513 ± 29	576 ± 11
Retroperitoneal fat, mg/mm tibial length	225 ± 17	257 ± 7
Epididymal fat, mg/mm tibial length	142 ± 8	138 ± 4
Omental fat, mg/mm tibial length	146 ± 11	181 ± 5*
Total fat mass, g	152 ± 8	118 ± 6*
Total lean mass, g	394 ± 10	368 ± 10
Bone mineral density, g/cm²	0.180 ± 0.003	0.176 ± 0.002
Bone mineral content, g	14.6 ± 0.3	12.6 ± 0.3*
Fasting blood glucose, mmol/L	4.1 ± 0.1	4.1 ± 0.2
Blood glucose 30 minutes, mmol/L	5.8 ± 0.2	5.9 ± 0.2
Blood glucose 120 minutes, mmol/L	4.8 ± 0.2	5.3 ± 0.1*
Area under the curve, mmol/L×minutes	671 ± 20	697 ± 16
Plasma non-esterified fatty acids, mmol/L	3.2 ± 0.2	2.6 ± 0.2
Plasma triglycerides, mmol/L	1.2 ± 0.1	0.7 ± 0.1*
Plasma total cholesterol, mmol/L	1.7 ± 0.2	1.6 ± 0.1
Systolic blood pressure, mmHg	125 ± 1	126 ± 2
LV diastolic stiffness constant (κ)	22.5 ± 1.0	25.8 ± 1.1*
LV + Septum wet weight, mg/mm tibial length	24.2 ± 1.4	22.8 ± 0.8
RV wet weight, mg/mm tibial length	4.49 ± 0.3	4.44 ± 0.22
Liver wet weight, mg/mm tibial length	349 ± 11	336 ± 14
Plasma alanine transaminase activity, U/L	24.0 ± 2.4	40.8 ± 1.2*
Kidney wet weight, mg/mm tibial length	66.5 ± 2.1	66.5 ± 1.9
Spleen wet weight, mg/mm tibial length	24.4 ± 0.6	24.0 ± 0.7
Small intestine length, cm	120 ± 3	122 ± 2
Colon length, cm	17.3 ± 0.7	23.2 ± 0.5*

All values are mean ± SEM, n = 7-10. Mean values within a row with a different superscript are significantly different, $P<0.05$. DSS, dextran sodium sulfate; LV, left ventricle; RV, right ventricle; DSS + water, dextran sodium sulfate + water; DSS + water group had replacement of DSS with water at 6 weeks.

Table S4. *Physical, dietary, metabolic, and organ weight parameters in rats treated with sulfasalazine*

Variables	***C***	***CS***	***D***	***DS***	***P-Value***		
					DSS	***Sulfasalazine***	***Interaction***
Body weight gain, g*	92 ± 7.9	68.1 ± 6.4	88.9 ± 5.7	71.8 ± 5.5	0.96	0.004	0.60
Energy intake, kJ/d*	511 ± 30	534 ± 34	503 ± 36	542 ± 54	1.00	0.44	0.84
Feed efficiency, g/kJ*	0.180 ± 0.014	0.131 ± 0.014	0.183 ± 0.017	0.138 ± 0.013	0.73	0.003	0.89
Blood glucose 30 minutes, mmol/L	6.8 ± 0.2	6.2 ± 0.1	6.6 ± 0.2	6.8 ± 0.5	0.50	0.50	0.18
Blood glucose 120 minutes, mmol/L	5.4 ± 0.1	5.2 ± 0.3	5.7 ± 0.2	5.8 ± 0.2	0.043	0.82	0.49
Retroperitoneal fat, mg/mm tibial length	398 ± 25^{a}	243 ± 33^{b}	349 ± 25^{a}	268 ± 29^{b}	0.67	0.0002	0.19
Epididymal fat, mg/mm tibial length	260 ± 22^{a}	157 ± 25^{b}	209 ± 18^{ab}	214 ± 14^{ab}	0.90	0.037	0.023
Omental fat, mg/mm tibial length	284 ± 23^{a}	168 ± 17^{b}	216 ± 13^{b}	193 ± 23^{b}	0.28	0.001	0.024
LV + Septum, mg/mm tibial length	23.5 ± 0.9	21.7 ± 0.8	22.4 ± 0.7	21.4 ± 0.7	0.38	0.08	0.61
RV, mg/mm tibial length	6.53 ± 0.61^{a}	4.53 ± 0.3^{b}	5.56 ± 0.41^{ab}	4.74 ± 0.42^{b}	0.41	0.004	0.20
Liver, mg/mm tibial length	403 ± 19	347 ± 17	379 ± 12	347 ± 7	0.42	0.005	0.42
Kidney, mg/mm tibial length	71.4 ± 1.4	67.4 ± 1.9	73.2 ± 2.6	70.6 ± 1.1	0.19	0.08	0.71
Spleen, mg/mm tibial length	27.3 ± 1.3^{a}	22.0 ± 1.4^{b}	25.5 ± 1.3^{ab}	24.2 ± 0.7^{ab}	0.87	0.011	0.11

All values are mean ± SEM, n = 6-8. Mean values within a row with a different superscript are significantly different, $P<0.05$. *Mean values for the last 6 weeks of treatment; C, control (0% DSS); D, 0.5% DSS; CS, C + sulfasalazine; DS, D + sulfasalazine; DSS, dextran sodium sulfate; LV, left ventricle; RV, right ventricle.

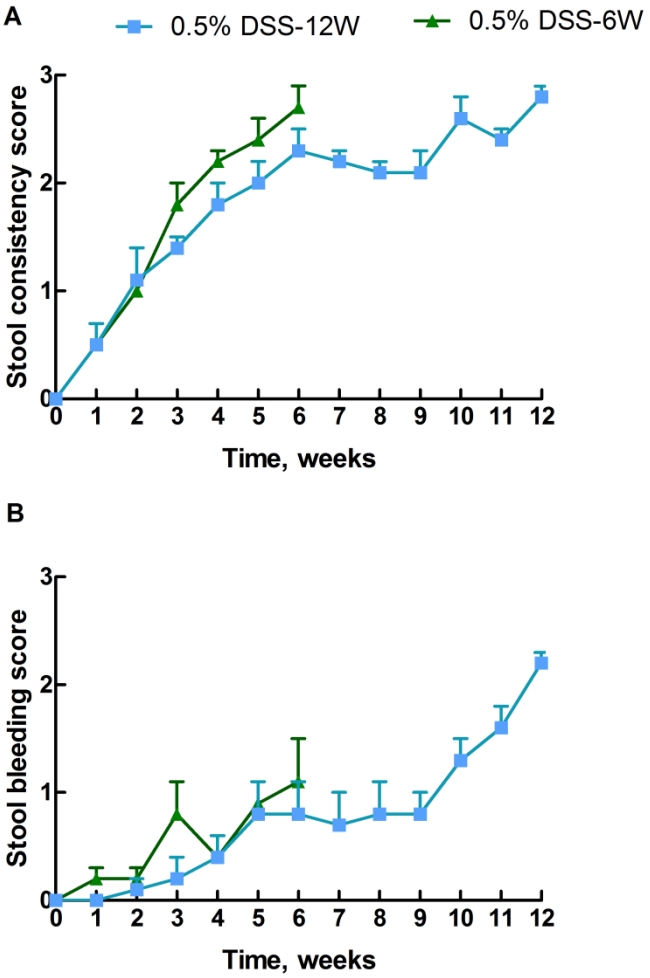

Fig. S1. *Effect of DSS on stool consistency (A) and stool bleeding (B) on rats given DSS for six weeks (0.5% DSS-6W) and for twelve weeks (0.5% DSS-12W). Values are mean ± SEM, n =10-12. Endpoint means with an asterisk differ, P < 0.05. DSS, dextran sodium sulfate.*

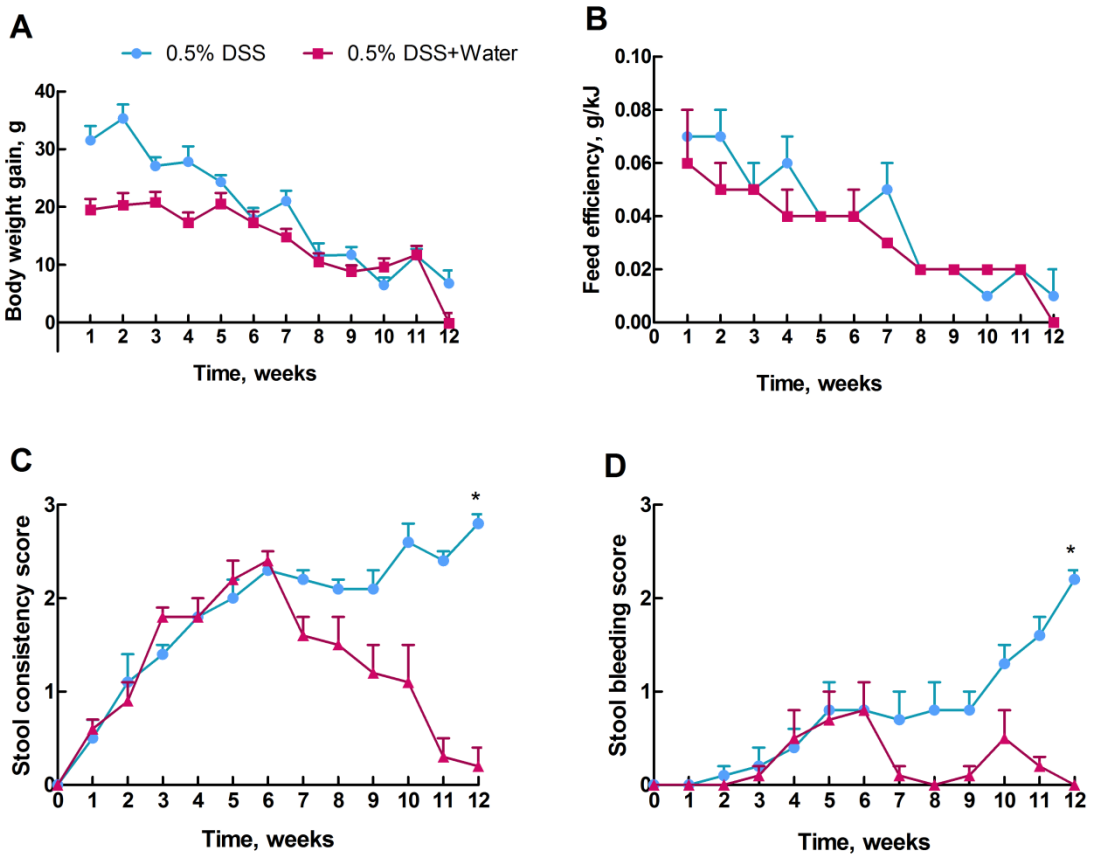

Fig. S2. *Effect of DSS on body weight gain (A), feed efficiency (B), stool consistency (C), and stool bleeding (D) on rats given 0.5% DSS or DSS + water for twelve weeks. DSS + water group had replacement of DSS with water at 6 weeks. Values are mean ± SEM, n = 12. Endpoint means with an asterisk differ, P<0.05. DSS, dextran sodium sulfate. Stool consistency score: 0-formed, 1-mild-soft, 2-very soft, 3-watery soft (diarrhea). Stool bleeding score: 0-normal color, 1-brown color, 2-reddish color, 3-bloody red.*

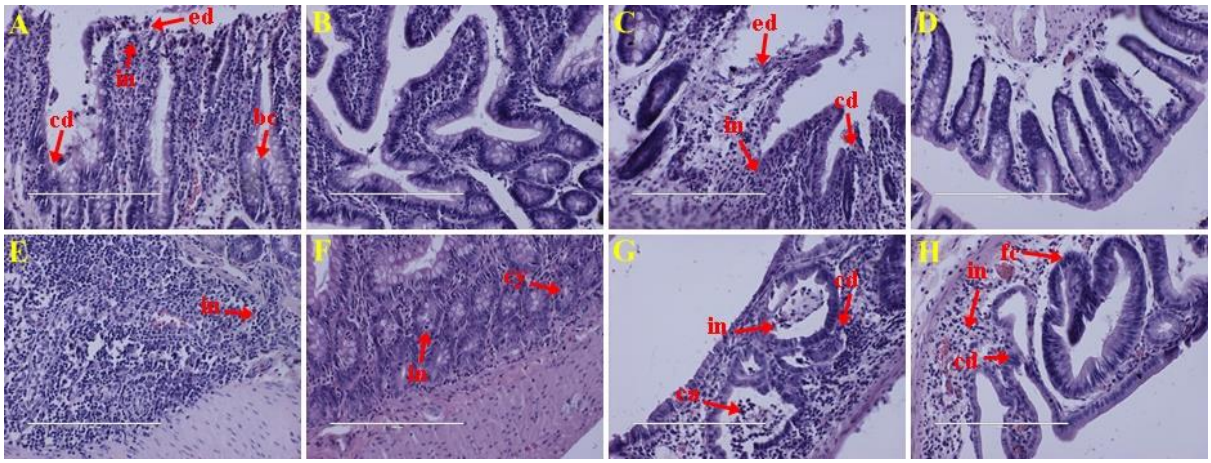

Fig. S3. *Effect of DSS on inflammation in the intestine. Hematoxylin and eosin staining of ileum and distal colon showing infiltration of inflammatory cells "in" (A, C, E, F, G, and H), epithelial disruption "ed" (A and C), crypt distortion "cd" (A, C, G, and H), cryptitis "cy" (F), crypt abscess "ca" (G), branched crypt "bc" (A), forked crypt "fc" (H), and mucosal atrophy (A, C, E, G, and H) (×20). Ileum of rats treated with 0.5% DSS water for 12 weeks (A, E, and F) and ileum of rats treated with 0.5% DSS water for 6 weeks and normal water for next 6 weeks (B). Colon of rats treated with 0.5% DSS water for 12 weeks (C, G, and H) and colon of rats treated with 0.5% DSS water for 6 weeks and normal water for next 6 weeks (D). DSS, dextran sodium sulfate. Intestinal inflammation shows signs of Crohn's disease in ileum with cryptitis and also signs of ulcerative colitis in colon with the crypt abscess.*

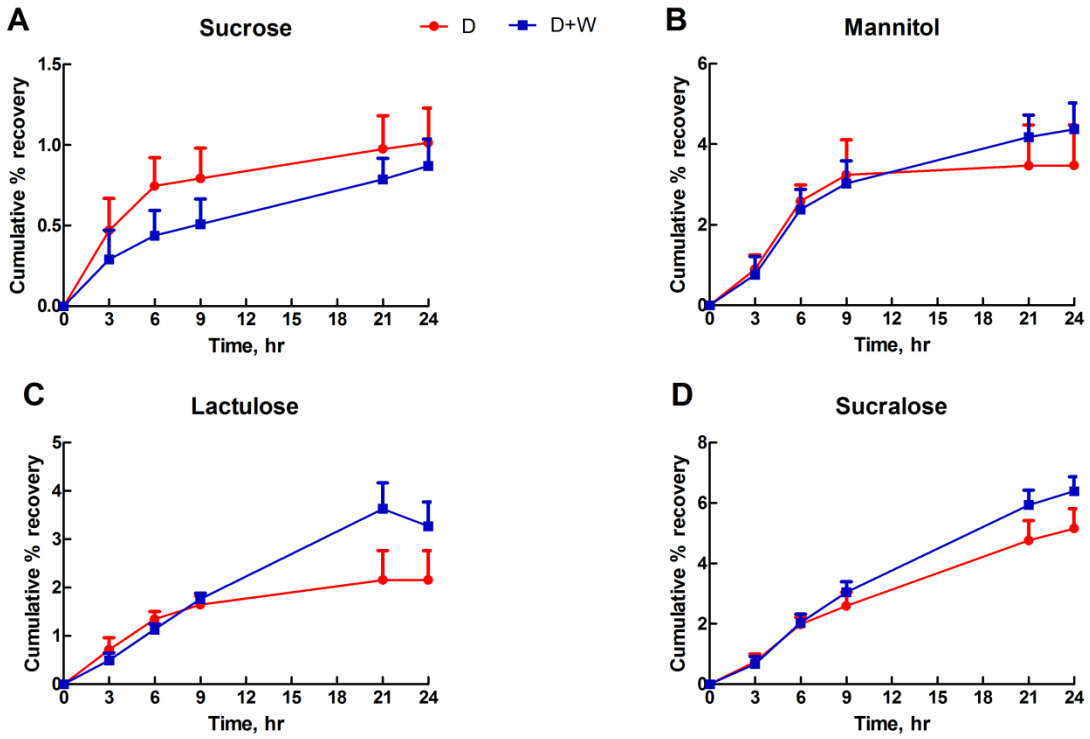

Fig. S4. *Effect of DSS on intestinal permeability in D and D+W rats. Cumulative percent recovery of sucrose (A), mannitol (B), lactulose (C), and sucralose (D) urinary excretion over 24 hours. DSS, dextran sodium sulfate; D, rats given 0.5% DSS for twelve weeks; D+W, 0.5% DSS for first 6 weeks followed by normal water for last 6 weeks.*

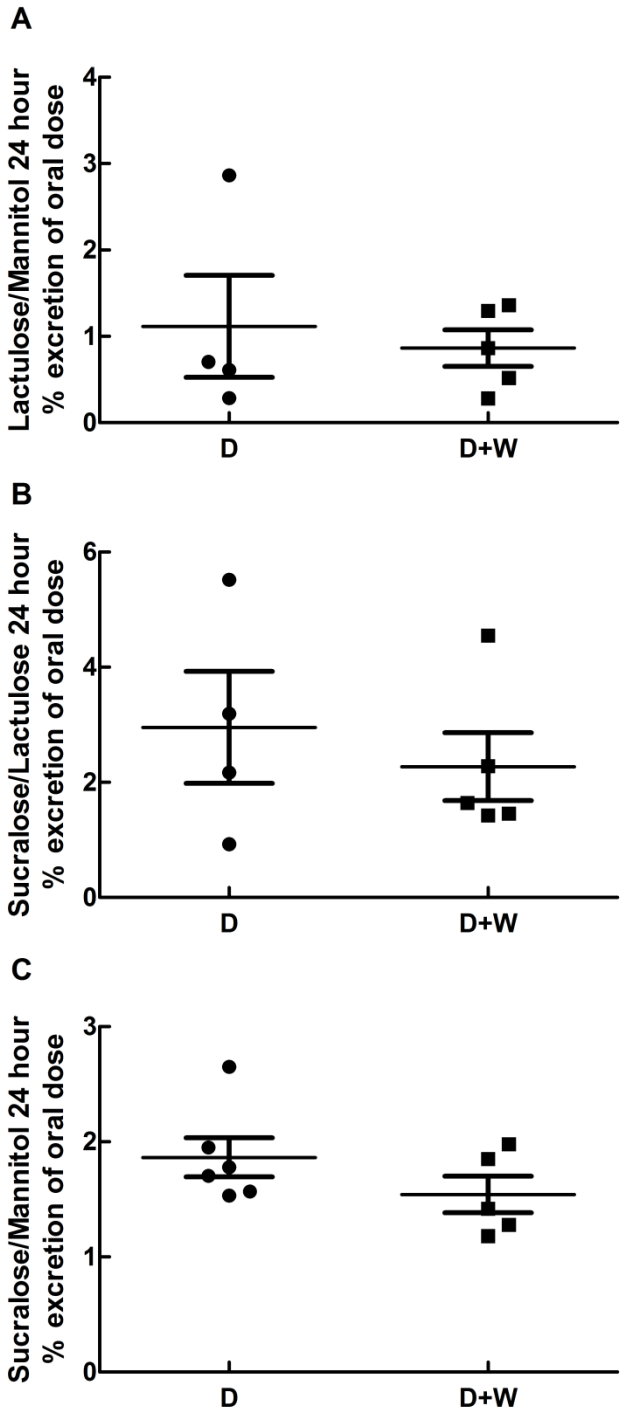

Fig. S5. *Effect of DSS on intestinal permeability in D and D+W rats. Scattered plot of 24 hour lactulose/mannitol ratio shows small intestinal permeability whereas sucralose/lactulose ratio and sucralose/mannitol ratio shows whole gut permeability. Values are mean ± SEM, n = 4-6. Endpoint means with an asterisk differ, P < 0.05. DSS, dextran sodium sulfate; D, rats given 0.5% DSS for twelve weeks; D+W, 0.5% DSS for first 6 weeks followed by normal water for last 6 weeks.*

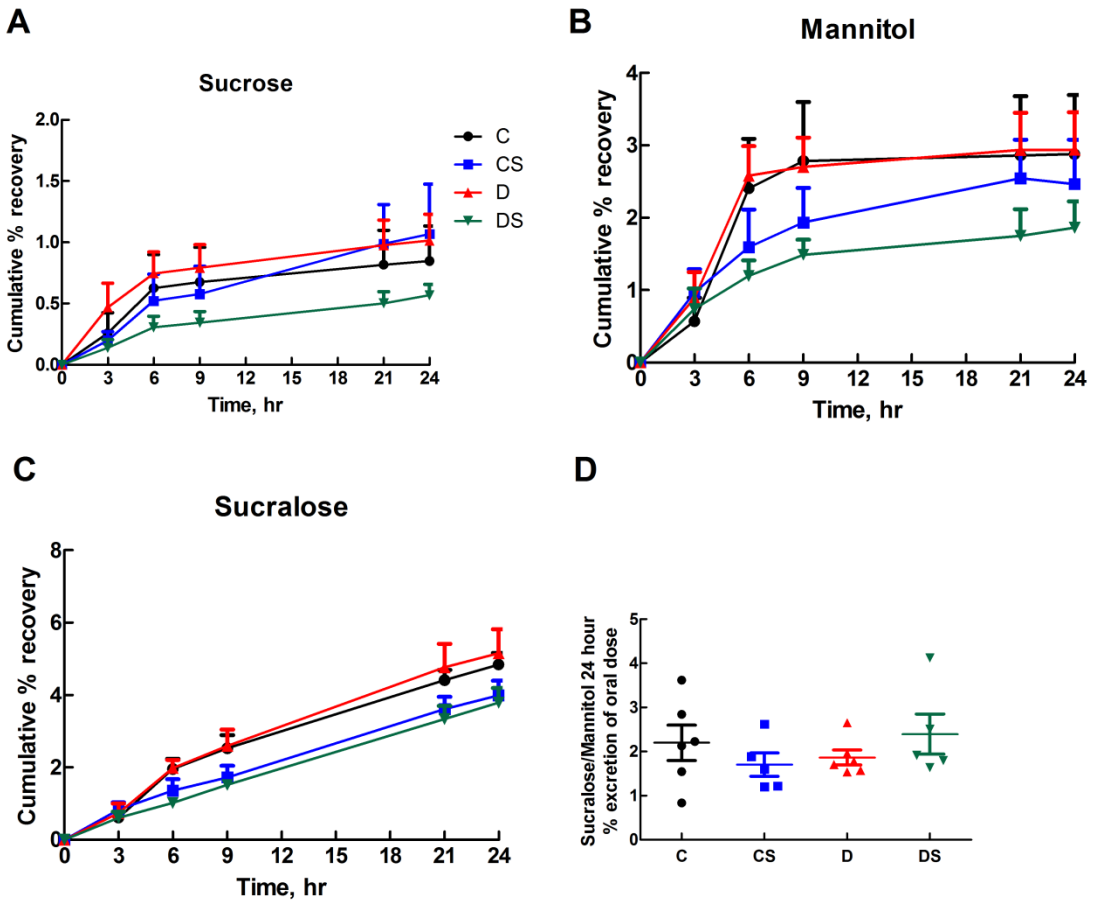

Fig. S6. *Effect of sulfasalazine on intestinal permeability in rats. 0.5% DSS was given for 12 weeks to D and DS rats. Cumulative percent recovery of sucrose (A), mannitol (B), and sucralose (C) urinary excretion over 24 hours; scattered plot of 24 hour sucralose/mannitol ratio for whole gut permeability (D). DSS, dextran sodium sulfate; D, rats given 0.5% DSS for twelve weeks; DS, 0.5% DSS for 12 weeks and last 6 weeks with sulfasalazine, C, rats given normal water for twelve weeks; CS, normal water for 12 weeks and last 6 weeks with sulfasalazine.*

Chapter 4. Cyanidin 3-glucoside attenuates inflammatory bowel disease in rats

Cyanidin 3-glucoside attenuates inflammatory bowel disease in rats

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Abstract

Inflammatory bowel disease (IBD) is a chronic gastrointestinal disease mainly affecting the ileum and colon. Anthocyanins may improve other chronic inflammatory states such as metabolic syndrome to reduce signs of the disease. Food sources of anthocyanins such as cyanidin 3-glucoside (C3G) include Queen Garnet plums (P) and purple carrots (PC). This study has investigated whether the chronic gastrointestinal changes in IBD induced in rats by dextran sodium sulphate (DSS) can be ameliorated by Queen Garnet plum juice, purple carrot juice or pure C3G powder. Rats were randomly divided into eight groups and were given powdered food with either normal water (C) or 0.5% DSS (D) in drinking water for 12 weeks to induce IBD. Queen Garnet plum juice, purple carrot juice or pure C3G powder at 8 mg C3G/kg body weight/day were added to the diet for the final 6 weeks to give CP, DP, CPC, DPC, CC and DC rats. No symptoms of IBD were observed in C, CP, CPC and CC rats. D rats had bloody diarrhoea and erosion of inner gut lining evident from crypt atrophy, and mucosal inflammatory cell infiltration. DP, DPC and DC rats had improved stool characteristics (stool consistency: C 0.0±0.0, D 2.4±0.4, DP 0.9±0.3, DPC 0.8±0.4, DC 0.6±0.4; stool bleeding: C 0.0±0.0, D 2.4±0.2, DP 0.4±0.2, DPC 0.6±0.4, DC 0.5±0.3), reduced ileum and colon inflammation. Dysbiosis was observed in D rats and C3G improved the gut homeostasis. Thus, supplementation of the diet with C3G-containing foods may ameliorate the symptoms of IBD by improving the gut microbiome.

Keywords: Inflammatory bowel disease, dextran sodium sulphate, cyanidin 3-glucoside, Queen Garnet plum, purple carrot, inflammation

1. Introduction

Inflammatory Bowel Disease (IBD) is characterized by chronic inflammation of all or part of the digestive tract (1). The gut microbiota is crucial to maintain gut health with a pivotal role in the metabolism of carbohydrates, amino acids, lipids, nucleotides and xenobiotics (2). IBD is aggravated due to dysbiosis which is one of the many interplaying factors in IBD including mucosal barrier disruption, immune dysregulation, host genetics and the environment (3). Functional foods may assist in reducing chronic inflammatory diseases such as obesity (4) and are now of increasing interest in the treatment of IBD (5). Currently, IBD patients rely on conventional drug therapy that may have adverse reactions. However, complementary options such as foods could improve control of the disease with fewer adverse effects and reduce cost of treatment but not as a sole therapy, despite favorable response seen in the DSS-model (6). Purple foods obtain their strong colour from natural anthocyanins, including cyanidin 3-O-β-D-glucoside (C3G), which have potential therapeutic responses in IBD due to their antioxidant and anti-inflammatory actions especially in intestinal cells, and also by modulation of the gut bacteria (7, 8). We have selected the C3G-containing purple foods, Queen Garnet plums and purple carrots, for comparison with pure C3G in this investigation.

The Queen Garnet plum (*Prunus salicina* Lindl.) is a Japanese variety developed by breeding techniques conducted by the Department of Primary Industry, Queensland Government, Australia (9). These plums contain C3G up to 272mg/100g of fresh fruit, around seven times higher than other anthocyanin-containing plums and berries (10). Our group reported that Queen Garnet plum juice given to diet-induced obese rats at 8mgC3G/kg bw/day reversed the symptoms of metabolic syndrome including inflammation of heart and liver (11). Our earlier studies on purple carrots (*Daucus carota* subsp *sativus*), another source of C3G, showed improved structural and functional changes of the heart and liver due to its anti-inflammatory action in rats with diet-induced metabolic syndrome as a model of chronic low-grade inflammation (12).

We hypothesize that purple foods with increased C3G can attenuate intestinal inflammation in a rat model of DSS-induced chronic IBD. We analysed the structure and function of the intestine including the gut bacteria from faecal samples following

treatment with either Queen Garnet plum juice, purple carrot juice or pure C3G powder given as food additives using a reversal protocol in these rats.

2. Materials and Methods

2.1 Diets and measurements in living rats

All experiments were approved by the University of Southern Queensland Animal Ethics Committee (approval no 14REA005) under the guidelines of the National Health and Medical Research Council of Australia. Male Wistar rats (8-9 weeks old, weighing 335.1 ± 0.4 g, n = 64) were purchased from the Animal Resource Centre, Perth. All rats were provided with free access to food and water and were individually housed in temperature-controlled (20 ± 2°C), 12-hour light-dark conditions at the University of Southern Queensland animal house.

The rat diet consisted of powdered rat food (Specialty Feeds, Glen Forest, WA, Australia). The rats were randomly divided into eight experimental groups (n=8 each). One group served as control (C) with normal drinking water and another group (D) with 0.5% dextran sodium sulphate (DSS, molecular weight: 36,000-50,000 Da, MP Biomedicals) in drinking water for 12 weeks of the protocol. Six groups of rats were fed with interventions in their diet for the last 6 weeks. Three groups of rats with normal drinking water were fed with Queen Garnet plum (CP) (65 ml/kg food with C3G 1.9 mg/ml of juice), purple carrot (CPC) (11.3 g/kg food with C3G 10.9 g/kg of juice) and cyanidin 3-glucoside (CC) (123 mg/kg food) and similarly three groups of rats with 0.5% DSS drinking water were fed with these interventions as DP, DPC and DC. The Queen Garnet purple plum juice was a gift from Nutrafruit, Australia. Purple carrot juice was a gift from Dr Red Nutraceuticals, Australia. Pure C3G was a gift from Biosynth AS, Sandnes, Norway.

Energy intake was calculated from the following values: powdered rat food, 13.8 kJ/g; purple plum juice, 0.243 kJ/g, purple carrot juice, 0.44 kJ/g. The energy density was calculated for powdered food diet, 13.8 kJ/g; purple plum juice diet, 14.043 kJ/g; purple carrot juice diet, 14.24 kJ/g. Rats were monitored daily for body weight, and food and water intakes. Daily anthocyanin intake was calculated from the daily food intake.

Rats were food-deprived for 12 hours and oral glucose tolerance tests were performed using Medisense Precision Q.I.D glucose meter (Abbott Laboratories, Bedford, USA) at 0, 6 and 12 weeks (13).

Stool characteristics were examined to assess the disease activity every day for 12 weeks. The stool consistency was scored as 0-formed, 1-mild-soft, 2-very soft, 3-watery soft (diarrhoea). The stool bleeding was scored as 0-normal colour, 1-brown colour, 2-reddish colour, 3-bloody red (14).

A cocktail of sucrose, mannitol, lactulose and sucralose (Sigma-Aldrich Australia, Sydney, Australia) was used to test the intestine permeability of the rats from their urine samples collected at various time points after 12 weeks of the study as mentioned in chapter 3.

Gastric transit was performed by charcoal method for the Queen Garnet plum study as described in chapter 3. The purple carrot and C3G groups had gastric emptying and gastric transit experiments performed by phenol red method. After 12 hours starvation, each rat was gavaged 3 mL of 0.05% phenol red solution (Sigma-Aldrich, Australia). After 20 min, the rat was euthanized and the stomach was ligated on the pyloric and cardiac ends and removed. The stomach was placed in 100 mL 0.1 M NaOH and was homogenized in a tissue homogenizer for 2 min at moderate speed. The homogenized mixture was kept aside for 1 hour. To 5 mL of the supernatant 1 mL of 33% trichloroacetic acid was added and centrifuged at 2500g for 20 min. The supernatant was mixed with 0.5M NaOH and the absorbance was recorded at 565 nm to determine the intensity of the colour that correlated to the intensity of the compound that remained in the stomach (15). Gastric emptying (%) = (1 − absorbance of test sample/absorbance of baseline control) × 100%.

The total intestine length was recorded and it was cut along its length longitudinally. Few drops of 0.5M NaOH were dropped on the lumen of the intestine from duodenum to ileum and the end point of the change to the pink colour was recorded. The length of intestine with pink colour divided by the total small intestine length was expressed as percentage of gastric transit (15).

2.2 Measurements after euthanasia

The rats were induced with terminal anaesthesia via i.p. injection of pentobarbitone sodium (Lethabarb, 100 mg/kg,Virbac, Milperra, Australia). Heparin

(200 IU; Sigma-Aldrich Australia, Sydney, Australia) was administered into the right femoral vein. The rats were dissected and the organs were excised (13).

The isolation and the preparation of the small intestine and large intestine to obtain distal ileum and distal colon (~1.5 cm) for histological examination and organ bath studies were performed as described in chapter 3.

As in chapter 3, after the blood collection from the abdominal aorta, the left ventricle along with septum and the right ventricle, kidney, spleen, liver and abdominal fat pads were collected, blotted dry and weighed from each rat. The organ weights were normalized to the tibial length at the time of organ isolation and expressed as mg/mm (13).

Examination of rat urine samples for sugars was performed at the Central Analytical Research Facility (CARF) of the Queensland University of Technology, Gardens Point, Brisbane, with the assistance of Dr Rajesh Gupta. The GCMS method as described in chapter 3 was used for the sugar analysis. The rat faecal samples collected at euthanasia were stored at -80 °C and later examined for gut microbiota diversity profiling at Australian Genomic Research Facility (AGRF), Adelaide, Australia, where the gDNA extraction of faecal samples and sequencing were performed (16). Venn diagrams for observed taxonomical units (OTUs) indicating richness of species, in not less than 50% of the samples of each group were prepared using the online plotting tool Venny 2.1.0 (http://bioinfogp.cnb.csic.es/tools/venny/index.html). Complex heatmaps of the bacterial phyla and species greater than 1% in abundance were generated by R software using R/bioconductor package (17).

2.3 Statistics

All data were expressed as mean ± SEM. Results from all the groups C, CP, CPC, CC, D, DP, DPC and DC were analysed for variance by using 1-way and 2-way ANOVA. The data were tested with Neumann-Keuls multiple comparison *post hoc* test and $P<0.05$ was considered as significant. The statistical analyses were run by using GraphPad Prism version 6 for Windows (GraphPad Software, San Diego, CA, USA) (13).

3. Results

3.1 Effects of Queen Garnet Plum

The stool consistency and stool bleeding scores of C and CP rats were minimal whereas D rats had increased stool consistency and bleeding which was reversed in DP rats indicating positive outcome of the intervention (Figure 1A & B). The small intestinal and colon lengths, and gastric transit of CP rats was unchanged to C rats (Table 1). These parameters of DP rats did not differ to D rats. The histology of ileum and colon of C and CP rats were normal (Fig. 2A & C; Fig. 3A & C). The ileum of D rats showed inflammation with inflammatory cells, epithelial disruption, crypt distortion and mucosal atrophy (Fig. 2B). The ileum of the DP rats showed regeneration of crypt and epithelial membrane with fewer inflammatory cells compared to D rats (Fig. 2D). The colon of D rats showed inflammation with inflammatory cells, epithelial disruption, crypt distortion, crypt atrophy and mucosal atrophy (Fig. 3B). The colon of DP rats improved when compared to the D rats with reduced inflammation, healthy crypts and mucosa (Fig. 3D). The forces of contraction of the isolated ileum and colon preparations in response to acetylcholine were not different among the C and CP rats (Ileum-C=27.3 ± 4.9, CP=39.1 ± 1.6; colon- C=64.9 ± 8.8, CP=61.9 ± 5.9). Similarly, D and DP rats did not differ in the ileum and colon force of contractions due to acetylcholine (Ileum-D=19.9 ± 4.1, DP=32.4 ± 4.6; colon-D=68.2 ± 7.8, DP=80.2 ± 12.3). The cumulative percent urinary recovery of sucrose and lactulose did not alter among all groups of rats at all time intervals (Fig 4A & B). The cumulative percent urinary recovery of mannitol and sucralose increased at 21 h and 24 h in the treatment groups of CP and DP rats (Fig 4C & D). The lactulose/mannitol ratio, an indicator of small intestine permeability did not alter among all groups whereas sucralose/mannitol ratio an indicator of whole gut permeability was decreased by the treatment in CP and DP rats compared to C and D rats, respectively (Fig 4E).

The final body weight of CP rats was lower than C however energy intake and feed efficiency did not differ (Supplementary table 1). D and DP rats did not show any difference in final body weight, energy intake and feed efficiency (Supplementary table 1). The anthocyanin intake of DP rats was higher than CP rats (Table 1). The quercetin intake of CP and DP rats was 1.41 ± 0.05 and 1.64 ± 0.12 (mg/kg/day)

respectively. Fasting blood glucose and AUC were similar among all the groups (Table 1). Total abdominal fat pads reduced in CP rats compared to C rats but not among D and DP rats (Table 1). However, retroperitoneal, epididymal and omental fat pads did not differ among C and CP rats or D and DP rats (Supplementary table 1). The wet weights of liver, left ventricle with septum, and right ventricle reduced in CP rats compared to C rats whereas the wet weights of kidney and spleen were similar in both groups (Supplementary table 1). The wet weights of left ventricle with septum, right ventricle, liver, kidney and spleen were similar in D and DP rats (Supplementary table 1).

3.2 Effects of purple carrot

The stool consistency and bleeding scores were unchanged among CPC and C rats whereas D rats had increased stool consistency and bleeding which was reversed in DPC rats (Fig. 1A & B). No change was observed in the small intestinal and colon lengths among C and CPC or D and DPC rats (Table 1). The gastric transit times and emptying were unchanged in C and CPC or D and DPC rats (Table 1). The histology of ileum and colon of C and CPC rats showed healthy mucosa (Fig. 2A & E; Fig. 3A & E). The ileum of D rats showed inflammation with inflammatory cells, epithelial disruption, crypt distortion and mucosal atrophy (Fig. 2B). The ileum of the DPC rats showed increased villi length and epithelial membrane with lesser inflammatory cells compared to D rats (Fig. 2F). The colon of D rats showed inflammation with inflammatory cells, epithelial disruption, crypt distortion, crypt atrophy and mucosal atrophy (Fig. 3B). The colon of the DPC rats improved with lesser inflammation, increased crypt number and depth compared to the D rats (Fig. 3F). The maximal forces of contraction of the isolated ileum and colon preparations in response to acetylcholine were not different in the C and CPC rats (ileum-C=27.3 ± 4.9, CPC= 16.7 ± 3.5; colon- C=64.9 ± 8.8, CPC=41.4 ± 10.3 mN). Similarly, D and DPC rats did not differ in the ileum and colon maximal force of contractions due to acetylcholine (ileum-D=19.9 ± 4.1, DPC= 16.0 ± 4.2; colon- D=68.2 ± 7.8, DPC= 58.4 ± 8.6 mN). The cumulative percent recovery of sucrose and lactulose did not differ among all groups of rats at all time intervals (Fig 4A & B). CPC and DPC rats had increased cumulative percent recovery of mannitol and sucralose increased at 21 h and 24 h (Fig 4C & D). The lactulose/mannitol ratio, an indicator of small intestine permeability and

the sucralose/lactulose or sucralose/mannitol ratios, indicators of whole gut permeability, did not alter among all groups (Fig 4E).

The final body weight, energy intake and feed efficiency of C and CPC rats were similar (Supplementary table 1). D rats and DPC rats did not differ in final body weight, energy intake and feed efficiency (Supplementary table 1). The anthocyanin intake of CPC and DPC rats were similar (Table 1). Fasting blood glucose and AUC were unchanged among all the groups (Table 1). Total abdominal fat pads including retroperitoneal, epididymal and omental fat pads did not differ among C and CPC rats or D and DPC rats (Table 1; Supplementary table 1). The wet weights of liver and right ventricle were reduced in CPC rats compared to C rats whereas the wet weights of left ventricle with septum, kidney and spleen were similar in both groups (Supplementary table 1). The wet weight of liver decreased in DP rats but the wet weights of left ventricle with septum, right ventricle, kidney and spleen were similar in D and DPC rats (Supplementary table 1).

3.3 Effects of cyanidin 3-glucoside (C3G)

The stool consistency and bleeding scores were unchanged in C and CC rats whereas D rats had increased stool consistency and bleeding which was reversed with C3G treatment in DC rats similar to treatments of Queen Garnet plum and purple carrot compared to D rats (Fig. 1A & B). The lengths of small intestine and colon were similar among C and CC or D and DC rats (Table 1), though 2-way analysis of variance indicated that treatment improved colon length. The gastric transit times and gastric emptying were similar in C and CC rats or D and DC rats (Table 1). The histology of ileum and colon of C and CC rats had normal mucosa (Fig. 2A & G; Fig. 3A & G). The ileum of D rats showed inflammation with inflammatory cells, epithelial disruption, crypt distortion and mucosal atrophy (Fig. 2B). In DC rats, C3G restored the villi length, epithelial membrane with lesser degree of inflammatory cells compared to D rats (Fig. 2H). The colon of D rats showed inflammation with inflammatory cells, epithelial disruption, crypt distortion, crypt atrophy and mucosal atrophy (Fig. 3B). Similarly, C3G also improved the colon of the DC rats with reduced inflammation, increased crypt number and depth compared to the D rats which had severely inflamed colons with crypt and mucosal atrophy (Fig. 3H). The maximal forces of contraction of the isolated ileum and colon preparations in response to

acetylcholine were not different among the C and CC rats (ileum-C=27.3 ± 4.9, CC= 13.1 ± 3.0; colon- C=64.9 ± 8.8, CC=43.5 ± 6.0 mN). Similarly, D and DC rats did not differ in the ileum and colon maximal force of contractions due to acetylcholine (Ileum-D=19.9 ± 4.1, DC= 14.0 ± 3.0; colon- D=68.2 ± 7.8, DC= 37.3 ± 5.8 mN). The cumulative percent recovery of sucrose and lactulose did not differ among all groups of rats at all time intervals (Fig 4A & B). The cumulative percent recovery of mannitol and sucralose increased at 21 h and 24 h in CC and DC rats compared to C and D rats, respectively (Fig 4C & D). The lactulose/mannitol ratio, an indicator of small intestine permeability and the sucralose/lactulose or sucralose/mannitol ratios, indicators of whole gut permeability, did not alter among all the groups (Fig 4E). Table 2 summarizes the intestinal effects of the interventions.

The gut bacterial diversity is shown in Fig. 5. The abundance of different phyla did not differ except for the increase in Cyanobacteria and Proteobacteria in DC rats compared to C, CC and D rats (Fig. 5A & B). The ratio of Firmicutes and Bacteroidetes and the Shannon diversity index, an indicator of alpha diversity did not differ among the groups (F/B ratio: C 4.96±1.45, CC 6.97±0.68, D 5.78±0.61, DC 3.89±0.40; Shannon diversity index (H): C 2.3±0.1, CC 2.4±0.1, D 2.4±0.1, DC 2.6±0.1). The observed taxonomical units (OTUs) representing number of species exclusively in C and CC rats were higher than D and DC rats (C-96, CC-110, D-51, DC-17), however 239 (26.8%) OTUs were common among all groups which constituted the maximum OTUs among the combinations of rat groups in Venn diagram (Fig. 5C). The relative abundances of order YS2 was higher in DC rats compared to C and CC rats; family Peptostreptococcacea was absent in C group and present in remaining groups; and genus *Sutteralla* was higher in DC rats (Fig. 5D). The heatmaps of species abundance clustered C and DC rats together, however CC and D rats were closer in the abundance of phyla (Fig. 6). C3G-treated DC and CC rats were clustered together and were closer to C rats than to D rats (Fig. 7). The cluster 1 of species showed increased abundance of , genus Allobaculum and order YS2 in DC rats compared to D rats whereas the cluster 2 showed C3G-treated rats had decreased abundance of family Lachnospiraceae and increased abundance of family S24-7 (Fig 8). Table 3 summarizes the metagenomics effects of C3G.

There was no difference in the final body weight, energy intake and feed efficiency in C and CC rats (Supplementary table 1). Similarly, D and DC rats did not

differ in final body weight, energy intake and feed efficiency (Supplementary table 1). The C3G intake of DC and CC rats were similar (Table 1). Fasting blood glucose and AUC were unchanged among all groups (Table 1). Total abdominal fat pads including retroperitoneal, epididymal and omental fat pads didn't differ among CC and C rats or DC and D rats, though 2-way analysis of variance indicated treatments reduced abdominal fat pads including retroperitoneal fat (Table 1; Supplementary table 1). The wet weights of right ventricle and liver reduced in CC rats compared to C rats whereas left ventricle with septum, kidney and spleen were similar in both groups (Supplementary table 1). D and DC rats had similar wet weights of left ventricle with septum, right ventricle, kidney and spleen but decreased wet weight of liver in DC rats (Supplementary table 1).

Table 1. Physical, dietary, metabolic, and gastrointestinal parameters in rats treated with Queen Garnet plum, purple carrot and C3G.

Variable	C	CP	CPC	CC	D	DP	DPC	DC	*P*-Value		
									DSS	Treatment	Interaction
Fasting blood glucose, mmol/L	4.2 ± 0.1	4.3 ± 0.1	4.1 ± 0.1	4.1 ± 0.2	4.5 ± 0.1	4.1 ± 0.2	4.0 ± 0.2	4.3 ± 0.2	0.6540	0.3116	0.3367
Blood glucose AUC, mmol/L/120 min	727 ± 18	768 ± 30	680 ± 32	702 ± 25	740 ± 9	730 ± 28	689 ± 28	709 ± 34	0.9057	0.0863	0.7525
Abdominal fat pads, mg/mm tibial length	942 ± 66^{a}	656 ± 46^{ab}	759 ± 52^{ab}	768 ± 70^{ab}	775 ± 44^{ab}	711 ± 56^{ab}	661 ± 74^{ab}	743 ± 86^{ab}	0.1944	0.0396	0.3415
Small intestinal length, cm	114 ± 3	114 ± 7	120 ± 1	121 ± 2	120 ± 2	120 ± 3	118 ± 2	119 ± 4	0.4236	0.7781	0.4639
Colon length, cm	22.6 ± 1.4^{a}	21.6 ± 1.3^{a}	20.9 ± 0.7^{ab}	22.3 ± 0.6^{a}	18.9 ± 0.9^{ab}	19.8 ± 0.6^{ab}	17.3 ± 0.9^{b}	19.2 ± 0.7^{ab}	<0.0001	0.2203	0.7327
Gastric transit (charcoal), %	76.8 ± 5.2	84.4 ± 3.0	-	-	86.6 ± 3.2	86.0 ± 3.2	-	-	0.3522	0.1382	0.2782
Gastric transit (phenol red), %	71.4 ± 3.9	-	70.9 ± 3.0	74.0 ± 4.9	69.6 ± 2.5	-	75.6 ± 1.4	74.2 ± 4.7	0.7242	0.5766	0.6491

Gastric emptying, %	46.8 ± 5.9	-	48.5 ± 6.7	50.8 ± 7.2	56.7 ± 6.6	-	51.0 ± 7.7	49.8 ± 8.2	0.4177	0.8922	0.7405
Anthocyanin intake, mg/kg bw/day	-	8.92 ± 0.32^{b}	8.33 ± 0.51^{b}	7.43 ± 0.36^{b}	-	10.33 ± 0.78^{a}	8.62 ± 0.34^{b}	7.83 ± 0.19^{b}	0.0658	0.0004	0.4171

All values are mean ± SEM, n = 4-8. Mean values within a row with a different superscript are significantly different, $P<0.05$. DSS, dextran sodium sulphate; C, normal water; CP, normal water + Queen Garnet plum; CPC, normal water + purple carrot; CC, Normal water + C3G; D, 0.5% DSS in drinking water; DP, 0.5% DSS in drinking water + Queen Garnet plum; DPC, 0.5% DSS in drinking water + purple carrot; DC, 0.5% DSS in drinking water + C3G; AUC, area under curve.

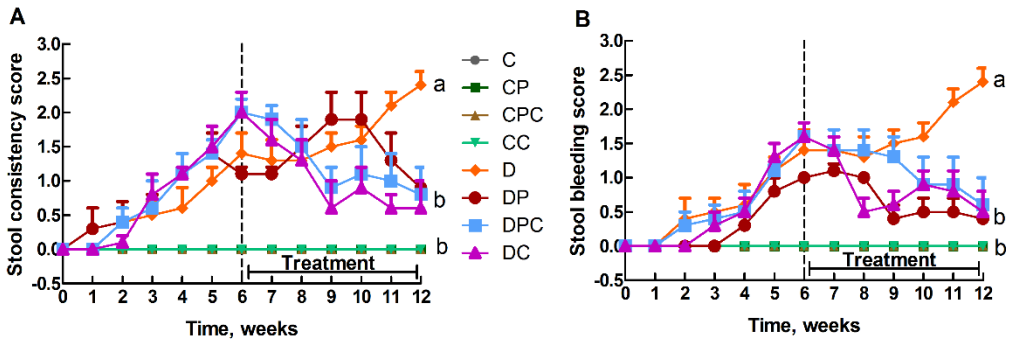

Fig.1. Effect of Queen Garnet plum, purple carrot and C3G on stool consistency (A) and stool bleeding (B) in rats given 0.5% DSS water (DP, DPC, DC) or normal water (CP, CPC, CC) in comparison to control (C) and 0.5% DSS (D) rats for twelve weeks. Values are mean ± SEM, n =8. Endpoint means with a different alphabet differ, $p < 0.05$. DSS, dextran sodium sulphate; C, normal water; CP, normal water + Queen Garnet plum; CPC, normal water + purple carrot; CC, normal water + cyanidin 3-glucoside (C3G); D, 0.5% DSS in drinking water; DP, 0.5% DSS in drinking water + Queen Garnet plum; DPC, 0.5% DSS in drinking water + purple carrot; DC, 0.5% DSS in drinking water + C3G. Stool consistency score: 0-formed, 1-mild-soft, 2-very soft, 3-watery soft (diarrhoea). Stool bleeding score: 0-normal colour, 1-brown colour, 2-reddish colour, 3-bloody red.

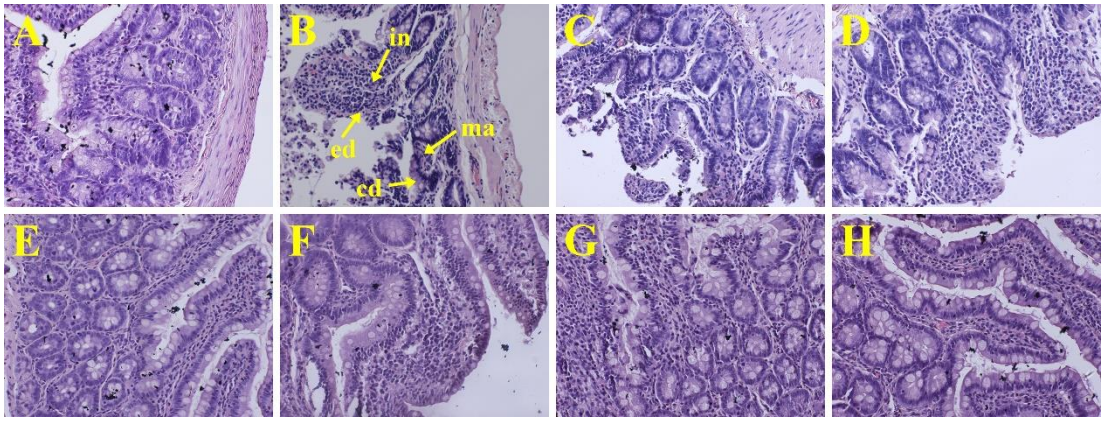

Fig. 2. Effect of anthocyanin on inflammation in the ileum. Haematoxylin and eosin staining of ileum showing infiltration of inflammatory cells “in” (B), epithelial disruption “ed” (B), crypt distortion “cd” (B), and mucosal atrophy “ma” (B) (×20).

Ileum of rats (n = 4) treated with normal water, 0.5% DSS water for 12 weeks (A and B); rats treated with normal water and 0.5% DSS water for 12 weeks with Queen Garnet plum juice (8mgC3G/kg BW/d) in the food for last 6 weeks (C and D), with purple carrot juice (8mgC3G/kg BW/d) in the food for last 6 weeks (E and F), with pure C3G powder (8mgC3G/kg BW/d) in the food for last 6 weeks (G and H). DSS, dextran sodium sulphate; C3G, cyanidin 3-glucoside. Intestinal inflammation in B shows signs of inflammatory bowel disease with epithelial disruption, crypt distortion and mucosal atrophy.

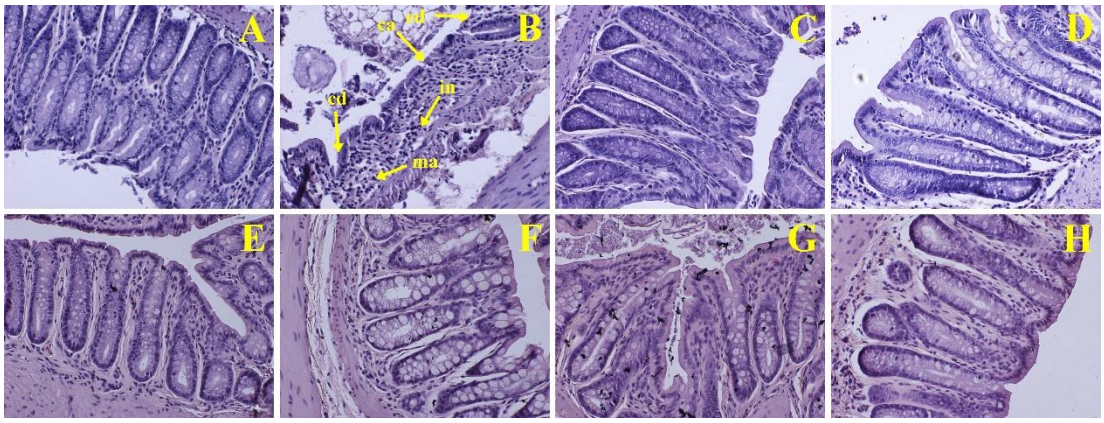

Fig. 3. Effect of anthocyanin on inflammation in the distal colon. Haematoxylin and eosin staining of distal colon showing infiltration of inflammatory cells “in” (B), epithelial disruption “ed” (B), crypt distortion “cd” (B), crypt atrophy “ca” (B) and mucosal atrophy “ma” (B) (×20). Colon of rats (n = 4) treated with normal water, 0.5% DSS water for 12 weeks (A and B); rats treated with normal water and 0.5% DSS water for 12 weeks with Queen Garnet plum juice (8mgC3G/kg BW/d) in the food for last 6 weeks (C and D), with purple carrot juice (8mgC3G/kg BW/d) in the food for last 6 weeks (E and F), with pure C3G powder (8mgC3G/kg BW/d) in the food for last 6 weeks (G and H). DSS, dextran sodium sulphate; C3G, cyanidin 3-glucoside. Intestinal inflammation in B shows signs of inflammatory bowel disease with epithelial disruption, crypt distortion, crypt atrophy and mucosal atrophy.

Table 2. Intestinal effects of Queen Garnet plum juice, purple carrot juice and C3G.

	DSS	Queen Garnet Plum juice	Purple carrot juice	C3G

Stool consistency	↑	↓	↓	↓
Stool bleeding	↑	↓	↓	↓
Inflammation	↑	↓	↓	↓
Epithelial disruption	↑	↓	↓	↓
Crypt distortion	↑	↓	↓	↓
Mucosal atrophy	↑	↓	↓	↓

DSS, dextran sodium sulphate; C3G, cyanidin 3-glucoside

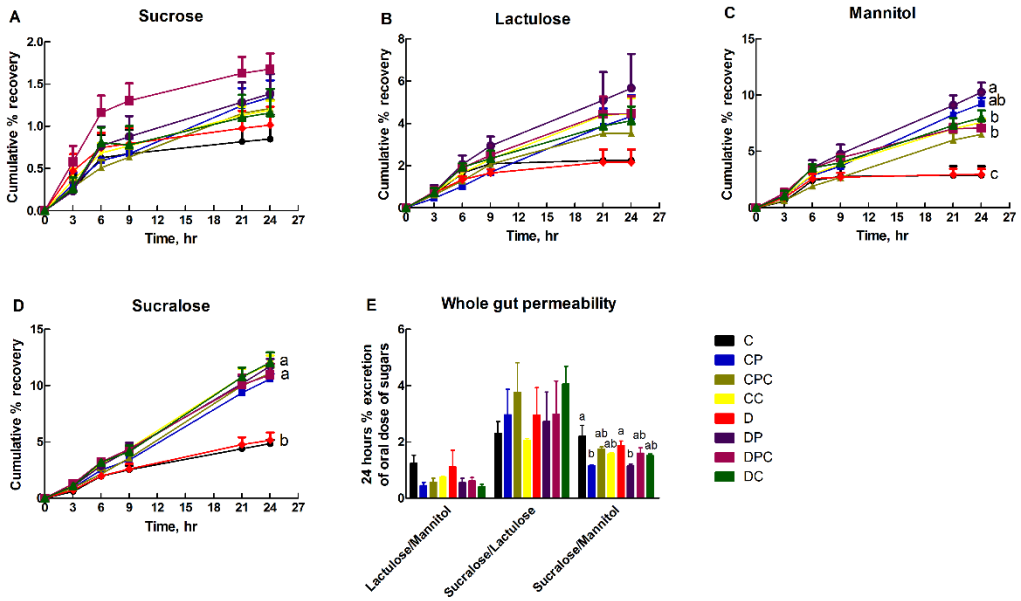

Fig. 4. Effect of Queen Garnet plum, purple carrot and C3G on the cumulative percent recovery of sucrose (A), lactulose (B), mannitol (C), sucralose (D) and whole gut permeability (E) over 24 h percent excretion of sugar probes in rats given 0.5% DSS water (DP, DPC, DC) or normal water (CP, CPC, CC) in comparison to control (C) and 0.5% DSS (D) rats for twelve weeks. Values are mean ± SEM, n =6. Endpoint means with a different alphabet differ, $p < 0.05$. DSS, dextran sodium sulphate; C, normal water; CP, normal water + Queen Garnet plum; CPC, normal water + purple carrot; CC, normal water + cyanidin 3-glucoside (C3G); D, 0.5% DSS in drinking

water; DP, 0.5% DSS in drinking water + Queen Garnet plum; DPC, 0.5% DSS in drinking water + purple carrot; DC, 0.5% DSS in drinking water + C3G.

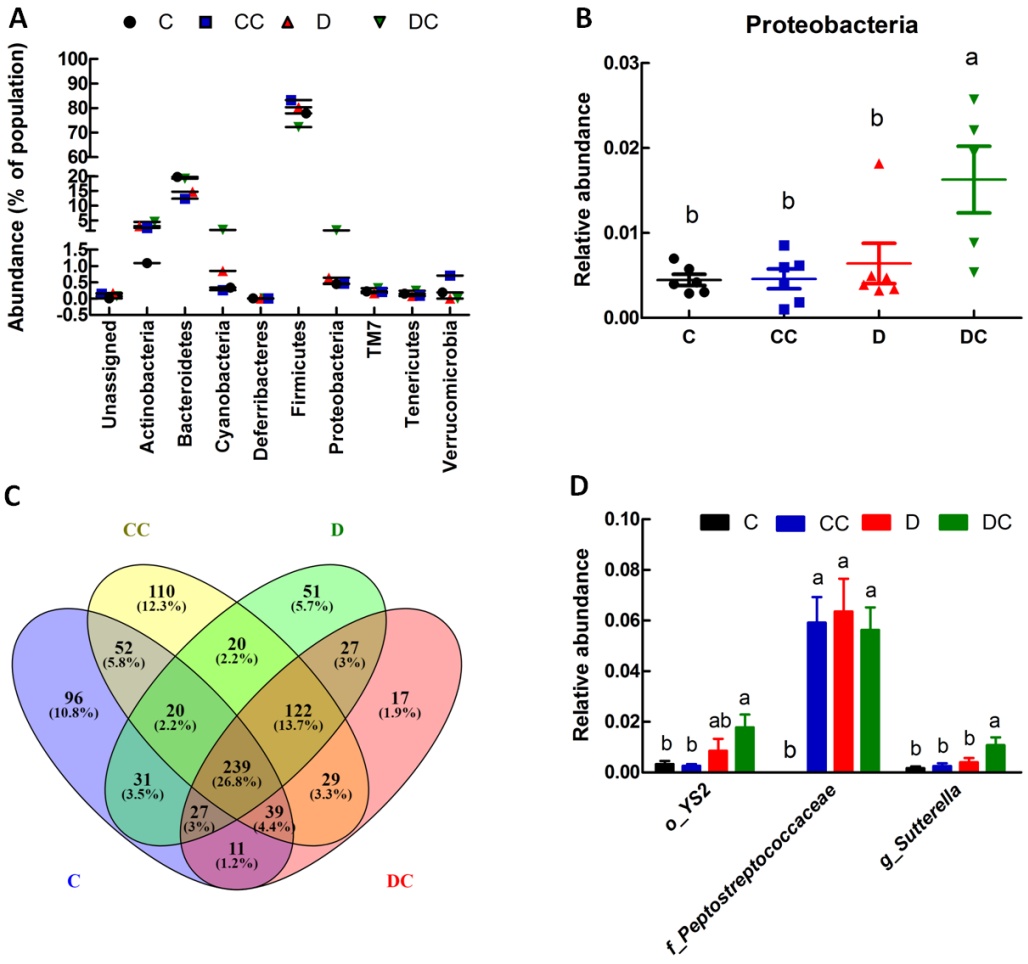

Fig. 5. Taxonomic diversity of faecal microbiota in rats given C3G with 0.5% DSS water (DC) or normal water (CC) in comparison to control (C) and 0.5% DSS (D) rats for twelve weeks. (A) Mean abundances of the major bacterial phyla as percentage of the total population among the four groups of rats. (B) Relative abundance of Proteobacteria phylum. (C) Venn diagram of observed taxonomical units (OTUs) among C, CC, D and DC rats. (D) Relative abundances of order YS2, family Peptostreptococcaceae and genus *Sutterella* among C, CC, D and DC rats.

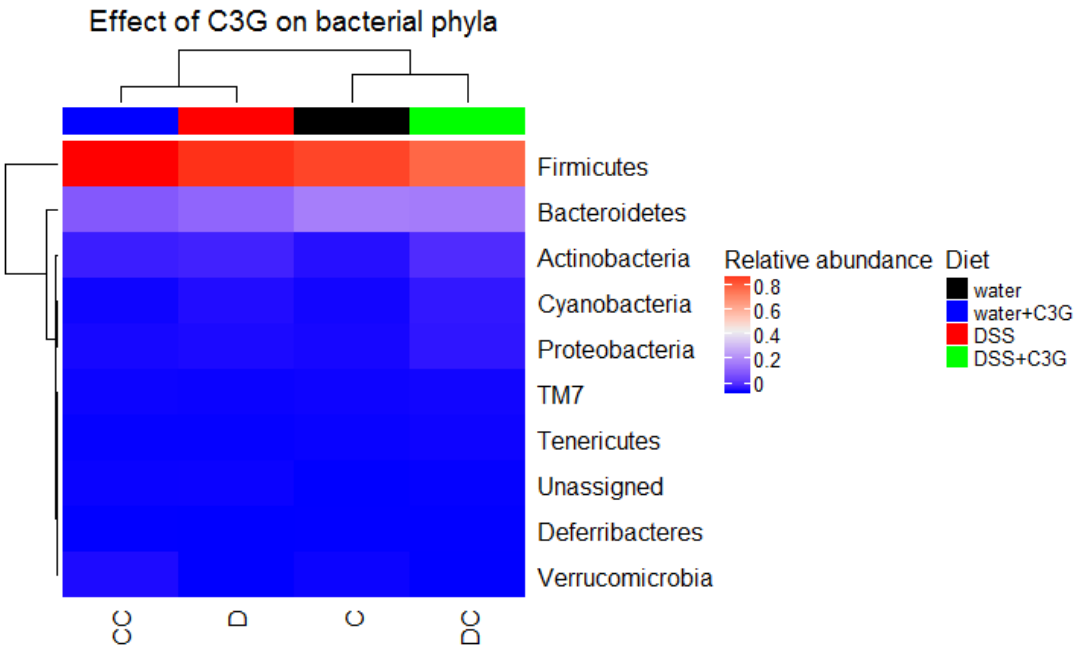

Fig. 6. Heat-map of bacterial phyla obtained from faecal microbiota in rats given C3G with 0.5% DSS water (DC) or normal water (CC) in comparison to control (C) and 0.5% DSS (D) rats for twelve weeks, n=6. The sample groups are on the horizontal axis and the bacterial phyla are on the vertical axis. Dark red- highest value, dark blue- lowest value.

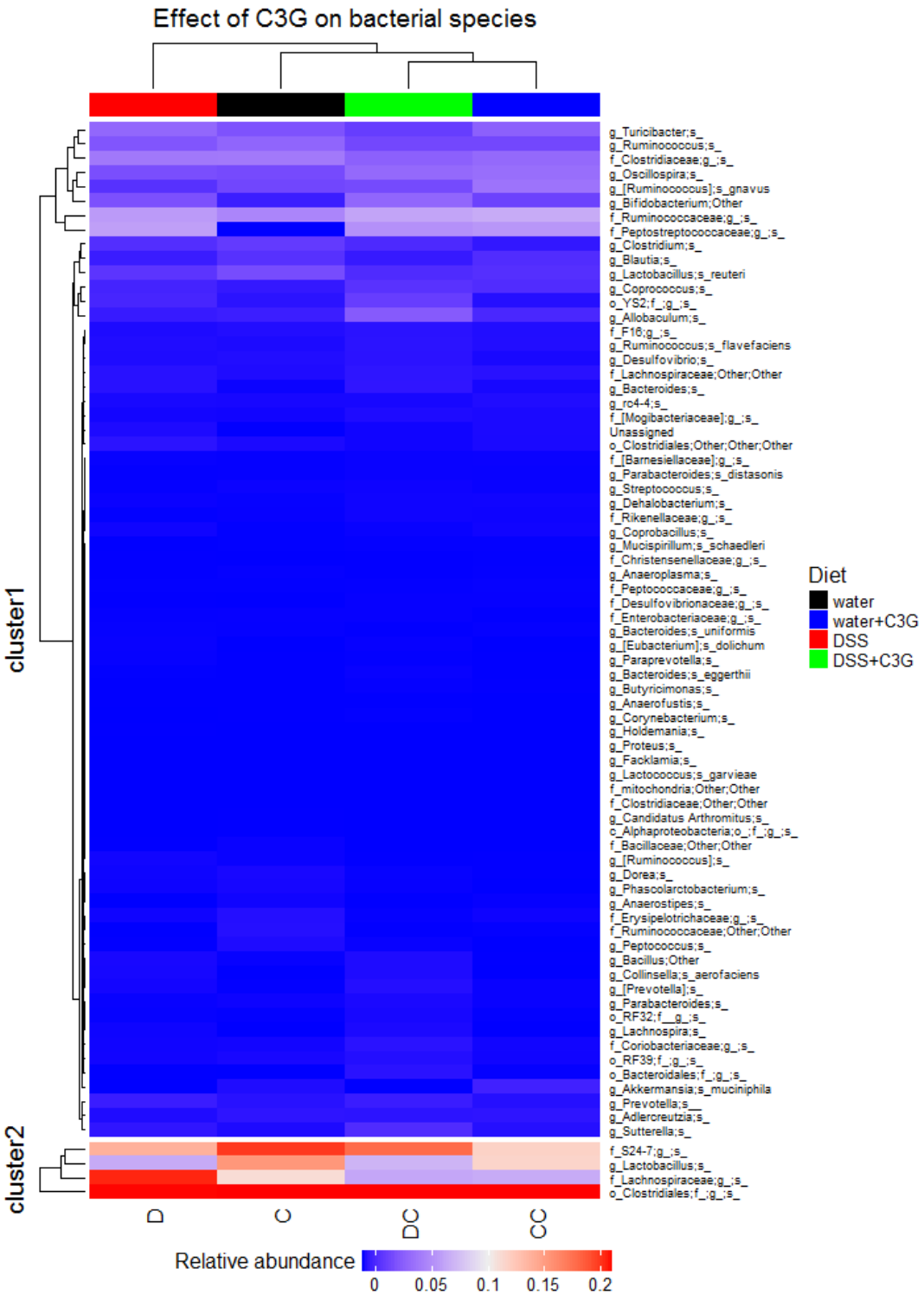

Fig. 7. Heat-map of bacterial species obtained from faecal microbiota in rats given C3G with 0.5% DSS water (DC) or normal water (CC) in comparison to control (C) and 0.5% DSS (D) rats for twelve weeks, n=6. The sample groups are on the horizontal axis and the bacterial species are on the vertical axis. Dark red- highest value, dark blue- lowest value.

Table 3. Metagenomics effects of C3G in DSS rats.

	Control	DSS	DSS+C3G
Cyanobacteria phylum	↓	↑	↑
Proteobacteria phylum	↓	↓	↑
YS2 order	↓	↓	↑
S24-7 family	↑	↓	↑
Lachnospiraceae family	↓	↑	↓
Allobaculum genus	↓	↓	↑
Sutterella genus	↓	↓	↑

DSS, dextran sodium sulphate; C3G, cyanidin 3-glucoside

4. Discussion

Anthocyanins, a major class of flavonoids abundant in fruits and vegetables, have shown anti-inflammatory activity in vitro, in vivo, and in clinical studies (18). One of the major anthocyanins, C3G, showed anti-inflammatory effects in human intestinal cell lines and thus could have a potential role in the treatment of IBD, a chronic inflammatory disease (7, 19-21). In this study, we showed that the C3G-containing foods, Queen Garnet plums and purple carrot, as well as pure C3G improved the stool characteristics in a rat model of chronic IBD. C3G and the C3G-containing foods reduced inflammation and induced regeneration of epithelial membranes, crypts and mucosal architecture in the ileum and colon of rats given 0.5% DSS. The gut bacteria in the C3G-treated DSS rats closely clustered to the control group in the heat-map of species abundance. The improvement in inflammation, ileal and colonic structure and gut bacteria was associated with improved clinical symptoms

of IBD, which as effective as the standard drug, sulphasalazine, as observed in chapter 3.

The limitations of our study include that only one dose of C3G was used for chronic treatment, thus no concentration-response curve was generated, and we did not analyse anthocyanins and their metabolites in intestinal, plasma or urine samples. The bioavailability of the C3G in the intestinal cells could give us a better understanding of the active ingredients of the foods available for the cell uptake and metabolism *in vivo* to deliver its pharmacological effect. Fecal samples from rats fed with QC juice and PC juice were not analyzed for microbiota composition and there could be synergistic effects of dietary fiber and other bio-actives with the C3G. Further, faecal samples were collected from the colon, rather than intestinal fluid, so we cannot define the products that reached the colon. Molecular markers of inflammation such as cytokines and cell adhesion molecules were not examined in this study which could have indicated the molecular mechanism of the treatments in attenuating IBD.

Functional foods are defined as foods that can prevent or reverse disease states in addition to providing nutrition; they may be useful to treat signs of metabolic syndrome such as obesity [22]. Anthocyanins extracted from blueberries showed protective effects on trinitrobenzene sulphonic acid (TNBS)-induced IBD model of mice (22). These blueberry anthocyanins at 40mg/kg bw/day reduced colonic neutrophil infiltration and histological score but were not effective at 10 mg/kg/day; however, both doses normalised stool characteristics and anti-inflammatory cytokine IL-10 concentrations and effectively inhibited the pro-oxidant and pro-inflammatory concentrations of nitric oxide (NO), tumour necrosis factor-alpha (TNF-α), interferon-gamma (IFN-γ) and interleukin-12 (IL-12) (22). This suggests that anthocyanin doses of 10 mg/kg bw/day induced anti-inflammatory responses but did not restore the tissue damage as the survival rate was 40% in 4 days of acute colitis induction, whereas the colitis control (TNBS 5mg/ml) had survival rate of 30% in 4 days. To test reversal of DSS-induced IBD, we treated rats with a low dose of 0.5% DSS for up to 12 weeks (chapter 3). Gastrointestinal symptoms were reversible with a replacement of the DSS solution with water for the final 6 weeks, or treatment for the final 6 weeks with approximately 300mg/kg sulphasalazine (chapter 3). We used C3G dose at 8 mg/kg bw/day which reduced the intestinal inflammation effectively as did sulphasalazine.

Quercetin can improve IBD symptoms as observed in acetic acid colitis model and the dose was 50 mg/kg and 100 mg/kg (23). However the amount of quercetin glycosides present in Queen Garnet plum juice is 0.3mg/ml (11), and the dose of quercetin glycosides in our study was around 1.5mg/kg so it is unlikely to show its effects at such low dose. Therefore, C3G, the main bioactive compound in Queen Garnet plum could improve the gut structure and function including whole gut permeability in our study.

Our previous studies on purple carrots (*Daucus carota* subsp *sativus*) at a C3G dose of 15 mg/kg showed improvement in the structural and functional changes of heart and liver in rats with diet-induced metabolic syndrome, a model of chronic low-grade inflammation (12). We have also reported that pure C3G and the juice of Queen Garnet plums (*Prunus salicina*) at a C3G dose of 8 mg/kg reversed the symptoms of metabolic syndrome in obese rats, including the structure and function of heart and liver (11). Further, anthocyanins in purple maize (*Zea mays*) and black chokeberry (*Aronia melanocarpa*) prevented heart and liver damage in diet-induced obese rats (24). C3G prevented heart and liver damage by inhibiting the infiltration of inflammatory cells. Moreover, both foods reduced body weight, abdominal fat pads including retroperitoneal fat, and weights of left ventricle with septum and liver in the metabolic syndrome rats. These results suggest that C3G has anti-inflammatory action indicating similar effects in IBD model too.

There are many potential benefits of C3G due to their anti-oxidant, anti-inflammatory and anti-carcinogenic effects (18). The severely inflamed colon in IBD can lead to colon cancer and C3G can attenuate symptoms in IBD or even in cancerous cells and tissues (25, 26). Different fruits and vegetables containing C3G such as purple carrot, bilberry, grape, purple corn and chokeberry inhibited the proliferation of human colorectal adenocarcinoma (HT29) cells (27). The reduction of colon length in inflammatory conditions and increased length due to the C3G and C3G-containing purple carrot and Queen Garnet plum suggests that C3G in foods can increase the healthy cells renewal against oxidative stress induced apoptosis as in inflammatory conditions (28). The oxidative colonic DNA damage was inhibited by the anti-oxidant activity of the purple carrot anthocyanins extract, quenching reactive oxygen species (ROS) in the human colonic mucosa cells (29). C3G rather than it's metabolites, phloroglucinol aldehyde and protocatechuic acid, inhibited DNA damage in colon

carcinoma cells (25). This could be the reason for the increase in colon length due to C3G and C3G-containing foods as seen in the current study. Moreover, it was also found that anthocyanin extract of purple carrot attenuated oxidative damage with reduction in the concentrations of malondialdehyde and carbonyl protein and increase in the activities of superoxide dismutase, glutathione peroxidase and total antioxidant capacity in liver, kidney, blood and heart of rats with D-galactose (400 mg/kg) induced oxidative stress (30).

The oral bioavailability of the anthocyanins is a major factor to consider when determining the anti-inflammatory activity in the tissues, especially the intestinal lining. C3G is rapidly and efficiently absorbed in jejunum and ileum (31), and its anti-inflammatory effects were seen in our ileum histology. It was observed that the digested purple carrot extract had less potency to quench ROS than the extract before digestion [13]. This suggests that sufficient anthocyanins were absorbed by the intestinal cells to induce their anti-oxidant activity and improve the gut structure and function as observed in the histology of DPC rats.

The anti-oxidant effect of anthocyanins from purple carrot attenuated liver damage due to cadmium toxicity by reducing the DNA destruction and oxidative stress (32). This shows that C3G could repair the DNA in the intestine of DSS-treated rats and increase the crypts, villi, goblet cells and epithelial membrane as seen in our study. C3G had cytoprotective properties when tested against cell damage induced by mycotoxins such as aflatoxin B1 (AFB1) and ochratoxin A (OTA) (33). The study highlighted the free radical scavenging property of anthocyanins which prevented DNA and protein damage in HepG2 and CaCo-2 cell lines (33). The role of C3G in promoting healthy tissue turnover is affirmed by their role in curbing tissue damage due to free radicals or antigens that can induce inflammation or even carcinogenesis.

Dysbiosis could be the causative factor or the consequence of gut inflammation depending on the interplaying factors (34). DSS induced imbalance of gut microbiota and mimicked human gut bacterial changes in IBD (35) as observed in our IBD model (chapter 3). C3G is readily absorbed in small intestine, and in colon is metabolized by gut microflora by cleavage of glycosidic linkages to produce protocatechuic acid, which has antioxidant and antimicrobial activities (8, 36, 37). In our study, C3G treatment groups clustered together suggesting that C3G had interaction with gut

microflora that could have improved the symptoms of inflammation. Family Peptostreptococcaceae of Firmicutes phylum increased in DSS-induced colitis mice fed with high cellulose diet (38), as seen in C3G-treated rats in our study; however, this family also increased in IBD patients (39), similar to our D rats indicating that gut microbiome is in a complex dynamic state that could be affected by many host and environmental factors other than DSS or treatment. Though Proteobacteria phylum increased in our DC rats as seen in IBD patients, the non-pathogenic genus *Sutterella* of the same phylum also increased in DC rats (40). Genus *Sutterella* are human gut commensal bacteria bound to epithelial barrier especially in duodenum, producing immunomodulatory effects (40). In DSS-induced colitis mice, gut commensal bacterial families Lachnospiraceae (butyrate-producing) and S24-7 decreased (41), and they increased when phenolics-containing lettuce were given to high fat diet-induced obese mice and in mice with remission of colitis mice during treatment phase, respectively (42, 43), as observed in our C3G-treated DSS rats (Fig. 7). This shows that C3G is efficient in modulation of gut microflora to reinstall homeostasis from dysbiosis, thus ameliorating IBD in DSS rats.

One of the possible mechanisms of C3G to modulate inflammation is that, after they are taken up by the cell by hexose transporters, they can inhibit NF-κB and MAPK-mediated inflammatory cell signalling pathways as observed in an inflamed Caco-2 BBe1/THP-1 co-culture cell model (44). Thereby, they reduce the concentrations of pro-inflammatory cytokines such as TNF-α and IL-8, and attenuate inflammation in the tissues. Gut microbial degradation of anthocyanins including C3G resulted in phenolic acids which are demethylated to release the hydroxyl group that imparts the antioxidant property (36, 46). This suggests that the metabolites of C3G could be the active molecules that exert their beneficial activity on the intestinal tissues. Though we showed DSS induced intestinal tissue damage, there was no effect on glucose uptake which is higher in duodenum and jejunum and declines in ileum under carbohydrate diet (47), indicating that, in our study, small intestinal inflammation is not continuous and we did not observe histology of duodenum and jejunum to confirm the tissue damage. Absorption of C3G in small intestine also depends on activities of sodium-dependent glucose transporter 1 and glucose transporter 2 (48), and the normal glucose tolerance in our study suggests that C3G absorption was not compromised. A recent study stated that intervention at early stage

of disease is better than later at a serious critical stage because high fibre diet did not reverse but prevented the DSS-induced intestinal damage (49). However, our study with C3G and C3G-containing foods with low fibre could reverse the intestinal damage and restore the gut environment towards normalcy.

In conclusion, the current study supports the role of C3G in attenuation of IBD symptoms. This study emphasizes the relevance of functional foods in treating chronic diseases such as IBD, which can be future clinical therapy with prospective clinical trials on these C3G-containing foods.

Conflicts of interest

The authors declare that there is no conflict of interest in the study.

Author contributions

N.K.R.G., S.K.P., and L.B. developed the original study aims. N.K.R.G. conducted the experiments. N.K.R.G., S.K.P., and L.B. analysed and interpreted the data; N.K.R.G., S.K.P., and L.B. prepared manuscript drafts and contributed to the final version. L.B. has been the corresponding author throughout the writing process. All authors read and approved the final manuscript.

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Supplementary Table S1. Physical, dietary, metabolic, and organ weight parameters in rats treated with Queen Garnet plum, purple carrot or C3G.

Variable	C	CP	CPC	CC	D	DP	DPC	DC	*P*-Value		
									DSS	Treatment	Interaction
Final body weight, g	632 ± 19[a]	552 ± 15[b]	584 ± 12[ab]	597 ± 14[ab]	612 ± 13[ab]	558 ± 17[b]	551 ± 18[b]	571 ± 15[ab]	0.1024	0.0004	0.6182
Energy intake, kJ/d	511 ± 30	486 ± 20	504 ± 26	468 ± 22	503 ± 36	564 ± 41	498 ± 28	473 ± 13	0.39	0.29	0.38
Feed efficiency, g/kJ	0.18 ± 0.014	0.169 ± 0.01	0.16 ± 0.018	0.17 ± 0.01	0.183 ± 0.017	0.141 ± 0.012	0.139 ± 0.016	0.158 ± 0.018	0.17	0.16	0.74
Retroperitoneal fat, mg/mm tibial length	398 ± 25	272 ± 20	321 ± 28	335 ± 35	349 ± 22	298 ± 28	277 ± 33	326 ± 43	0.3756	0.0237	0.5727
Epididymal fat, mg/mm tibial length	260 ± 22	179 ± 17	190 ± 14	211 ± 16	209 ± 18	206 ± 17	172 ± 22	199 ± 32	0.3546	0.0660	0.3099
Omental fat, mg/mm tibial length	284 ± 23	204 ± 16	249 ± 16	222 ± 22	216 ± 13	207 ± 18	212 ± 23	218 ± 16	0.0501	0.1243	0.2177
LV + Septum wet weight, mg/mm tibial length	23.5 ± 0.9[a]	19.7 ± 1.2[b]	20.2 ± 0.9[ab]	20.4 ± 0.6[ab]	22.4 ± 0.7[ab]	21.9 ± 0.8[ab]	20.3 ± 0.5[ab]	21.3 ± 0.6[ab]	0.3592	0.0076	0.2271

RV wet weight, mg/mm tibial length	6.53 ± 0.61^{a}	4.2 ± 0.52^{b}	4.85 ± 0.31^{b}	4.68 ± 0.26^{b}	5.56 ± 0.41^{ab}	4.3 ± 0.36^{b}	3.91 ± 0.3^{b}	4.02 ± 0.4^{b}	0.0382	<0.0001	0.5364
Liver wet weight, mg/mm tibial length	403 ± 19^{a}	326 ± 10^{b}	335 ± 9^{b}	330 ± 16^{b}	379 ± 12^{a}	372 ± 17^{a}	326 ± 10^{b}	348 ± 9^{b}	0.4129	0.0001	0.0544
Kidney wet weight, mg/mm tibial length	71.4 ± 1.4^{ab}	62.9 ± 1.6 ^{b}	70.9 ± 1.9^{ab}	69.2 ± 2^{ab}	73.2 ± 2.6^{a}	69 ± 2.1^{ab}	67 ± 2.7^{ab}	70.8 ± 2.8^{ab}	0.3702	0.0433	0.1677
Spleen wet weight, mg/mm tibial length	27.3 ± 1.3	22.6 ± 1.4	25.7 ± 1.1	22.9 ± 1.4	25.5 ± 1.3	23.2 ± 1.2	24.4 ± 2.5	25.6 ± 2.1	0.9650	0.1843	0.4920

All values are mean ± SEM, n = 8. Mean values within a row with a different superscript are significantly different, $P<0.05$. DSS, dextran sodium sulphate; C, normal water; CP, normal water + Queen Garnet plum; CPC, normal water + purple carrot; CC, normal water + Cyanidine-3-glucoside (C3G); D, 0.5% DSS in drinking water; DP, 0.5% DSS in drinking water + Queen Garnet plum; DPC, 0.5% DSS in drinking water + purple carrot; DC, 0.5% DSS in drinking water + C3G; LV, left ventricle; RV, right ventricle.

Chapter 5. Pelargonidin 3-glucoside from strawberry improves chronic inflammatory bowel disease in rats

Pelargonidin 3-glucoside from strawberry improves chronic inflammatory bowel disease in rats

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Abstract

The global prevalence of inflammatory bowel disease (IBD), mainly ulcerative colitis and Crohn's disease affecting primarily the colon and small intestine, is increasing. There is a need for complementary treatments to improve the effectiveness and adverse effect profile of current drug therapy. Functional foods can prove beneficial for the treatment of chronic diseases. Pelargonidin 3-glucoside (P3G), the major anthocyanin in strawberry, has anti-inflammatory and antioxidant effects. Therefore, we tested P3G-containing strawberry in our chronic IBD rat model induced by dextran sodium sulphate (DSS). Rats were randomly placed into four groups and were given powdered food with either normal water (C) or 0.5% DSS (D) in drinking water for 12 weeks to induce IBD. Strawberry (SB) as freeze-dried powder was added to the diet for the final 6 weeks to give a dose of 8 mg P3G/kg body weight/day in CSB and DSB rats. Body weight and stool characteristics were assessed daily for 12 weeks. C and CSB rats had no symptoms of IBD. D rats had severe diarrhoea, bloody stools, erosion of mucosal epithelium, crypt atrophy, loss of villi and goblet cells, and inflammatory cell infiltration. P3G-containing strawberry treatment in DSS rats reversed the observed symptoms of IBD with healthy stools (stool consistency: C 0.0±0.0, D 2.5±0.2, DSB 1.3±0.3; stool bleeding: C 0.0±0.0, D 2.4±0.2, DSB 0.6±0.2) and mucosal lining of ileum and colon including increased villi, crypts and goblet cells and reduced inflammation compared to standard drug, sulphasalazine. Our study suggests that addition of P3G in diet from strawberry may help mitigate the symptoms of IBD and improve the quality of life in IBD patients.

Keywords: Inflammatory bowel disease, dextran sodium sulphate, pelargonidin 3-glucoside, strawberry, inflammation

1. Introduction

Inflammatory bowel disease (IBD) is a chronic intestinal disease affecting people in both the developed and developing world (1, 2). IBD therapy requires chronic administration of drugs, and this increases the risk of adverse effects (3-5). Moreover, treatment with biologics and even surgery can be expensive (6). An effective, cheaper and non-toxic therapy is always sought for chronic diseases including IBD. Therefore, functional foods that prevent or reverse disease as well as providing nutrition may be a valuable complementary chronic treatment for IBD (7). Anthocyanins from berries and other foods are well-known for their anti-oxidant and anti-inflammatory activities which may be potential therapeutic mechanisms in treating chronic diseases (2, 8). Strawberry (*Fragaria* × *ananassa*) contains pelargonidin 3-glucoside (P3G) which is an important anthocyanin present in many fruits (9, 10). P3G has anti-inflammatory actions and radical scavenging properties as shown in human whole blood cell cultures (11). In human skin cells, strawberry extract improved regeneration of cells, decreased cell and DNA damage and reduced reactive oxygen species (ROS) in the cell after exposure to pro-oxidant stimuli demonstrating the anti-oxidant properties of P3G (12). Another study in which ethanol was used to induce damage in the lining of the stomach (also known as gastric mucosa) in rats showed that strawberry in food was able to increase anti-oxidant enzymes in the stomach lining (13). There was a correlation between anthocyanin intake and reduction of gastric damage (13). Inflammatory bowel disease is a gastrointestinal disease involving any part of the gut, predominantly the small and large intestine, so that strawberry could prove to be a beneficial product in reducing gastric mucosal erosion that leads to bleeding and diarrhoea. Freeze-dried strawberry powder intake by adults improved insulin sensitivity for those resistant to insulin (14). A study using human colon cancer cells showed that a crude strawberry extract as well as pure anthocyanin including P3G decreased the proliferation of cancer cells (15). These studies strengthen the potential of P3G-containing strawberry as a possible intervention for IBD.

Our laboratory has conducted rat studies on cyanidin 3-glucoside (C3G), a major anthocyanin in purple-coloured foods, at a C3G dose of 8mg/kg body weight/day following addition to the food. In these studies, C3G attenuated inflammatory bowel disease in DSS-induced IBD rats (chapter 4) and metabolic

syndrome in diet-induced obese rats (16, 17). We hypothesized that P3G at the same dose will also have efficacy and therapeutic potential in IBD. This dose is not toxic for the rats as other studies have used a much higher dose of 100mg/kg body weight (18).

The present study aims to understand the responses to pelargonidin 3-glucoside (P3G) in the chronic IBD rat model. P3G showed anti-carcinogenic effects on colonic cells (19) suggesting its responses in IBD are worth exploring. A comparison of P3G with C3G and sulphasalazine from our earlier studies was made. We will be able to ascertain the therapeutic effects of P3G-containing strawberry in chronic IBD rats, which could suggest a dietary intervention to improve the gut health of IBD patients.

2. Materials and Methods

2.1 Diets and measurements in living rats

The animal experiments conducted in this study were authorized by the University of Southern Queensland Animal Ethics Committee under the guidelines of the National Health and Medical Research Council of Australia. Male Wistar rats (8-9 weeks old, weighing 335 ± 1 g, n = 32) were purchased from the Animal Resource Centre, Perth. All rats were provided free access to food and water and were individually housed in temperature-controlled (20 ± 2°C), 12-hour light-dark conditions at the University of Southern Queensland animal house.

The rats were given standard diet of powdered rat food (Specialty Feeds, Glen Forest, WA, Australia). They were randomly divided into four experimental groups (n=8 each). The first group served as healthy control (C) with normal drinking water and the second group was IBD control (D) with 0.5% dextran sodium sulphate (DSS, molecular weight: 36,000-50,000 Da, MP Biomedicals) in drinking water. P3G-containing strawberry powder was given as treatment for last 6 weeks to the third group of rats with normal water (CSB) and the fourth group of rats with 0.5% DSS drinking water (DSB). The concentration of pelargonidin 3-glucoside (P3G) was 108 mg/kg of food. The freeze-dried strawberry powder with its anthocyanin content analysis was prepared and analysed by Queensland Department of Agriculture and Fisheries (DAF), Brisbane, Australia (Table 1).

Table 1. Anthocyanin content of freeze-dried strawberry powder .

Anthocyanin	Content – mg/100 g powder

Cyanidin 3-glucoside	33
Pelargonidin 3-glucoside	1048
Pelargonidin 3-rutinoside	66
Pelargonidin 3-malonylglucoside	58
Total	1205

- 87% pelargonidin 3-glucoside
- 97% pelargonidin-based anthocyanins

Energy intake was calculated from the value of powdered rat food of 13.8 kJ/g. Rats were monitored daily for body weight, and food and water intakes. Daily anthocyanin (P3G in strawberry powder) intake was calculated from the daily food intake.

Rats were food-deprived overnight for 12 hours and oral glucose tolerance tests were performed with Medisense Precision Q.I.D glucose meter (Abbott Laboratories, Bedford, USA) at 0, 6 and 12 weeks (20).

Rat stools were examined to assess the disease activity daily for 12 weeks. The stool consistency was scored as 0-formed, 1-mild-soft, 2-very soft, 3-watery soft (diarrhoea). The stool bleeding was scored as 0-normal colour, 1-brown colour, 2-reddish colour, 3-bloody red (21).

A cocktail of sucrose, lactulose, mannitol and sucralose (Sigma-Aldrich Australia, Sydney, Australia) was utilized to assess the rats' intestinal permeability after 12 weeks of the study as described in chapters 3 and 4. Gastric transit and gastric emptying experiments were performed using 0.05% phenol red solution (Sigma-Aldrich, Australia) as described in chapter 3.

2.2 Measurements after euthanasia

Terminal anaesthesia was induced in the rats via intraperitoneal injection of pentobarbitone sodium (Lethabarb, 100 mg/kg,Virbac, Milperra, Australia). Heparin (200 IU; Sigma-Aldrich Australia, Sydney, Australia) was injected into the right femoral vein. Further, the rats were dissected and the organs were collected (20).

The lengths of small intestine and colon were measured. Distal ileum and distal colon (~1.5 cm) were collected for histological examination and organ bath studies as described in chapter 3. The tissues were formalin-fixed, processed, embedded in paraffin wax, and 5 µm sections were cut. The tissues were stained with haematoxylin and eosin as well as Periodic acid–Schiff (PAS) stains and combined Alcian blue-PAS

stain. All stained slides were digitally scanned at 20x magnification using the Leica SCN400 slide autoscanner. Images were viewed in Image Scope with software provided by Leica Biosystems to determine the infiltration of inflammatory cells and damage to the intestinal tissue (20).

As in chapter 3, following the blood collection from the abdominal aorta, the left ventricle along with septum and the right ventricle, kidney, spleen, liver and abdominal fat pads were isolated, blotted dry and weighed for each rat. The organ weights were normalised to the tibial length at the time of organ isolation and expressed as mg/mm (20).

Examination of rat urine samples for sugars was performed at the Central Analytical Research Facility (CARF) of the Queensland University of Technology, Gardens Point, Brisbane, with the assistance of Dr. Rajesh Gupta. The GCMS method as described in chapters 3 and 4 was used for the sugar analysis, which was modified from other study (22). Cumulative percent urinary recovery of each sugar and small intestine and whole gut permeability were calculated (23-25).

The whole blood analysis was performed by collecting blood in EDTA tubes. The samples were sent to The University of Queensland, Gatton where they were analysed using an autoanalyser. The parameters tested were WBC, RBC, haemoglobin, platelets, haematocrit, MCV, MCH and MCHC.

2.3 Statistics

The data were expressed as mean ± SEM. Results from all groups C, CSB, D and DSB were analysed for variance by using 1-way and 2-way ANOVA. The data were tested with Neumann-Keuls multiple comparison *post hoc* test and $P<0.05$ was considered as significant. Statistical analyses was performed using GraphPad Prism version 6 for Windows (GraphPad Software, San Diego, CA, USA) (20).

3. Results

The stool consistency and stool bleeding scores of C and CSB rats were zero and D rats increased the stool consistency and stool bleeding scores, which were reduced in DSB rats indicating a positive outcome of the intervention (Fig. 1A & B). The small intestinal and gastric transit did not differ among C and CSB rats or DSB and D rats. DSS treated rats had reduced colon length and gastric emptying was reduced in

strawberry-treated rats (Table 2). The histology of ileum and colon of C and CSB rats showed healthy mucosal epithelium and goblet cells (Fig. 2A, B, E & F; Fig. 3A, B, E & F; Fig. 4A, B, E & F). The ileum and colon of the D rats had crypt and mucosal atrophy, loss of goblet cells and villi whereas the DSB rats improved with reduced infiltration of inflammatory cells, villi and crypts and regeneration of epithelial membrane compared to D rats (Fig. 2C, D, G & H). D rats had decreased number of goblet cells leading loss of the protective mucus layer in ileum and colon (Fig. 3C & G; Fig. 4C & G). P3G-containing strawberry increased the presence of goblet cells in ileum and colon which helps to maintain mucous membranes in DSB rats (Fig. 3D & H). The forces of contraction of the isolated ileum and colon preparations in response to acetylcholine were not different among the C and CSB rats (Ileum-C=10.9 ± 3.1, CSB=19.9 ± 5.9; colon- C=48.7 ± 5.6, CSB=68.0 ± 3.9) (Fig. 1C &D). D rats had lower acetylcholine-induced force of contraction of ileum than DSB rats but not of colon (ileum-D=11.0 ± 2.2, DSB=31.0 ± 4.3; colon- D=45.7 ± 6.5, DSB=58.1 ± 8.2 mN) (Fig. 1C &D). Overall the strawberry treatment increased the ileum and colon force of contraction compared to C and D rats. The cumulative percent recovery of sucrose did not differ among C or CSB rats or D and DSB rats (Fig. 5A), however CSB rats had higher value than C and DSB rats at 12h and 24h. Lactulose cumulative percent recovery was higher in CSB rats than in C, D, DSB rats at 6 h, 9 h, 21 h and 24h but was unaltered among D and DSB rats (Fig. 5B). The cumulative percent recovery of mannitol was similar among C and CSB rats or D and DSB rats but DSB rats had lower value than C and CSB rats at 24 h (Fig. 5C). Sucralose cumulative percent recovery of strawberry treated rats at 21 h and 24 h increased compared to C and D rats (Fig 5D). The lactulose/mannitol ratio, an indicator of small intestine permeability increased in CSB rats compared to C rats but did not alter among C, D and DSB rats (Fig. 5E). The indicators of whole gut permeability, sucralose/lactulose ratio was similar among all the groups of rats whereas the sucralose/mannitol ratio increased in DSB rats compared to D rats but did not alter among C, CSB and D rats (Fig. 5E). Table 3 summarises the intestinal effects of the interventions.

None of the groups of rats showed any difference in final body weight, energy intake and feed efficiency (Table S1). Total abdominal fat, retroperitoneal fat, epididymal fat, omental fat, fasting blood glucose and blood glucose AUC also remained unchanged among the four groups of rats (Table 2 & S1). The wet weights

of left ventricle with septum, right ventricle, liver, kidney and spleen were similar among all the groups of rats (Table S1). WBC, RBC, haemoglobin, platelets, haematocrit, MCV, MCH and MCHC had no change either due to DSS or the intervention on the rats (Table S1).

Table 2. Metabolic and gastrointestinal parameters in rats treated with strawberry.

Variable	C	CSB	D	DSB	*P*-Value		
					DSS	Treatment	Interaction
Fasting blood glucose, mmol/L	4.3 ± 0.1	3.8 ± 0.2	4.3 ± 0.2	4.0 ± 0.1	0.0173	0.5322	0.5322
Blood glucose AUC, mmol/L/120 min	700 ± 14	674 ± 28	688 ± 22	709 ± 21	0.5958	0.9080	0.2823
Abdominal fat pads, mg/mm tibial length	555 ± 26	585 ± 85	473 ± 27	556 ± 45	0.2914	0.2830	0.6117
Small intestine length, cm	111 ± 4	113 ± 4	113 ± 3	108 ± 4	0.6941	0.6941	0.3618
Colon length, cm	21.0 ± 1.0	18.5 ± 0.7	19.0 ± 1.0	17.9 ± 0.6	0.0419	0.1348	0.4140
Gastric transit, %	80.0 ± 3.0	77.0 ± 3.0	73.1 ± 3.8	77.8 ± 4.5	0.4248	0.8230	0.3155
Gastric emptying, %	63.9 ± 4.8	53.5 ± 5.7	67.0 ± 2.8	55.7 ± 6.3	0.6053	0.0417	0.9299
Anthocyanin intake, mg/kg bw/day	0.0 ± 0.0	6.9 ± 0.2	0.0 ± 0.0	7.1 ± 0.1	0.3787	<0.0001	0.3787

All values are mean ± SEM, n = 8. Mean values within a row with a different superscript are significantly different, $P<0.05$. C, control (0% DSS); D, 0.5% DSS; CSB, C + strawberry; DSB, D + strawberry; DSS, dextran sodium sulphate; AUC, area under curve; CSB and DSB rats were treated with strawberry for last 6 weeks of the 12 weeks' protocol.

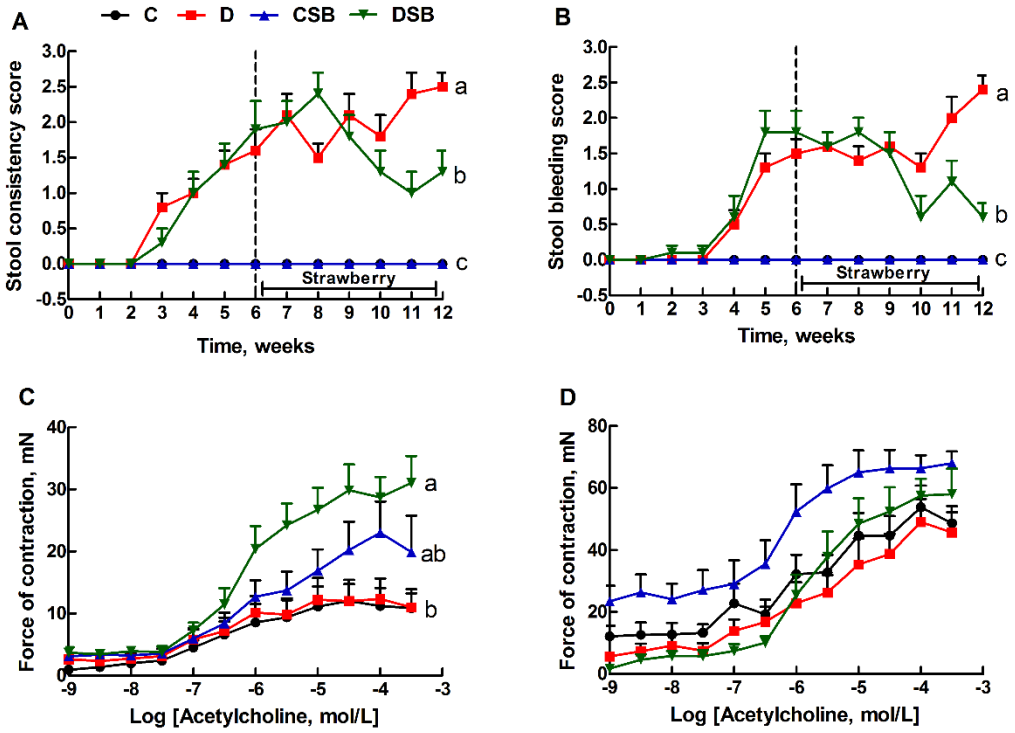

Figure	*P*-Value		
	DSS	**Treatment**	**Interaction**
A	<0.0001*	0.0025	0.0025
B	<0.0001*	<0.0001	<0.0001
C	0.1633	0.0011	0.1729
D	0.3143	0.0192	0.5869

Fig. 1. Effect of P3G-containing strawberry powder on stool consistency (A), stool bleeding (B), acetylcholine-induced force of contraction on ileum (C) and colon (D) preparations, on rats. Values are mean ± SEM, n =8. Endpoint means with an asterisk differ, $p < 0.05$. DSS, dextran sodium sulphate. Stool consistency score, 0-formed, 1-mild-soft, 2-very soft, 3-watery soft (diarrhoea). Stool bleeding score, 0-normal colour, 1-brown colour, 2-reddish colour, 3-bloody red. Interventions in the diet from week 7-12.

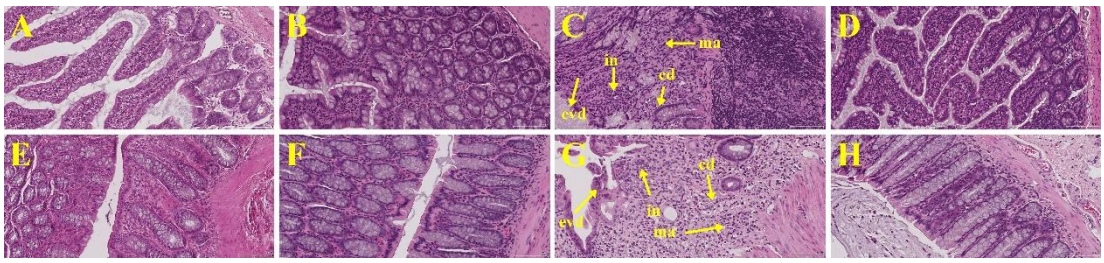

Fig. 2. Effect of anthocyanin P3G-containing strawberry powder on inflammation in the ileum and distal colon. Haematoxylin and eosin staining of ileum and distal colon showing infiltration of inflammatory cells “in” (C, G), epithelial and villi disruption “evd” (C, G), crypt distortion “cd” (C, G), and mucosal atrophy “ma” (C, G) (×20). Ileum of rats treated with normal water and 0.5% DSS water for 12 weeks (A and C respectively); rats treated with normal water and 0.5% DSS water for 12 weeks with P3G-containing strawberry powder (8mgP3G/kg BW/d) in the food for last 6 weeks (B and D respectively. Colon of rats treated with normal water and 0.5% DSS water for 12 weeks (E and G respectively); rats treated with normal water and 0.5% DSS water for 12 weeks with P3G-containing strawberry powder (8mgP3G/kg BW/d) in the food for last 6 weeks (F and H respectively). DSS, dextran sodium sulphate; P3G, Pelargonidin-3-glucoside. Intestinal inflammation in C shows signs of inflammatory bowel disease with epithelial disruption, crypt distortion and mucosal atrophy and in G shows signs of ulcerative colitis with sever colon inflammation. The treatment with P3G-containing strawberry powder improved epithelial membrane and crypts in ileum (D) and colon (H).

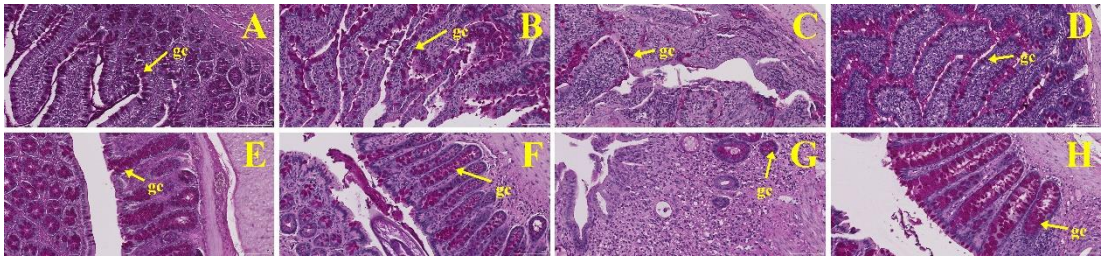

Fig. 3. Effect of P3G-containing strawberry powder on mucin-secreting goblet cells in the ileum and distal colon. Periodic acid–Schiff (PAS) staining of ileum and distal colon showing decreased number of goblet cells stained dark pink for neutral mucin. D rats had few goblet cells “gc” (C, G) (×20). Ileum of rats treated with normal water and 0.5% DSS water for 12 weeks (A and C respectively); rats treated with normal water and 0.5% DSS water for 12 weeks with P3G-containing strawberry powder (8mgP3G/kg BW/d) in the food for last 6 weeks (B and D respectively). Colon of rats treated with normal water and 0.5% DSS water for 12 weeks (E and G respectively); rats treated with normal water and 0.5% DSS water for 12 weeks with P3G-containing

strawberry powder (8mgP3G/kg BW/d) in the food for last 6 weeks (F and H respectively). DSS, dextran sodium sulphate; P3G, Pelargonidin-3-glucoside. Intestinal inflammation and loss of goblet cells in C and G indicates active inflammatory bowel disease. The treatment with P3G-containing strawberry powder improved goblet cells and epithelial membrane in ileum (D) and colon (H).

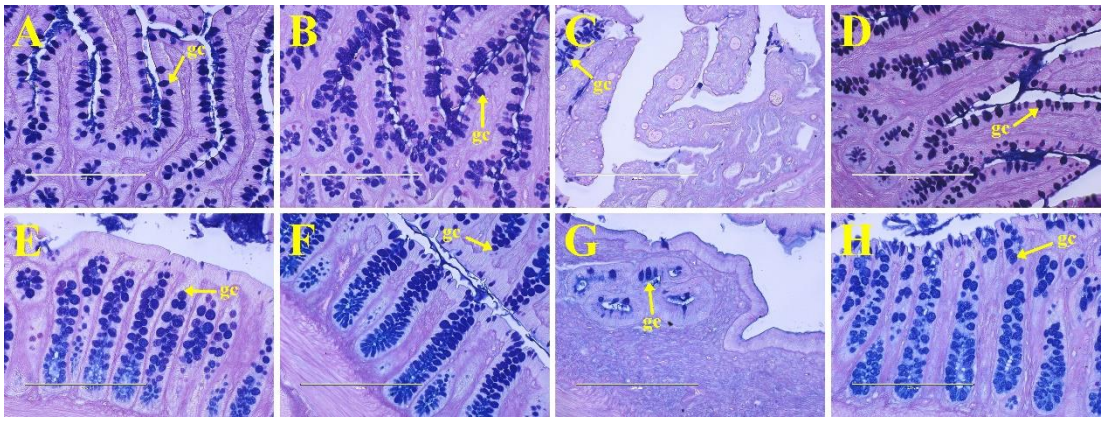

Fig. 4. Effect of P3G-containing strawberry powder on mucin-secreting goblet cells in the ileum and distal colon. Alcian Blue-Periodic acid–Schiff staining of ileum and distal colon showing decreased number of goblet cells stained purple indicating presence of neutral mucin (dark pink) stained by PAS and acidic mucin (light blue) stained by Alcian Blue. D rats had few goblet cells "gc" (C, G) (×20). Ileum of rats treated with normal water and 0.5% DSS water for 12 weeks (A and C respectively); rats treated with normal water and 0.5% DSS water for 12 weeks with P3G-containing strawberry powder (8mgP3G/kg BW/d) in the food for last 6 weeks (B and D respectively). Colon of rats treated with normal water and 0.5% DSS water for 12 weeks (E and G respectively); rats treated with normal water and 0.5% DSS water for 12 weeks with P3G-containing strawberry powder (8mgP3G/kg BW/d) in the food for last 6 weeks (F and H respectively). DSS, dextran sodium sulphate; P3G, Pelargonidin-3-glucoside. Intestinal inflammation and loss of goblet cells in C and G indicates active inflammatory bowel disease. The treatment with P3G-containing strawberry powder improved goblet cells and epithelial membrane in ileum (D) and colon (H).

Table 3. Intestinal effects of strawberry powder.

	DSS	DSS + strawberry powder

Stool consistency	↑	↓
Stool bleeding	↑	↓
Inflammation	↑	↓
Epithelial disruption	↑	↓
Crypt distortion	↑	↓
Mucosal atrophy	↑	↓
Goblet cells with acidic and neutral mucin	↓	↑

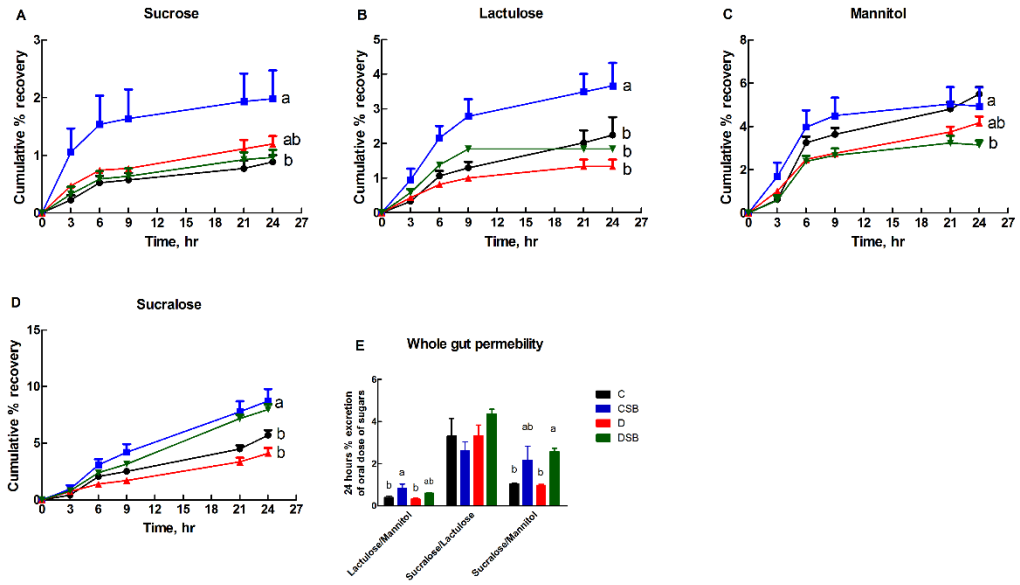

Fig. 5. Effect of P3G-containing strawberry powder on the cumulative percent urinary recovery of sucrose (A), lactulose (B), mannitol (C), sucralose (D) and whole gut permeability (E) over 24 h percent excretion of sugar probes in rats given 0.5% DSS water (DSB) or normal water (CSB) in comparison to control (C) and 0.5% DSS (D) rats for twelve weeks. Values are mean ± SEM, n =6. Endpoint means with a different alphabet differ, $p < 0.05$. DSS, dextran sodium sulphate; C, Normal water; CSB, Normal water + strawberry; D, 0.5% DSS water; DSB, 0.5% DSS water + strawberry.

Discussion

In our study, the strawberry freeze-dried powder enriched with P3G improved the stool characteristics by reducing diarrhoea and bleeding. The treatment improved gastric smooth muscle contractility and gastric emptying. The histology of ileum and colon also showed reduced infiltration of inflammatory cells. Further, there were increases in goblet cells, mucin layer, epithelial and crypt cell turnover. Overall, the mucosal layer of ileum and colon improved from the inflammatory insult caused by DSS. The limitations of this study are that the mechanistic investigations with regard to inflammatory cytokines and gut bacteria were not conducted.

DSS causes epithelial membrane breakdown which encourages bacterial pathogens to invade the intestinal tissue (26). This stimulates a cascade of events that attracts innate immune cells and antigen-presenting cells. Neutrophils and lymphocytes are the major inflammatory cells that invade the intestinal tissue and they release cytokines including TNFα and IL-1β which further trigger the release of other pro-inflammatory cytokines (27). Thus, the DSS model mimics human IBD (28). Anthocyanins and C3G in particular are well-known for their antioxidant and anti-inflammatory activity by inhibiting the production of TNFα, IL-6 and IL-1β (29). We wanted to ascertain the responses to P3G, a closely-related anthocyanin to C3G, in an inflammatory condition such as IBD in our DSS rat model. We observed that P3G had considerable effect in similar fashion as C3G as observed in chapter 4 in reducing the intestinal inflammation. P3G is found in strawberries, unlike C3G which is quite low as in our sample (Table 1). Therefore, the positive effects of strawberry powder to counterattack the action of DSS could be due to P3G. Similarly, from our previous experiments in chapter 3, P3G was as effective as sulphasalazine, the standard drug for IBD.

The health benefits of P3G from strawberry have been reported. Strawberry extract acted as free radical scavenger in human skin cells and promoted regeneration of cells (12). P3G and one of its metabolites, phloroglucinaldehyde, increased the concentrations of IL-10 in diluted whole blood cells after the cells were stimulated with lipopolysaccharide (11). IL-10 is known for its anti-inflammatory activity and is a vital cytokine to prevent intestinal inflammation (30). Promotion of IL-10 and inhibition of free radicals prevents DNA damage, in turn promoting cell turnover. This

further enhances the cell proliferation in nontoxic environment and this was observed in our study by the increase in mucosal epithelium, villi length, crypt depth and also goblet cells. Increase in goblet cells promotes the mucin secretion which forms a protective layer on the intestinal lining that prevents infiltration of gut microbiota that induces inflammation (31). DSS rats without treatment had extensive loss of epithelial membrane goblet cells which led to loss of protective barrier thus the gut microbiota may invade and induce inflammation leading to intestinal tissue inflammation. The P3G-containing strawberry powder reversed these changes owing to its anti-oxidant and anti-inflammatory activity and it is supported by another study on hydroalcoholic extract of strawberry treatment in acetic acid-induced colitis in rats (32). Though DSS can cause increased intestine permeability (27), we did not observe such effects indicating the inflammation was not severe enough to damage intestinal wall function.

Strawberry extract also elevated the activities of anti-oxidant enzymes in the stomach lining thus reducing gastric damage (13). We observed that there was improvement of gastric emptying which suggests that the strawberry powder had better absorption. One study reported that the absorption of C3G and P3G, with different aglycones but similar sugars, was different however they both had high conversion to metabolites with the total urinary recovery of P3G and its metabolites much higher than that of C3G (33). This suggests that P3G has better bioavailability than C3G. The main metabolite of P3G is pelargonidin monoglucuronide which had higher urinary excretion in humans than the sulpho-conjugate of pelargonidin and the parent compound, P3G itself which was the least (34). Not long after the above studies, another group published a study on pelargonidin with regard to its absorption, distribution and excretion in rats (35). They reported that pelargonidin was predominantly present in the stomach after 2h of ingestion and dropped down to 1.2% only after 18h of ingestion. This supports our results on gastric emptying and emphasises the role of P3G in ameliorating intestinal damage by increased absorption to enhance the systemic antioxidant and anti-inflammatory activity.

Further down the intestine, the P3G passes to the colon where it undergoes microbial fermentation to release one of the major metabolites, 4-hydroxybenzoic acid (36). 4-hydroxybenzoic acid was also observed in plasma and urine of rats after 2h and 18h of pelargonidin ingestion (35). In a randomised, crossover, controlled interventional trial testing red wine or dealcoholized wine or gin, it was observed that

Bifidobacteria increased with increases in 4-hydroxybenzoic acid in the urine of participants that took red wine and dealcoholized wine (37). We observed improved ileum and colon smooth muscle contractility due to the P3G-containing strawberry powder in healthy and DSS rats. It is understood that this improves intestinal function to allow the treatment to reach the colon and be metabolised by gut bacteria. Moreover, the above studies indicate that anthocyanins can act as prebiotics and modulate gut microbiota towards gut homeostasis as observed in our previous C3G study in IBD.

The gastric emptying can also be delayed when strawberry is taken with cream as observed by researchers from UK and Italy. The excretion of P3G metabolites, mainly pelargonidin-O-glucuronide, was delayed in the first two hours of ingestion and increased during 5 to 8 h after ingestion in participants that had strawberry with cream and the opposite effect was seen with participants that had only strawberry (38). This suggests that the bioavailability of P3G in strawberry can be enhanced by ingesting with cream and this allows greater absorption which in turn will promote higher anti-inflammatory activity in the intestinal tissue. Strawberry when taken with yogurt was tested for *in vitro* gastrointestinal digestion to determine its stability and bio-accessibility of the anthocyanin metabolites in the gastrointestinal tract (39). The study highlighted that the anti-oxidant activity of the strawberry yogurt was higher than the undigested yogurt suggesting that the P3G is more active *in vivo*. However, these anthocyanins, primarily pelargonidin 3-glucoside and pelargonidin 3-rutinoside, were more prevalent in the gastric compartment with lesser amounts in the small intestine comparatively and this correlated with decreased gastric emptying as observed in treatment groups in our study.

The health benefits of strawberry have been reported in cardiovascular disease and cancer due to its anti-oxidant and anti-carcinogenic activity (2, 40). In a healthy group of individuals, frozen strawberry intake reduced the lipid peroxidation in their serum and highlighted the anti-oxidant activity of strawberry (41). In human umbilical vein endothelial cells, it was observed that pelargonidin showed antithrombotic activity by prolonging activated partial thromboplastin time, prothrombin time, and inhibited the thrombin and activated factor X (FXa) indicating its benefits in preventing thrombus formation and thereby enhancing blood circulation (42). DSS or the strawberry treatments did not affect haematological parameters in our study. To test the antitumor effect of strawberry, rats were fed for 5 weeks with N-

nitrosomethylbenzylamine (NMBA) to induce tumorigenesis in the rat oesophagus. For the next 25 weeks, the rats were fed with freeze-dried strawberries which reduced the tumorigenesis (43). Similarly, other studies on strawberry, blueberry, blackberry, red raspberry, black raspberry and cranberry extracts showed anti-carcinogenic effects on cervical (CaSki, SiHa), breast (MCF-7, T47-D), human oral (KB, CAL-27), colon (HT-29, HCT116), and prostate (LNCaP) tumour cell lines (44, 45). These properties of strawberries could be of benefit for IBD patients who may suffer from cardiovascular diseases or are at increased risk of colon cancer which may manifest in advanced stages of IBD. Based on these observations, we are currently investigating its effects on metabolic syndrome at the same dose in our diet-induced obesity.

In conclusion, P3G-enriched strawberry powder can be considered as a treatment choice for IBD. There is a clear need for clinical trials and more mechanistic studies to understand the role of P3G in curbing the inflammatory insult at a molecular level. Strawberry, rich in nutrition, can also be labelled as a cost-effective functional food.

Conflicts of interest

The authors declare that there is no conflict of interest in the study.

Author contributions

N.K.R.G., S.K.P., and L.B. developed the original study aims. N.K.R.G. and F.L. conducted the experiments. N.K.R.G., F.L., S.K.P., and L.B. analysed and interpreted the data; N.K.R.G., F.L., S.K.P., and L.B. prepared manuscript drafts and contributed to the final version. L.B. has been the corresponding author throughout the writing process. All authors read and approved the final manuscript.

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Table S1. Metabolic and gastrointestinal parameters in rats treated with strawberry.

Variable	C	CSB	D	DSB	*P*-Value		
					DSS	Treatment	Interaction
Final body weight, g	562 ± 8	551 ± 18	537 ± 9	550 ± 14	0.3220	0.9387	0.3600
Energy intake, kJ/d	475 ± 22	456 ± 14	469 ± 15	474 ± 15	0.7238	0.6802	0.4812
Feed efficiency, g/kJ	0.146 ± 0.009	0.156 ± 0.012	0.145 ± 0.009	0.145 ± 0.011	0.5661	0.6322	0.6322
Retroperitoneal fat, mg/mm tibial length	266 ± 17	294 ± 47	214 ± 10	275 ± 29	0.2361	0.1403	0.5781
Epididymal fat, mg/mm tibial length	118 ± 8	122 ± 20	107 ± 8	121 ± 7	0.6213	0.4599	0.6804
Omental fat, mg/mm tibial length	171 ± 7	169 ± 20	151 ± 14	160 ± 12	0.3107	0.8050	0.6983
LV + Septum wet weight, mg/mm tibial length	23.4 ± 1.1	24.1 ± 1.4	23.2 ± 1.3	22.1 ± 0.7	0.3497	0.8639	0.4430
RV wet weight, mg/mm tibial length	4.33 ± 0.22	4.47 ± 0.31	3.70 ± 0.31	4.19 ± 0.26	0.1124	0.2661	0.5335
Liver wet weight, mg/mm tibial length	287 ± 9	299 ± 11	288 ± 10	312 ± 7	0.4661	0.0649	0.5270
Kidney wet weight, mg/mm tibial length	65.8 ± 1.1	71.7 ± 3.5	68.8 ± 1.5	72.2 ± 1.7	0.4239	0.0398*	0.5668
Spleen wet weight, mg/mm tibial length	19.8 ± 1.1	23.5 ± 2.0	21.1 ± 1.0	22.4 ± 1.2	0.9429	0.0814	0.3929

WBC, x 10^9/L	4.23 ± 0.81	4.09 ± 0.76	4.72 ± 0.7	2.75 ± 0.6	0.5626	0.1594	0.2194
RBC, x 10^9/L	7.90 ± 0.20	8.40 ± 0.23	8.05 ± 0.27	7.89 ± 0.12	0.4064	0.4326	0.1357
Haemoglobin, g/L	143 ± 3	149 ± 4	143 ± 4	141 ± 3	0.2713	0.5779	0.2713
Haematocrit, L/L	0.41 ± 0.01	0.42 ± 0.01	0.42 ± 0.01	0.41 ± 0.00	1.0000	1.0000	0.2618
MCV, fL	51.5 ± 0.6	50.2 ± 0.7	51.7 ± 0.7	51.3 ± 0.6	0.3306	0.2071	0.4980
MCH, Pg	18.0 ± 0.3	17.8 ± 0.2	17.8 ± 0.3	18.0 ± 0.4	1.0000	1.0000	0.5238
MCHC, g/L	348 ± 1	354 ± 2	345 ± 2	347 ± 6	0.1516	0.2470	0.5577
Platelets, x 10^9/L	906 ± 30	860 ± 41	910 ± 28	943 ± 84	0.3849	0.8956	0.4292

All values are mean ± SEM, n = 6-8. Mean values within a row with a different superscript are significantly different, $P<0.05$. C, control (0% DSS); D, 0.5% DSS; CSB, C + strawberry; DSB, D + strawberry; DSS, dextran sodium sulfate; WBC, white blood cell; RBC, red blood cell; MCV, mean corpuscular volume; MCH, mean corpuscular hemoglobin, MCHC, mean corpuscular hemoglobin concentration. CSB and DSB rats were treated with strawberry for last 6 weeks of the 12 weeks' protocol.

Chapter 6. Discussions and conclusions

1. Summary of results

My thesis investigated functional foods that may be used as therapeutic interventions in IBD. With regard to this, I started my thesis with the first chapter on introduction of IBD followed by review on nutraceuticals in IBD and, in the following chapters, I validated a chronic IBD rat model and tested prospective functional foods for their ability to attenuate IBD in my rat model. Here I summarise the key take-home messages from each of the chapters, followed by the limitations of my study and future directions of my study.

Chapter 1 titled "Introduction" discussed briefly the overview of IBD, the possible molecular players in the development of IBD and the role of diet in IBD. I emphasized the need to develop a chronic IBD rat model, along with possible nutraceuticals that may be used to test in that rat model.

Chapter 2 titled "Nutraceuticals in rodent models as potential treatments for human Inflammatory Bowel Disease" discussed the functional role of naturally occurring bioactive compounds such as polyphenols in attenuating IBD. Initially, I briefly discussed the epidemiology of IBD which has increased in incidence from western developed countries to developing countries in east. Further, the current drug treatments with their adverse effects and cost is a real burden for IBD patients. So the need to validate new therapeutics led to natural products owing to their reduced adverse effects and relatively lower cost. These advantages are only useful if the natural products are effective in reducing IBD symptoms. Natural products showed their efficacy in modulating the key pathogenic pathways of IBD, such as dysbiosis, oxidative stress, pro-inflammatory cytokines, immune system dysregulation and inflammatory cell signalling pathways. Fructo-oligosaccharides and fibre-rich foods such as germinated barley act as prebiotics that increased the commensal gut bacteria pre-clinical and clinical studies proving to be prospective component of IBD therapy. Curcumin, anthocyanins, punicalagin, 6-gingerol and 6-shoagol inhibited pro-inflammatory cytokines and inflammation. Curcumin modulated several of these pathways including oxidative stress, pro-inflammatory cytokines, immune system dysregulation and inflammatory cell signalling pathways to inhibit the production of pro-inflammatory cytokines. The NF-κB inflammatory cell signalling pathway is

linked to the production of TNF which is suppressed by many functional foods, thereby improving the intestinal homeostasis and ameliorating the inflammation.

Chapter 3 titled “An improved rat model for chronic inflammatory bowel disease” discussed the development of a rat model for chronic IBD. To validate the potential of the natural products in IBD treatment, a well-established animal model is needed. I conducted this study to develop a chronic reversible IBD model as the literature abounds in acute and prevention therapy animal models of IBD, however IBD is a chronic lifelong disease. So I tested low concentrations of dextran sodium sulphate (DSS; 0, 0.25, 0.5 and 1%) for 6 weeks of which 0.5% DSS was selected for the development of relatively stable changes in the stool consistency and bleeding characteristics, including the ileum and colon histology. Further, I extended the timeline to 12 weeks with 0.5% DSS and also replaced 0.5% DSS with normal water after 6 weeks. This study established that 0.5% DSS for 12 weeks is a good model of chronic IBD mimicking human IBD with characteristics of diarrhoea, bloody stools, impaired ileum and colon tissue architecture including mucosal inflammation, loss of epithelial layer, villi and crypts, infiltration of inflammatory cells and dysbiosis including increases in phylum Proteobacteria, *Ruminococcus gnavus*, *Oscillospira* sp and *Streptococcus* sp. Further, DSS-induced changes were limited to the gastrointestinal system, with minor changes in cardiovascular, liver and metabolic parameters. These gastrointestinal symptoms were improved in the DSS replacement group suggesting that my model was suitable for testing treatments for reversal therapy. Therefore, I tested sulphasalazine, a standard first line of drug therapy for IBD patients, in my rat IBD model. Sulphasalazine treatment also improved the gastrointestinal IBD symptoms.

Chapter 4 titled “Cyanidin 3-glucoside attenuates inflammatory bowel disease in rats” discussed the findings of my study on anthocyanin-containing foods in my chronic IBD rat model. Considering that functional foods containing anthocyanins have anti-inflammatory activity, I investigated the effects of Queen Garnet plum and purple carrot juices as sources of the anthocyanin, cyanidin 3-glucoside (C3G). Further, I tested pure C3G as well as the functional foods. The key findings are that the 0.5% DSS rats on Queen Garnet plum, purple carrot and C3G had improved gastrointestinal symptoms of IBD. During the treatment phase, the stool consistency and bleeding scores reduced dramatically and the gut histology supported the physical

characteristics. The ileum and colon had improved epithelial membrane, villi and crypts. These changes correlated with the gut bacteria analysis for the C3G-treated rats which clustered closer to healthy control rats, including increases in family Lachnospiraceae and genus *Sutterella*. The responses to the functional foods and C3G treatments was comparable to sulphasalazine of my previous chapter, suggesting that C3G is efficient in ameliorating IBD.

Chapter 5 titled "Pelargonidin 3-glucoside from strawberry improves chronic inflammatory bowel disease in rats" discussed the responses to the anthocyanin, pelargonidin 3-glucoside (P3G) with its anti-inflammatory action on the intestine. I examine whether P3G has similar effects to C3G on my IBD model. The freeze-dried strawberry powder enriched with P3G when added to the diet of the 0.5% DSS rats for the last six weeks of the 12 resulted in improved gut motility observed through increased ileum and colon force of contraction and improved gastric emptying. Reduction of watery and bloody stools, improved epithelium lining of ileum and colon, increased villi height, crypt depth and goblet cells were key observations with the treatment. P3G powder improved gut characteristics in chronic IBD rat model through its anti-inflammatory activity. The P3G treatment was comparable with the C3G and sulphasalazine treatments as well indicating that C3G and P3G at optimum doses can attenuate IBD as well as the standard drug.

2. Limitations

Limitations of my investigations are that systematic and meta-analysis reviews of the functional foods in the treatment of chronic diseases in pre-clinical and clinical trials was not done. This may provide deeper understanding on the impact of functional foods on our health and how to effectively benefit with their positive features(1). Although DSS is the causative agent in my IBD model, the cause of the human disease is unknown (2). Therefore, the cause of IBD is not mimicked in my rat IBD model. Moreover, the IBD model was specific to gut inflammation and no systemic inflammation was observed. So, a longer protocol in rats could manifest extra-intestinal changes due to chronic systemic inflammation following increased intestinal permeability and produce cardiovascular changes as observed in IBD patients (3, 4). Though functional and structural changes have been characterised in my IBD model and the treatment groups, the molecular changes were not investigated. Molecular

mechanisms by which the bioactive food-derived compounds modulated the intestinal inflammation would give an in-depth understanding of the key signal transduction pathways that trigger cytokines to improve or worsen inflammation (5). My studies used only a single dose of C3G and P3G (8 mg/kg bw/d) and higher doses can have more efficacy as in blueberry anthocyanin extract (6). Heat maps generated for the gut microbiota in my studies were limited with taxonomic relative abundance. However, metagenomics analysis for microbial function would give information on the metabolic pathways altered based on the relative abundance of the gut microflora due to the treatments in healthy or IBD rats (7-9). In my studies, faecal samples were analysed for gut bacteria abundance rather than mucosal bacteria. Many bacteria are in close proximity to mucosal lining of the gut and the mucosal inflammation is key in IBD, so there are differences in the composition of faecal and mucosal bacteria between healthy and IBD patients (10). It may be worth collecting both faecal and mucosal samples for gaining a better understanding of the gut bacteria and estimate the short chain fatty acids produced by gut bacteria. Fecal lipocalin, a marker for inflammation caused by DSS was not evaluated but it could be done in future to confirm the histology that showed gut inflammation.

3. Future directions

The incidence of IBD is growing around the world, inflicting a younger population than ever before, who are at higher risk of colorectal cancer and the growing need of biological drug therapy is taking a huge toll on the lower socio-economic populations especially in developing countries (11). Thus there is a growing need for complementary therapies for IBD. There is growing evidence that functional foods with anti-inflammatory and anti-oxidant activities can ameliorate IBD (12, 13). The outcome of my research provides the evidence for Queen Garnet plum, purple carrot rich in C3G and strawberry rich in P3G as functional foods that mitigate intestinal inflammation. However, more mechanistic studies need to be done with regard to the absorption, bioavailability, absorption, distribution, metabolism and excretion of the bioactive compounds from the tested foods.

Anthocyanins activate anti-oxidant transcription factor nuclear factor E2-related factor 2 (Nrf2) and lead to improved cell survival (14, 15). However, they can also inhibit NF-κB proinflammatory signalling pathways independent of Nrf2

mechanism (16). Therefore, a better understanding of the effect of C3G and P3G on Nrf2 and NF-κB signalling pathways will enrich our understanding on their anti-oxidant and anti-inflammatory activities. Three dimensional co-culture cell models with epithelial cells, macrophages and bacteria may be helpful to determine the molecular mechanisms of inflammation regulation with treatment of different concentrations of C3G and P3G (17). Nrf2-deficient mice are more sensitive to DSS-induced colitis (18) and testing these anthocyanins in such a model may give an idea of their Nrf2-independent anti-inflammatory activity. NOD2 and ATG16L1 are the major IBD susceptible genes which regulate intracellular bacteria sensing and elimination, respectively (19). The NLRP3 inflammasome, (NLRP3, an IBD susceptible gene), interlinked with NOD2 and ATG16L1, has mucosal protective effects against pathogenic bacteria (20, 21). It will be worthwhile to test the C3G and P3G foods for their responses on these key players in intestinal inflammation. A novel cytokine, IL-38, reduced IL-17A and IL-22 secreted by Th17 cells but not IFN-gamma secreted by Th1 cells in human PBMCs infected by *C. albicans* that induces production of IL-17 (22). Th17 is known to be involved in the pathogenesis of IBD for 1L-17A which makes it interesting to explore the role of IL-38 in intestinal cells as well (23). IFN-gamma has negative feedback regulation by PTPN2, an IBD susceptible gene, in absence of which IFN-gamma increases the expression of claudin-2 leading to increased intestine permeability (24). However, IFN-gamma selectively increased the porosity for large molecules such as *E. coli*-derived LPS that leads to systemic inflammation as well and not to small molecules across the barrier (25). Localized effects of DSS caused inflammation of the gut, gut bacterial changes but did not cause permeability changes. These bacterial changes are likely to lower production of short chain fatty acids as protectors of the gut mucosa, and probably increase LPS production, causing local damage, rather than systemic responses. The gut bacterial changes due to P3G will be studied as we did with C3G. In the interventions study, I can correlate the effect of intestinal permeability results with not only physiology of the gut relating to which part of it is more permeable and damaged/inflamed but also why it could have happened by analysing the tight junction proteins, occludin and claudin 2, along with molecular regulators of these proteins IFN-gamma and PTPN2 including STAT1 and STAT3. Before venturing into clinical trials, pharmacokinetic and mechanistic studies in preclinical trials are necessary to ensure safety and efficacy of the treatments (26). Different IBD animal models including those deficient in the

IBD susceptible genes have gut inflammation and anthocyanins are anti-inflammatory compounds so they are expected to produce symptomatic relief in genetic models as well. Therefore, these animal models may be treated with C3G and P3G to further evaluate their antioxidant and anti-inflammatory activities, mucosal barrier protection, gut homeostasis and modulation of cell signalling pathways.

In future, the C3G concentrate of Queen Garnet plum and purple carrot and P3G concentrate of strawberry could be combined at optimum concentrations based on the results of the studies mentioned earlier; this mixture may be able to be patented. This anthocyanin cocktail of C3G and P3G could be tested in a prospective randomised placebo-controlled double-blind multicentre crossover clinical trials in healthy and IBD people including those who have single nucleotide polymorphisms (SNPs) of NOD2, ATG16L1, NLRP3, PTPN2 of different age groups to ascertain the potential as IBD treatment. Since dysbiosis is a major player in IBD, the human biopsies or tissue samples can be studied for genetic and molecular markers to have better idea on the molecular mechanisms that can lead to better therapy. My PhD research would be fruitful in long run by paving way for cost-effective treatments for millions of IBD patients across the globe to improve their health and well-being.

4. Conclusions

A stable reversible chronic IBD rat model was established with low dose of 0.5% DSS for 12 weeks unlike in other published studies. This is the first research to evaluate Queen Garnet plum, purple carrot and strawberry in as reversal treatment in a model of chronic IBD. Queen Garnet plum and purple carrot attenuated the gastrointestinal IBD symptoms similar to C3G and sulphasalazine in this IBD rat model. Further P3G-containing strawberry powder also ameliorated IBD similar to C3G and sulphasalazine, suggesting that both anthocyanins are efficient in treating IBD. The take-home message of my PhD research is that optimum intake of anthocyanins such as C3G and P3G restores intestinal homeostasis and thereby may be helpful in chronic IBD management.

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