# Improving animal models and investigating a raspberry-derived treatment for leaky gut diseases

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## ABSTRACT

The intestinal epithelium is a selective mass transport barrier where nutrients from our food are absorbed into the bloodstream while bacteria and other pathogens remain in the intestine. When intestinal barrier function is diminished pathogens can cross the epithelium, initiating a chronic cycle of immune responses. A growing portion of the population has intestinal barrier dysfunction, commonly called a leaky gut. This leaky gut is implicated in diseases such as inflammatory bowel disease, but we lack an understanding of how the disease manifests or how to treat it most effectively. This work focuses on improving the animal models used to study inflammatory bowel disease and introduces an innovative, fruit-derived approach to treating leaky gut diseases.

The DSS colitis animal model is the most commonly used inflammatory bowel disease model, but the typical administration procedures result in rapid and severe colitis. This leads to symptoms, including increased intestinal permeability, that are more severe than those found in clinical cases. Because this animal model is used in a wide variety of applications to study inflammatory bowel disease, it is important to understand how to better implement it to produce more disease-relevant symptoms. We developed an attenuated DSS model that still produces the hallmarks of chemically induced colitis, but results in intestinal permeability increases that better recapitulate what occurs in patients. We also demonstrate methods for reducing variability when administering DSS, which increases usability of the model and reduces the need for repeat experiments.

To address the growing need for inflammatory bowel disease treatments, we examined how fruits and vegetables affect intestinal permeability. Fruits and vegetables are rich in potentially active natural products, and these metabolites are generally well-handled by the

intestine. We show that raspberry reduces intestinal permeability in vitro and in vivo, which could offer patients an option for disease treatment and prevention. Because this treatment is derived from raspberry, it should not be associated with any of the undesirable side effects of traditional inflammatory bowel disease treatments, improving patient compliance and quality of life. This could offer a safe therapeutic approach for treating inflammatory bowel disease by directly targeting one of the suspected causes: increased intestinal permeability.

## TABLE OF CONTENTS

## LIST OF TABLES

## LIST OF FIGURES

## INTRODUCTION

The human body contains countless examples of selective mass transport barriers, each of which is critical for our overall function and health. Generally, mass transport barriers in the body allow for the selective uptake of nutrients, oxygen, and other required compounds, while blocking passage of harmful or toxic substances.[1–4] One such barrier is the intestine, which is made up of a single layer of intestinal epithelial cells connected by a complex set of proteins called tight junctions (Fig I.1). These junctions and epithelial cells work together to regulate mass transport. For a compound to cross the intestinal barrier, it must pass either a) through the intestinal cells (transcellular) or b) in the spaces between cells (paracellular), which are controlled by tight junctions.[4] The result is a selective barrier that allows nutrients to cross the intestinal lining and pass into the bloodstream, after which they travel to various places in the body.

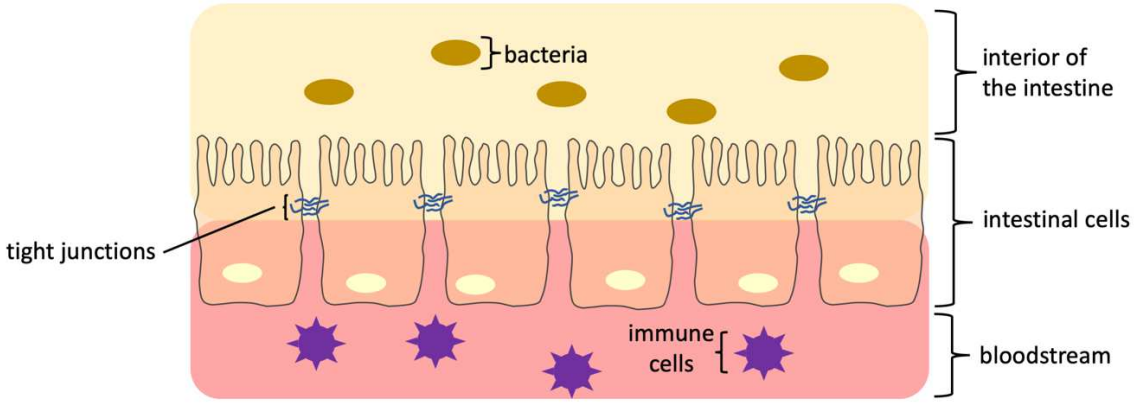



**Figure I.1. A healthy intestinal epithelium provides a selective mass transport barrier.** This allows nutrients to pass into the blood stream but blocks passage of bacteria and other pathogens.

As food transits through the gastrointestinal tract, it is slowly broken down and the nutrients are absorbed. An important aspect of this process is the naturally-occurring bacteria in

the intestine, which aide in the digestion of this food and regulate the intestinal environment.[5] These bacteria are imperative but present a substantial problem if they are able to cross the intestinal barrier into the bloodstream, where they can interact with the immune system. In most healthy people, these bacteria remain largely in the interior of the intestine and serve their intended purpose. However, a growing portion of the population is experiencing dysregulation of the intestinal barrier, or increased intestinal permeability. This is colloquially referred to as a "leaky gut" and is associated with diseases including Crohn's disease and ulcerative colitis, which are types of inflammatory bowel disease (IBD).[1,2]

### I.1 Inflammatory Bowel Disease

IBD is a set of painful and debilitating disorders that affects approximately 1.6 million Americans, with upward of 70,000 new diagnoses each year.[6–8] Although an increasing amount of research is being devoted to studying disease mechanisms and treatments, our understanding of the etiology of these diseases is lacking. Patients with IBD suffer from persistent diarrhea, rectal bleeding, weight loss, fatigue, and abdominal pain, which dramatically impacts their health and quality of life.[9]

Overwhelming evidence points towards a leaky gut being implicated in IBD, but it is unclear if a leaky intestinal barrier is a symptom or a cause of the disease.[10,11] People with decreased intestinal barrier function (or increased intestinal permeability), are unable to properly regulate their intestinal barrier. This results in bacteria and other toxins inside the intestine crossing the intestinal lining and entering the blood stream (Fig I.2).[12] Here, the immune system reacts to the pathogens and a signaling cascade is triggered.[1] This cascade results in the recruitment of inflammatory and other immune compounds at the site, which is known to further

inflame and damage the intestinal barrier. The endless feedback loop is responsible for the chronic nature of IBD and causes substantial health and well-being issues for patients.

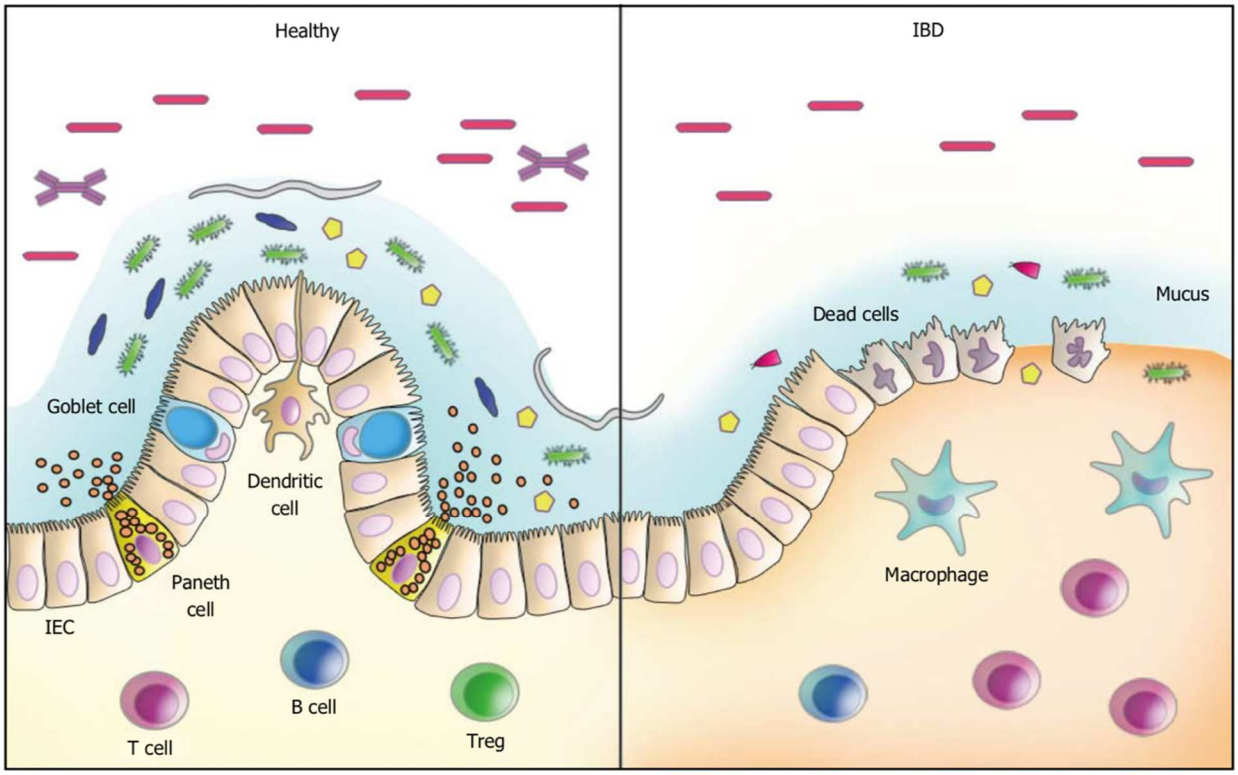



**Figure I.2. Loss of barrier function in IBD results in interactions between gut bacteria and the immune system, leading to inflammation.** Schemetic from Rapozo, Bernardazzi, De Souza 2017.

## I.2 Treatments for IBD

There are limited viable IBD therapeutics, likely due in part to our lack in understanding of the disease. Both genetic and environmental factors are suspected to contribute to the development of IBD, which complicates research aiming to cure the disease.[6,7] A wide variety of drugs are available to treat IBD (Table I.1), and they all manage the disease rather than provide a cure.[13] These include antibiotics, steroids, and immunosuppressants, but each comes with substantial, and often systemic, side effects.[14] Use of antibiotics can lead to digestion issues, while prolonged use of steroids leaves patients with joint problems.

Immunosuppressants, which mitigate the interaction between gut bacteria that leak across the intestine and the immune system, can result in patients being susceptible to infection because of their suppressed immune system.[14] These therapeutics, along with the others on the market (Table I.1), are not always effective and patients often have to cycle through them to retain efficacy and mitigate side effects. Given the severe and chronic nature of IBD, there is an urgent need for novel therapeutics. Because direct testing in humans is typically not possible, studies of new therapeutics are reliant on the use of an animal model.

**Table I.1. Current therapeutics used to manage and treat IBD.** Table from Triantafillidis, Merikas, Georgopoulos 2011.

| Drug group | Drugs |
|---|---|
| Anti-inflammatory | • Mesalazine<br>• Corticosteroids (prednisolone, methylprednisolone, butesonide) |
| Immunosuppressives | • Azathioprine, 6-mercaptopurine, methotrexate, cyclosporin, tacrolimus |
| Antibiotics | • Metronidazole, ornidazole, clarithromycin, rifaximin ciprofloxacin, anti-TB |
| Probiotics | |
| Biologics | • Infliximab, adalimumab, certolizumab pegol |

**Abbreviation:** TB, tuberculosis.

## I.3 IBD Animal Models

Many animal models have been developed for studying IBD, varying in cost and complexity. These include transgenic mice, knockout mice, and mouse models using chemically induced colitis.[15,16] Although each of these models have some characteristics that are similar to the human disease, none fully recapitulate human IBD. For this reason, the animal model must be matched to the particular aspects of the disease being analyzed; no single model is suitable for all studies.[17] The most widely used IBD model is the administration of dextran sulfate sodium

(DSS) in the drinking water of mice to chemically induce colitis.[18] This model is the current standard in many laboratories because it is relatively reproducible, customizable, and does not require the spontaneous development of colitis in transgenic or knockout mice. DSS inflicts damage to the intestinal epithelium that results in inflammation and permeability increases, hallmarks of IBD.[17]

While this model is relatively cheap and easy to use, it induces a rapid and severe colitis that is problematic for studies interested in intestinal permeability. This is because the permeability increases observed with typical DSS studies are many-fold greater than those observed in IBD patients.[19] Fortunately, previous research suggests that it is possible to induce milder colitis symptoms by reducing DSS administration duration.[20] We were motivated to expand upon these findings by evaluating a broad array of colitis metrics, including intestinal permeability, in an attenuated DSS mouse model. Chapter 1 of this thesis focuses on finding DSS administration conditions that result in more disease-relevant increases in intestinal permeability.

### I.4 Reducing Intestinal Permeability

With incidence of IBD on the rise and limited therapeutic options, more innovative approaches to treating the disease are vital. One such approach is to develop therapeutics that directly target intestinal permeability, reducing bacterial passage and consequently inflammatory response.[21] To be an improvement on already available therapeutics, the drug must be both efficacious and well-tolerated. Fruits and vegetables are an intriguing source of new therapeutics because they are already part of the human diet and contain potentially active natural products.[22]

Natural product drugs are a broad category of drugs that were each originally derived from a living organism (plant, bacteria, fungi, etc.). Given that fruits and vegetables contain thousands of natural products, we screened a library to determine their effect on intestinal permeability. Chapter 2 focuses finding a fruit that reduces intestinal permeability, and determining which natural product is responsible for this effect.

## CHAPTER 1: Expanding the utility of the dextran sulfate sodium (DSS) mouse model to induce a clinically relevant loss of intestinal barrier function

Kyle E. Cochran, Dr. Nicholas G. Lamson, and Dr. Kathryn A. Whitehead

### 1.1 Introduction

Inflammatory bowel disease (IBD) is a set of painful and debilitating disorders that affects approximately 1.6 million Americans, with upward of 70,000 new diagnoses each year.[6–8] IBD includes ulcerative colitis, Crohn's disease, and indeterminant colitis, and our understanding of the etiology of these diseases is lacking. Patients with IBD suffer from persistent diarrhea, rectal bleeding, weight loss, fatigue, and abdominal pain, which dramatically impacts their health and quality of life.[9] Current treatments manage, rather than cure, IBD and include antibiotics, steroids, and immunosuppressants.[13,14] Although the full mechanisms through which the disease develops remain uncertain, evidence points to loss of intestinal barrier function as a key aspect of clinical IBD. It is unclear if barrier loss is a symptom or a cause of the disease.[10,11] Thus, reducing intestinal permeability has been the goal of some therapeutic candidates,[23] and the success of such studies is dependent on the use of appropriate animal models.

The most widely used IBD model uses dextran sulfate sodium (DSS) that is introduced into the drinking water of mice to chemically induce colitis. Although its exact mechanism for inducing colitis is unknown, DSS administered in the drinking water of mice inflicts damage to the intestinal epithelium.[18,19] This results in colitis symptoms including loose and bloody stool, significant weight loss, shortening of the colon, and decreased intestinal barrier function.[17,18,24] This model is the current standard in many laboratories because it is relatively reproducible, customizable, and doesn't require the spontaneous development of colitis that models using

transgenic or knockout mice do.[17] However, a significant downfall of the model is the rapid and widespread damage to the intestinal epithelium, which doesn't reflect the degree of decreased barrier function in patients.[19]

Studies of potential IBD therapeutics often use 3.0-5.0% DSS for longer durations of six to eight days.[25,26] While this falls within the recommendations of administration protocols,[18] the rapid and severe colitis and epithelial damage induced by these models does not resemble clinical IBD.[19] Specifically, these models result in loss of intestinal barrier function that is far more severe than the losses observed in clinic. Lactulose/mannitol tests have demonstrated intestinal permeability increases of 2- to 18-fold in colitis patients, while the standard DSS model can increase permeability to macromolecule markers by more than 100-fold.[16,17] Studies of therapeutics that restore intestinal barrier function have been limited, likely in part because the standard DSS model destroys the intestinal epithelial monolayer. Fortunately, previous research suggests that it is possible to induce milder colitis symptoms by reducing DSS administration duration.[20] We were motivated to expand upon these findings by evaluating a broad array of colitis metrics, including intestinal permeability, in an attenuated DSS mouse model.

To identify DSS colitis protocol conditions that better recapitulate the loss of barrier function present in clinical IBD, we fixed the DSS dose at 3.0% and varied exposure length from four to seven days. We tracked the onset and progression of weight loss, fecal samples, colon length, spleen weight, and intestinal permeability and scored colon histological samples. In addition to identifying DSS administration conditions that result in clinically relevant levels of intestinal permeability, we also examined how environmental factors, such as acclimation to experimental conditions, affected colitis onset.

## 1.2 Materials and Methods

### 1.2.1 Animals

Animal protocols were approved by the Institutional Animal Care and Use Committee at Carnegie Mellon University (Pittsburgh, PA), and all experiments were conducted in accordance with approved protocols. Female C57BL/6 mice six weeks of age were purchased from Charles River Laboratories and acclimated to facility conditions for two weeks before studies. Mice were housed under controlled temperature (25ºC) on 12 hour light-dark cycles. Animals were given access to standard diet and water, with experimental groups receiving 3% DSS in their water. Animals were sacrificed early if they lost more than 30% of their initial body weight or if they showed severe signs of distress.

### 1.2.2 Materials

Dextran sodium sulfate colitis grade (DSS, 36,000-50,000 MW) was purchased from MP Biomedicals (Santa Ana, CA). Fluorescein isothiocyanate-dextran 4 kDa (FITC-DX4) was purchased from Sigma Aldrich (St. Louis, MO). Hemoccult Guaiac Fecal Occult Blood Test slides were purchased from VWR (Radnor, PA). Phosphate buffered saline (PBS) was purchased from Thermo Fisher LifeTech (Carlsbad, CA). Water bottles, spouts, cages, and bedding were provided by Mellon Institute Centralized Vivarium.

### 1.2.3 DSS Administration

Glass water bottles with stainless steel drinking spouts were filled with 100 mL of chlorine treated water and placed in each group's cage. For groups receiving DSS, 3.0 grams of DSS was fully dissolved in the water before the bottle was placed the cage. Water level for each

cage was recorded daily and the water was fully changed every two days. Animals received DSS for 7, 6, 5, or 4 days, after which they were switched to fresh water for 7 days unless early sacrifice was required. Groups were designed so that DSS administration was staggered and all animals were switched to fresh water on the same day.

### 1.2.4 Weight and Fecal Scores

Weight was recorded and a small fecal sample was collected for each animal daily. Fecal samples were examined for consistency, tested for blood using Hemoccult slides, and scored accordingly.

### 1.2.5 Intestinal Permeability

Mice were rectally administered FITC-DX4 as a colonic permeation marker (60 mg/mL in PBS, 600 mg/kg animal). Three hours later, blood samples were taken via the submandibular gland and the serum was examined for fluorescence at 495/519 nm on a BioTek Synergy2 plate reader. Application of a calibration curve yields a blood concentration of FITC-DX4.

### 1.2.6 Evaluation of Colons and Spleens

Upon sacrifice, the spleen and colon of each animal were excised. Colons were measured to the nearest half millimeter then fixed in 4% formaldehyde for 24 hours. Fixed colons were washed twice with PBS and subsequently stored in 70% ethanol at 25ºC. Hematoxylin and eosin (H&E) staining was performed on colon cross-sections by University of Pittsburgh Medical Center Tissue and Research Pathology Services (Pittsburgh, PA). Stained slides were scored and

imaged by Dr. Lora Rigatti, University of Pittsburgh (Pittsburgh, PA). Spleens were weighed on an Ohause Adventure Pro analytical balance.

### 1.2.7 Statistical Analysis

Animals were placed in group of 6 per cage, and premature animal deaths were accounted for in analysis. Error is displayed in all plots as the standard error of the mean and experimental groups are compared to the control individually using ordinary t-test, without correction for multiple comparisons.

## 1.2 Results

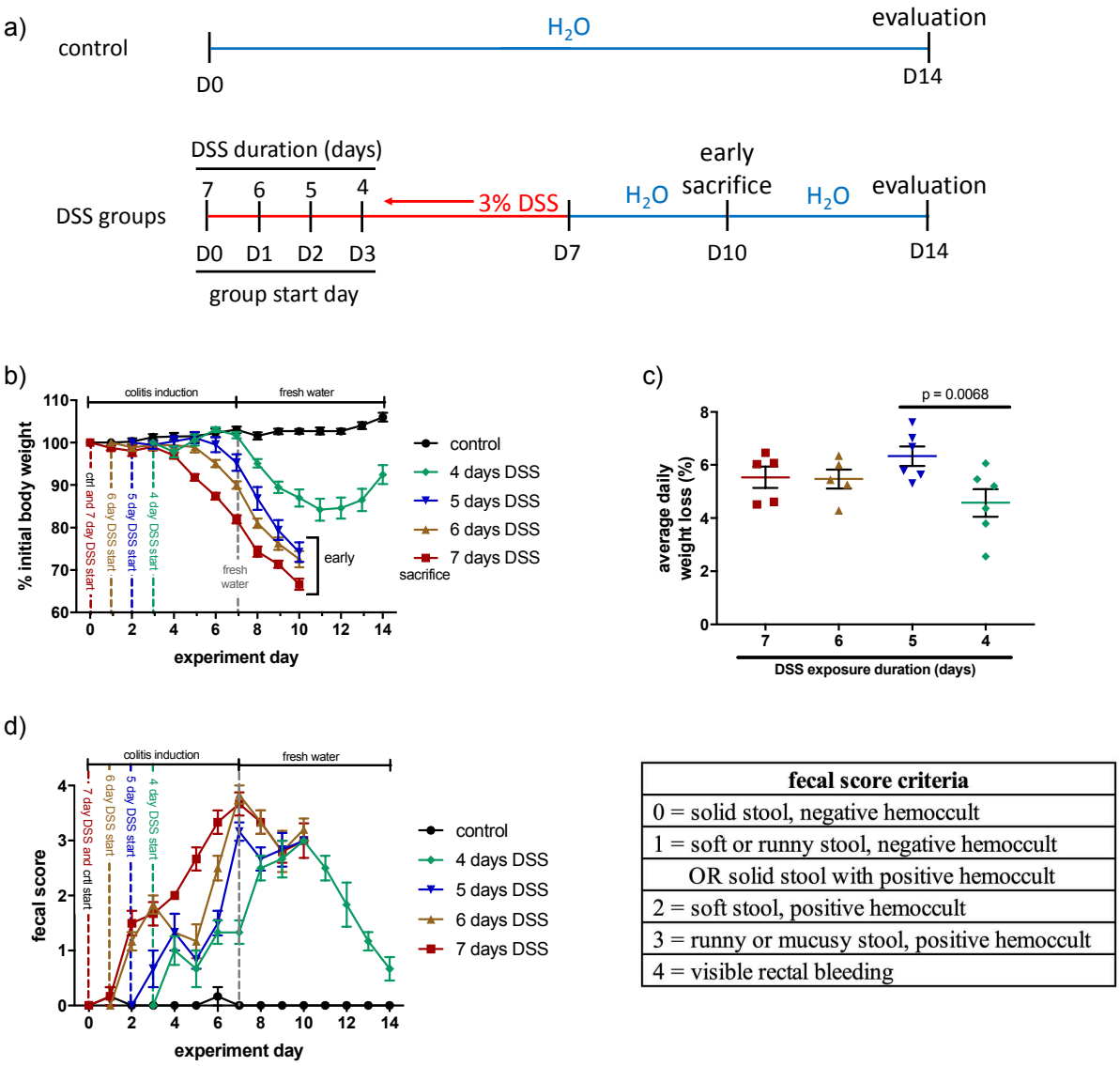
The use of an animal model that recapitulates key aspects of colitis is crucial to the study of disease mechanisms and evaluation of potential IBD therapeutics. One of the most common colitis models, the DSS model, is highly tunable and can be used to induce colitis of varying severities. We reasoned, therefore, that it would be possible to induce colitis symptoms without destroying intestinal barrier function.

### 1.3.1. Establishing a Model with Less Severe Symptoms

To identify a chemically-induced colitis protocol that results in less severe, yet measurable, symptoms, we examined how altering the administration duration of DSS affected symptom onset. To accomplish this, groups received 3.0% DSS in their drinking water for either seven, six, five, or four days. After the DSS administration period, they were switched to fresh water for seven days, or four days if early sacrifice was required. Starting with the seven-day group on Day 0, each group began receiving DSS on subsequent days, such that all animals were

switched to fresh water on Day 7 (Fig 1.1a). Each day, mice were weighed, and fecal samples were collected and scored. All DSS animals began losing substantial weight four to five days after the start of DSS administration (Fig 1.1b), but only the 4-day DSS group made any recovery before sacrifice was required. The seven, six, and five-day groups were unable recover body weight, and each required early sacrifice because they reached 30% weight loss (a predetermined endpoint of the study). Average weight loss per day was calculated between the fourth and eighth day of DSS administration. Average daily weight loss was similar between colitis groups, but animals from the four-day DSS group lost less weight per day than animals from the five-day DSS group ($p = 0.0068$, Fig 1.1c).

Each day, fecal samples were taken and scored for consistency and blood content (Fig 1.1d). Colitis is indicated by the increasing fecal scores observed for all DSS groups. Similar to weight loss, the onset of increasing fecal scores was similar across all colitis groups, but only the 4-day DSS group recovered after returning to fresh water. Together, these results indicate that shortening the DSS administration time affected the severity, rather than the onset, of colitis symptoms such as weight and fecal score.




| fecal score criteria |
|---|
| 0 = solid stool, negative hemoccult |
| 1 = soft or runny stool, negative hemoccult |
| OR solid stool with positive hemoccult |
| 2 = soft stool, positive hemoccult |
| 3 = runny or mucusy stool, positive hemoccult |
| 4 = visible rectal bleeding |

**Figure 1.1. Weight loss and fecal scores were affected by the duration of DSS exposure.** a) DSS was administered to the 7-day DSS group on D0, followed by the 7-day group on D1, and so on. Early sacrifice was required for mice in the 7-, 6-, and 5-day DSS groups. b) Mice from each group began losing weight four to five days after the start of their DSS regimen. Weight loss from all groups continued after switching to fresh water, with all but the 4-day DSS group reaching the 30% weight loss cutoff. Only the 4-day DSS group recovered body weight. c) Average daily weight loss was calculated for each animal, between the fourth and eighth days of their DSS regimen. Weight loss was similar across groups, with the four-day group losing the least weight per day. d) Fecal samples were scored daily and ranged from 0-4, with 4 indicating the most severe symptoms. Error bars for all panels display s.e.m. (n = 5 – 6).

### 1.3.2 Effect of DSS Administration Duration on Intestinal Barrier Function

Decreased intestinal barrier function is a symptom of clinical IBD and IBD animal models.[18,29] To demonstrate the loss of intestinal barrier function during colitis development, we rectally administered the permeability marker FITC-DX4 (4 kDa) on Days 7, 10, and 14 and collected blood samples three hours later. The concentration of this fluorescent permeability marker in serum samples was analyzed to assess the barrier function of the animals' colons.

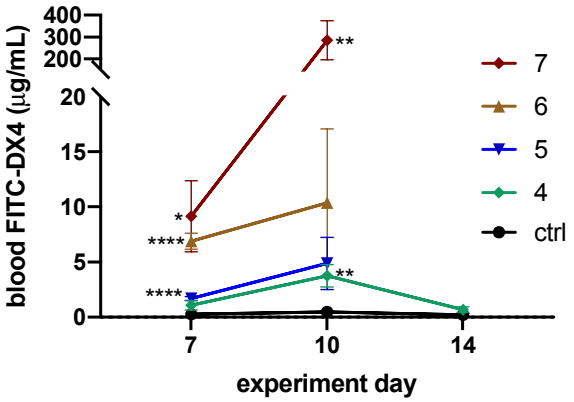



**Figure 1.2. DSS colitis increased intestinal permeability, the severity of which depended on DSS exposure duration.** Intestinal permeability measured at Day 7 was significantly increased for all groups except the four-day DSS group. By Day 10, the permeability of the seven-day DSS group was nearly 28-fold higher than any other group. Seven days after being switched to fresh water (Day 14), the intestinal permeability of the four-day DSS group returned to normal. Mice that received DSS for only four days experienced a significant increase in permeability but recovered to baseline after seven days of fresh water. * $p < 0.05$, ** $p < 0.01$ **** $p < 0.0001$ compared to the PBS control, error bars display s.e.m. ($n = 5 – 6$).

On Day 7, the day on which each group completed their DSS regimen and was switched to fresh water, the seven ($p = 0.005$), six ($p < 0.0001$), and five-day ($p < 0.0001$) DSS groups each had significantly elevated intestinal permeabilities (Fig 1.2). The four-day DSS group had permeabilities that were slightly higher than the control, while the seven-day DSS group showed the most intense increase in permeability, increasing 35-fold compared to the control. Permeability measurements on Day 10, three days after animals were switched to fresh water,

showed that barrier function continues to decline even when DSS is discontinued. At this point, the seven-day group showed a greater than 600-fold increase in intestinal permeability compared to the control ($p = 0.0063$), while the six ($p = 0.1364$), five ($p = 0.0936$), and four-day ($p = 0.0089$) DSS groups all had less than a 22-fold increase (Fig 1.2).

Because animals from the seven, six, and five-day DSS groups reached greater than 30% body weight loss around Day 10, they required early sacrifice. Only the control and 4-day DSS groups continued through the entire 14-day experiment. On Day 14, the final intestinal permeability measurement for the four-day DSS group showed that animals had returned to near control values, indicating a restoration of colonic barrier function (Fig 1.2). Overall, the abbreviated 4-day DSS protocol results in a measurable increase in intestinal permeability without permanently destroying intestinal barrier function.

### 1.3.3 Effect of DSS Administration Duration on Spleen Weight and Colon Length

As colitis progresses and the epithelium begins to erode, the colon thins and becomes shorter and immune cells infiltrate the lamina propria.[18] Infiltration of immune cells results in inflammation that is often assessed by measuring spleen weight. These phenomena provide two additional metrics, colon length and spleen weight, by which the severity of chemically-induced colitis can be measured.[18] To examine these aspects of the disease model, mice were sacrificed on the final day of the experiment and their colons and spleens were harvested. Spleen weights from all groups differed significantly from the control ($p = 0.0003$ for seven-day, $p = 0.0008$ for six-day, $p = 0.0127$ for five-day, $p = 0.0047$ for four-day), but only the four-day DSS group experienced enlarged spleens (Fig 1.3a). The seven, six, and five-day DSS groups each had spleen weights that were significantly less than the control. Dehydration and lack of a recovery

(requiring early sacrifice) are likely the reasons that groups receiving DSS for longer durations had lower spleen weights.

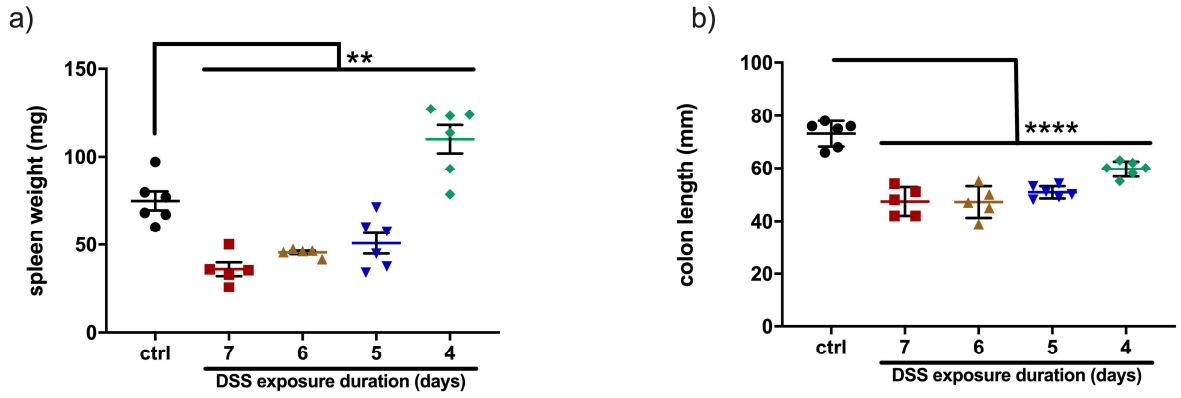



**Figure 1.3. Only animals from the 4-day DSS group experienced an increase in spleen weight, but all DSS groups had significant shortening of the colon.** a) The spleens from all other groups weighed significantly less than the control group, likely due to the animals' severe dehydration and a lack of recovery from colitis. b) Although a four-day DSS regimen resulted in less severe symptoms by most metrics, it nonetheless produced measurable colitis. For both panels, * $p < 0.05$, **** $p < 0.0001$, error bars display s.e.m. ($n = 5 – 6$).

The colons of animals from all DSS groups were significantly shorter than control animals ($p < 0.0001$ for all groups, Fig 1.3b). Although the symptoms of the four-day DSS protocol were less severe, thinning and shortening of the colon nonetheless occurs. This indicates that colonic shortening, one of the hallmarks of chemically induced colitis, is retained in this attenuated four-day model and provides a viable metric against which to compare IBD therapeutics.

### 1.3.4 Histological Evaluation of Colon Samples from the Colitis Model

To assess the effect that DSS administration duration has on the degree of damage to the intestinal epithelium, three colon samples from each group were stained with hematoxylin and eosin (H&E) and scored. Each sample was blindly scored by a trained pathologist for four

criteria shown in Fig 1.4a: inflammation (0-3), extent (0-3), regeneration (3-0), and crypt damage (0-4). The score from each criterion was multiplied by a score for the involvement (0-3) to indicate how much of the sample section was affected. Possible scores ranged from 0-39, with higher scores indicating increased damage to the intestinal epithelium.

a)

| Scoring System | | |
|---|---|---|
| Inflammation | 0 | None |
| | 1 | Slight |
| | 2 | Moderate |
| | 3 | Severe |
| Extent | 0 | None |
| | 1 | Mucosa |
| | 2 | Mucosa and submucosa |
| | 3 | Transmural |
| Regeneration | 3 | No tissue repair |
| | 2 | Minimal regenerative change |
| | 1 | Partial regeneration of gland |
| | 0 | Complete regeneration or normal tissue |
| Crypt Damage | 0 | None |
| | 1 | Basal 1/3 damaged |
| | 2 | Basal 2/3 damaged |
| | 3 | Only surface epithelium intact |
| | 4 | Entire crypt and epithelium lost |
| Involvement | 0 | None |
| | 1 | up to 1/3 of the section |
| | 2 | 1/3 to 2/3 of the section |
| | 3 | 2/3 of the section to diffuse |

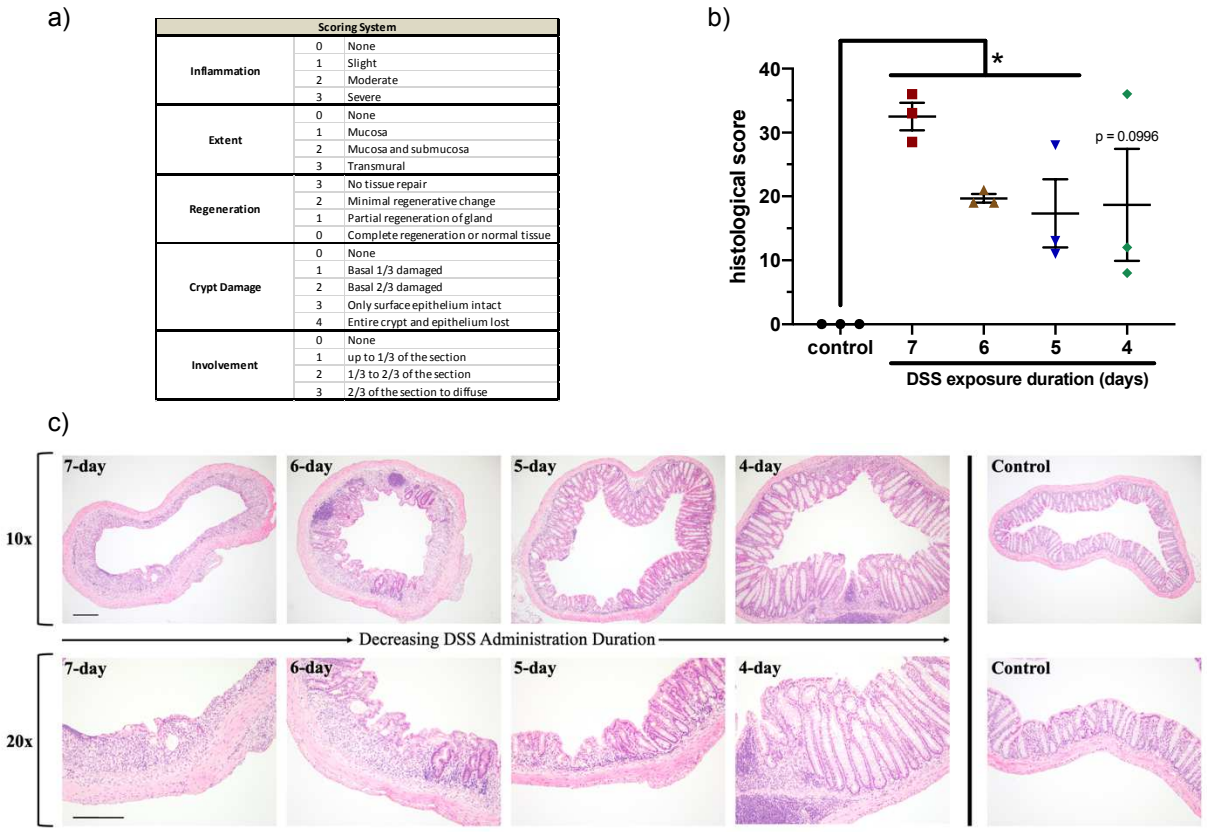



**Figure 1.4. DSS administration duration dramatically affected the integrity of the colonic epithelium.** a) Colon samples were evaluated for inflammation, extent, regeneration, crypt damage, and involvement and scored 0-39. Increasing scores indicate increasing damage inflicted from DSS administration. b) Epithelial and crypt structure were absent from the colon samples of animals exposed to 7 days of DSS. Colonic structure gained integrity as the duration of DSS exposure was reduced. c) Colon samples were H&E stained and representative images were captured at 10x and 20x magnification (scale bars represent 100 µm). * $p < 0.05$, error bars display s.e.m. (n = 3).

Control mice had an average score of 0, indicative of the fully intact epithelium found in healthy mice (Fig 1.4b). Mice that received DSS had individual scores ranging from 8 to 36, and scores generally increased with increasing length of DSS administration. The 7-day DSS group

had the highest score with an average of 32.5 ($p < 0.0001$), followed by the 6-day DSS group with an average of 19.7 ($p < 0.0001$). The five- and four-day DSS groups had the lowest average histological scores, 17.3 ($p = 0.0319$) and 18.7 ($p = 0.0996$), respectively. Images of selected colons in Fig 1.4c show the increasing loss of epithelial structure as DSS administration duration increased. In the seven- and six-day DSS groups, lack of crypts further confirms the severity of the colitis associated with longer DSS administration regimens. Similar to other metrics, the histological scores show that the full seven-day DSS model resulted in substantial damage, unrecoverable to the intestinal epithelium. Reducing DSS administration time decreased the severity of the inflicted damage, although there was some variability within groups.

### 1.3.5 Avoiding Pitfalls: Acclimation to Experimental Conditions

Chemically-induced colitis affects each animal differently, which manifests as highly variable results in these models. To reduce variability, animals should be given ample time to acclimate to experimental conditions before observations begin. In our animal facility, DSS administration required removal of the standard Lixit drinking system, which was replaced with individual water bottles in each cage. Animals must be properly acclimated to this switch before experimental observations begin.

To demonstrate this, we set up control and DSS cages that were switched from the Lixit system to water bottles five days before observations (Day -5) or on the day that observation began (Day 0) (Fig 1.6a and b). For control animals that were switched to water bottles on Day 0 (Fig 1.5a), there was a significant drop off in weight of about 3% on Day 1 ($p = 0.0080$), likely due to hesitation by the mice to drink water from an unfamiliar system. In general, the weight of these mice was less consistent than that of the mice that were acclimated to water bottles five

days before observations began. A similar pattern was observed for mice receiving DSS. On Day 1, mice that were not acclimated to the water bottle lost an average of 5% of their body weight (blue squares, Fig 1.5b). Although these mice did recover that weight, their initial lack of water consumption resulted in a measurable delay in the onset of colitis related weight loss compared to acclimated mice (green circles). On each day after Day 4, acclimated mice had significantly lower weights compared to the control, ($p = 0.0060$ for Day 4, $p = 0.0065$ for Day 5, $p = 0.0288$ for Day 6, and $p = 0.0129$ for Day 7) indicating the faster and more consistent colitis onset. For this reason, we recommend that mice be acclimated to experimental conditions, including drinking from water bottles, at least five days prior to the experiment to allow them ample time to adjust.

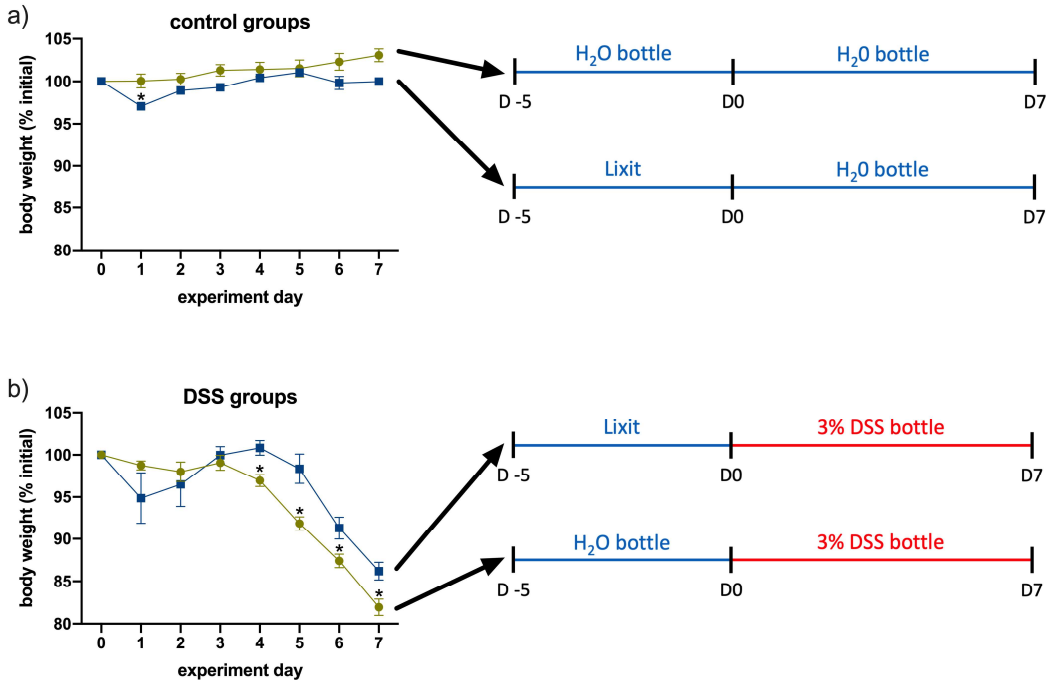



**Figure 1.5. Failing to acclimate animals to experimental conditions resulted in significant differences in symptom onset.** a) When not acclimated to water bottles, control mice (receiving only water) lost up to 3% of their body weight when they were switched from Lixit to bottle water consumption. b) DSS-treated mice not acclimated to water bottles experienced greater fluctuations in body weight and delayed onset of symptoms compared to acclimated mice. For all panels, n = 5 – 6, with error bars representing s.e.m., * = $p < 0.05$.

## 1.4 Discussion

As IBD becomes increasingly prevalent and ever-growing efforts are devoted to understanding and treating the disease, it is imperative to fully understand IBD models and how to best implement them. DSS is often administered at high doses for longer durations to induce an acute colitis.[25,26] Our results confirm that this induces a sweeping loss of intestinal barrier function, which precludes the testing of therapeutics designed to repair the intestinal epithelium.

Decreased intestinal barrier function is a critical factor in clinical IBD and the DSS colitis model due to the subsequent immune cell infiltration and inflammatory response.[17,29] However, DSS administration inflicts widespread damage to the intestinal epithelium that results in permeability increases far higher than those observed in clinical IBD.[27,28] Permeability increased in all groups receiving DSS, but the magnitude of the increase was predicated on administration duration. The four-day DSS protocol produced intestinal permeability changes that best mimic what is observed clinically. First, the 8-fold increase in intestinal permeability for the four-day group best recapitulates the 2- to 18-fold increases observed in IBD patients[27,28]. Furthermore, permeability in this group returned to baseline before sacrifice. These results are more representative of many cases of clinical IBD where a patient experiences bouts of inflammation from which they can recover. Although the 5-day group experienced similar increases in permeability (10-fold compared to control animals), their body weight loss required early sacrifice. In experiments in which epithelial treatments are evaluated, this early endpoint would limit the assessment of the therapeutic since it cannot be administered through symptom resolution. It will be necessary for other research laboratories to run their own attenuated protocols to assess whether 5, 4, or potentially even 3-day DSS administration regimens are most

appropriate for their specific mouse colonies and their specific batch of DSS, which can vary in potency.

Although not a direct measure of inflammation, recording spleen weight at sacrifice is a quick way to correlate inflammation to DSS administration.[18] As colitis progresses in the animal, the resulting immune response leads to an increase of immune cells and thus an increase in spleen weight. The increased weight of the spleens from the four-day DSS group is indicative of the robust immune response present despite abbreviating the colitis induction period. Conversely, the decrease in spleen weight observed in the seven, six, and five-day DSS groups is likely the result of the severe dehydration and weight loss experienced by these animals.

Shortening of the colon is a hallmark of chemically induced colitis and an often-used metric to compare IBD treatments to controls.[18] All groups from this work demonstrated significant colonic shortening, regardless of administration duration. These data indicate that, despite inducing less severe colitis by most metrics, the four-day DSS model retains measurable colitis symptoms against which to compare therapeutics.

Histological scoring also confirmed that the 4-day DSS model produced measurable symptoms of colitis. All DSS groups showed epithelial damage and inflammation compared to the control group, with severity corresponding to administration duration. Histological scores indicate that shorter DSS administration duration generally results in less severe damage to the intestinal epithelium.

Although the etiology of clinical IBD remains unclear, it is generally accepted that genetic predisposition is not the only contributing factor. Diet and lifestyle of western societies are inferred to have some responsibility for the increased prevalence of IBD in countries such as the United States.[6–8] The DSS colitis model is similar in that environmental factors, including

diet and animal facility conditions, impact the induced colitis.[17] This makes comparing results between different studies difficult and leads to increased variability of the model, but measures can be taken to ensure that experiments are reproducible with steady colitis induction. All animals in the experiment should be properly acclimated to the animal housing facility before colitis induction begins. Because DSS must be administered via drinking water, the acclimation period should include training animals to drink from the water bottle, rather than the system-wide fresh water supply. Failing to properly acclimate animals results in altered disease model symptoms, which confounds weight loss due to colitis, stress, and/or dehydration.

## 1.5 Conclusion

The DSS colitis model is a powerful tool for studying disease mechanisms and potential therapeutics for IBD. Administering DSS for seven days results in the rapid onset of severe colitis that precludes the testing of epithelium-focused treatments. Here, we demonstrate that an attenuated four-day model induces increases in intestinal permeability that better mimic those of IBD patients while retaining the hallmark signs of chemically-induced colitis. Because this model does not destroy the intestinal epithelium, it will enable the screening of therapeutics that aim to restore or improve barrier function in colitis patients.

## 1.6 Acknowledgements

The authors also thank M. Oudhoff and J. Gleeson for their helpful discussions and input.

## CHAPTER 2: Raspberry fortifies intestinal barrier function

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### 2.1 Introduction

The intestinal epithelium plays a critical role in the human body, serving as a selective mass transport barrier. In a healthy person, the single layer of epithelial cells that form the intestinal lining allows for the uptake of nutrients from digested food while blocking the passage of bacteria and other pathogens.[30] Intestinal barrier dysfunction is implicated in diseases including celiac disease and inflammatory bowel disease (IBD)[2], and in many cases results in painful and debilitating chronic inflammation of the gastrointestinal tract.[31] Colloquially referred to as a "leaky gut", decreased intestinal barrier function allows naturally occurring gut bacteria to interact with the immune system, resulting in further inflammation.[30] In the case of IBD, which currently affects more than 1.6 million Americans,[6,7] it is well understood that decreased intestinal barrier function plays a role in the disease, though it is unclear if it is a symptom or a cause.[10,11]

Due to the complex nature and unknown etiology of IBD, currently available therapeutics offer disease treatment rather than cure. These treatments include antibiotics, steroids, and immunosuppressants, the most appropriate of which depends on the type and severity of the patient's colitis.[13] While these drugs can offer patients relief from the disease, the side effects they produce often lead to further health complications.[13,14] IBD treatment is an active area of research, but there has been limited successful work to develop novel therapeutics that target intestinal barrier dysfunction.[23] Such drugs would offer the benefit of directly treating a

suspected cause of IBD and may not induce the adverse side effects of current therapeutics, and likely requires exploring novel sources of potential therapeutics.

Natural products are a broad class of chemical compounds originating from living organisms. These compounds are often biologically active and make up a wide-ranging class of therapeutics known as natural product drugs.[32] One such example is paclitaxel (or Taxol), which is an effective and clinically proven chemotherapeutic first isolated from Pacific Yew trees.[33] Fruits and vegetables are an intriguing source of natural product drugs to treat intestinal diseases because they are already part of the human diet and are known to contain thousands of natural product metabolites.[22]

To examine the ability of fruits to affect intestinal barrier function, we first screened a small library using the Caco-2 intestinal monolayer model. After finding that raspberry reduced permeability of the Caco-2 monolayers, we further purified raspberry samples and tested them in mice. Our findings show that raspberry extract reduces the permeability of two different in vitro intestinal models and reduces intestinal permeability in mice. Further, we identified cyanidin, a prominent natural product component of raspberry, as being at least partially responsible for this barrier function modulation. This work provides evidence that natural products from fruits can reduce intestinal permeability and offer potential therapeutic value in the expanding field of intestinal barrier dysfunction research.

## 2.2 Materials and Methods

### 2.2.1 Materials

Conventionally grown red raspberries were purchased from local grocery stores (Giant Eagle Marketplace, Trader Joe's, and Costco, Pittsburgh, PA). Cyanidin was purchased from ChromaDex (Los Angeles, CA). Fluorescein isothiocyanate-dextran 4 kDa (FD4) was purchased from Millipore Sigma (St. Louis, MO) and calcein was purchased from ThermoFisher Lifetech (Waltham, MA). Phosphate buffered saline (PBS) and PrestoBlue Cell Viability Reagent were purchased from ThermoFisher LifeTech. Plastic feeding tubes (oral gavage needles) were purchased from Instech Labs (Plymouth Meeting, PA).

For cell culture and in vitro experiments, Dulbecco's Modified Eagle Medium (DMEM), DMEM-F12/HAM, penicillin/streptomycin, trypsin-ethylenediaminetetraacetic acid (trypsin-EDTA), fetal bovine serum (FBS), Falcon 225 $cm^2$ and 75 $cm^2$ tissue culture flasks, Corning BioCoat HTS 1.0 μm porous support Transwell plates, 0.4 μm polycarbonate Transwell plates, Falcon 24-well plates, Corning CellBIND 96-well microplates Corning 96-well clear bottom plates, sodium butyrate, and MITO+ serum extender were purchased from VWR (Radnor, PA). Porcine serum was purchased from ThermoFisher (Waltham, MA), and epidermal growth factor (EGF) and insulin/transferrin/selenium (ITS) were purchased from Millipore Sigma. Hanks Balanced Salt Solution (HBSS) was purchased from MilliporeSigma. Caco-2 cells were purchased from American Type Cell Culture (ATCC, Manassas, VA) and IPEC-J2 cells were purchased from DSMZ (Braunschweig, Germany).

### 2.2.2 Animals

Animal protocols were approved by the Institutional Animal Care and Use Committee at Carnegie Mellon University (Pittsburgh, PA), and all experiments were conducted in accordance with approved protocol number AR201900009. Female C57BL/6 mice between 6 and 20 weeks of age were purchased from Charles River Laboratories or bred in our facility. All purchased animals were acclimated to facility conditions for at least one week before studies. Mice were housed under controlled temperature (25ºC) on 12-hour light-dark cycles. Animals were given access to standard diet and water. After completion of an experiment, animals were euthanized by carbon dioxide inhalation and death was confirmed via cervical dislocation.

### 2.2.3 Cell Culture

Caco-2 cells were cultured in DMEM supplemented with 10% FBS and 1% penicillin/streptomycin. IPEC-J2 cells were cultured in DMEM-F12/HAM supplemented with 10% PS, 1% penicillin/streptomycin, 5 ng/mL EGF, and 1% ITS. Cultures were incubated at 37°C in a fully humid, 5% $CO_2$ environment. Cells were passaged every 3-4 days at 1:3-8 ratios by applying 0.25% trypsin-EDTA. Caco-2 cells used for experiments were between passage numbers 25-60. IPEC-J2 cells used for experiments were between passage numbers 10-25.

### 2.2.4 Caco-2 Monolayer Experiments

For Caco-2 transepithelial electrical resistance (TEER) and permeation experiments, monolayers were grown using the rapid 3-day method.[34] Cells were suspended in DMEM supplemented with MITO+ serum extender (basal seeding medium, BSM) and seeded onto collagen coated HTS 1.0 μM pore 0.33 $cm^2$ Transwell plates at $2 \times 10^5$ cells/well. Forty-eight

hours later the media was removed and replaced with fresh media composed of DMEM, MITO+ serum extender, and 2 mM sodium butyrate (enterocyte differentiation medium, EDM) and the cells were incubated for 24-48 hours. TEER was monitored for formation of a proper barrier and only monolayers with initial TEER values of at least 150 $\Omega \times cm^2$ were used for experiments.

Transwell inserts containing Caco-2 monolayers were transferred to 24-well plates containing 1 mL of DMEM per well and allowed to equilibrate for 30 minutes before initial TEER values were recorded using a Millicell voltohmmeter. Raspberry extract treatments reconstituted in EDM were applied to the apical side of the monolayers. Negative control well received fresh EDM. TEER measurements were recorded at 15, 30, 60, 120, and 180 minutes.

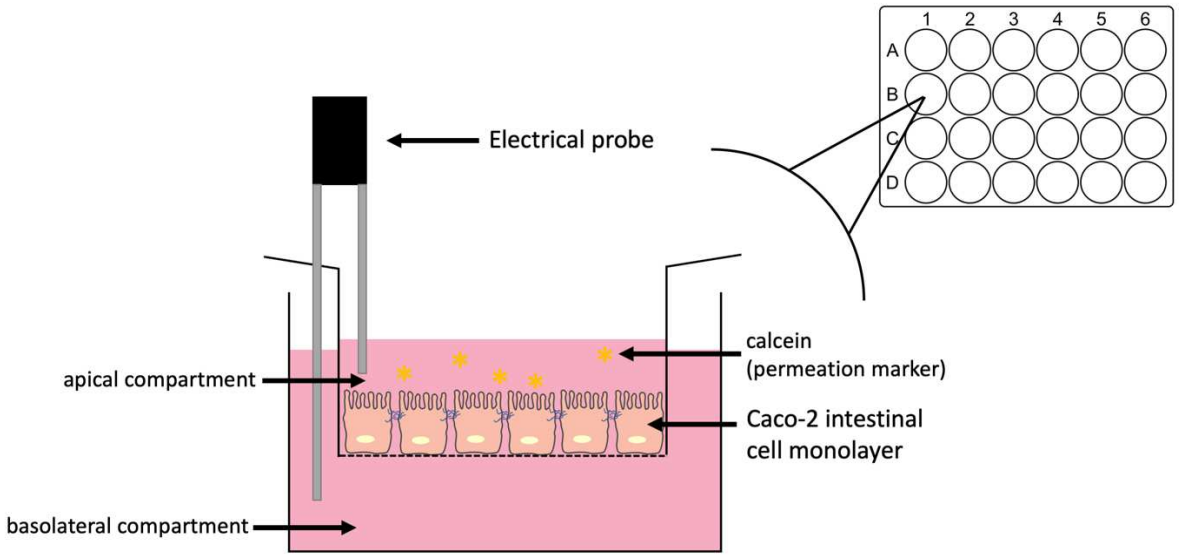


**Figure 2.1. Caco-2 monolayers grown on semi-permeable membranes (Transwell plates) provide a model to test the permeability changing effect of compounds.** Both TEER and calcein permeation are used to assess permeability.

Calcein (0.5 mM) was used to assess molecular permeability of the monolayers by applying the marker with treatments (in EDM) to the apical side of the monolayers. At 1, 2, and

3 hours, the basal chamber was changed to maintain sink conditions and the basal medium was examined for fluorescence at 495/515 nm using a BioTek Synergy2 plate reader. Application of a calibration curve produced the concentration of calcein in the basal compartment, and apparent permeability was calculated by:

$$P_{app} = \frac{\Delta M}{C_a A \Delta t}$$

where $P_{app}$ is the apparent permeability through the monolayer, ΔM is the marker mass in the basal compartment, $C_a$ is the apical marker concentration, A is the area of the monolayer, and Δt is the time between sampling. Permeability measurements are expressed as the ratio of the permeation across the monolayer between hours 2 and 3 of the experiment, normalized to the untreated control.

### 2.2.5 Raspberry Extraction

To produce a crude raspberry product, fresh raspberries were blended in a household blender until a homogenous mixture was formed. After blending, the fruit pulp was filtered from the liquid via vacuum filtration through a Büchner funnel with No. 1 filters and a household coffee filter. The resulting liquid was lyophilized and stored at -80°C until use.

A more potent raspberry extract was produced by lyophilizing fresh raspberries before grinding the fruit to a powder with a household food processor. The powder was extracted with a 60/40 solution of ethanol/water (1 L of liquid per 300g of fresh fruit) for twenty-four hours before being filtered via vacuum filtration through a Büchner funnel with No. 1 filters and a household coffee filter. The resulting liquid was rotary evaporated to remove ethanol and leave an aqueous product. This aqueous product was then mixed with acetone (1 L acetone per 300 grams fresh fruit) and stirred for twenty-four hours to precipitate out excess sugars in the extract.

The resulting mixture was filtered via vacuum filtration through a Büchner funnel with No. 1 filters and a household coffee filter and rotary evaporated to remove acetone. The aqueous extraction product was lyophilized and stored at -80°C until use.

### 2.2.6 In Vivo Permeability Experiments

Before all in vivo intestinal permeability experiments, mice were fasted for 12 hours but given assess to water ad libitum. Raspberry extract was reconstituted in PBS at 60 mg/mL and rectally administered to mice at 10 mL/kg (600 mg/kg). Control mice received enemas of PBS. Rectal administration was performed using a lubricated 1 mL pipette on mice anesthetized briefly with isoflurane. One to eight hours after raspberry extract or PBS administration, mice received an enema of the fluorescent permeation marker, calcein (40 mg/kg). Three hours after administration of calcein, blood draws were taken via the submandibular gland and serum samples were examined for fluorescence at 495/515 nm using a BioTek Synergy2 plate reader. Application of a calibration curve yielded calcein concentration in the blood, which was compared to control animals to assess intestinal permeability.

To examine the effect of raspberry when administered orally, mice were orally gavaged with raspberry extract at 120 mg/mL (10 mL/kg, 1200 mg/kg) using either flexible plastic gavage needles or rigid stainless-steel gavage needles. Control mice received PBS gavages and the rectal group received enemas as described above. Six hours later for the oral group and three hours later for the rectal group, calcein was rectally administered as a permeation marker (40 mg/kg). Three hours after calcein administration, blood draws were taken via the submandibular gland and serum samples were examined for fluorescence at 495/515 nm using a BioTek

Synergy2 plate reader. Application of a calibration curve yields calcein concentration in the blood, which is compared to control animals to assess intestinal permeability.

### 2.2.7 Repeat Dosing Experiments

To study the effect of repeated raspberry doses, mice were orally gavaged raspberry extract each day for nine consecutive days. On the $10^{th}$ day, twenty-four hours after the most recent dose, FD4 was rectally administered as a permeation parker (600 mg/kg). Three hours after FD4 administration, blood draws were taken via the submandibular gland and serum samples were examined for fluorescence at 485/515 nm using a BioTek Synergy2 plate reader. Application of a calibration curve yields FD4 concentration in the blood, which is compared to control animals to assess intestinal permeability.

### 2.2.8 IPEC-J2 Monolayer Experiment

For IPEC-J2 TEER experiments, monolayers were grown using the 21-day method.[35] Cells were suspended in in IPEC-J2 growth medium and seeded onto polycarbonate 0.4 μm pore size 0.33 $cm^2$ Transwell plates at $0.66 \times 10^5$ cells/well. Twenty-four hours later the media was removed and replaced with fresh media, and subsequent media changes were performed every 48 hours. TEER was monitored for formation of a proper barrier and only monolayers with initial TEER values of at least 15 $\Omega \times cm^2$ were used for experiments.

At the start of the experiment, cell culture media was removed and replaced with transport buffer composed of HBSS, 25 mM HEPES, and and 12.5 mM D-glucose (0.1 mL in the apical compartment, 0.7 mL in the basolateral compartment). Monolayers were equilibrated for 60 minutes before initial TEER values were recorded using a Millicell voltohmmeter. Raspberry

extract and cyanidin treatments reconstituted in transport buffer were applied to the apical side of the monolayers. Negative control wells received fresh transport buffer. The plate was incubated at 37°C and 70 RPM, and TEER measurements were recorded at 60, 120, and 180 minutes.

### 2.2.9 Statistical Analysis

Error is displayed in all plots as the standard error of the mean and experimental groups are compared to the control individually using ordinary t-test (two-tailed), without correction for multiple comparisons.

## 2.3 Results

### 2.3.1 Raspberry reduces permeation across Caco-2 Monolayers

To study the effect of raspberry on intestinal permeability, we first examined its effect on Caco-2 monolayers. The Caco-2 monolayer model is a well-established and commonly used screening tool because the monolayers provide a physiological representation of the intestinal epithelium.[36,37] Moreover, the integrity of the monolayer can be quickly assessed by measuring electrical resistance, referred to as the transepithelial electrical resistant (TEER). A decrease in TEER generally corresponds to an increase in intestinal permeability, while an increase in TEER indicates a decrease in intestinal permeability.[38] The barrier function of the monolayers is also assessed by applying a fluorescent marker, in this case calcein, to the apical side of the cells and tracking its permeation across the monolayers.

Experiments on Caco-2 monolayers were initially performed with crude raspberry, which was produced by blending fresh raspberries, filtering out colloidal debris, and lyophilizing the product (Fig 2.2a). When crude raspberry was applied at 15 mg/mL to the apical side of the

Caco-2 monolayers, TEER values increased significantly over the control within 30 minutes. (Fig 2.2b). The increase in TEER persisted for the remainder of the three-hour experiment and suggested that the crude raspberry extract strengthened intestinal barrier function.

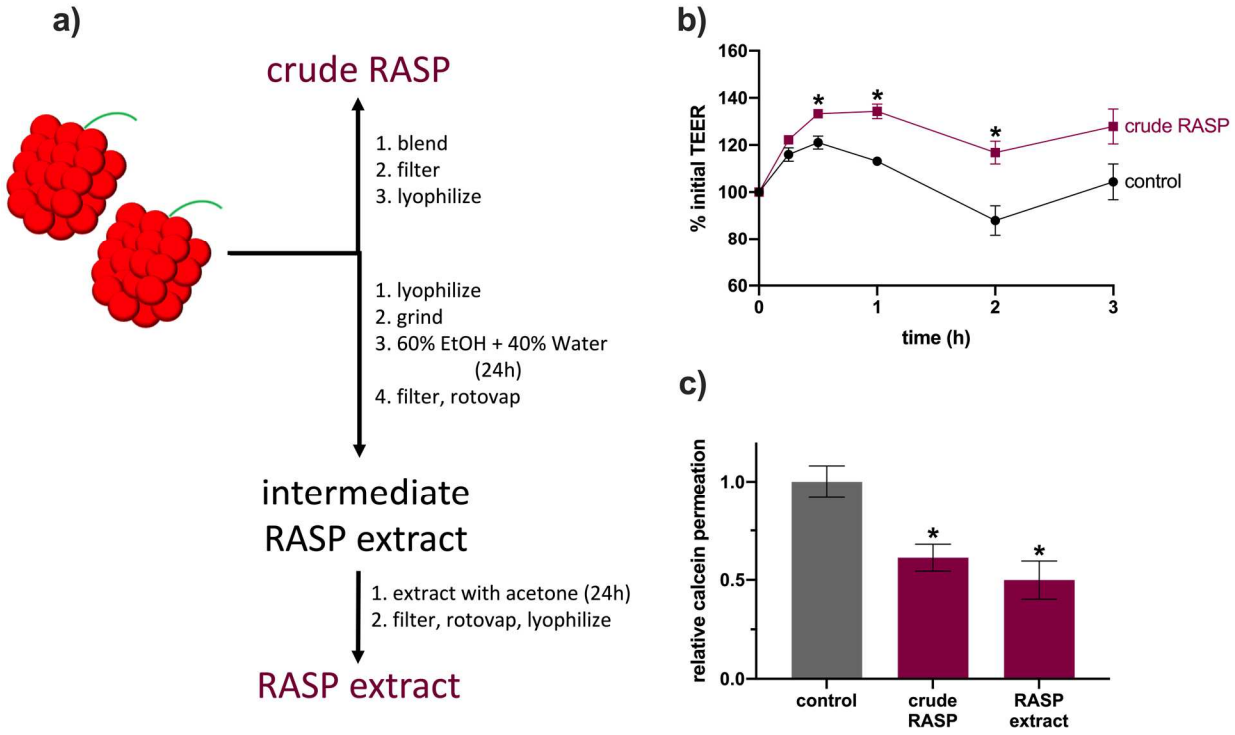


**Figure 2.2. Raspberry increases TEER and reduces fluorescent marker permeation across the Caco-2 intestinal monolayer model.** a) Raspberry was processed to minimally to produce crude RASP or further extracted with ethanol and water, followed by acetone, to produce RASP extract. b) Crude raspberry extract increases TEER values significantly above the control by 30 minutes, and effect that lasts for the duration of the three-hour experiment. c) Raspberry extract (15 mg/mL) and crude raspberry (15 mg/mL) were similarly efficacious in reducing calcein permeation across Caco-2 monolayers. For all panels, error bars represent s.e.m. (n = 3) and * $p < 0.05$

Following this preliminary experiment, we produced a more concentrated raspberry product (RASP extract) using the extraction scheme outlined in Fig 2.2a. Briefly, raspberries were dried and extracted with ethanol and water to remove the majority of soluble natural product metabolites. This intermediate product was mixed with acetone to remove as much sugar as possible and the resulting aqueous product was dried to produce a raspberry extract.

We then analyzed this raspberry extract, together with the crude raspberry, for their effect on calcein permeation across Caco-2 monolayers. Crude RASP and RASP extract significantly reduced permeation by 39% and 50%, respectively, across the monolayers when compared to control monolayers (Fig 2.2c). Although there was no statistical difference between samples, the RASP extract further reduced permeation by 11% compared to the crude RASP. This confirms that raspberry maintains its activity after the more involved extraction procedure and is suitable for further experiments. Moreover, this extraction removes sugar and produces a more concentrated product, meaning that it can be dosed at higher concentrations and thus lower volumes.

### 2.3.2 Raspberry reduces intestinal permeability in mice

Based on these promising in vitro results, we asked whether raspberry extract could improve intestinal barrier function in mice. Initially, we administered raspberry rectally, to the colon, using a lubricated pipet tip. Rectal delivery was chosen over oral delivery to eliminate the possibility of the active raspberry component degrading in the low pH environment of the stomach or by digestive enzymes. To assess the duration of raspberry effect in the colon, raspberry extract was rectally administered at 600 mg/kg, followed by the rectal administration of calcein between one and eight hours later (Fig 2.3a). Three hours after calcein administration, calcein concentration was measured in the systemic circulation to determine its permeation across the intestine. Raspberry extract began reducing intestinal permeability within one hour and continued to act through the eight-hour time point, which was the longest time point considered. At the two hour time point, we observed the maximal permeability reduction of

43%. These results indicate that raspberry extract improves colonic barrier function in the more complex in vivo environment for a considerable time period (at least eight hours).

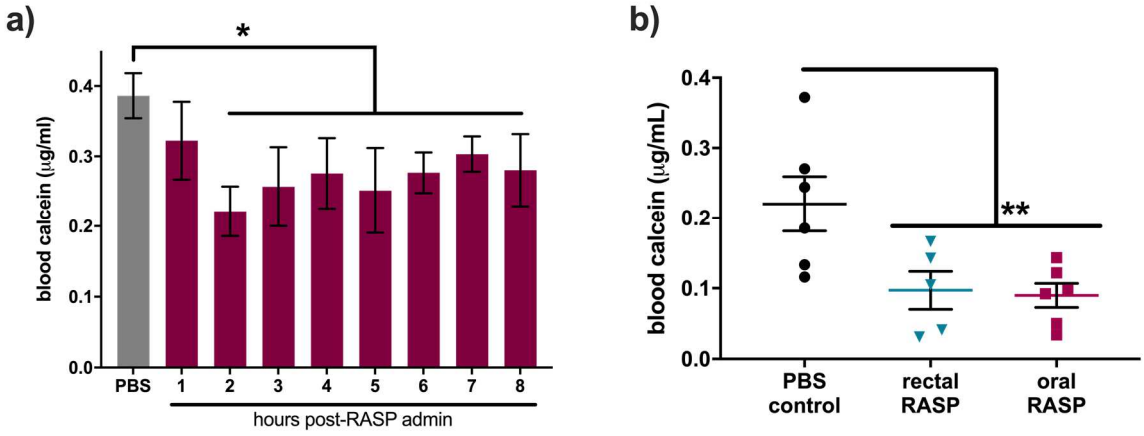


**Figure 2.3. Raspberry extract reduced intestinal permeability in mice.** a) Rectally administered raspberry extract (600 mg/kg) reduced the intestinal permeability of the marker molecule, calcein, from two to at least eight hours post-administration. b) Orally and rectally administered raspberry (1200 mg/kg) both reduced intestinal permeability, suggesting that the active component survives digestion in the stomach. For all panels, * $p < 0.05$ and ** $p < 0.005$ compared to the control, error bars represent s.e.m. ($n = 5 – 6$)

Encouraged by the colonic data, we next examined if the extract would remain effective after passing through the harsh environment of the stomach. To test oral delivery of raspberry extract, mice were gavaged raspberry extract and then rectally administered calcein (three hours later) to track permeability. Control mice were gavaged PBS, and another group was given raspberry enemas to enable direct comparison between oral and rectal administration. Whether delivered orally or rectally, raspberry extract (1200 mg/kg) significantly reduced intestinal permeability by an average of 56% (Fig 2.3b). These data suggest that the active component in the raspberry extract survives the pH extremes and digestive enzymes of the gastrointestinal tract. The notable differences between PBS groups in different experiments (Fig 2.3a and 2.3b) highlights the innate variability of intestinal permeability in healthy mice. It is important to use

mice from the same cohort/colony and of similar age so this will not affect groups within a single experiment.

### 2.3.3 Repeated doses have a lasting effect

To examine the effects of repeat dosing, mice were orally gavaged raspberry extract (1200 mg/kg) once a day for nine days (Fig 2.4). On the tenth day, 24 hours after the most recent dose, fluorescein isothiocyanate dextran (4 kDa, FD4) was rectally delivered to examine permeability. FD4 was used in these experiments to gauge the effect of raspberry extract on the intestinal permeability of a small macromolecule intended to model peptides. We were encouraged to observe an 65% reduction in FD4 permeability 24 hours following the end of raspberry treatment. Statistical significance was difficult to achieve due to the innate variability in the baseline FD4 permeability of control mice.

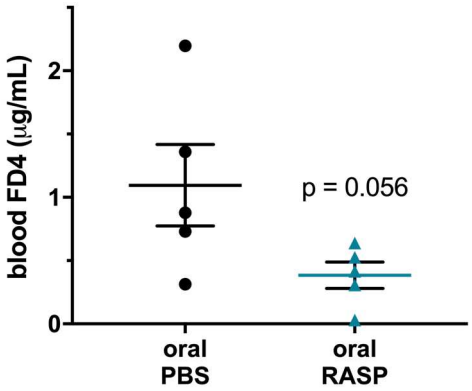


**Figure 2.4. Daily repeated oral doses of raspberry extract reduced intestinal permeability.** After nine daily doses of raspberry, a reduction in the intestinal permeability of the marker molecule, FITC-dextran 4000, was still present 24 hours after the last dose. Error bars represent s.e.m. (n = 5)

### 2.3.4 Comparing the activity of cyanidin and raspberry extract

Raspberry contains thousands of natural product metabolites, many of which have been well-characterized.[39,40] Previous work in our laboratory suggests that anthocyanidins, which are polyphenolic compounds that contribute to the pigmentation in fruits, can mediate intestinal permeability. Given that the most abundant anthocyanidin in raspberry is cyanidin, we examined its effect on intestinal permeability. These experiments were performed using an IPEC-J2 intestinal monolayer cell culture model, which is similar to the Caco-2 model but produces TEER values that are more representative of human small intestinal tissue.[35] When these cells were treated with either raspberry extract or cyanidin, barrier function improved over the course of the experiment (Fig 2.5). Specifically, following three hours of treatment, raspberry extract and cyanidin produced statistically identical increases in resistance of 28% and 25% over the control, respectively. Although there may be many components responsible for raspberry's permeability-reducing effect, these results indicate that cyanidin is an active component in raspberry. Due to its pure nature, a 5-fold lower dose of cyanidin is required to achieve the same effect as raspberry. Identification of an active component is important for potential applications of raspberry treatment, as the active component can be purchased or directly synthesized instead of being isolated from raspberries using complex natural product chemistry techniques.

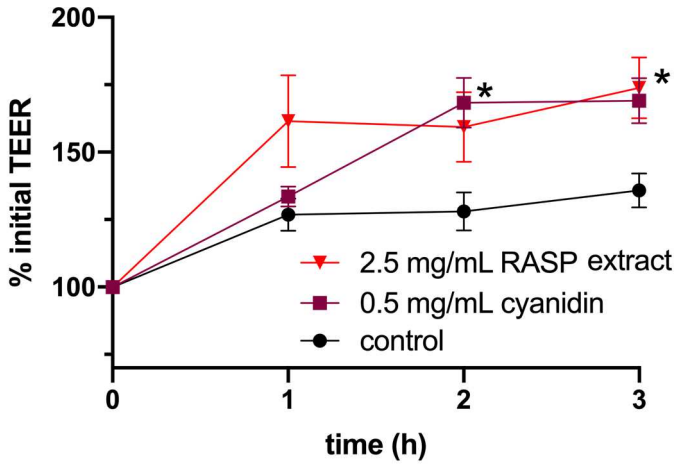


**Figure 2.5. Cyanidin and raspberry extract both improved barrier function of IPEC-J2 intestinal monolayers.** Cyanidin, the most abundant anthocyanidin natural product found in raspberries, increased TEER as effectively as raspberry and is likely a key component in its efficacy. * $p < 0.05$, error bars represent s.e.m. ($n = 4$)

## 2.4 Discussion

Modulation of intestinal barrier function is a highly active research area, but it focuses mostly on increasing permeability for oral delivery of protein drugs. Reducing intestinal permeability could help to prevent or mitigate symptoms of a leaky gut, but it remains a relatively unexplored topic.[21] Considering the lack of tolerable therapeutic options for diseases like IBD, more innovative and unconventional approaches such as restoring or increasing intestinal barrier merit consideration.

Caco-2 cell monolayers are often used as a high-throughput in vitro model for the intestine.[36,37] In particular, these monolayers are a good choice for permeability studies because their relative permeability is readily assessed both by measuring electrical resistance (TEER) and the permeation of a fluorescent marker compound (calcein). After screening a library of fruits and vegetables, we found that raspberry significantly reduced the permeability of Caco-2 monolayers. In this regard, raspberry was an anomaly as most fruits and vegetables increased

the permeability of the monolayers or destroyed them altogether due to high concentrations. After processing raspberry with a more elaborate extraction method, raspberry remained efficacious in reducing permeability of the Caco-2 monolayers. Raspberry contains a unique set of natural products that are biologically active in reducing permeability without being toxic to intestinal cells.

Although Caco-2 monolayers are a prolific screen tool, they lack the complexity of in vivo intestinal tissue. For a compound to remain efficacious in vivo, it must be unaffected by the harsh environment of the gastrointestinal tract and pass through the mucus layer to reach intestinal cells. Despite not being preferred by patients, rectal administration of drugs (enema) is a viable and clinically used therapeutic option for patients with IBD because it allows drugs quickly reach inflamed intestinal tissues.[41] When administered rectally, our raspberry extract reduced intestinal permeability in healthy mice in as little as two hours. The reduction in permeability was significant at least eight hours later, which would provide a more patient friendly administration regimen. Even considering these lower suggested dosing requirements, oral delivery is easier and more patient friendly. When orally delivered to healthy mice, raspberry was equally efficacious in reducing intestinal permeability (compared to rectal delivery). This indicates the natural product in raspberry that is responsible for reducing intestinal permeability survives digestion in the stomach and remains active during its transit through the gastrointestinal tract.

Diseases associated with a leaky intestinal barrier are chronic in nature, meaning any therapeutic must be effective and tolerable over repeated doses. For example, antibiotics are commonly prescribed to manage IBD, but they cannot be used full time without serious repercussions on the sensitive intestinal environment.[42] Therapeutics that can be given for

extended time periods without side effects offer significant advantage over those that require dosing interruptions. Mice that were orally gavage raspberry extract once a day for nine days saw no ill effects, maintaining a steady weight and healthy fecal samples. Moreover, on the tenth day (a full 24 hours after the most recent dose), there was evidence of reduced intestinal permeability. Achieving statistical significance between treatment groups is difficult given the innate variability in intestinal permeability between healthy animals. This variability is exacerbated in a long-term experiment, as daily handling of mice stresses and affects each animal differently. Further, the permeation of a macromolecule such as FD4 is already relatively low, so our results may actually underestimate the barrier-function-fortifying effect that raspberry has on the intestine.

Raspberry contains hundreds of natural product metabolites,[40] many of which could exhibit some form of biological activity. This makes identifying the specific compound responsible for raspberry's permeability-reducing effect challenging. However, doing so is important because the natural product metabolite profile is affected by cultivar and environment factors,[40,43,44] and dosing of the therapeutic is imperative for clinical applications. Anthocyanins, called anthocyanidins in their unglycosylated form, are a prominent natural product group in most fruits and are the compounds that provide pigment to the fruit's flesh.[45] Of the anthocyanins present in raspberry cyanidin-glycoside is widely documented as the most abundant, making it the foremost candidate to examine for permeability-reducing effects.[43,44] To test cyanidin, we used the unglycosylated form because in vitro testing lacks enzymes to break down the glycoside to leave free cyanidin. We examined cyanidin on the IPEC-J2 cell monolayer model because it more closely resembles the physiological permeability of the human intestine and is naturally less tight than Caco-2 monolayers.[35] Cyanidin and raspberry each

increased TEER significantly compared to the control, indicating that cyanidin is at least partially responsible for the barrier function enhancing effect of raspberry.

Testing cyanidin in vivo would provide further proof of its efficacy, but cyanidin poorly soluble in water. Cyanidin in raspberries is found in its glycosylated form,[43,44] which increases solubility making it a more feasible choice for in vivo experiments. The sugar should be cleaved in the stomach to leave free cyanidin, but free cyanidin would be difficult to deliver directly because it is nearly insoluble in aqueous buffers. It is likely that the glycosylation affects bioactivity, so evaluating both free cyanidin and cyanidin-glycoside would be valuable.[46] Given that both forms of cyanidin are costly and would be required at relatively high concentrations to demonstrate efficacy in healthy animals, it was not practical to do further in vivo experiments.

## 2.5 Conclusion

Intestinal barrier function is a crucial aspect of our digestive and overall health, but a substantial portion of the population has increased intestinal permeability. We have shown that raspberry extract decreases permeation across in vitro intestinal models and reduces permeability by more than 50% in healthy mice. Although there may be many natural products in raspberry responsible for this effect, cyanidin has similar activity to raspberry extract at 5-fold lower doses. This novel approach to reducing intestinal permeability may offer patients suffering from a leaky gut reprieve from their symptoms without the side effects associated with traditional drugs.

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## CONCLUSIONS AND OUTLOOK

Leaky gut diseases are chronic afflictions that already affect millions of people, but evidence points towards increasing disease incidence. Our lack in understanding of the disease and dearth of therapeutic options means that vast resources are being devoted to studying these diseases. However, there is still a pressing need to better understand the animal models used to study these diseases and to develop novel, patient-friendly treatments. The work in this thesis offers improvements to a commonly used IBD animal model and introduces an innovative approach to treating leaky gut diseases.

The DSS animal model work presented in Chapter 1 offers methods and recommendations for improving the commonly used model. While the model is affordable and reproducible, it induces severe colitis that isn't representative of clinical IBD. We shortened the induction period of the model to generate more physiologically relevant losses of intestinal permeability, which will allow researchers to use DSS for a broader range of applications. With our attenuated model, the slight increase in intestinal permeably due to the induced colitis is a more appropriate analog for the loss of barrier function seen in IBD patients. Moreover, we provide suggestions for reducing the variability of the model by properly acclimating animals to experimental conditions, which mitigates the need for repeated experiments and helps to keep animal counts as low as possible. Future work should include more robust characterization our of attenuated DSS model, including a full analysis of the inflammatory and cytokine response.

Modulation of intestinal permeability is typically focused on increasing permeability to orally delivered macromolecule therapeutics. However, our work uncovered a compound that reduces intestinal permeability, an anomaly in the field and an intriguing option to treat leaky gut diseases. Currently available therapeutics for diseases like IBD offer limited therapeutic benefit

and often come with significant side effects. Chapter 2 of this thesis presents our work in the area of natural product research, isolating a compound from raspberries that reduces intestinal permeability. Raspberry and its isolated active component, cyanidin, reduce permeability in multiple in vitro models and in vivo. Repeated administrations offer lasting reduction in permeability, which enhances its potential as a clinical treatment for leaky gut diseases. Given the food-derived nature of our therapeutic, the intestine is already well-adjusted to these compounds which increases safety and reduces the boundary to a clinical application. Future work should examine the effect of orally dosed cyanidin in an animal model for IBD and include a study on long-term safety when used in vivo.

IBD and other leaky gut disease are challenging to study, due in part to the diverse and mostly unknown nature of the disease etiology. The work in this thesis offers improvements to the way we study the disease, which will allow others to pursue new studies. Our novel outlook to treating leaky gut diseases, by administration of raspberry-derived compounds that reduce permeability, will offer patients a therapeutic option that does not come with the side effects of traditional options.

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