HAS GENETIC IMPROVEMENT OF WHEAT

RESULTED IN NEGATIVE EFFECTS ON

GUT HEALTH?

By

BRYANT H. KEIRNS

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Oklahoma Christian University

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Thesis Approved:

Dr. Brenda J. Smith
Thesis Adviser

Dr. Edralin Lucas

Dr. Dingbo Lin

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EFFECTS ON GUT HEALTH?

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Abstract:

Objectives: Wheat consumption has recently declined amid growing concerns about gluten-sensitivity. To determine if genetic manipulation of wheat contributes to gut inflammation, we investigated the effects of modern and heirloom wheat on gut health under normal and western diet (WD) conditions.
Methods: 6-week, C57BL/6 male mice were utilized in a 2x3 factorial with diet (control [AIN-93G] or WD, 45% fat and high sucrose) and wheat flour (no wheat, 10% heirloom [Turkey] or 10% modern [Gallagher]) as factors. After 6-weeks, body composition was assessed, and tissues were collected to evaluate metabolic parameters, systemic and intestinal indicators of inflammation, structural changes in different regions of the intestine and gut barrier integrity. Additionally, cecal short-chain fatty acids (SCFAs) were assessed. Data were analyzed using 2-way ANOVA (SAS, Version 9.4) unless tests of normality failed, in which case Friedman's test was performed. Fischer's least square means was run for post-hoc analysis.
Results: Body weight, % fat, fasting glucose, total cholesterol, and NEFA were increased with WD and wheat did not affect these parameters. Serum C-reactive protein (hsCRP) and lipopolysaccharide binding protein (LPS BP) were unchanged by WD or wheat. WD decreased the SCFA, acetic acid, but adding Gallagher to WD restored levels to control ($P_{WD*Wheat}<0.05$). No other SCFA were altered. Histological evaluation revealed reduced villi height ($P<0.05$) and area ($P<0.05$) in the jejunum with WD and wheat did not alter this response. Within the ileum, Gallagher increased villi area ($P<0.01$) relative to control; no other changes were noted. No effects of WD or wheat on crypt hyperplasia, lymphocyte infiltration, or goblet cell number within the jejunum, ileum or colon were observed. Overall, gene and protein abundance of tight junction proteins were unaffected by WD or wheat, except for a reduction in junction adhesion molecule-3 (*JAM-3*) with WD+T ($P<0.05$). Within the ileum lamina propria, WD increased interferon-γ (*IFN-γ*) ($P<0.05$). Adding Turkey suppressed interleukin-17 (*IL-17*) ($P_{WD*Wheat}<0.05$) within the context of WD. No other inflammatory cytokines (i.e., tumor necrosis factor-α [*TNF-α*], *IFN-γ*, or *IL-6*) were altered by wheat.
Conclusion*: These findings indicate Gallagher did not compromise barrier function or contribute to gut inflammation relative to Turkey.

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CHAPTER I

INTRODUCTION

Wheat is a staple food not only the United States, but in countries worldwide [1]. This is due in part to the general convenience and low cost of grain-based products, as well as the World Health Organization's recommendation that whole wheat products are a health promoting food [2]. Despite these factors, wheat consumption per capita in countries such as the U.S. dropped sharply in the mid-2000s from 146.3 to 133.4 pounds per capita, and has continued to steadily decline in recent years reaching as low as 131.8 in 2017 [3]. This trend likely results from one or more of the following factors: 1) popularity of carbohydrate-restricted diets; 2) perception that genetic modification of crops known as GMOs (genetically modified organisms) has negative health consequences; 3) increasing concern about gluten sensitivity; and 4) increasing popularity and availability of gluten-free products [4-6].

Coinciding with decreased wheat consumption are reports of increased incidence of wheat related disorders (e.g., celiac disease) [7-9]. Rubio-Tapia and colleagues [10] estimated that prevalence of celiac disease (CD), an autoimmune disease triggered by specific proteins found in wheat (e.g., gluten, gliadin), has increased 4-5 fold since the 1950s, resulting in 1-3% of the population having CD, depending on the country analyzed [11]. In addition to CD, a population has emerged that reports negative gastrointestinal symptoms (e.g., bloating, diarrhea) following wheat or gluten consumption, but lack the genotype and severity of symptoms classically associated with CD [12]. This population is referred to as non-celiac gluten sensitive

(NCGS), and is estimated to comprise 4-7% of the population worldwide [11]. At this time, there are no known biomarkers for NCGS, although some diagnostic criteria have been proposed, including results from a gluten challenge and increased number of intraepithelial lymphocytes in intestinal biopsies [12]. While some studies support the notion that gluten is the primary trigger in NCGS individuals, this has recently been questioned due to evidence that other wheat components (e.g. fructans and fermentable short-chain carbohydrates or FODMAPS) may contribute to these negative symptoms [13-15]. Alongside the emergence of NCGS, it has been estimated that 21% of Americans include gluten-free products as part of their diet [16]. Even with the most liberal estimates of CD and NCGS, this number is larger than would be expected based on the prevalence of wheat-related conditions.

A common criticism in popular media is that genetic manipulation used to improve properties of modern wheat (e.g., yield and drought resistance) has made the immune system more likely to recognize wheat as an antigen relative to its heirloom predecessor [17]. These effects are often attributed to the wheat protein gluten, one of its constituent proteins gliadin, and peptides derived from their metabolism; however, other candidates have been proposed as well (e.g., fructans, FODMAPS) [18, 19]. Attempts to quantify the abundance of potential wheat antigens in heirloom compared to modern wheat cultivars have yielded mixed results [20, 21]. Some work has been done comparing the effects of heirloom and modern wheat in both humans and animals, showing a reduction in various circulating inflammatory mediators (e.g., tumor necrosis factor (TNF)-α, interleukin (IL)-6, interferon (IFN)-γ) with consumption of heirloom wheat relative to modern wheat [22-24]. With respect to gut health, limited data also suggests heirloom wheat may lead to positive outcomes (i.e., increased villi height, reduced lymphocyte

infiltration) in animals [25]. This data suggests that heirloom wheat may possess anti-inflammatory properties relative to modern varieties.

While wheat products comprise a significant portion (i.e., ~20% of daily calories) of the United States' diet, there are other substantial components of the standard western diet (WD) that are capable of contributing to inflammation [26, 27]. The 2015-2020 U.S. Dietary Guidelines reports that on average 11% and 13% of individuals' daily calories come from saturated fat and added sugars, respectively [28]. Both of these intakes exceed U.S. dietary recommendations and are distinguishing characteristics of the typical WD, which contribute to the pathophysiology of the most common chronic diseases (e.g., obesity, cardiovascular disease and certain cancers) with an underlying inflammatory etiology [26, 28]. Indeed, women consuming a WD exhibited higher serum inflammatory markers, C-reactive protein (CRP), IL-6 and E-selectin, relative to women following a more prudent diet lower in saturated fat and refined sugar [29]. Further, intake of a single high-fat meal has been associated with increased circulating inflammatory cytokines (i.e., IL-6, TNF-α) in both men and women [30]. Similar findings have been reported experimentally with animal models fed a WD, with the gut being a major site of inflammation [31-33]. Due to the inflammatory nature of the WD, one may question whether foods, such as wheat, that would normally be benign to the general population could potentially exacerbate an underlying inflammatory state when consumed in the context of the WD.

Recently, there has been a great deal of interest in the interplay between the contents of the gastrointestinal tract and the underlying mucosal immune system. Sections of the lower gastrointestinal tract, especially the ileum and colon, provide a dynamic environment where the mucosal immune system, the gut microbiota, and ingested food continually interact with one another. Interestingly, approximately 70% of the body's immune cells reside within the gut

associated lymphoid tissue (GALT) at any given time [34]. In addition, these immune cells (e.g., T cell and dendritic cell populations) can traffic to and from an elaborate network of lymphoid tissues (i.e., lamina propria, Peyer's patches, and mesenteric lymph nodes). Under normal conditions, professional antigen presenting cells known as dendritic cells extend between the intestinal epithelial cells that line the villi to survey contents of the intestinal lumen [35]. Dendritic cells then present found antigens from food, bacteria, or environmental toxins to naïve CD4+ T helper cells (Th cells) that, depending on the local cytokine environment, differentiate into one of four subtypes: Th1, Th2, Th17, or T regulatory cells (Tregs) [36]. Th1, Th2, and Th17 cells have distinct roles within the inflammatory response, while Tregs are important in maintaining immunotolerance, particularly in the gut where there is considerable exposure to non-self (e.g., digested food, bacteria) [37]. Each of these subtypes have important roles in health, but when dysregulated can contribute to pathological inflammation [38].

In both CD and NCGS, a gut mucosal immune response occurs, but there are unique features characteristic of each condition. One defining characteristic of CD is that in 95% of cases, a genetic background is present (i.e., human leukocyte antigen (HLA)-DQ2 and/or HLA-DQ8 variants) that increases antigen presenting cells' recognition and presentation of gluten-derived peptides to naïve CD4+ T cells in the lamina propria and secondary lymphoid organs (e.g., lymph nodes, Peyer's patches) [39-41]. This leads to over activation of naïve CD4+ T cells in response to wheat-derived peptides, especially by the inflammatory Th1 and Th17 cells, and subsequent tissue damage [42, 43]. Th2 cell activation is also implicated in CD pathophysiology, as their effector functions results in production of auto-antibodies [40]. Compounding the mucosal inflammatory response, Tregs in CD patients are less able to suppress the activity of inflammatory immune cells (i.e., Th1, Th17) [44, 45]. In the case of NCGS, the major

immunological features are less clearly defined. There is evidence of an adaptive immune response mediated by Th cells, as shown by elevated duodenal cytokine expression (i.e., *IFN-γ*) following a gluten challenge [46]. Additionally, increased CD3+ intraepithelial lymphocytes have been observed in NCGS, but to a lesser extent than in CD [46, 47]. It has also been suggested that NCGS symptoms may be in part due to an innate immune response, as evidenced by increased toll-like receptor-2 (*TLR-2*) gene expression [47]. Overall, CD is characterized by pronounced inflammation driven by the adaptive immune system (i.e., Th helper cells, B cells), while NCGS shows weaker signs of both an innate and adaptive immune response.

Another biological influence on the lower gastrointestinal tract is the gut microbiota, which rivals the number of host cells in the human body [48]. The gut microbiota is necessary for development of an adequate immune system and promotes induction of different Th subtypes [49-53]. Dietary patterns and the microbiota also are intimately connected. For example, consuming foods rich in non-digestible carbohydrates increase bacterial diversity in the gut, which is associated with positive health outcomes (e.g., improved weight regulation, reduced type 2 diabetes or T2D) [54-56]. Conversely, the WD is capable of inducing microbiota dysbiosis in animal models, altering the balance between pathogenic bacteria and commensals [31, 32, 57]. Active CD and CD patients in remission have been associated with shifts in bacterial populations (e.g., decreased *Bifidobacteria* and *Lactobacillus*, increased *Staphylococcus*), although no definitive mechanism by which the microbiota may contribute to CD is clear. [58-61].

Intestinal epithelial cells are traditionally thought of as absorptive cells, but also serve important roles in gastrointestinal health by preventing bacterial overgrowth, regulating intestinal permeability, and participating in the mucosal immune response. Small intestinal epithelial cells

are organized in such a way to provide a large surface area to maximize nutrient absorption through finger-like projections in the lumen known as villi. Additionally, grooves in between villi known as crypts house intestinal stem cells and produce anti-microbial peptides, which are responsible for regulation of bacterial growth [62]. Related to their role as a physical barrier, intestinal epithelial cells are connected by an elaborate array of proteins that complex to form tight and adherens junctions [63]. Tight junctions are of particular importance and are comprised of transmembrane proteins that connect epithelial cells (e.g. claudins, occludin, junction adhesion molecules or JAMs) and intracellular scaffolding proteins that stabilize the transmembrane proteins (e.g. ZO, actins) near the apical surface of intestinal epithelial cells [64]. The primary role of tight junctions is to form a selectively permeable epithelial barrier, allowing small molecules (e.g. ions, water) to pass through, yet preventing entry of larger peptides with antigenic potential [64, 65]. Intestinal epithelial cells also express various innate immune system pattern recognition receptors (PRRs), highlighting their ability to participate in the mucosal immune response.

In the context of CD, disruption of tight junctions in the gut, known as intestinal permeability, is well characterized [66, 67]. This is likely attributable to several mechanisms but is mainly credited to a gluten-induced immune response (e.g., Th cell activation) resulting in epithelial cell and tight junction protein damage [68-71]. Additionally, there is some evidence to suggest that certain gluten derived peptides damage intestinal epithelial cells directly [72, 73]. With respect to NCGS, there is mixed evidence suggesting that intestinal permeability is increased, however this has not been definitively established [47, 74]. In regards to villi and crypt structure in CD, activated mucosal Th cells producing IFN-γ and TNF-α lead to blunted villi height or atrophy and deepening of crypts, which is known as hyperplasia [40]. Although

increased intraepithelial lymphocytes have been observed in NCGS, this population does not display villous atrophy or crypt hyperplasia [46, 47, 75]. Structural characteristics of the gut are also negatively regulated by habitual high-fat diet consumption. For example, intestinal permeability has been shown to increase in animals chronically fed high-fat diets [32, 76, 77]. Additionally, villi and crypt structure can also be negatively affected by a high fat diet, resulting in villi atrophy and crypt hyperplasia [78, 79]

Since there is a disparity between estimates of individuals who have CD and NCGS and those choosing to consume gluten-free products occurring alongside increased rates of both of these conditions, several questions arise. For one, why are these individuals outside of the CD and NCGS populations choosing to eat gluten-free? Secondly, has some aspect of wheat changed in a manner that has resulted in unintended health consequences and decreased wheat consumption? Finally, do these potential changes have a negative impact on the health of the average individual consuming an otherwise unhealthy diet? These questions have not been adequately explored.

For these reasons, the **<u>purpose</u>** of this study was to determine if genetic manipulation of wheat has led to increased gut inflammation. This was accomplished by comparing the dietary impact of an heirloom variety of wheat (Turkey) to a modern variety (Gallagher) in the context of a normal diet and a WD in a healthy C57BL/6 mouse model. This will allow for the assessment of wheat variety on gut inflammation, as well as the potential influence of an inflammatory western style diet high in saturated fat and refined sugar. The **<u>hypothesis</u>** that was tested is that modern wheat will exacerbate WD induced intestinal inflammation and permeability relative to heirloom cultivars but will not induce an inflammatory response in the context of a normal diet.

Aim 1: To characterize the effect of WD and wheat cultivar on body composition and serum metabolic parameters (i.e., glucose, total cholesterol, triglycerides, non-esterified fatty acids).

Working hypothesis: Western diet consumption will increase body composition and parameters related to metabolic health and wheat will not affect these markers.

Aim 2: To determine the effect of wheat cultivar on villi and crypt structures (e.g., villi length, villi area, villi perimeter, crypt depth) within the jejunum, ileum, and colon under normal and WD conditions.

Working hypothesis: Gallagher will have no effect on measures of villi and crypt structures in the context of a normal diet, but will have negative effects on these structural parameters compared to Turkey when consumed with the WD.

Aim 3: To evaluate the effect of wheat cultivar on barrier integrity via gene expression and protein levels of tight junction proteins (e.g., claudins, occludin) in the colon under normal and WD conditions.

Working hypothesis: Gallagher will not contribute to decreased barrier integrity when consumed with a normal diet, however when combined with a WD will decrease barrier integrity relative to Turkey.

Aim 4: Evaluate the effect of wheat cultivar and WD on short chain fatty acids within the cecum.

Working hypothesis: Western diet consumption will decrease concentrations of cecal SCFA and wheat will not affect these parameters.

Aim 5: To determine if systemic markers of inflammation (i.e., CRP) and intestinal permeability (i.e., lipopolysaccharide binding protein or LPS BP), are altered in animals consuming a modern wheat cultivar relative to an heirloom variety under normal and WD? conditions.

Working hypothesis: Gallagher will have no effect on systemic indicators of inflammation and intestinal permeability in the context of a normal diet, but will negatively impact these parameters in the context of a WD compared to Turkey.

Aim 6: To determine the effect of wheat cultivar on indicators of local gut inflammation based on alterations in gene expression of inflammatory mediators (e.g. IL-17, IL-1β), levels of fecal calprotectin, and lymphocyte infiltration using histopathological analysis.

Working hypothesis: Gallagher will have no effect on measures of local gut inflammation in the context of a normal diet, but will negatively impact these parameters when consumed with a WD compared to Turkey.

Limitations

As is the case with any research, the present study is not without limitations. These limitations include evaluation of a single time point, only studied one gender, and lack of functional assessment of barrier function. At this time, our study can only speak to the effects of Gallagher after consumption for 6 weeks. Therefore, no insight into any potential acute response (e.g., 1 week) or more chronic response (e.g., 6 months) can be gained. This study also only characterized the response of young male mice to Gallagher consumption relative to Turkey. Lastly, although our data provides strong evidence that there were no signs of reduced barrier

integrity in the gut, we did not perform functional analysis (e.g., transepithelial resistance,

lactulose mannitol test) that would provide further insight.

CHAPTER II

LITERATURE REVIEW

Introduction to Wheat-related Disorders

Wheat products are a staple of not only the U.S. diet, but in countries worldwide [1]. This

may be attributable to the general convenience, low cost of grain-based products, and recognition

that whole wheat products are a health-promoting food [2]. In the United States, wheat

consumption increased dramatically between 1972 to 2000, from 110 to 146.3 pounds per capita

[3]. Shortly after this peak, wheat consumption dropped sharply from 2000-2004 from 146.3 to

133.4 pounds per capita, and has continued to steadily decline in recent years reaching 131.8 in

2017 [3, 80]. This decline in wheat consumption has been suggested to result from one or more

of several factors, including popularity of carbohydrate restricted diets, concern that GMO foods

have a negative impact on health, increasing perception or concern for gluten sensitivity, or the

increased popularity and availability of gluten-free products [4-6].

Coinciding with decreased wheat consumption are reports of increased incidence of

wheat-related disorders (e.g., celiac disease) [7-9]. Up until the 1970s, it was thought that CD

was a rare disease, present in only 0.03% of the population [81]. More recent estimates are much

higher, indicating the prevalence is approximately 1% of the population [10, 11, 82]. An analysis

performed by Myeus and colleagues [83] found an even higher prevalence of 3% in a cohort of

Swedish children. Patients suffering from CD endure from acute symptoms related primarily to

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the gastrointestinal tract, including chronic diarrhea and/or constipation, abdominal bloating, and weight loss with gluten consumption [84]. If CD is left untreated or undiagnosed for extended periods of time, pathologies outside of the GI tract such as nutrient malabsorption leading to anemias and bone loss can occur [84, 85].

There are a number of established diagnostic tests suggestive of CD, but the presence of more than one positive test is needed for a diagnosis. One important test is screening for serum autoantibodies to tissue transglutaminase 2, an enzyme that deamidates gluten in the small intestine [86, 87]. While tissue transglutaminase 2 is most common autoantibody screened, other antibodies against gliadin, a glycoprotein found in gluten, and connective tissue around muscle cells known as the endomysium are useful diagnostically as well. [88]. Another screening method used by itself or in conjunction with antibody titers is genetic testing for the presence of variants in the HLA system, which encode for MHC II proteins. Specifically, HLA DQ2 and DQ8 variants are seen in over 95% of CD cases and increase these individual's antigen presenting cells' (e.g., dendritic cells) propensity to recognize gluten-derived peptides [89]. If one or more of these screening tests is positive, a series of duodenal biopsies are then examined for the presence and severity of villous atrophy, crypt hyperplasia, and intraepithelial lymphocyte infiltration as measured by the Marsh classification to confirm the diagnosis [84, 87, 90]. Due to the other factors influencing these histological parameters (e.g., western diet consumption, severe bacterial infection, inflammatory bowel disease) this test cannot stand alone either [78, 91]. Several of these technologies have been improved alongside increased estimates of CD prevalence (i.e., serum autoantibody assay, intestinal biopsy techniques), and this provides some explanation for increased CD incidence. However, one study applied current diagnostic standards to two cohorts of serum samples, one from the 1950s and another from the early 2000s,

and saw that undiagnosed CD rates were 4 to 5 times greater in the more recent cohort [10]. This data, paired with the understanding that there are modern environmental risk factors (e.g., infections, hygiene) suggests diagnostic improvements are not the sole explanation for increased CD diagnosis [82].

In addition to CD, a population has emerged that reports negative gastrointestinal symptoms (e.g., bloating, diarrhea) following wheat or gluten consumption, but lack the genotype and severity of symptoms associated with CD [12]. This population is referred to as non-celiac gluten sensitive (NCGS), and is estimated to comprise 4-7% of the population [11]. Common symptoms of NCGS include bloating, abdominal pain, and diarrhea [12]. Extra-gastrointestinal symptoms typically reported are headaches, joint pain, and fatigue. Long-term effects of NCGS are not well characterized due to inherent difficulties of studying it (e.g., less severe symptoms overall). Some research has focused on establishing biomarkers for NCGS, but with limited success. Volta and colleagues [92] performed serological examination for antibodies typically seen elevated in CD in a population of NCGS individuals but with little success. Specifically, among anti-tissue transglutaminase 2, anti-endomysial, and anti-gliadin antibodies, the only marker elevated was anti-gliadin antibodies; however, only in 56% of the cohort. Udhe et al., [74] identified several markers suggestive of decreased gut barrier function (i.e., lipopolysaccharide binding protein or LPS BP, soluble cluster of differentiation 14 or CD14, anti-flagellin antibodies, anti-LPS antibodies) in an NCGS cohort. In addition, fatty-acid binding protein 2 (FABP2), a protein specific to intestinal epithelial cells and indicative of damage, was elevated in the serum of these individuals. A patent was recently filed based on these findings for technology allowing simultaneous testing of soluble CD14, LPS BP, anti-LPS antibodies, anti-flagellin antibodies, anti-gliadin antibodies, and FABP2 with the intent of establishing a panel of

markers to diagnose NCGS [93]. Although, many of these markers are related to gut

inflammation and barrier integrity, most are non-specific to a wheat-induced immune response.

From a histological standpoint, several immune cell subset have been identified as elevated in

NCGS, but to a lesser extent than CD [94]. These include intraepithelial CD3+ T cells, lamina

propria CD45+ cells, and eosinophils in the duodenum and rectal mucosa. Although validation

of biomarkers indicative of NCGS is lacking, in 2014 experts in the field proposed diagnostic

criteria referred to as the Salerno criteria that included a score on the self-reported

Gastrointestinal Symptom Rating Scale of Reflux, abdominal pain, indigestion, diarrhea and

constipation, as well as increased intraepithelial lymphocytes infiltration following a gluten

challenge [12]. Assessment of serum anti-gliadin antibodies was still recommended for NCGS,

but its limitations were noted. While some studies support the notion that gluten is the primary

trigger in NCGS, this has been questioned recently. Some existing evidence suggests that other

wheat components (e.g. FODMAPS) may contribute to these negative symptoms [13-15]. A

recently published double-blind crossover trial showed that in an NCGS cohort, fructans and not

gluten contributed to the negative symptomology [15]. Following the gluten challenge arm of the

trial, symptoms were not significantly different than placebo. Similar results were reported from

another double-blind crossover trial in 2013 that evaluated the effects of gluten in the context of

a diet with reduced FODMAP intake, a broader category of non-digestible carbohydrates that

includes fructans [14]. In that study, participants already adhering to a gluten-free diet who

reduced FODMAPS exhibited further improvement in their symptoms. Additionally, only 8% of

the subjects exhibited signs of gluten specific side-effects and 92% of the subjects were most

responsive to FODMAP reduction. An additional confounder that has been pointed out related to

NCGS is that reported symptoms closely resemble irritable bowel syndrome, making this condition even more difficult to study and potentially underdiagnosed [95].

In 2015, 21% of Americans were reported to include gluten-free products as part of their diet [16]. Even with the most liberal estimates of CD and NCGS, only 10% of the population would be expected to be gluten intolerant. This 11% margin of individuals who choose to avoid gluten suggest either underdiagnoses of wheat-related conditions, or a subset of the population that is experiencing symptoms that are subclinical even to NCGS. Other claims should also be considered, such as eating gluten-free will result in health benefits independent of wheat-related conditions and that genetic changes in wheat are driving this phenomena [17].

Comparison of Heirloom and Modern Wheat

A common criticism in popular media is that genetic manipulation used to improve properties of modern wheat (e.g., yield and drought resistance) has increased the propensity of the immune system to recognize wheat-derived peptides as antigen relative to its heirloom predecessors [17]. These effects are often attributed to the wheat protein gluten and one of its constitutive proteins gliadin [18]. Interestingly though, the National Wheat Improvement Committee reports that, on average, modern varieties (e.g., Gallagher, Jagger) contain less gluten than heirloom varieties (e.g., Turkey, Khorasan), but there is variation among cultivars [96]. Despite the possibility that modern wheat may have less total gluten, Kasarda and colleagues [97] estimated that total dietary gluten consumption has increased since 1977, driven in large part by the consumption of gluten as a food additive in products such as soups, potato chips, and processed meats having tripled [98]. Koning [99] has made the case that higher exposure to gluten may increase the probability of a susceptible individual developing CD, therefore it may be feasible that increased overall gluten intake is contributing to increased wheat-sensitivity.

Within the protein gluten, specific peptides that are formed from digestion are known to initiate

an immune response in CD (e.g., the "33mer") [19]. One analysis of characterized immunogenic

peptides (i.e., α-gliadins, γ-gliadins) by Prandi and colleagues [21] found that older varieties of

wheat contain more than modern varieties following *in vitro* digestion. Conversely, Van den

Broeck and colleagues [20] concluded that gluten-derived epitopes are found in higher

frequencies in modern wheat compared to heirloom varieties in Europe [20]. Importantly,

differences in these studies may be due to factors outside of cultivar age and purposeful genetic

modification such as innate genetic differences among varieties, growing environment, and

processing techniques [100].

A handful of studies have been conducted in humans comparing the effects of heirloom

and modern wheat verities under various conditions, several of which focus on the heirloom

variety Khorasan. For example, in type 2 diabetic patients, Khorasan wheat supplementation led

to increased total antioxidant status (i.e., all hydrophilic reactive oxygen species (ROS)

scavengers) and decreased IL-1 receptor antagonist (IL-1RA) and ROS production [101].

Additionally, modern wheat consuming participants displayed decreased antioxidant status and

increased circulating IL-8. In patients with acute coronary syndrome it was found that ROS

production and serum TNF-α were reduced after the Khorasan wheat intervention [24].

Similarly, in healthy individuals with an elevated risk for CVD, consumption of Khorasan wheat

decreased measures of oxidative stress (i.e., TBARS) and circulating IL-6, IL-12, MCP-1, MIP-

1β, TNF-α, and VEGF [22]. Khorasan wheat supplementation also reduced inflammation

associated with NAFLD (i.e., serum TNF-α, IL-1RA, IL-8, and IFN-γ) relative to modern wheat

[23]. Within the context of NCGS, *ex vivo* stimulation of immune cells with modern wheat

protein extracts yielded a greater inflammatory response (i.e., CXCL10 secretion) relative to

control, however there was no difference between heirloom and modern extracts on this parameter [102].

Related to gastrointestinal health, individuals with irritable bowel syndrome reported improvements in subjective measures of abdominal discomfort (e.g., abdominal pain and bloating) and experienced decreased serum inflammatory markers (i.e., IL-17, IL-6, IFN-γ and MCP-1) with consumption of Khorasan heirloom wheat, but not with a modern wheat variety [103]. Another study evaluated gut microbiota composition and aspects of the fecal metabolome (e.g., SCFAs) in healthy volunteers consuming Khorasan heirloom wheat relative to modern control [104]. Interestingly, no differences were observed in microbiota composition at the phylum level with the two wheat varieties. When assessing fecal SCFA, modern wheat elevated butyrate, while heirloom consumption increased methyl-butyrate [104]. A recent crossover study compared the effects of the traditional Italian durum wheat variety known as Senatore Cappelli with a modern commercial variety in the context of NCGS as defined by the Salerno Criteria [105]. Interestingly, after 2 weeks of consumption of Senatore Cappelli, participants reported reduced symptomology based on the Gastrointestinal Symptom Rating Scale relative to the modern variety. Taken together, these clinical studies suggest that some heirloom varieties (i.e., Khorasan, Senatore Cappelli) heirloom wheat may have greater anti-inflammatory properties relative to modern varieties, although it is difficult to discern if this is a positive effect of heirloom or a negative effect of modern varieties due to the lack of a control group not consuming wheat.

Several *in vivo* studies have also directly compared heirloom and modern wheat varieties under various experimental conditions. One study that fed rats exclusively wheat products focused on the hepatic response and saw that rats fed heirloom bread had higher glutathione

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activity, lower ROS production, and decreased inflammation when compared to the modern bread control [106]. Gorelick et al. [107] reported that in non-obese diabetic (NOD) mice supplementing with 3 different heirloom varieties in the *Triticum* family reduced circulating IFN-γ and increased IL-10 relative to control and a modern wheat containing group.

With respect to gut health, rats were fed exclusively pasta made from Khorasan wheat or a modern wheat pasta for 7 weeks, prior to administration of doxorubicin, a chemotherapeutic agent that induces oxidative stress [25]. Animals consuming the Khorasan wheat exhibited longer duodenal villi, decreased lymphocyte infiltration in the duodenum, decreased lymphatic follicle diameter, and increased propionate relative to the modern wheat group under both normal and oxidative stress conditions. In a porcine model, it was observed that Einkorn heirloom wheat increased the *Bacteroides*:*Firmicutes* ratio, while standard wheat increased *Verrucomicrobia* [108]. Both varieties increased fecal acetate, propionate, and butyrate, however this response was more pronounced with the standard wheat for acetate and propionate. An *in vitro* study in the colon cancer cell line HCT116 [109] suggested that gliadin has antigenic potential despite the degree of genetic manipulation of wheat, as treating with gliadin from several modern and heirloom varieties led to elevated ROS, myeloperoxidase (MPO), and gene expression of cytokines (i.e., *IL-1β*, *IL-15*). Overall, these data support findings from clinical studies that suggest heirloom varieties have anti-inflammatory properties, but only one study in NOD mice was able to show improvements with heirloom consumption relative to a non-wheat containing control group.

Western diet and Inflammation

While wheat products comprise around 20% of daily calories on average, there are other substantial components of the western diet (i.e., saturated fat, sucrose) that are capable of

contributing to inflammation [26, 27]. The 2015-2020 U.S. Dietary Guidelines report that on average 11% and 13% of daily calories come from saturated fat and added sugars, respectively [28]. This level of consumption of both saturated fat and added sugars exceeds recommendations based on the U.S. Dietary Guidelines and are distinguishing characteristics of the typical western diet. As a result of the overconsumption of saturated fat and refined sugar, the western diet is understood to contribute to the pathophysiology of a number of common chronic diseases (e.g., obesity, cardiovascular disease and certain cancers) with an underlying inflammatory etiology [26, 28]. In support of this, the WHO reports that the United States has the highest rates of obesity of any country and ranks tenth in cases of cardiovascular disease [110, 111]. Observationally, women from the Nurses' Health Study cohort consuming a western diet had higher serum inflammatory markers (i.e., C-reactive protein or CRP, IL-6, E-selectin) relative to women following a more prudent diet lower in saturated fat and refined sugar [29]. Similarly, NHANES data from 1999-2000 revealed a positive association between markers of saturated fat intake (i.e., saturated fatty acids in serum phospholipids) and the inflammatory markers CRP and fibrinogen [112].

Similar findings have been reported experimentally in clinical trials. For example, elevation of IL-6, IL-8, and measures of immune cell signaling (i.e., antigen presentation and TLR signaling) were observed following consumption of a western style diet rich in saturated fat relative to subjects consuming primarily monounsaturated fat [113]. Further, Nappo and colleagues [30] reported increased inflammatory cytokines (i.e., IL-6, TNF-α) after intake of single high-fat meal [30]. Several studies utilizing animal models have also been performed and provide potential mechanisms by which western style diets contribute to inflammation. Work from Christ and colleagues [114] suggested that consumption of the western diet stimulates

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innate immune system signaling (i.e., nucleotide oligomerization domain or NOD and IFN signaling pathways) leading to granulocyte and monocyte proliferation. The adaptive immune system also appears to be activated by high-fat diets, evidenced by three times more splenic Th17 cells in animals fed a high fat diet compared to control and increased T cell receptor activity in humans consuming a western diet [113, 115].

Locally in the gut, de La Serre et al. [31] initially showed that consumption of a western diet increased *TLR-4* expression and MPO in the ileum of rats [31]. Kyung-Ah and colleagues [33] went on to demonstrate that upregulated TLR-4 was associated with increased nuclear factor kappa-light-chain-enhancer of activated B cells (*NF-kB*) expression and colonic pro-inflammatory cytokines (i.e., TNF-α, IL-1β, and IL-6) of mice on a high-fat diet. The importance of this pathway in high-fat diet-induced gut inflammation was demonstrated through amelioration of this response in TLR-4 knockout mice. Notably, the saturated fatty-acid palmitate, which is found in common western foods (e.g., animal products) and is the predominant product of de novo lipogenesis, can act as a TLR-4 ligand [116]. Moreover, specific immune cell populations in the gut have been shown to respond to western diet consumption. For example, mice fed a high saturated fat diet display increased TNF-α production by lamina propria macrophages and increased lymphocyte adherence to mucosal microvessels on the small intestine, consistent with an increased immune response [117]. Overall, western dietary patterns clearly stimulate both the adaptive and innate immune systems, with signs of inflammation being detected systemically and locally in the gut. Due to the inflammatory nature of the western diet, one may speculate that foods that would normally be benign to the general population (e.g., wheat) may exacerbate an underlying inflammatory state.

Gut Microbiota

The gut microbiota has received increasing attention in recent years due to its importance in gut and overall host health. Therefore, it is unsurprising that dramatic shifts from an individual's normal microbiota or dysbiosis are associated with a variety of disease states [118]. Relatively speaking, the details by which the microbiota regulates host health are still poorly understood, however one mechanism that has been identified is production of metabolites from ingested food that impact overall health [119-121]. Specifically, short chain fatty-acids (SCFA) derived from non-digestible carbohydrate fermentation by the gut microbiota are known to contribute to host health by acting as a fuel source and through their roles as signaling molecules. Predominant SCFA produced in the gut include butyrate, propionate, and acetate, while others such as valerate are produced in smaller quantities [122]. In the lower GI, SCFA are important in supplying energy to colon epithelial cells and by regulating mucosal immune homeostasis through induction of colonic Tregs [123]. Additionally, butyrate is known to increase claudin-1 and claudin-2 expression, promoting gut barrier integrity [124, 125]. The consumption of non-digestible carbohydrates also appears to increase bacterial diversity in the gut, which is associated with positive health outcomes (e.g., reduced body weight and type 2 diabetes) [54-56].

The gut microbiota also interacts with the mucosal immune system (e.g., Th cells and dendritic cells), and it has been demonstrated that the gut microbiota is necessary for maturation of GALT and normal levels of CD4+ T cells [49, 126]. Further, certain strains of bacteria can promote the induction of different Th cell subsets [50-52]. Specifically, segmented filamentous bacteria in the intestinal microbiota have been shown to increase induction of inflammatory

Th17 cells [127]. On the other hand, *Clostridia* species have been implicated in the induction of Tregs, which contribute to mucosal tolerance [50, 52].

Unlike consumption of non-digestible carbohydrates, certain dietary patterns can negatively shape microbiota composition. Specially, western diet consumption induces gut microbiota dysbiosis in humans and animal models [31, 32, 57, 128]. Carmody and colleagues [129] provided compelling evidence that consuming a western diet is a more powerful modulator of the gut microbiome than other factors (i.e., genetics), as chronic intake reproducibly altered gut microbiota composition in over 200 strains of mice. Chronic consumption of a western diet also causes changes in the gut microbiota that are associated with obesity (i.e., increased *Firmicutes*, decreased *Bacteroidetes*) [57]. One mechanism that may explain the ability of a western diet to alter gut microbiota composition is suppression of antimicrobial peptides [130]. It was recently demonstrated that in western diet-induced obesity, shifts in the microbiota and decreased expression of antimicrobial peptides preceded elevation of circulating inflammatory mediators. Although some changes in the gut microbiota occurred prior to antimicrobial peptides suppression, decreased levels of antimicrobial peptides may exacerbate microbiota dysbiosis and subsequent inflammation. Another mechanism by which western diet-induced dysbiosis may negatively impact health is increased production of the bacterial toxin LPS. LPS is known to activate the immune system primarily by TLR-4 recognition, which subsequently activates NF-kB stimulation and upregulation of many inflammatory cytokines (i.e., IL-1β, IL-6, TNF-α) in the gut and systemically [113, 117, 131, 132]. Importantly, germ-free mice do not develop obesity or gut inflammation when fed a diet resembling the western diet, highlighting the role of the gut microbiota in western diet-induced inflammation [133].

Wheat consumption in the context of CD also has been associated with gut microbiota dysbiosis. Since around 40% of the population has the genetic background for CD and only 1% go on to develop it, environmental factors such as the microbiota are hypothesized to play a role in the disease process [134]. Indeed, several studies have characterized decreased numbers of bacterial populations that are generally considered beneficial in CD (e.g., *Bifidobacteria*, *Lactobacillus*) [58-61]. Additionally, several species are increased in CD that are associated with negative symptoms (i.e., *Candida*, *Escherichia* and *Helicobacter*) [135]. Interestingly, these changes may be partially reversible after adoption of a gluten free diet in some patients [60]. A related observation is that breast feeding and vaginal delivery of infants have been associated with a decreased risk of developing CD later in life, two factors known to influence the infant microbiota composition [58, 136].

A potential mechanism for the direct involvement of bacterial species in CD pathogenesis was provided by Caminero and colleagues [120], when certain bacterial strains (i.e. *Pseudomonas aeruginosa*) metabolized gluten in a manner that increased translocation through the epithelial barrier and activated T cells from CD patients. Notably, the immunogenic peptides produced by *Pseudomonas aeruginosa* didn't elicit as strong of a T cell response when metabolized further by *Lactobacillus*, suggesting a protective effect of some bacterial species. Additionally, the SCFA produced by microbes have been shown to be altered in active CD. Data regarding total and major types of SCFA (i.e., acetate, propionate, butyrate) produced in CD is mixed, but there is reason to believe that overall SCFA production is suppressed in this patient population [60, 61, 137-139]. Reduced SCFA production is consistent with intestinal permeability associated with CD, as SCFAs (i.e., butyrate) promote gut barrier integrity. Regarding NCGS, some data suggests improvements in microbiota composition following a

gluten-free diet (i.e., reduced *Firmicutes* and increased *Bacteroides* species) [140]. Further, Natividad and colleagues [141] showed in an animal model of NCGS, that manipulating the gut microbiota (i.e., increasing Gram-negative bacteria and a decreasing Gram-positive *Lactobacillus*) could trigger an inflammatory response to gluten. Further, one can speculate bacterial populations may have a role in NCGS, based on data showing innate immune system receptors that recognize microbial antigen (i.e., TLR-2) are upregulated in the small intestine of NCGS individuals [47]. However, aside from this data the potential role of the microbiota in NCGS is largely unknown.

Although lower than fruits and vegetables, wheat flour is a non-trivial source of dietary fiber with one slice of bread containing 2 grams of fiber [142]. Indeed, limited data suggests that wheat consumption in the absence of CD and NCGS may contribute to beneficial changes in the gut microbiota and related metabolites. For example, healthy individuals who consumed whole-wheat breakfast cereal for 3 weeks displayed increased fecal *Bifidobacteria* and *Lactobacillus* relative to a bran cereal control [143]. Similarly, pigs consuming wheat displayed improvements in microbiota composition (i.e., increased *Bacteroides*, *Verrucomicrobia* and decreased *Firmicutes*) and increased fecal SCFAs such as acetate, propionate, and butyrate [108]. Overall, the gut microbiota is subject to regulation by many factors including the western diet and wheat, and when dysbiotic may contribute to inflammation and the pathogenesis of a variety of disease states.

Gut Barrier Function

Intestinal epithelial cells are traditionally thought of absorptive cells, but also serve important roles in gastrointestinal health by regulating intestinal permeability, preventing bacterial overgrowth, and participating in the mucosal immune response. Intestinal epithelial

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cells are connected by an elaborate array of proteins that complex to form tight and adherens

junctions, forming a selectively permeable physical barrier [63]. Tight junctions are comprised of

transmembrane proteins that connect adjacent epithelial cells (e.g. claudins, occludins, JAM) and

intracellular scaffolding proteins that stabilize the transmembrane proteins (e.g. ZO, actins) near

the apical surface of intestinal epithelial cells (**Figure 1**) [64].
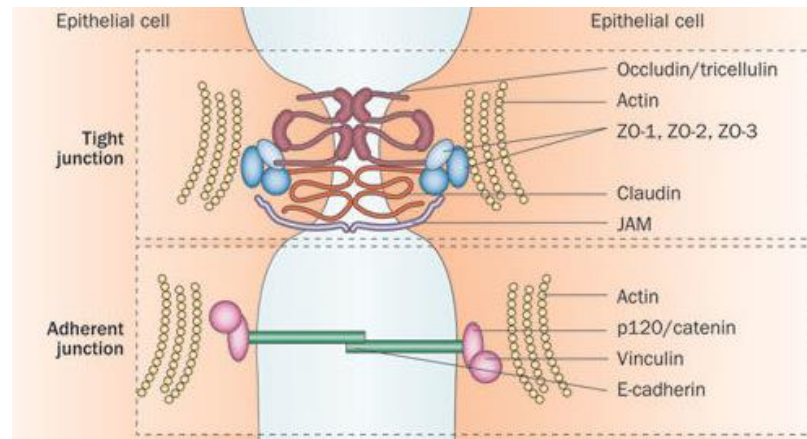
Figure 1: Tight and Adherens Junction Structure [144]

The primary role of tight junctions is to regulate paracellular transport, allowing small molecules

(e.g. ions, water) to pass through, yet preventing entry of larger peptides (e.g., dietary peptides,

bacterial products) with antigenic potential [64, 65]. In contrast, adherens junctions are located

basolaterally to tight junctions and initiate cell-to-cell adhesion, playing an important role in

overall junction maintenance [63]. Like tight junctions, adherens junctions consist of

transmembrane proteins (e.g., e-cadherin) and scaffolding proteins (e.g., vinculin, p120 catenin,

and actin). Other protein complexes known as desmosomes and gap junctions are also involved

in gut integrity and are located near the basolateral surface between intestinal epithelial cells.

When these connections between epithelial cells are disrupted, the intestinal barrier is

compromised and bacterial products such as LPS leak into circulation. In response, the liver releases an acute phase protein known as LPS BP. LPS BP complexes with soluble CD14, and together they initiate an immune response by stimulating TLR-4 on leukocytes [145]. For this reason, LPS BP is considered an indicator of intestinal permeability, and may be preferable over direct measurement due to the likelihood of assay contamination from environmental LPS. Overall, tight and adherens junctions are critical in preventing luminal contents from entering the lamia propria and ultimately circulation, which can initiate an immune response and subsequent host damage [146].

The gut barrier is subject to damage under conditions of chronic inflammation such as active CD and chronic western diet consumption [146-148]. In the context of CD, increased intestinal permeability due to damaged or reduced expression of tight junction proteins (e.g., claudins, occludin, zonula occludens or ZOs) is well characterized [66, 67, 149, 150]. This compromise in barrier integrity associated with active CD is thought to be a major contributor to GALT immune cell stimulation and subsequent inflammation [95]. Loss of gut barrier integrity in CD is likely due to a series of events, beginning with the relative resistance of gluten peptides to digestion [151]. This is then exacerbated by reduced and less active brush border enzymes (e.g., aminopeptidase N, glycyl-leucine dipeptidase), resulting in larger, more antigenic gluten peptides reaching the small intestine [152]. Regarding the specific mechanism by which gluten-derived peptides cross the intestinal epithelium, several hypotheses have been proposed. For example, Fasano and colleagues [153, 154] report that gliadin peptides stimulate the release of a molecule known as zonulin, which increases intestinal permeability via displacement of the scaffolding protein ZO-1, which leads to destabilization of tight junctions. In support of this theory, pharmacological zonulin inhibitors have provided promising results in refractory CD

patients [154, 155]. Another proposed mechanism independent of tight junction damage is increased transcellular transport of gliadin peptides in CD patients. In enterocyte cell lines derived from adenocarcinomas (i.e., HT29, Caco-2), gliadin peptides are taken up into endosomes and are excreted basolaterally only partially degraded [156, 157]. Notably, this activity is increased in the presence of IFN-γ, a cytokine upregulated in CD. Matysiak-Budnik and colleagues [158] provided additional insight into this mechanism, showing that anti-gliadin IgA is involved in this process (**Figure 2**). Anti-gliadin IgA is secreted onto the mucosal surface, where it complexes with gliadin derived peptides and is transported back into the lamina propria [152, 158]. These complexes travel to the lamina propria by binding to the apical enterocyte receptor CD71, which allows for unimpeded transport back across the intestinal epithelium. Interestingly, CD71 expression is dramatically upregulated in active CD [158].
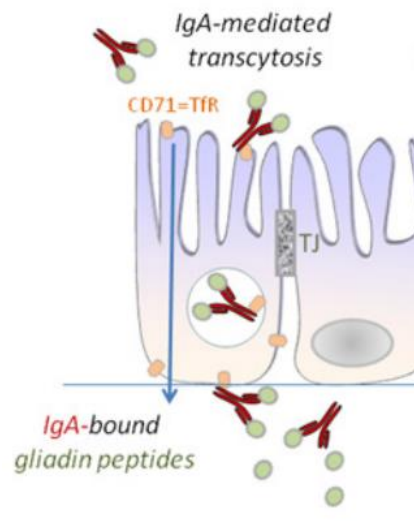

Figure 2: IgA mediated transport of gliadin peptides into gut lamina propria [152]

 Regardless of mechanism, the result of increased gliadin entering the lamina propria increased immune cell activation (e.g., dendritic cells, macrophages) and inflammation [159-161].

Few studies have evaluated barrier integrity in the context of NCGS and findings are mixed. Hollon and colleagues [162] reported that intestinal biopsies from NCGS individuals displayed increased permeability relative to CD patients in remission. Similarly, several markers suggestive of gut permeability (i.e., soluble CD14, LPS binding protein, anti-flagellin antibodies, and anti-LPS antibodies) are elevated in the serum of individuals with NCGS [74]. Theoretically, increased *IFN-γ* expression reported in NCGS could contribute to intestinal permeability in a similar mechanism to CD, but this has yet to be demonstrated directly [46]. On the other hand, cross-sectional data suggests that NCGS individuals have improved barrier integrity, evidenced by increased claudin-4 expression relative to healthy controls and CD [47].

Intestinal permeability is also increased with chronic consumption of a western diet, which may be driven by diet-induced microbial dysbiosis and subsequent inflammation [32, 163, 164]. Specifically, Martinez-Medina and colleagues [164] demonstrated that western diet consumption (60% fat) increased mucin degrading (i.e., *Ruminococcus torques*) and pathogenic (i.e., *Bacteroides-Prevotella, E. coli*) bacterial populations. This shift in microbiota composition was associated with elevated *NOD2* and *TLR-5* expression, TNF-α secretion, and intestinal permeability. Consistent with these findings, LPS and LPS BP are elevated with chronic consumption of a western diet [165, 166]. Further, antibiotic treatment rescued western diet-induced intestinal permeability, emphasizing the interplay between western diet consumption, the gut microbiota, and the intestinal barrier [32]. In summary, evidence suggests that active CD, chronic western diet consumption, and potentially wheat consumption in NCGS can induce intestinal permeability, likely by altering the gut microbiota and increasing inflammation.

In addition to providing a physical barrier, intestinal epithelial cells in the small intestine are organized in such a way to provide a large surface area to maximize nutrient absorption. This

is accomplished with finger-like projections in the lumen known as villi, which are also lined with microvilli. Another advantageous structural characteristic of both the small and large intestine is the presence of grooves between each villus known as crypts that contain specialized Paneth cells that are an important source of anti-microbial peptides (e.g. regenerating islet-derived proteins (Regs), defensins) and intestinal stem cells [62]. Antimicrobial peptides serve as an innate protection from bacteria, protozoa, fungi, and viruses, by suppressing their growth while intestinal stem cells are important in maintaining the constantly regenerating intestinal epithelium [167]. Within the context of CD, crypt and villi structures are damaged, resulting in blunted villi height (i.e., atrophy) and deepening of crypts (i.e., hyperplasia) [40, 168]. The elongation of crypts precedes villous atrophy and is due to an increase in the proliferative compartment of crypts [169, 170]. This reflects increased rates of mitosis of intestinal stem cells (i.e., hyperplasia), which can renew damaged enterocytes and goblet cells [169-171]. Also contributing to crypt hyperplasia are increased stromal cell proliferation and lamina propria expansion, as well as increased influx of inflammatory cells (e.g., Th1 cells) and subsequent tissue remodeling. The occurrence of villous atrophy is thought to be largely due to damage by activated Th cells producing the inflammatory cytokines IFN-γ and TNF-α [40, 168]. Importantly, villous atrophy is not only a biomarker for CD, but can also lead to nutrient deficiencies (e.g., iron, calcium) in CD due decreased surface area for absorption, and can contribute to secondary pathologies (e.g., anemia, osteoporosis) [84]. In the case of NCGS, villus atrophy and crypt hyperplasia are not observed, suggesting a milder inflammatory response relative to CD [12, 75]. Outside of chronic inflammatory diseases, villi and crypt structure is also impacted by dietary patterns resembling the western diet. With respect to villi height, data is mixed. In one study, rats on a diet consisting of 40% butter fat displayed reduced villi height in

the jejunum and ileum [172]. In partial agreement, rats fed a 70% fat diet based on polyunsaturated fat for 1 week led to increased villi height, but reduced microvilli height [173]. Another report, in C57BL/6 mice fed a 42% fat diet found that ileal villi length where increased as well, although this diet was also based on polyunsaturated fat (i.e., soybean oil) rather than saturated fat [174]. Also consistent with an inflammatory response, Hamilton et al. [79] reported increased ileal crypt depth with western diet consumption. Overall, there is strong evidence that villi and crypt structure are altered in CD, and reason to believe that high saturated fat diets may promote a similar phenotype.

In addition to structural roles, intestinal epithelial cells have an active role in mucosal immunity. This is evidenced by their array of pattern recognition receptors (PRRs), including NODs and TLRs [175, 176]. These innate immune system receptors recognize bacterial antigens, known broadly as pathogen associated molecule patterns (PAMPs). In addition to PRRs, epithelial cells sample luminal contents via microfold cells (M cells) located over Peyer's patches [177]. Within Peyer's patches, antigen is taken up by dendritic cells which can then be presented to all Th cell subtypes (i.e., Th1, Th2, Th17, or Tregs). Further, intestinal epithelial cells express MHC II and are capable of presenting antigen directly to Th cells [178, 179]. In sum, intestinal epithelial cells have a wide range of functions beyond their role of absorbing nutrients that includes regulating gut barrier integrity, regulating bacterial populations, and participating in the mucosal immune response.

Mucosal Immune System

Recently, there has been a great deal of interest in the interplay between the contents of the gastrointestinal tract and the underlying mucosal immune system. The lower gastrointestinal tract (i.e., small intestine and colon) is a dynamic environment where the mucosal immune

system continually interacts with luminal contents (i.e., gut microbiota, digested food). Interestingly, approximately 70% of an individual's immune cells reside in the gut associated lymphoid tissue (GALT) at any given time [34]. In addition, these immune cells (e.g., T cells, dendritic cells) can traffic to and from an elaborate network of lymphoid tissues (i.e., lamina propria, Peyer's patches, and mesenteric lymph nodes). Under normal conditions, professional antigen presenting cells known as dendritic cells extend between intestinal epithelial cells and survey the intestinal lumen [35]. Dendritic cells then present found dietary antigens, bacteria, or environmental toxins to naïve CD4+ Th cells through the surface protein known as MHC II [36]. Interactions between dendritic cells and naïve Th cell including MHC II recognition, binding of other co-receptors, and the local cytokine environment lead to differentiation of the naïve Th cell into one of four subtypes: Th1, Th2, Th17, or Tregs. Th1 cells are induced by IL-12 and IFN-γ signaling and are classically known for providing defense against intracellular pathogens by activation of phagocytes (e.g., macrophages) through IFN-γ and IL-12 signaling. Importantly, hyper-responsive Th1 cells have been linked to a host of chronic inflammatory diseases (e.g., CD, IBD) [38, 40, 180]. IL-4 signaling is known to differentiate naïve Th cells into Th2 cells, which can then activate antibody producing B lymphocytes through their own production of IL-4 [181]. Th17 cells are pro-inflammatory cells induced by the combination of IL-6 and TGF-β or IL-21 alone [182, 183]. Under normal conditions, Th17 cells secrete cytokines (i.e., IL-17) to recruit neutrophils to the site of an extracellular bacterial infection [182, 183]. However, overproduction of IL-17 has been shown to increase other inflammatory cytokines (e.g., IL-6, IL-1β) *in vitro* and contribute to tissue damage and autoimmunity *in vivo* [184-186]. Finally, Tregs are differentiated from naïve CD4+ T cells by TGF-β signaling, and produce cytokines (e.g. IL-10, TGF-β) that suppress the activity of inflammatory immune cells – an important

function in the gut where many molecules present are innocuous (i.e., food, commensal bacteria) and an immune response is unnecessary [187]. Due to the inflammatory nature of Th17 cells and the immunosuppressive function of Tregs, the ratio of Th17: Tregs cells and their characteristic cytokines are of particular interest as indicator for gut health [188, 189]. Production of IgA by lamina propria B cells is also considered an important feature of the mucosal immune system. Specifically, it is secreted into the lumen where it neutralizing pathogenic bacteria and toxins [190].

CD and NCGS both are characterized by a gut mucosal immune response; however, there appear to be unique features characterizing each condition. Specifically, over 95% of CD patients have a genetic background (i.e., HLA-DQ2 and/or HLA-DQ8 variants) that increase antigen presenting cells' (i.e., dendritic cells) propensity to recognize and present gluten-derived peptides to naïve CD4+ T cells in the lamina propria and secondary lymphoid organs (e.g., lymph nodes, Peyer's patches) [39-41]. After activation by dendritic cells, Th cells secrete primarily Th1 cytokines (e.g. IFN-γ, TNF-α), which increase fibroblast production of matrix metalloproteases (MMPs) that damage intestinal structure (i.e., villous atrophy and crypt hyperplasia) [40]. Another cytokine over produced by CD4+ Th cells in CD is IL-21, which also stimulates MMPs and increases mucosal intraepithelial lymphocyte infiltration into the intestinal mucosa [40, 191]. In addition to these functions, IL-21 signaling perpetuates the Th1 response. This was illustrated by decreased IFN-γ and Th1 cell-inducing transcription factor (i.e., T-bet) expression when gliadin stimulated intestinal organ cultures were treated with an anti-IL-21 antibody [192]. Th2 related cytokines (e.g., IL-4, IL-10) are also present in intestinal biopsies from CD patients following activation of naïve CD4+ T cells by gluten peptides *ex vivo* [193]. These Th2 cytokines are implicated in B cell activation, which produce autoantibodies against

enzymes involved in gluten metabolism (i.e., tissue transglutaminase 2 or TG2) and gluten peptides themselves [40]. In addition to a common biomarker, tissue transglutaminase 2 antibodies play an active role in the disease process of CD by increasing the propensity of TG2 to create gluten derived epitopes, increasing intestinal permeability, and activating monocytes [194-196]. Although the evidence is mixed, there is also reason to believe that anti-tissue transglutaminase 2 antibodies inhibit intestinal epithelial cell proliferation [196].

In addition to Th1 and Th2 cells, two other Th cells subsets, Th17 cells and Tregs, are implicated in CD pathogenesis [44, 197, 198]. Indeed, gliadin specific Th17 cells have been identified and elevated IL-17 production is well characterized in CD [197, 199-201]. In addition to IL-17, gliadin specific Th17 cells also produce IFN-γ, potentially contributing to tissue remodeling in a manner similar to Th1 cells [201]. Importantly, IL-21 signaling, originating from CD4+ intraepithelial lymphocytes, contributes to Th17 cell activation in CD and is overproduced in the mucosa of patients [197, 202]. Notably, IL-21 production is dependent on IL-15 signaling produced primarily by dendritic cells and intestinal epithelial cells in the context of gut inflammation [203, 204]. Exacerbating mucosal inflammation, Treg's immunosuppressive function is impaired in CD patients. Specifically, inflammatory Th cell subsets (i.e., Th1, Th17) are more resistant to downregulation by Treg signaling and this effect appears to be mediated by IL-15 signaling *in vivo* [44, 45]. Overall, CD is characterized by a complex network of cytokines resulting in a strong adaptive immune response by Th cells that is not dampened by Tregs.

The innate immune system is also involved in the pathogenesis of CD. For example, macrophages are activated by tissue transglutaminase 2 (TG2) antibodies and gliadin directly *in vitro*, leading to production of TNF-α and IL-12 [159]. Similarly, CD intestinal biopsies treated with gliadin derived peptides increased cyclooxygenase-2 (COX-2) [205]. Further, dendritic cells

elaborate a host of inflammatory cytokines (i.e., IL-6, IL-8, TNF-α) in an NF-kB dependent manner in response to gluten peptides in addition to their role in activating Th cells [161]. Overall, CD pathogenesis is dominated by a Th response, but the innate immune system is implicated as well.

In the case of NCGS, the major immunological players are less clearly defined. There is evidence of an adaptive immune response mediated by Th cells, as shown by elevated cytokines (i.e., IFN- γ) following a gluten challenge in at least one study [46]. Additionally, increased CD3+ intraepithelial lymphocytes and eosinophils have been identified in biopsies from individuals with NCGS, but to a lesser extent than CD [46, 47, 94]. Notably, certain cytokines that contribute to CD pathogenesis (i.e., IL-17) are not upregulated in intestinal biopsies of NCGS individuals [199]. It has also been suggested that NCGS symptoms may be related to an innate immune response, evidenced by increased toll-like receptor (TLR) 2 gene expression [47]. In sum, the immune response to NCGS is less clear, with some evidence of both an adaptive and innate immune system activation, although much less pronounced than CD.

The characterization of NCGS and broader population that is intentionally avoiding wheat products raises the question as to whether genetic changes to wheat relative to its heirloom predecessors have led to negative health effects in a larger population than celiac disease and NCGS. Despite some research that compares the health effects of heirloom and modern wheat, no studies have evaluated indicators of gastrointestinal health in-depth in a model that is representative of the general population. Additionally, whether the effect of wheat on gut health is dependent on the overall dietary context it is consumed in (i.e., normal or western diet) is also unknown. These findings are important in beginning to understand whether modern wheat varieties are initiating a more pronounced inflammatory response compared to heirloom varieties

to either inform nutrition recommendations or to alleviate concerns around GMO wheat products

and gluten-sensitivity.

CHAPTER III

METHODS

Animal Care

Six-week old C57BL/6 male mice (n=80; Charles River) were housed at Oklahoma State University's environmentally controlled Laboratory Animal Research Facility (4-5 mice/cage) and were acclimated for 2 weeks before initiation of the study. Mice (n=12-13/group) were then randomized to the following treatment groups in a 2 x 3 factorial design with diet (AIN93-G control diet or western diet) and wheat (no added wheat, the heirloom variety Turkey, or the modern wheat variety Gallagher) as factors. Gallagher was chosen as the modern variety because it is widely grown. The western diet (WD) was formulated to consist of 45% fat kcal primarily from lard and was high in refined sugar (**Table 1**). Both Turkey and Gallagher wheat were milled to flour and then subsequently analyzed for their protein, fat, fiber, calcium, and phosphorus content (NPAL Analytical Laboratories, St. Louis, MO). Diets containing wheat were supplemented at 10% (w/w) and adjusted to match macronutrient, fiber, calcium and phosphorus content to the AIN93-G diet (control) or WD diet. Mice were had *ad libitum* access to their respective diets and water daily. Food intake was assessed daily, and body weights recorded weekly throughout the 6-week study period. After 5 weeks on treatments, fasting blood glucose was assessed, and the following week mice were anesthetized using a ketamine/xylazine

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cocktail (100 mg/10 mg/ kg body weight) and exsanguinated by the carotid artery. Blood was collected. A 25 µl sample of whole blood was transferred to microcentrifuge tube containing Türk's solution (1:20) for quantifying total white blood cell counts and blood smears were made to assess leukocyte differential counts. After flushing the small and large intestine with ice cold PBS, small sections of the jejunum, ileum, and colon were fixed in 10% NBF for histological examination. The remaining ileum lamina propria and colon were snap frozen in liquid nitrogen and stored at −80°C for gene expression and protein analyses. Cecal contents were flushed and weighed for SCFA analysis. Liver, white adipose (WAT), spleen, heart, thymus, cecum weights were recorded and these tissues as well as Peyer's patches were stored at −80°C. All procedures were approved by the Oklahoma State University Institutional Animal Use and Care Committee.

Body Composition Assessment

At the time of necropsy, whole body PixiMus scans (GE Medical Systems Lunar, Madison, WI) were performed to assess body composition (i.e., lean mass, fat mass, and body fat percentage).

Histological Analysis

Jejunum, ileum, colon, and liver were dehydrated (Shandon Citadel 2000 Waltham, MA) using a graded ethanol series and toluene. Tissues were then embedded in paraffin and 5 um section were cut (Leica Biosystems Wetzlar, Germany). H&E staining was performed to view structural changes in villi height, villi width, villi area, villi perimeter, crypt depth with BZ-X800 software (Keyence Osaka, Japan). Slides were then subjected to histopathological analysis by the study pathologist. For gut sections, overall scoring was based on the criteria set by Erben, et al. [206], which encompassed lymphocyte infiltration (subscale 1-4), villous atrophy and crypt hyperplasia (subscale 1-5), and goblet cell number (subscale 1-4). Subscales were added together

for a total score, with higher scores representing negative outcomes with respect to these parameters. For liver sections, a steatosis score was given based on the criteria proposed by Brunt, et al. [207, 208] where a score of 0 represented <5% and 4 indicated >75% steatosis.

RNA Extraction and Gene Expression Analysis

Total RNA was extracted from the colon and ileum lamina propria using Trizol (Life Technologies, Carlsbad, CA). RNA (2 µg; n=6/group) was reverse transcribed (Superscript II, Invitrogen, Carlsbad, CA) to make complimentary DNA (cDNA) and real-time quantitative reverse transcription polymerase chain reaction (qRT-PCR) (7300 Real-Time PCR System, Applied Biosystems, Foster City, CA) was performed using SYBR green as the detector (Roche, Penzberg, Germany). In the colon, genes regulating barrier integrity were assessed (e.g., claudins, occludin, mucin-2) and in the ileum lamina propria genes involved in immune cell activity (e.g., TNF- α, IL-17, IL-10) and anti-microbial peptides (e.g., Reg3) were assessed (**Table 2**).

Protein Analysis

The tight junction proteins claudin-4, occludin, and ZO-1 were assessed in the colon using western blotting (n=5). Total protein was extracted from the colon using radioimmunoprecipitation assay (RIPA) buffer and quantified using the bicinchoninic acid (BCA) assay. Protein (20-30 µg) was then boiled for initial denaturation and separated on a denaturing sodium dodecyl sulfate (SDS) polyacrylamide gel and transferred to a polyvinylidene fluoride (PVDF) membrane. Best results for ZO-1 were obtained when samples were not boiled prior to SDS-page. Equal transfer was confirmed with Ponceau staining before blocking for 1 hour (claudin-4, occludin) or 8 hours (ZO-1) with 5% nonfat milk in 0.1% TBST. Primary antibodies for claudin-4 (Thermo Fisher catalog #36-4800, 1:1,000), occludin (Thermo Fisher

catalog #33-1500, 1:5,000), and ZO-1 (Thermo Fisher catalog #61-7300, 1:10,000) were

incubated with the membrane overnight at 4° C. Next, the membranes were washed, incubated

with the secondary antibody for 1 hour at room temperature, and imaged using SuperSignal West

chemiluminescent substrate (Thermo Fisher Waltham, MA). Blots were developed using the

ProteinSimple Fluorchem R (San Jose, CA). Data were normalized to β-actin and quantified

using Image J software (NIH Rockville, MD).

Serum Analysis

A commercially available ELISA was used to assess serum lipopolysaccharide binding

protein (LPS BP) (Hycult Biotech Uden, Netherlands). Serum metabolic parameters (i.e., total

cholesterol, triglycerides, non-esterified fatty acids) were assessed using the BioLis 24i

automated chemistry analyzer (Carolina Chemistries Greensboro, NC).

SCFA Analysis

To assess cecal SCFA concentration, samples were suspended in ice-cold Millipore H_2O

and spiked with internal standard (1 mM 2-ethylbutyric acid in 12% formic acid). The pH for

each sample was adjusted to 2-3 using 5 M HCl. Samples were then homogenized for 1 minute

and centrifuged for collection of the supernatant. Supernatants were filtered using 0.45-mm

polytetrafluoroethylene syringe filters (Agilent Technologies). Gas chromatographic analysis

was performed using an Agilent 6890N GC system with a flame ionizable detector and an

automatic liquid sampler (Agilent Technologies Santa Clara, CA). Samples concentration were

determined using a 5-point calibration curve, with each standard containing solutions of acetic,

propionic, butyric, valeric, isovaleric, isobutyric, caproic and heptanoic acids (Sigma-Aldrich).

Statistical Analysis

Data was analyzed using SAS Version 9.4 statistical analysis software (SAS Institute Inc., Cary, NC). First, a Shapiro Wilks test was performed to assess whether data for continuous variables was normally distributed. Normally distributed data was analyzed using 2-way ANOVA, with diet and wheat as factors. When data were not normally distributed, Friedman's test was performed. When $p < 0.05$, Fischer's least square means was run for post-hoc analysis. Histological scoring was evaluated using Fisher's exact for categorical data. All data is presented as mean ± standard error, and the alpha was set at 0.05.

Table 1: Diet Formulation

Ingredients	Control	Control + Turkey	Control + Gallagher	Western Diet	Western + Turkey	Western + Gallagher
Wheat	0	100	100	0	100	100
Carbohydrates						
Cornstarch (g)	397.5	323.9	323.5	88.5	14.9	14.5
Maltodextrin (g)	132	132	132	115	115	115
Sucrose (g)	100	100	100	200	200	200
Wheat (g)	0	73.6	74	0	73.6	74
Total (g)	**629.5**	**629.5**	**629.5**	**403.5**	**403.5**	**403.5**
Protein						
Casein (g)	200	189.6	189.9	245	234.6	234.9
Wheat (g)	0	10.4	10.1	0	10.4	10.1
Total (g)	**200**	**200**	**200**	**245**	**245**	**245**
Fat						
Soybean Oil	70	68.91	68.88	30	28.91	28.88
Lard	0	0	0	195	195	195
Wheat	0	1.09	1.12	0	1.09	1.12
Total (g)	**70**	**70**	**70**	**225**	**225**	**225**
Fiber						
Cellulose (g)	50	49.698	49.654	58	57.698	57.654
Wheat (g)	0	0.302	0.346	0	0.302	0.346
Total (g)	**50**	**50**	**50**	**58**	**58**	**58**
Vitamin mix (g)	10	10	10	19	19	19
Mineral						
Mineral mix (Ca-P Deficient) (g)	13.4	13.4	13.4	16.46	16.46	16.46
Calcium Carbonate (40.04%ca) (g)	12.5	12.45	12.45	15.35	15.32	15.31
Calcium Phosphate	0	0	0	3.4	3.4	3.4
Calcium from Wheat (g)	0	0.0141	0.0152	0	0.0141	0.0152
Sodium Phosphate, monobasic (25.81% P) (g)	4.38	4.35	4.38	5.39	5.37	5.4
Potassium Phosphate, monobasic (22.76% P) (g)	1.88	1.87	1.88	2.32	2.31	2.32
Phosphorus from Wheat (g)	0	0.0834	0.0717	0	0.0834	0.0717
Sucrose (g)	2.84	2.93	2.89	0.08	0.14	0.11
Total (g)	**35.00**	**35.00**	**35.00**	**43.00**	**43.00**	**43.00**
L-Cysteine (g)	3	3	3	3.5	3.5	3.5
Choline Bitartrate (g)	2.5	2.5	2.5	3	3	3
TBHQ	0.014	0.014	0.014	0.014	0.014	0.014
Total (g)	**1000**	**1000**	**1000**	**1000**	**1000**	**1000**

Table 2: Primer Sequence List for qRT- PCR

Symbol	Name	Sequence
Cyclo	Cyclophilin	QF 5'- GGTCTTTGGGAAGGTGAAAGAA -3' QR 5'- GCCATTCCTGGACCCAAAA -3'
IL-6	Interleukin 6	QF 5'- GAGGATACCACTCCCAACAGACC -3' QR 5'- AAGTGCATCATCGTTGTTCATACA -3'
TGFβ	Transforming growth factor beta	QF 5'- CCCTATATTTGGAGCCTGGA -3' QR 5'- CTTGCGACCCACGTAGTAGA -3'
IFN-γ	Interferon gamma	QF 5'- TGGCATAGATGTGGAAGAAAAGA -3' QR 5'- TGCAGGATTTTCATGTCACCAT -3'
IL-1β	Interleukin 1 beta	QF 5'- CAACCAACAAGTGATATTCTCCAT -3' QR 5'- GATCCACACTCTCCAGCTGCA -3'
IL-17	Interleukin 17	QF 5'- ATCCCTCAAAGCTCAGCGTGTC -3' QR 5'- GGGTCTTCATTGCGGTGGAGAG-3'
TNF-α	Tumor necrosis factor alpha	QF 5'- CTGAGGTCAATCTGCCCAAGTAC -3' QR 5'- CTTCACAGAGCAATGACTCCAAAG-3'
ROR-γ	Retinoic acid-related orphan receptor gamma	QF 5'- GAAGGCAAATACGGTGGTGT -3' QR 5'- GGGCAATCTCATCCTCAGAA -3'
IL-10	Interleukin 10	QF 5'- GGTTGCCAAGCCTTATCGGA-3' QR 5'- ACCTGCTCCACTGCCTTGCT -3'
Cldn2	Claudin 2	QF 5'- TCTCAGCCCTGTTTTCTTTGG -3' QR 5'-GGCGAGCAGGAAAAGCAA -3'
Cldn15	Claudin 15	QF 5'- TGGAGCCTGTGGGATGGT-3' QR 5'- GTGGGTTGAAGAAGTCAGTAGTGATG-3'
Muc2	Mucin-2	QF 5'- CTGACCAAGAGCGAACACAA -3' QR 5'- CATGACTGGAGGCAACTGGA -3'
Ocln	Occludin	QF 5'- ACCCGAAGAAAGATGGATCG-3' QR 5'- CATAGTCAGATGGGGGTGGA -3'
JAM3	Junction adhesion molecule 3	QF 5'- CACTACAGCTGGTACCGCAATG -3' QR 5'- CTGGGATTGGCTCTGGAATC -3'
ZO-1	Zonula Occluden 1	QF 5'- AGCCTGGTTGTTTAGGAGCA -3' QR 5'- CAGAATACGGCTCCTTCCTG -3'
Reg3β	Regenerating islet derived protein 3 beta	QF 5'- TGGGAATGGAGTAACAAT -3' QR 5'- GGCAACTTCACCTCACAT-3'
Reg3γ	Regenerating islet derived protein 3 gamma	QF 5'- CCATCTTCACGTAGCAGC -3' QR 5'- CAAGATGTCCTGAGGGC -3'

CHAPTER IV

RESULTS

Body Weight, Body Composition and Tissue Weight

First, the effects of the WD and wheat were assessed on body weight and composition. At baseline, there was no statistically significant difference in body weight between groups; however, animals fed WD displayed increased body weight beginning after 1 week of treatment until the end of the study (**Figure 3**). Analysis of body composition revealed that animals consuming WD exhibited increased percent fat and lean mass ($p <0.01$; **Table 3**). Likewise, the abdominal fat depot was increased by 48% in the groups consuming the WD. Neither variety of wheat affected these indicators of body composition (**Table 3**).

Metabolic Parameters

In addition to changes in body composition, metabolic indicators were also assessed. As would be expected, fasting blood glucose, total cholesterol and NEFA were significantly elevated ($p <0.05$) by WD treatment (**Table 3**). The WD had no effect on serum triglycerides, but the addition of both wheat varieties reduced triglycerides relative to groups not consuming wheat (Table 3, $p <0.05$). No significant effect of wheat on fasting blood glucose, total cholesterol and NEFA occurred in response to wheat under either normal of WD conditions.

Histopathological analysis of the liver was performed to assess the effect of the WD and wheat on liver steatosis. No alterations in liver steatosis were noted due to WD diet or wheat over the course of this 6-week study (**Figure 3**).

Gut Structural Analysis and Histopathology Scores

Histological analyses of villi and crypt structures were performed on the jejunum, ileum and colon. **Figure 4** shows representative histological sections for each of these regions of the intestine. WD treatment significantly reduced villi height, area and perimeter in the jejunum, but no structural changes were noted with this diet in the villi of the ileum ($p <0.05$, **Table 4**). Wheat had no effect on any parameter in the jejunum, yet wheat increased villi width, area, and perimeter in the ileum. Post hoc analysis revealed that groups consuming Gallagher were higher than groups not consuming wheat and an intermediate effect of Turkey was seen in all these ileal parameters ($p <0.05$, **Table 4**). Increased crypt depth is associated with chronic gut inflammation; however, in this study we saw no effect of WD or wheat on this parameter in any region of the small and large intestine. From a histopathological standpoint, no differences amongst treatment groups were observed on scores that encompassed structural parameters such as villous atrophy, crypt hyperplasia. Similarly, no detectable changes were observed on mucus producing goblet cells in any region of the gut.

Gut Barrier Integrity and Function

To determine the effects of wheat alone and in combination with the WD, we assessed colonic expression of genes related to barrier integrity. Expression of the intracellular scaffolding protein *ZO-1*, was unaffected by treatment. Similarly, genes encoding for tight junction transmembrane proteins (i.e., *Cldn2*, *Cldn15*, *Ocln*) and proteins important in the mucus layer formation (i.e., *Muc2*) were unchanged relative to control (**Table 5**). One exception was

suppression of *JAM3* with addition of Turkey to the WD relative to all other groups ($p < 0.05$, Table 5).

In the colon, relative abundance of the tight junction proteins, ZO-1, occludin, and claudin-4, were assessed by western blotting. For all of these proteins, no statistical difference was detected with WD and wheat, alone and in combination (**Figure 4A-B**).

Serum LPS BP was analyzed as indirect measure of circulating LPS, which is known to leak into circulation when barrier integrity is compromised. After six weeks of treatment, serum LPS BP was not altered with the WD or wheat diets (**Figure 4C**).

Assessment of Inflammatory Markers

Serum hsCRP and white cell counts were assessed as indicators of systemic inflammation. No effect of WD or wheat were seen (**Figure 5**). Interestingly, main effects were observed by WD and wheat for total WBC count. With both treatments, total WBC were reduced suggesting increased extravasation towards a site of inflammation, although all groups were still within the normal range ($p < 0.05$).

To determine if wheat contributed to a local inflammatory response in the gut and whether WD affected this response, gene expression of inflammatory mediators was evaluated in ileum lamina propria samples. Expression of cytokines considered inflammatory in the gut (i.e., *TNF-α, IL-6, IL-1β*) were unaffected by treatment. Similarly, anti-inflammatory cytokines involved in maintaining gut immunotolerance (i.e., *IL-10, TGF-β*) were unaffected by treatment (**Table 6**). Among those altered, *IFN-γ* expression was increased with WD, consistent with an inflammatory response. Further, *IL-17* expression was suppressed by Turkey in the context of WD relative to all other groups ($p < 0.05$, Table 6). Interestingly expression of *ROR-γ,* a transcription factor involved in induction of IL-17 producing Th17 cells, was unchanged.

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SCFA and Anti-Microbial Peptide Analysis

Cecal SCFAs are known to play an import role in epithelial cell health and immune regulation. For many SCFAs analyzed (i.e., i-butyric, n-butyric, propionic, i-valeric, and n-valeric), no effect of WD or wheat variety was observed (**Table 7**). However, a significant interaction was observed cecal acetate content, with the WD group suppressing acetate relative to control ($p < 0.05$, Table 7). Interestingly, addition of Gallagher to the WD restored cecal acetate to that of the control group.

Neither WD nor wheat affected gene expression for the antimicrobial peptides, *Reg3β* and *Reg3γ*, which are important in regulating bacterial growth in the gut (**Table 6**).

Figure 3.
Body Weights Over Time
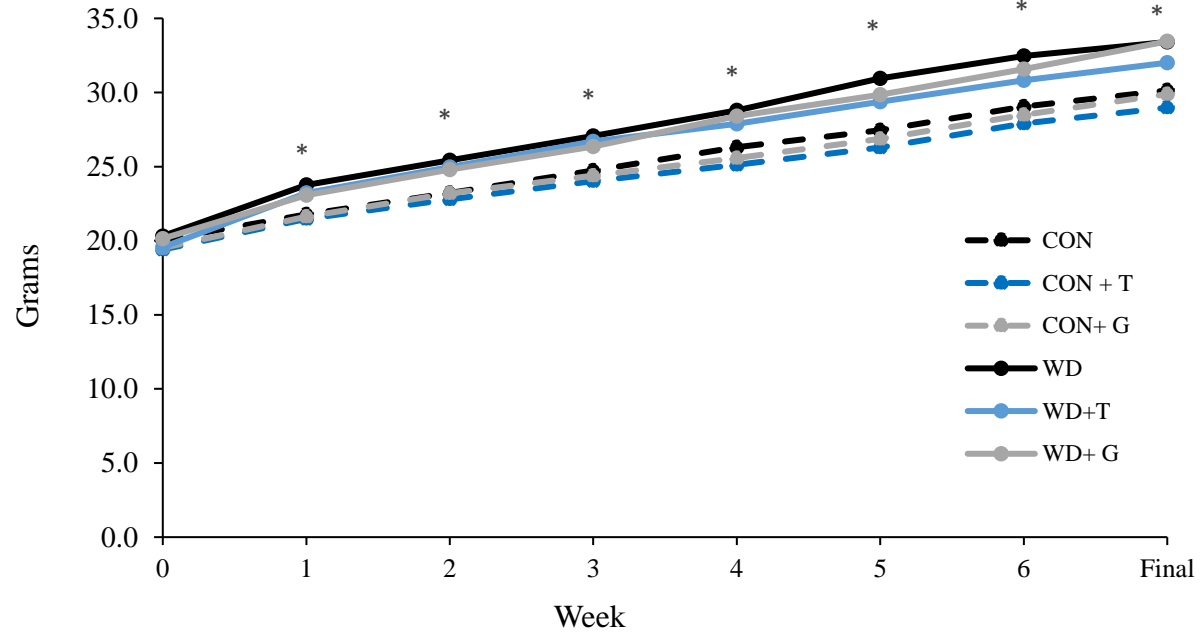
Figure 3. Body weights over the course of the 6-week study. Animals were assigned to six different treatment groups in a 2x3 factorial design. Factors were diet (control or WD) and wheat (no added wheat, Turkey, or Gallagher). Control diets are indicated by dashed lines and solid lines represent western diet groups. Groups with the same color line had the same wheat variety added: no added wheat (black), Turkey (blue), or Gallagher (grey). Baseline weights are indicated by week 0. Asterisk denote main effect by WD.

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Table 3

Anthropometric Data, Tissue Weights, and Metabolic Parameters

	Con	Con + T	Con + G	WD	WD + T	WD + G	P-Value WD	P-value Wheat	P-value WD*Wheat
Body Weights									
Baseline (g)	20.06 ± 0.30	19.40 ± 0.31	19.51 ± 0.45	20.31 ± 0.43	19.55 ± 0.15	20.14 ± 0.30	0.2197	0.1131	0.7519
Final (g)	30.14 ± 0.39	28.98 ± 0.64	29.89 ± 0.71	33.41 ± 0.76	32.01 ± 0.66	33.45 ± 1.14	**<0.0001**	0.1650	0.9388
Body Composition									
Lean mass (g)	20.93 ± 0.25	20.93 ± 0.23	20.94 ± 0.33	22.18 ± 0.40	21.61 ± 0.23	21.60 ± 0.37	**0.0011**	0.5910	0.5622
Percent Fat (%)	31.32 ± 0.64	29.27 ± 0.68	30.61 ± 1.18	34.31 ± 1.05	34.37 ± 1.26	36.28 ± 1.44	**<0.0001**	0.3346	0.4488
Visceral WAT (mg)	502 ± 30	469 ± 22	489 ± 42	767 ± 045	649 ± 48	742 ± 51	**<0.0001**	0.1613	0.5487
Tissue Weight									
Spleen (mg/g BW)	3.69 ± 0.29	3.34 ± 0.13	3.87 ± 0.24	3.70 ± 0.17	3.45 ± 0.15	3.40 ± 0.16	0.9999	0.4403	0.1354
Thymus (mg/g BW)	1.72 ± 0.14	2.02 ± 0.14	1.88 ± 0.12	1.85 ± 0.14	1.71 ± 0.12	1.75 ± 0.09	0.3833	0.8282	0.1945
Heart (mg/g BW)	3.97 ± 0.11	4.01 ± 0.13	3.94 ± 0.09	3.81 ± 0.07	3.75 ± 0.10	3.85 ± 0.15	0.0654	0.9998	0.7931
Cecum (mg/g BW)	3.42 ± 0.21	3.33 ± 0.13	3.32 ± 0.15	3.11 ± 0.21	2.78 ± 0.11	2.51 ± 0.14	**0.0003**	0.1632	0.4476
Liver (mg/g BW)	42.53 ± 0.88	42.57 ± 0.94	42.21 ± 0.73	41.37 ± 0.74	40.93 ± 1.12	41.20 ± 0.58	0.0747	0.8484	0.9894
Pancreas (mg/g BW)	4.61 ± 0.15	4.61 ± 0.25	4.16 ± 0.16	4.24 ± 0.15	4.05 ± 0.16	4.10 ± 0.11	**0.0221**	0.2087	0.3862
Metabolic Parameters									
Glucose (mg/dL)	150 ± 3	146 ± 5	135 ± 3	176 ± 9	184 ± 3	178 ± 8	**<0.0001**	0.3105	0.3644
Total Cholesterol (mg/dL)	129 ± 6	138 ± 4	140 ± 4	180 ± 5	179 ± 6	187 ± 9	**<0.0001**	0.3368	0.6415
Triglycerides (mg/dL)	47 ± 4	46 ± 3	46 ± 3	58 ± 4	43 ± 5	43 ± 3	0.4644	**0.0354**	0.0825
NEFA (mEq/L)	0.97 ± 0.15	1.25 ± 0.11	0.80 ± 0.21	1.31 ± 0.16	1.47 ± 0.07	1.00 ± 0.20	**0.0390**	0.0940	0.4331

Data are presented as mean ± SE. P values <0.05 are considered statistically different.

Figure 4. Representative Images of Gut Histological Sections and Histopathological Scoring of the Gut and Liver

A.

| | Con | Con + T | Con +G | WD | WD + T | WD + G |

Jejunum

Ileum

Colon

B. Gut Histopatholgical Scores
☐ Jejunum
■ Ileum
■ Colon

C. Liver Steatosis Score   p = 0.6215
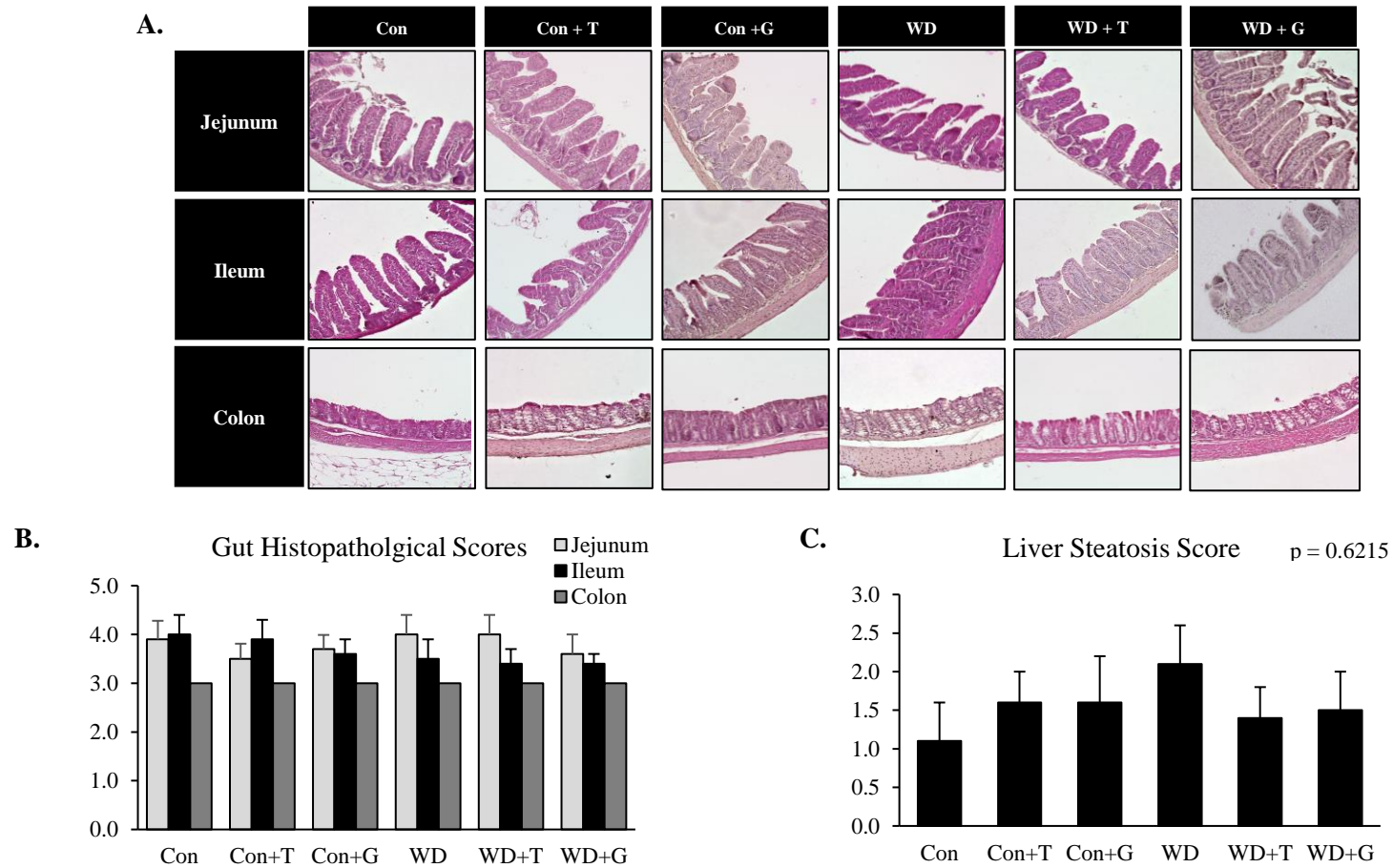
Figure 4. **(A)** Representative images of hematoxylin and eosin (H&E) stained cross-sections of the jejunum, ileum, and colon. Tissue sections of the jejunum, ileum and colon (n=10/group) were subjected to histopathological scoring which encompassed villous atrophy, crypt hyperplasia, lymphocyte infiltration, and goblet cell number **(B)**. Scores ranged from 3 to 13, with higher scores represented negative outcomes with respect to these parameters. **(C)** Sections of the liver were scored for degree of steatosis, with 0 representing <5% and a maximum of 4 indicating >75% steatosis. Data are presented as mean ± SE.

Table 4

Villi and Crypt Structural Parameters in the Jejunum, Ileum, and Colon

	Con	Con + T	Con + G	WD	WD + T	WD + G	P-Value WD	P-value Wheat	P-value WD*Wheat
Jejunum									
Villi Height (μm)	196.9 ± 9.3	213.0 ± 7.4	199.5 ± 10.9	197.2 ± 11.8	179.6 ± 5.7	187.7 ± 6.9	**0.0448**	0.9375	0.1729
Villi Width (μm)	93.5 ± 3.5	94.4 ± 3.4	94.7 ± 3.1	91.5 ± 4.0	85.0 ± 2.9	92.6 ± 3.5	0.1071	0.4765	0.4757
Villi Area (mm^2)	11.5 ± 0.8	12.8 ± 0.7	11.9 ± 1.0	11.0 ± 1.0	9.5 ± 0.5	10.7 ± 0.6	**0.0162**	0.9760	0.2125
Villi Perimeter (μm)	520.3 ± 22.3	551 ± 19.9	524.8 ± 28.8	518.3 ± 28.2	465.7 ± 12.4	493.5 ± 17.6	**0.0323**	0.8677	0.1778
Crypt Depth (μm)	59.1 ± 2.7	60.0 ± 3.2	62.1 ± 2.2	63.1 ± 2.8	62.0 ± 3.2	61.8 ± 2.9	0.4185	0.9276	0.7573
Ileum									
Villi Height (μm)	158.0 ± 6.6	160.8 ± 3.9	162.2 ± 4.6	144.8 ± 5.6	159.0 ± 6.1	158.2 ± 3.7	0.1210	0.1521	0.5002
Villi Width (μm)	82.8 ± 3.5	85.0 ± 2.5	94.1 ± 3.8	84.8 ± 2.4	87.2 ± 3.9	90.7 ± 4.1	0.9601	**0.0408**	0.6386
Villi Area (mm^2)	8.0 ± 0.5	8.5 ± 0.3	9.2 ± 0.4	7.5 ± 0.2	8.5 ± 0.5	8.9 ± 0.4	0.3593	**0.0096**	0.8192
Villi Perimeter (μm)	413.8 ± 15.3	425.7 ± 10.0	438.0 ± 11.7	384.8 ± 9.1	420.7 ± 15.9	425.5 ± 8.3	0.0974	**0.0218**	0.5912
Crypt Depth (μm)	68.8 ± 2.3	63.8 ± 4.9	75.3 ± 3.0	64.1 ± 2.3	65.6 ± 2.6	65.2 ± 2.8	0.0966	0.1901	0.1736
Colon									
Crypt Depth (μm)	99.9 ± 8.8	95.1 ± 7.4	97.5 ± 6.1	90.8 ± 3.8	101.3 ± 7.7	105.2 ± 6.6	0.7454	0.7233	0.4277

Data are presented as mean ± SE. P values <0.05 are considered statistically different.

Table 5

Relative Expression of Genes Related to Barrier Integrity and Mucous Layer Formation in the Colon

	Con	Con + T	Con + G	WD	WD + T	WD + G	P-Value WD	P-value Wheat	P-value WD*Wheat
Claudin-2	1.00 ± 0.06	1.29 ± 0.17	1.27 ± 0.12	0.97 ± 0.12	0.99 ± 0.13	1.09 ± 0.17	0.1144	0.2981	0.5856
Claudin-15	1.00 ± 0.18	1.02 ± 0.09	1.05 ± 0.15	0.79 ± 0.17	1.06 ± 0.09	0.95 ± 0.30	0.5322	0.7332	0.7852
Occludin	1.00 ± 0.07	1.13 ± 0.08	1.13 ± 0.08	1.07 ± 0.08	0.99 ± 0.08	1.12 ± 0.12	0.7478	0.5718	0.4647
JAM-3	1.00 ± 0.05[a]	1.13 ± 0.09[a]	1.10 ± 0.03[a]	1.00 ± 0.02[a]	0.82 ± 0.03[b]	1.02 ± 0.08[a]	**0.0123**	0.3543	**0.0213**
ZO-1	1.00 ± 0.08	1.40 ± 0.15	1.31 ± 0.05	1.10 ± 0.10	1.21 ± 0.13	1.28 ± 0.21	0.6587	0.0703	0.4997
Mucin-2	1.00 ± 0.17	2.14 ± 0.47	1.60 ± 0.36	1.30 ± 0.39	2.31 ± 0.63	1.49 ± 0.68	0.8018	0.0782	0.8975

Data are presented as mean ± SE. Within a given row, values that share the same superscript letter are not statistically different from each other.
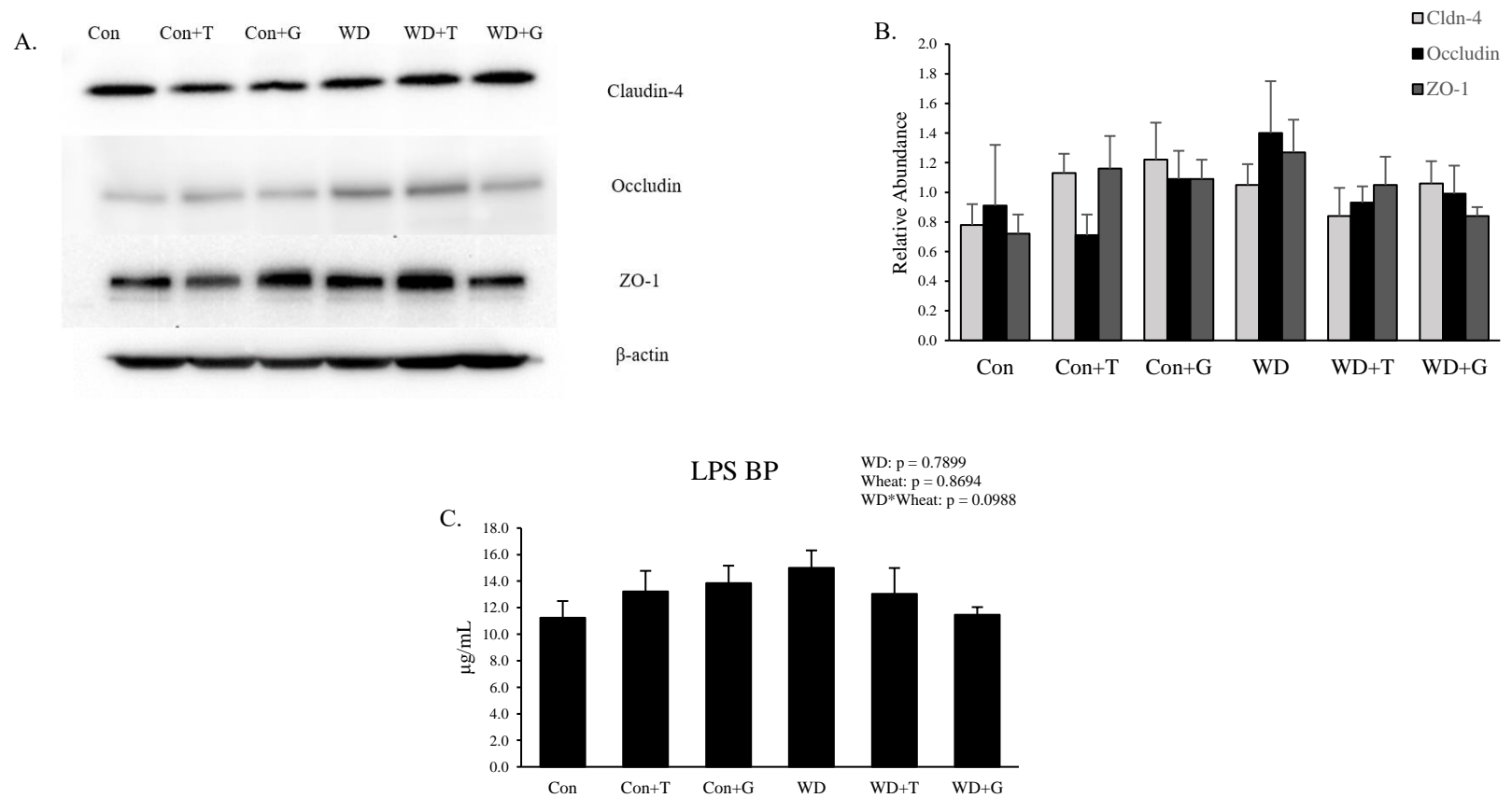
Figure 5

Indicators of Gut Integrity in the Colon

Figure 5. (A) Representative images (n= 5) of western blots probed for claudin-4, occludin, and ZO-1 in the colon. **(B)** Relative quantification of proteins claudin-4, occludin, and ZO-1 in the colon. **(C)** Serum LPS BP. Abbreviations: **Cldn-4** claudin-4**; ZO-1** zonula occluden-1; **LPS BP** lipopolysaccharide binding protein. Data are presented as mean ± SE.

Figure 6
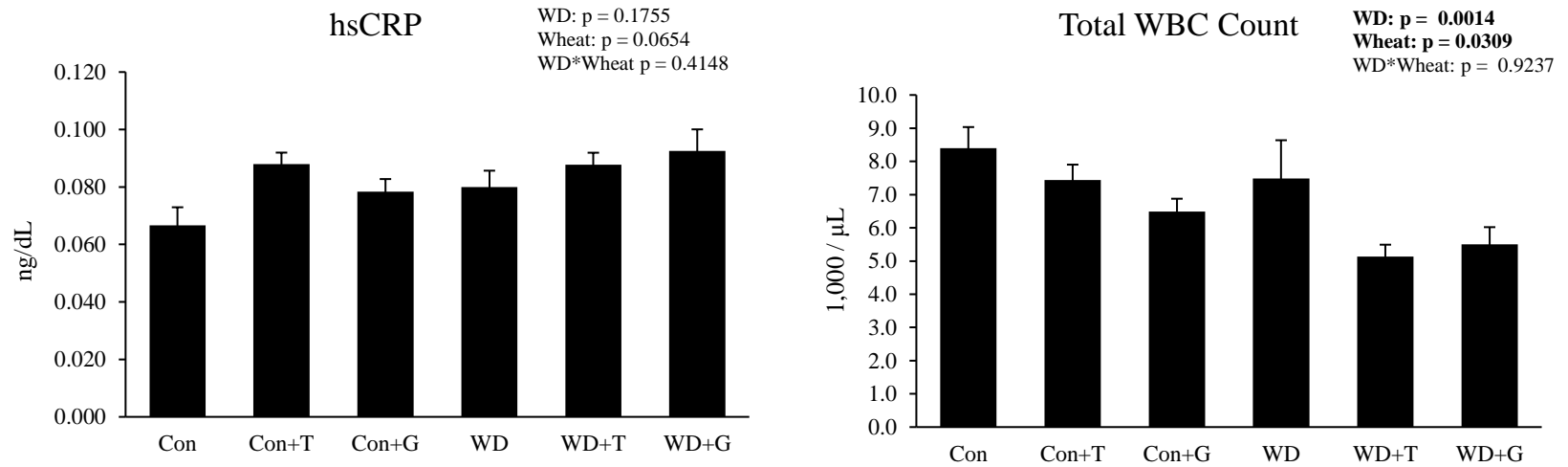Indicators of Systemic Inflammation

Figure 6. Effect of western diet and wheat variety on indicators of systemic inflammation. **(A)** Serum hsCRP **(B)** Blood total white blood cell counts. Abbreviations: **hsCRP** high sensitivity C-reactive protein; **WBC** white blood cell. Data are presented as mean ± SE. P values <0.05 are considered statistically different.

Table 6

Relative Expression of Genes Related to the Immune Response and Antimicrobial Peptides in the Ileum Lamina Propria

	Con	Con + T	Con + G	WD	WD + T	WD + G	P-Value WD	P-value Wheat	P-value WD*Wheat
Inflammatory Mediators									
IFN-γ	1.00 ± 0.19	1.11 ± 0.24	1.31 ± 0.57	2.07 ± 0.45	1.61 ± 0.72	2.89 ± 1.00	**0.0290**	0.4061	0.6628
IL-1β	1.00 ± 0.09	0.87 ± 0.08	1.35 ± 0.35	1.05 ± 0.21	0.66 ± 0.10	0.72 ± 0.12	0.0989	0.0792	0.6039
IL-6	1.00 ± 0.23	0.68 ± 0.17	1.58 ± 0.52	0.70 ± 0.12	0.96 ± 0.29	1.74 ± 0.77	0.8855	0.1125	0.7819
IL-17	1.00 ± 0.12[abc]	1.01 ± 0.09[abc]	1.21 ± 0.37[bc]	1.15 ± 0.14[ab]	0.99 ± 0.28[c]	1.39 ± 0.30[a]	0.4982	0.2197	**0.0247**
TNF-α	1.00 ± 0.17	1.35 ± 0.32	0.91 ± 0.13	1.05 ± 0.27	0.89 ± 0.12	1.43 ± 0.43	0.8842	0.9855	0.4525
ROR-γ	1.00 ± 0.11	0.78 ± 0.08	0.82 ± 0.03	0.79 ± 0.11	0.86 ± 0.08	0.80 ± 0.09	0.4350	0.6118	0.2870
IL-10	1.00 ± 0.24	0.97 ± 0.25	1.24 ± 0.28	0.57 ± 0.23	0.54 ± 0.15	1.21 ± 0.28	0.1151	0.1209	0.6383
TGF-β	1.00 ± 0.22	0.71 ± 0.14	0.75 ± 0.13	0.60 ± 0.14	0.42 ± 0.09	0.76 ± 0.25	0.1577	0.2303	0.2328
Antimicrobial Peptides									
Reg3-β	1.00 ± 0.49	1.16 ± 0.31	1.54 ± 0.57	0.91 ± 0.27	1.14 ± 0.35	1.16 ± 0.74	0.6847	0.5534	0.7864
Reg3- γ	1.00 ± 0.53	0.91 ± 0.13	1.37 ± 0.43	0.81 ± 0.25	1.33 ± 0.26	0.96 ± 0.35	0.7262	0.3643	0.3575

Data are presented as mean ± SE. Within a given row, values that share the same superscript letter are not statistically different from each other.

Table 7
Cecal Short Chain Fatty-Acid Levels

	Con	Con + T	Con + G	WD	WD + T	WD + G	P-Value WD	P-value Wheat	P-value WD*Wheat
Acetate (mM)	1.20 ± 0.30^a	0.64 ± 0.10^{ab}	0.68 ± 0.08^{ab}	0.54 ± 0.04^b	0.87 ± 0.11^{ab}	0.97 ± 0.08^a	0.6124	0.7823	**0.0239**
Propionate (mM)	0.13 ± 0.03	0.10 ± 0.02	0.10 ± 0.02	0.07 ± 0.01	0.10 ± 0.01	0.11 ± 0.01	0.4558	0.6124	0.1614
i-Butyrate (mM)	0.02 ± 0.005	0.01 ± 0.004	0.01 ± 0.004	0.01 ± 0.003	0.02 ± 0.003	0.02 ± 0.003	0.8440	0.3877	0.0812
n-Butyrate (mM)	0.10 ± 0.02	0.08 ± 0.02	0.08 ± 0.01	0.08 ± 0.01	0.11 ± 0.02	0.13 ± 0.02	0.1319	0.5887	0.1109
i-Valerate (mM)	0.03 ± 0.008	0.02 ± 0.006	0.02 ± 0.003	0.02 ± 0.003	0.02 ± 0.004	0.03 ± 0.004	0.9494	0.7269	0.2716
n-Valerate (mM)	0.02 ± 0.008	0.02 ± 0.004	0.02 ± 0.004	0.02 ± 0.003	0.02 ± 0.003	0.03 ± 0.004	0.3381	0.3237	0.3789

Data are presented as mean ± SE. Within a given row, values that share the same superscript letter are not statistically different from each other.

CHAPTER V

DISCUSSION

The purpose of this study was to investigate whether genetic modification of wheat to widely grown modern varieties such as Gallagher contributes to gut inflammation and to determine whether consuming these new varieties in the context of the western diet affects this response. Interest in this topic stemmed from the disconnect between public concerns around GMOs and gluten sensitivity, the prevalence of CD/NCGS and recent declines in wheat consumption. Previous studies [22-24, 101, 106, 107] have compared the effects of heirloom and modern wheat varieties in humans and animals in various disease states (e.g., NAFLD, CVD, T2D); however, main outcomes are largely related to markers of systemic inflammation (i.e., circulating inflammatory makers) and antioxidant capacity. Several studies have evaluated aspects of gut health [25, 103-105, 108], but to date there hasn't been a study specifically focusing on factors affecting gut health such as local inflammation and permeability. Furthermore, no animal model has been utilized that is representative of the general population in the U.S., characterized as free of NCGS and CD, but consuming a typical western diet. Within this population, it is important to understand whether modern wheat varieties are initiating an inflammatory response to either inform nutrition recommendations or to alleviate concerns around GMO wheat products and gluten-sensitivity.

Gut villi are important for nutrient absorption and intestinal crypts house stem cells that support the continually regenerating epithelium and antimicrobial peptide-secreting Paneth cells.

Reduced villi height (i.e., atrophy) and increased crypt depth due increased stem cell division (i.e., hyperplasia) are classically observed in duodenal biopsies in CD [168, 171]. Both of these pathological features are associated with inflammation and are in part due to activated Th cell producing inflammatory cytokines such as IFN-γ and TNF-α in CD [40, 168]. NCGS has not been linked to changes in mucosal architecture, likely due to less severe symptomology compared to CD [12, 75]. Interestingly, in our study we observed a positive effect of Gallagher on villi structure. Specifically, Gallagher consumption led to increased villi area in the distal small intestine (i.e., ileum) when combined with both control and western diets relative to non-wheat containing groups, and an intermediate effect of Turkey was observed. This finding differs from the report by Carnveli et al., [25] which found longer villi in the proximal small intestine (i.e., duodenum) with consumption of the heirloom variety Khorasan relative to an unspecified modern wheat variety when rats were treated with doxorubicin. This is the only other study we are aware of that has evaluated gut mucosal architecture when comparing an heirloom and modern varieties of wheat outside of CD and NCGS. With respect to WD consumption, we observed that mucosal architecture was negatively altered (i.e., reduced villi area) in the jejunum. This is similar to a report where feeding a 40% fat diet based on saturated fat for 8 weeks reduced villi height in the jejunum and ileum [172]. In partial agreement, Goda et al., [173] reported reduced microvilli height, yet increased villi height with a 70% fat diet based on polyunsaturated fat. The partial discrepancy in the latter study may be due to difference in fat source and the fact that the study only lasted 1 week. Also consistent with an inflammatory response, Hamilton et al. [79] reported increased ileal crypt depth with a similar western diet to ours (i.e., 45% fat, high in saturated fat), however we did not observe this effect. We next evaluated the effect of WD and wheat using histopathological scoring on sections of the jejunum,

ileum, and colon. Our study pathologist assessed villous atrophy and crypt hyperplasia, which commonly occurs as intestinal stem cells undergo mitosis more rapidly in an attempt to regenerate the damaged epithelium [171]. For both villous atrophy and crypt hyperplasia, no changes were observed amongst any treatment group, consistent with the absence of an inflammatory response. Although villi height was evaluated by the aforementioned study by Carnevali and colleagues [25], this is the first study to our knowledge evaluating the effect wheat cultivar on clinical histological parameters (i.e., crypt hyperplasia and villous atrophy). Related to wheat intake, crypt hyperplasia and villous atrophy have only been studied in patients with active CD and NCGS [46, 47, 75]. Specifically, it is well established that active CD patients display both villous atrophy and crypt hyperplasia, while the mucosa of NCGS appears normal with respect to these structural parameters. Importantly, our study is the first to ask whether wheat intake and variety affects these parameters in an animal model resembling an otherwise healthy population consuming a semi-purified or a typical western diet. Our findings suggest that with the modern wheat variety Gallagher, there were not negative, and in some cases positive effects, on measures of gross histological structure in the ileum.

Reduced gut barrier integrity has been implicated in a variety of disease states and is an important indicator of gastrointestinal health. This effect is mediated by several factors, including downregulation of tight junction proteins and a diminished mucus layer [146, 209]. One consequence of increased gut permeability is the translocation of bacterial products into circulation (e.g., LPS), which contributes to an inflammatory response [210]. In the context of CD, several laboratories have shown that wheat consumption reduces barrier integrity directly by upregulating zonulin signaling and indirectly by stimulating an immune response [66, 67, 149, 150, 153, 154]. In patients with NCGS, some evidence suggests that intestinal permeability is

increased (i.e., increased transepithelial electrical resistance (TEER), circulating bacterial products); however, cross sectional data revealed that *Cldn-4* expression was upregulated in this population, consistent with improved barrier integrity [47, 74, 162]. Similarly, chronic western diet consumption is known to contribute to intestinal permeability, potentially through gut microbiota dysbiosis and subsequent inflammation [32, 163, 164]. In our animal study, we evaluated serum LPS binding protein as a proxy for LPS leakage and found no effect of wheat variety or WD on this parameter. Due to the fact our animals were free of NCGS, it is not surprising that this finding differed from Udhe and colleagues' report [74] that LPS BP is elevated in NCGS individuals. Unaltered LPS binding protein in our study partially contrasts with other studies [33, 165], suggesting that diets resembling our western diet induce intestinal permeability indicated by increased LPS and LPS binding protein. However, this discrepancy may be explained by relatively low calories from fat in our western diet compared to other formulations (i.e., 45% vs. 60-70%) and shorter study duration [33, 165].

To investigate indicators of local gut integrity, we evaluated genes and proteins important in tight junction and mucus layer formation in the colon. With respect to gene expression, we saw no effect of wheat variety or WD on tight junction scaffolding proteins such as *ZO-1* or transmembrane proteins (i.e., *Cldn2*, *Cldn15*, and *Ocln*). Conversely, one transmembrane protein (i.e., *JAM3*) was reduced when Turkey was added to the WD. This finding appears to stand alone in terms of negative impacts of Turkey in the context of WD consumption, and no other negative effects of heirloom wheat relative to a modern variety have been reported on measures of gut integrity. We also evaluated expression of *Muc-2* as an indicator of the gut mucus layer and no impact of western diet or wheat were seen. Similarly, we assessed goblet cell number as indirect measure of mucus layer status and no changes were observed across treatment groups. The

proteins that were assessed were chosen based on reports that they are reduced in CD (i.e., ZO-1, occludin), upregulated in NCGS (i.e., claudin-4) or suppressed with WD consumption (i.e., occludin) [33, 47, 211-213]. For all three of these proteins, the relative abundance was unchanged with wheat, western diet, or the combination. This data may appear somewhat surprising with respect to WD treatment as there have been other reports of WD reducing tight junction proteins in the gut (e.g., occludin, claudin-1) [33, 77]. However, these studies either used a higher percentage of fat (i.e., 60%), found no effect of western diet on proteins we assessed (i.e., ZO-1) or the literature is mixed on the effect of western diet on that protein (i.e., occludin) [33, 77, 213]. With respect to wheat variety, these results are consistent with the idea that barrier integrity is unaltered when wheat is consumed in the absence of CD and NCGS. In sum, no negative effects of Gallagher were seen on indicators of barrier integrity alone or in combination with WD consumption.

In the context of CD, systemic inflammation is evident, and an aberrant immune response by Th cells locally in the gut is well-characterized [42, 43, 214]. With respect to NCGS, there is little evidence of a systemic immune response, but in the gut *IFN-γ*, *TLR2*, and gut CD3+ intraepithelial lymphocytes are increased [13, 46, 47]. When directly comparing the effects of heirloom and modern wheat varieties on circulating inflammatory indicators (e.g., serum TNF-α, IFN-γ, IL-17, IL-6, IL-8), several clinical studies have shown beneficial effects of heirloom relative to modern varieties in disease states other than CD and NCGS (i.e., IBS, NAFLD, T2D, at risk for CVD) [22, 23, 101, 103]. Similarly, existing animal studies suggest that heirloom wheat, in particular Khorasan, may have anti-inflammatory properties in various tissues (i.e., liver, gut) relative to several modern wheat varieties [25, 106]. To investigate the local inflammatory response in the gut, we evaluated expression of similar cytokines evaluated in

human trials in the ileum lamina propria. For most inflammatory indicators assessed (i.e., *TNF-α, IL-6, ROR-γ, IL-1β*), we saw no effect of wheat or WD. Importantly, comparing results of our study with those done in disease states should be done cautiously as no disease state was present from the onset of treatment in our model. Further, this model may be more informative for the general population as the incidence of CD and NCGS are 1-3% and 4-7%, respectively [10, 11]. Nonetheless, these results differ somewhat from previous reports [22, 24], where circulating TNF-α and IL-6 were downregulated in humans fed heirloom wheat with acute coronary syndrome and a cohort at risk for CVD. Regarding expression of anti-inflammatory cytokines such as *IL-10* and *TGF-β,* we also saw no effect of western diet or wheat. Among genes altered in our study, *IFN-γ* expression was increased with WD consumption, which is similar to other reports that WD stimulates TLR-4/ NF-κB signaling and cytokine production (i.e., TNF-α, IL-1β, IL-6) in the colon [31, 33, 215]. In addition, Turkey suppressed *IL-17* expression in the context of WD relative to all other groups. Likewise, Sofi et al. [103] observed reduced serum IL-17 is with consumption of heirloom wheat, but not with modern wheat in the context of IBS. As another indicator of local inflammation, at least one study has evaluated the effect of heirloom and modern wheat on duodenal lymphocyte infiltration and lymphatic follicle diameter following treatment with the pro-oxidant doxorubicin. They reported reduced lymphocyte infiltration and lymphatic follicle diameter in the duodenum with heirloom relative to modern wheat, consistent with decreased gut inflammation [25]. We also evaluated lymphocyte infiltration in the jejunum, ileum, and colon, but no differences were observed among treatment groups in our hands. To investigate whether wheat variety alone or in combination with a western diet induced a systemic immune response, we evaluated serum hsCRP and total WBC counts. With respect to hsCRP neither wheat variety nor WD consumption had an effect. This is

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similar to the report that hsCRP is not upregulated in NCGS, but this is the first study we are aware of that assessed this marker when directly comparing an heirloom and modern cultivar outside of an established condition such as CD and NCGS. Interesting, main effects by wheat and western diet were observed for total WBC (suppressed), however all groups remained in the normal range for C57BL/6 mice. Altogether, our data suggests that there is no increased inflammatory response with Gallagher consumption, but limited data suggests Turkey may have anti-inflammatory properties (i.e., *IL-17* suppressed under WD conditions). Apart from favorably effects on *IL-17* expression, our data deviates somewhat from studies showing an overall anti-inflammatory effect of heirloom wheat in disease states (e.g., CVD, NAFLD) and animal models where oxidative stress is induced, however, this is not surprising given our animals were fed wheat in the context of a normal diet or a western diet more consistent with actual intake in the U.S. (i.e., 45% fat). Importantly, nearly all studies reporting benefits of heirloom wheat versus modern wheat do not include a non-wheat containing control group making it difficult to discern whether these findings can be attributed to positive properties or heirloom, negative of modern, or some combination of the two. With respect to WD treatment, we did not see a major effect on markers of inflammation, but again the relatively low kcal from fat utilized and study duration may explain some of these discrepancies.

Metabolic disturbances such as elevated blood lipids, fasting glucose, visceral adiposity, and body weight are hallmarks of WD consumption [33, 163]. There is also evidence to suggest that within the context of T2D, consuming heirloom wheat may positively impact some of these parameters (i.e., total cholesterol, LDL-cholesterol, triglycerides, glycemic control) relative to modern varieties [216, 217]. As expected, WD consumption elevated fasting glucose, total cholesterol, NEFA, percent body fat, and body weight. In contrast to other studies using diabetic

models, neither Turkey nor Gallagher affected these parameters. Interestingly, both wheat varieties lowered triglycerides relative to groups not consuming wheat. Another common metabolic response to chronic high-fat diet consumption is hepatic lipid accumulation [218]. For this reason, we performed histological analyses on degree of liver steatosis, however, this parameter was not affected by our 45% fat western diet or wheat after 6 weeks of treatment. Together, WD induced expected metabolic changes and wheat had either no effect or a positive effect on these measures.

SCFAs are important in overall gastrointestinal health due to their preferential use by epithelial cells as a fuel source and regulation of immune cell populations such as Tregs [122, 123]. In addition, some evidence suggests that SCFA production is suppressed during active CD and with high fat diet consumption [60, 61, 219]. For these reasons, we assessed the cecal SCFAs acetate, propionate, butyrate, and valerate. In our hands, cecal acetate concentrations were reduced with WD diet control, however, addition of Gallagher to the WD reversed this effect. A similar finding by Barone and colleagues was reported in a porcine model, where fecal acetate was increased with both heirloom and modern wheat varieties, but this response was greater with modern wheat [108]. The same study also reported beneficial effects of wheat on levels of propionate and butyrate, but apart from acetate, we saw no effect of WD or wheat on any other SCFA assessed. Consistent with our other data, this suggests that modern wheat did not lead to abnormal gut health through alterations in SCFA levels.

In our study, the modern wheat variety Gallagher did not negatively impact indices of gastrointestinal health such as gut structure, barrier integrity, inflammation, and SCFAs in the context of a normal or western diet. While in most cases there were no statistical differences between Gallagher and Turkey, for some parameters (i.e., ileum villi area and cecal acetate) a

positive effect of Gallagher was observed. On the other hand, Turkey displayed a positive effect on ileal *IL-17* expression in the context of WD (suppressed), but Gallagher did not affect this parameter. These benign and in some cases positive findings with Gallagher consumption should be evaluated in other modern wheat varieties and confirmed in future human trials. The 10% w/w dosage of wheat used in this study is a feasible quantity for human consumption (high-normal consumption), supporting potential for translation into clinical studies. We conclude, that the modern wheat variety Gallagher did not negatively impact indicators of gastrointestinal health relative to the heirloom variety Turkey in mice consuming a normal or typical western diet.

CHAPTER VI

SUMMARY, CONCLUSIONS, RECOMMENDATIONS

Summary

The purpose of this study was to determine whether genetic modification of wheat from heirloom compared to the modern variety Gallagher has led to increased gut inflammation and permeability, and whether consuming a typical western diet (WD) affects this response. Male 6-week old C57BL/6 mice were assigned to treatment in a 2x3 factorial design, with factors of diet (control or WD) and wheat (no added wheat, heirloom wheat [Turkey], modern wheat [Gallagher] at 10% w/w). After 6 weeks on their respective diets, body composition was assessed, and blood and tissue specimens were collected to evaluate metabolic parameters, as well as systemic and local intestinal indicators of inflammation and gut barrier integrity. Cecal short-chain fatty acids (SCFAs) were also assessed. Findings indicated that body weight, percent body fat, fasting glucose, total cholesterol, and NEFA were increased with WD and wheat had no effect on these metabolic parameters. Regarding measures of villi and crypt structure, WD decreased villi area in the jejunum and the mice consuming the Gallagher variety exhibited an increase villi area in the ileum relative to mice not consuming wheat. Histopathological scoring revealed no effect of WD or wheat on villous atrophy, crypt hyperplasia, lymphocyte infiltration, and goblet cell number. Indicators of barrier integrity, including genes encoding for protein, protein expression, and serum LPS BP were unaffected by WD or wheat, with the exception of

JAM3 suppression with WD+Turkey. Measures of systemic (i.e., CRP) and local gut inflammation (i.e., cytokine gene expression) were largely unaffected by treatment. Among genes altered, WD increased *IFN-γ* and wheat did not affect this response. Additionally, Turkey suppressed *IL-17* in the context of WD. WD decreased the SCFA, acetic acid, but addition of Gallagher wheat to WD restored levels to control. No other SCFA were altered. Overall, Gallagher consumption did not promote damage to villi and crypt structure or gut integrity relative to Turkey and no evidence of an inflammatory response was observed beyond that of WD control. Gallagher had favorable effects on several parameters relative to Turkey wheat (i.e., villi area in the ileum, concentration of cecal acetate).

Conclusions

The following is a list of specific aims and working hypothesis that were proposed for this study:

Specific Aim 1: To characterize the effect of western diet and wheat cultivar on body composition and serum metabolic parameters (i.e., glucose, total cholesterol, triglycerides, non-esterified fatty acids).

Working hypothesis: Western diet consumption will increase body composition and parameters related to metabolic health and wheat will not affect these markers.

As anticipated, the western diet increased body weight, body fat percentage, and several metabolic parameters (i.e., fasting glucose, total cholesterol, NEFA), however addition of both wheat varieties reduced serum triglycerides. Based on these results, we accept the hypothesis that western diet will negatively affect these metabolic parameters and reject the hypothesis that wheat will have no effect.

Specific Aim 2: To determine the effect of wheat cultivar on villi and crypt structures (e.g., villi length, villi area, villi perimeter, crypt depth) within the jejunum, ileum, and colon under normal and western diet conditions.

Working hypothesis: Gallagher will have no effect on measures of villi and crypt structures in the context of a normal, diet but will have negative effects on these structural parameters compared to Turkey when consumed with the western diet.

No negative effects of Gallagher on villi and crypt structure were observed under normal or western diet conditions. The western diet alone reduced villi area in the jejunum and Gallagher exhibited a positive effect on ileal villi area in the context of both control and western diet. From a histopathological standpoint, no differences in villous atrophy, crypt hyperplasia, or goblet cell number were observed among treatment groups. As a result of these findings, we reject the hypothesis.

Specific Aim 3: To evaluate the effect of wheat cultivar on barrier integrity via gene expression and protein analysis of tight junction proteins (e.g., claudins, occludin) in the colon under normal and western diet conditions.

Working hypothesis: Gallagher will not contribute to decreased barrier integrity when consumed with a normal diet, however when combined with a western diet will decrease barrier integrity relative to Turkey.

No effect of Gallagher was observed on indices of local barrier integrity (i.e., genes regulating barrier function, tight junction proteins) in the colon. Based on these results we reject the hypothesis.

Specific Aim 4: Evaluate the effect of wheat cultivar and western diet on short chain fatty acids within the cecum.

Working hypothesis: Western diet consumption will decrease concentrations of cecal SCFA and wheat will not affect these parameters.

Acetate was suppressed with western diet control, but the western diet did not affect any other SCFA (i.e., propionate, i-butyrate, n-butyrate, i-valerate, n-valerate). With respect to wheat, addition of Gallagher to the western diet restored acetate levels to that of control. Wheat did not impact concentrations of any other SCFA. With the exception of reduced cecal acetate by western diet, we reject the hypothesis.

Specific Aim 5: To determine if systemic markers of inflammation (i.e., CRP) and intestinal permeability (i.e., lipopolysaccharide binding protein or LPS BP), are altered in animals consuming a modern wheat cultivar relative to an heirloom variety under normal and western diet conditions.

Working hypothesis: Gallagher will have no effect on systemic indicators of inflammation and intestinal permeability in the context of a normal diet, but will negatively impact these parameters in the context of a western diet compared to Turkey.

No effects of western diet, wheat, or the interaction were observed for circulating CRP or LPS BP. Therefore, we reject the hypothesis.

Specific Aim 6: To determine the effect of wheat cultivar on indicators of local gut inflammation based on alterations in gene expression of inflammatory mediators (e.g. *IL-17*, *IL-1β)* and lymphocyte infiltration using histopathological analysis.

*Working hypothesis***:** Gallagher will have no effect on measures of local gut inflammation in the context of a normal diet, but will negatively impact these parameters when consumed with a western diet compared to Turkey.

Consumption of Gallagher did not impact expression of inflammatory mediators or lymphocyte infiltration in the context of a normal or western diet. The only changes observed were increased *IFN-γ* with western diet consumption and reduced expression of *IL-17* with consumption of Turkey in the context of the western diet. As a result of these findings, we reject the hypothesis.

Recommendations

This study evaluated the effect of genetic modification of wheat from heirloom to modern varieties on measures of gastrointestinal health in the context of a normal and western diet in animals free of wheat-related conditions. Results from this study provide strong evidence that the modern wheat variety, Gallagher, does not invoke an inflammatory response or impair measures of gut integrity. From an experimental design perspective, evaluating similar outcomes at

additional timepoints would help establish if there is an acute inflammatory response (e.g., 1 week) to Gallagher that later normalizes or, conversely, a longer study duration (e.g., 6 months) could provide insight into whether > 6 weeks is necessary for signs of an inflammatory response to develop. Additionally, the amount of wheat in our diets (i.e., 10% w/w) represented high-normal intake in humans. To test if there are detrimental effects when modern wheat is consumed at a dose representing more extreme intakes, diets containing higher amounts of weight (e.g., 20-30% w/w) could be administered. Similarly, a western diet that has a higher percentage of fat (e.g., 60-70% kcal) than the 45% kcal we used could be administered to represent the most extreme scenario. These changes to diet formulations would answer the question of if it is possible for Gallagher to elicit an inflammatory response under conditions that may be beyond what would be deemed normal.

Our data provide compelling evidence that Gallagher did not negatively impact gut health and was an appropriate first step in assessing these outcomes; however, future studies could be done utilizing more advanced techniques to confirm the findings of this study. For example, our work demonstrates that genes and proteins regulating barrier integrity (e.g., claudins, occludin) and serum LPS binding protein are not affected by Gallagher or western diet. This provides strong evidence that gut barrier integrity is unaltered, but to further demonstrate this, more functional analysis could be performed pre-necropsy (i.e., the lactulose mannitol test) or immediately post-necropsy (i.e., TEER). Similarly, we found no effect of Gallagher on gene expression of inflammatory mediators in the gut, however, to further demonstrate this point immunohistochemistry of gut sections, flow cytometry of inflammatory immune cells, or *ex vivo* immune cell stimulation with wheat protein extracts (e.g., gliadin, gluten) would strengthen our findings related to the local inflammatory response in the gut. Further, although one could argue

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that the response of different regions of the lower gastrointestinal tract would be similar, for the most thorough investigation, local inflammatory mediators and indicators of barrier integrity would both be assessed in the small and large intestine.

Another avenue that should be explored is whether the effects observed in rodent models are observed in humans. Clinical studies could be designed that assess indicators of gut integrity and inflammation that also take advantage of insightful measures that cannot be assessed in mice (i.e., serum or fecal zonulin). Finally, our data has extreme relevance to individuals living in the Midwest as Gallagher is widely grown in this region, however, it is impossible to know whether all modern and heirloom varieties have similar effects on gut health. To address the question of variation among wheat cultivars, studies like ours should be performed with additional study arms for other modern and heirloom varieties. Overall, this study provides evidence that consumption of the modern wheat cultivar Gallagher does not lead to increased measures of gut inflammation or intestinal permeability relative to Turkey.

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VITA

BRYANT KEIRNS

Candidate for the Degree of

Master of Science

Thesis: HAS GENETIC IMPROVEMENT OF WHEAT RESULTED IN NEGATIVE
         EFFECTS ON GUT HEALTH?

Major Field: Nutritional Sciences

Biographical:

    Education:

    Completed the requirements for the Master of Science in Nutritional Sciences at
    Oklahoma State University, Stillwater, Oklahoma in July, 2019.

    Completed the requirements for the Bachelor of Science in Biology
    at Oklahoma Christian University, Edmond, Oklahoma in May, 2016.

Experience: Employed by Oklahoma State University, Department of
    Nutritional Sciences as a graduate research assistant; Oklahoma State
    University, Department of Nutritional Sciences, 2017 to present.