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A Comparative Study of Modern and Heirloom Wheat on Indicators of Gastrointestinal Health

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Supporting Information

ABSTRACT: Wheat consumption has declined amid growing concerns about gluten-sensitivity. To determine if genetic manipulation of wheat contributes to systemic and localized gut inflammation, we compared the effects of the modern variety Gallagher and a blend of two heirloom varieties, Turkey and Kharkof, on measures of gut inflammation, structural characteristics, and barrier integrity under normal and Western diet (WD) conditions in C57BL/6 mice. Indicators of gut inflammation, including lymphocyte infiltration and cytokine expression, were largely unaffected by WD or wheat, although WD elevated interferon-γ (*Ifng*) and heirloom varieties modestly reduced interleukin-17 (*Il17*) in the context of WD. WD negatively affected jejunal villi structure, while the modern variety improved villi structure in the ileum. Relative mRNA and tight junction proteins and serum lipopolysaccharide binding protein were unaltered by WD or wheat. These findings indicate that the modern variety did not compromise barrier function or contribute to gut inflammation compared to its heirloom predecessor.

KEYWORDS: *wheat, gastrointestinal tract, inflammation, gut permeability, gluten, mucosal immunity, Western diet*

INTRODUCTION

Wheat is a staple food in many countries; however, consumption in the United States has declined in recent years.[1] These changes in wheat intake have occurred alongside increased public concerns about the health effects of genetically altered foods and growing awareness of gluten sensitivity.[2,3] Interestingly, 2−4 times the number of Americans report avoiding gluten-containing food products than have a wheat-related medical condition, suggesting perceived health benefits.[4−6] Wheat undoubtedly has immunogenic properties in patients with celiac disease (CD), wheat allergy, and the newly identified nonceliac gluten sensitivity (NCGS) that cause varying degrees of gastrointestinal distress and inflammation. Although wheat is poorly tolerated by these patients, there is no compelling evidence to date that wheat has inherent antigenic properties in otherwise healthy individuals.

One possible explanation as to why wheat is being increasingly avoided is that selective breeding of heirloom wheat to modern varieties has altered it in such a way that has led to negative health outcomes. From the late 19th century until the 1940s, "Turkey" (*Triticum aestivum* L.) was the predominant hard red winter wheat cultivar in the United States and is considered an heirloom variety or landrace introduction. However, around the turn of the 20th century, other varieties began to be introduced without the use of artificial or induced hybridization. This included selections from Turkey, such as "Kharkof" (*T. aestivum* L.), which demonstrated improved resilience to harsh growing conditions.[7] Over a century, selective breeding of the Turkey lineage preceded the creation of "Gallagher" (*T. aestivum* L.), which was released in 2012. Since this time, Gallagher has become one of the most widely grown bread wheat varieties in wheat-intensive states of the U.S. Great Plains region (i.e., Oklahoma and Kansas), due to its favorable yield and pathogen/pest resistance.[8,9]

It is well-known that the typical dietary pattern of Westernized societies is characterized as high in saturated fat and refined sugar. This diet, referred to as the Western diet (WD), is well-known for increasing the risk of chronic diseases, including obesity, cardiovascular diseases, type 2 diabetes, and certain cancers.[10−12] One of the central features of the underlying pathogenesis of these diseases is a chronic inflammatory state, with patients displaying elevated acute phase proteins (e.g., C-reactive protein) and inflammatory cytokines [e.g., interleukin-6 (IL-6) and tumor necrosis factor alpha (TNF-α)].[13] Postprandial studies have provided further evidence of the link between the WD and the inflammatory response, in that 4−6 h following the consumption of a high fat meal, circulating IL-6 and TNF-α are elevated.[14] However, it is animal studies that have provided insight into the WD's effects on the gastrointestinal tract itself. Specifically, C57BL/6 mice consuming the WD exhibit increased activation of nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) and inflammatory cytokine production (i.e., TNF-α, IL-1β, IL-6) within the ileum and colon.[15,16] This inflammatory response

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was accompanied by a decrease in tight junction proteins (i.e., occludin and claudin-1) and an increase in plasma endotoxin, both of which are indicative of compromised gut barrier function.[15] Similarly, increased plasma endotoxin has been reported in human studies following the consumption of a high-fat or Western-style diet.[17]

While WD consumption has been associated with negative effects on gut health, it is unknown whether WD-induced gut inflammation creates a mucosal immune environment that is hyperresponsive to foods (e.g., modern wheat). Thus far, the majority of work comparing heirloom and modern wheat has been performed by an Italian research group studying wheat in the context of chronic diseases (e.g., type 2 diabetes, nonalcoholic fatty liver disease). These studies suggest that heirloom wheat has anti-inflammatory properties and improves gastrointestinal symptoms compared to modern wheat.[18−21] Similarly, Wistar rats fed heirloom wheat displayed improved indicators of gut health (i.e., duodenal villi height, lymphocyte infiltration) relative to modern wheat when subjected to oxidative stress.[22] Despite this evidence that heirloom wheat has beneficial properties relative to modern wheat in disease states, it is not known whether similar effects are observed in an otherwise healthy population consuming the WD.

For these reasons, this study aimed to determine whether consumption of a modern wheat variety, Gallagher, in the context of the WD, led to negative effects on gastrointestinal health (e.g., increased inflammation and gut permeability) relative to a blend of Turkey and Kharkof heirloom wheat. In an animal model, with the C57BL/6 mouse representing a healthy population, we evaluated the effect of wheat cultivar on (a) the histological structure of the jejunum, ileum, and colon; (b) measures of inflammation in the ileum lamina propria; and (c) indicators of intestinal permeability in the colon in order to determine if modern wheat impairs gut health.

EXPERIMENTAL PROCEDURES

Animal Care and Dietary Interventions. Six-week old C57BL/6 male mice (n = 80; Charles River Laboratories, Wilmington, MA) were housed at Oklahoma State University's environmentally controlled Laboratory Animal Research Facility in cages (4 or 5 mice/cage) and were acclimated for 2 weeks before initiation of the study. Mice (n = 12 or 13/group) were then randomized to treatment in a 2 × 3 factorial design with diet (AIN-93G control diet or WD) and wheat (no added wheat, Heirloom, or Modern) as factors. Treatment groups included control, control + Heirloom, control + Modern, WD, WD + Heirloom, and WD + Modern. The WD was formulated to consist of 45% kilocalories from fat, primarily from lard, and was high in refined sugar (Supplementary Table 1, Supporting Information). A mixture of heirloom varieties Turkey/Kharkof (Heirloom) was selected to represent the genetic foundation for hard red winter wheat, the most widely grown U.S. wheat market class and principal source of all-purpose wheat flour and bread flour.[1] Gallagher was chosen as the modern cultivar (Modern) due to the fact it has been widely and commercially grown in the hard red winter wheat region of the southern Great Plains for many years.[8] The wheat lineage from Turkey to Gallagher is shown in Figure 1.

Both Heirloom and Modern were grown in the same research trials in Oklahoma under the same environmental conditions, simulating commercial agriculture during the 2017 crop season. The harvested grain was milled to flour at a 65% extraction rate, consistent with typical white flour, and then subsequently analyzed for its protein, fat, fiber, calcium, and phosphorus content (NPAL Analytical Laboratories, St. Louis, MO) and wet gluten content as a percentage of total protein as previously described[23] (Table 1). Diets containing wheat were supplemented at 10% (w/w), a dose resembling normal to high human consumption, and adjusted to match macronutrient, fiber, calcium, and phosphorus content to the control or WD diet. Mice had ad libitum access to their respective diets and water daily. Food intake was assessed daily, and body weights were recorded weekly throughout the 6-week study period. All procedures were approved by the Oklahoma State University Institutional Animal Use and Care Committee.

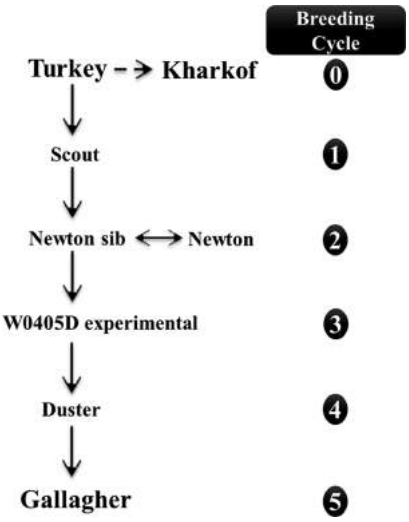

Figure 1. Lineage from the heirloom wheat variety Turkey (breeding cycle 0) to the variety Gallagher (breeding cycle 5), which was used as the modern wheat comparison. Grain from Kharkof (breeding cycle 0), a close relative of Turkey, was mixed in equal proportion with grain from Turkey to represent the genetic foundation for hard red winter wheat in the Great Plains.

Table 1. Nutrient Analysis and Gluten Content of Wheat Varieties

	wheat variety	
	Heirloom	Modern
carbohydrate (%)	73.60	74.00
protein (%)	10.40	10.10
wet gluten (% total protein)	33.05	26.80
fat (%)	1.09	1.12
fiber (%)	0.30	0.35
other[a] (%)	14.61	14.43

[a]Other includes moisture, ash, vitamins, and minerals.

Sample Collection. After 6 weeks on treatment, fasting blood glucose was assessed, and then mice were anesthetized using a ketamine/xylazine cocktail (100 mg/10 mg/kg body weight) followed by exsanguination via the carotid artery. Blood was collected and a 25 μL aliquot was transferred to a microcentrifuge tube containing Türk's solution (1:20) for quantifying total white blood cell (WBC) counts. After flushing the small and large intestine with ice cold PBS, small sections of the jejunum, ileum, and colon were fixed in 10% neutral buffered formalin for histological examination. The remaining ileum lamina propria and colon were placed in RNALater (Invitrogen, Carlsbad, CA) and stored at −80 °C for subsequent gene expression and protein analyses. Cecal contents were flushed, weighed, and stored at −80 °C for short-chain fatty acid (SCFA) analysis. Tissue weights were recorded for the thymus, spleen, cecum, liver, and white adipose tissue and then stored at −80 °C.

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 Analyses. Fixed jejunum, ileum, colon, and liver specimens were dehydrated (Shandon Citadel 2000, Waltham, MA) using a graded ethanol series and toluene. Tissues were then

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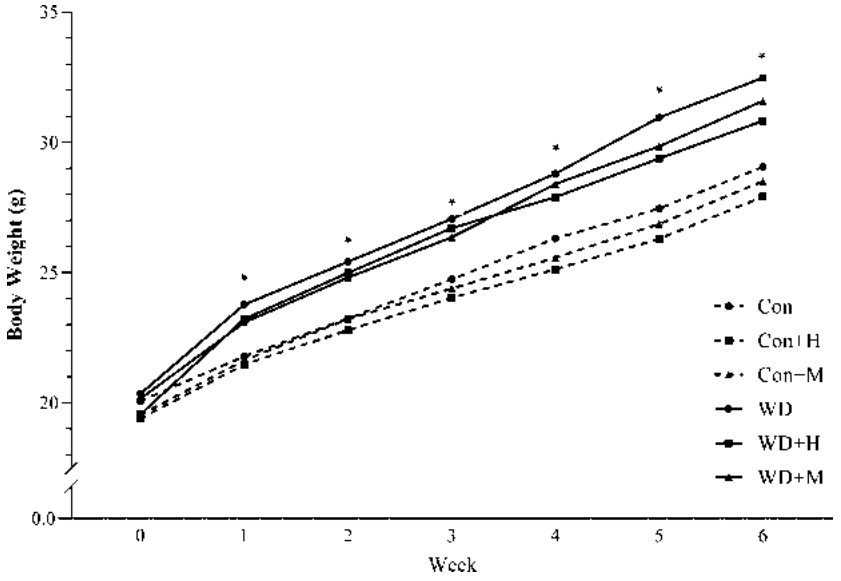

Figure 2. Body weights over the course of the 6-week study. Animals were assigned to six different treatment groups in a 2 × 3 factorial design. Factors were diet (control or WD) and wheat (no added wheat, Heirloom, or Modern). Control diets are indicated by dashed lines and solid lines represent WD groups. Groups with the same symbol consumed the same wheat variety: no added wheat (circle), Heirloom (square), or Modern (triangle). Baseline weights are indicated by week 0. Asterisks denote time points in which a main effect of diet (Con diet groups vs WD groups) was observed. Abbreviations: Con, control; WD, Western diet; H, Heirloom; M, Modern.

embedded in paraffin and 5 μm sections were cut (Leica Biosystems, Wetzlar, Germany). Hematoxylin and eosin (H&E) staining was performed to view structural changes in villi height, width, area, perimeter, and crypt depth with BZ-X800 software (Keyence, Osaka, Japan). Slides were then subjected to histopathological analysis by the study pathologist. For gut sections, lymphocyte infiltration (subscale 1−4), villous atrophy and crypt hyperplasia (subscale 1−5), and goblet cell number (subscale 1−4) were scored as previously described.[24] Subscales were summed for a total score, with higher scores representing negative outcomes relative to control samples. For liver sections, a steatosis score ranging from 0 to 4 was given with a score of 0 representing <5% steatosis and a 4 indicated >75% steatosis, as reported elsewhere.[25]

RNA Extraction and Gene Expression Analyses. Total RNA was extracted from the colon and ileum lamina propria (n = 6/group) using Trizol (Life Technologies, Carlsbad, CA). RNA (2 μg) was reverse-transcribed (Superscript II, Invitrogen, Carlsbad, CA) to complementary DNA (cDNA) and quantitative real-time 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, such as *Cldn2* and *Ocln*. In the ileum lamina propria, genes involved in immune cell activity, including *Tnf*, *Il17a*, *Il10*, and the antimicrobial peptides *Reg3b* and *Reg3g*, were assessed. All genes of interest were analyzed using the ddCT method and were normalized to peptidylprolyl isomerase B (*Ppib*). Primer sequences are shown in Supplementary Table 2 (Supporting Information).

Protein Extraction and Analysis. The tight junction proteins, claudin-4, occludin, and zonula occludens-1 (ZO-1), were assessed in the colon using immunoblotting techniques (n = 5). Total protein was extracted from the colon using radioimmunoprecipitation assay (RIPA) buffer and quantified using the bicinchoninic acid (BCA) assay. Protein samples (30 μg) were then boiled for initial denaturation and separated on a denaturing sodium dodecyl sulfate−polyacrylamide electrophoresis (SDS−PAGE) gel and transferred to a polyvinylidene fluoride (PVDF) membrane. Samples used for ZO-1 immunoblots were not boiled prior to SDS−PAGE. Equal transfer was confirmed with Ponceau staining before blocking for 1 h (claudin-4, occludin) or 8 h (ZO-1) with 5% nonfat milk in 0.1% Tris-buffered saline with Tween 20 (TBST). Primary antibodies for claudin-4 (1:1000), occludin (1:5000), and ZO-1 (1:10 000) (Thermo Fisher, Waltham, MA) were incubated with the membrane overnight at 4 °C. Next, the membranes were washed, incubated with the secondary antibody for 1 h at room temperature, and imaged using SuperSignal West chemiluminescent substrate (Thermo Fisher, Waltham, MA). Blots were developed using a ProteinSimple Fluorchem R (San Jose, CA). The area under the curve corresponding to the intensity and area of each protein band of interest was quantified using ImageJ software (NIH, Rockville, MD) and then normalized to β-actin, which was quantified in the same manner (Santa Cruz Biotechnology, Dallas, TX).

Serum Analyses. A commercially available ELISA kit was used to assess serum lipopolysaccharide binding protein (LPS BP) (Hycult Biotech, Uden, Netherlands). Serum metabolic parameters [i.e., total cholesterol, triglycerides, and nonesterified fatty acids (NEFA)] and high-sensitivity C-reactive protein (CRP) were assessed using a BioLis 24i automated analyzer (Carolina Chemistries, Greensboro, NC).

SCFA Analyses. 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 min and supernatants were collected and filtered using 0.45 mm polytetrafluoroethylene syringe filters (Agilent Technologies, Santa Clara, CA). Gas chromatographic analysis was performed using an Agilent 6890N GC system with a flame ionizable detector and an automatic liquid sampler. Sample concentrations were determined using a five-point calibration curve, with each standard containing solutions of acetate, propionate, isobutyrate, butyrate, isovalerate, and valerate (Sigma-Aldrich, St. Louis, MO).

Statistical Analyses. Data were 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 were analyzed using two-way ANOVA, with diet and wheat as factors. Main effects of WD signified a statistically significant difference between the control-diet-based (Con) treatments and WD-based treatments, irrespective of wheat. Similarly, a main effect for wheat indicates a difference explained by wheat variety, regardless of the diet (Con or WD) being consumed. When there was a significant interaction (p < 0.05) with a combination of diet and wheat treatments, Fisher's least-squares means was performed for

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Table 2. Anthropometric Data, Relative Tissue Weights, and Metabolic Parameters[a]

							p-value		
	Con	Con + H	Con + M	WD	WD + H	WD + M	WD	wheat	WD × wheat
Body Composition									
% lean mass	69.47 ± 0.75	72.36 ± 0.95	70.30 ± 1.21	67.87 ± 1.11	66.89 ± 1.30	65.08 ± 1.51	**<0.0001**	0.2584	0.1925
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
% fat mass	31.67 ± 0.61	29.90 ± 0.86	30.99 ± 1.22	35.42 ± 1.09	35.61 ± 1.38	37.00 ± 1.47	**<0.0001**	0.5956	0.5719
visceral WAT (mg)	502 ± 30	469 ± 22	489 ± 42	767 ± 045	649 ± 48	742 ± 51	**<0.0001**	0.1613	0.5487
Relative Tissue Weights (mg/g BW)									
spleen	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	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
cecum	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	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
Metabolic Parameters									
glucose (mg/dL)	150 ± 3	146 ± 5	135 ± 3	176 ± 9	184 ± 3	178 ± 8	**<0.0001**	0.3105	0.3644
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 (mequiv/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

[a]Data are presented as mean ± SE; *p*-values <0.05 are considered statistically significant and are shown in bold. Abbreviations: BW, body weight, WAT, white adipose tissue; NEFA, nonesterified fatty acids; Con, control; WD, Western diet; H, Heirloom; M, Modern.

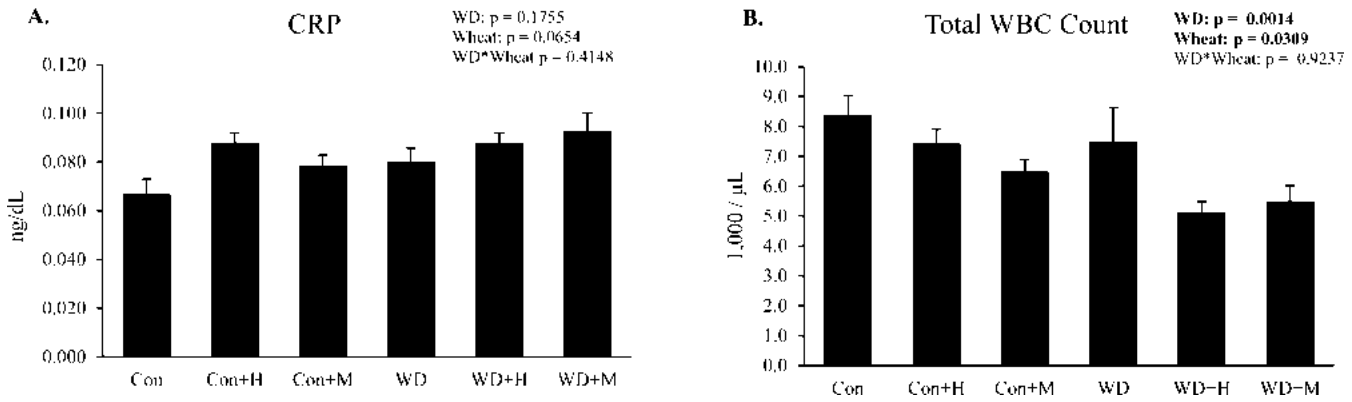

Figure 3. Effect of WD and wheat variety on indicators of systemic inflammation: (A) serum CRP and (B) blood total white blood cell counts. Data are presented as mean ± SE; *p*-values <0.05 are considered statistically different. Abbreviations: CRP, C-reactive protein; WBC, white blood cell. Con, control; WD, Western diet; H, Heirloom; M, Modern.

posthoc analysis, and appropriate superscripts were added to designate differences between treatment groups. When data were not normally distributed, Friedman's test was performed. Histological scoring was evaluated using Fisher's exact test. Data are presented as mean ± standard error, and the α was set at 0.05.

RESULTS

Body Weight, Body Composition, and Relative Tissue Weight. First, the effects of the WD and wheat on body weight and composition were assessed. At baseline, there was no statistically significant difference in body weight between groups; however, mice fed the WD exhibited increased body weight ($p < 0.05$) after 1 week of treatment and the effect persisted until the end of the study (Figure 2). Analysis of body composition revealed that mice consuming the WD had greater percent body fat and lean mass, as well as a reduced percent lean mass ($p < 0.01$; Table 2). 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 2). Spleen, thymus, and liver weights were unaffected by treatment when expressed relative to body weight; however, relative cecum weight was suppressed by the WD ($p < 0.001$; Table 2).

Metabolic Parameters. In addition to changes in body composition, serum metabolic indicators were assessed to determine if either variety of wheat altered the metabolic response to the WD. As expected, fasting blood glucose, total cholesterol, and NEFA were significantly elevated ($p < 0.05$) by WD treatment (Table 2). The WD had no effect on serum triglycerides, but the addition of both wheat varieties reduced triglycerides relative to groups not consuming wheat (Table 2; $p < 0.05$). No significant effect on fasting blood glucose, total cholesterol, and NEFA occurred in response to wheat consumption under normal or WD conditions.

Hepatic lipid accumulation is a common consequence of metabolic disturbances. For this reason, 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 the WD or wheat over the course of this 6-week study.

Assessment of Inflammatory Markers. Serum CRP and white cell counts were assessed as indicators of systemic inflammation. No effect of WD or wheat was observed on CRP (Figure 3A). Interestingly, main effects of WD and wheat were observed for total WBC counts (Figure 3B). With both

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Table 3. Relative Expression of Genes Related to the Immune Response and Antimicrobial Peptides in the Ileum Lamina Propria[a]

							p-value		
	Con	Con + H	Con + M	WD	WD + H	WD + M	WD	wheat	WD × wheat
Inflammatory Mediators									
Ifng	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
Il1b	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
Il6	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
Il17	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	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
Rorc	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
Il10	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
Tgfb	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									
Reg3b	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
Reg3g	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

[a]Data are presented as mean ± SE. *p*-values <0.05 are considered statistically significant and are shown in bold. Superscripts are utilized when two-way ANOVA revealed a significant interaction, and values within a given row that share the same letter are not statistically different from each other. Abbreviations: *Ifng*, interferon-γ; *Il1b*, interleukin-1β; *Il6*, interleukin-6; *Il17*, interleukin-17; *Tnf*, tumor necrosis factor-α; *Rorc*, retinoic acid-related orphan receptor gamma; *Il10*, interleukin-10 *Tgfb*, transforming growth factor-β; *Reg3b*, regenerating islet derived protein 3 gamma; *Reg3g*, regenerating islet derived protein 3 gamma; Con, control; WD, Western diet; H, Heirloom; M, Modern.

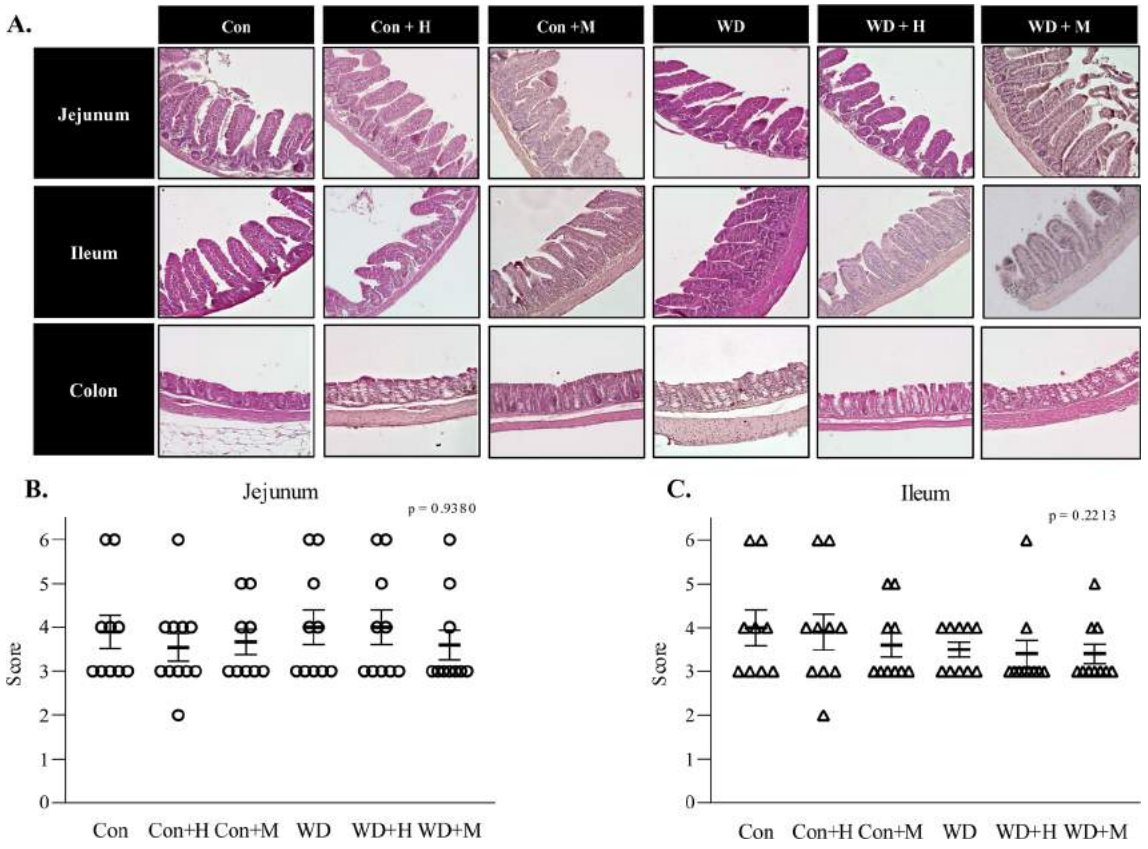

Figure 4. (A) Representative images of H&E-stained cross sections of the jejunum, ileum, and colon. Tissue sections of the (B) jejunum and (C) ileum (n = 10/group) were subjected to histopathological scoring, which encompassed villous atrophy, crypt hyperplasia, lymphocyte infiltration, and goblet cell number. Scores ranged from 3 to 13, with higher scores represented negative outcomes relative to the control. Data are presented as scatter plots for individual samples with bars indicating mean ± SE. Abbreviations: Con, control; WD, Western diet; H, Heirloom; M, Modern.

treatments, total WBCs were reduced ($p < 0.05$), but mean values for all groups were still within the normal range.

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. Expression of cytokines considered inflammatory in the gut (i.e., *Tnf*, *Il6*, *Il1b*) were unaffected by treatment. Similarly, anti-inflammatory cytokines involved in maintaining gut immunotolerance (i.e., *Il10*, *Tgfb*) were unaffected by treatment (Table 3). Among genes that were altered, *Ifng* expression was increased with WD, consistent with an inflammatory response. Further, *Il17* expression was

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Table 4. Villi and Crypt Structural Parameters in the Jejunum, Ileum, and Colon[a]

	Con	Con + H	Con + M	WD	WD + H	WD + M	*p*-value		
							WD	wheat	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

[a]Data are presented as mean ± SE. *p*-values <0.05 are considered statistically significant and are shown in bold. Abbreviations: Con, control; WD, Western diet; H, Heirloom; M, Modern.

Table 5. Relative Expression of Genes Related to Barrier Integrity and Mucous Layer Formation in the Colon[a]

	Con	Con + H	Con + M	WD	WD + H	WD + M	*p*-value		
							WD	wheat	WD × wheat
Cldn2	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
Cldn15	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
Ocln	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
Jam3	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**
Zo1	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
Muc2	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

[a]Data are presented as mean ± SE. *p*-values <0.05 are considered statistically significant and are shown in bold. Superscript letters are utilized when two-way ANOVA revealed a significant interaction, and values within a given row that share the same letter are not statistically different from each other. Abbreviations: *Cldn2*, claudin-2; *Cldn15*, claudin-15; *Ocln*, occludin; *Jam3*, junction adhesion molecule-3; Zo1, zonula occludens-1; Muc2, mucin-2; Con, control; WD, Western diet; H, Heirloom; M, Modern.

modestly suppressed ($p < 0.05$) by Heirloom in the context of WD relative to all other groups, but the expression of *Rorc*, a transcription factor involved in induction of IL-17-producing Th17 cells, was unchanged.

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 ($p < 0.05$), but no structural changes were noted with this diet in the villi of the ileum (Table 4). Wheat had no main effect on any villi parameters in the jejunum, but Modern increased villi width, area, and perimeter in the ileum. Post hoc analysis revealed that Modern improved ileal villi width, area, and perimeter relative to groups not consuming wheat ($p < 0.05$) and Heirloom was not statistically different than groups not containing wheat or Modern-consuming groups (Table 4). Increased crypt depth is associated with chronic gut inflammation; however, in this study we saw no effect of WD or wheat on crypt depth in any region of the small and large intestines. From a histopathological standpoint, no differences among treatment groups were observed for scores that encompassed structural parameters, such as villous atrophy, crypt hyperplasia, and the number of mucus-producing goblet cells in any region of the gut (Figure 4).

Gut Barrier Integrity and Function. To determine the effects of wheat alone and in combination with WD on indicators of gut permeability, we assessed colonic expression of genes related to barrier integrity. Expression of the intracellular scaffolding protein *Zo1* 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 the control (Table 5). One exception was suppression of *Jam3* with the addition of Heirloom to the WD relative to all other groups ($p < 0.05$; Table 5).

In the colon, relative protein abundance of the tight junction proteins, ZO-1, occludin, and claudin-4, was assessed by Western blotting, and representative Western blots are shown (Figure 5A). No statistically significant difference was detected for any of these tight junction proteins with WD and wheat, alone and in combination (Figure 5B).

Serum LPS BP was analyzed as an indirect measure of circulating LPS, which can translocate into circulation when barrier integrity is compromised. After 6 weeks of treatment, serum LPS BP was not altered with the WD or wheat diets (Figure 5C).

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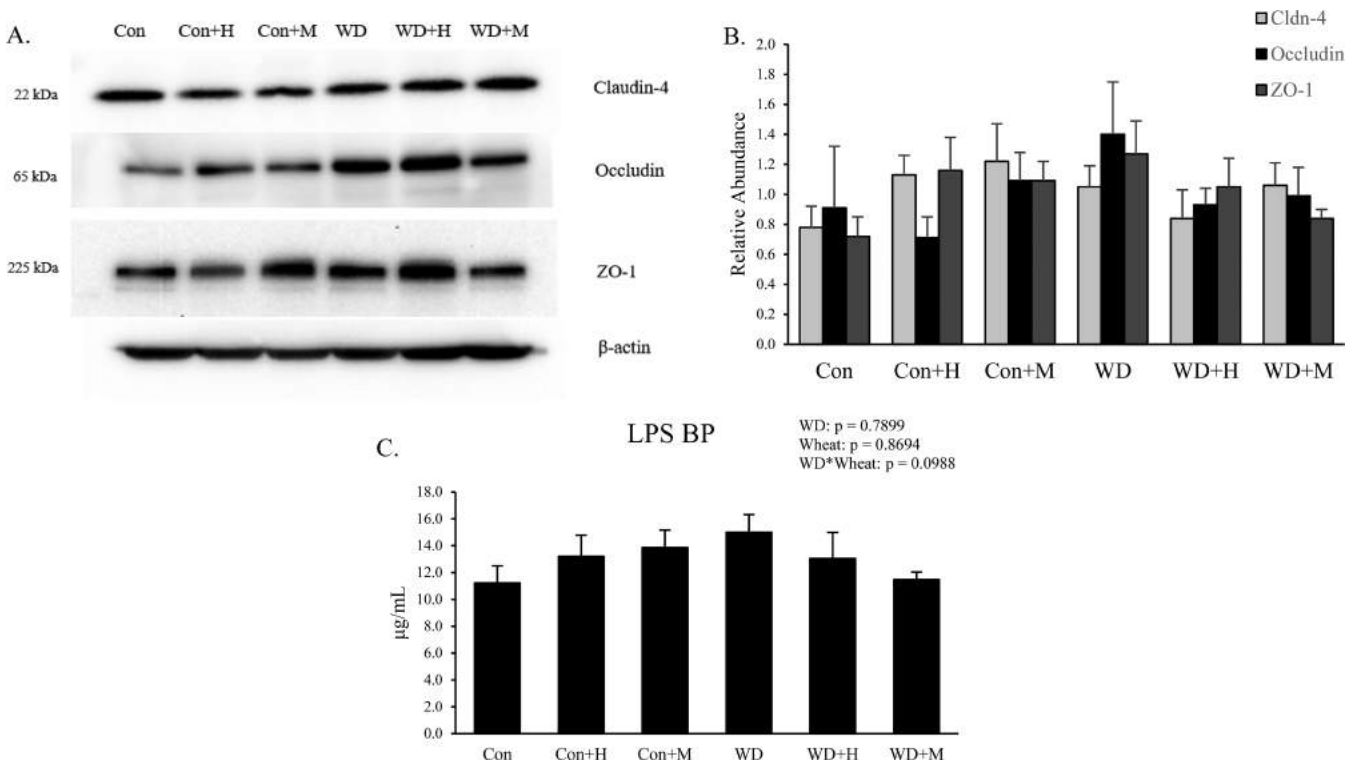

Figure 5. (A) Representative images (n = 5/group) of immunoblots probed for claudin-4, occludin, and ZO-1 in the colon. (B) Relative quantification of the proteins claudin-4, occludin, and ZO-1 immunoblotted in the colon. (C) Serum LPS BP (n = 10/group). Data are presented as mean ± SE. Abbreviations: Cldn-4, claudin-4; ZO-1, zonula occluden-1; LPS BP, lipopolysaccharide binding protein; Con, control; WD, Western diet; H, Heirloom; M, Modern.

Table 6. Cecal Short-Chain Fatty Acid Levels[a]

							p-value		
	Con	Con + H	Con + M	WD	WD + H	WD + M	WD	wheat	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
isobutyrate (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
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
isovalerate (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
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

[a]Data are presented as mean ± SE. p-values <0.05 are considered statistically significant and are shown in bold. Superscript letters are utilized when two-way ANOVA revealed a significant interaction, and values within a given row that share the same letter are not statistically different from each other. Abbreviations: Con, control; WD, Western diet; H, Heirloom; M, Modern.

SCFA and Anti-Microbial Peptide Analyses. Due to the importance of SCFAs in gut epithelial cell health and immune regulation, we assessed cecal acetate, propionate, isobutyrate, butyrate, isovalerate, and valerate levels. For many SCFAs analyzed (i.e., isobutyrate, butyrate, propionate, isovalerate, and valerate), no effect of WD or wheat variety was observed (Table 6). However, a significant interaction occurred for cecal acetate content, with the WD group suppressing acetate relative to the control (p < 0.05; Table 6). Interestingly, addition of Modern to the WD restored cecal acetate to the level of the control group.

Neither WD nor wheat affected gene expression for the antimicrobial peptides, *Reg3b* and *Reg3g*, which are important in regulating gut bacterial growth (Table 3).

DISCUSSION

This study investigated whether repeated, cyclic breeding of wheat for yielding ability, as represented by the widely grown modern variety Gallagher, induces gastrointestinal inflammation when consumed within the context of a typical WD. The majority of research done comparing heirloom and modern wheat cultivars has been performed within a clinical disease state (e.g., type 2 diabetes, NCGS) or animal models of oxidative stress (i.e., doxorubicin injection), leaving questions as to whether inherent properties of wheat drive reported changes or if these effects are only observed within pre-existing pathologies.[21,22,26−28] Additionally, these studies largely utilize crossover designs and/or lack a negative control, making it difficult to know if modern wheat has negative properties or if heirloom wheat has beneficial properties. Also, the health effects of modern compared to heirloom wheat are not well characterized in a model more representative of the general

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population. To achieve this objective, we investigated the effects of modern and heirloom wheat in normal C57BL/6 mice fed the optimal AIN-93G diet[29] or a diet formulated to mimic the WD. The 10% w/w wheat dosage used resembles normal to high intake in humans. The WD is well-known for increasing the risk for metabolic disturbances that can include elevated fasting glucose and lipids, along with increasing visceral adiposity that can contribute to an acute and chronic inflammatory state.[14,30] In the current study, mice on the WD exhibited these body composition and metabolic features, which provided an important testing ground to investigate whether intestinal mucosa structural, inflammatory, or gut barrier integrity is negatively affected by the modern compared to heirloom wheat varieties.

The gastrointestinal tract is known to be highly responsive to the dietary patterns of the host, and the resident stem cells within the crypts provide a means of ongoing regeneration of the epithelial lining of the villi. Reduced villi height or atrophy and increased crypt depth due to increased stem cell hyperplasia are classically observed with wheat consumption in CD and are in part due to inflammatory stated induced by activated Th1 and Th17 cells.[31] The reduced villi area, height, and perimeter within the jejunum that we observed in this study with the WD are similar to those previously reported for animals fed a high saturated fat diet (40%) and the reduced microvilli height with a 70% fat diet high in polyunsaturated fat.[32,33] Interestingly, consumption of the modern wheat Gallagher improved villi structural parameters (i.e., area, perimeter, and width) in the ileum of mice consuming the control and WD compared to nonwheat consuming groups. This response was not observed with the heirloom blend of Turkey/Kharkof wheat. In contrast, Carnevali et al.[22] reported increased villi height with consumption of the heirloom variety Khorasan relative to an unspecified modern wheat variety in an animal model of chemically induced oxidative stress, but their focus was within the duodenum. Improvements in ileum villi structure in this study show clear divergence from wheat-related pathologies and suggests that neither wheat variety led to negative gut structural characteristics in normal mice.

We next evaluated the effect of WD and wheat on gut architecture using histopathological scoring on sections of the jejunum, ileum, and colon. Our study pathologist assessed villous atrophy and crypt hyperplasia, which can occur as intestinal stem cells undergo division more rapidly in an attempt to regenerate the damaged epithelium.[34] Neither atrophy nor crypt hyperplasia was altered by WD or wheat treatment, providing evidence of immune homeostasis. Although villi height was evaluated by Carnevali and colleagues,[22] our study is the first study to evaluate the effect of wheat cultivar on clinical histological parameters (i.e., crypt hyperplasia and villous atrophy) outside of CD or models of chemically induced oxidative stress. This is an important distinction, as our findings may more closely approximate the effect of wheat cultivar on gut health in an otherwise healthy population consuming a typical WD. Taken together, our histopathological findings indicate that a modern variety had no negative effects on gut structure and positively affected ileal villi parameters.

Wheat-induced intestinal inflammation in CD is characterized by a robust T helper cell response, and individuals with NCGS display elevated *Ifng*, *Tlr2*, and gut $CD3^+$ intraepithelial lymphocytes.[35−37] When comparing the effects of heirloom and modern wheat on circulating cytokines (e.g., 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 that have included irritable bowel syndrome, nonalcoholic fatty liver disease, type 2 diabetes, and at risk for cardiovascular disease (CVD).[18,20,26,38] However, the absence of control arms in these studies are a limitation that leaves questions as to whether these improvements in circulating cytokines are due to the beneficial properties of heirloom wheat or negative effects of modern wheat. Similar to clinical reports, existing animal studies suggest that heirloom wheat, in particular Khorasan, has anti-inflammatory properties in the liver and gut.[22,39] Our gene expression data in the ileum lamina propria suggest there was no evidence of the T-cell-mediated or innate immune response to WD or wheat, as evidenced by no changes in *Tnf*, *Il6*, *Rorc*, and *Il1b*. These data differ somewhat from previous reports,[19,38] where circulating TNF-α and IL-6 were downregulated in humans with acute coronary syndrome and a cohort at risk for CVD who were fed heirloom compared to modern wheat. Further, there were no alterations of *Il10* and *Tgfb* in the ileum lamina propria, suggesting that there was no effect of WD or wheat on T regulatory cell populations. Among the genes that were altered in our study, *Ifng* expression was increased with WD consumption, which is consistent with reports that WD increases TLR-4/NF-κB signaling and cytokine production (i.e., TNF-α, IL-1β, IL-6) in the colon.[15] The heirloom wheat did mildly suppress *Il17* in the context of WD relative to all other groups. Interestingly, this was not accompanied by changes in *Rorc*, a transcription factor inducing IL-17-producing Th17 cells. Likewise, Sofi et al.[26] observed reduced serum IL-17 with consumption of heirloom wheat, but not modern wheat, in individuals with irritable bowel syndrome. Another indicator of gut inflammation, increased lymphocyte infiltration and lymphatic follicle diameter, has been reported in animals consuming heirloom wheat vs modern wheat following treatment with the pro-oxidant doxorubicin.[22] We also evaluated lymphocyte infiltration in the jejunum, ileum, and colon, but no differences were observed among treatment groups in our study. When evaluating the systemic immune response, there was no effect of treatment on CRP, which is similar to findings in patients with NCGS.[40] We did observe a statistically significant decrease in WBC with the WD and both wheat varieties, but all treatment groups remained within the normal range for C57BL/6 mice. Together, our data suggests that there was little effect of either wheat variety or WD on markers of gut and systemic inflammation. There is some indication that Heirloom may have anti-inflammatory properties (i.e., *Il17* suppressed under WD conditions), consistent with previous studies of heirloom wheat varieties.[20,22,38]

To further explore whether wheat affects gut barrier integrity, which is an important indicator of gastrointestinal health and implicated in a variety of disease states, we evaluated whether changes in tight junction proteins or diminished gut mucus layer occurred in these animals.[41] First, we evaluated serum LPS BP as a proxy for LPS translocation from the gut into circulation, but no treatment effect for wheat or WD was observed. Unaltered LPS BP is consistent with intact barrier integrity and is distinct from NCGS[42] and with WD consumption in rats.[43] The lack of treatment effect by WD could be due to lower calories from fat in our WD compared to other formulations utilized (i.e., 45% vs 60−70%), shorter study duration, or different species used. To investigate indicators of local gut integrity, we evaluated

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genes and proteins important in tight junction and mucus layer formation in the colon. We saw no transcriptional changes or differences in protein abundance with tight junction intracellular scaffolding proteins or transmembrane proteins with the exception of reduced *Jam3* when Heirloom was added to the WD. This suppression of *Jam3* appears to stand alone in terms of negative impacts of Heirloom on indicators of gut integrity, and similar reports are lacking. With respect to markers of gut mucus layer status (i.e., *Muc2* expression, goblet cell number), no impact of WD or wheat was seen. Certain tight junction proteins important in maintaining barrier integrity are reduced with WD consumption (i.e., occludin).[15] We assessed claudin-4, occludin, and ZO-1, and for all of these proteins, relative abundance was unchanged by any treatment. Similar to our LPS BP data and analysis of genes encoding tight junction proteins, our lack of a WD effect may be explained by our lower fat content and shorter study duration relative to other reports.[15,44] With respect to wheat variety, these results are consistent with the notion that barrier integrity is unaltered when wheat is consumed in the absence of CD and NCGS. In sum, there were largely no differences between the two wheat varieties on indicators of barrier integrity alone or in combination with WD consumption.

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 T regulatory cells.[45,46] In addition, some evidence suggests that SCFA production is suppressed with a high-fat diet.[47] For these reasons, we assessed the cecal SCFAs acetate, propionate, isobutyrate, butyrate, isovalerate, and valerate. Interestingly, cecal acetate was reduced with the WD diet control; however, the addition of Modern to the WD reversed this effect. A similar finding 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.[28] The same study also reported beneficial effects of wheat on levels of propionate and butyrate, but we failed to see any other treatment effects on SCFAs. Consistent with our other data, this suggests that neither Heirloom nor Modern led to abnormal gut health via changes in SCFAs.

Our study provides evidence that neither Gallagher nor the blend of Turkey/Kharkof wheat varieties negatively impacted gut mucosal architecture, intestinal permeability, or markers of gut inflammation compared to the control diet with no wheat added. Importantly, our findings also demonstrate that the modern wheat variety Gallagher had no negative effects on gastrointestinal health relative to its heirloom predecessors Turkey/Kharkof. While in most cases there were no statistical differences between Modern and Heirloom, for some parameters (i.e., ileum villi structure, cecal acetate) a positive effect of Modern was observed. Conversely, animals consuming the WD with heirloom wheat added displayed a positive effect on *Il17* expression within the lower small intestine (modest suppression), but this was not the case with the modern wheat. The overall lack of response we observed with both wheat varieties and, in some cases, positive findings with Modern consumption should be evaluated by utilizing other modern wheat varieties and confirmed in human trials. The 10% w/w dosage of wheat used in this study is a reasonable quantity for human consumption, supporting potential for translation into clinical studies. We conclude that neither the modern wheat variety Gallagher nor the heirloom blend of Turkey/Kharkof negatively impacted indicators of gastrointestinal health in a normal, C57BL/6 mouse model consuming a control or typical Western diet.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acs.jafc.9b05851.

Tables listing the diet formulations and the primer sequence list for qRT-PCR (PDF)

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Notes

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■ ABBREVIATIONS USED

H, Heirloom; M, Modern; IFN, interferon; IL, interleukin; LPS BP, lipopolysaccharide binding protein; CD, celiac disease; NCGS, nonceliac gluten sensitivity; TNF, tumor necrosis factor; WBC, white blood cell; Cldn, claudin; Ocln, occludin; ZO, zonula occludens; CRP, C-reactive protein; NEFA, nonesterified fatty acid; Rorc, RAR-related orphan receptor gamma; Tgf, transforming growth factor; Reg, regenerating islet-derived protein; Muc, mucin; Jam, junction adhesion molecule; SCFA, short-chain fatty acid; Tlr, toll-like receptor.

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