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Macronutrients in diets differentially affect gastrointestinal cytokine and tight junction protein levels

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Abstract

Western diet is known to result in intestinal inflammation and loss of barrier function. In the present work, we investigated whether other macronutrients contribute to inflammation and destabilising barrier function in mice, without using any inflammatory agents, to see the sole effects of dietary intervention. The present work was designed to determine the direct effects of diet on the intestinal barrier function and inflammation, using eight diets that differed on carbohydrate, fat, and fibre ratios for 17 weeks. At the end of the study, a distinct difference in mRNA expressions of cytokines and tight junction proteins was observed between intestinal and colon samples. Small intestinal cytokine expressions showed no difference among different diets, and tight junction protein expressions were only significant for occludin and ZO-1 in high carbohydrate diets. Colon samples had significantly different TNFα and IL-6 expressions among diets, especially in high carbohydrate diets. Tight junction protein expressions also differed significantly among diets, and low carbohydrate zero fibre diet had the lowest expression levels compared to the rest of diets. The present work reveals that not only western diet, but also diets high in carbohydrate negatively affect intestinal health, resulting in significant changes in inflammation markers. The role of carbohydrate and fiber contents are also observed in regulating tight junction protein expression. Based on these findings, adjusting macronutrient ratios can be used as a potential approach to help manage intestinal inflammation, though further research is needed.

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Introduction

Intestines are mainly responsible for water and nutrient uptake, but they also act as a barrier, keeping luminal contents away from internal organs. In addition to nutrient absorption, intestines host a multitude of microorganisms with great diversity (Gebrayel *et al.*, 2022). The microbiota confers health benefits to the host, including the development of the immune system, and the production of vitamins and neurotransmitters. However, it also poses a health risk when dysbiosis occurs. Intestinal epithelium, along with the mucin layer, constitutes the epithelial barrier, and is responsible for the selective permeation of water and solutes. This barrier also prevents the passage of proinflammatory factors such as toxins, antigens, and pathogens from the lumen to the intestinal mucosa (Lee, 2015). When this barrier cannot properly function, a variety of diseases take place, mainly through driving inflammation. The first organ affected by the inflammation is the intestines; however, non-GI organs are also affected. The diseases associated with an improper intestinal barrier include type I diabetes, asthma, rheumatoid arthritis, and neurological diseases, including Alzheimer's disease (Martel *et al.,* 2022). The barrier function of the epithelium is regulated by tight junction proteins: occludin and claudins, which are transmembrane proteins, while ZO-1 functions to attach transmembrane proteins to actin cytoskeleton (Lee, 2015).

The gut is considered the body's largest immune organ. Inflammatory diseases of the intestines, such as ulcerative colitis and Crohn's disease are known to increase the likelihood of developing colorectal cancer, which is one of the most common cancers. The tumour formation in colorectal cancer is mainly driven by mutations of the genes *Apc*, *K-ras*, and *p53*. However, tumour promotion and progression depend on the tumour

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microenvironment (Borowczak *et al.*, 2022). Inflammatory cytokines can be produced by the tumour cells themselves or by other cells, such as mast cells and macrophages, recruited to the tumour site, and promote tumour growth. Thus, cytokines such as TNFα, IL-1β, and IL-6 are targets for cancer treatment. Non-steroidal anti-inflammatory drugs are known to lower the mortality from sporadic colon cancer, and cause regression of adenomas in familial adenomatous polyposis (FAP) patients (Borowczak *et al.*, 2022). Earlier studies indicated that proinflammatory cytokines (TNFα, IL-1β, and IL-6) caused an increase in intestinal permeability, and contributed to inflammation by allowing passage of luminal contents. TNFα plays a central role in intestinal inflammation, and exerts its proinflammatory effects in colitis through activating NF-κB, and causes augmenting of angiogenesis, inducing death of Paneth cells through necroptosis, and damaging barrier function by inducing myosin light chain kinase (MLCK), thereby increasing permeability of intestinal epithelial tight junction proteins (Mitoma *et al.*, 2018; Aardoom *et al.*, 2019). Treatments that target TNFα antibodies have been successfully used in colitis. Anti-TNFα antibodies are effectively used for the treatment of CD and UC. IL-6, on the other hand, has proinflammatory and homeostatic functions in the intestine. IL-6 production by macrophages of the lamina propria and CD4+ T cells is increased in experimental colitis models and patients. Blocking of IL-6 signalling resulted in suppression of chronic inflammation in mouse models (Mitoma *et al.*, 2018). The IL-6 produced by the lamina propria and by the IECs themselves is also involved in the proliferation and expansion of IECs, aiding in the closure of ulcers and improving barrier function. IL-10 and TGF-β are produced by T_{reg} cells, and function to suppress the proinflammatory cytokine production. Mice lacking IL-10 or TGF-β failed to suppress proinflammatory cytokines produced by antigen-presenting cells (APC) and T_{reg} cells, and developed chronic inflammation (Noth *et al.*, 2012).

Diet is a major factor in intestinal health and disease. The dietary changes, along with other factors such as lifestyle and lack of physical exercise coincide with the increased incidence of inflammatory diseases (Martel *et al.*, 2022). Western diet (WD) is mostly consumed in developed countries, and is assumed to be one of the major drivers for the progression of inflammatory bowel diseases (IBD) (Statovci *et al.*, 2017; He *et al*., 2022). It is composed of high amounts of fats and simple sugars. Interestingly, the incidence of IBD in Turkey is also high (Ozin *et al*., 2009; Can *et al.*, 2019), even though the macronutrient composition of the Turkish diet is quite different from the WD: Turkish diet is heavily based on refined grains (40% of caloric intake is from bread or other grains), but with lower amounts of fats and sugars. The present work was thus designed to understand the impact of different macronutrients on intestinal inflammation and tight junction protein levels. Mice were subjected to seven different diets for four months. The diets differed in their fat, carbohydrate, and fibre contents. The microbiota is known to mediate dietary effects on inflammation; however, a microbiota analysis was not included in the present work, which may limit our understanding of the complex interplay between dietary macronutrients, microbial community shifts, and the resultant inflammatory responses. Therefore, the results presented herein took into account the effects of both the nutrition and the microbiota, and future investigations will aim to incorporate such assessments. The inflammatory cytokine and tight junction proteins mRNA levels from small and large intestinal tissues were compared. The results suggested that carbohydrate and fibre contents of diets could also be determining factors in intestinal inflammation, and they should be considered as additional parameters in nutrition studies. This is in contrast to the popular belief that only high fat- and simple sugar-rich WD are responsible for increased bowel inflammation.

Materials and methods

Animals and diets

Five-week-old male Balb/c mice were kept at Erciyes University Dekam (Kayseri, Türkiye). The animals were kept in three animals per cage, and under controlled temperature, air humidity, and regulated light-dark cycles. Animal procedures were performed upon permission of Erciyes University Central Ethics Committee and Animal Experimentation (CECAE) (approval no: 04/16/067). Mice groups were fed *ad libitum.* The diets were purchased from TestDiet (Land O' Lakes, Inc., MO, USA). They included a standard chow diet (S) to be used as a control, and compared to diets high in carbohydrates (HC and HC-0F), low in carbohydrates (LC-0F and ketogenic), and western diets (WD and

HP) (0F: diets that contained zero fibre). The diets were specifically selected to mimic different types of human diets, and to allow for comparative analysis of macronutrients using different diets. The HC-0F and LC-0F diets contained no fibre, and the remaining diets (HC, K, W, and HP) contained the same amount of micro-nutrients (vitamins and minerals), protein, fibre, and cholesterol per calorie. The macronutrient compositions of diets are listed in Table 1.

Each experimental group consisted of six mice which were selected by power analysis. Adherence to ethical guidelines for animal research while ensuring reliable statistical data were factored into the decision-making. To adapt to experimental diets, mice were fed a mixed diet for a week, where standard mice chow was provided along with respective experimental diets, *ad libitum.* At the end of the first week, all mice groups were switched to and fed with 100% *ad libitum* experimental diets until the end of the study. At weeks 0, 1, 2, 3, 5, 9, 13, and 17, mice were weighed, and faecal samples were taken and stored at -80°C. At the end of week 17, euthanisation of mice was performed using a ketamine/xylazine cocktail, and tissue samples were collected and placed in RNAlater (Thermo Fisher Scientific CA, USA) for qPCR.

Table 1. Macronutrient composition of diets (% Cal).

Diet	Protein	Fat	Carbohydrate	Fibre
Standard chow (S)	21	10	70	5.0
High carbohydrate zero-fibre (HC - 0F)	15	11	74	0
Low carbohydrate zero-fibre (LC - 0F)	15	59	26	0
High carbohydrate (HC)	16	12	72	5.8
Ketogenic (K)	33	65	2	7.8
Western (WD)	16	39	45	6.7
High protein (HP)	38	40	22	6.8

RNA extraction and qPCR

Briefly, 100 mg ileum and colon samples were stored in RiboX™ (GeneAll Biotechnology, South Korea). RNA extraction was performed following the user manual (GeneAll Hybrid-R RNA purification kit, South Korea). A Nanodrop 2000 spectrophotometer was used to measure RNA concentrations of samples. High-Capacity cDNA Reverse Transcription kit (Applied Biosystem, Thermo Fisher Scientific, CA, USA) was used to produce cDNA.

qRT-PCR was performed using Maxima SYBR green qPCR master mix (Thermo Scientific, MA, USA) in triplicates on a CFX96 Real-Time PCR Detection System (Table 2). Primers used in the analysis were as follows: IL-1β (5'-GCAACTGTTCCTGAACTCAACT-3' and 5'-ATCTTTTGGGGTCCGTCAACT-3'); IL-6 (5'-AGTTGCCTTCTTGGGACTGA-3' and 5'-CAGAATTGCCATTGCACAAC-3'); TNFα (5'-GAACTGGCAGAAGAGGCACT-3' and 5'-AGGGTCTGGGCCATAGAACT-3'); IL-10 (5'-TCAAGGATGCACATCAAAAGGC-3' and 5'-AGGCAGCAACTTCCTCCCT-3'); and TGF-β (5'-CAGAGCTGCGCTTGCAGAG -3' and 5'-GTCAGCAGCCGGTTACCAAG-3'). Tight junction protein gene expressions were studied for occludin (5'-TTGAAAGTCCACCTCCTTACAGA-3' and 5'- CCGGATAAAAAGAGTACGCTGG-3'), claudin-1 (5'-CTGGGTTTCATCCTGGCTTC-3' and 5'-TTGATGGGGGTCAAGGGGTC-3'), ZO-1 (5'-CCAGTCCCTTACCTTTC-3' and 5'-CTCCTCCAGTCTGACATTAG-3'), and housekeeping gene GAPDH (5'-CATCACTGCCACCCAGAAGACTG-3' and 5'-ATGCCAGTGAGCTTCCCGTTCAG-3').

Statistical analyses

Data were presented as means with standard errors. Mean differences were considered significant at $p < 0.05$. Statistical analyses were performed on GraphPad Prism using One-way ANOVA with *post hoc* Tukey test ($n \geq 3$ for gene expression analyses).

Results

The mRNA levels of pro-inflammatory cytokines TNFα, IL-1β, IL-6, and anti-inflammatory were analysed to elucidate the intestinal inflammatory state. No significant differences in cytokine gene expressions were observed among diet groups in the small intestine (Figure 1). A slight

Table 2. 2(Δ ΔCt) ± SEM expression levels of cytokines and tight junction proteins across all diets.

Gene	Small intestine 2(Δ ΔCt) ± SEM							Colon 2(Δ ΔCt) ± SEM						
	S	**HC - 0F**	**LC - 0F**	**HC**	**K**	**WD**	**HP**	**S**	**HC - 0F**	**LC - 0F**	**HC**	**K**	**WD**	**HP**
TNFα	1 ± 0.12	1.78 ± 0.40	1.04 ± 0.15	1.36 ± 0.38	0.59 ± 0.17	0.62 ± 0.20	0.87 ± 0.21	1 ± 0.12	1.26 ± 0.19	0.73 ± 0.07	2.1 ± 0.19	0.68 ± 0.23	0.51 ± 0.04	0.54 ± 0.06
IL-1β	1 ± 0.26	1.59 ± 0.21	0.74 ± 0.04	1 ± 0.09	0.83 ± 0.08	0.72 ± 0.15	0.84 ± 0.09	1 ± 0.29	1.15 ± 0.16	0.56 ± 0.16	1.73 ± 0.43	0.78 ± 0.35	0.54 ± 0.03	0.84 ± 0.30
IL-6	1 ± 0.27	1.57 ± 0.69	0.48 ± 0.08	2.16 ± 0.50	0.57 ± 0.16	0.40 ± 0.13	0.72 ± 0.17	1 ± 0.31	2.05 ± 0.59	0.56 ± 0.04	4.47 ± 0.94	0.98 ± 0.49	0.45 ± 0.20	0.36 ± 0.07
IL-10	1 ± 0.16	0.98 ± 0.46	0.94 ± 0.34	0.61 ± 0.17	0.67 ± 0.06	1.08 ± 0.36	1.05 ± 0.23	1 ± 0.03	1.47 ± 0.23	1.08 ± 0.26	0.89 ± 0.02	0.72 ± 0.11	0.72 ± 0.10	1.32 ± 0.09
TGF-β	1 ± 0.05	1.33 ± 0.23	1.16 ± 0.17	0.88 ± 0.10	1.71 ± 0.20	0.80 ± 0.01	0.79 ± 0.09	1 ± 0.27	1.46 ± 0.20	0.69 ± 0.05	0.58 ± 0.02	0.59 ± 0.10	0.81 ± 0.03	0.63 ± 0.03
Occludin	1 ± 0.12	1.45 ± 0.27	0.02 ± 0.01	1.28 ± 0.17	0.65 ± 0.14	0.97 ± 0.19	0.81 ± 0.17	1 ± 0.39	0.92 ± 0.06	0.04 ± 0.01	0.93 ± 0.14	0.45 ± 0.05	0.68 ± 0.02	0.32 ± 0.04
Claudin-1	1 ± 0.07	1.96 ± 0.42	1.16 ± 0.29	1.42 ± 0.13	1.99 ± 0.48	0.56 ± 0.17	0.85 ± 0.26	1 ± 0.44	0.71 ± 0.02	0.05 ± 0.01	0.56 ± 0.02	0.96 ± 0.15	0.05 ± 0.01	0.28 ± 0.08
ZO-1	1 ± 0.10	0.80 ± 0.11	1.12 ± 0.14	1.38 ± 0.19	0.85 ± 0.14	0.50 ± 0.17	0.12 ± 0.02	1 ± 0.03	1.15 ± 0.08	1.99 ± 0.16	0.87 ± 0.14	0.66 ± 0.06	0.51 ± 0.01	0.20 ± 0.06

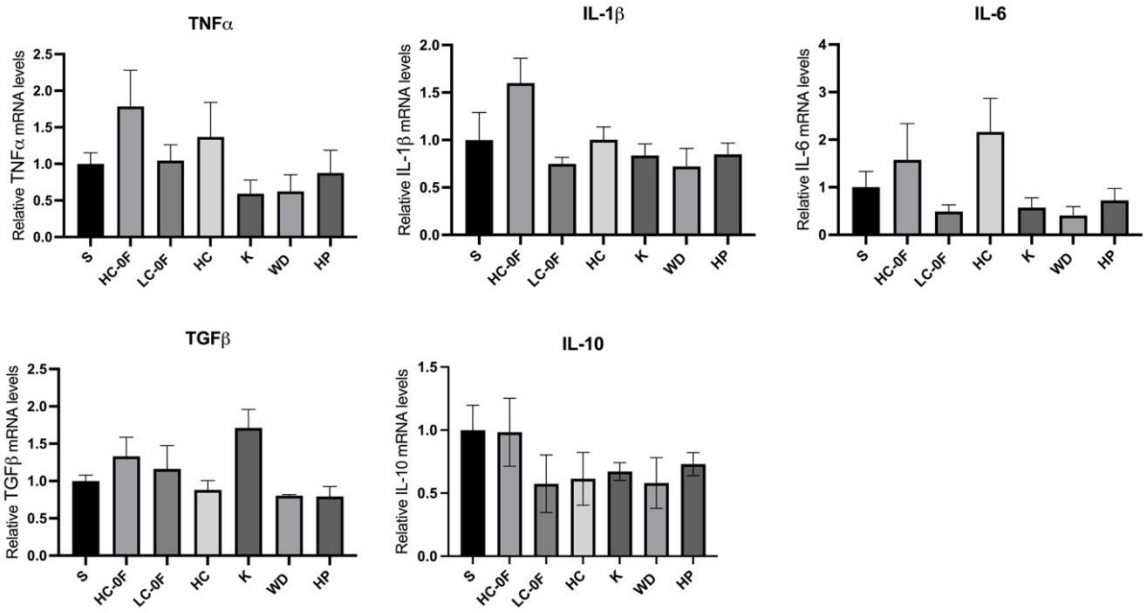

Figure 1. Gene expressions of pro- and anti-inflammatory cytokines in small intestine for different diets (S, HC-0F, LC-0F, HC, K, WD, and HP). Data are mean ± SEM ($n \geq 3$), analysed *via* One-way ANOVA with Tukey's HSD.

increase in TNFα, IL-1β, and IL-6 levels for HC and HC-0F diets was observed; however, they were neither statistically nor biologically significant. On the contrary, in colon samples, HC diet displayed a significant increase in colon TNFα, as well as IL-6 levels (Figure 2). HC diet resulted in a significantly higher relative TNFα expressions, compared to LC-0F ($p < 0.01$), K ($p < 0.01$), HP ($p < 0.01$), and WD ($p < 0.005$) diets, while no difference was observed when compared to the standard chow diet. Compared to standard chow diet ($p < 0.05$), as well as LC-0F ($p < 0.05$), K ($p < 0.05$), WD ($p < 0.01$), and HP ($p < 0.01$) diets, HC diet caused significantly increased relative IL-6 gene expressions. No significant difference was observed for IL-1β gene expressions between the same diets.

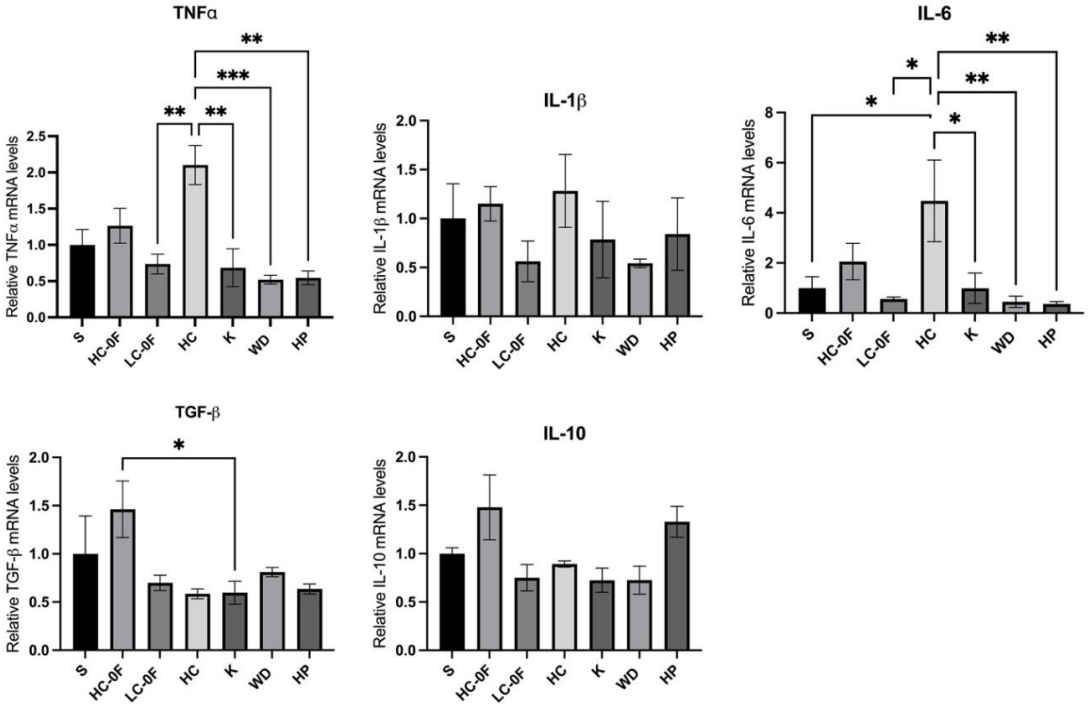

Figure 2. Gene expressions of pro- and anti-inflammatory cytokines in colon for different diets (S, HC-0F, LC-0F, HC, K, WD, and HP). Data are mean ± SEM ($n \geq 3$), analysed *via* One-way ANOVA with Tukey's HSD. Significance: *$p < 0.05$, **$p < 0.01$, and ***$p < 0.001$.

The tight junction protein gene expressions in small intestine samples were observed to be similar among diet groups: No diet group caused significantly different tight junction protein gene expressions against the standard diet (Figure 3), indicating that the integrity of the barrier was less influenced by diet alone. Occludin gene expressions in LC-0F diet revealed significant differences against that of HC ($p < 0.05$) and HC-0F ($p < 0.01$) diets. For ZO-1 gene expressions, the only significant difference was observed between HC *vs*. WD ($p < 0.05$) diets, and HC *vs*. HP ($p < 0.01$) diets. Differences in occludin and ZO-1 expressions (*e.g*., between HC and other diets) were statistically significant but biologically limited, as they did not correlate with changes in inflammatory markers. Claudin-1 gene expressions were non-significant among different diets.

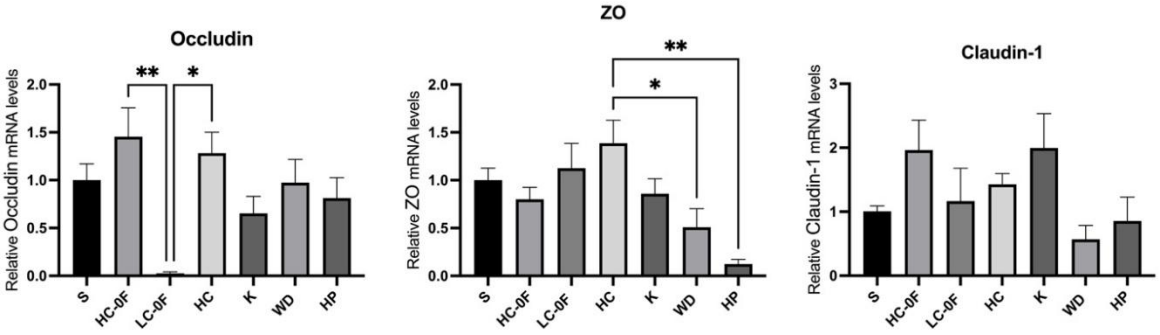

Figure 3. Tight junction protein gene expressions in small intestine for different diets (S, HC-0F, LC-0F, HC, K, WD, and HP). Data are mean ± SEM ($n \geq 3$), analysed *via* One-way ANOVA with Tukey's HSD. Significance: *$p < 0.05$, and **$p < 0.01$.

The TJ protein gene expressions in the colon samples revealed more noticeable results; but, these were not biologically significant, except for LC-0F diet. LC-0F diet had the lowest levels for occludin and claudin-1 proteins, and the highest levels for ZO-1 (Figure 4). The difference in occludin gene expression was most significant between LC-0F and HC-0F ($p < 0.01$) diets, and between LC-0F and HC ($p < 0.005$) diets. For claudin-1 gene expression, K diet had significantly higher expression than S diet ($p < 0.05$), and resulted in higher expression levels for most of the remaining diets. LC-0F and WD diets had the lowest claudin-1 levels among the diets, and both were significantly lower than HC-0F ($p < 0.05$) and K ($p < 0.005$) diets. For ZO-1 gene, expressions were highest for LC-0F diet, and lowest for HP diet.

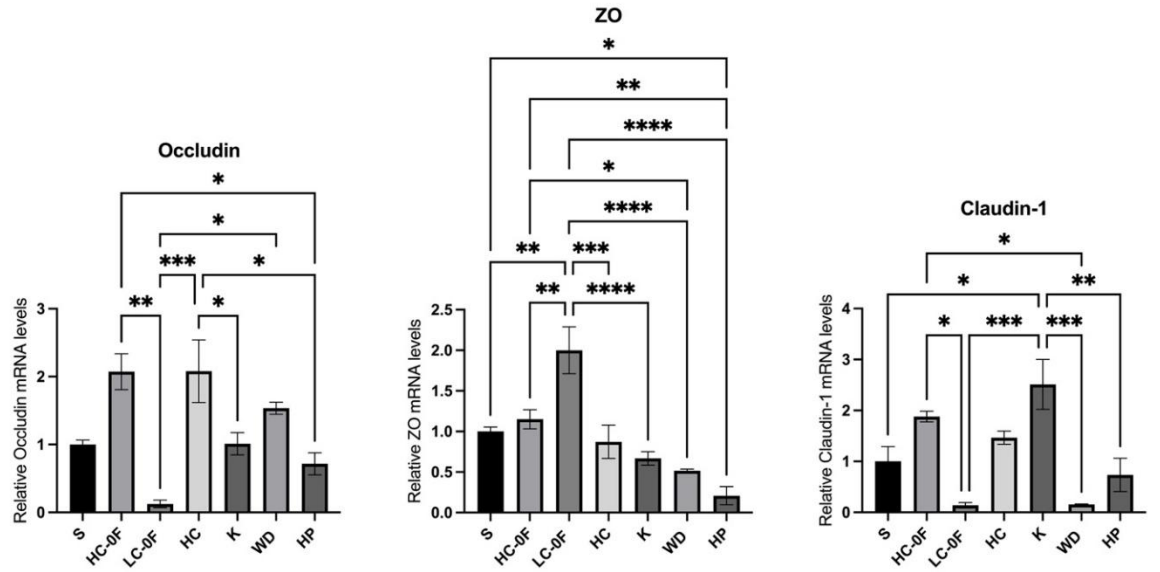

Figure 4. Tight junction protein gene expressions in colon for different diets (S, HC-0F, LC-0F, HC, K, WD, and HP). Data are mean ± SEM ($n \geq 3$), analysed *via* One-way ANOVA with Tukey's HSD. Significance: *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, and ****$p < 0.0005$.

Discussion

Diet is closely associated with intestinal health and disease. Epidemiological studies have shown that dietary patterns high in processed carbohydrates or "Western-style" eating habits are associated with an increased prevalence of inflammatory disorders, including inflammatory bowel diseases (IBD) and metabolic dysregulations in humans (Rizzello *et al*., 2019; Malesza *et al*., 2021). For instance, high glycaemic load and rapidly digestible carbohydrates in human diets have been linked to alterations in the gut microbiome and intestinal barrier function, potentially contributing to chronic low-grade inflammation. Likewise, systematic reviews have found that fibre intake and balanced macronutrient profiles can be protective against gastrointestinal and systemic inflammatory conditions (Hou *et al*., 2011; Rosa *et al*., 2018). In addition, probiotics may be used to increase the number of protective bacteria, increase the anti-inflammatory response, and may result in remission of different IBDs (Cheng *et al*., 2020). We have observed an increase in inflammatory disease incidence in Türkiye as well as the developed countries, where macronutrient contents differ considerably. In Türkiye, traditional diets are very high in processed carbohydrates, namely grains. We have observed in our study that, high carbohydrate diets were associated with increased inflammatory marker levels in mice colon but not in small intestine. We further noted that HC diet resulted in significantly higher levels of proinflammatory cytokines TNFα and IL-6 in the colon of mice compared to other diets (LC-0F, K, HP, WD, and S for only IL-6) (Malesza *et al*., 2021). These results agreed with the hypothesis that higher processed carbohydrate intake may exacerbate inflammatory markers. Despite these parallels, interspecies differences in gut physiology, immune system responses, and dietary habits necessitate caution when extrapolating from mice models to humans.

The statistically different levels in proinflammatory cytokine expression may not be reflected in the biologically observable levels; however, it may dispose to or exacerbate inflammatory disorders under additional stressors or susceptible individuals. Regarding high-fat diets and inflammation, the results from the literature are conflicting and no change in pro-inflammatory cytokine expression was observed in high-fat diets in this study (LC-0F and K) (Anderson and Van Itallie, 2009; Le Chatelier *et al*., 2013; Malesza *et al.*, 2021). For the anti-inflammatory cytokine gene expressions, no biologically important difference was observed among diet groups. The only significant expression among anti-inflammatory cytokines was observed in TGF-β levels between HC-0F and K diets ($p < 0.05$). No significance was observed among any of the diet groups for IL-10 mRNA expression.

Tight junction proteins are responsible for maintaining integrity of the inter-epithelial barrier, and are very important regulators of solute permeability across epithelia. Impaired structure and integrity of tight junction proteins can result in enhancing inflammation by activation of helper T-lymphocytes and antigen-presenting cells, as a result of increased passage of bacteria and other antigens (Anderson and Van Itallie, 2009; Le Chatelier *et al*., 2013; Chelakkot *et al*., 2018).

Previous studies reported the effects of diets on inflammation, but these studies almost always incorporate inflammatory agents, *e.g.*, DSS in experimental design. However, in the present work, we intended to see the sole effects of macronutrients, and subjected mice to these diets for a longer duration (17 weeks). Our results suggested that diet itself was capable of manipulating tight junction protein expressions, with no additional inflammatory agent present. Regarding how diets change tight junction protein expressions, literature presents conflicting results in animal models and human biopsy samples. Occludin, claudin-1, and ZO-1 levels tend to decrease in an inflammatory environment, while there are also reports of no change or increase (Nighot *et al.*, 2015; Zhu *et al.*, 2019; Zuo *et al.*, 2020; Cheng *et al.*, 2020).

The difference in occludin gene expression was most significant for LC-0F diet compared to HC-0F and HC diets. The high occludin levels for HC were accompanied by the high IL-6 levels. This could imply that the HC diet might lead to changes in the integrity of the epithelial barriers, potentially increasing permeability, and the cells may be compensating for inflammation-driven barrier dysfunction by upregulating occludin to maintain epithelial integrity. LC-0F diet resulted in significant downregulations in occludin and claudin-1 levels, and these were independent of the inflammatory cytokine levels. The observed reduction in tight junction

protein expressions in LC-0F diet, despite unchanged inflammatory cytokine levels, highlighted the complexity of intestinal barrier regulation. Tight junction proteins may respond to nutrient signalling, microbial metabolites like short-chained fatty acids (SCFAs), or physical changes in the gut environment rather than to inflammatory cytokines only (Peng *et al*., 2009; Liu *et al*., 2021). Tight junction proteins are highly dynamic, and subject to regulation by multiple physical and chemical signals. These include mechanical stress, shifts in gut motility, and a number of transcriptional and post-translational regulations that modulate barrier function independently of cytokine-driven pathways (Günzel and Yu, 2013). Another finding to note is that when zero-fibre HC diet was compared to HC diet, decreased proinflammatory TNFα and IL-6 expressions, and increased anti-inflammatory cytokines TGF-β and IL-10, were observed. This can possibly be explained by the absence of dietary fibre, and the changes in microbiota, which in turn resulted in amelioration of inflammation associated with HC diet.

ZO-1 gene expressions were highest for LC-0F diet, and lowest for HP diet. The increase in ZO-1 expression in LC-0F diet could have been due to the contribution of ZO-1 in epithelial repair (Kuo *et al*., 2021), and high protein diets were associated with lower ZO-1 levels (Hussain *et al*., 2019).

Gut microbiota also plays a critical role, as fibre deficiency alters the microbial composition, reducing the production of barrier-enhancing metabolites (Chelakkot *et al*., 2018). This highlights the complex interaction between diet, microbiota, and intestinal barrier function, and requires the need for further studies. Although the present work was focused on a murine model, it is important to highlight the translational implications of these findings for human health.

Future studies are needed to confirm whether the dietary-induced changes in tight junction protein expression and proinflammatory cytokine levels observed in our murine model similarly occur in humans. A key focus could be investigating the mechanisms linking dietary carbohydrates to the expression of tight junction proteins, and determining whether these effects are directly mediated by microbiota and SCFAs. High-throughput sequencing, functional metagenomics, and metabolomics studies could help elucidate the microbial populations and pathways responsible for modifications in barrier function.

Conclusion

The present work demonstrated that diet, by itself, was able to influence inflammatory cytokine levels and tight junction protein expressions in the mice colon. The small intestine was relatively unaffected by the diet, suggesting that microbiota had an active role in inflammation and tight junction protein expression in the colon. Future studies may help understand the role of macronutrients and the associated changes in the microbiota in modulating the inflammatory state of the colon, and discovering alternative anti-inflammatory therapies.

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