

ORIGINAL ARTICLE

Broccoli consumption attenuates inflammation and modulates gut microbiome composition and gut integrity-related factors in mice fed with a high-fat high-cholesterol diet

Gil Zandani¹, Sarit Anavi-Cohen², Noa Sela³, Abraham Nyska⁴ and Zecharia Madar^{1*}

¹The Faculty of Agriculture, Food and Environment, The Hebrew University of Jerusalem, Rehovot, Israel; ²Peres Academic Center, Rehovot, Israel; ³Department of Plant Pathology and Weed Research, Volcani Center, Rishon LeZion, Israel; ⁴Sackler School of Medicine, Tel Aviv University, Tel Aviv, Israel

Popular scientific summary

- The effect of whole broccoli (florets and stalks) was examined in high-fat high-cholesterol diet-fed mice.
- Whole broccoli supplementation improved the glycemic response and ameliorated the liver inflammatory state.
- Broccoli consumption led to changes in microbiome composition; however, it did not affect gut barrier integrity.
- These results could provide a new perspective on the role of dietary broccoli in the development of NAFLD.

Abstract

Background: Nonalcoholic fatty-liver disease (NAFLD) is a global health problem associated with gut dysbiosis and intestinal permeability. Broccoli is a natural source of bioactive phytochemicals, characterized by health-promoting properties.

Objective: This study evaluated the effect of broccoli florets and stalks on liver fat accumulation, inflammation, gut microbiome, and intestinal barrier integrity.

Design: Male C57BL/6J mice ($n = 32$, 8-week-old) were fed with a high-fat high-cholesterol diet (HFCD) with/without 15% broccoli (florets or stalks) for 7 weeks. Liver damage was evaluated by changes in glucose response and histological and biochemical parameters. Protein and gene expressions related to liver inflammation were examined. The effect of broccoli on microbiota population together with genes related to barrier integrity in the gut was investigated.

Results: Dietary broccoli improved the glycemic response assessed by oral glucose tolerance test (OGTT). Histological evaluation showed no change in hepatic steatosis. Broccoli consumption also attenuated inflammation as revealed by lower inducible nitric oxide synthase (iNOS) and serum amyloid A1 (SAA1) expression levels in broccoli-supplemented groups. Gut microbiota analysis demonstrated elevated Acidifaciens and reduced *Mucispirillum schaedleri* abundance in the stalks group, whereas Proteobacteria strains abundance was increased in the florets group. Gut integrity remained unchanged.

Conclusion: Broccoli supplementation improves glucose tolerance, attenuates liver inflammation, and alters microbial composition, but does not affect gut integrity. This research provides new evidence on the effects of dietary broccoli under HFCD.

Keywords: *broccoli; inflammation; NAFLD; gut microbiome; barrier integrity*

To access the supplementary material, please visit the article landing page

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Nonalcoholic fatty liver disease (NAFLD) is currently the most common cause of chronic liver disease in the western world (1). NAFLD encompasses a wide spectrum of liver diseases ranging from simple steatosis through non-alcoholic steatohepatitis (NASH) to advanced fibrosis, cirrhosis, and finally hepatocellular carcinoma (2). The global prevalence of NAFLD is estimated to be 25% (1), while it is believed that 10–20% of all those with NAFLD develop NASH (3). In the progressive transition from steatosis to NASH, two mechanisms are considered to be pivotal: oxidative stress and inflammation (4).

NAFLD prevalence is positively correlated with the prevalence of type 2 diabetes (5), metabolic syndrome, and insulin resistance (4, 6). It has been suggested that bacterial translocation from gut microbiota is a new environmental factor implicated in the pathophysiology of NAFLD (7).

Currently, therapeutic options for NAFLD are limited due to the complexity of this disease and its high individual variability. Nutritional interventions are still one of the most successful strategies for managing NAFLD and NASH (8). Thus, identifying bioactive food compounds is of great interest and a major goal for preventing and treating NAFLD and NASH.

Numerous health promoting and chronic disease prevention effects are attributed to members of the brassica genus. This genus includes vegetables that have bioactive compounds, such as broccoli (9). Broccoli is low in calories and rich in vitamins, minerals, and antioxidants. Thus, this vegetable is considered highly nutritional (10–12). Harvested broccoli usually consists of florets and stalks, which comprise similar nutrient values. Yet, broccoli stalks are mostly discarded during industrial processing or by home usage (13). Epidemiological studies suggest that dietary broccoli may elicit protection against different types of cancers (11). Additionally, the efficaciousness of broccoli consumption in ameliorating several chronic diseases has been demonstrated in several clinical studies (10, 14).

Beneficial health outcomes associated with broccoli are due to its high glucosinolates content as well as their degradation products, primarily glucoraphanin and isothiocyanate sulforaphane (15). Glucoraphanin has been reported to affect lipid metabolism, gut microflora, and inflammatory factors in clinical and in vivo studies (16, 17), while sulforaphane has been shown to have antioxidant functions and can reduce oxidative stress (18). Additionally, it exerts anti-inflammatory properties and induces apoptosis (19).

Specific dietary fibers and isolated phytonutrients from broccoli have been shown to exert beneficial effects on the host's metabolism (10). However, the influences of whole broccoli have not been elucidated yet. The

present study has been undertaken to elucidate the effects of whole ('as is') dietary broccoli supplementation (florets or stalks) on metabolic parameters, inflammatory markers, gastrointestinal microbiome composition, and epithelial barrier integrity in mice fed with a high-fat high-cholesterol diet (HFCD).

Materials and methods

Broccoli analysis

Frozen broccoli florets and stalks were kindly provided by Sunfrost, Israel. Florets and stalks (15% by weight) were ground into a mash and incorporated as an addition to the experimental diets. The broccoli composition analysis is presented in Table 1.

Experimental animals and diets

Male C57BL/6J mice, 7–8 weeks old, were purchased from Harlan Laboratories (Jerusalem, Israel). All experimental protocols used in the animal experiments were performed according to the guidelines of the Authority for Biological and Biomedical Models and were approved by the Institutional Animal Care Ethics Committee of the Hebrew University of Jerusalem (AG-19-15838-3). Mice were housed under a controlled atmospheric environment (12/12 h light/dark cycle, 18–24°C, and humidity 60%) and provided with food and water at all times. Following the acclimatization period, the mice ($n = 32$) were randomly divided into four groups ($n = 8$ per group): 1) normal diet (ND), 2) high-fat (55%) + 1% (w/w) cholesterol + 0.5% (w/w) cholate (HFCD), 3) HFCD + 15% broccoli florets (HFCD+F), 4) HFCD + 15% broccoli stalks (HFCD + S) for 7 weeks. The diet compositions are presented in Table 2. All mice were allowed free access to food and water throughout the experiment. Their body weight (BW) and food intake were recorded weekly.

Oral glucose tolerance test

Glucose-loading tests were conducted in week 5 of the experimental period. The mice fasted overnight before the oral glucose tolerance test (OGTT), and then they were weighed and marked. At time 0, an initial baseline glucose measurement was taken. The mice were then given D-glucose

Table 1. Macronutrients, fiber, and moisture provided by broccoli

Compounds	100 g broccoli florets	100 g broccoli stalks
Carbohydrate (g)	4.9	3.4
Fiber (g)	3.36	2.87
Protein (g)	2.73	0.86
Fat (g)	0.36	0.33
Moisture (g)	91.34	94.99
Dry weight (g)	8.66	5.01

Table 2. Compositions of animal diets

Ingredients	Normal diet (ND)		High-fat cholesterol diet (HFCD)	
	Gr	kcal	Gr	kcal
Casein	140	560	248	992
L-Methionine	1.8	7.2	4	16
Corn starch	496	1,984	185	740
Dextrose	125	500	74	296
Sucrose	100	400	61	244
Lard	0	0	260	2,340
Soybean oil	40	360	48	432
Cellulose	50	0	50	0
Cholesterol	0	0	10	0
Cholic acid	0	0	5	0
Mineral mix	35	0	43	0
Vitamin mix	10	0	13	0
Choline chloride	2.5	0	3	0
Butylated hydroxytoluene	0.014	0	0.014	0
Total kcal	1,000	3,811	1,000	5,052

(3 g/kg BW) via gavage. Glucose levels in blood samples were obtained from tail veins and measured at 30, 60, and 120-min after glucose loading with a glucometer (handheld Optimum Xceed Glucometer, Abbott Diagnostic Care Ltd.).

Animal sacrifice and organ collection

At the end of the experiment, the mice fasted overnight, their BW was recorded, and they were sacrificed in random order by isoflurane (Minard Inc., USA) anesthesia. Blood was collected from the vena cava, centrifuged at 8,000 rpm at 4°C for 10 min, and stored at -80°C. Adipose tissue was removed, weighed, placed in liquid nitrogen, and stored at -80°C. Liver tissue was collected and weighed. A small sample from the right liver lobe was placed in 4% formaldehyde, and the remaining liver tissue was minced in liquid nitrogen and stored at -80°C. Intestines were collected, weighed, and placed in ice. The ceca were separated from the large intestines, and their contents were collected for microbiota analysis. The ceca and large intestines were rinsed in cold saline. A small piece of each was placed in 4% formaldehyde, and the remaining tissues were placed in liquid nitrogen and stored at -80°C.

Biochemical analysis of serum parameters

Liver enzymes, such as serum alanine aminotransferase (ALT) and serum aspartate aminotransferase (AST), were measured by an automated clinical chemistry analyzer along with total cholesterol, high-density lipoprotein (HDL), and total triglycerides (American Laboratories Ltd., Herzliya, Israel). Concentrations of plasma insulin were determined by a Rat/Mouse Insulin ELISA Kit (Cat #EZRMI-13K), supplied by Merck.

Automated quantification of fatty vacuoles in the liver by AI

In each liver section, five different microscopic fields (magnification $\times 600$) were selected by a board-certified pathologist, all located in the centrilobular regions. These microscopic fields were photographed with the microscope-based digital pathology system and analyzed in real-time using the artificial intelligence (AI) application for automated quantification of fatty vacuoles in the liver. The AI application (AIRA Matrix, Mumbai, India) used in this study is based on a semi-automated algorithm.

Liver and intestine histology examination

Histological slides were prepared by Patholab (Rehovot, Israel). Livers and colons were macro-dissected, placed in plastic cassettes, and dehydrated. The dehydrated samples were embedded in paraffin blocks by an automatic apparatus. Serial sections 3–5 μm thick were cut from each block, placed on glass slides, stained with hematoxylin and eosin (H&E), and covered by an automatic apparatus. The histopathological examinations were performed by Dr. Abraham Nyska, DVM, Dipl. ECVP, Fellow IATP, board certified in toxicologic pathology – <https://ebvs.eu/colleges/ECVP/members/prof-abraham-nyska>. Histopathological changes were described and scored by the study pathologist, using semiquantitative grading of five grades (0–4), taking into consideration the severity of the changes. The scoring reflects the predominant degree of the specific lesion seen in the entire field of the histology section. A generic grading criterium was used (20): zero (0) = no lesion; 1 = minimal change; 2 = mild change; 3 = moderate change; and 4 = marked change.

Hepatic liver extraction

Quantification of total lipids in the liver was carried out using the Folch method (21). Frozen liver tissue (100 mg) was homogenized using 700 μL methanol. Then, 1,400 μL of chloroform was added (2:1, v/v) and lightly shaken overnight to separate the two phases. On the next day, the vials were centrifuged at 3,000 rpm for 10 min at room temperature. The upper aqueous phase was removed, and the lower organic phase (containing the lipids) was transferred to a clean, previously weighed tube. The samples were evaporated until complete dryness, measured to determine the lipid fraction weight, and normalized to initial tissue sample weight.

Western blot analysis

Liver tissues were lysed with lysis buffer containing 20 mM Tris-HCl (pH 7.4), 145 mM NaCl, 10% glycerol, 5 mM Ethylenediaminetetraacetic acid (EDTA), 1% Triton X-100, 0.5% NP-40, 100 mM phenylmethylsulfonyl fluoride (PMSF), 200 mM NaVO_4 , 5 mM NaF, and 1% protease inhibitor cocktail. Lysates were centrifuged at 14,000 rpm for 15 min, and the protein concentration was determined by the Bradford method using bovine

serum albumin as a standard. The samples were then separated by SDS-PAGE gel (10%) and transferred to nitrocellulose membranes. Blots were incubated with dilutions of the primary antibody inducible nitric oxide synthase (iNOS) 1:1,000 (bd610432 BD-Biosciences) at 4°C overnight. After several washes, the membranes were incubated using a secondary goat antibody (Jackson Immuno-Research Laboratories, West Grove, PA, USA). The immune reaction was detected by enhanced chemiluminescence, with bands being quantified by densitometry and expressed as arbitrary units. β -Actin was used as a control protein.

Quantitative real-time polymerase chain reaction

Total RNA was isolated from liver and colon tissues by using Tri-Reagent (Sigma-Aldrich, Rehovot, Israel), according to the manufacturer's protocol. Complementary DNA was prepared using the High-Capacity cDNA Reverse Transcription Kit (Quanta BioSciences, Gaithersburg, MD, USA). Real-time polymerase chain reaction (PCR) was performed using the 7300 Real-Time PCR System (Applied Biosystems, Foster City, CA, USA), with specific primers. Quantitative changes in gene expression were determined by normalizing to 18S. The primer sequences are listed in Table 3.

Gut microbiota analysis

The effects of each diet on the bacterial population in the gut microbiome were examined by analyzing the prokaryotic 16S ribosomal RNA gene (16S rRNA), which is approximately 1,500 bp long and contains nine variable regions interspersed among conserved regions. These variable regions were subjected to phylogenetic classification according to genus or species, in diverse microbial populations.

The following protocol describes a two-step PCR-based method for preparing samples for sequencing the variable V3 and V4 regions of the 16S rRNA gene. Bacterial DNA was extracted from fecal samples by using DNeasy Powersoil kit (Qiagen), according to the manufacturer's instructions. Each sample was then quantified using a Qubit 2.0 Fluorometer (ThermoFisher Scientific, Waltham, MA, USA) and diluted to a final concentration of 5 ng μ L⁻¹ in 10 mM Tris at pH 8.5. The 16S library preparation was carried out as described in Illumina's 16S sample preparation guide with minor modifications. PrimeStar HS DNA Polymerase Premix (Takara-Clontech, Mountain View, CA, USA) was used instead of the PCR enzyme. Sequences with 97% similarity were assigned to the same operational taxonomic units (OTUs). OTUs of representative sequences at a similarity of 97% and their relative abundances were used to calculate and analyze rarefaction curves. Bacterial richness and diversity within samples were classified by α diversity (Pielou's index, observed-species indices, and Shannon index).

Statistical analysis

Results are presented as mean \pm standard error of mean (SEM). Data were analyzed using the JMP 14 Pro software suites (SAS Institute, Cary, NC, USA). Comparisons between groups were made by one-way analysis of variance (ANOVA), followed by a Tukey–Kramer test or by a unpaired two-tailed Student's *t*-test. Statistical significance was defined at $P < 0.05$.

Results

Dietary broccoli did not affect BW, food intake, or tissues weight

Table 4 summarizes the changes in BW, daily food intake, and adipose and liver tissue weight in each group. There

Table 3. Primers sequences

Name	Reverse	Forward
18s	5'-CCTCAGTTCCGAAAACCAAC-3'	5'-ACCGCAGCTAGGAATAATGG-3'
iNOS	5'-TCTCTGCTCTCAGCTCCAAG-3'	5'-AGCTCCCTCCTTCTCCTTCT-3'
SAA-1	5'-GGTCAGCAATGGTGTCTCA-3'	5'-GATGAAGCTACTCACCAGCCT-3'
AdipoR1	5'-CACATCTACGGGATGACTCTCCA-3'	5'-AGTTCATGTATAAGGTCTGGGAGG-3'
AdipoR2	5'-CATGATGGGAATGTAGGAGC-3'	5'-TTCTATTATGAAAATAGCCCGGA-3'
ZO-1	ACTCCCACTTCCCCAAAAC-3'-5'	5'-CCACAGCTGAAGGACTCACA-3'
Occludin	5'-GTCCGTGAGGCCTTTTGA-3'	5'-GGTGCATAATGATTGGGTTTG-3'
JAM-A	5'-TGATCTTTGACCCCGTGAC-3'	5'-ACCAGACGCCAAAATCAAG-3'
E-cadherin	5'-ATCCTCGCCCTGCTGATT-3'	5'-ACCACCGTTCTCCTCCGTA-3'
Claudin-2	5'-TGAACACGGACCACTGAAAG-3'	5'-TTAGCAGGAAGCTGGGTCAG-3'
Muc1	5'-CTGTTCAACCACCACCATGAC-3'	5'-CTTGAAGGGCAAGAAAACC-3'
Muc2	5'-CAACAAGCTTCAACCACAATCTC-3'	5'-CAGACCAAAAAGCAGCAAGGTA-3'
Reg3 γ	5'-ACCATCACCATCATGTCCTG-3'	5'-GGCATCTTTCTTGCCAACCTT-3'
IgA	5'-CGTCCAAGAATTGGATGTGA-3'	5'-AGTGACAGGCTGGGATGG-3'

Table 4. Effects on body weight, food intake, and tissues weight

Parameters	Group			
	ND	HFCD	HFCD+F	HFCD+S
Initial body weight (g)	20.02 ± 0.29 ^a	19.87 ± 0.39 ^a	19.92 ± 0.37 ^a	19.95 ± 0.29 ^a
Final body weight (g)	28.24 ± 0.58 ^a	25.11 ± 0.64 ^b	25.15 ± 1.13 ^b	24.21 ± 0.38 ^b
Food intake (g/day)	3.39 ± 0.03 ^a	2.85 ± 0.63 ^b	2.95 ± 0.52 ^b	2.72 ± 0.22 ^b
Adipose tissue weight (g)	0.75 ± 0.04 ^a	0.48 ± 0.06 ^b	0.57 ± 0.09 ^b	0.40 ± 0.04 ^b
Liver tissue weight (g)	0.95 ± 0.02 ^b	1.21 ± 0.06 ^a	1.36 ± 0.10 ^a	1.17 ± 0.04 ^{ab}
Liver/body weight ratio	0.04 ^a	0.05 ^b	0.05 ^b	0.05 ^b

Male C57BL/6J mice were fed with normal diet (ND), high-fat cholesterol diet (HFCD), high-fat cholesterol diet + 15% broccoli florets (HFCD+F), and high-fat cholesterol diet + 15% broccoli stalks (HFCD+S) for 7 weeks. Values are expressed as mean ± SEM (*n* = 8). Means without a common letter are statistically different (*P* < 0.05).

Table 5. Effects on serum lipid profile and liver enzymes

Parameters	Group			
	ND	HFCD	HFCD+F	HFCD+S
Lipid profile				
Total triglycerides (mg/dL)	188.14 ± 15.81 ^a	36.57 ± 3.47 ^b	39.50 ± 1.63 ^b	42.57 ± 3.88 ^b
Total cholesterol (mg/dL)	145.57 ± 6.58 ^a	147.57 ± 8.73 ^a	142.71 ± 3.34 ^a	146.00 ± 3.39 ^a
HDL (mg/dL)	119.03 ± 3.94 ^a	76.79 ± 1.38 ^b	74.49 ± 3.78 ^b	76.24 ± 4.48 ^b
Liver enzymes				
AST (IU/L)	45.00 ± 2.41 ^c	89.71 ± 6.96 ^{bc}	172.86 ± 25.19 ^a	111.71 ± 11.66 ^{ab}
ALT (IU/L)	19.86 ± 1.82 ^c	68.67 ± 5.37 ^{bc}	187.00 ± 38.88 ^a	124.00 ± 18.83 ^{ab}

Male C57BL/6J mice were fed with normal diet (ND), high-fat cholesterol diet (HFCD), high-fat cholesterol diet + 15% broccoli florets (HFCD+F), high-fat cholesterol diet + 15% broccoli stalks (HFCD+S) for 7 weeks. Values are expressed as mean ± SEM (*n* = 8). Means without a common letter are statistically different (*P* < 0.05).

were no differences in final body weight and in food intake between the HFCD-fed groups. Unexpectedly, ND-fed mice exhibited the highest final BW of all groups, which was in accordance with the increased food intake observed by this group. A similar pattern was found in adipose tissue weight, which was similar among HFCD-fed mice but significantly highest in the ND mice. Conversely, compared with ND, liver tissue weight was significantly elevated in the HFCD and HFCD+F groups but did not reach statistical significance in the HFCD+S group. Given the observed changes in BW, the ratio between liver and BW was measured. Liver/BW ratio was statistically higher in all HFCD groups compared with the ND group.

Broccoli supplementation did not change serum lipid profile but elevated liver enzymes

Serum triglycerides and HDL levels were significantly elevated in the ND group but were comparable in all HFCD groups (Table 5). There was no significant change in total serum cholesterol in all groups. Serum AST levels significantly increased in the HFCD+F group compared with ND and HFCD groups and were only mildly elevated in

the HFCD+S group. Serum ALT showed the same tendency as AST in all the experimental groups.

Broccoli intake improves glucose tolerance

A glucose tolerance test was conducted after 5 weeks of the feeding regime. The area under the curve (AUC) was calculated as a measure of the overall glycemic response. The AUC was significantly attenuated in the broccoli-fed groups compared with ND and HFCD groups (Fig. 1B). In comparison to the ND group, fasting blood glucose and insulin levels were higher in the HFCD+F group, while only insulin was significantly elevated in the HFCD and HFCD+S groups. However, the addition of broccoli stalks to HFCD ameliorated the increase in serum insulin levels observed in this group (Fig. 1C, D).

Broccoli consumption did not affect the liver lipid content

Further examination addressed the ability of dietary broccoli to affect hepatic lipid accumulation. Whereas the HFCD significantly increased hepatic lipid content, the addition of broccoli to this diet statistically mitigated this effect. However, physiologically, this

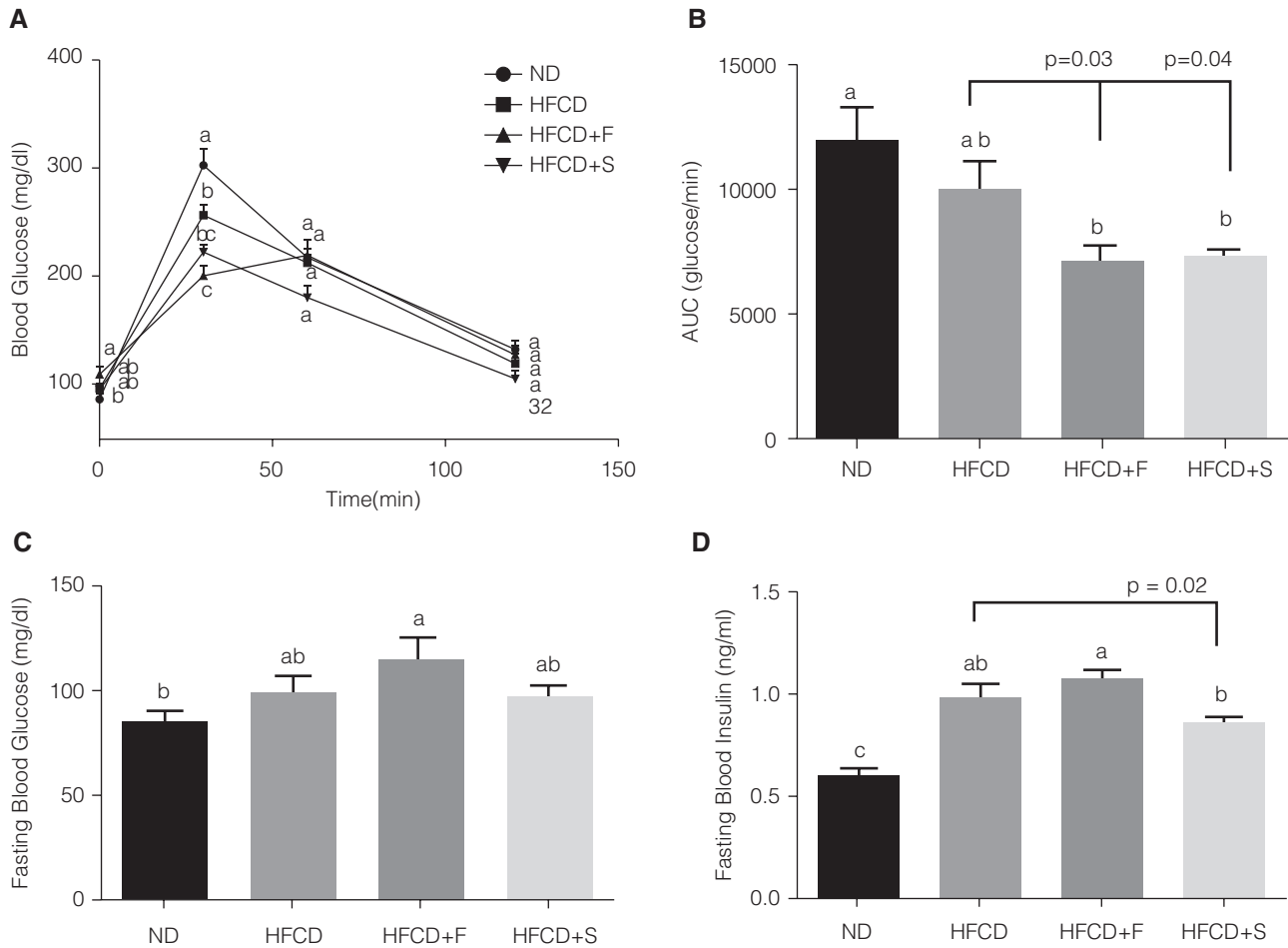


Fig. 1. Effects of broccoli intake on glucose homeostasis. Male C57BL/6J mice were fed with normal diet (ND), high-fat cholesterol diet (HFCD), high-fat cholesterol diet + 15% broccoli florets (HFCD+F), high-fat cholesterol diet + 15% broccoli stalks (HFCD+S) for 7 weeks. An oral glucose tolerance test for 120 min was performed at week 5 (A). Glucose tolerance test was measured as the area under the curve (AUC) (B). Mean fasting blood glucose concentration at sacrifice (C) and insulin serum levels at sacrifice (D). Values are expressed as mean \pm SEM ($n = 7-8$). Means without a common letter are statistically different ($P < 0.05$).

reduction was minuscule and did not markedly affect liver steatosis (Supplementary Fig. 1A). These findings were corroborated by liver histology using H&E stain and AI. Results showed no significant effect for broccoli addition to HFCD on liver lipid accumulation (Supplementary Fig. 1B and Supplementary Table 1).

Dietary broccoli affects adiponectin receptors

The expression of adiponectin receptors was determined. Adiponectin receptor 1 (AdipoR1) mRNA levels were not significantly altered in all HFCD-fed groups compared with ND (Fig. 2A). In contrast, the adiponectin receptor 2 (AdipoR2) expression was markedly greater in the group supplemented with broccoli stalks compared with the HFCD+F group and tended to increase compared with the HFCD group (Fig. 2B).

Broccoli supplementation decreased inflammatory markers in the liver

The effects of broccoli addition to HFCD on liver inflammation were investigated. HFCD significantly upregulated iNOS mRNA and protein expression levels. However, this increase was blunted by the addition of broccoli to this diet (Fig. 3A). Changes in iNOS levels were paralleled by similar fluctuations in the serum amyloid A1 (SAA1) gene expression in all the experimental groups (Fig. 3B, C).

Dietary broccoli did not impact microbiota richness and diversity indexes

To evaluate the differences within the samples, alpha diversity indexes were calculated. The Shannon index represents community diversity and richness. No significant differences were noticed among HFCD groups compared with the ND group (Table 6). OUTs, which represent the rare species in each group, and Pielou's index, which

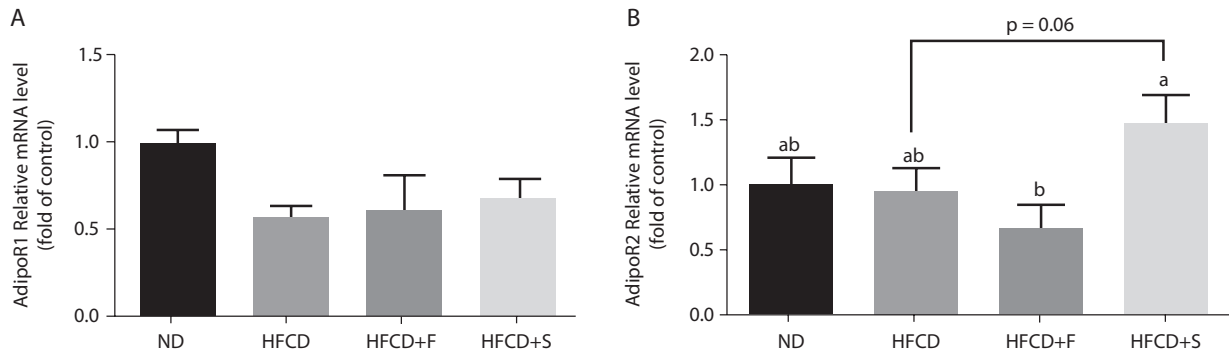


Fig. 2. The effect of broccoli supplementation on liver adiponectin receptors. Male C57BL/6J mice were fed with normal diet (ND), high-fat cholesterol diet (HFCD), high-fat cholesterol diet + 15% broccoli florets (HFCD+F), high-fat cholesterol diet + 15% broccoli stalks (HFCD+S) for 7 weeks. Gene expressions of AdipoR1 (A) and AdipoR2 (B) were measured. Values are expressed as mean \pm SEM. Means without a common letter are statistically different ($P < 0.05$).

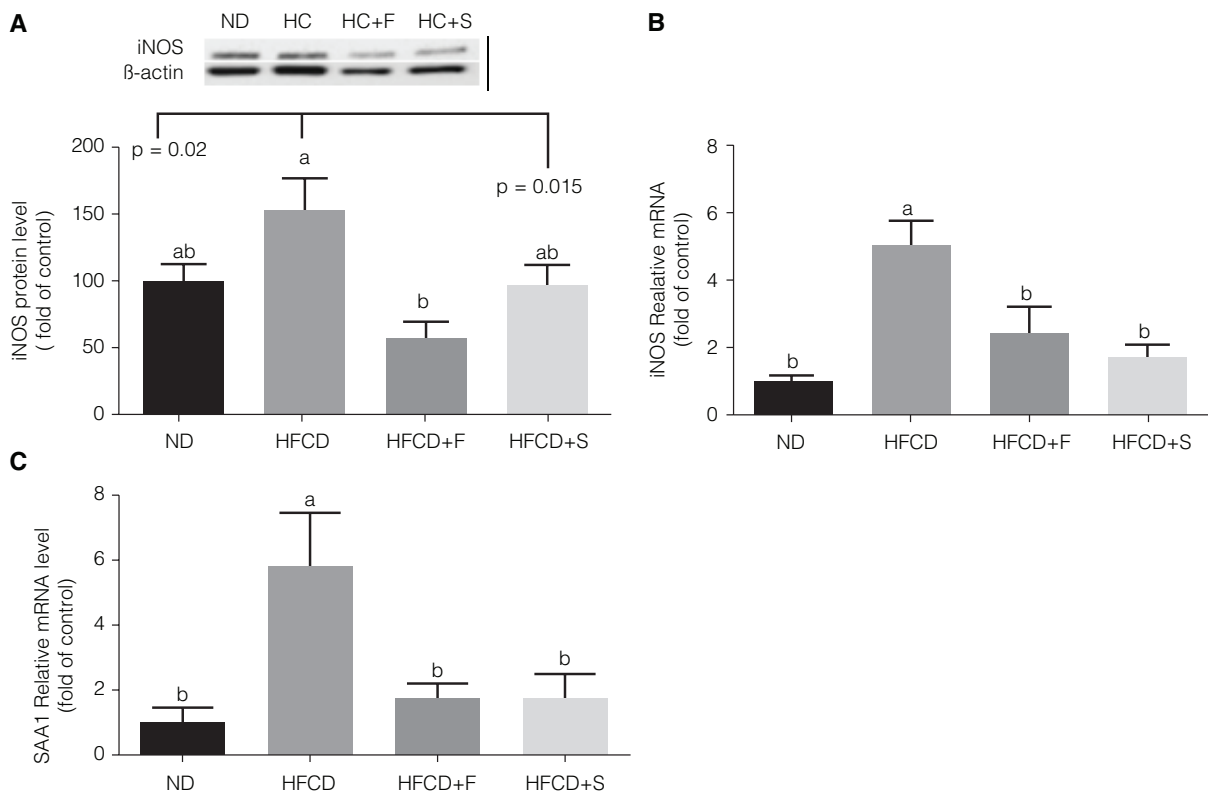


Fig. 3. Effects of broccoli supplementation on inflammatory markers in the liver. Male C57BL/6J mice were fed with normal diet (ND), high-fat cholesterol diet (HFCD), high-fat cholesterol diet + 15% broccoli florets (HFCD+F), high-fat cholesterol diet + 15% broccoli stalks (HFCD+S) for 7 weeks. Protein and gene expressions of iNOS (A+B) and SAA1 gene (C) were examined. Values are expressed as mean \pm SEM. Means without a common letter are statistically different ($P < 0.05$).

indicates species evenness, were substantially diminished by the HFCD consumption, irrespective of broccoli supplementation (Table 6).

Dietary broccoli impacts on mice bacterial microbiome

Microbiota composition was comprehensively characterized at all taxonomic levels. At the phylum level,

Firmicutes and Cyanobacteria abundances were lower in all HFCD groups compared with the ND group (Fig. 4A). Deferribacteres abundance was markedly attenuated by broccoli stalks added to HFCD (Fig. 5A). Proteobacteria abundance was significantly raised in the group supplemented with broccoli florets compared with HFCD and ND groups (Fig. 4A). The Bacteroidetes/Firmicutes

Table 6. Dietary broccoli impact on microbiota richness and diversity

Diversity	ND	HFCD	HFCD+F	HFCD+S
Shannon index	5.65 ± 0.05 ^a	4.57 ± 0.27 ^b	4.72 ± 0.07 ^b	4.83 ± 0.11 ^b
Observed OUTs	134.25 ± 2.63 ^a	78.75 ± 13.86 ^b	85.40 ± 2.44 ^b	86.25 ± 4.79 ^b
Pielou's index	0.8 ^a	0.72 ± 0.02 ^b	0.73 ± 0.01 ^b	0.75 ± 0.01 ^b

Male C57BL/6J mice were fed with normal diet (ND), high-fat cholesterol diet (HFCD), high-fat cholesterol diet + 15% broccoli florets (HFCD+F), high-fat cholesterol diet + 15% broccoli stalks (HFCD+S) for 7 weeks. Values are expressed as mean ± SEM (n = 5). Means without a common letter are statistically different (P < 0.05).

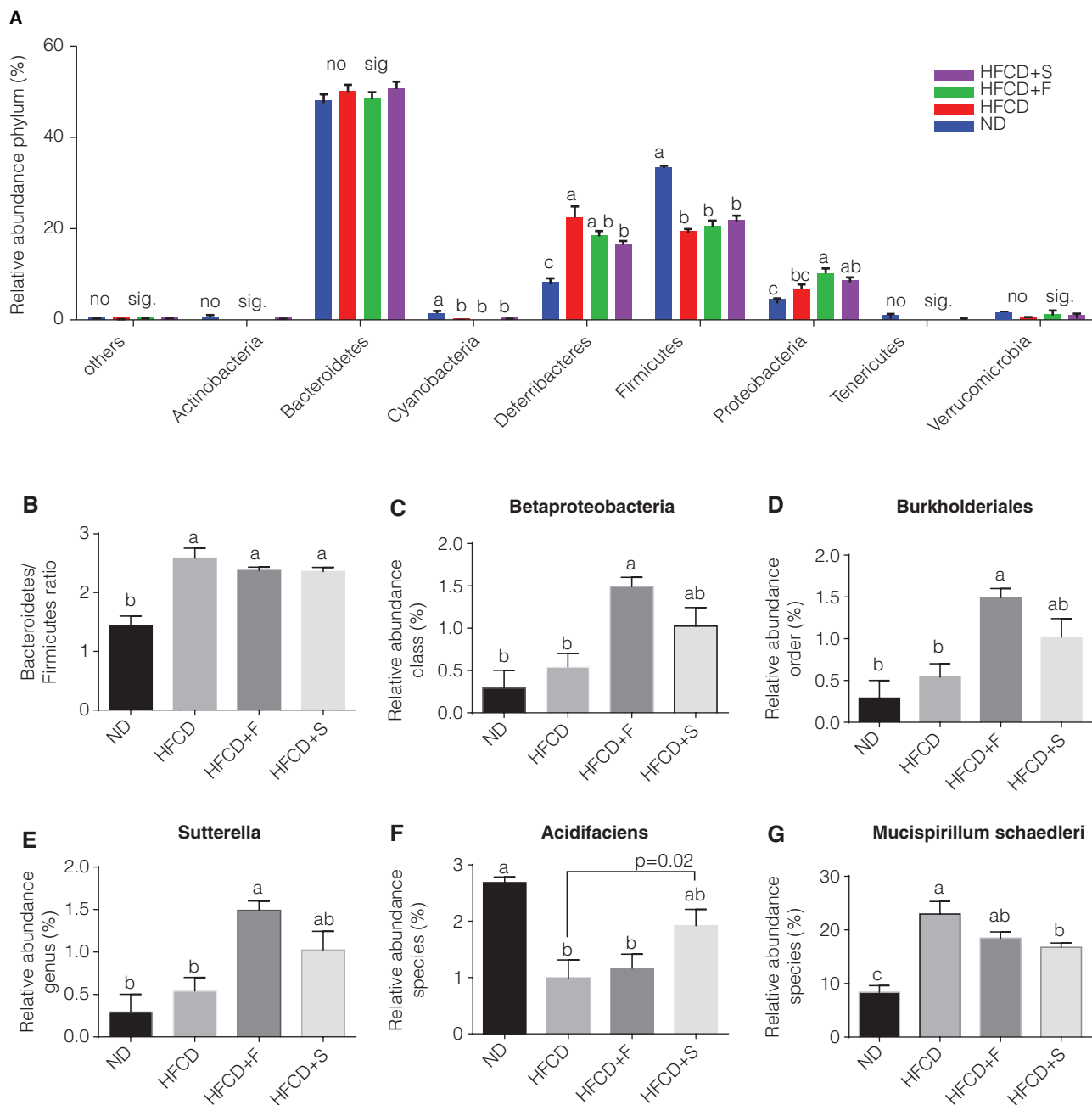


Fig. 4. The effect of broccoli supplementation on gut microbiota composition evaluated at all taxonomic levels. Male C57BL/6J mice were fed with normal diet (ND), high-fat cholesterol diet (HFCD), high-fat cholesterol diet + 15% broccoli florets (HFCD+F), high-fat cholesterol diet + 15% broccoli stalks (HFCD+S) for 7 weeks. Phylum level abundance (A), Bacteroidetes/Firmicutes ratio (B), class level abundance (C), order level abundance (D), genus level abundance (E), and species level abundance (F and G). Values are expressed as mean ± SEM (n = 5). Means without a common letter are statistically different (P < 0.05).

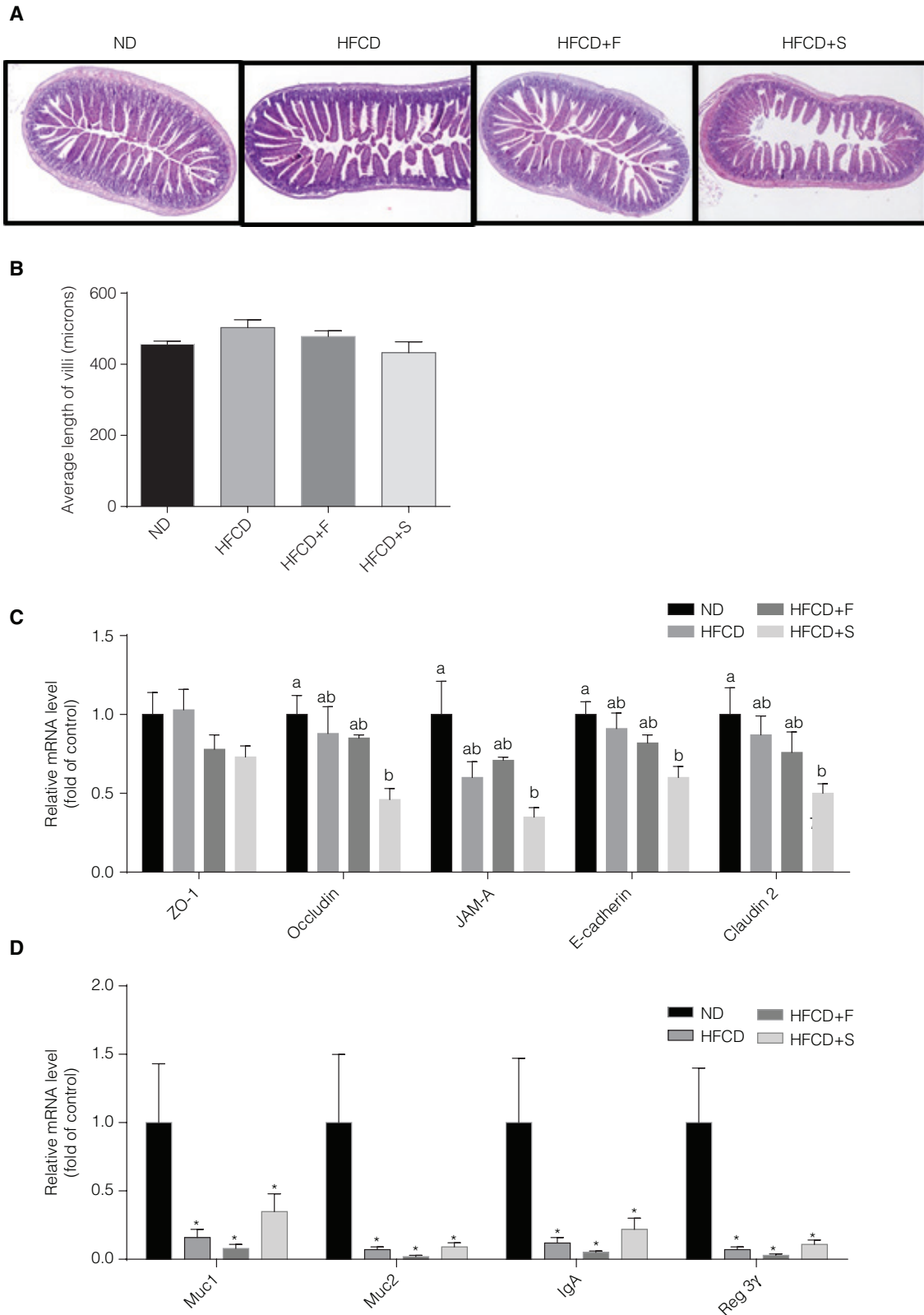


Fig. 5. The effect of broccoli supplementation on colon histology and genes related to intestinal barrier integrity. Male C57BL/6J mice were fed with normal diet (ND), high-fat cholesterol diet (HFD), high-fat cholesterol diet + 15% broccoli florets (HFCD+F), high-fat cholesterol diet + 15% broccoli stalks (HFCD+S) for 7 weeks. Colon histology (A), colon crypt length (the length of 3 villi was measured in each section) (B), and genes related to intestinal barrier integrity (C + D). Values are expressed as mean \pm SEM ($n = 5$). Means without a common letter are statistically different ($P < 0.05$). *Versus ND group.

ratio was decreased in the ND group compared with all HFCD groups (Fig. 4B). At the class level, Betaproteobacteria abundance was significantly elevated in mice fed with broccoli florets compared with HFCD and ND groups (Fig. 4C). At the order level, Burkholderiales abundance was significantly increased in the HFCD+F group (Fig. 4D). At the genus level, a significant increase in *Sutterella* abundance was observed in the florets-supplemented group (Fig. 4E). At the species level, a decrease in the abundance of *Mucispirillum schaedleri* was registered in the HFCD+S group compared with the HFCD group, while the *Acidifaciens* community was elevated compared with the HFCD group (Fig. 4F, G).

Dietary broccoli effects on colon histology and integrity

A histopathological evaluation of the colon did not show any alterations by HFCD or its combination with broccoli. Measurements of the colon crypt length also remained unchanged by the experimental diets (Fig. 5A, B). The expression of key proteins that govern tight junction permeability was assessed, namely, zonula occludens-1 (ZO-1), occludin, junctional adhesion molecule A (JAM-A), epithelial cadherin (E-cadherin), and claudin-2. Except for ZO-1 gene expression that showed no significant difference, all tested genes exhibited a higher expression level in the ND group compared with the HFCD+S group. Although no significant difference was observed between HFCD-fed groups, there was an almost uniform pattern of decrease between the HFCD and HFCD+S groups (Fig. 5C). The ZO-1 gene expression tended to be reduced by broccoli addition, regardless of the diet lipid content. The examination of mucin-1 (Muc1), mucin-2 (Muc2), regenerating islet-derived protein 3 γ (Reg3 γ), and immunoglobulin A (IgA) expression demonstrated higher levels in the ND group compared with all HFCD-fed groups (Fig. 5D).

Discussion

HFCD has been shown to effectively promote liver fat accumulation and, as such, is an animal model for NAFLD with hallmarks of steatosis and inflammation detected within 6 weeks (22). The present study investigated the effects of dietary broccoli supplementation to HFCD for 7 weeks. In agreement with previous data, our results demonstrate that the HFCD consumption leads to lower body and adipose tissue weight along with reduced serum triglycerides levels while increasing liver lipids accumulation (23).

Hepatocyte steatosis is the main histological manifestation of NAFLD with NASH being additionally characterized by the addition of intralobular inflammation (24). Although lipid accumulation was greater in the HFCD group, liver histological evaluation and AI examination found a comparable increase in liver steatosis

in all HFCD-fed groups. These results further indicate the coexistence of hepatic inflammation in HFCD and HFCD+F groups compared with ND. Although the histological evaluation indicated that dietary broccoli enrichment did not affect the steatosis grade, plasma levels of the liver enzymes, AST and ALT, were significantly higher in the broccoli-florets-supplemented group. Elevated ALT and AST levels are usually believed to reflect nonspecific hepatocellular damage (25). However, Contreras-Zentella et al. (26) reported that a drastic increase in liver transaminase activity in the serum does not necessarily reflect liver cell death. Alternatively, lower-free thyroid hormones may be implicated in the exacerbation of liver injury observed in the broccoli-added groups. Cruciferous vegetables, like broccoli, naturally contain a high concentration of glucosinolates, of which some are considered to be goitrogens. Therefore, augmented consumption of broccoli in the present study may have compromised the thyroid gland's function and subsequently elicited a negative effect on the liver (27). However, it is important to emphasize that broccoli, particularly if cooked, does not constitute much of a threat to thyroid hormones synthesis in humans (28).

The liver inflammatory state appears to be ameliorated by adding broccoli to HFCD. Among other stressors, the iNOS expression is known to be markedly induced by inflammatory stimuli and to play a pivotal role in the pathophysiology of inflammatory-related diseases (29). iNOS was significantly upregulated in HFCD-fed mice, indicating the existence of inflammation in this organ. This is corroborated by the increased expression of SAA1, an acute inflammatory response factor, in the liver by HFCD. The addition of dietary broccoli to HFCD resulted in a significant reduction in these inflammatory markers. These results are in agreement with previous *in vitro* studies performed with broccoli floret extract or isolated sulforaphane from broccoli (30–32).

Impaired glucose regulation is very common in NAFLD patients (33). In the present study, dietary broccoli consumption facilitated glucose tolerance, as revealed by OGTT. The ability of broccoli addition to ameliorate the glycemic response has been previously shown. Axelson et al. (34) showed sulforaphane administration, and broccoli extract could separately improve hepatic glucose metabolism in rodents under HFD as well as in type 2 diabetic patients. This effect might be attributed to natural dietary fibers, which are known to improve glucose tolerance to achieve adequate glycemic control (35). The observed reduction in serum insulin levels along with the trend of elevated AdipoR2 expression with broccoli stalk feeding reinforces this notion. AdipoR2 is most abundant in the liver, even though both adiponectin receptors regulate insulin sensitivity in insulin targeted tissues and are important in the pathophysiology of insulin resistance (36). Savard et al. (37) reported 30 weeks of HFCD led

to a reduction in adiponectin sensitivity. Thus, it can be inferred that short-term exposure to HFCD, like in the present experiment, is not severe enough to cause considerable disruptions in adiponectin sensitivity.

Recent evidence indicates that metabolites generated by intestinal microbiota play an essential role in NAFLD etiology (38). Dietary fibers preserve gut integrity and host physiology, interact directly with gut microbes, and lead to the production of key metabolites (39). In the present study, a shift in gut microbiome profile, indeed, occurred due to HFCD feedings, while alpha diversity indexes remained unaffected. Changes in Bacteroidetes/Firmicutes ratio were noticed, with all HFCD-fed groups presenting a higher ratio compared with the ND group. Our results are similar to previous studies showing elevated Bacteroidetes/Firmicutes ratio in NASH patients and animal models (40–43).

Proteobacteria abundance was significantly augmented by the addition of broccoli florets to HFCD. Proteobacteria were proposed to represent a possible microbial signature of disease (44). However, high abundance is also observed in non-disease conditions, thus discouraging its use as a surrogate marker for existence pathology. This phylum has been acknowledged as the most unstable over time in the gut microbiota. It is possible that Proteobacteria act as front-line responders, which are highly sensitive to the host's diet and environmental factors (45). The role of Proteobacteria in gut inflammation and metabolic disorders has also been described (44). However, this phylum consists of several bacterial classes, whose division is related to different conditions (45). Gammaproteobacteria were reported to be more abundant in NAFLD patients (44). Conversely, an increase in the abundance of Betaproteobacteria strains (Burkholderiales, Sutterella), as observed in the present study, was found in genetically obese mice (ob/ob mice) compared with same genetic background mice supplemented with prebiotics (46).

Reduction in *Mucispirillum schaedleri* (Deferribacteres phylum) abundance in the stalks group was detected. High microbial dominance of *Mucispirillum* was found to be associated with proinflammatory responses in the gut (47). More research is needed to comprehensively define its role during inflammation and diseases (48). The Acidifaciens (Bacteroidetes phylum) community was elevated in the stalk group compared with the HFCD group. Yang et al. (49) demonstrated that Acidifaciens feeding improved hepatic and peripheral insulin sensitivity and glucose tolerance. These findings give rise to the possibility that this species may be beneficial in metabolic diseases prevention, such as type 2 diabetes and obesity.

Recent data imply that changes in intestinal microbiota composition can contribute to the pathogenesis of liver diseases through the 'gut-liver' axis. According to this

theory, disruption of the intestinal barrier integrity leads to pathological bacterial translocation and subsequently the initiation of an inflammatory response in the liver (50). Our study further evaluated the dietary effects on gut barrier integrity. Occludin, JAM-A, E-cadherin, and claudin-2 represent key apical junctional complex components that function to maintain epithelial barrier integrity (51, 52). The expression levels of these factors decreased in the combination of HFCD and broccoli stalks. This result is in contrast to the absence of inflammation in the livers of mice in this group. Thus, reduced expression in this group did not necessarily correlate with changes in gut permeability.

Mucins are glycoproteins expressed in various epithelial cell types like the colon. Membrane-bound MUC1 is involved in cell signaling, immuno-regulation, and the inhibition of cell-cell and cell-matrix adhesions, while the gel-forming MUC2 produces the mucus that protects and lubricates epithelial surfaces (53). ND presented higher expression of Muc1 and Muc2 compared with all HFCD groups. The antimicrobial peptides Reg3 γ (54) and IgA, a non-inflammatory antibody specialized in mucosal protection (55), presented higher expression in the ND group compared with all HFCD groups. Taken together, these data suggest that the HFCD consumption compromises epithelial barrier permeability and/or the intestinal inflammatory state. The addition of broccoli to this diet failed to prevent or restore adequate intestinal function.

Conclusion

Under the setting of HFCD, the addition of dietary broccoli led to a reduction in the hepatic inflammatory state and consequent damage, and further improved glucose tolerance. Although broccoli florets increased intestinal Proteobacteria strains abundance, no harmful effect was observed. This study reveals preliminary information on the effects of broccoli stalks consumption. Broccoli stalks increased insulin sensitivity, which was accompanied by changes in microbiome composition toward species known to contribute to this metabolic effect. Further comprehensive research is needed to further characterize the effects of dietary broccoli during NAFLD and to identify the underlying mechanisms responsible for these effects.

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*Zecharia Madar

Institute of Biochemistry
 Food Science and Nutrition
 Robert H. Smith Faculty of Agriculture, Food and Environment
 The Hebrew University of Jerusalem
 Rehovot
 Israel
 Email: zecharia.madar@mail.huji.ac.il