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Dietary Antioxidants and Intestinal Barrier Protection During Inflammation

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SUMMARY

The intestinal barrier helps the body absorb nutrients while protecting it from harmful substances. Inflammation can damage this barrier by weakening the connections between intestinal cells. We hypothesized that digested spinach would better protect intestinal barrier function during inflammation than digested bread, particularly when applied before inflammatory damage occurred. Spinach and bread were first passed through a laboratory digestion process that simulated the mouth, stomach, and small intestine. Their antioxidant capacities were measured before and after digestion, with vitamin E used as a positive control. The digested food samples were then added to Caco-2 intestinal cell layers either before or after inflammation was induced. Barrier health was measured using TEER, ZO-1 staining, and scanning electron microscopy. Spinach had a higher antioxidant capacity than bread, even after digestion. Cells treated with spinach before inflammation maintained stronger barrier function, more organized tight junctions, and better-preserved surface structures than cells treated with bread. Spinach added after inflammation was less effective. These results suggest that digested spinach may help protect the intestinal barrier during inflammation, especially when it is present before damage begins. However, spinach contains many nutrients and natural compounds, so the protective effects cannot be linked to antioxidants alone.

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INTRODUCTION

Antioxidants are compounds that can reduce damage caused by unstable molecules called free radicals (1). Many fruits, vegetables, and other foods contain antioxidants, but not all of these compounds are immediately available to the body. Before nutrients can interact with intestinal cells, they must first be released from the structure of the food during digestion. The portion that is released and becomes available for absorption is called the bioaccessible fraction (2). This is important for whole foods such as spinach and bread because their fibers, proteins, starches, and fats may affect how much antioxidant material is released. Therefore, understanding antioxidant activity in foods requires consideration not only of the orginal food, but also of how digestion changes the compounds that become available to the intestinal lining.

To study these changes, researchers often use the INFOGEST 2.0 in vitro digestion method (3). This standardized method simulates the oral, gastric, and intestinal phases of digestion using controlled pH values, digestive enzymes, and incubation times. Studies using simulated digestion have shown that the release of antioxidant compounds depends on the food (4, 5). For example, digestion and food processing can change the amount of carotenoids, flavonoids, and phenolic compounds released from spinach. Bread also contains antioxidant compounds, but their release can depend on the type of flour and the way the bread is processed or fermented. Therefore, digestion may decrease antioxidant capacity in some foods but increase the release of certain compounds in others. One method used to quantify these changes is the oxygen radical absorbance capacity, or ORAC, assay (6). This test measures how well a sample protects against oxidation and compares its activity with Trolox, a vitamin E-like antioxidant standard. Vitamin E is a well-known antioxidant and can therefore be used as a positive control to confirm that the ORAC assay is working properly (7) Importantly, antioxidant capacity measured before digestion may not represent what intestinal cells are actually exposed to after food passes through the digestive system.

The intestinal epithelium forms a barrier between material inside the digestive tract and the rest of the body. Tight junction proteins, including ZO-1, occludin, and claudins, help connect neighboring intestinal cells and control what can pass between them. During inflammation, these tight junctions may become damaged, causing the intestinal barrier to become more permeable. Previous studies using Caco-2 intestinal cell models have shown that vitamin E, flavonoids, and antioxidant-rich food extracts can help maintain transepithelial electrical resistance, or TEER, and preserve tight junction organization during inflammatory stress (9, 10). However, many previous studies have tested isolated antioxidant compounds or food extracts rather than digested whole

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foods (11, 12). Fewer studies have directly compared digested whole foods and connected their antioxidant capacity after digestion with their effects on intestinal barrier function. It also remains unclear whether exposure to food-derived compounds before inflammatory injury is more protective than treatment after barrier damage has already occurred.

In this study, we compared spinach and bread to determine how digestion affected antioxidant capacity and whether their digesta differently influenced intestinal barrier function during inflammation. We hypothesized that spinach would retain greater antioxidant capacity after digestion than bread, that spinach digesta would better preserve intestinal barrier integrity during inflammatory stress, and that preventive treatment before inflammation would be more effective than treatment after inflammation. We found that spinach had greater antioxidant capacity than bread both before and after digestion, although digestion reduced antioxidant capacity. Spinach digesta, particularly when we applied it before inflammation, better preserved TEER, ZO-1 organization, and epithelial surface structure than bread digesta or treatment initiated after inflammation. Together, these results suggested that digestion-derived components of spinach helped protect intestinal barrier integrity during inflammatory stress and that the timing of food exposure influenced the degree of protection. Because whole-food digesta contained many nutrients and phytochemicals, our results did not establish that antioxidant activity alone caused the protective effects.

RESULTS

Antioxidant capacity decreased after digestion

We first digested spinach and bread using the INFOGEST 2.0 protocol, as described in the Materials and Methods section and Figure 1. We then evaluated the antioxidant capacities of vitamin E, spinach, and bread before and after digestion to determine how digestion affected antioxidant activity (Figure 2). We used vitamin E as a positive control because of its known antioxidant activity. As expected, fresh vitamin E showed the highest antioxidant capacity, with a mean value of approximately 390 µmol Trolox equivalents per gram (µmol TE/g). After digestion, its antioxidant capacity decreased to approximately 190 µmol TE/g. Among the food samples, fresh spinach showed a higher antioxidant capacity than fresh bread. The antioxidant capacity of spinach decreased from approximately 18 µmol TE/g before digestion to approximately 13 µmol TE/g after digestion. Bread showed the lowest antioxidant capacity, decreasing from approximately 6 µmol TE/g before digestion to approximately 5 µmol TE/g after digestion. Overall, both spinach and bread showed lower antioxidant capacity after digestion, although the decrease was greater for spinach than for bread.

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Spinach digesta better preserved intestinal barrier integrity during inflammation

Next, we measured TEER over time to evaluate intestinal barrier integrity under control, inflamed, preventive, and post-inflammation treatment conditions (Figure 3). In both the spinach and bread groups, control monolayers showed stable TEER values, indicating successful formation of a polarized epithelial barrier before experimental treatments. Following inflammation induction on Day 19, TEER values in the inflamed groups decreased rapidly, demonstrating disruption of barrier integrity. In the spinach digesta condition (Figure 3A), preventive treatment maintained significantly higher TEER values compared with the inflamed control. Although TEER declined after inflammation, the reduction was less severe than in untreated inflamed monolayers. In contrast, spinach treatment applied after inflammation showed only partial preservation of TEER. For bread digesta (Figure 3B), preventive treatment did not maintain TEER to the same extent as spinach pretreatment. TEER values declined more rapidly after inflammation and approached levels similar to those of the inflamed group. Post-inflammation treatment with bread digesta resulted in minimal improvement in TEER values compared with the inflamed group. Statistical analysis showed significant differences between treatment groups during the post-inflammation phase (***, $p < 0.001$). In general, spinach digesta, particularly when applied before inflammation, preserved TEER more effectively than bread digesta.

Spinach pretreatment better preserved ZO-1 organization

We also performed ZO-1 immunofluorescence staining on Day 22 to evaluate tight junction organization and epithelial barrier structure (Figure 4). Control monolayers (Figure 4A) showed continuous ZO-1 localization outlining well-defined cell borders, indicating intact tight junction organization. In contrast, inflamed samples (Figure 4B) displayed fragmented and discontinuous ZO-1 staining, consistent with disruption of epithelial junctions. Preventive treatment with spinach digesta (Figure 4C) preserved a more continuous ZO-1 network compared with the inflamed condition. Spinach treatment applied after inflammation (Figure 4D) showed partial restoration of junctional organization, although staining remained less uniform than in controls. Bread digesta showed weaker effects overall. Bread pretreatment (Figure 4E) resulted in faint and uneven ZO-1 staining, while post-inflammation treatment (Figure 4F) showed minimal recovery of junctional continuity. These qualitative observations were consistent with the TEER measurements, where spinach digesta better maintained barrier integrity than bread digesta.

Spinach treatment partially preserved apical microvilli structure

Finally, we used scanning electron microscopy (SEM) to examine apical surface ultrastructure and microvilli organization in Caco-2 monolayers under different treatment conditions (Figure 5). At low magnification, the control group (Figure 5A) showed a continuous and confluent epithelial

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surface. At higher magnification, the control condition (Figure 5B) showed dense and organized brush-border microvilli. Spinach-treated inflamed samples (Figure 5C) retained partially organized microvilli structures compared with the inflamed condition. In contrast, the inflamed group (Figure 5D) exhibited disrupted and flattened surface morphology with reduced microvilli density. These observations showed that spinach treatment partially preserved apical epithelial structure during inflammatory stress.

DISCUSSION

This study tested whether digested spinach could better protect an inflamed intestinal cell barrier than digested bread. We first found that antioxidant capacity decreased after digestion in both spinach and bread, although spinach maintained a higher antioxidant capacity than bread both before and after digestion. Across the three measurements of intestinal barrier integrity, the results showed the same general pattern. Spinach digesta helped the Caco-2 cell layer maintain higher TEER values, more continuous ZO-1 staining, and better-preserved microvilli structure. These results suggested that compounds released from spinach during digestion may have helped protect intestinal cells during inflammatory stress. The timing of treatment also appeared to matter. Spinach was more effective when we added it before inflammation than when we added it after the barrier had already been damaged. Preventive treatment helped slow the loss of barrier strength, while post-inflammation treatment led to only limited recovery. Overall, these findings supported our hypothesis that spinach would have greater antioxidant capacity than bread, that spinach digesta would better preserve intestinal barrier integrity, and that preventive treatment would be more effective than treatment after inflammation. These results suggested that supporting the intestinal barrier before or during inflammation may be easier than restoring it after substantial damage had already occurred.

The TEER, ZO-1, and SEM results supported each other. TEER measured the strength of the cell barrier, ZO-1 staining showed the organization of tight junctions, and SEM showed changes in the surface structure of the cells. Because all three methods showed better preservation with spinach treatment, the findings were stronger than they would have been if we had used only one measurement. This study also showed why it was important to test foods after digestion. Previous studies showed that digestion could change the amount and type of compounds released from food before they reached intestinal cells (12, 13). Spinach and bread were both complex foods, and their digesta contained many different nutrients and molecules. For this reason, the protective effect associated with spinach could not be explained by antioxidants alone. Vitamins, minerals, fiber-related compounds, and other phytochemicals may also have contributed to the results . In addition, although spinach had both higher antioxidant capacity and greater barrier-protective

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effects than bread, this association did not demonstrate that higher antioxidant capacity caused the improved barrier protection.

There were several limitations. We used a Caco-2 cell model, which was useful for studying intestinal barrier function but did not include all the cell types, immune responses, microorganisms, and physical conditions found in the human intestine (14). The ZO-1 and SEM results were mainly qualitative, and we used a limited number of samples. In addition, we compared only spinach and bread, so the results could not be applied to all vegetables or grain-based foods. Future studies could test a wider range of foods and include additional inflammatory markers, such as IL-6, IL-8, TNF-α, and MCP-1 (15). Tight junction changes could also be measured more quantitatively. For example, researchers could calculate a tight junction continuity index from ZO-1 images (16), use quantitative reverse transcription PCR, or qRT-PCR, to measure the expression of tight junction-related genes, and use ELISA or Western blotting to measure the amounts of tight junction proteins (17). More advanced intestinal models could also be used to better represent the complexity of the human intestine. These models could include stromal cells, immune cells, and neuronal cells in addition to intestinal epithelial cells. The gut microbiome is also important because intestinal microorganisms can break down food components and produce metabolites that may affect inflammation and barrier function (18). Future studies could therefore combine simulated digestion with intestinal cell models and gut microbial communities to better understand how whole foods influence human gut health.

Overall, this study provided a useful method for connecting food digestion with intestinal barrier testing. By combining simulated digestion, antioxidant measurements, and a human intestinal cell model, the study provided a more realistic way to examine how digested whole foods may influence the intestinal barrier during inflammatory stress. The results suggested that spinach digesta provided greater barrier protection than bread digesta, particularly when we applied it before inflammation, while also showing that further studies were needed to identify the specific compounds and mechanisms responsible for these effects.

MATERIALS AND METHODS

Research setting and compliance

All experiments were conducted in Professor David Kaplan's lab in the Department of Biomedical Engineering at Tufts University under trained supervision. An established Caco-2 cell line obtained from the American Type Culture Collection (ATCC, cat. CRL-2102) was used. The study did not involve human participants, newly collected human tissue, or vertebrate animals. All cell culture work and chemical handling were performed according to Tufts University laboratory safety and biosafety procedures.

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In vitro digestion

Spinach and bread samples were digested using the standardized INFOGEST 2.0 (3) in vitro digestion method with minor modifications. This method simulated the oral, gastric, and intestinal stages of human digestion, as summarized in Figure 1. Briefly, 1 g of each food sample was mixed with 1 g of simulated salivary fluid at pH 7 containing salivary amylase at 75 U/mL. The samples were mixed and incubated at 37 °C for 2 min (Figure 1A). For the gastric phase, the oral mixture was combined with an equal amount of simulated gastric fluid at pH 3 containing porcine pepsin (Sigma-Aldrich, cat. P7000) at 2,000 U/mL and gastric lipase (Lipolytech, cat. REG20) at 60 U/mL. The samples were incubated at 37 °C for 2 h (Figure 1B). For the intestinal phase, the gastric mixture was combined with an equal amount of simulated intestinal fluid at pH 7 containing trypsin (Sigma-Aldrich, cat. T4799), chymotrypsin (Sigma-Aldrich, cat. 1134007), pancreatic α-amylase (Sigma-Aldrich, cat. A3176), and pancreatic lipase (Sigma-Aldrich, cat. L3126) at final activities of 100, 25, 200, and 2,000 U/mL, respectively. The samples were incubated at 37 °C for an additional 2 h (Figure 1C). Following digestion, samples were collected and stored at -80 °C until further analysis.

Antioxidant quantification

The antioxidant capacities of vitamin E, spinach, and bread were measured before and after digestion using an oxygen radical absorbance capacity (ORAC) assay kit (Abcam, cat. ab65329). Vitamin E was included as a positive control. Before analysis, fresh vitamin E, spinach, and bread samples were homogenized on ice and centrifuged at 10,000 × g for 10 min at 4 °C. Digested samples were thawed on ice and centrifuged under the same conditions. The liquid supernatant was collected and used for the ORAC assay according to the manufacturer's instructions. A Trolox standard curve was used to calculate antioxidant capacity. Results were normalized to the original mass of each food sample and reported as micromoles of Trolox equivalents per gram of food (μmol TE/g).

Caco-2 cell culture

Human colorectal adenocarcinoma Caco-2 cells were maintained under standard mammalian cell culture conditions in a humidified incubator at 37 °C with 5% CO_2. Cells were handled using sterile technique in a biosafety cabinet. Routine cultures were monitored by light microscopy for confluency and morphology, and cultures showing signs of contamination were discarded.

Caco-2 cells were cultured in high-glucose Dulbecco's Modified Eagle Medium (DMEM; ThermoFisher, cat. 11965092) supplemented with fetal bovine serum (FBS; ThermoFisher, cat. A5256701) and 1x antibiotic-antimycotic (ThermoFisher, cat. 15240062). Complete medium was warmed to room temperature or 37 °C before use.

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Frozen Caco-2 cells were thawed rapidly in a 37 °C water bath until only a small ice crystal remained. The exterior of the vial was disinfected with ethanol, and the cell suspension was transferred into pre-warmed complete medium to dilute the cryoprotectant. Cells were collected by gentle centrifugation, resuspended in fresh complete medium, and plated into tissue-culture-treated flasks. Medium was replaced after the cells had attached and recovered and was changed regularly thereafter.

Cells were maintained below over-confluence and passaged at approximately 70-90% confluence. Spent medium was removed, and cells were rinsed with sterile phosphate-buffered saline (PBS; ThermoFisher, cat. 10010023). 0.25% Trypsin-EDTA (ThermoFisher, cat. 25200072) was added to detach the cells. Once the cells had detached, enzyme activity was neutralized with complete medium, and the cell suspension was gently triturated to obtain a single-cell suspension. Cells were then re-seeded into fresh culture vessels. When required, cells were counted using a hemocytometer, and viability was assessed using 0.4% trypan blue dye exclusion (ThermoFisher, cat. 15250061) before experimental seeding.

Caco-2 seeding on Transwell inserts and inflammation induction

Caco-2 cells were harvested from routine culture, counted, and resuspended in complete growth medium. Cells were seeded onto the apical surface of 24-well Transwell inserts with a 0.4 µm pore size (ThermoFisher, cat. 140620) at a density of 33,000 cells per insert to establish a polarized epithelial barrier. Complete medium was added to both the apical and basolateral compartments. Medium was replaced every 2 days in both chambers. Monolayer formation and cell morphology were monitored by light microscopy, and barrier development was evaluated by measuring transepithelial electrical resistance (TEER) until stable polarization was achieved.

To model inflammatory injury to the intestinal barrier, polarized Caco-2 monolayers were exposed to lipopolysaccharide (LPS; ThermoFisher, cat. 00-4976-93) and interferon-gamma (IFNγ; ThermoFisher, cat. 300-02). On Day 19 after seeding, fresh complete medium was added, and monolayers were treated with 100ng/mL LPS and 20ng/mL IFNγ under standard incubation conditions at 37 °C with 5% CO_2.

Two treatment approaches were used to determine whether digested food samples could preserve or restore epithelial barrier function. For the preventive condition, digested spinach or bread samples were applied beginning on Day 2 after seeding and were maintained before inflammatory induction with LPS and IFNγ on Day 19. For the post-inflammation treatment condition, inflammation was induced first, and digested food samples were subsequently applied to evaluate recovery of barrier integrity. Control monolayers that did not receive inflammatory

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treatment and inflamed monolayers that did not receive food digesta were included for comparison. Digested spinach and bread samples were applied at 100µg/mL.

Transepithelial electrical resistance measurement

TEER was measured to evaluate the integrity of the polarized Caco-2 epithelial barrier. Before each measurement, Transwell plates were equilibrated at room temperature for approximately 10-15 min to minimize temperature-related variability. TEER was recorded using a Epithelial voltohmmeter equipped with chopstick electrodes (WPI, EVOM2) according to the manufacturer's instructions. The shorter electrode was placed in the apical chamber and the longer electrode was placed in the basolateral chamber without disturbing the cell monolayer.

Background resistance from a blank insert containing culture medium without cells was measured and subtracted from all readings. Final TEER values were normalized to the surface area of the insert and expressed as Ω·cm² using the following equation: TEER (Ω·cm²) = (measured resistance - blank resistance) x membrane area. Measurements were collected at regular intervals during monolayer formation and before and after experimental treatments to monitor changes in barrier integrity. Care was taken to avoid bubbles or mechanical disruption during electrode placement.

ZO-1 immunofluorescence staining

Immunofluorescence staining was performed to visualize the tight junction protein ZO-1 and evaluate epithelial barrier organization. Polarized Caco-2 monolayers cultured on Transwell inserts were stained using an Alexa Fluor 594-conjugated anti-ZO-1 monoclonal antibody (Invitrogen, clone ZO1-1A12, cat. 33-9100). All staining steps were performed at room temperature unless otherwise specified, and samples were protected from light after antibody addition.

Culture medium was aspirated from the apical and basolateral chambers, and monolayers were fixed with 4% paraformaldehyde (PFA), pH 7.4 (ThermoFisher, cat. J61899.AK). A volume of 300 µL was added to the apical chamber and 700 µL to the basolateral chamber. Samples were incubated for 30 min at room temperature and washed three times with 1× PBS. Cells were then permeabilized with 0.1% Triton X-100 (ThermoFisher, cat. A16046.AE) in PBS for 30 min and washed twice with PBS for 5 min per wash. Nonspecific binding was blocked with 3% bovine serum albumin (BSA; Sigma-Aldrich, cat. A9418) in PBS for 30–60 min.

ZO-1 staining was performed using Alexa Fluor 594-conjugated anti-ZO-1 antibody diluted 1:200 in PBS containing 0.1% BSA. Nuclear counterstaining was performed with DAPI (ThermoFisher, cat. 62248) according to the manufacturer's recommendations. A total of 200 µL of staining solution was added to the apical chamber of each insert, while PBS was maintained in the

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basolateral chamber. Samples were protected from light and incubated for 3 hours at room temperature. Following incubation, inserts were washed three times with PBS to remove unbound antibody. Fluorescence images were acquired directly in PBS using a fluorescence microscope (KEYENCE, BZ-XLC1) to visualize ZO-1 localization and nuclear staining.

Scanning electron microscopy

Scanning electron microscopy (SEM) was used to examine the apical surface ultrastructure and microvilli organization of Caco-2 monolayers. Samples were prepared using a modified glutaraldehyde fixation and graded ethanol dehydration procedure. All steps were performed gently to preserve epithelial morphology.

Samples were fixed in 1% glutaraldehyde (ThermoFisher, cat. A17876.0F). Fixation was performed at room temperature for approximately 1 hour per millimeter of sample thickness. Following fixation, samples were rinsed in the same buffer without glutaraldehyde for approximately 10 min per millimeter of sample thickness or overnight at 4 °C. Samples were then washed twice in distilled water for 10 min per wash on ice, followed by three additional 5-min washes on ice.

Samples were dehydrated through a graded ethanol series. Samples were incubated in 30% ethanol for 10 min, 50% ethanol for 10 min, 70% ethanol for 15 min at −20 °C, 90% or 95% ethanol for 15 min at −20 °C, and 100% ethanol for 15 min at −20 °C. Samples were then transferred to fresh 100% ethanol for at least 20 min at −20 °C, followed by a final exchange with fresh 100% ethanol for 20 min. After dehydration, ethanol was carefully removed and samples were treated with hexamethyldisilazane (HMDS; ThermoFisher, cat. L16519.AP) to replace ethanol and minimize surface-tension artifacts during drying. Samples were air-dried in a chemical fume hood, mounted on SEM stubs, and coated with a conductive layer using Pt/Pb. Samples were imaged using a SEM (Zeiss, Ultra Plus).

Statistical analysis

All quantitative data were analyzed using GraphPad Prism (11.0, GraphPad Software). Results were presented as mean ± standard deviation (SD) unless otherwise indicated. Individual data points were included in graphs when possible to show experimental variability. Comparisons among experimental groups were performed using one-way analysis of variance (ANOVA). A p-value < 0.05 was considered statistically significant. Sample sizes (n) represented independent biological replicates unless otherwise stated. Statistical testing, graph preparation, and quantitative data visualization were performed using GraphPad Prism.

CONFLICTS OF INTEREST

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The authors declare that they have no conflicts of interest and no financial interests that could have influenced the research reported in this manuscript.

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FIGURES AND TABLES

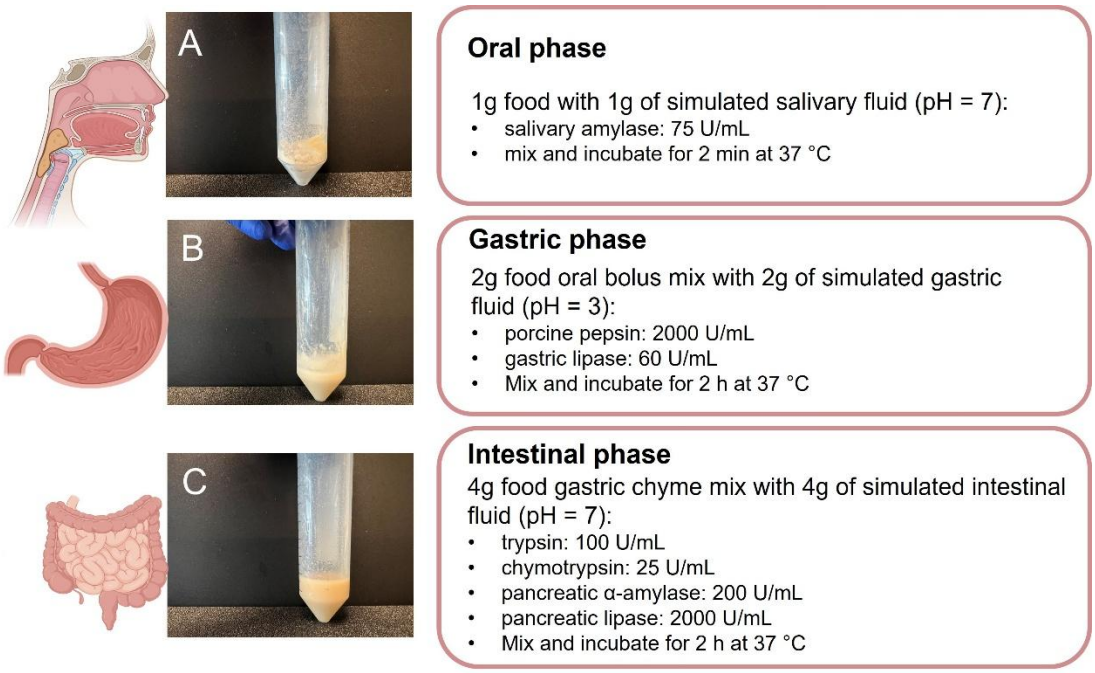


Figure 1. Overview of the in vitro gastrointestinal digestion procedure. Food samples were digested using a modified INFOGEST 2.0 protocol that simulated the oral, gastric, and intestinal phases of human digestion. (A) During the oral phase, 1 g of food was mixed with 1 g of simulated salivary fluid containing salivary amylase and incubated at 37 °C for 2 min. (B) During the gastric phase, the oral bolus was mixed with an equal amount of simulated gastric fluid containing porcine pepsin and gastric lipase and incubated at 37 °C for 2 h. (C) During the intestinal phase, the gastric chyme was mixed with an equal amount of simulated intestinal fluid containing trypsin, chymotrypsin, pancreatic α-amylase, and pancreatic lipase and incubated at 37 °C for 2 h. Representative images show the appearance of the samples after each digestion phase. The schematic was created using BioRender.com.

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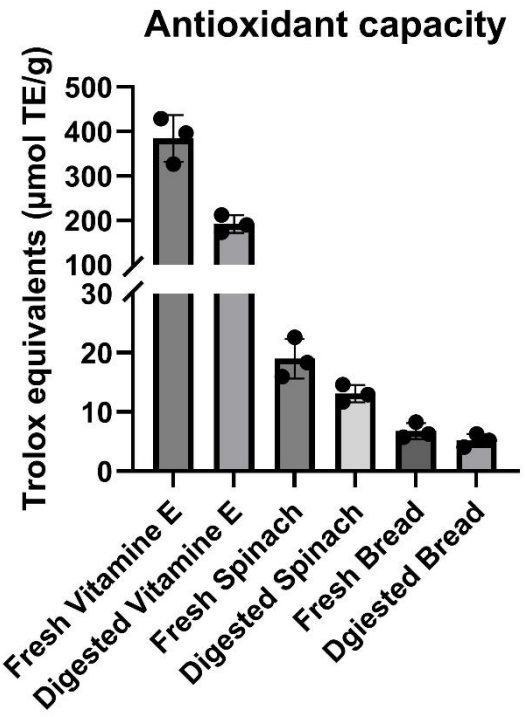


Figure 2 Antioxidant capacity of vitamin E, spinach, and bread before and after in vitro digestion. Antioxidant capacity was measured using an oxygen radical absorbance capacity, ORAC, assay and expressed as micromoles of Trolox equivalents per gram of sample, µmol TE/g. Vitamin E showed the highest antioxidant capacity, followed by spinach and bread. For all three samples, antioxidant capacity was lower after digestion than before digestion. Bars represent the mean, and individual data points represent independent measurements. Error bars indicate standard deviation of the mean. n=3.

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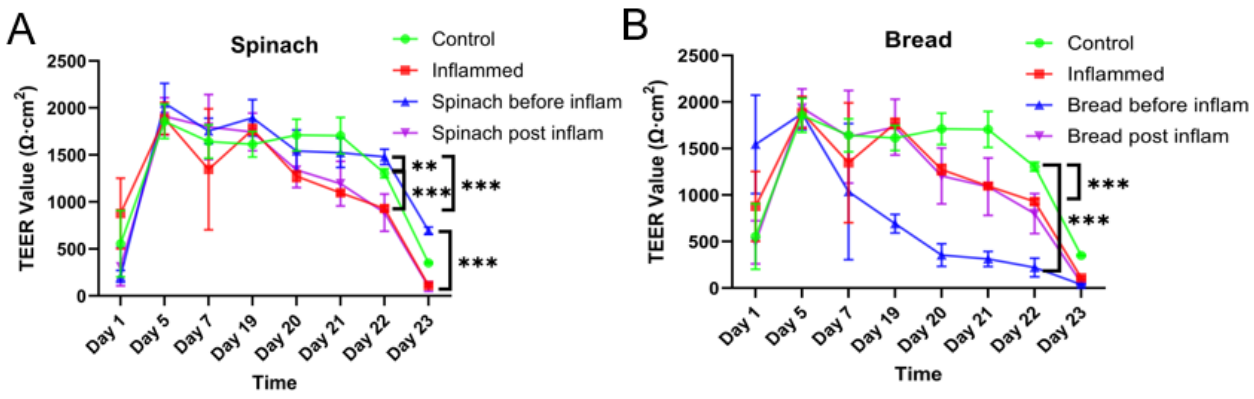


Figure 3. **Effects of spinach and bread digesta on intestinal barrier integrity during inflammation**.(A) TEER measurements of Caco-2 monolayers treated with spinach digesta under control, inflamed, preventive (pre-inflammation), and therapeutic (post-inflammation) conditions over time. Spinach pretreatment maintained higher TEER values and delayed barrier decline compared with inflamed controls, indicating a protective effect on epithelial integrity. Post-inflammation treatment showed limited restoration of barrier function. (B) TEER measurements for bread digesta under the same experimental conditions. Bread pretreatment did not preserve TEER stability to the same extent as spinach, and post-treatment resulted in minimal recovery following inflammation. Data are presented as mean ± SD, n=3. Statistical significance is indicated by *** ($p < 0.001$). Experimental timeline: Day 2, pretreatment; Day 19, inflammation induction; Day 19 onward, post-treatment phase.

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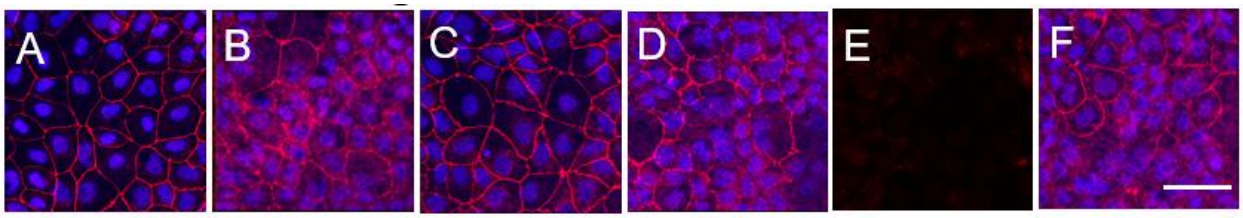


Figure 4. **ZO-1 immunostaining reveals differential preservation of tight junction integrity in Caco-2 monolayers at Day 22**.(A) Control monolayers exhibit continuous junctional ZO-1 outlining intact epithelial borders.(B) Inflamed samples display disrupted and fragmented ZO-1 organization, consistent with barrier damage. (C) Spinach pretreatment maintains more continuous ZO-1 localization, indicating protection of tight junction structure. (D) Spinach post-treatment shows only partial restoration. (E) Bread pretreatment results in weak cell staining. (F) Bread post-treatment shows minimal improvement. Scale bar: 20 μm.

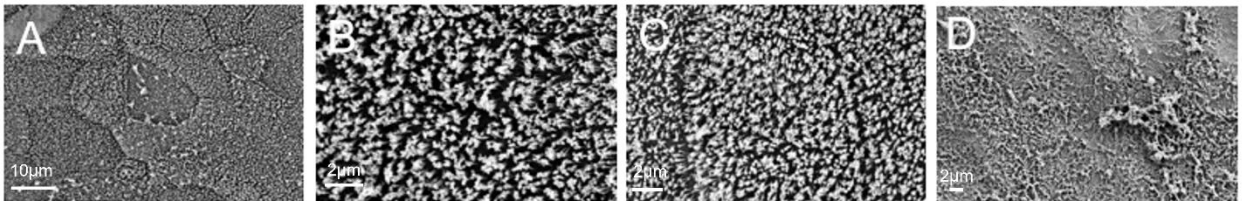


Figure 5. **SEM analysis of apical microvilli structure in Caco-2 monolayers**. Representative scanning electron microscopy images showing surface ultrastructure under different conditions. Dense, polarized hair-like microvilli indicate functional epithelial organization. (A) Control, low-magnification overview of a confluent monolayer. (B) Control, higher magnification showing organized brush-border microvilli. (C) Spinach-treated inflamed samples display partially preserved microvilli architecture. (D) Inflamed group shows disrupted and flattened microvilli consistent with surface damage. These observations support a protective effect of spinach treatment on epithelial ultrastructure during inflammatory stress.

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