Prebiotic Modulation of Intestinal Permeability

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Abstract

In this study, we work to characterize the effects of dietary galacto-oligosaccharides (GOS) and "humanized" galacto-oligosaccharides (hGOS) on intestinal permeability. Previous work shows that both GOS and hGOS modulate the gut microbiota, increasing the abundance of beneficial microorganisms including *Bifidobacterium*, *Lactobacillus*, and *Akkermansia*. We used physiological assays to determine that both dietary GOS and hGOS were able to facilitate the restoration of intestinal barrier function in aging animals. We then developed and utilized a novel histology approach to visualize and quantify mucin within intestinal sections, revealing that GOS-fed animals had more mucus than hGOS or control-fed animals. Finally, we performed reverse transcription qPCR to characterize gene expression of mucin genes, tight junction proteins, and inflammatory signals within GOS and hGOS-fed animals. This work, in conjunction with previously published work provides valuable insights into how intestinal permeability can be modulated by dietary prebiotics.

Background

The maintenance of a functional intestinal barrier is critical to overall health of the host, as a "leaky gut" often results in systemic pathologies, including dysregulated inflammation and introduction of infections. Age-associated defects in intestinal barrier integrity can lead to systemic health problems, reducing quality of life in aging individuals. Gut barrier is modulated by host factors including expression of tight junction proteins and mucus, as well as through direct and indirect interactions with members of the gut microbiota. While use of dietary prebiotics, complex carbohydrates selectively fermented by microorganisms in the gut, is becoming a popular method to beneficially modulate the gut microbiota, it was not known whether and how intestinal permeability is modulated by prebiotics GOS and hGOS.

Experimental Design

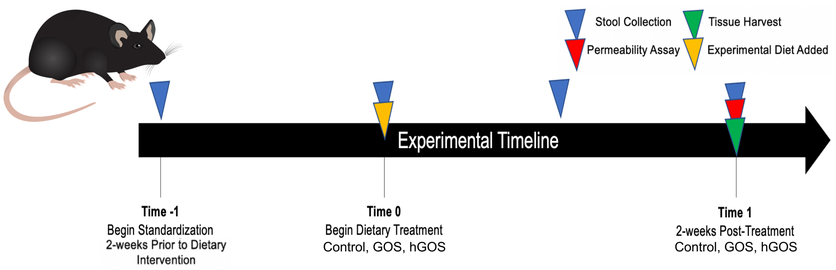

Figure 1: Workflow of Live Animal Experiment: Wild-type C57BL/6J mice were randomly co-housed based on age group (6-weeks old or 60-weeks old). Mice were fed a control diet without supplemental prebiotics, for 14 days in order to standardize the gut microbiome composition. Following standardization period, mice were transferred into paired-housing that consisted of one young mouse and one old mouse per cage. The mice in each cage were either fed the control diet, a GOS diet, or an hGOS diet for 14 days. Throughout the study, stool samples were collected from each animal at different time points. The mice were sacrificed in a humane manner and their intestinal tissue were harvested for further physiological analysis.

Prebiotic Modulation of Intestinal Barrier

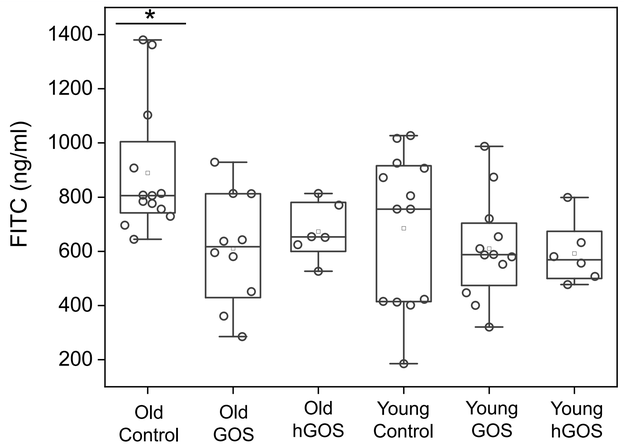

Figure 2: Prebiotics Improve Intestinal Barrier Function: Box-plots for concentration of FITC-Dextran found in the serum of each animal treatment group. When treated with prebiotics, the gut barrier is tighter, letting less FITC-Dextran to pass from the intestinal lumen into the blood. The results indicate that there is significantly less concentration of FITC-Dextran in serum of old animals fed the prebiotic diets.

GOS Induces Muc2 Expression

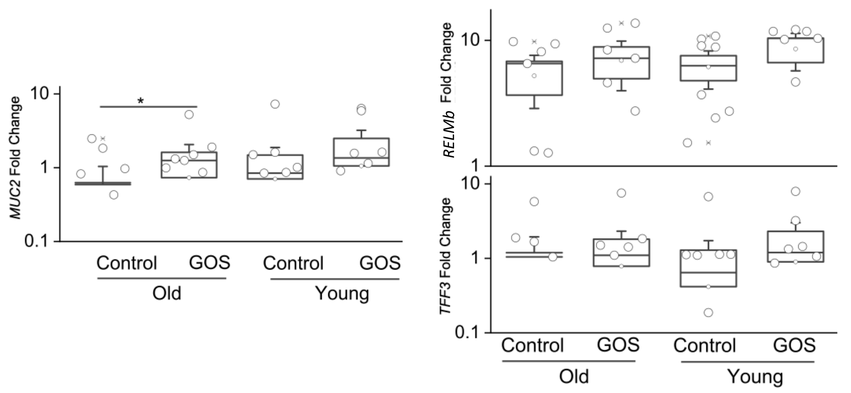

Figure 3: Expression of Muc2 is induced by prebiotic GOS. Total RNA isolated from mouse colon was subject to RT-qPCR to quantify differential expression of *MUC2*, *RELMb*, and *TFF3* between GOS and control-fed animals. While no differences were observed between *RELMb* or *TFF3*, *MUC2* expression was induced by GOS feeding in old animals. *p<0.05

Mucus Imaging

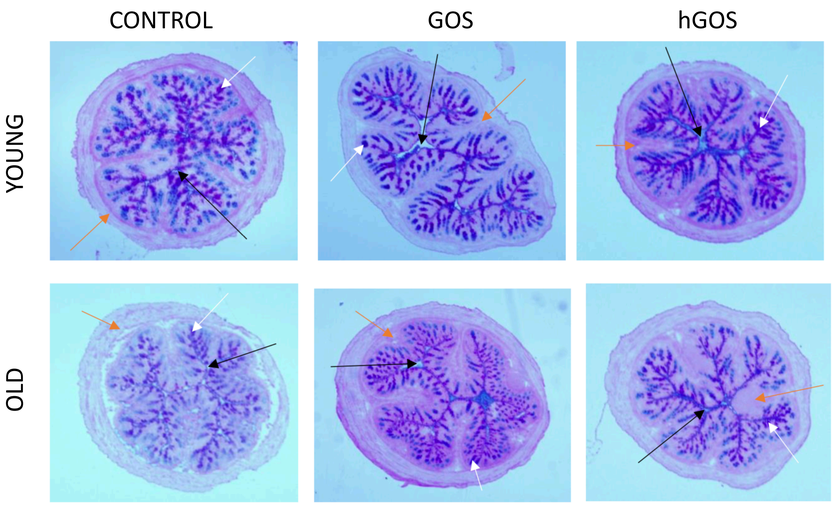

Figure 4: A.) Images of distal colon tissue sections from young and old mice on control diet, GOS diet, and hGOS diet. Orange arrows indicate epithelial cells, white arrows indicate mucin-producing goblet cells, and black arrows indicate the intestinal lumen. Darker colors within the lumen represent mucus.

Mucus Image Analysis

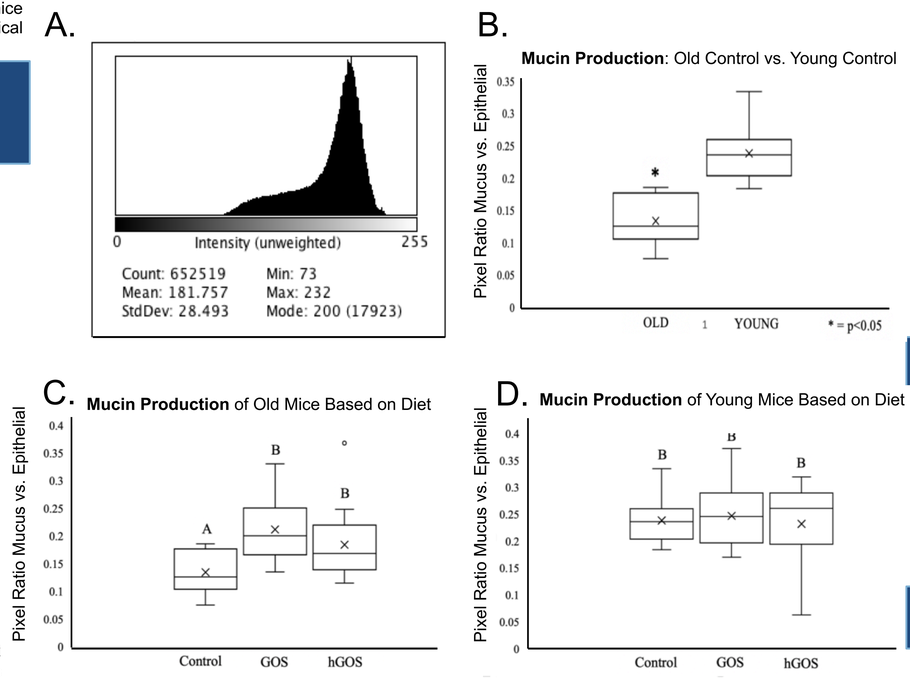

Figure 5: GOS Increases Mucin Abundance. A.)The software ImageJ (version 1.52q) was used to analyze the images at a pixel-level. Each tissue sample image was isolated from the background by using the software tools to circumscribe only the pixels specific to the tissue sample. A histogram of the frequency of pixels (y-axis) at each color value (x-axis) was generated using the software for each image, where a value of 0 represented black and a value of 255 represented white. B-D.) Box and whisker plots for mucin production based on mucin pigment : epithelial cell pigment ratios for animals in each group. Plots denoted "A" and "B" statically different (p<0.05). B.) Mucin production for old control mice compared to mucin production for young control mice. C.) Mucin production for old mice on control diet, GOS diet, and hGOS diet. D.) Mucin production for young mice on control diet, GOS diet, and hGOS diet.

Prebiotic Modulation of Permeability-Associated Genes

Gene Name	Function	Primer Sequence
MUC 2	Mucin	**F**: GCTGACGAGTGGTTGGTGAATG **R**: GATGAGGTGGCAGACAGGAGAC
MUC 4	Mucin	**F**: CCC CCA TCT TTC TGT CTC AA **R**: AGG ATG GAA TTG GTG TT TG
MUC 6	Mucin	**F**: 5′-TCGAAACACGTAGCAAGTAT-3 **R**: 5′-TCATTTCAAGACCAGTCTGG-3
TFF3	Mucin Biosynthesis Regulator	**F**: CAGATTACGTTGGCCTGTCTCC **R**: ATGCTTGCTACCCTTGGACCAC
RELMb	Mucin Biosynthesis Regulator	**F**: CCATTTCCTGAGCTTTCTGG **R**: AGCACATCCAGTGACAACCA
Ocln	Tight junction protein	**F**: CGG TAC AGC AGC AAT GGT AA **R**: CTC CCC ACC TGT CGT GTA GT
ZO-1	Tight junction protein	**F**: ACT ATG ACC ATC GCC TAC GG **R**: GGG GAT GCT GAT TCT CAA AA
KLF4	Tight junction protein	**F**: CCA AAG AGG GGA AGA AGG TC **R**: CTG TGT GAG TTC GCA GGT GT
Cldn4	Tight junction protein	**F**: GGG GAT CAT CCT GAG TTG TG **R**: CAC TGC ATC TGA CCT GTG CT
TNFa	Inflammatory marker	**F**: ACG GCA TGG ATC TCA AAG AC **R**: GTG GGT GAG GAG CAC GTA GT
IL-6	Inflammatory marker	**F**: CTG CAA GAG ACT TCC ATC CAG TT **R**: GAA GTA GGG AAG GCC GTG G

Table 1: Gene targets associated with intestinal permeability. Reverse transcription qPCR was performed to characterize gene expression of mucin genes, tight junction proteins, and inflammatory signals within GOS and hGOS-fed animals.

Materials and Methods

Animal Husbandry: Old and young female C57BL/6J mice (6-weeks old and 60-weeks old) were co-housed and fed a control diet for 2-weeks prior to being paired off (1 old/1 young) from different co-housing groups and switched to prebiotic GOS or hGOS diets for an additional 2-week period. A control group was included, which was not administered prebiotics throughout the study. Stool samples were collected prior to each change in diet or treatment. After two weeks, each animal was subject to FITC-dextran permeability assay and subsequently euthanized via CO_2 asphyxiation and cardiac puncture for the harvest of serum, tissue and intestinal contents.

Mouse Intestinal Permeability Assay: Mice were administered 100mg fluorescein isothiocyanate (FITC) dextran/100g body weight via oral gavage 4-hours prior to sacrifice. Serum was collected post-sacrifice via cardiac puncture, and FITC signal was quantified with TECAN Infinite M200 Plate Reader (ex:485nm, em:528nm).

RNA Extraction: Total RNA was isolated from a 1cm section of mouse colon using Qiagen RNeasy mini kit (Qiagen, Valencia, CA) following manufacturer's instructions. Total RNA was subsequently quantified using Quant-iT RiboGreen RNA Reagent (Molecular Probes, Thermo Fisher Scientific, Waltham, MA) and stored at -80C.

Reverse Transcription qPCR: 1µg of total RNA was subject to reverse transcription using qScript cDNA synthesis kit (QuantaBio). Specific target amplification was performed on cDNA using target gene primers (refer to Table 1) and house-keeping genes *GapDH* (Fwd: 5'-TGCACCACCACCAACTGCTTAG-3', Rev: 5'-GGATGCAGGGATGATGTTC-3'), and *Actin* (Fwd: 5′-GCTCCTCCTGAGCGCAAT-3′, Rev: 5´-GTGGACAGTGAGGCCAGGAT-3'). qPCR reactions were performed using Power SYBR Green Master Mix on the QuantStudio Q6 instrument (Thermo Fisher Scientific, Waltham, MA).

Mucin Staining and Tissue Imaging: Sections of mouse distal colon were harvested, embedded in optimal cutting temperature (OCT) compounding agent and flash-frozen. Blocks were cut at -20ºC on a cryostat and mounted onto slides subject to immediate paraformaldehyde vapor fixation (4%PFA, 60ºC) for 8-hours prior to PAS staining and imaging on Nikon 2000-E inverted widefield microscope.

Mucin Quantification from Histological Images: The software ImageJ (version 1.52q) was used to analyze the images at a pixel-level. Each tissue sample image was isolated from the background by using the software tools to circumscribe only the pixels specific to the tissue sample. An area of the tissue representing pigment from epithelial cells as well as an area representing pigment from mucin cells were isolated and the pigment values were recorded. This was used for reference to locate the range of pigment values that corresponded to epithelial cells and the range that corresponded to mucin cells. The pigment values for the whole tissue sample that was originally encompassed was totaled for mucin cell pixels, epithelial cell pixels, and total tissue pixels. These values were used to calculate a mucin to epithelial cell ratio for each sample. Higher values for the mucus: epithelial ratio indicate more mucus production. Statistical analysis was performed on pairwise comparisons using a Student's T-Test with a P- value cutoff of 0.05

Conclusions and Future Directions

Old mice experience decreased barrier function and produce less mucus in the gut compared to young mice

- Dietary prebiotic GOS and hGOS restore barrier function in mice, most notably in old mice
- Mucin production is upregulated in the presence of GOS prebiotic dietary intervention
- Abundance of mucus in the gut in increased as a result of GOS diet, but not due to hGOS
- Further exploration of potential mechanisms leading to decreased intestinal permeability (other mucin proteins, tight junction proteins, inflammatory signals) still need to be studied

Acknowledgements

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