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Ramsha Nabihah Ghazali Khan April 9, 2021

Analyzing Probiotic-Induced Shifts to the Microbiome in a Murine Model of Atherosclerosis

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An abstract of
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of Emory University in partial fulfillment
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Abstract

Analyzing Probiotic-Induced Shifts to the Microbiome in a Murine Model of Atherosclerosis

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Atherosclerosis is a leading cause of death worldwide, as the build-up of fatty deposits in blood vessels can lead to both ischemic strokes and ischemic heart attacks**.** Recently, the gut microbiome has been implicated in both driving and inhibiting atherogenesis. Thus, probiotics have emerged as novel therapeutic paradigms to lessen atherosclerotic disease burden. Notably, *Lactococcus lactis* subspecies *cremoris* (*L. cremoris*; LLC), a probiotic commonly utilized in dairy fermentation processes, has been shown to elicit protection against detrimental cardiometabolic phenotypes in mice fed a Western-style (high-fat, high-sugar) diet. Therefore, in order to better characterize the potential mechanisms by which *L. cremoris* drives its protective phenotypes, this study aims to compare the effects of *L. cremoris* and *Lactobacillus rhamnosus GG* (*L. rhamnosus*; LGG), another widely-used probiotic, on gut microbial composition in a murine model of atherosclerosis. To assess the impact of probiotic administration on bacterial communities, the QIIME bioinformatics pipeline was utilized to analyze alpha diversity, beta diversity, and taxa abundance metrics following 16S rRNA sequencing of fecal samples at the V3-V4 region. In the results of this study, we have found that in comparison to treatment with either *L. rhamnosus* or a vehicle saline solution, *L. cremoris* significantly increased the abundance of *Odoribacter*, a butyrate-producing bacterium with beneficial anti-inflammatory activity. Additionally, *L. cremoris* treatment resulted in a significant reduction of microbes typically associated with weight gain, increased gut permeability, and atherogenesis. In mice administered *L. cremoris*, decreases in the abundance of the *Marvinbryantia* and *Akkermansia* genera, as well as the *Clostridiaceae* and *Ruminococcaceae* bacterial families were observed. Furthermore, while an increase in the Firmicutes/Bacteroidetes ratio has been previously identified as a marker of diet-induced obesity, *L. cremoris* treatment resulted in a reduction of this ratio. Thus, dietary supplementation of *L. cremoris* results in differential alterations to the gut microbial profile, relative to those induced by either *L. rhamnosus* or vehicle treatments. Because *L. cremoris* is generally regarded as safe for consumption and has previously been shown to attenuate metabolic complications, this bacterium’s impact on the microbiome serves as an exciting avenue for future therapeutic exploration.

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Acknowledgements

I am incredibly grateful to have had the opportunity to study in Dr. Rheinallt Jones's lab, as well as for the many lessons and techniques each member of the Jones Lab has taught me to further enrich my understanding of biology. I would like to thank Dr. Rheinallt Jones for giving me the chance to delve into the field of microbiome studies, and I am continually inspired by his remarkable contributions to the field. I am forever indebted to the kind mentorship of Dr. Crystal Naudin, whose tireless efforts in research continue to motivate me further in my own studies. I would also like to thank Dr. Simbarashe Nkomo and Dr. Roger Deal for supporting me on my academic journey in numerous capacities, as well as the invaluable insight they have offered throughout this process. Lastly, I would like to express my gratitude to all my professors, including Dr. Florian Pohl and Dr. Arri Eisen, who have continually supported and encouraged my exploration of many different research projects over the past four years.

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Abbreviations

Abbreviation	***Explanation***
ApoE-/-	Apolipoprotein E knockout mice; atherosclerotic-prone mice
CVD	cardiovascular disease
FXR	farnesoid X receptor
L. cremoris, LLC	*Lactococcus lactis* subspecies *cremoris*
L. rhamnosus, LGG	*Lactobacillus rhamnosus GG*
OTU	operational taxonomic units
QIIME	Quantitative Insights into Microbial Ecology, a bioinformatics pipeline
SEM	standard error of the mean
TMA	trimethylamine
TMAO	trimethylamine *N*-oxide
WT	wild-type mice

Introduction

The Gut Microbiome's Impact on Host Health

As Antonie van Leeuwenhoek peered through his self-made single-lens microscopes, he noted the presence of 'living animalcules' in oral and fecal samples, which appeared to differ in both morphology and abundance at their respective body sites. Hailed the 'Father of Microbiology,' Leeuwenhoek's discoveries of the first human-associated bacteria date back to the late 17th century, ushering in a field of study that aimed to better understand the connections between these bacterial groups and disease progression. [1] Today, it is well-accepted that the human body hosts a distinct array of microorganisms, such as viruses, fungi, protozoa, archaea,

and bacteria. While the relative bacterial compositions in individuals vary due to distinct genetic and environmental factors, similarities in the microbiome composition have been observed at higher taxonomic levels. Notably, the Firmicutes and Bacteroidetes phyla typically comprise over 90% of the human gut, while other phyla like Actinobacteria, Proteobacteria, and Verrucomicrobia tend to occupy a lesser percentage of the overall gut microbiome. [2]

Over time, a mutualistic relationship has been fostered between humans and their gut microbiota in which the microbes benefit from human substrates to drive their proliferation and life cycles, while also aiding the host by metabolizing dietary carbohydrates, 'training' the immune system, producing essential vitamins (e.g. Vitamin B), and generating important signaling molecules like secondary bile acids and short-chain fatty acids. [3], [4] Thus, it is evident that the host microbiome influences host physiology through various avenues, and these microorganisms themselves are impacted by the host and their environment, as genetics, hygiene, diet, and medications have also been found to distinctly affect the gut microflora. However, despite the aforementioned beneficial effects associated with the microbiota, the gut microbiome has also been implicated in a wide array of inflammatory, infectious, and metabolic pathologies, including obesity, Type 2 diabetes mellitus, and cardiovascular disease (CVD). [5], [6]

Tales of Men in Mice: Experimental Approaches for Studying the Microbiome in Disease States

To date, many studies have identified associations between microbiome shifts and disease progression. In order to understand the complex mechanisms by which the microbiota affect host health, two leading experimental models have emerged in microbiome studies: germ-free animal models and antibiotic regimens. The utilization of these small animal models offers an exciting

avenue to establish causative vs. correlative factors. In particular, these experimental innovations provide researchers better ways to categorize the impact that various subsets of microbes have on disease pathologies. [7]

Germ-free (gnotobiotic) animal models are bred in isolated conditions to prevent their exposure to viruses, germs, and other pathogens. While this approach allows for the selective colonization of specific microorganisms to study particular disease states, it is often costly and labor-intensive to maintain these germ-free colonies as they must be regularly monitored via culturing, sequencing, and microscopy assays. [8] Thus, broad-spectrum antibiotic treatment regimens have emerged as a more convenient alternative for depleting the gut microbiome. [7] Notably, in gnotobiotic models, the use of fecal transplants offer an avenue to study the onset of distinct pathologies. In fecal transplant methodologies, germ-free mice are colonized by intestinal microbiota derived from a donor stool sample, allowing for recipient animals to develop a microbiome similar to that of the human donor. Notably, this model has indicated causal links between the microbiota and certain diseases, such as non-alcoholic fatty liver disease and diet-induced obesity. [9], [10], [11]

However, much of the gut microbiota remains to be isolated, cultured, and identified. Thus, innovations in sequencing methodologies offer exciting avenues to study gut microbial compositions. For example,16S rRNA gene sequencing allows for the amplification of bacterial 16S sequences via polymerase chain reactions. These sequences are then aligned and grouped based on degree of similarity (e.g. a 95% threshold for genus identification, a 97% similarity threshold for species designation, and so forth). These grouped sequences are then used to produce clusters of closely-related organisms in groups known as operational taxonomic units (OTUs). [13] OTUs are then cross-checked against reference genomes to determine their

taxonomies. Additionally, shotgun metagenomic sequencing offers an even more comprehensive window into the microbiome's configuration, as it allows for the characterization of all genes present in a sample. [13] Thus, the advancement of microbiota experimental techniques provides invaluable insight into investigating the role of the microbiome in host health.

At the Heart of the Matter: The Gut Microbiome in Atherosclerosis

Atherosclerosis is a leading cause of death worldwide, leading to both ischemic strokes and ischemic heart attacks. It is characterized by the build-up of fatty deposits (plaques) that constrict blood flow in the arteries, and its progression can be driven by a variety of risk factors, including diet, cigarette smoking, diabetes, and high blood pressure. [14] The links between the gut microbiome and atherogenesis are of particular interest, as bacteria found in atherosclerotic plaques have also been enriched in the gut. [14] Notably, fecal samples from healthy individuals and atherosclerotic patients were found to differ in bacterial composition, as *Roseburia* and *Eubacterium* were abundant in healthy subjects, while *Collinsella* was increased in individuals with atherosclerosis. [15] Moreover, the predominant bacterial phyla present in atherosclerotic plaques appear to be from both Proteobacteria and Firmicutes. [16] Bacterial DNA from *Neisseriaceae* and *Helicobacteraceae* are also enriched in the plaques of patients with symptomatic atherosclerosis. However, while *Helicobacter pylori* and *Porphyromonas gingivalis* have been found to exacerbate lesion formation in murine models, further studies must be conducted to determine if such a trend is also applicable in humans. [17], [18]

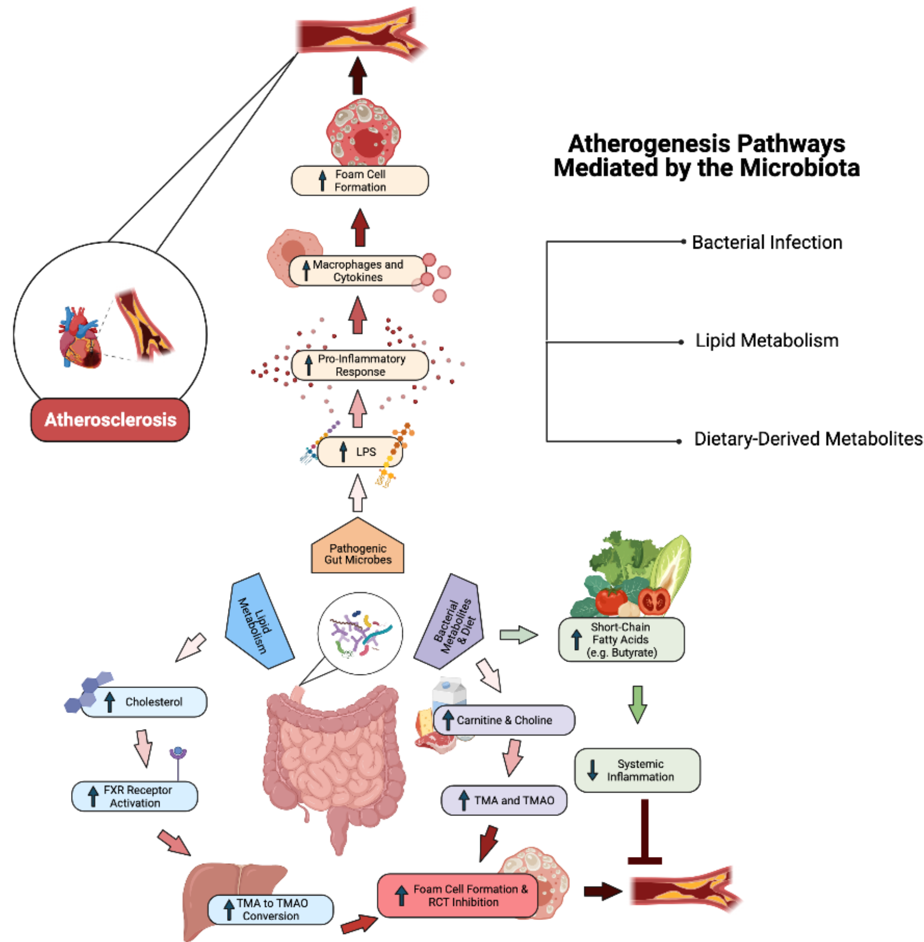

Figure 1. The various atherogenesis pathways that are mediated by the gut microbiota. Bacterial infection can lead to an increase in the pro-inflammatory response, which can catalyze the formation of foam cells, lipid-laden macrophages that drive the onset of atherosclerosis. The gut microbiota also metabolize lipids to yield cholesterol, and increased cholesterol levels can lead to the overexpression of the FXR receptor. This receptor's downstream signaling effects include the conversion of TMA to TMAO, a key bacterial metabolite known to drive atherogenesis. Carnitine and choline, molecules metabolized from various foods (e.g. red meat, dairy, and eggs) also serve as TMAO precursors. Notably, other dietary derivatives, such as the short-chain fatty acids sourced from leafy greens, have been found to decrease systemic inflammation. This reduction in inflammatory tone may be critical in hindering atherogenesis. *Diagram made using BioRender*.

Thus far, the microbiome has been found to drive atherogenesis via several distinct mechanisms (Figure 1). The presence of bacterial pathogens and endotoxins trigger the immune response and induce inflammatory states that can drive atherosclerotic onset. Furthermore, increased levels of Gram-negative bacterial components like lipopolysaccharides (LPS) can have devastating consequences, leading to increased gut and intestinal permeability. This LPS leakage can catalyze the onset of metabolic endotoxemia which can then exacerbate metabolic disorders

like insulin resistance and diabetes, as well as CVD complications. [19] High-fat diets, which have been found to drive atherogenesis, have also been implicated in altering and impairing gut permeability and may catalyze the onset of systemic inflammation through the translocation of toll-like receptor (TLR) bacterial ligands to sub-epithelial compartments.

The gut bacteria have also been found to metabolize lipids to produce cholesterol. These bacteria can further emulsify lipids to yield secondary bile acids, which are important signaling molecules that act via the farnesoid X receptor (FXR). This receptor activates the conversion of trimethylamine (TMA) to trimethylamine *N*-oxide (TMAO), a bacterial metabolite implicated in atherogenesis. [20], [21] Lastly, the gut microbiota also metabolize dietary-derived carnitine and choline, a process that can lead to the formation of TMAO precursors that drive atherosclerotic lesion formation. However, dietary interventions, like an increased intake of compounds that yield short-chain fatty acids (e.g. butyrate) via bacterial metabolism, may play a remedial, anti-inflammatory role in mitigating atherosclerosis. [22], [23]

From Food to Factory to Pharmacy: The Potential of Probiotic-Based Therapeutic Interventions for Atherosclerosis

The gut microbiome has been implicated in both driving and hindering the progression of atherosclerosis, and so the role of probiotics in treating associated complications remains a viable avenue of exploration. Probiotics are live bacterial cultures that can confer protective health benefits to the host, though they have had varying levels of success in treating CVD thus far. For example, the administration of a specific *Lactobacillus plantarum* strain decreased inflammatory phenotypes in atherosclerotic patients, while three different *Lactobacillus reuteri* strains attenuated diet-induced obesity complications, but did not appear to have any effect on lesion formation in atherosclerotic-prone mouse models (*ApoE-/-*). [24], [25]

Certain bacteria that can act as microbial factories by synthesizing lactate have emerged as areas of interest in probiotic studies. *Lactobacillus rhamnosus GG* (*L.* rhamnosus; LGG) is a colonizing, Gram-positive, facultatively-anaerobic, rod-shaped bacterium. [26] *L. rhamnosus* produces lactic acid, deriving energy from the fermentation of carbohydrates to yield lactate via homofermentative metabolic pathways. [27] It is also a widely-studied probiotic that has been shown to attenuate atherosclerotic complications, as well as improve gut permeability and tissue healing in models of intestinal injury. [28], [29]

Lactococcus lactis subspecies *cremoris* (*L. cremoris*; LLC) is another lactic-acid-producing bacteria that promotes cytoprotection in intestinal injury models. [31] Initially isolated from plants, this non-colonizing, Gram-positive, facultatively-anaerobic, spherical bacterium also uses homofermentative mechanisms to produce lactate from lactic acid. It has also long been used to ferment dairy products, often preferred over other bacterial strains as it can impart a particularly pleasant taste to various cheeses. *L. cremoris* has emerged as an exciting probiotic strain to study, as it has a generally regarded as safe (GRAS) status and exhibits extracellular secretion capabilities. [32]

Notably, the Jones Lab has shown that the probiotic, *Lactococcus lactis* subspecies *cremoris* ATCC 19257 (*L. cremoris*), elicits greater protection against cardiometabolic complications caused by a Western (high-fat, high-sugar) diet, especially in comparison to *L. rhamnosus*. Mice administered *L. cremoris* had lower mean serum cholesterol levels, reduced body mass index, and decreased hepatic steatosis and inflammation. (Figures 2A-2C). [33] In another recent study in the Jones Lab, Western diet-fed mice were treated with either a vehicle saline solution or the aforementioned probiotic strains. It was found that mice administered *L.*

lactis cremoris exhibited decreased lesion formation in both wild-type (WT) and apolipoprotein 1 knockout (*ApoE-/-*) models (Figure 2D).

Thus, in an effort to better understand the various mechanisms by which *L. cremoris* elicits protective effects in Western-diet fed mice, the present study aims to characterize any probiotic-induced shifts to the microbiome in a murine model of atherosclerosis. It is critical to characterize the microbiome-associated pathways employed by *L. cremoris,* as the probiotic may be producing metabolites that drive these observed remedial effects, or actively competing for or supplementing nutrients that promote beneficial changes to the gut's microbial composition.

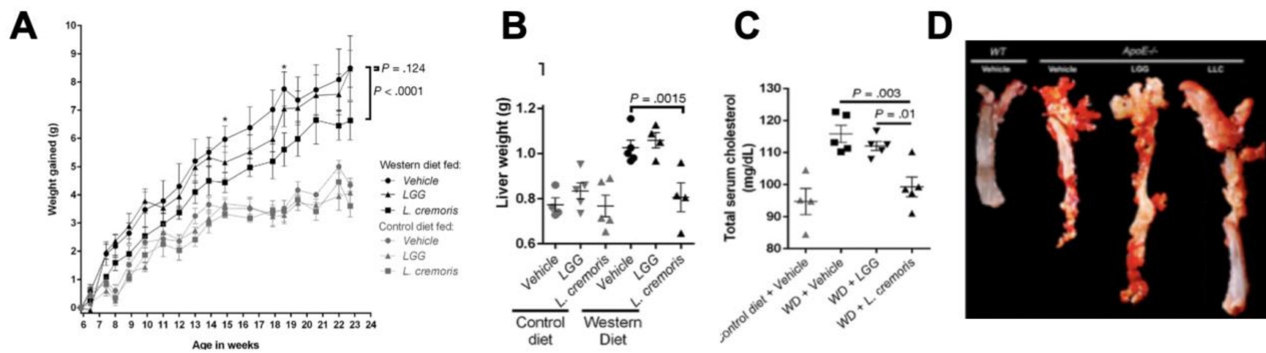

Figure 2. Dietary supplementation of *L. cremoris* induces beneficial phenotypes that protect against metabolic complications caused by a Western-style diet. (A) The weights of female C57BL/6 gavaged three times weekly with either 2 x10^9 CFU of *L. cremoris, L. rhamnosus*, or an equivalent dosage of vehicle HBSS solution, from five weeks of age. Mice were fed either a control diet or a Western-style diet from six weeks of age onwards. **(B)** Final liver weights of mice at 23 weeks of age (16 weeks of dietary and probiotic regimen). **(C)** Serum cholesterol levels from buccal bleed samples at 8.5 weeks of age. **(D)** Aortic dissections of *ApoE-/-* mice treated with vehicle, *L. rhamnosus*, or *L. cremoris*. Lipid was stained red with Oil Red O. (*All data in Figure 2 are courtesy of Dr. Jones, Dr. Naudin, and other members of the Jones Lab. Figures A-C have been recently published.* [32] *Figure D is sourced from an ongoing study*).

Materials and Methods

Dietary Regimen and Probiotic Administration in Animal Models

The impact of probiotic treatment on the microbiome of male C57BI/6 wild-type (WT) and apolipoprotein E knockout (*ApoE-/-*) mice was analyzed in this study. All mice were housed

in cohorts of 3-5 animals per cage, on a 12:12-hour artificial light cycle at a humidity of 55 ± 5% and a temperature of 21 ± 1°C. For one week in the lead-up to the study, soiled cage bedding was routinely mixed between cages to normalize the baseline microbiome. All mice were given free access to drinking water and a standard chow diet (Research Diet D14042707) before being switched to a Western-style (high-fat, high sugar) diet (Research Diet D12079B) at 13 weeks of age (Table 1). Prior to this dietary shift, 12-week-old mice were also given oral gavage treatments of either 100 µL of Hank's Balanced Salt Solution (HBSS), *Lactobacillus rhamnosus GG* ATCC 53103 (2×10^8 CFU per 100 µL HBSS), or *Lactococcus lactis* subspecies *cremoris* ATCC 19257 (2×10^8 CFU per 100 µL HBSS). All mice were administered either vehicle or probiotic treatments five times a week for 13 weeks, with the first oral gavage administrations occurring at 12 weeks of age (1 week prior to the Western diet regimen shift). Prior to sacrifice, fecal samples were collected at 25 weeks of age (following 12 weeks of Western diet feeding) and sent for processing to the ZymoBIOMICS Targeted Sequencing Core in Irvine, California.

Six experimental and control groups were analyzed in this study: *L. cremoris* + WT mice (n = 3), *L. cremoris* + *ApoE-/-* mice (n = 3), *L. rhamnosus* + WT mice (n = 3), *L. rhamnous* + *ApoE-/-* mice (n = 2), HBSS + WT mice (n = 4), and HBSS + *ApoE-/-* mice (n = 2). All aspects of the study complied with the animal care and use guidelines set by the Institutional Animal Care and Use Committee (IACUC) at Emory University.

Table 1. Research Diet Composition

	Research Diet Ingredients			
	D14042707 (Control Chow Diet)		D12079B (Western-Style Diet)	
	g	kcal	g	kcal
Cholesterol	0	0	1.5	0
Choline Bitartrate	2	0	2	0
Corn Starch	695	2780	50	200
Sucrose	0	0	341	1364
Maltodextrin 10	150	600	100	400
Milk Fat, Anhydrous	42.5	383	200	1800
Vitamin Mix V10001	10	40	10	40
Corn Oil	10	90	10	90
Casein, 80 mesh	195	780	195	780
Calcium Carbonate	4	0	4	0
Mineral Mix S10001	35	0	35	0
FD&C Red Dye, #40	0.05	0	0	0
Cellulose, BW200	50	0	50	0
Ethoxyquin	0.04	0	0.04	0
	Macronutrient Summary			
	g	kcal	g	kcal
Fat	4	10	21	40
Carbohydrates	71	73	50	43
Protein	17	17	20	17
Total	92	100	91	100

Probiotic Culture

L. cremoris and *L. rhamnosus* were cultured in Gibco MRS media at 37° C. The concentration of bacteria was calculated by measuring the absorbance at a wavelength of 550 nm. Next, 2 x 10^8 CFU of *L. cremoris* and *L. rhamnosus* in 100 µL of HBSS was administered by oral gavage to designated treatment groups at 12 weeks of age.

DNA Extraction and Sample Preparation

At the ZymoBIOMICS Targeted Sequencing Core, DNA was extracted from fecal samples using the ZymoBIOMICS®-96 MagBead DNA Kit, followed by amplification of the V3-V4 region of the 16S rRNA region. The ZymoBIOMICS® Microbial Community Standard was used as a positive control for the DNA extractions. All samples were sequenced on Illumina® MiSeq™ with a v3 reagent kit.

Bioinformatics Analysis

All sequence reads obtained from the ZymoBIOMICS facility in Irving, California, were then processed using the open-source, bioinformatics pipeline, Quantitative Insights Into Microbial Ecology (QIIME, Version 1.9.1). [53] Prior to downstream sequencing analysis, a mapping file containing sample metadata was constructed to include details of treatment type and experimental subgroups. All raw sequence reads were then denoised and checked for quality control parameters to prevent an artificial display of OTU inflation. Forward and reverse primers were also removed to trim sequence length, with trimmed reads later demultiplexed to remove sample ID barcodes. Using QIIME alignment scripts, sequences were aligned based on 97% similarity to each other and clustered using UCLUST. Closed-reference OTU picking was then employed to compare OTU groups against the GreenGenes reference database in order to apply taxonomic identifications to representative sequences. Following the construction of an OTU table, which serves as a matrix that describes the number of reads per OTU in each sample, QIIME scripts were utilized to generate alpha diversity, beta diversity, bacterial heat maps, and abundance metrics. The generated OTU tables were also analyzed using the open-source Linear discriminant analysis effect size (LEFse) platform, which compares the relative abundances of bacterial groups across treatment groups, in order to provide insight into bacterial populations that are significantly enriched in certain experimental groups. [54]

Statistical Analysis

Data are expressed as mean ± standard error of the mean (SEM). Statistical differences were determined by two-way ANOVA, followed by Tukeys multiple comparisons test (n=3-5),

in which a P-value < 0.05 was considered significant. One-way ANOVA tests were also utilized to analyze comparisons between multiple experimental groups, followed by the Brown-Forsythe test and Bartlett's test (*n*=3-5), in which a P-value < 0.05. All statistical tests were conducted using GraphPad Prism 6.

Note: *Due to the onset of the COVID-19 pandemic, I conducted the bioinformatics analyses for this study while Dr. Crystal Naudin administered the probiotic and vehicle treatments to the mice.*

Results

L. cremoris treatment significantly altered the abundance of certain genera

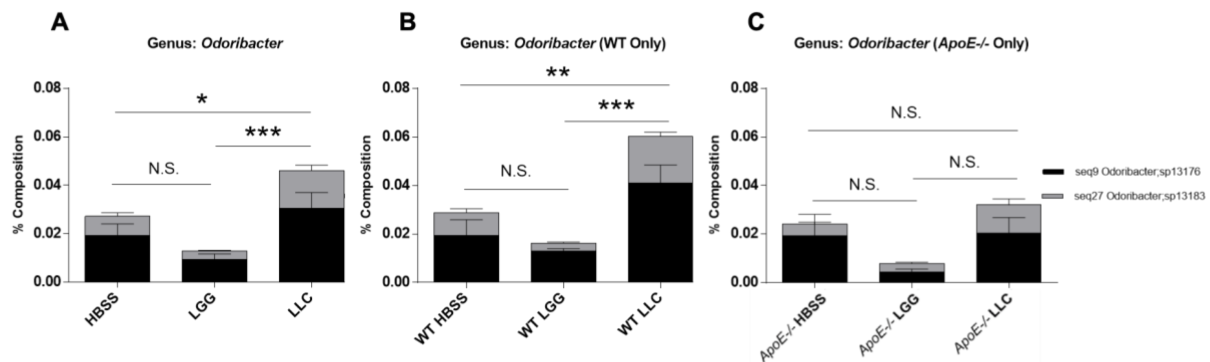

Figure 3. ***L. cremoris*** **treatment increases the abundance of the genus** ***Odoribacter*** **in mice fed a Western-style diet. (A)** Percent composition plot depicting the abundance of the genus *Odoribacter* in both WT and *ApoE-/-* mice, treated five times weekly with probiotic administration (2 x 10^8 CFU) of either *L. rhamnosus* (LGG) or *L. cremoris* (LLC), or an equal volume of HBSS saline solution from 12 weeks of age. All mice were also started on a Western-style diet from 13 weeks of age in tandem with vehicle and probiotic treatments. **(B)** Percent composition plot depicting the abundance of the genus *Odoribacter* in WT mice only. **(C)** Percent composition plot depicting the abundance of the genus *Odoribacter* in *ApoE-/-* mice only. Data are depicted as the mean ± SEM. **P* < 0.05, ***P* < 0.01, *** *P* < 0.001, *** *P* < 0.0001, N.S. = not significant. *P*-values were obtained by two-way ANOVA, followed by Tukey's multiple comparisons tests; *n* = 3-5 per mouse group.

L. cremoris administration significantly increased the abundance of the genus *Odoribacter*, compared to *L. rhamnosus* and HBSS when assessing the percent composition changes across all experimental and control groups (Figure 3A). This trend was again noted when analyzing only WT mice, but failed to reach significance in the *ApoE-/-* mice (Figures 3B and 3C).

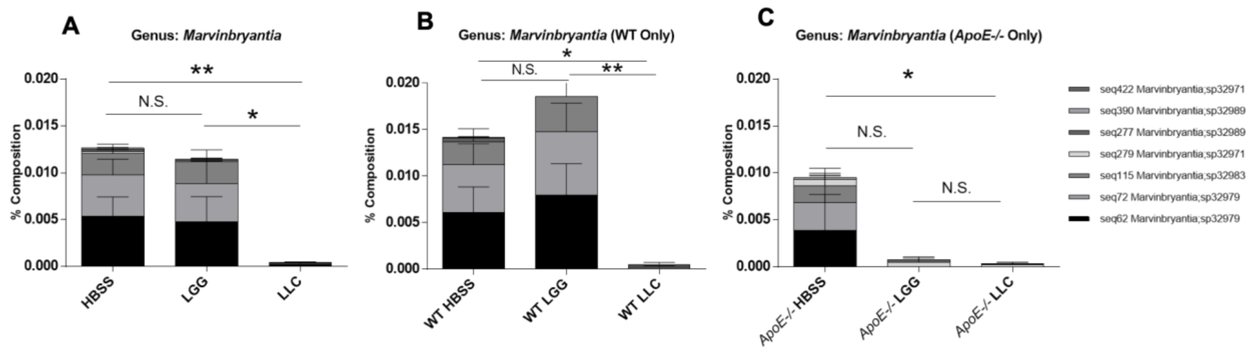

Figure 4. *L. cremoris* treatment decreases the abundance of the genus *Marvinbryantia* in mice fed a Western-style diet. (A) Percent composition plot depicting the abundance of the genus *Marvinbryantia* in both WT and *ApoE-/-* mice, treated five times weekly with probiotic administration (2 x 10^8 CFU) of either *L. rhamnosus* (LGG) or *L. cremoris* (LLC), or an equal volume of HBSS saline solution from 12 weeks of age. All mice were also started on a Western-style diet from 13 weeks of age in tandem with vehicle and probiotic treatments. **(B)** Percent composition plot depicting the abundance of the genus *Marvinbryantia* in WT mice only. **(C)** Percent composition plot depicting the abundance of the genus *Marvinbryantia* in *ApoE-/-* mice only. Data are depicted as the mean ± SEM. *$P < 0.05$, **$P < 0.01$, *** $P < 0.001$, *** $P < 0.0001$, N.S. = not significant. *P*-values were obtained by two-way ANOVA, followed by Tukey's multiple comparisons tests; n = 3-5 per mouse group.

L. cremoris administration significantly reduced the abundance of the genus *Marvinbryantia*, compared to *L. rhamnosus* and HBSS treatments when assessing the percent composition changes across all experimental and control groups (Figure 4A). Furthermore, *L. cremoris* administration significantly decreased *Marvinbryantia* levels when compared to *L. rhamnosus* and HBSS treatments in WT mice (Figure 4B). The amount of *Marvinbryantia* present in *ApoE-/-* mice was also significantly decreased when comparing the effects of HBSS and *L. cremoris* treatment (Figure 4C).

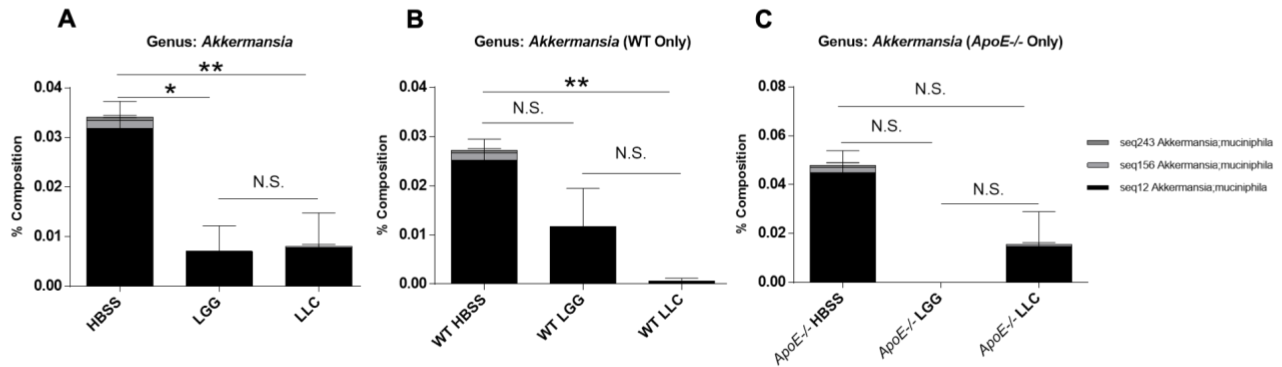

Figure 5. ***L. cremoris*** **treatment decreases the abundance of the genus** ***Akkermansia*** **in mice fed a Western-style diet. (A)** Percent composition plot depicting the abundance of the genus *Akkermansia* in both WT and *ApoE-/-* mice, treated five times weekly with probiotic administration (2 x 10^8 CFU) of either *L. rhamnosus* (LGG) or *L. cremoris* (LLC), or an equal volume of HBSS saline solution from 12 weeks of age. All mice were also started on a Western-style diet from 13 weeks of age in tandem with vehicle and probiotic treatments. **(B)** Percent composition plot depicting the abundance of the genus *Akkermansia* in WT mice only. **(C)** Percent composition plot depicting the abundance of the genus *Akkermansia* in *ApoE-/-* mice only. Data are depicted as the mean ± SEM. *$P < 0.05$, **$P < 0.01$, *** $P < 0.001$, *** $P < 0.0001$, N.S. = not significant. *P*-values were obtained by two-way ANOVA, followed by Tukey's multiple comparisons tests; n = 3-5 per mouse group.

L. cremoris administration significantly decreased the abundance of the genus *Akkermansia* ($P < 0.01$), compared to HBSS treatment when assessing the percent composition changes across all experimental and control groups, while *L. rhamnosus* also decreased *Akkermansia* levels, but to a lesser extent (P < 0.05; Figure 5A). This trend was also noted when comparing WT mice treated with either HBSS or *L. cremoris,* but *L. rhamnosus* administration failed to elicit a significant decrease in WT groups (Figure 5B). In *ApoE-/-* mice, no probiotic treatments reached significance in altering *Akkermansia* abundance(Figure 5C).

L. cremoris treatment significantly impacted the abundance of certain bacterial families

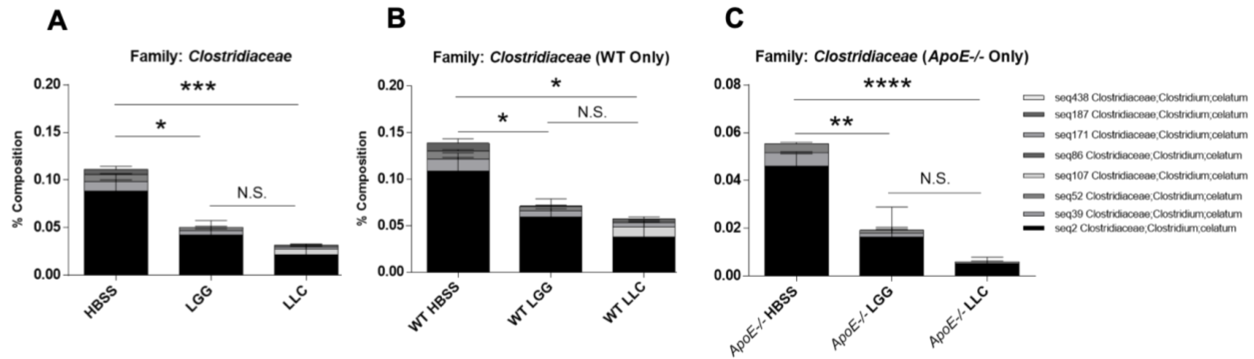

Figure 6. ***L. cremoris*** **treatment decreases the abundance of the family** ***Clostridiaceae*** **in mice fed a Western-style diet. (A)** Percent composition plot depicting the abundance of the genus *Clostridiaceae* in both WT and *ApoE-/-* mice, treated five times weekly with probiotic administration (2 x 10^8 CFU) of either *L. rhamnosus* (LGG) or *L. cremoris* (LLC), or an equal volume of HBSS saline solution from 12 weeks of age. All mice were also started on a Western-style diet from 13 weeks of age in tandem with vehicle and probiotic treatments. **(B)** Percent composition plot depicting the abundance of the genus *Clostridiaceae* in WT mice only. **(C)** Percent composition plot depicting the abundance of the genus *Clostridiaceae* in *ApoE-/-* mice only. Data are depicted as the mean ± SEM. *$P < 0.05$, **$P < 0.01$, *** $P < 0.001$, *** $P < 0.0001$, N.S. = not significant. *P*-values were obtained by two-way ANOVA, followed by Tukey's multiple comparisons tests; n = 3-5 per mouse group.

L. cremoris treatment significantly reduced the abundance of the family *Clostridiaceae* ($P < 0.001$), compared to HBSS when assessing the percent composition changes across all experimental and control groups, while *L. rhamnosus* also decreased *Clostridiaceae* levels but to a lesser degree ($P < 0.05$; Figure 6A). Both *L. cremoris* and *L. rhamnosus* treatments significantly decreased *Clostridiaceae* levels in WT mice (Figure 6B). In *ApoE-/-* mice, *L. cremoris* significantly decreased *Clostridiaceae* ($P < 0.0001$), to an even greater degree than *L. rhamnosus* ($P < 0.01$; Figure 6C).

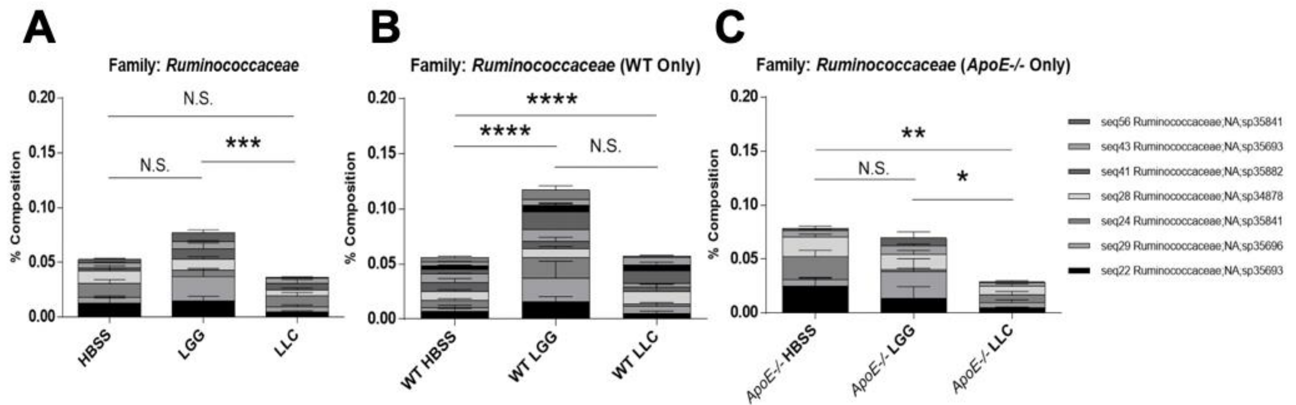

Figure 7. ***L. cremoris*** **treatment decreases the abundance of the family** ***Ruminococcaceae*** **in mice fed a Western-style diet. (A)** Percent composition plot depicting the abundance of the genus *Ruminococcaceae* in both WT and *ApoE-/-* mice, treated five times weekly with probiotic administration (2 x 10^8 CFU) of either *L. rhamnosus* (LGG) or *L. cremoris* (LLC), or an equal volume of HBSS saline solution from 12 weeks of age. All mice were also started on a Western-style diet from 13 weeks of age in tandem with vehicle and probiotic treatments. **(B)** Percent composition plot depicting the abundance of the genus *Ruminococcaceae* in WT mice only. **(C)** Percent composition plot depicting the abundance of the genus *Ruminococcaceae* in *ApoE-/-* mice only. Data are depicted as the mean ± SEM. *$P < 0.05$, **$P < 0.01$, *** $P < 0.001$, *** $P < 0.0001$, N.S. = not significant. *P*-values were obtained by two-way ANOVA, followed by Tukey's multiple comparisons tests; n = 3-5 per mouse group.

L. cremoris treatment significantly decreased the abundance of the family *Ruminococcaceae* ($P < 0.01$), compared to both *L. rhamnosus* and HBSS when assessing the percent composition changes across all experimental and control groups (Figure 7A). Both *L. cremoris* and *L. rhamnosus* treatments significantly decreased *Ruminococcaceae* levels in WT mice (Figure 7B). In *ApoE-/-* mice, *L. cremoris* significantly decreased *Ruminococcaceae* ($P < 0.01$), to an even greater degree than *L. rhamnosus* ($P < 0.05$; Figure 7C).

Probiotic treatments alter the composition of the gut microbiome

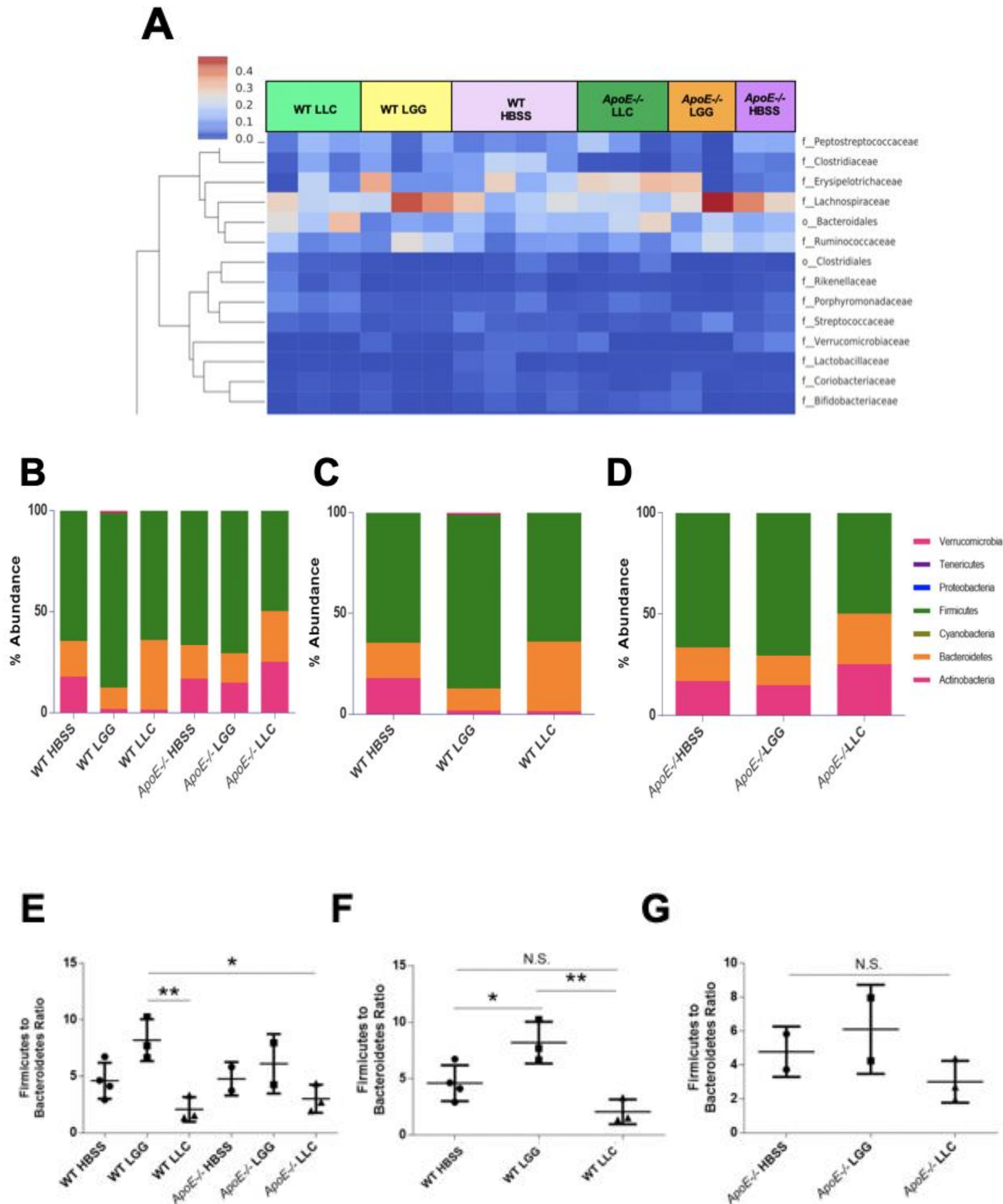

Figure 8. Probiotic treatment alters gut microbial composition. **(A)** Heat map depicting changes in bacterial taxa in both WT and *ApoE-/-* mice, treated five times weekly with probiotic administration (2 x 10^8 CFU) of either *L. rhamnosus* (LGG) or *L. cremoris* (LLC), or an equal volume of HBSS saline solution from 12 weeks of age. All mice were also started on a Western-style diet from 13 weeks of age in tandem with vehicle and probiotic treatments. **(B)** Percent abundance of bacterial phyla in both WT and *ApoE-/-* mice. **(C)** Percent abundance of bacterial phyla in WT mice only. **(D)** Percent abundance of bacterial phyla in *ApoE-/-* mice only. **(E)** Firmicutes to Bacteroidetes ratio in both WT and *ApoE-/-* mice **(F)** Firmicutes to Bacteroidetes ratio in WT mice only **(G)** Firmicutes to Bacteroidetes ratio in *ApoE-/-* mice only. Data are depicted as the mean ± SEM. *$P < 0.05$, **$P < 0.01$, *** $P < 0.001$, *** $P < 0.0001$, N.S. = not significant. *P*-values were obtained by one-way ANOVA, followed by the Brown-Forsythe test and Bartlett's test; n = 3-5 per mouse group.

L. cremoris and *L. rhamnosus* treatment led to differential alterations to the gut microbiome at various bacterial taxa levels, such as by impacting the family, *Ruminococcaceae*, and the order, Bacteroidales (Figure 7A). Notably, *L. cremoris* treatment significantly decreased the Firmicutes/Bacteroidetes ratio, while *L. rhamnosus* significantly increased this ratio in WT mice (Figure 8F).

Analyzing the impact of probiotic treatment on alpha and beta diversity

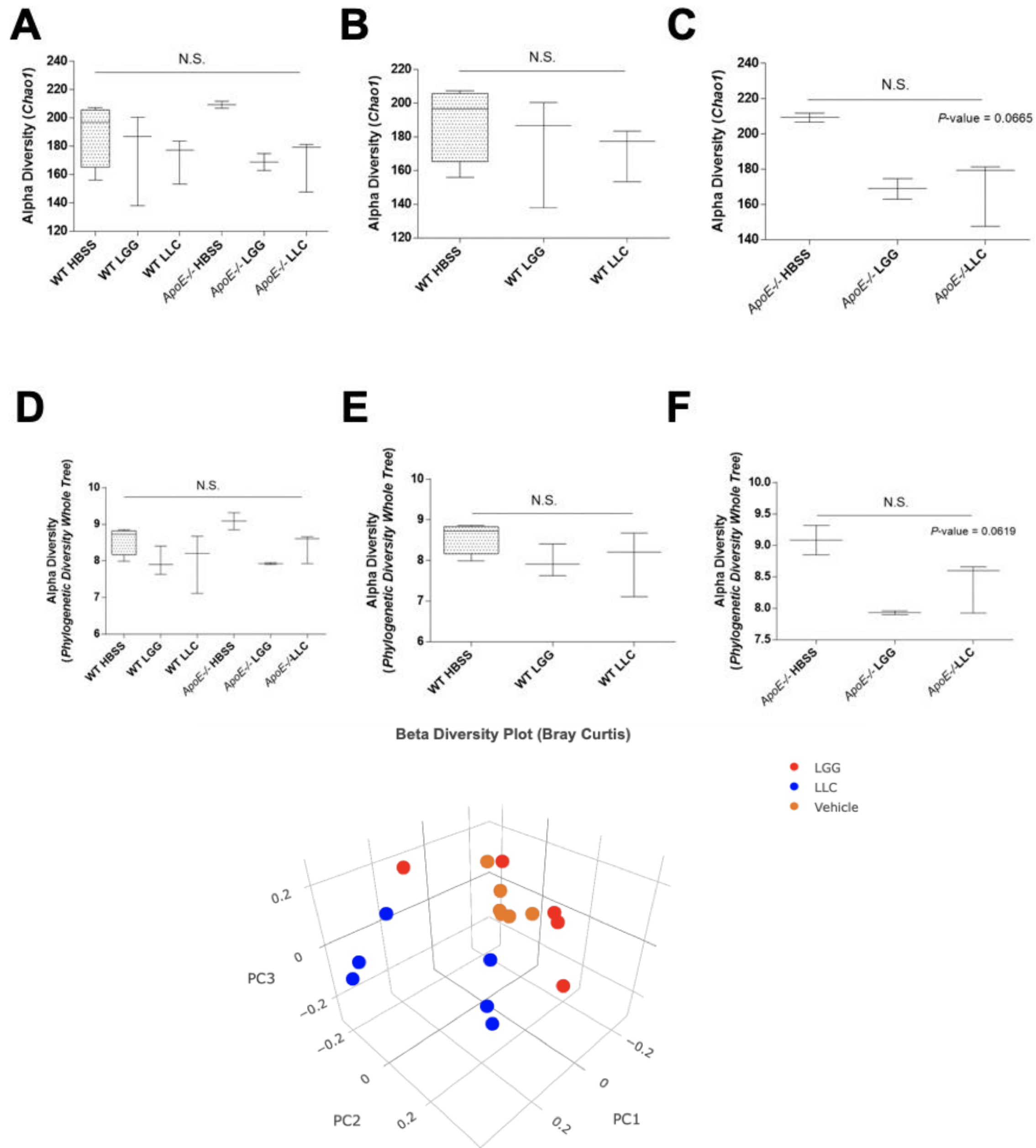

Figure 9. Alpha and beta diversity metrics across treatment groups. (A) Alpha diversity metrics based on the *Chao1* index for both WT and *ApoE-/-* mice, treated five times weekly with probiotic administration (2 x 10^8 CFU) of either *L. rhamnosus* (LGG) or *L. cremoris* (LLC), or an equal volume of HBSS saline solution from 12 weeks of age. All mice were also started on a Western-style diet from 13 weeks of age in tandem with vehicle and probiotic treatments. **(B)** Alpha diversity metrics based on the *Chao1* index for WT mice only. **(C)** Alpha diversity metrics based on the *Chao1* index for *ApoE-/-* mice only. **(D)** Alpha diversity metrics based on the *PD Whole Tree* index for both WT and *ApoE-/-* mice. **(E)** Alpha diversity metrics based on the *PD Whole Tree* index for WT mice only. **(F)** Alpha diversity metrics based on the *PD Whole Tree* index for *ApoE-/-* mice only. **(G)** Principal coordinates analysis plot using the Bray-Curtis dissimilarity index to depict beta diversity metrics for both WT and *ApoE-/-* mice. *$P <$

0.05, **$P < 0.01$, *** $P < 0.001$, *** $P < 0.0001$, N.S. = not significant. P-values were obtained by one-way ANOVA, followed by the Brown-Forsythe test and Bartlett's test; $n = 3\text{-}5$ per mouse group.

Alpha diversity metrics, including the *Chao1* and the *PD Whole Tree* indices, were used to assess the overall bacterial diversity within treatment groups. There were no significant differences in sample diversity across all experimental and control groups, though the impact of *L. cremoris* treatment on alpha diversity trended towards significance in *ApoE-/-* mice (Figures 9C and 9F). The Bray-Curtis dissimilarity index was utilized to analyze beta diversity across groups, and samples with the same genotype and treatment tended to loosely cluster together based on the similar compositions of their respective gut bacterial communities (Figure 9G).

Discussion

Past studies have indicated the correlation between the enrichment of particular bacteria in atherosclerotic plaques and their associated abundance in the oral and gut microbiomes. [14] Coupled with emerging data that implicate dietary-derived molecules such as carnitine and choline, as well as bacterial metabolites like TMAO, in promoting atherogenesis, the microbiome has emerged as a viable avenue of exploration for developing novel therapeutics (Figure 2). [33]

In this study, we have explored the impact that probiotic administration has on the composition of the gut microbiome in a murine model of atherosclerosis. Notably, *L. cremoris* treatment significantly impacts the abundance of bacterial taxa that have been associated with atherogenesis and the progression of cardiometabolic diseases. For example, *L. cremoris* significantly increases the abundance of the genus *Odoribacter* relative to both HBSS and *L. rhamnosus* treatments (Figure 3). Notably, a reduction in *Odoribacter* and other similar short-chain fatty acid producers has been observed in a murine model of inflammatory atherosclerosis.

[34] Decreased levels of *Odoribacter* have also previously been implicated in microbiota-associated disease etiologies, including cystic fibrosis, inflammatory bowel disease, and non-alcoholic fatty liver disease. [35], [36], 37] The outer membrane vesicles of an *Odoribacter* strain also exhibit anti-inflammatory activity, a characteristic that is particularly important in reducing atherosclerotic burden. [38] *Odoribacter* produces butyrate, a short-chain fatty acid that has been shown to prevent atherogenesis. [22] Notably, *L. cremoris* is a lactic-acid-producing bacteria that synthesizes lactate, a precursor molecule necessary for the production of butyrate. [31] Thus, although *L. cremoris* does not colonize the gut microbiome, it may serve as a microbial cell factory that manufactures the lactate needed by other intestinal microbiota for the eventual fermentation and synthesis of butyrate, a compound that has been shown to have beneficial, anti-atherosclerotic activity. [38] However, *L. rhamnosus* can also metabolize lactate from lactic acid, but the same increase in *Odoribacter* abundance was not observed in LGG-treated mice, thus, implicating the potential for a more specific crosstalk that occurs between *L. cremoris* and *Odoribacter* (Figure 3). [27]

Additionally, compared to *L. rhamnosus* and HBSS, *L. cremoris* treatment significantly reduced the abundance of the genus *Marvinbryantia* (Figure 4), a bacterium that has been positively correlated with weight gain, and decreased in models of weight loss. [40] In past studies, *L. cremoris*-treated mice exhibited a reduction in body mass even while being fed a Western-style diet. [32] Thus, this decrease in *Marvinbryantia* abundance may indicate a potential avenue by which *L. cremoris* prevents weight gain, a significant risk factor for atherogenesis. [41] Furthermore, an increase in the Firmicutes/Bacteroidetes ratio was observed in *L. rhamnosus* WT mice, but not in *L. cremoris* mice (Figure 8F). [42], [43] A greater proportion of Firmicutes to Bacteoidetes has been associated with obesity in human and animal studies, and it has previously

been shown that *L. rhamnosus*-treated mice have similar visceral fat storage levels to that of vehicle-treated mice in Western diet studies. [32] However, *L. cremoris*-treated mice exhibited a decrease in adiposity, which is reflected by the observed decrease in this bacterial abundance ratio.

Additionally, in mice treated with *L. cremoris,* a significant decrease in the presence of the genus *Akkermansia* was observed, in comparison to *L. rhamnosus* and HBSS-treated mice. This is notable as previous studies have shown *Akkermansia muciniphila* (*A. muciniphila*) to be protective against atherogenesis by reducing the burden of metabolic endotoxemia-associated inflammation in *ApoE-/-* mice, while other studies have indicated that *A. muciniphila* can improve glucotoxicity, lipotoxicity, and oxidative stress outcomes in diabetic rats. [44], [45] However, *A. muciniphila* blooms in mucus-rich environments as it degrades mucin, biomolecules found in a large family of glycosylated proteins that play an important role in maintaining the gut mucosal layer. The gut membrane and mucosal layer are one of the first barriers of protection against pathogens and inflammatory disease states, and more mucus is secreted by goblet cells in times of physiological stress. [46] *L. cremoris* has previously been shown to prevent gut permeability and the detrimental impact of a leaky gut in models of intestinal injury. [30] A more balanced concentration of mucus, as seen in such models of improved gut barrier function, would likely not be conducive to *A. muciniphila* proliferation. Thus, this indicates that *L. cremoris* potentially elicits its protective effects in a mechanism that is distinct from that of *A. muciniphila*.

While both *L. cremoris* and *L. rhamnosus* significantly decreased the abundance of the *Clostridiaceae* and *Ruminococcaceae* families, *L. cremoris* treatment did so to a much greater degree (Figures 6 and 7). These bacterial families are associated with high TMAO production, a

bacterial-derived metabolite that is a significant driver of atherosclerosis (Figure 1). [47], [48], [49] In a study that involved depleting the gut microbiome in human test subjects via an antibiotic regimen and a subsequent implementation of increased red meat consumption, which served as a source of dietary L-carnitine, bacterial taxa that belonged to *Clostridiaceae* were positively correlated with increased TMAO production. [33] This study further implicates the role of *Clostridiaceae* in TMAO metabolism, which can then subsequently promote atherosclerosis onset. Additionally, serum TMAO levels were found to be positively correlated with the enrichment of *Ruminococcaceae* and *Clostridiaceae* in other animal and human studies. [50], [51] Furthermore, when analyzing the impact of Western-diet feeding on *ApoE-/-* mice, *Ruminococcaceae* was positively correlated with atherosclerotic lesion size. [52] In other experiments conducted at the Jones Lab, *L. cremoris* has been shown to decrease fatty deposits and lesions in the blood vessels of *ApoE-/-* mice (Figure 2D). This reduction of *Ruminococcaceae* and *Clostridiaceae* by *L. cremoris* appears to be beneficial in attenuating atherosclerotic-associated complications.

Thus, in this pilot study, we have shown that *L. cremoris* treatment elicits notable alterations to the gut microbiome in Western-diet-fed mice. Some experimental limitations include the relatively small sample size (n = 3-5) and the inclusion of only male mice as test subjects. Additional studies with expanded experimental groups must be conducted in order to determine any potentially dimorphic phenotypes. Furthermore, many of the genera and families that were significantly altered as a result of this probiotic administration only comprise a relatively small percentage of the total gut microbiome composition. However, *L.* cremoris-associated decreases in the Firmicutes/Bacteroidetes ratio in WT mice indicate that more abundant bacterial taxa are also affected (Figure 8F). Thus, these probiotic-induced shifts in the

gut microbiome warrant further study in order to better characterize and understand the bacterial crosstalk that can both promote and inhibit atherogenesis.

Conclusions and Perspectives

Through this pilot study, we have shown that microbial intervention with *L. cremoris* drives significant changes in the abundance of certain bacterial taxa that have been implicated in atherosclerotic onset. These microbiota-induced alterations are distinct from those elicited by treatments of either vehicle solution or *L. rhamnosus*, another widely-used probiotic with beneficial anti-inflammatory activity. In the future, we intend to study associations between bacterial enrichment and other atherosclerotic phenotypes (e.g. serum cholesterol levels, endotoxin circulation, and lesion size), as well as correlations between *L. cremoris* treatment changes in lipid profiles, endocannabinoid signaling, and apelin gene expression. Furthermore, we hope to expand upon this study by analyzing microbiota changes at distinct intervals throughout the probiotic regimen in order to better characterize the dynamics of a changing gut microbiome in models of atherosclerosis. In the future, these microbial conversations between probiotics and gut commensals may provide important insight into the mechanisms driving cardiovascular disease etiologies, and thus offer an exciting avenue for novel therapeutic exploration.

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