DIET INFLUENCE OF LACTOBACILLUS UCC118 COLONIZATION

By

Jessica Gutgsell, RDN

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Jessica Gutgsell, RDN

has been approved

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Mentor/Advisor	Date
	06-14-16
Member	Date
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Abstract

Lactobacillus salivarius salivarius UCC118 (UCC118) is found in the human gut in small numbers and it has been shown to successfully colonize the human intestine when taken as a supplement at 10^9 colony-forming units per day. Colonization of bacteria in the *Lactobacillus* genus has been associated with diets high in protein and refined carbohydrate, but evidence for associations of UCC118 supplement colonization with specific diet variables is lacking. We compared evidence of colonization of UCC118, given at a dose of 10^8 colony forming units per day, in two groups: one group (n=20) taking the supplement in pill form, the other group (n=20) taking the supplement mixed into a yogurt medium. The effectiveness of delivery methods has not been compared for this strain and dose. In fecal colony samples, the group taking the supplement in yogurt medium showed significantly greater odds of colonization compared to the pill-supplemented group. This could be due to a symbiotic relationship between the yogurt medium and supplement strain, by way of prebiotics or natural probiotics in yogurt. The diets of the two groups, via three 24-hour recalls, were also compared to explore if diet variables were associated with colonization. A higher intake of percent calories from protein and grams of animal protein was associated with decreased colonization in the group that consumed the supplement in pill form while the other diet variables and the pill plus yogurt group showed no associations. This is contrary to the previous research on the *Lactobacillus* genus showing increased colonization in diets high in protein, although it may not be accurate to generalize the behaviors of one species to others in the genus. Dietary variables were similar across the 40 participants and this lack of diet diversity may have limited our ability to observe significant associations.

Specific Aims

The human gut microbiome influences systemic immunity, nutritional status, digestion and absorption, inflammatory state, and gene expression in addition to having the ability to alter many disease processes. Many health professionals promote probiotics in various forms to "improve the gut microbiome" without adequate supportive studies on colonization of specific probiotic strains, dose, or delivery methods. Humans ingest probiotics in multiple oral forms including in yogurt, fermented foods, and dietary supplements. Numerous supplements are available in powder-filled capsule form and they vary in heat-sensitivity, strain mix, dosage and quality. Many of the probiotic formulas available to the public include *Lactobacillus* strains. The *Lactobacillus* group of bacteria comprises a comparatively small population of the natural human gut microbiome but is important for production of short chain fatty acids (primarily acetate, butyrate, and propionate) from fermentable carbohydrate substrate. *Lactobacillus salivarius salivarius* strain UCC118 has been shown to effectively colonize the gut in doses at or above 10^9 colony-forming units (CFU) per day delivered in yogurt or fermented dairy products. The UCC118 strain we used is the clinically available dose of 10^8 CFU per day in room-temperature stable capsules. This specific dose in capsule form has not been studied for colonization effectiveness in healthy humans.

The strains and number of microbes in the human gut depend on numerous factors including gut luminal and mucosal pH, available nutrient substrate, individual patient gene profile, age, gender, and medication use. The types of foods ingested, especially the types of fermentable carbohydrates (prebiotics), can influence the form and quantity of probiotic colonization. Studies on associations of *Lactobacillus* strain abundance with diets such as

high or low protein, high or low fat, or vegetarian diets, have shown mixed results. Processed carbohydrates, food additives, and artificial sweeteners have been correlated with unfavorable influences on human gut *Lactobacillus* colonization. Given what is known about the impact of substrate on gut colonization, habitual diet could greatly affect supplemental UCC118 colonization.

The primary goal of this study was to determine if encapsulated shelf-stable UCC118 at the dose of 10^8 CFU/day would colonize the intestine of healthy human adults. Secondary goals included identifying which delivery form and/or diet variables affect colonization. We hypothesized that colonization of UCC118 in the gut would be greater when suspended in yogurt rather than the same dose in capsule form. We tested this hypothesis with the following specific aims:

Specific Aim 1: Determine the presence of UCC118 colonies in fecal samples before, during, and after 3 weeks of daily supplements via room-temperature stable capsules for ingestion alone or for mixing with yogurt prior to ingestion. We hypothesized UCC118 colonization would be greater when delivered in yogurt as compared to capsules alone.

Specific Aim 2: Examine the interaction between dietary intake during the study and incidence of UCC118 colonization. Dietary intake was assessed using three 24-hour diet recalls, including two weekdays and one weekend day, while participants were taking the supplement. We expected that if the groups of subjects include significantly different diet variables such as types of carbohydrate and protein, some associations with colonization would be present. We hypothesized that diets high in fruits and vegetables and low in red meat and simple carbohydrates would promote greater UCC118 growth.

Introduction

Probiotics, including those in the *Lactobacillus* group, are commonly used by the public for a variety of reasons. They are taken for general health benefit, as part of a disease management plan, or as treatment for specific diseases. In the current study, we compare colonization between supplementation of *Lactobacillus salivarius salivarius* UCC118 powder in pill form to supplementation of the same powder mixed with yogurt. We also looked for diet variables that may be correlated with colonization. Although there are only a handful of studies on *Lactobacillus salivarius* UCC118 specifically, there is evidence to believe there may be colonization differences between our study groups, as well as differences in colonization based on diet components.

Using bacterial cultures from stool samples is common practice for predicting the composition of the human microbiome. It is a relatively simple and non-invasive way to collect clues as to which bacteria may be present in the mucosal microflora of the intestine. Fecal microbiota can be a combination of shed mucosal bacteria as well as bacteria that have not adhered to the colon. Since intestinal mucosa cells shed at a rapid rate, it is assumed that specimens taken from fresh fecal samples will reflect the major diversity; however it is hard to isolate exactly where in the colon, and in what proportions, the microflora occur [1]. Studies comparing intestinal site samples to fecal samples tend to be small. Generally, fecal populations and diversity correspond with luminal samples when using rDNA analysis but reliability can vary between individuals [2].

Gut microbiome and probiotic activity

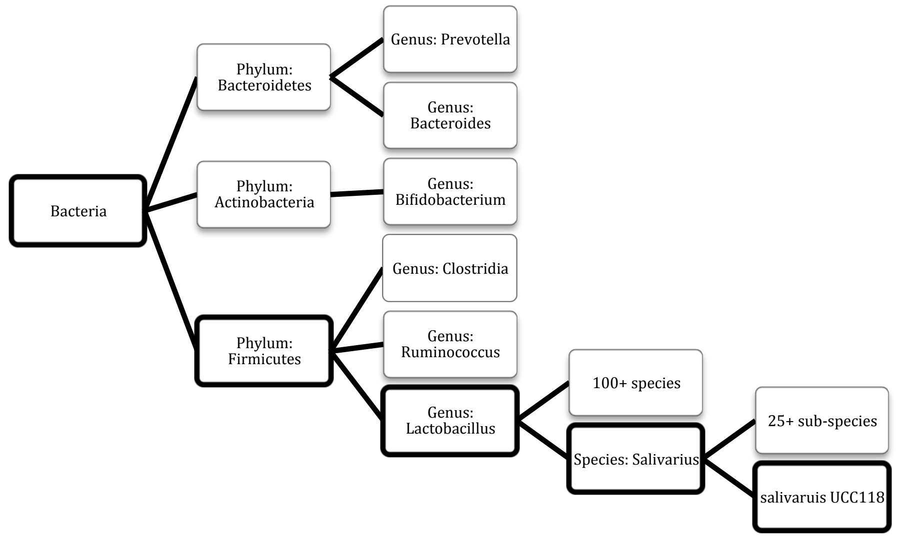
A mutualistic relationship exists between host and microflora. The gut microbiome provides multiple benefits to human hosts including protection from pathogen insult and growth, immune modulation, digestion aid, production of necessary metabolites, and nutrient scavenging [3]. Probiotics also may be able to affect gene expression [4]. Without direct modulation, microbiomes tend to be fairly stable within a specific individual but inter-individual variation is high [5, 6]. The strain and number of microbes in the gut depends on factors including the host's culture, stress, genetics, physical activity, disease, age, medication use, diet, intestinal pH, and available substrate. Not only does the host's diet affect gut microflora, but also the microflora present in the gut can affect how diet components are utilized. Gut microbes are able to ferment diet components such as insoluble fibers and these fermentation end products contribute to the total energy of the host [6]. In addition, the microbiome can alter the function and availability of vitamins, minerals, and drugs. The specific composition of an individual's microbiome could influence their susceptibility to disease or weight gain due to these changes in microbial functionality. Host diets have the potential to have cascading effects on health in countless ways, but many specifics about the diet-microbiome relationship are still unknown.

With so many factors affecting the microbiome composition, it is very difficult to predict how specific diet components or changes will affect specific probiotic species. Each substrate from the diet has a different structural make-up and utilization rate by microbes. Some bacterial species show greater modulation with changes to diet than others [5], so that changes in diet can create environments that allow certain species to outcompete others. It is thought that species that resist the effect of diet may either have a better

ability to utilize multiple human diet components for energy, or small changes in a particular species may not be detectable.

Fecal microbes can reach numbers of 10^{14}, with up to 160 different species within an individual [7]. The three phyla found in largest numbers in the human colon include *Bacteroidetes, Actinobacteria,* and *Firmicutes,* to which *Lactobacillus salivarius* belongs (**Figure 1**). Three dominant enterotypes have been seen in gut microbial analysis [8]: those high in *Bacteroides* or *Prevotella,* of the *Bacteroidetes* phyla; and those high in *Ruminococcus,* of the *Firmicutes* phyla. The *Lactobacillus* genus contains over 100 species, while the *Lactobacillus salivarius* clade has been shown to contain at least 25 sub-species [4, 9]. Bacteria of the *Lactobacillus* genus are most often found in the small intestine, although they can also be found in large numbers in the colon. Overall, they comprise a comparatively small population of the human gut microbiome; *Lactobacilli* are found in much smaller populations of total fecal bacteria than those of the *Bacteroidetes* phyla (<1-3% vs 12-60% respectively) [4, 9]. *Lactobacilli* have pro-adhesion properties, which make them likely to colonize the gut when supplemented, although long term increases in colonies after supplementation have not been seen [10, 11].

Figure 1. Bacterial Classification of UCC118 in terms of Phylum, Genus, and Species.



To the host's benefit, probiotic supplementation can help prevent intestinal infections and potentially decrease antibiotic use [9, 12]. When taken orally, probiotics must survive gastric acid and bile to colonize the large intestine. *Lactobacillus salivarius* species have the ability to bypass inherent host defenses, such as antimicrobials and acidic environments, to successfully colonize the human gut and prevent growth of pathogenic bacteria [9, 12]. One way that they inhibit growth of pathogenic bacteria is by creating an acidic environment in the gut via lactic acid production [11, 13]. *Lactobacillus salivarius* can also benefit the host by producing bacteriocins, which inhibit pathogenic bacteria and protect gut barrier integrity [9, 14]. The bacteriocin ABP118 has been shown to protect against Listeria monocytogenes in mice when supplemented with *Lactobacillus salivarius salivarius* UCC118 at 10^9 CFU [15]. Along with decreasing pathogenic viability, this strain could also decrease inflammatory activity and stimulate the gut immune processes [9, 14]. UCC118 has shown potential to boost gut immunity by modulating epithelial pro-inflammatory responses. When added to a colonic epithelial cell culture medium at 10^7

[16], UCC118 induced regulatory cytokine secretion from dendritic cells and reduced inflammatory Interleukin-8 secretion. Other epithelial cell interactions include increasing tight-junction integrity. Weakening of the tight junctions allows antigen penetration, stimulates the inflammatory response, and is a common trait of gastrointestinal disorders like Irritable Bowel Syndrome. The UCC118 strain has been shown to protect against hydrogen peroxide-induced decreases in barrier function as well as other threats to tight junctions [9, 14]. All of these benefits support optimal health by maintaining gut homeostasis. If the intestinal lumen is healthy, it will function at its best ability to digest and absorb nutrients, provide a protective barrier, and maintain a healthy immune system.

Diet and microbiome: Fermentable carbohydrate

Up to 95 percent of carbohydrates (CHO), protein, and fat are absorbed in the human GI tract before they reach the large intestine [4, 6]. The term "prebiotics" is used to define human diet components that pass undigested and unabsorbed to the large intestine, where they are fermented by microbes and used to promote growth of those populations[4, 6]. These include non-digestible materials like inulin, fructo-oligosaccharides (FOS), lactulose, galacto-oligosaccharides, resistant starch, and plant fibers. Most bacteria can use CHO as an energy source but bacterial groups vary in what other substrates can be used for energy [7]. Variations in nutrient composition of the host diet influence the diversity and composition of the microbiome in the large intestine. This also makes it likely that the microbiome will change in response to diet. Products of microbial fermentation in the gut include short chain fatty acids (SCFA), acetate, propionate, and butyrate. The *Firmicutes* phylum can utilize resistant starches and/or breakdown lactate and produce butyrate,

while *Lactobacilli* specifically are important for the production of short chain fatty acids (SCFA) from fermentable carbohydrate which can be used by the host for energy [5, 17].

There is a positive association between dietary fiber intake and SCFAs in the intestine [12]. A study of high CHO vs. low CHO diets on microbiome composition by Brinkworth et al. [18] found that high CHO diets increased fecal SCFA. This large cohort study was conducted on 91 overweight and obese participants. All were assigned to weight loss diets including either high CHO or low CHO foods for 8 weeks. The group on the high CHO diet showed increased fecal SCFA, indicating more fermentation by probiotics. The low CHO diet, which included very few fruits, vegetables, and grains, led to a decrease in acetate, butyrate, and total fecal SCFA; indicating less fermentation of CHO. Specifically, butyrate concentration was 30-60% lower in those on the low CHO diet. Counts of *Lactobacilli* did not change significantly with either diet, although there is a possibility that a change occurred but was undetected. This study showed that type and amount of non-digestible CHO in the diet could impact the probiotic fermentation activity in the gut.

Weight loss diets consisting of high or low CHO were compared in another study of 23 obese subjects by Duncan et al. [17]. All subjects spent four weeks on each of two diets: high-protein, low-CHO or high-protein, moderate-CHO. While on the low-CHO diet, there were reductions in key butyrate-producing bacteria of the *Roseburia* and *Eubacterium rectale* groups within the *Firmicutes* phyla, as well as *Bifidobacteria*, but no reduction of total *Firmicutes*. Butyrate is used as an energy source by colonocytes, so reductions of these butyrate-producing bacteria could have negative effects on function and health of the colon. Butyrate also regulates anti-inflammatory responses in the colon and may help

protect against colon cancer [16]]. Diets higher in fermentable CHO could be beneficial in many ways, including supporting a healthy gut microbiome.

We hypothesized that colonization of UCC118 would be greater in the group that ingested it suspended in yogurt because the yogurt could provide not only physical protection, but also provide an energy substrate for the *Lactobacillus* bacteria. Prebiotics have been shown to be helpful when ingested alongside probiotic supplements. Rajkumar et al. [19] studied the effect of *Lactobacillus salivarius* UBLS22 (UBLS22) supplementation with or without accompanying prebiotic fructo-oligosaccharides on 45 healthy young adults. Each adult was on one of three interventions for 6 weeks: UBLS22 supplement alone, the probiotic group; UBLS22 supplement with prebiotic fructo-oligosaccharides, the symbiotic group; or placebo. Fecal counts of UBLS22 were significantly increased in both probiotic (7.23 ± 0.11 log_{10}CFU/g) and symbiotic groups (7.96 ± 0.10 log_{10}CFU/g) compared to placebo (6.61 ± 0.12 log_{10}CFU/g) and baseline, although a greater effect was seen in the symbiotic group.

Collins et al. [10] also studied the effect of prebiotics along with probiotic supplementation. They found that UCC118 at a concentration of 10^{10} CFU/day significantly colonized the ileum in participants who received the probiotic in fermented milk, as well as those who received it in fresh milk. Probiotic colonization was compared between groups receiving UCC118 in either pasteurized fresh or fermented milk and control groups receiving one of the two milks only. Both groups receiving the supplement showed significant colonization of UCC118 of the large intestine after 21 days of supplementation and there was no UCC118 colonization seen in either of the control milk-only groups. The group receiving the supplement delivered in fresh milk showed 15 times more fecal counts

of UCC118 than the group receiving it in fermented milk. The authors hypothesized that this could be due to the increased gastric transit time of fermented milk. In contrast, fermented milk delivery methods showed an 11.5-fold increase in total *Lactobacilli* populations and a 200-fold increase in total enterococci populations compared to baseline, while the total numbers of *Lactobacillus* and enterococci in fresh milk were not significantly different compared to baseline. Five participants still showed colonization of UCC118 21 days after supplement stopped (four from the fresh milk group and one from the fermented milk group). Those who received the supplement in fermented milk showed a greater increase in total probiotic growth, indicating a symbiotic relationship between delivery substrate and resident gut probiotics.

Diet and microbiome: Macronutrient composition

Macronutrient composition of the host diet can also affect microflora type. *Lactobacillus* species have consistently been found to be more numerous in diets typical of the western world: those high in fat, high in protein, and low in fiber. After four weeks, *Lactobacillus* populations in a small cohort study (n=8) [20] were found to be significantly greater when they were fed an omnivorous diet of 23% protein, 45% fat, and 32% CHO for four weeks than when they were fed a nonmeat diet of 20% protein, 30% fat, and 50% CHO for the same amount of time. Stool samples were collected for four days after the study diet and analyzed for bacteria, including *Lactobacillus* species. On the nonmeat diet, average values of *Lactobacilli* were 9.42 $\log_{10}$ per gram dry feces and on the omnivorous diet, stool values were 10.14 $\log_{10}$ per gram dry feces. Van Faassen et al. [11, 21] did a similar study on 12 men where it was found that *Lactobacilli* were significantly lower on a

vegan diet and highest on a lacto-ovo diet. Participants followed each diet for 20 days: a western-type diet, lacto-ovo diet (consuming milk, cheese, and eggs), and vegan diet (excluding all animal products). This shows that components of the animal proteins could be influencing *Lactobacillus* colonization.

Drastically different cultural diet patterns have also been shown to alter microbiome diversity. A significantly different microbiome was detected in a cross-sectional study by De Filippo et al. [22], who recruited 29 children, ages 1-6 years, from rural Africa or Florence, Italy. The African children's diet was mostly vegetarian, higher in fiber (total fiber content 10-14 g/d), and included minimally processed foods. The Italian children consumed diets high in animal protein, refined carbohydrates, and fat, while low in fiber (5-8 g/d). The diet of the African children was high in whole grains, which provide fiber, oligosaccharides, resistant starches, and fermentable carbohydrates. The *Firmicutes* to *Bacteroidetes* ratio was four times higher in Italian children although overall microbial diversity and richness were higher in the African children. It is hypothesized that the microbiome differences are due to evolution of the GI tract environment to best utilize the materials ingested for energy. *Firmicutes* populations, to which *Lactobacillus salivarius* belongs, seem to thrive better on more omnivorous diets.

Even though changes in diet variables seem to affect species of microflora, diet changes may not have an effect on enterotypes. A controlled feeding study by Wu et al. [8] found that diets containing different ratios of macronutrients were able to change the composition of probiotic species within an enterotype (those dominated by *Bacteroides*, *Prevotella*, or *Ruminococcus*), but not change the main enterotypes in the gut. For ten days, ten participants were given either diets of high-fat/low-fiber foods or low-fat/high fiber

foods and stool samples were collected every day. Differences within resident taxa of individuals were seen within 24 hours, although the taxa that changed were different between subjects. All subjects were dominated by the *Bacteroides* enterotype at start and remained so throughout, indicating that major enterotype groups did not change significantly within any of the individuals, despite the extreme diets. There was still a great deal of inter-individual variation within enterotype. This was a short-term diet change; perhaps greater variations or shifts in enterotypes would be seen in longer interventions.

To support the theory that enterotypes are affected by longer-term diet patterns, a cross sectional survey of major fecal enterotypes and diet associations in 98 individuals found that diet patterns were associated with specific enterotypes [23]. Diet patterns were assessed using 24-hour diet recalls and Food Frequency Questionnaires (FFQ) and compared to fecal sample data analyzed by rDNA sequencing. Significant associations between diet and enterotype were seen only with the FFQ data, which could indicate that long-term diet may have influenced enterotype composition more than short-term diet. Inverse associations were found in total microbes with fat and protein foods versus carbohydrates and plant products. *Bacteroidetes* and *Actinobacteria* phyla were positively associated with fat and negatively associated with fiber intake. *Firmicutes* and *Proteobacteria* were positively associated with fiber and negatively associated with fat. *Lactobacillus* species were not studied specifically. A dominant *Bacteroides* enterotype was associated with intake of animal protein, amino acids, and saturated fats. The *Prevotella* enterotype was positively associated with carbohydrates and simple sugars and negatively associated with animal protein and saturated fat. Lower-level taxa within each phyla group did not correlate significantly with diet patterns.

A high-fat diet was studied in mice by Hildebrandt et al. [24] to see how it affected the microbiome composition of wild-type vs. RELM-B knock-out type. RELM-B is a colonic goblet cell gene involved in gut immune function; intestinal microflora environments, as well as high-fat diets, have been shown to increase expression of RELM-B. Changes in the microbiome of wild type vs. RELM-B knockout type mice were studied when both groups were fed standard chow followed by a high-fat diet for 3 months. The wild type became obese on the high-fat diet while the knockout mice on the high-fat diet did not, allowing the researchers to compare changes in the obese microbiome to that of non-obese. Fecal samples were analyzed on both diets. Both groups showed similar bacterial colonies while on the standard chow diet (*Bacteroidetes* dominated) and both groups showed changes in the microbiome when they were switched to high-fat diets; total levels of *Proteobacteria*, *Firmicutes*, and *Actinobacteria* increased. When further analyzed on the DNA level, changes in gene expression of bacteria were seen, including: increased genes involved in membrane transport, transcription, cell motility, and sugar assimilation; and decreased genes involved in CHO and energy metabolism. The high-fat diet caused gene expression changes in the entire bacterial community. Both obese and non-obese mice on the same diet had similar microbiome changes. This shows that diet can affect the microbiome more significantly than the presence of obesity and it can cause microflora and gene expression changes.

When the genome of UCC118 was analyzed by Claesson et al. [25], they found signs that this species has the ability to adapt to a variety of intestinal environments and host diets. *Lactobacillus salivarius salivarius* UCC118 is unable to produce eight amino acids, which makes it more dependent on taking up amino acids from the environment. For this

reason, they have a broader set of peptide and amino acid transport functions [26]. This could increase the bacteria's affinity for higher-protein gut environments. UCC118 is also able to ferment many monosaccharaides and disaccharides [25], which could influence its growth in high carbohydrate environments.

Diet and microbiome: Vegetarian diets

Studies of plant-based diets compared to animal-based diets show significant differences in microbiomes of subjects. To study short-term diet influences, David et al. [27] followed a group of 10 healthy American subjects that were fed either a plant-based or animal-based diet for 5 days and observed for 6 days afterwards. RNA sequencing was used to measure bacterial colonies. In the animal-based diet group, diet-associated gut microbiota changed within a day of the diet initiation and reverted back to baseline two days after it ended. Bile-tolerant bacteria such as *Bacteroides* increased, while bacteria of the *Firmicutes* group decreased. The animal-based diet was considerably higher in fat and protein and sparse in fiber. High-fat diets have been shown to cause more bile acid secretion. Similarly, the animal-based diets had lower levels of carbohydrate fermentation products and higher levels of amino acid fermentation products, showing that the bacteria present changed to utilize the diet substrate best. There were also gene expression changes, such as an increased expression of genes responsible for degrading polycyclic aromatic hydrocarbons, carcinogenic compounds found on charred meat, on the animal-based diet. The rapid changes in the bacteria found in human fecal samples provide evidence that the microbiome is sensitive to changes in diet. The decrease in *Firmicutes* in the human population on a high-fat diet is in contrast to the previously mentioned increase

in murine concentrations of *Firmicutes* on high-fat diets [24]. This could be due to the different subject species or study design.

Vegan and vegetarian diets were studied by Zimmer et al. [11] in a case-control study. Omnivorous controls were matched to 144 lacto-ovo vegetarians and 105 vegans. Different species were seen between the three groups but the total numbers of fecal bacteria were not significantly different. Higher CHO diets that included more fiber produced more SCFA and lower pH in the gut. Numbers of *Lactobacillus* species were not significantly different between groups. Vegans had a significantly lower stool pH than either the vegetarian or control group. This pH difference could be due to the fact that vegan diets included significantly more CHO and fiber. The lack of difference in *Lactobacillus* species conflicts with earlier-cited studies on higher *Lactobacillus* and *Firmicutes* seen with omnivorous diets.

Diet and microbiome: Other diet factors

Less-studied diet factors may also affect the gut microbiome. Gluten-free diets have been seen to reduce *Lactobacillus* species in small cohorts [6]. Few studies have looked at omega-3 fatty acids and *Lactobacilli* specifically but in those that have, omega-3 fatty acids have been positively associated with *Lactobacilli* in mice and humans [4]. Artificial sweeteners may also affect colonization since components of artificial sweeteners cannot be digested by the human gut but can be fermented by bacteria. *Lactobacillus salivarius salivarius* UCC118 has the ability to catabolize the sugar alcohol sorbitol [25]. This could promote its ability to colonize in individuals who consume this non-nutritive sweetener. The sugar alcohol maltitol has been shown to increase species of *Lactobacilli* as well as

amounts of SCFA after 6 weeks. Contrarily, sucralose supplementation for 12 weeks has increased gut pH and reduced some species, including *Lactobacilli* [6, 23].

Summary

Bacterial populations, and specifically *Lactobacillus* strains, have shown changes or differences in counts and types when different diet types have been compared in a variety of study designs. The different diets are usually characterized by level or type of protein, fat, or carbohydrates. Although studies on UCC118 are few, multiple studies have shown that *Lactobacilli* populations increase with an increased intake of low-fiber, refined CHO, high fat, and high protein foods. We know that each species can have a different reaction to various diet components. The benefits of probiotic supplementation are vast and include reduced inflammation, improved gut function, and increased immune response. Knowing which diets best support growth of these bacteria will help individuals gain maximum health benefits from supplementation.

Methods and Procedures

Research Design

This study was a randomized two-arm trial "Intestinal colonization of a probiotic strain, *Lactobacillus salivarius salivarius* UCC118, delivered in yogurt or encapsulated format". To enroll, potential participants attended a screening visit at Oregon Health & Science University (OHSU). At the screening visit, participants received a detailed description of the study aims and procedures, inclusion and exclusion criteria (**Table 1**), study instructions, and needed materials. They also received a short description of the 24-

hour recall procedure from a Registered Dietitian. Forty healthy volunteers who met the inclusion and exclusion criteria were randomly assigned to one of two groups. Both received UCC118 at a dose of 10^8 once per day for 3 weeks; one group of 20 participants received the supplement in capsule form and the other received the capsule contents delivered in 6 ounces of yogurt. Randomization was stratified by sex. There was no blinding. Diet patterns were assessed using three 24-hour diet recalls collected during the 21-day supplementation period. Fecal samples were collected by participants and delivered to OHSU to determine colonization levels of *L. salivarius salivarius* UCC118.

Recruitment

Study recruitment posters were displayed throughout the OHSU campus. Participants were also recruited by emails and word-of-mouth from study staff. Recruitment was open to OHSU staff and students as well as to the public.

Consent forms included a detailed description of the study purpose and procedures, schedule, risks, benefits, liability, participant rights, and confidentiality agreement. To be included, participants had to be between the ages of 18 and 65 and be able to give informed consent. Use of non-study probiotic supplements or yogurts during the study or within one day of beginning the study also excluded participants. In addition, participants could not have changes in over-the-counter supplements or medications, use narcotics, participate in other investigational studies, or be involved in weight loss programs within 30 days of this study. Use of corticosteroids, stimulant drugs, antibiotics, nicotine products, or significant changes in exercise habits 15-45 days prior to or during the study were also prohibited. Prospective participants were also excluded if they had a significant medical history, a

current significant diagnosis (i.e. Hepatitis B or C, diabetes, congestive heart failure), clinically significant lab results, or allergies or hypersensitivities to the products.

The participants were randomized using a computer generated randomization scheme, which produced a list of participants that could be assigned to study arms as they arrived at visit one. Each participant was assigned a coded label as enrolled. The label contained identification of the treatment arm (P= probiotic only, PY= probiotic and yogurt) and consecutive numbering (01, 02, 03, etc.).

Table 1. Inclusion and Exclusion Criteria.

Inclusion Criteria
Ability to give informed consent.
Subject is between 18 and 65 years of age.
Subjects that are pregnant or become pregnant during the study period will be included.
Exclusion Criteria
Use of oral probiotic dietary supplements or probiotics-containing yogurts for one day prior to and for the duration of the study.
Initiation of therapy, or change of dose of OTC preparations, nutritional supplements, and/or medical foods for treatment of dysglycemia within 30 days prior to Day 0 and for the duration of the study.
Use of medications, classified as narcotics, 30 days prior to screening, and for the duration of the study.
Use of an investigational drug or participation in an investigational study within 30 days prior to Day 0, and for the duration of the study.
Use of oral or injectable corticosteroids in the 45 days prior to Day 0, and for the duration of the study.
Use of any antibiotics within 4 weeks of study enrollment through the conclusion of study participation.
Known allergy or hypersensitivity to study products.
Clinically significant abnormalities in medical history, physical examination, or laboratory results.
No initiation of a new or significant change of an existing exercise regimen within 15 days prior to Day 0, and for the duration of the study.
No current involvement or within 30 days of Day 0 of a significant diet or weight loss program, such as Atkin's or other low-carb diet programs, very low calorie liquid diet programs (such as Optifast, Medifast, and/or HMR), or any diet that has led to a weight loss of 5% of body weight over a period of ten weeks.
No serious, unstable illnesses including cardiovascular, hepatic, renal, gastroenterologic, respiratory, endocrinologic, neurologic, immunologic, or hematologic disease.
Known infection with Human Immunodeficiency Virus (HIV), Tuberculosis, or Hepatitis B or C.
Smoking or use of nicotine or cannabis-containing products 30 days prior to screening, and for the

duration of the study.
Use of drugs, such as cocaine, phencyclidine [PCP], and methamphetamine within 15 days prior to Day 1, and for the duration of the study.
Inability to comply with study and/or follow-up visits.
Any other concurrent condition which, in the opinion of the Investigator, would preclude participation in this study or interfere with compliance.
Subjects with a current diagnosis or personal history of: a. Any cardiovascular disease including myocardial infarction (MI), angina, cardiovascular surgery, congestive heart failure (CHF), cardiac arrhythmias or conduction abnormalities, cerebrovascular accident, transient ischemic attack (TIA), or peripheral vascular disease, deep vein thrombosis (DVT), or pulmonary embolus (PE). b. Any personal history of pre-diabetes, diabetes mellitus Type 1 or 2, or hypoglycemia. c. Any autoimmune disease, such as inflammatory bowel disease (including Crohn's Disease and/or ulcerative colitis), multiple sclerosis, rheumatoid arthritis, systemic lupus erythematosus, polymyositis, scleroderma and/or thyroiditis. d. Any significant liver or kidney disease, such as cirrhosis or non-alcoholic fatty liver disease, glomerulonephritis, and/or undergoing dialysis treatment. e. Any malignancy (with the exception of basal or squamous cell carcinoma of the skin if adequately treated and no recurrence for >five years). f. Any serious mental illness, including a history of attempted suicide. g. Any subject with known history of bacterial endocarditis

Participant visits

After recruitment, participants met with the study coordinator a total of six times. Visit one was the lengthiest and included screening, consenting, enrollment, and distribution of study materials. Materials included supplements and fecal sample collection kits. One week later, at visit two, participants met with the study coordinator to deliver the first stool sample. Visit three was three weeks after the second visit and participants returned any unused supplies, the second fecal sample, and study questionnaires. At the subsequent three visits, one week apart, participants delivered fecal samples only.

Supplementation

L. salivarius salivarius UCC118 was taken by the participants in a capsule or yogurt medium at 10^8CFU/day within two hours of eating. Participants were encouraged to take the supplement at the same time each day. Depending on which study arm they were assigned to, supplements were given alone or with one six-ounce container of yogurt per pill with mixing instructions. Participants received the entire 21-day supply of supplements and yogurt, if applicable, at the first visit. They were instructed to follow their normal diet, excluding non-study probiotic supplements or yogurts. Yogurt used was low-fat Tillamook brand flavors strawberry or vanilla. This yogurt does not have probiotics added besides *S. Thermophilus*, *L. Bulgaricus*, *L. Acidophilus*, and *L. Bifidus* used in the production process.

Sample Collection

Participants collected fecal samples five times during the study; at baseline, within 12 hours of the final probiotic dose, seven days after the final probiotic dose, 14 days after the final probiotic dose, and 21 days after the final probiotic dose. Participants brought samples to OHSU, where the samples were stored in a -15 degree freezer until all samples were received. They were then sent off-site for analysis of UCC118 composition using strain-specific polymerase chain reaction techniques (PCR) with strain specific primers. Both groups were compared to each other and participants were compared to themselves for pre and post levels of UCC118 in the feces. Fecal collection kits were purchased from DNAGenotek and all samples were sent to Research and Testing Laboratories in Lubbock,

Texas. Colonization of UCC118 was expressed semi-quantitatively using an ordinal scale of none, weak, moderate, strong, and very strong based on the PCR signal.

Diet analysis

A Registered Dietitian conducted three random 24-hour recalls over the phone during the three weeks of UCC118 supplementation. Recalls included two weekdays and one weekend day. Recalls were conducted with pen and paper using the multiple-pass method. Data was then entered into the Nutrition Data System for Research (NDS-R) software program version 2014 for analysis. Data from the three recalls was averaged, then analyzed and compared to colonization rates by a Registered Dietitian with the assistance of a statistician.

Statistical Analysis

The statistical analysis was done using the categorical data, provided by Research and Testing Laboratories, of the samples' UCC118 colonization level. Diet analysis data from the NDS-R software was used as continuous and categorical data. Results were expressed as the mean +/- standard deviation and 95% confidence interval for all variables.

For Specific Aim 1: We used a Pearson chi-square test and non-parametric rank sum tests to determine if colonization levels of UCC118 throughout supplementation are associated with treatment group (Pill or Pill + Yogurt). Fecal sample detection levels of undetectable ("none") and detectable ("weak", "moderate", "strong", or "very strong")were given the numerical values 0 and 1 respectively.

For Specific Aim 2: Each continuous diet variable was treated as the response in a two-way analysis of variance (ANOVA) with treatment group (Pill or Pill + Yogurt), colonization status (colonization or no colonization), and the interaction between these two variables as explanatory factors of interest. Significance ($p < 0.10$) of the overall F-test indicated that mean response was associated with at least one of the explanatory variables. All ANOVA models used robust standard errors for estimation and testing to guard against potential problems of measurements exhibiting non-constant variance or lack of normality.

Results

Each treatment group included 20 participants, evenly matched by sex. Mean age and BMI were similar between groups. In the Pill group, ages ranged from 23-54 years, with a mean age of 34±8 years. In the Pill + Yogurt group, ages ranged from 26-50, with a mean of 34±7 years. Mean Body Mass Index (BMI) was 27±6 (range 19-46) in the Pill group and 26±5 (range 19-40) in the Yogurt + Pill group (**Table 2**). None of the participants met the classification for underweight based on BMI. Five participants in the Pill group met classification for overweight based on BMI (BMI 25.0-29.9) and four met classifications for obese (BMI ≥30). In the Pill + Yogurt group, seven participants met classification for overweight and four were obese. All participants were healthy adults and met criteria to participate. All participants completed the study.

Table 2. Participant Characteristics.

Group	Sex	Age (years) Mean ± SD	BMI (kg/m^2) Mean ± SD
Pill n= 20	Male: n= 10 Female: n= 10	34 ± 8	26.8 ± 6
Pill + Yogurt n= 20	Male: n= 10 Female: n= 10	34 ± 7	26.0 ± 5

Total participant numbers per group and by sex. Mean age and BMI ± standard deviation (represented by SD) by group.

Lactobacillus salivarius salivarius UCC118 colonies were not detected in any participant fecal samples at the baseline sample collection (sample one). Sample two, which was within 12 hours of the final probiotic dose, did show colonization levels, although it was the only sample out of the five that had detectable levels of UCC118 colonization. Fecal samples three (seven days after the final probiotic dose) through five did not show colonization retention. This indicates that, in this population, supplementation of UCC118 is not effective at creating colonies that last more than 7 days after probiotic supplements are stopped. The following results are based on colonization values for the second time point, within 12 hours of the last UCC118 dose.

In the pill treatment group, there were fourteen participants with no colonization and six with some level of colonization: weak, moderate, strong, or very strong (**Figure 2**). More participants had detectable levels of colonization in the Pill + Yogurt treatment group; seven participants showed no signs of colonization and thirteen showed a detectable level of colonization.

Figure 2. Colonization Levels by Treatment Group

Colonization Detection Level	Pill	Pill + Yogurt
none	14	7
weak	0	4
moderate	1	2
strong	1	0
very strong	4	7

Categorical designations of fecal colonization detection at sample two by treatment group.

The raw data of the five levels of colonization detection ("none" through "very strong") were condensed for statistical purposes due to the small number of participants in each category. When separated into two groups, no colonization or colonization, the proportion with colonization was found to be significantly different between the two treatment groups using a Pearson chi squared test (p=0.03) (**Table 3**). There was a greater amount of colonization with UCC118 among the Yogurt + Pill group compared to the Pill only group.

Table 3. Detected Colonization by Treatment Group.

	No Colonization	Colonization	n
Pill	14	6	20
Pill + Yogurt	7	13	20
Total	21	19	40

Condensed categories of fecal detection at sample two by treatment group (p= 0.03).

Diet variables included in the analysis were means of: total calories, grams of protein, grams of vegetable protein, grams of animal protein, total servings of meat, grams of total sugar, milligrams of artificial sweetener, grams of sugar alcohol, total servings of artificial sweeteners, grams of fiber, total servings of grains, ounces of whole grains, percent of calories from fat, percent of calories from CHO, percent of calories from protein, percent of calories from saturated fat, total servings of fruit, and total servings of vegetables (**Table 4**). The ANOVA of diet variables by treatment group and colonization status produced a significant F-test ($p<0.10$) for total percent protein, total percent fat, grams of fiber, and grams of animal protein. This indicated that the mean response was associated with at least one explanatory variable: treatment group, colonization status, or interaction between treatment group and colonization status. With these diet variables, we performed more specific tests ($p<0.05$) to identify the source and structure of the association, beginning with the treatment-colonization interaction and proceeding to main effects for treatment or colonization separately only if the main interaction was not statistically significant ($p>0.10$).