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Coaggregation patterns and surface characteristics of Lactobacillus rhamnosus GG under varying nutrient conditions

Amanda Rielinger

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Coaggregation patterns and surface characteristics of Lactobacillus rhamnosus GG under varying nutrient conditions

Abstract
Lactobacillus rhamnosus GG (LGG) is one of the best studied probiotic organisms. The ability of probiotics to adhere to other microorganisms and the intestinal epithelium is thought to play a major role in their protective functions. Coaggregation is an important mechanism for biofilm formation by microorganisms. The goals of this study were to examine the ways in which nutrient variation affects intercellular interactions between LGG and other gut microbes and the hydrophobic character of LGG. We hypothesized that nutritional variation may affect the ability of LGG to coaggregate and form biofilms and thus affect its probiotic characteristics and ability to colonize the gastrointestinal tract. Lactobacillus rhamnosus GG was cultured anaerobically in different formulations of tryptone, yeast extract, and glucose (TYG) medium in order to simulate a milk-based, plant-based, and meat-based diet. Since the gut is mainly colonized by microorganisms belonging to the Firmicutes and Bacteroidetes phyla, we tested the coaggregation ability between LGG and Bacteroides spp. and Parabacteroides spp. Our results suggest that coaggregation varies by media type, with LGG cultured in BeYG [Beef extract (meat protein), yeast extract, and glucose] medium exhibiting the highest coaggregation scores. Hydrophobicity values of LGG cells cultured in varying nutrient conditions differed significantly by media type, and LGG cultured in BeYG exhibited the highest hydrophobicity scores. These results indicate that different nutrient conditions may enhance the ability of LGG to colonize the human gut microbiome and enhance its ability to act as a successful probiotic.

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COAGGREGATION PATTERNS AND SURFACE CHARACTERISTICS
OF LACTOBACILLUS RHAMNOSUS GG UNDER VARYING
NUTRIENT CONDITIONS

By
Amanda Rielinger
A Senior Thesis Submitted to the
Eastern Michigan University
Honors College
in Partial Fulfillment of the Requirements for Graduation
with Honors in Biology

Approved at Ypsilanti, Michigan, on this date April 17, 2019

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Abstract

*Lactobacillus rhamnosus* GG (LGG) is one of the best studied probiotic organisms. The ability of probiotics to adhere to other microorganisms and the intestinal epithelium is thought to play a major role in their protective functions. Coaggregation is an important mechanism for biofilm formation by microorganisms. The goals of this study were to examine the ways in which nutrient variation affects intercellular interactions between LGG and other gut microbes and the hydrophobic character of LGG. We hypothesized that nutritional variation may affect the ability of LGG to coaggregate and form biofilms and thus affect its probiotic characteristics and ability to colonize the gastrointestinal tract. *Lactobacillus rhamnosus* GG was cultured anaerobically in different formulations of tryptone, yeast extract, and glucose (TYG) medium in order to simulate a milk-based, plant-based, and meat-based diet. Since the gut is mainly colonized by microorganisms belonging to the Firmicutes and Bacteroidetes phyla, we tested the coaggregation ability between LGG and *Bacteroides* spp. and *Parabacteroides* spp. Our results suggest that coaggregation varies by media type, with LGG cultured in BeYG [Beef extract (meat protein), yeast extract, and glucose] medium exhibiting the highest coaggregation scores. Hydrophobicity values of LGG cells cultured in varying nutrient conditions differed significantly by media type, and LGG cultured in BeYG exhibited the highest hydrophobicity scores. These results indicate that different nutrient conditions may enhance the ability of LGG to colonize the human gut microbiome and enhance its ability to act as a successful probiotic.
Introduction

The human gut microbiome is thought to play a predominant role in the regulation of immune response, protection against pathogens, and the breakdown and synthesis of nutrients (Xu and Knight, 2015). In addition, dysbiosis of the normal gut microbiota has been linked to a number of diseases, including inflammatory bowel disease, colorectal cancer, obesity, insulin resistance, metabolic syndrome, allergy, and autoimmune diseases (Rios-Covian et al., 2017; Valdes et al., 2015). However, colonization of the gut by microorganisms is not well understood. One hypothesis is that gastrointestinal microorganisms may develop as attached microbial communities, known as biofilms. Evidence of plasmid transfer, the expression of colonization factors, and the adherence of bacteria to mucin proteins suggests that biofilm formation occurs in the gastrointestinal tract. Gastrointestinal biofilms have been observed to adhere to the mucin layers both \textit{in vitro} in artificial mucin gels and \textit{in vivo} in the proximal large bowel of mice, rats, baboons, and humans. In addition, alteration in mucin production and biofilm formation has been linked to inflammatory bowel disease and colorectal cancer (Sicard et al., 2017).

Studying the mechanisms behind the cellular recognition and interaction involved in biofilm development will improve our understanding of how the gastrointestinal tract is colonized and maintained by various microbial species and how these microbial species impact human health.

Colonization of the human microbiome and biofilm development are affected by multiple environmental factors. In infants, who are colonized by microorganisms at birth, a number of factors, including delivery method, breastfeeding, and age of weaning are thought to influence the development of the microbiota (Nicholson et al., 2012). Diet is
another important factor known to influence the composition of the gut microbiome (Albenberg and Wu, 2014; Gentile and Weir, 2018; Xu and Knight, 2015). The presence of certain nutrients may also affect biofilm development, as oral biofilms are disrupted by the presence of the amino acid L-arginine (Kolderman et al., 2015). Thus, it is important to discern how nutritional shifts affect the underlying mechanisms of biofilm development in the gut in order to elucidate their overall effects on human gastrointestinal health.

One essential mechanism for biofilm formation is coaggregation (Stevens et al., 2015). First observed in oral bacteria, coaggregation is the cell-cell interaction that involves specific recognition and adherence of genetically different microorganisms to each other (Katharios-Lanwermeyer et al., 2014; Stevens et al., 2015). Coaggregation is thought to contribute to biofilm formation through two pathways (Figure 1). In the first pathway, individual cells in suspension recognize and adhere to cells in a developing biofilm. In the second, individual cells undergo coaggregation in suspension, followed by adhesion of the entire coaggregate or autoaggregate to the developing biofilm (Rickard et al., 2003). One study found that coaggregation between gut microorganisms may be weak, and thus may not play a major role in gut biofilm development (Ledder et al., 2008). However, as only 10 intestinal species were selected for this study, this may not be representative of all the interactions that occur in the gut. Others suggest that coaggregation occurs between a broad range of gut microorganisms (Schotten et al., 2016). Therefore, coaggregation may occur between gastrointestinal microbial species and influence biofilm formation in the gut.
Figure 1: Possible models for multi-species biofilms. Each different color corresponds to a different unknown, but genetically distinct, member of a microbial species. Identical colors indicate that they are members of the same species. After primary colonization of the substratum, which is covered in a “conditioning film” made of polysaccharides and proteins, single cells [pathway 1], coaggregates (2 or more species) [pathway 2], and autoaggregates (1 species) [pathway 2] may recognize and adhere to cells in the developing biofilm. Figure adapted from Katharios-Lanwermeyer et al. (2014).

Gut microorganisms predominantly belong to five phyla (Firmicutes, Bacteroidetes, Actinobacteria, Proteobacteria, and Verrucomicrobia), with more than 80% of species belonging to the Firmicutes and Bacteroidetes phyla (Rios-Covian et al., 2017). Of these species, those microorganisms that benefit the host when given in sufficient amounts are considered probiotics. Most probiotics are *Bifidobacterium* and *Lactobacillus* species, and can be found in foods and dietary supplements (Valdes et al., 2018). Probiotics are thought to benefit gastrointestinal health through excluding or inhibiting pathogens, enhancing epithelial barrier function, and/or modulating immune response (Jensen et al., 2012). One of the best studied probiotic microorganisms is *Lactobacillus rhamnosus* GG (LGG), which has been shown to provide a variety of health benefits to the host, including prevention of diarrhea, eczema, respiratory tract
disease, milk allergy, and dental carries in children and infants (Koskenniemi et al., 2009; Lebeer et al., 2018).

LGG may be a successful probiotic because of its ability to adhere to gastrointestinal surfaces. In addition, the ability to coaggregate is thought to be an important characteristic of probiotic microorganisms because the binding of large amounts of probiotic bacteria to the intestinal epithelium is thought to both prevent contact between pathogens and intestinal cells and modulate immune response (Polak-Berecka et al., 2014). A number of long pili adhesins on LGG’s cellular surface are thought to be responsible for this adherence ability (Velez et al., 2010). One of the best studied of these pili is the SpaCBA pili, which allows LGG to adhere to the intestinal mucus and epithelium and modulates immunoregulatory interactions (Lebeer et al., 2018). Since coaggregation involves microbial cell-surface proteins, or adhesins, that recognize and attach to complementary polysaccharide receptors found on the partner microbial species (Katharios-Lanwenneyer et al., 2014), it is possible that the adhesins on the surface of LGG, or even its pilus structures, may also allow it to coaggregate with other microorganisms.

In *Lactobacillus rhamnosus* strains E/N and PEN, adhesion to human intestinal cells is related to specific surface proteins, exopolysaccharides, and the hydrophobic character of the cell surface ((Polak-Berecka et al., 2014). Both adhesion and coaggregation are thought to be mediated by cell-surface proteins, or adhesins, and polysaccharide receptors (Katharios-Lanwenneyer et al., 2014). Therefore, any changes to the cell surface in terms of either protein or exopolysaccharide expression could affect a microorganism’s ability to coaggregate or adhere to the intestinal epithelium. In addition, cell-surface structures are related to the hydrophobic nature of the cell surface,
which influences the adhesion of microorganisms through electrostatic and van der Waals forces (Polak-Berecka et al., 2014). Examining how the cell surface of LGG changes in terms of protein expression and hydrophobicity in response to nutrient shifts will be helpful for determining the role that these properties play in adherence and coaggregation.

The aim of this study was to examine how nutritional variation affects cell surface properties of LGG and the intercellular interactions between LGG and other native gastrointestinal microorganisms. Specifically, this study examined coaggregation between interactions between LGG and *Bacteroides* spp. and *Parabacteroides* spp., both of which belong to the order Bacteroidales, the most abundant Gram-negative bacteria in the gut (Rios-Covian et al., 2017). In addition, to test the effects of different capsular polysaccharides on intercellular interactions between LGG and other microorganisms, coaggregation between LGG and ten capsular types of *B. thetaiotaomicron* will be examined (each *B. thetaiotaomicron* capsular type exhibits a different capsular polysaccharide) (Porter et al., 2017). We hypothesized that nutritional variation leads to changes in the expression of cell-surface structures by LGG. Since coaggregation is thought to be mediated by protein adhesins and polysaccharide receptors on the cell surface (Katharios-Lanwermeyer et al., 2014), any change to the cell surface, either in LGG or in *B. thetaiotaomicron*, may lead to changes in coaggregation patterns. Furthermore, these structural changes may also affect the hydrophobic nature of the cell surface.
Materials and Methods

**Culture Growth and Processing**

Twenty-two native human gastrointestinal *Bacteroides* and *Parabacteroides* strains and eight different mutant capsular types of *Bacteroides thetaiotaomicron* VPI-5482 were selected to determine the effects of nutrient shifts on the ability of *L. rhamnosus* GG ATCC 53103 (LGG) to coaggregate with Gram-negative gut microorganisms (Table 1). All bacterial strains were recovered from frozen stocks. All *Bacteroides* spp. and *Parabacteroides* spp. were grown anaerobically [GasPack jars, Gas Pack EZ (BD)] at 37°C in tryptone (Fisher Biotech, Fairlawn, NJ), yeast extract [Becton, Dickinson and Company (BD), Sparks, MD], and glucose (Acumedia, Lansing, MI) (TYO) broth media. TYO broth media was prepared in the laboratory according to the formula provided by Holdeman *et al.* (Holdeman *et al.*, 1977)

To test for the effects of nutrient variation on LGG, LGG was cultured anaerobically at 37°C in four different variations of tryptone (milk peptone), yeast extract, and glucose (TYG) broth media. TYG broth media variations were created to simulate dietary variation by exchanging the tryptone with soytone (BD, Sparks, MD) (SYG), a plant peptone, or beef extract (BD, Sparks, MD) (BeYG), a meat peptone, or glucose with fructose (Sigma-Aldrich, St. Louis, MO) (TYF). All cultures were harvested by centrifugation at 14,480 x g (10,000 rpm with Sorvall SA-600 fixed angle rotor in Sorvall RC5B Plus Centrifuge) at 4°C for 10 minutes. The cells were washed three times with coaggregation buffer and resuspended in coaggregation buffer prepared according to the formula provided by Kolenbrander and Williams ((Kolenbrander and Williams, 1981).
Table 1: Bacterial Strains used for this study. Strains were sourced from Eric Martens and Nicole Koropatkin in the University of Michigan Department of Microbiology and Immunology. Twenty-two native human gastrointestinal *Bacteroides* and *Parabacteroides* strains and mutant capsular types of *Bacteroides thetaiotaomicron* VPI-5482 were selected.

<table>
<thead>
<tr>
<th>Strain name</th>
<th>Designation</th>
<th>Properties</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Bacteroides caccae</em></td>
<td>ATCC 43185</td>
<td>Human gut bacterium</td>
</tr>
<tr>
<td><em>Bacteroides cel lulosilyticus</em></td>
<td>DSM 14838</td>
<td>Human gut bacterium</td>
</tr>
<tr>
<td><em>Bacteroides claus</em></td>
<td>DSM 22519</td>
<td>Human gut bacterium</td>
</tr>
<tr>
<td><em>Bacteroides dorei</em></td>
<td>DSM 17855</td>
<td>Human gut bacterium</td>
</tr>
<tr>
<td><em>Bacteroides eggerthii</em></td>
<td>DSM 20697</td>
<td>Human gut bacterium</td>
</tr>
<tr>
<td><em>Bacteroides finegoldii</em></td>
<td>JCM 13345</td>
<td>Human gut bacterium</td>
</tr>
<tr>
<td><em>Bacteroides fluxus</em></td>
<td>DSM 22534</td>
<td>Human gut bacterium</td>
</tr>
<tr>
<td><em>Bacteroides fragilis</em></td>
<td>ATCC 25285</td>
<td>Human gut bacterium</td>
</tr>
<tr>
<td><em>Bacteroides intestinai s</em></td>
<td>DSM 17393</td>
<td>Human gut bacterium</td>
</tr>
<tr>
<td><em>Bacteroides nordii</em></td>
<td>DSM 18764</td>
<td>Human gut bacterium</td>
</tr>
<tr>
<td><em>Bacteroides oleciplenus</em></td>
<td>DSM 22535</td>
<td>Human gut bacterium</td>
</tr>
<tr>
<td><em>Bacteroides ovatus</em></td>
<td>ATCC 8483</td>
<td>Human gut bacterium</td>
</tr>
<tr>
<td><em>Bacteroides sayersiae</em></td>
<td>DSM 18765</td>
<td>Human gut bacterium</td>
</tr>
<tr>
<td><em>Bacteroides stercor is</em></td>
<td>ATCC 43183</td>
<td>Human gut bacterium</td>
</tr>
<tr>
<td><em>Bacteroides uniformis</em></td>
<td>ATCC 8492</td>
<td>Human gut bacterium</td>
</tr>
<tr>
<td><em>Bacteroides vulgar us</em></td>
<td>ATCC 8481</td>
<td>Human gut bacterium</td>
</tr>
<tr>
<td><em>Bacteroides xylanisolvens</em></td>
<td>XBIA</td>
<td>Human gut bacterium</td>
</tr>
<tr>
<td><em>Parabacteroides goldsteinii</em></td>
<td>DSM 19448</td>
<td>Human gut bacterium</td>
</tr>
<tr>
<td><em>Parabacteroides gordonii</em></td>
<td>DSM 23371</td>
<td>Human gut bacterium</td>
</tr>
<tr>
<td><em>Parabacteroides johnsonii</em></td>
<td>JCM 3406</td>
<td>Human gut bacterium</td>
</tr>
<tr>
<td><em>Parabacteroides distasonis</em></td>
<td>ATCC 8503</td>
<td>Human gut bacterium</td>
</tr>
<tr>
<td><em>Parabacteroides merdae</em></td>
<td>ATCC 43184</td>
<td>Human gut bacterium</td>
</tr>
<tr>
<td><em>Bacteroides thetaiotaomicron</em></td>
<td>ATCC 29148 (VPI-5482)</td>
<td>Human gut bacterium, wild-type strain expressing all 8 capsules</td>
</tr>
<tr>
<td><em>Bacteroides thetaiotaomicron</em></td>
<td>ATCC 29148 (VPI-5482)</td>
<td>Human gut bacterium, strain expressing capsule 1</td>
</tr>
<tr>
<td><em>Parabacteroides thetaiotaomicron</em></td>
<td>ATCC 29148 (VPI-5482)</td>
<td>Human gut bacterium, strain expressing capsule 2</td>
</tr>
<tr>
<td><em>Parabacteroides thetaiotaomicron</em></td>
<td>ATCC 29148 (VPI-5482)</td>
<td>Human gut bacterium, strain expressing capsule 3</td>
</tr>
<tr>
<td><em>Parabacteroides thetaiotaomicron</em></td>
<td>ATCC 29148 (VPI-5482)</td>
<td>Human gut bacterium, strain expressing capsule 4</td>
</tr>
<tr>
<td><em>Parabacteroides thetaiotaomicron</em></td>
<td>ATCC 29148 (VPI-5482)</td>
<td>Human gut bacterium, strain expressing capsule 5</td>
</tr>
<tr>
<td><em>Parabacteroides thetaiotaomicron</em></td>
<td>ATCC 29148 (VPI-5482)</td>
<td>Human gut bacterium, strain expressing capsule 6</td>
</tr>
<tr>
<td><em>Parabacteroides thetaiotaomicron</em></td>
<td>ATCC 29148 (VPI-5482)</td>
<td>Human gut bacterium, strain expressing capsule 7</td>
</tr>
<tr>
<td><em>Parabacteroides thetaiotaomicron</em></td>
<td>ATCC 29148 (VPI-5482)</td>
<td>Human gut bacterium, strain expressing capsule 8</td>
</tr>
<tr>
<td><em>Parabacteroides thetaiotaomicron</em></td>
<td>ATCC 29148 (VPI-5482)</td>
<td>Human gut bacterium, strain with deletion in all 8 capsule loci</td>
</tr>
</tbody>
</table>
Coaggregation Assay

Qualitative coaggregation assays were conducted to assess the coaggregation ability of LGG under different nutritional variation. In order to control for the amount of bacteria used for the assay, optical density of bacterial suspensions was measured by a DU800 spectrophotometer (Beckman Coulter) and adjusted to OD$_{600}$ = 1.0 ± 0.1 through dilutions in coaggregation buffer. Bacterial suspensions of two partner strains (100 µL each) were combined in standard glass test tubes (10 x 75 mm). Control tubes were prepared to control for the effect of autoaggregation, and consisted of 200 µL of a single partner strain in each tube. The tubes were rocked back-and-forth 100 times to encourage cell-cell contact and coaggregation. Coaggregation was visualized using a colony counter magnifying lens with backlight (Quebec Colony Counter) and scored based on measures of suspension turbidity and cell clumping according to a qualitative 4-point scale (Kolenbrander and London, 1992) (Table 2). Coaggregation assays were conducted at least three times for each coaggregation pair.

Table 2: Descriptions of Coaggregation Scores. Adapted from Kolenbrander and London (1992)

<table>
<thead>
<tr>
<th>Score</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>Even, turbid suspension of bacteria (no coaggregation)</td>
</tr>
<tr>
<td>1</td>
<td>Finely dispersed clumps in turbid background (weak)</td>
</tr>
<tr>
<td>2</td>
<td>Definite clumps of bacteria are easily seen but do not settle immediately and remain in turbid background (moderate)</td>
</tr>
<tr>
<td>3</td>
<td>Clumps settle immediately with a slightly turbid background (moderate-strong)</td>
</tr>
<tr>
<td>4</td>
<td>Clumps settle immediately and supernatant is completely clear (strong)</td>
</tr>
</tbody>
</table>
**Protease Assay**

Protease assays were conducted to determine the presence of protein adhesins on partner bacterial strains. Coaggregating partner strains that scored 2 or above were selected for further testing using the protease inhibition assay to evaluate the presence of protein adhesin on either partner strain. Bacterial suspensions (900 µL) were combined with 2 µg pronase E [100 µL of pronase E (20µg/mL)] (Sigma-Aldrich, St. Louis, MO) in a 1.5 mL microcentrifuge tube and then incubated in a dry heat bath at 37°C for 75-120 minutes. The samples were washed three times in 500 µL of coaggregation buffer in a microcentrifuge (Eppendorf Centrifuge 5417C) at 11,000 x g (10,000 rpm), room temperature (RT), for 2-3 minutes each cycle. The pelleted cells were resuspended in 800 µL coaggregation buffer. Bacterial suspensions were mixed in the same ratios as in the standard coaggregation assay, except the combinations of partner strains were as follows: both untreated suspensions in one tube, both protease-treated suspensions in another tube, and one tube each with one treated partner and one untreated partner, for four total combinations. Control tubes containing 200 µL of a single treated and untreated partner strain were also prepared. All tubes were scored according to the standard coaggregation assay protocol (see previous section). Protease assays were conducted three times for each strong coaggregation pair.

**Hydrophobicity Assay**

Hydrophobicity assays were conducted to examine the effects of nutrient shifts on the hydrophobic nature of the cell-surface of LGG. Bacterial samples were centrifuged, resuspended in coaggregation buffer, and adjusted to OD600=1.0±0.1, which was recorded as the pre-OD. One milliliter of the bacterial sample was mixed with 1 mL of
hexadecane and vortexed for a minute to encourage interaction between the hexadecane and the bacterial cells. The mixture was left to sit at room temperature for 15 minutes to allow the phases to separate. The bacterial cells will remain in either the aqueous phase or the organic hexadecane phase depending on the level of hydrophobicity (Pembrey et al., 1999). The OD of the aqueous (bottom) phase was taken as the post-OD. The relative hydrophobicity was expressed as a percent drop in OD between the pre-and post-readings, which was calculated using the following formula:

\[ \text{Relative hydrophobicity} = \left( \frac{\text{PreOD} - \text{PostOD}}{\text{PreOD}} \right) \times 100\% \]

This assay was repeated twenty times for each culture condition.

**Statistics**

To test for significant differences between coaggregation scores of LGG cultured in different media types, a Pearson’s Chi-squared test was conducted ($\alpha = 0.05$). A Kruskal-Wallis Post-Hoc analysis was conducted using SPSS to make pair-wise comparisons. Results where $p<0.05$ were considered significant.

**Results**

**Autoaggregation ability of strains**

Visual autoaggregation was observed for *L. rhamnosus* GG (LGG) cultured TYG, TYF, and BeYG media. LGG had an autoaggregation score of 1, with the exception of LGG grown in SYG, which had a score of 0, and LGG grown in BeYG, which had a score of 2. Seven out of 31 *Bacteroides* and *Parabacteroides* strains exhibited visual autoaggregation. *B. fragilis*, *B. eggerthii*, *B. cellulosilyticus*, *B. fluxus*, and *B. xylanisolvens* had an autoaggregation score of 1. *B. stercoris* and *B. intestinalis* had an autoaggregation score of 2. (Table 3).
Coaggregation ability of LGG with wild-type Bacteroides and Parabacteroides strains

The ability of LGG to coaggregate with wild-type Bacteroides and Parabacteroides strains was media dependent. A significant difference in coaggregation scores was observed when compared by media type ($X^2 = 83.130, p<0.0001, n=3$). Both TYG and TYF cultivation were more likely to exhibit weak coaggregation scores when compared to LGG cultured in other media formulations. LGG cultured in SYG was more likely to exhibit no coaggregation, and LGG cultured in BeYG was more likely to exhibit moderate to strong coaggregation. When cultured in TYG, LGG coaggregated with 17 of the 22 wild-type strains, of which twelve had a score of 1, three had a score of 2, and two had a score of 3 (Table 3, Figure 2a, Figure 3). LGG cultured in TYF coaggregated with 15 of the 22 wild-type strains. Of these 15 pairings, eight had a coaggregation score of 1, five had a score of 2, and two had a score of 3 (Table 3, Figure 2b, Figure 3). When cultured in SYG, LGG coaggregated with eight of the 22 wild-type strains. Five of these eight pairings had a visual coaggregation score of 1, two of the pairings had a score of 2, and one pairing had a coaggregation score of 3 (Table 3, Figure 2c, Figure 3). LGG cultured in BeYG coaggregated with 19 out of 22 wild-type strains. Two of the 19 pairings had a coaggregation score of 1, twelve had a coaggregation score of 2, and five had a coaggregation score of 3 (Table 3, Figure 2d, Figure 3). Of the 22 wild-type Bacteroides and Parabacteroides strains tested, three of the strains consistently coaggregated at a score of 2 or above with LGG across all media types: B. fragilis, B. vulgatus, and P. goldsteinii (Table 3)
Table 3: Visual coaggregation scores of LGG in different media types (TYG, TYF, SYG, BeYG) with wild-type Bacteroides/Parabacteroides. Coaggregation was observed to be growth medium dependent.

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</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>LGG (TYG)</td>
<td>3 0 1 1 2 1 1 1 1</td>
<td>2 0 1 2 1 1 0 1 0 1</td>
<td>3 1 1 1 0 0 1 0 1 3</td>
<td>1</td>
<td></td>
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<td></td>
</tr>
<tr>
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<td>LGG (TYF)</td>
<td>3 0 0 1 2 1 1 0 1 2 0 1 2 1 2 1 0 1 0 3 2</td>
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Figure 2: Comparison of coaggregation scores between wild-type *Bacteroides/Parabacteroides* spp. and LGG cultured in TYG (A), TYF (B), SYG (C), BeYG (D). For each score (0-3), the percentage of wild-type *Bacteroides/Parabacteroides* spp. that coaggregated with LGG is given. A significant difference was observed between coaggregation scores when compared by media type ($X^2 = 83.130$, $p < 0.0001$, $n=3$).
Thickness of bands corresponds to coaggregation score

**Figure 3:** Alluvial diagram of coaggregation interactions between TYG, TYF, SYG, BeYG LGG and wild-type *Bacteroides/Parabacteroides*. From left to right, beginning bands represent division of LGG into growth medium types (from top to bottom, SYG, TYG, BeYG, TYF), with each middle node representing a different medium. Bands between the middle and end nodes represent coaggregation scores between LGG and partner strains, with thicker bands indicating stronger coaggregation scores, and end nodes representing partner strains.
Coaggregation ability of LGG with mutant B. thetaotaomicron capsular types

Coaggregation between LGG and mutant B. thetaotaomicron capsular types was media dependent ($X^2 = 45.975, p<0.0001, n=3$). TYG and TYF cultivation was more likely to lead to moderate coaggregation scores. LGG cultured in SYG was more likely to exhibit no coaggregation or weak coaggregation, and LGG cultured in BeYG was more likely to exhibit moderate to strong coaggregation. Weak to moderate coaggregation occurred in all cases, except for SYG-cultured LGG and B. thetaotaomicron ΔCPS and B. thetaotaomicron CPS 1, of which no coaggregation was observed (Table 4). Weak to moderate coaggregation was observed between TYG and TYF LGG and all capsular types (Table 4, Figure 4a/b, Figure 5). Weak or no coaggregation was observed between SYG LGG and all B. thetaotaomicron capsular types (Table 4, Figure 4c, Figure 5), while moderate coaggregation was observed between BeYG and all B. thetaotaomicron capsular types (Table 4, Figure 4d, Figure 5).

Table 4: Visual coaggregation scores of LGG in different media types (TYG, TYF, SYG, and BeYG) with wild-type and mutant B. thetaotaomicron capsular types. Coaggregation was observed to be growth medium dependent.

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<td>B. thetaotaomicron CPS 3</td>
<td>B. thetaotaomicron CPS 4</td>
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Figure 4: Comparison of coaggregation scores between *B. thetaiotaomicron* capsular types and LGG cultured in TYG (A), TYF (B), SYG (C), BeYG (D). For each score (0-3), the percentage of *B. thetaiotaomicron* capsular types that coaggregated with LGG is given. A significant difference was observed between coaggregation scores when compared by media type ($X^2 = 45.975$, $p<0.0001$, $n=3$).
Figure 5: Alluvial diagram of coaggregation interactions TYG, TYF, SYG, and BeYG LGG and mutant *B. thetaiotaomicron* capsular types. From left to right, beginning bands represent division of LGG into growth medium types (from top to bottom, BeYG, TYF, TYG, SYG), with each middle node representing a different medium. Bands between the middle and end nodes represent coaggregation scores between LGG and partner strains, with thicker bands indicating stronger coaggregation scores, and end nodes representing partner strains.
Adhesin identification by protease assay

Moderate to strong coaggregation was reduced to no coaggregation after treatment of LGG with protease for most interactions, suggesting unimodal adhesion, with the adhesin located on LGG. In addition, coaggregation interactions between TYG LGG, TYF LGG, SYG LGG, and BeYG LGG and *B. vulgatus* were reduced from a score of 2 or 3 to 1 upon treatment of either LGG or *B. vulgatus* with protease, suggesting bimodal adhesion with each partner possessing an adhesin. Coaggregation between LGG and *B. vulgatus* was completely eliminated only after protease treatment of both partners (Table 5).

Table 5: Protease inhibition of coaggregation between untreated (UT) and treated (T) LGG and *Bacteroides/Parabacteroides* strains. Unimodal adhesion mediation was observed for most cases. However, some bimodal interactions were also observed.

|                  | LGG (TYG) UT | T | LGG (TYF) UT | T | LGG (SYG) UT | T | LGG (BeYG) UT | T | B. fragilis UT | T | B. vulgaris UT | T | P. goldsteinii UT | T | B. thetaiotaomicron WT UT | T |
|------------------|--------------|---|--------------|---|--------------|---|--------------|---|--------------|---|---------------|---|-----------------|---|------------------|---|------------------|
| LGG (TYG) UT UT | 2            |   |              |   |              |   |              |   |              |   |               |   |                 |   |                 |   |                 |
| T                | 0            |   |              |   |              |   |              |   |              |   |               |   |                 |   |                 |   |                 |
| LGG (TYF) UT UT |               |   |              | 2 |              |   |              |   |              |   |               |   |                 |   |                 | 3 |                 |
| T                | 0            |   |              |   | 3            |   | 2            |   | 1            |   | 3             |   | 2               |   |                 | 2 |                 |
| LGG (SYG) UT UT |               |   |              |   |              | 0 |              |   |              |   |               | 0 |                 | 2 |                 | 2 |                 |
| T                | 0            |   |              |   |              |   |              |   |              |   |               |   |                 |   |                 | 3 |                 |
| LGG (BeYG) UT UT|               |   |              |   |              |   |              | 2 |              |   | 3             |   | 3               |   |                 | 3 |                 |
| T                | 0            |   |              |   |              |   |              |   |              |   | 0             |   |                 |   |                 | 0 |                 |

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**Hydrophobicity**

Average hydrophobicity (represented as a percent drop in OD) was the highest for LGG cultured in BeYG media (45.40 ± 15.87%) and the lowest for LGG cultured in TYF media (8.34 ± 7.30%). LGG cultured in TYG media and SYG media had similar hydrophobicity values, at 12.67 ± 5.24% and 11.42 ± 5.36%, respectively. A significant difference was observed among the 4 groups (p<0.0001). LGG cultured in BeYG was significantly different from all other media types (p<0.01), but LGG cultured in TYG, TYF, and SYG were not significantly different from each other (Figure 6).

**Figure 6:** Box and whisker plot of the relative hydrophobicity (%) of LGG grown in TYG, TYF, BeYG, and SYG. For each media type, the “whiskers” represent the upper and lower extremes, and the three lines that make up the box represent the lower quartile, the median, and the upper quartile. Plotted stars and circles represent outliers in the data. The letter “A” indicates that the groups did not significantly differ from each other. The letter “B” indicates that the group is significantly different from the other groups. There was a significant difference observed among the 4 groups (P<0.0001; n=20).
Discussion

Evidence suggests that gut microorganisms exist in biofilms that adhere to the mucin layer of the gastrointestinal tract (Sicard et al., 2017). Coaggregation is considered an essential mechanism for biofilm development (Stevens et al., 2015). Although a previous study indicated that coaggregation does not occur extensively between gut microorganisms (Ledder et al., 2008), others have found the opposite (Schotten et al., 2016). This study examined the effects of nutrient variation on coaggregation and cellular surface properties of probiotic *Lactobacillus rhamnosus* GG (LGG). The results of this study indicate that moderate coaggregation occurs between several representative gastrointestinal bacterial species. Not only was LGG able to coaggregate with a variety of microorganisms, but this coaggregation was growth-medium dependent. Of the media types tested, LGG cultured in BeYG (meat peptone) was more likely to exhibit moderate coaggregation, and LGG cultured in SYG (plant peptone) was more likely to exhibit no or weak coaggregation. However, this may be due in part to differences in autoaggregation observed with changes in media type. Examination of the role of autoaggregation in coaggregation between microorganisms through visualization by confocal microscopy of coaggregates may help to elucidate the role of autoaggregation. Since the adherence of cells in suspension to form coaggregates and autoaggregates is thought to contribute to biofilm development (Rickard et al., 2003; Stevens et al., 2015), it is possible that changes in the ability of an organism to coaggregate due to nutritional shifts could affect biofilm development. Therefore, analyzing biofilm formation under varying nutrient conditions using fluorescent imaging and confocal microscopy will be helpful for understanding how biofilms in the gut may be affected by diet.
One reason for the difference in coaggregation patterns may be changes in cell surface structures, because cell surface protein adhesins and polysaccharide receptors are thought to mediate coaggregation interactions (Katharios-Lanwermeyer et al., 2014). Unimodal adhesin mediated coaggregation was observed for most LGG and Bacteroides/Parabacteroides spp. interactions, suggesting a surface protein on LGG is responsible for coaggregation interactions. Previous studies have shown that the proteome of LGG varies under differing nutrient and media conditions due to different metabolic demands (Bove et al., 2012; Koskenniemi et al., 2009). For example, LGG cultured in cheese-like conditions had modified amounts of proteins responsible for a variety of metabolic pathways compared to LGG cultured in Man, Rogosa, and Sharpe (MRS) broth (Bove et al., 2012). Furthermore, vaginal Lactobacillus rhamnosus strains BGHV719 and BGHV954 exhibited changes in cell wall protein composition under varying carbohydrate conditions (Begovic et al., 2010). It is possible that changes in diet leads to changes in metabolic demands of gastrointestinal microorganisms, which in turn leads to changes in protein expression. This may also affect the presence of surface proteins/adhesins responsible for mediating coaggregation. Determining which surface molecules and/or proteins are responsible for coaggregation in LGG as well as how these surface structures are affected by nutritional shifts would provide valuable information on the mechanism of coaggregation between LGG and other microbial species and how these mechanisms are affected by changes in nutritional resources.

Since hydrophobicity is related to the proteins and sugars presented on the surface of cells (Polak-Berecka et al., 2014), changes in the expression of cell surface structures responsible for coaggregation will correlate with changes in hydrophobicity.
Hydrophobicity is thought to aid in the initial adherence of microbial cells to host cells, and high hydrophobicity values have been correlated with better colonization of mucosal surfaces (Schillinger et al., 2005). One study found that LGG cultured in a yogurt food model was more hydrophobic and exhibited higher adherence to Caco-2 (human epithelial colorectal adenocarcinoma) cells than LGG cultured in an ice cream food model, which suggested that there is a relationship between food, hydrophobicity, and the adherence ability of LGG (Deepika et al., 2011). Similarly, our study found that the hydrophobicity of LGG was affected by media composition. LGG cultured in BeYG had significantly greater hydrophobicity values when compared to the other media types, but the hydrophobicity values of LGG cultured in the other media types did not significantly differ from each other. Given that BeYG-cultured LGG exhibited high hydrophobicity and higher autoaggregation and coaggregation scores, our results suggest that high hydrophobicity is correlated with higher coaggregation ability. Since both coaggregation patterns and relative percent hydrophobicity of LGG differed under varying nutrient conditions, it is likely that nutrient variation affects the expression of cell surface structures of LGG and other gut microorganisms. Changes in hydrophobicity may also influence the ability of LGG to interact with the mucin layer of the gastrointestinal tract and food particles as they move through the gut (Deepika et al., 2011).

Carbohydrates and amino acids in solution may also reverse coaggregation by binding to competitive receptor sites (Katharios-Lanwermeyer et al., 2014). L-arginine has been found to inhibit coaggregation between oral bacterial species and destabilize oral biofilms (Kolderman et al., 2015). While it is possible that certain amino acids or carbohydrates in the different media formulations may have bound to competitive
receptor sites and reversed coaggregation, the amino acids and carbohydrates of the 
media formulations that may have been responsible were not determined. In the gut, it is 
possible that ingestion of foods high in certain amino acids or carbohydrates may reverse 
coaggregation between microorganisms. A more thorough examination the inhibition of 
coaggregation between gut microorganisms by specific amino acids and carbohydrates 
may reveal important information pertaining to the ability of amino acids and 
carbohydrates to reverse coaggregation between gastrointestinal microorganisms.

The effect of media type on the coaggregation ability of LGG also has 
implications for the efficacy of LGG as a probiotic. It has been suggested that the ability 
of microorganisms to adhere to the intestinal epithelium and mucin layer provides a 
protective barrier against pathogens. In addition, both autoaggregation and coaggregation 
to pathogens have been proposed as important characteristics of probiotic organisms 
(Collado et al., 2007). As the results presented here suggest, autoaggregation and 
coaggregation ability of LGG vary by media type. Therefore, changes in media type may 
affect the efficacy of probiotics grown in labs for human consumption. Optimizing 
culture techniques to improve adherence and aggregation ability of probiotic organisms 
may enhance their success in the gastrointestinal tract.

One source of nutrient variation is diet. Diet is a well-known factor thought to 
influence the composition of the gut microbiome (Xu and Knight, 2015). In non-human 
primates, African green monkeys (Chlorocebus aethiops sabaeus) fed a Western diet 
were observed to have microbiomes with increased microbial richness and relative 
abundance of Prevotella (Amato et al., 2015b). In addition, the gut microbiome of black 
howler monkeys (Alouatta pigra) undergoes changes as a result of seasonal diet variation
Similar results have been observed in human subjects. A previous study on healthy individuals found that agrarian, carbohydrate-rich diets were associated with a gut microbiome with high amounts of bacteria belonging to the *Prevalent* taxa. In contrast, diets high in animal fats and proteins were associated with an intestinal microbiome high in *Bacteroides* (Wu et al., 2011). Furthermore, the gut microbiome of the Hadza hunter-gatherers of Tanzania experiences taxonomic shifts due to seasonal changes in diet (Smits et al., 2017). Nutrient variation also influences aggregation ability in rhizobacterium *Azospirillum brasilense* strain Cd. (Burdman et al., 1999) and biofilm formation of LGG (Lebeer et al., 2007). As the evidence provided here suggests, one mechanism through which diet may alter the composition of gastrointestinal microflora is by impacting intercellular interactions, such as coaggregation. Future studies examining how nutritional variation affects biofilm composition *in vitro* and *in vivo* would lead to a better understanding of how diet affects the biofilm formation and colonization of the gastrointestinal epithelium and mucin layer.

In the gastrointestinal tract microorganisms are thought to form biofilms on mucosal surfaces, mucus, and food particles. The ability of LGG to form biofilms on surfaces is affected by pH, osmolarity, and the presence of bile. Interestingly, the effect of these factors is dependent on the nutritional environment. This may be because of changes in metabolic demands of the bacterial cells under varying nutrient conditions. In addition, the inclusion of polysaccharides into the extracellular matrix produced by bacterial cells may increase biofilm formation (Lebeer et al., 2007). The results of our study suggest that in addition to affecting cellular metabolism and the composition of the extracellular matrix, dietary variation may also affect intercellular interactions, such as...
coaggregation and autoaggregation. However, diet is not the only known factor thought to influence the composition of the gut microbiome. Other factors, such as medications, antibiotics, food additives, and probiotic intake may also influence the composition of the gastrointestinal ecosystem, which has been implicated in a number of diseases, including inflammatory bowel disease, diabetes, obesity, arthritis, and eczema (Valdes et al., 2018). As the evidence provided here suggests, further studies are needed to investigate the effects of environmental factors, such as diet, on microbial colonization of the gastrointestinal tract and intercellular interactions between microorganisms and host cells. This will provide valuable information on the relationship between environmental factors, the gut environment, and human health.
References


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