The Effects of Inulin and Galactooligosaccharides on the Production of Reuterin by *Lactobacillus Reuteri*

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ABSTRACT

The microbiome is a dynamic community that can positively and negatively influence host health. *Lactobacillus reuteri* is a probiotic that has received much attention for its ability to inhibit pathogens such as *Salmonella typhimurium*, *Escherichia coli,* and *Clostridium difficile*. It does so by its unique ability to metabolize glycerol into the antimicrobial compound 3-HPA, which is commonly referred to as reuterin. The ability to secrete reuterin is dependent not only on glycerol availability but also the concentration of glucose. In fact, there appears to be a "goldilocks" ratio between glucose and glycerol as either too much or too little glucose significantly diminishes reuterin production. Since *L. reuteri* primarily resides in distal regions of the intestine and colon where most of the glucose has already been absorbed, it seems unlikely that reuterin production would be promoted at the physiological level via this mechanism.

Prebiotics are carbohydrates that are indigestible by the host and remain for enzymatic digestion by intestinal probiotics. Inulin and galactooligosaccharides (GOS) are two widely studied prebiotics that are known for their ability to promote the growth of a wide range of *Lactobacilli*, and have been shown to promote *L. reuteri* growth to varying degrees. Here, we asked if prebiotics such as inulin and GOS promote the production of reuterin in the absence of glucose. *L. reuteri* were cultured in TSB with or without glycerol in the presence of glucose, inulin, or GOS and assessed for their ability to produce reuterin. While inulin did not enhance the production of reuterin, GOS induced reuterin production, although 45% less than that of glucose. Moreover, unlike the dose-dependence observed with glucose, incubation with GOS induced similar reuterin production regardless of concentration. This suggests that an enzymatic equilibrium may exist where glucose/galactose is cleaved from GOS only as needed by *L. reuteri*.

Finally, to confirm the biopotency of reuterin production, we cultured *S. typhimurium* with supernatants from *L. reuteri* that were grown with various carbohydrates. Supernatant dilutions as low as 1:15 were able to significantly retard growth of S. typhimurium with ratios of 1:1 completely inhibiting growth. Together, these results suggest that prebiotics such as GOS may be able to elicit physiologically relevant production of reuterin, which may shape the flora of the microbiome and reduce incidence and severity of pathological infections. Further, as GOS are particularly abundant in breast milk, it suggests a possible link for early immunoprotection from intestinal pathogens while the infant is still immunologically naïve.

Keywords: *Lactobacillus reuteri*, *Salmonella typhimurium*, inulin, *Galactooligosaccharides* (GOS), reuterin, metabolism, prebiotics, probiotic

INTRODUCTION

Microbiome: Influence on health

The interaction and effects between human health and the microbiota have been widely studied (D'Argenio and Salvatore, 2015; Gibson and Roberfroid, 1995). Improper balances in the composition of the microbiome can lead to deleterious effects on the host. For example, obesity has been correlated with elevated levels of pathogenic bacteria in the gut (Fei and Zhao, 2013). One study in particular looked at the ratio of one specific probiotic, *Bifidobacteria*, and one specific pathogen, *Escherichia coli*. They found that a group of obese school aged children had a significantly lower ratio of probiotics to pathogens (Gao et al., 2015). Other negative outcomes associated with unhealthy gut bacterial composition are inflammatory bowel disease (D'Argenio and Salvatore, 2015) and chronic heart failure (Pasini et al., 2016). In contrast to poor gastrointestinal conditions, symbiotic bacteria have been seen to ameliorate certain conditions such as constipation (Bekkali, Bongers, Van den Berg, Liem, and Benninga, 2007; Coccorullo et al., 2010; Ojetti et al., 2014), obesity (Chen et al., 2014; National Human Genome Research Institute, n.d.), and can inhibit pathogens in vitro (De Weirdt et al., 2012) and in vivo (Uraipan and Hongpattarakere, 2015). These beneficial bacteria are referred to as probiotics which can be defined as living organisms that convey beneficial health to the host or support a proper equilibrium of autochthonous microbes within the gastrointestinal tract (Uraipan and Hongpattarakere, 2015). Two genera most commonly studied for their favorable effects upon human health are *Bifidobacteria* and *Lactobacilli*.

Probiotics generally exert their positive shift in a balanced microbiome in one of two ways. The first is through occupying a physical niche within the gut by which they are able to limit any pathogen adherence to intestinal walls. The second mechanism involved in probiotic activity is in the secretion of an antimicrobial substance that inhibits pathogen (De Weirdt et al., 2012; Kšonžeková et al., 2016). The secreted antimicrobial typically either kills pathogenic organisms, or it prevents adherence to intestinal walls thereby preventing niche establishment; both mechanisms effectively shift the gastrointestinal equilibrium. Probiotics produce microbial inhibition by excreting exopolysaccharides, which are polysaccharides on the cell surface of gram positive bacteria that then can be released into the adjacent vicinity (Chapot-Chartier and Kulakauskas, 2014), or other compounds with antimicrobial properties including bacteriocin peptides (De Weirdt et al., 2012; Kšonžeková et al., 2016; Schaefer et al., 2010; Silva Sabo, Converti, Todorov, Domínguez, and Souza Oliveira, 2015). Because of probiotics' anti-pathogenic properties and correlation with health, much research has focused on elucidating how they may be promoted.

In the pursuit of enhancing the endogenous probiotic populations of the gut, a new category of compounds was outlined. Gibson and Roberfroid first coined the term prebiotics to describe these compounds, and characterized them as nondigestible food stuffs that selectively promote the growth or activity of bacteria that beneficially impact the health of the host (Gibson and Roberfroid, 1995). Further delineation can be made between fibers and prebiotics by clarifying that fibers cannot be fermented in the gut while prebiotics are fermentable compounds (Stewart, Savarino, and Slavin, 2009). There are a number of prebiotics that have been studied including different types of fructans and oligosaccharides. These prebiotics selectively promote the growth of probiotics (Chung et al., 2016), mainly the genera *Bifidobacteria* and *Lactobacillus* (Kneifel, 2000; Kolida, Tuohy, and Gibson, 2002).

Changes in the microbiome have also been observed in human subjects due to prebiotics. For example, Costabile et al. administered different prebiotics to humans to see how they would impact the microbiota. While the overall number of bacteria remained the same, specific probiotics such as *Bifidobacteria* and *Lactobacillus* increased depending on the particular prebiotic used. At the same time, bacteria associated with an unhealthy gut, such as *Clostridia* and *Bacteroides*, decreased in number (Costabile et al., 2010). Not only do prebiotic effect bacterial distribution, but Tarr et al. have also observed a significant impact on health. Mice were subjected to a variety of conditions involving stress and prebiotics, and not only did the addition of prebiotics help modulate the gut composition to keep it within a range of normal, but it also prevented the mice from becoming anxious when placed in stressor situations. (Tarr et al., 2015).

Lactobacillus reuteri and the antimicrobial production of reuterin

Lactobacillus reuteri (*L. reuteri*) is a probiotic with promising potential. It has already been demonstrated to have health benefits in human studies, and studies like the ones conducted by Ojetti et al. and Coccorullo et al. have shown the potential advantageous outcomes associated with the *L. reuteri* (Coccorullo et al., 2010; Ojetti et al., 2014). In a double-blind, randomized, placebo controlled study by Coccorullo et al., supplementation of *L. reuteri* was investigated for its ability to alleviate the functional chronic constipation that some infants experience. Not only did they see a significant increase in bowel movements, but there was also no observed adverse effect resulting from the added probiotic (Coccorullo et al., 2010). Another essential benefit of *L. reuteri's* probiotic capacity is in its inhibition of pathogenic activity. One mechanism of inhibition is by limiting the ability of pathogens to adhere to gut epithelial cells. One group of researchers demonstrated that *L. reuteri* produced an exopolysaccharide that limited E. coli's ability to adhere to porcine epithelial cells in vitro (Kšonžeková et al., 2016). This is in addition to the general characteristic of probiotics ability to occupy space that could otherwise be utilized by pathogens (Uraipan and Hongpattarakere, 2015). Another intriguing aspect of *L. reuteri's* probiotic ability is its unique metabolism of glycerol into reuterin. De Weirdt et al. observed that the supernatant from *L. reuteri* produced antimicrobial effects against *Salmonella typhimurium*. In this study, they subjected *S. typhimurium* to a variety of supernatant concentrations from *L. reuteri* cultures. A 1:10 concentration of supernatant from *L. reuteri* grown in the presence of glycerol not only limited the adhesion of *S. typhimuirum* to other cells, but it also inhibited the actual growth of *S. typhimurium* in vitro. Moreover, it was determined that reuterin was the active antimicrobial compound that was secreted by *L. reuteri* (De Weirdt et al., 2012).

Reuterin **(Figure 1)** is also known as 3-hydroxyproponal or 3-hydroxypropionaldehyde (Schaefer et al., 2010), and is produced by a glycerol dehydratase that is B12 dependent (Talarico and Dobrogosz, 1990). It also is clear that reuterin is metabolized from glycerol and not due to an overall increased metabolism. The precise mechanism of reuterin's inhibition is not fully understood though it has been seen to have a wide range of antimicrobial inhibition (Talarico and Dobrogosz, 1989), including the pathogenic *Clostridium difficile*, which is responsible for many extended hospitalizations (Schaefer et al., 2010). There are currently two hypotheses to explain its broad range of inhibition. The first is that reuterin inhibits the activity of ribonucleotide reductase (Cleusix, Lacroix, Vollenweider, Duboux, and Le Blay, 2007; Talarico, Casas, Chung, and Dobrogosz, 1988), the enzyme that synthesizes ribonucleotides in bacteria (Torrents, 2014). The other proposed, and more widely accepted, hypothesis is that reuterin introduces oxidative stress to the cells. Schaefer et al. investigated the mechanism by which reuterin may exert its potential oxidative stress. They showed that *E. coli* that had mutations for a specific gene involved with oxidative stress were more susceptible to reuterin's effect. Moreover, they also showed that excess cysteine in the media could mitigate the antimicrobial effects of reuterin, suggesting that retuerin is interacting with thiol groups (Schaefer et al., 2010).

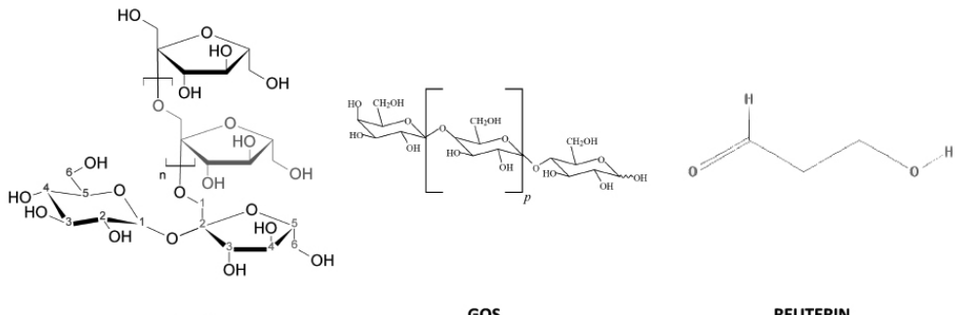

Figure 1: Structures of Inulin, GOS, and Reuterin. Each of the three compounds of interest for this research is depicted in this image. The β-glycosidic bonds between the monomers in inulin and GOS can be seen. As well, the varying degree of polymerization for both carbohydrates is illustrated with the brackets. Reuterin is easily observed as a modified version of glycerol by its molecular structure. (Fisch, 2006; Klaas, n.d.; Pubchem, n.d.)

Inulin and Galactooligosaccharides

Inulin **(Figure 1)** is a known prebiotic that can promote the growth of probiotics. Found in foods such as chicory root, Jerusalem artichoke, banana, onion, and garlic, inulin is a polymer of carbohydrate that varies in its degrees of polymerization (Costabile et al., 2010). It is mainly composed of fructose monomers linked by 1-2 β-glycosidic bonds of repeating length. This β-glycosidic linkage is what makes it indigestible by human gastrointestinal enzymes. An α-glucose moiety sometimes starts the polymer (Roberfroid, 2005). Chung et al. meticulously verified the prebiotic activity of inulin for various probiotics in vitro (Chung et al., 2016). These prebiotic effects have been observed in vivo as well. One such study was conducted with humans where half of the subjects were given inulin and half were given a placebo of maltodextrin. Fecal samples taken before and after revealed that the number of *Lactobacilli* and *Bifidobacteria* increased in the experimental group. This suggests that prebiotics, and specifically inulin, can be used to actively modulate the composition of the microbiome (Costabile et al., 2010). Not only can inulin be given directly, but it also can be incorporated into baked goods while still retaining its prebiotic activity, thus expanding its realistic use (Kleessen B

et al., 2007). *L. reuteri* is known to respond to various prebiotics of which inulin is one (Kassim, Baijnath, and Odhav, 2014).

Galactooligosaccharides **(Figure 1)** (GOS) are naturally occurring carbohydrates in breast milk that are synthesized via a β-galactosidase enzyme (Macfarlane, Steed, and Macfarlane, 2008). GOS have β-glyosidic linkages between galactose moieties of repeating length, and most often terminate with a single glucose monomer (Austin, Bénet, Michaud, Cuany, and Rohfritsch, 2014). GOS can often vary in degrees of polymerization from 2 to 10 (Macfarlane et al., 2008), and are known to be metabolized by at least two different strains of *L. reuteri* (Kneifel, 2000).

Bacterial metabolism

Although prebiotics such as inulin and GOS are known to enhance the growth of some strains of *L. reuteri*, it is uncertain how, or if, the metabolism of these prebiotics impacts the overall metabolic state of the organism. Some evidence demonstrates that prebiotics have increased the amount of antimicrobial substances produced by probiotics. If this is true, the ingestion of prebiotics could become a medicinally important part of a person's diet. Our research set out to determine if metabolism of prebiotics by *L. reuteri* significantly increase reuterin production. Specifically, we hypothesized that the addition of prebiotics such as inulin or GOS to *L. reuteri* would increase reuterin secretion and inhibit *S. typhimurium* growth.

METHODS

Bacteria, compounds, and storage

Lactobacillus reuteri PTA 6475 were obtained from the American Type Culture Collection (Manassas, VA) and were maintained by anaerobic culture on Tryptic Soy Agar plates. Salmonella typhimurium was obtained from Presque Isle Cultures (Erie, PA), and incubated aerobically on TSA plates. Plated cultures were stored at 4° C for use throughout experiments. Further, 10% and 20% glycerol stocks were created for long-term storage of cultures. Inulin from chicory root was obtained from Chem-Impex International, while GOS was acquired from Bimuno. Tryptic Soy Broth (TSB) was supplemented with 0.1% Tween 80 in all conditions as a surfactant to aid the growth of *L. reuteri* [2].

Reuterin production by supplementation of inulin

L. reuteri was inoculated in TSB and twelve hours later, tubes were measured by spectrophotometry (OD 596). Next, 180 μL of working solution (OD596 close to 0.1) was inoculated in triplicate into Falcon tubes containing 9 mL of either TSB, TSB with 5.0% inulin (w/v), TSB with 20 mM glycerol, or TSB with 20 mM glycerol and 5.0% inulin. Cultures were incubated with shaking for sixteen hours at 37°C. Following this incubation, *L. reuteri*, were pelleted by centrifugation at 1500 rcf for ten minutes. The supernatant was removed, and the pellets were suspended in 9 mL of the corresponding broth (TSB, TSB with 5.0% inulin, TSB with 100 mM glycerol, and TSB with 100 mM glycerol and 5.0% inulin). They were then incubated again for two hours at 37°C with shaking. Subsequently, each sample was centrifuged at 8000 rcf for ten minutes. From every tube, 4.5 mL of the supernatant was placed into another tube that had 4.5 mL of TSB to render a 1:1 ratio of supernatant to TSB. Additional controls included 9mL of broth that had not been cultured with *L. reuteri* (TSB, TSB with 100mM glycerol, and TSB with 5.0% inulin). All samples were inoculated with 180 μL from the *S. typhimurium* working solution which consisted of *S. typhimurium* culture with an OD596 of 0.1. Initial experiments were then incubated in a 37°C shaking water bath for two, three, four, five, six, and seven hours. At each time point, a 1 mL aliquot was taken from each of the samples and measured by spectrophotometry for their OD596. All conditions were performed in triplicate.

Titration of Reuterin

To titrate reuterin production, the previously described protocols were repeated except the supernatant was diluted to 1:1, 1:5, 1:10, and 1:15. Further, a 1 mL aliquot was removed only after four hours (during *S. typhimurium* growth) for spectrophotometric analysis.

GOS as primary carbon source

Assays were carried out in a similar manner as stated previously. However, carbon restricted (glucose free) TSB media was used to make the GOS media. In the carbon restricted TSB, the following four concentrations of GOS (w/v) were added: 0.25% GOS, 0.25% GOS with glycerol, 2.0% GOS, and 2.0% GOS with glycerol. As previously noted, glycerol concentration started at 20 mM and then was brought up to 100 mM for each control that had glycerol. The positive control was standard TSB, which contains 13.9 mM of glucose, supplemented with glycerol. The negative control in this experiment lacked *L. reuteri* supernatant, and it was used to assess uninhibited growth of *S. typhimurium*. A 1:5 ratio of supernatant to TSB was used to incubate the *S. typhimurium*. After four hours, the *S. typhimurium* cultures were assessed by the spectrophotometer at 596 nm.

Statistical Analysis

The statistical analysis for all experiments was done with a student's t test to determine significance of results. A p value < 0.05 was considered to be statistically significant.

RESULTS

Effect of inulin on reuterin production

S. typhimurium was first grown in the experimental supplements inulin and glycerol to determine if in the absence of *L. reuteri*, any inhibition of growth would be observed. As shown in Figure 2, inulin and glycerol did not inhibit *S. typhimurium* growth. Furthermore, as others have reported, Figure 2 demonstrates that when *L. reuteri* was grown without glycerol, and the supernatant was used to culture *S. typhimurium*, there is an absence of any detectable antimicrobial effect. Each *S. typhimurium* sample was measured for growth with the spectrophotometer at an OD596. These results indicate that inulin alone does not inhibit *S. typhimurium* growth **(Figure 2)**. However, as shown in Figure 3, supernatants at a 1:1 dilution from *L. reuteri* cultured in the presence of glycerol inhibit *S.*

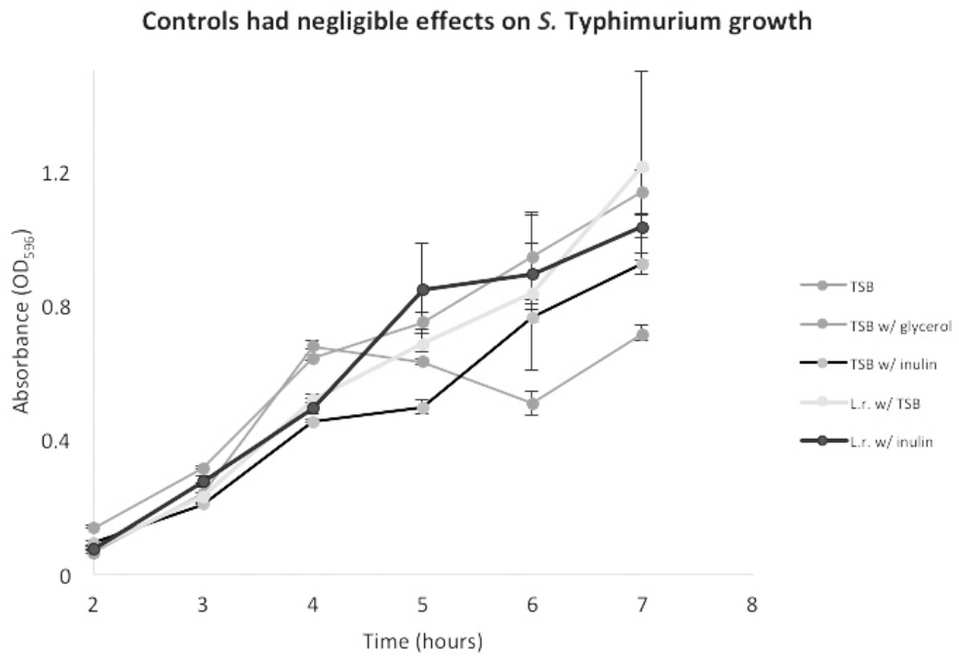

Figure 2: Glycerol and inulin alone do not inhibit the growth of *S. typhimurium*. *S. typhimurium* was cultured with or without glycerol and inulin and assessed for growth over seven hours of log growth. Supernatants from *L. reuterin (L.r.)* that were cultured with or without inulin were also cultured with *S. typhimurium* to assess inhibitory capacity. All samples were completed in triplicate.

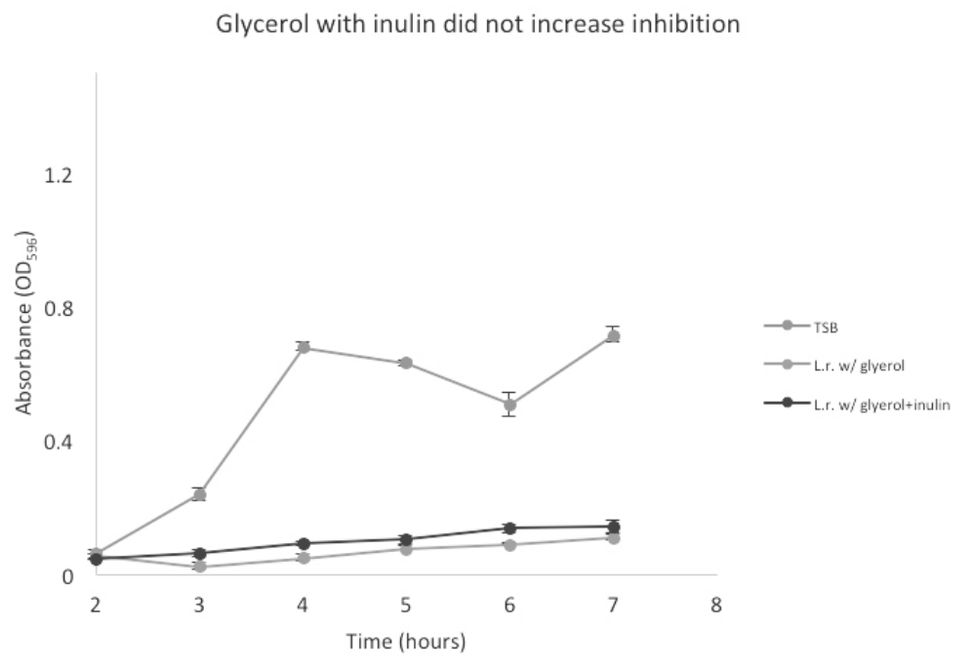

Figure 3: Glycerol with inulin supplementation did not increase inhibition of *S. typhimurium*. *S. typhimurium* growth was significantly inhibited by both supernatants that were extracted from *L. reuteri* cultures incubated with glycerol. However, there was no difference in inhibition between supernatants with glycerol vs.glycerol with inulin at a 1:1 ratio.

typhimurium growth for every time point after hour 2 ($p<0.001$). And while the addition of glycerol significantly inhibited the growth of *S. typhimurium*, the addition of inulin did not further affect this response at a 1:1 dilution as can be seen in **Figure 3**.

Dilution assay

As a 1:1 dilution of supernatants from *L. reuteri* cultures resulted in complete inhibition of *S. typhimurium*, we asked if effects of inulin would be observed over smaller dilutions in order to more precisely understand reuterin production. Dilutions for glycerol cultured *L. reuteri* with and without inulin were made at 1:1, 1:5, 1:10, and 1:15. *S. typhimurium* was then cultured in these varied supernatant dilutions, and the optical density of *S. typhimurium* was assayed after four hours of incubation. The steady increase of absorbance indicates that the dilutions were within the sensitive range of our assay's measurable limits. There was no significant difference observed between supernatant from *L. reuteri* grown with glycerol or glycerol plus inulin for any of the dilutions **(Figure 4)**. These data further suggest that the addition of inulin does not increase reuterin production. Moreover, the dose dependent increase of absorbance indicates that the dilutions were within the sensitive range of our assay's measurable limits. The data obtained from **Figure 4** further suggests to reject the first hypothesis that suggested the addition of inulin would increase reuterin production.

GOS as a primary carbon source

The supernatant of *L. reuteri* grown with GOS as the primary carbon source was used to determine whether *L. reuteri* can produce reuterin while metabolizing other carbohydrate sources other than glucose. As expected, the supernatants without any glycerol supplementation did not inhibit *S. typhimurium* growth significantly **(Figure 5).**

While not as potent as TSB that contains glucose as a carbon source, the addition of glycerol to GOS as a primary carbon source, at both concentrations tested, significantly inhibited the growth of *S. typhimurium* ($p<0.01$). This suggests that *L. reuteri* can secrete reuterin while metabolizing carbohydrates other than glucose. Also, the negative control of the supernatant from glucose with glycerol was significantly lower than every other sample ($p<0.05$).

DISCUSSION

We were able to consistently recreate the study of reuterin's inhibitory effects on *S. typhimurium* by De Weirdt et al. (De Weirdt et al., 2012). While no inhibitory ability was observed by the addition of inulin, it cannot be entirely ruled out that the metabolism of inulin does not promote any inhibitory reuterin production. Even though it is known that some *L. reuteri* species can use inulin (Kassim et al., 2014), metabolism was not confirmed in this study, and it is possible that the *L. reuteri* did not metabolize

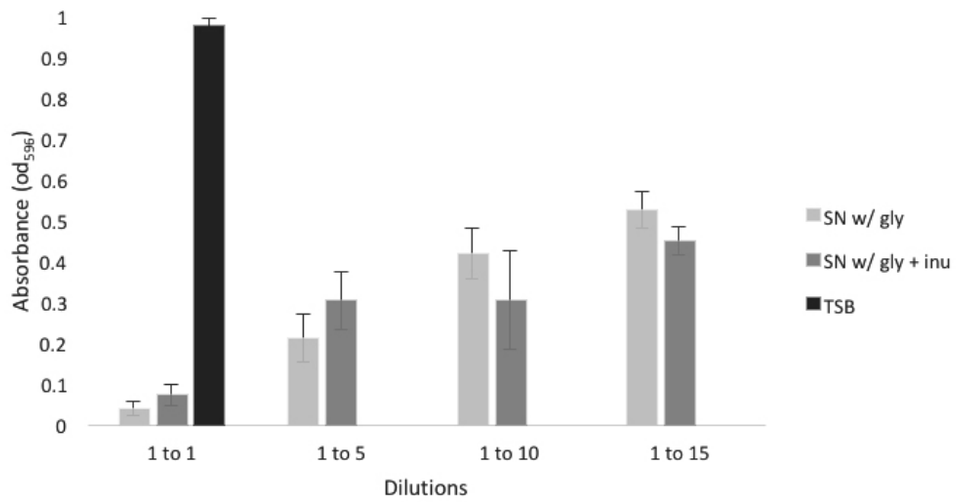

Figure 4: Titration of reuterin production is not influenced by inulin supplementation. Supernatants were prepared from *L. reuteri* grown with glycerol and glycerol with inulin. Each were diluted accordingly before use in the assay. All dilutions inhibited *S. typhimurium* growth. There was no significant difference between glycerol and glycerol with inulin at any dilution ($p>0.05$).

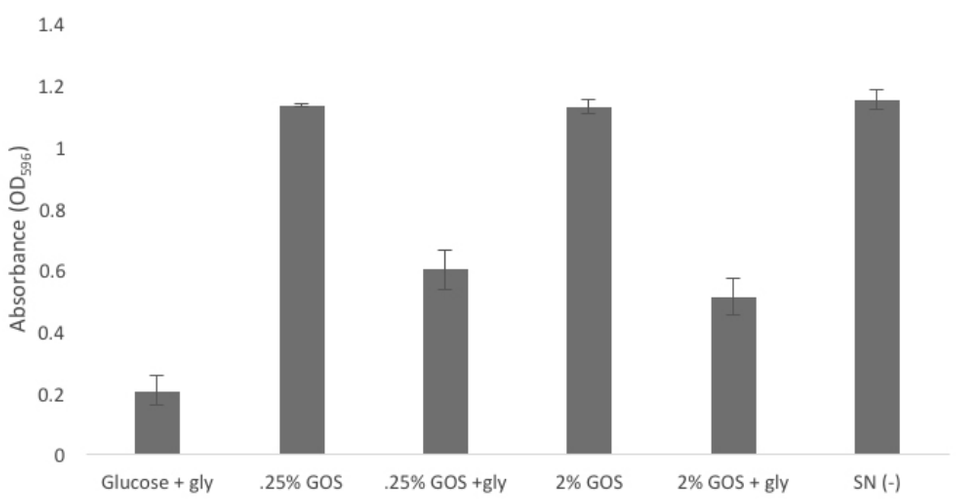

Figure 5: Reuterin production with GOS as primary carbon source is sufficient to inhibit *S. typhimurium* growth. Both concentrations of GOS at 0.25% or 2% allowed for reuterin production to occur thus the inhibition. Though significant inhibition was observed, there was also significant difference between the negative control and the GOS with glycerol
supernatants.

the inulin much, if at all, because it was in the glucose rich carbon environment of the TSB. Furthermore, other studies have shown that certain strains of *L. reuteri* do not metabolize inulin at all (Adebola, Corcoran, and Morgan, 2014; Kneifel, 2000). So while this research demonstrated for the first time that an addition of inulin does not increase the production of reuterin with or without glycerol, it did not answer the question of whether it would have if inulin were the sole carbon source in the media. To better construct a more thorough assay would require a carbon restricted media, which would shift *L. reuteri t*o metabolize the inulin. However, the promise of continuing this line of experimentation may be limited. Two studies looked at the effect that a variety of prebiotcs had on different probiotics. These studies seem to imply that prebiotic utilization is strain specific. For example, inulin was not well metabolized by the strains used in at least two studies (Adebola et al., 2014; Kneifel, 2000). Contrarily, Kassim et al. showed that some *L. reuteri* strains did metabolize inulin (Kassim et al., 2014). Nevertheless, the PTA 6475 strain used in this study has not been tested for its ability to metabolize inulin in any literature reviewed. Therefore, the PTA 6475 strain cannot yet be said with great confidence to metabolize inulin when in a carbon restricted media. This is one area of study yet to be explored.

A new direction to be followed is how other carbohydrate sources effect reuterin production. One study particularly has already shown that carbohydrate sources can act synergistically to impact antimicrobial production (Tzortzis, Baillon, Gibson, and Rastall, 2004). A logical continuation of this would be to extend a study to incorporate different prebiotics to see if they impact antimicrobial output. This is precisely what was done with the GOS assay. The carbon restricted media (no glucose) was supplemented with GOS as the primary carbon source for *L. reuteri*. A study conducted by Lüthi-Peng, Dileme, and Puhan demonstrated that reuterin synthesis is optimized at a particular glycerol to glucose ratio. Following this line of thought, their study would seem to insinuate that carbohydrate metabolism does indeed impact reuterin output (Lüthi-Peng, Dileme, and Puhan, 2002). Supported by this study, the GOS assay was completed in order to better determine the relationship between reuterin synthesis and carbohydrate metabolism. This research showed for the first time that reuterin can be metabolized without glucose being the primary carbon source.

Discovering *L. reuteri's* ability to continue to synthesize reuterin with GOS as a primary carbon source brought great insight into this unique form of metabolism (**Figure 5**). Going forward, there is still much to continue to study. For one, there was a significant difference in inhibition between glucose and GOS as the carbon source. It is known that different factors impact the production of different metabolites. (Årsköld et al., 2007). One study by Liu and Yu revealed that the ratio of glucose to glycerol can be optimized for the production of reuterin. (Liu and Yu, 2015). Future studies should determine whether there is an optimal ratio of GOS to glycerol in the same way that others have previously found the ideal glucose to glycerol ratio.

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