Ecological importance of cross-feeding of the intermediate metabolite 1,2-propanediol between bacterial gut symbionts

by

Christopher C. Cheng

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Department of Biological Sciences
University of Alberta

Abstract

Cross-feeding of intermediary metabolites such as 1,2-propanediol (1,2-PDO) has been proposed to have an important role in the establishment of syntrophic interactions among gut symbionts, but its ecological importance has not been empirically established. This thesis shows that growth of *L. reuteri* ATCC PTA 6475 in media is enhanced through 1,2-PDO produced by *Bifidobacterium breve* UCC2003 and *Escherichia coli* MG1655 from substrates (fucose and rhamnose) that cannot be utilized by *L. reuteri*. This syntrophy is strictly dependent on the *pdu-cbi-cob-hem* gene (*pdu*) cluster in *L. reuteri*, which encodes for the ability to utilize 1,2-PDO as an electron acceptor to enhance their growth rates, and it requires the L-fucose permease (*fucP*) gene in *B. breve*, which is needed for the metabolite formation of 1,2-PDO from fucose. Experiments in gnotobiotic mice revealed that ecological performance of *L. reuteri* ATCC PTA 6475 in the gastrointestinal tract was enhanced through trophic interactions with *B. breve* UCC2003. Use of isogenic mutants confirmed that this advantage was dependent on the *pdu* cluster in *L. reuteri* and *fucP* in *B. breve*, indicating that this interaction is specifically based on 1,2-PDO. These findings establish the ecological importance of syntrophic relationships based on 1,2-PDO for the fitness of a bacterial symbiont in the vertebrate gut.

Cross-feeding of 1,2-PDO between non-pathogenic members of the gut microbiota has been inferred from the metabolic pathways of gut microbes and metagenomic analyses, but its ecological ramifications remain unexplored. In this thesis, we determined the importance of 1,2-PDO cross-feeding using isogenic mutants of both the bacterium that produced the metabolic intermediate (*Bifidobacterium breve*) and the species that utilized it (*Lactobacillus reuteri*). The results indicate that trophic interactions based on 1,2-PDO are important for the ecophysiology

of the gut as they influence both growth and *in vivo* performance of the microbe able to utilize the intermediate. The findings from this study improve our understanding of how metabolic networks establish within the gut microbiota and are relevant for the design of strategy to modulate gut ecosystems, which might benefit from the use of mixtures of bacterial strains that establish syntrophic interactions in probiotic applications.

Preface

This thesis is an original work by Christopher Cheng.

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Cheng CC designed and conducted the experiments, collected and analyzed data, and wrote the manuscript. Duar RM and Lin XB designed the experiments and contributed to manuscript editing. Perez Munoz ME contributed to manuscript editing, produced and provided artistic drawings, and designed animal experiments. Tollenaar S was responsible for animal husbandry. Oh JH and van Pijkeren JP generated the mutant *L. reuteri* ATCC PTA 6475 Δ*pduCDE* strains and contributed to manuscript editing. Li F contributed to manuscript editing. van Sinderen D provided the *Bifidobacterium bifidum* PRL2010 and *Bifidobacterium breve* UCC2003 strain and gave technical and conceptual advice. Gänzle MG oversaw analytical analysis and contributed to manuscript editing. Walter J conceptualized the experiments, supervised data analysis, and wrote the manuscript.

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1. Chapter 1: Literature review

1.1 Introduction

Microorganisms are found in virtually every environment, including multicellular eukaryotic organisms which act as habitats for microbial communities. For mammals, the microbes that reside in the gastrointestinal tract represent the largest population, with cell densities reaching as high as 10^{12} cells/mL (Clemente *et al.*, 2012) . In humans, the abundance of microbial cells alone has been estimated to be equivalent to the number of human cells at 1.3:1 trillion (Sender *et al.*, 2016). Comprised of thousands of species, the diverse microbial communities that inhabit their hosts are collectively known as the microbiota and their genomes the microbiome. These microbes are crucial to the overall health of the host, as they contribute to host digestive functions, immune tolerance and modulation, pathogen exclusion, neurodevelopment, and social development (Talarico *et al.*, 1988; Taranto *et al.*, 2003; Walsham *et al.*, 2016; Mu *et al.*, 2018a). The health benefits conferred by the microbiota to humans have warranted enough importance that large-scale interdisciplinary projects, such as the European Metagenomics of the Human Intestinal Tract (MetaHIT) (Lozupone *et al.*, 2012) and the Human Microbiome Project (Ley *et al.*, 2007), were conceived with the intention to characterize the basis of microbiotas associated with healthy individuals and the diseases in relation to the disruption of these microbial communities.

In this chapter, the dynamics of the gut microbiota in the gastrointestinal tract were discussed by identifying critical host and diet derived factors, as well as the introduction of the ecological interaction known as cross-feeding. The ecology of *Lactobacillus reuteri* in different vertebrate hosts and how the conservation of the *pdu-cbi-cob-hem* gene (*pdu)* cluster, in the

context of *L. reuteri,* may be used as a model to observe the intricate metabolic interactions that occur in the gut microbiota will also be discussed.

1.2 Role of the gut microbiota in host health

The gut microbiota has been shown to be fundamental to the well-being of its host. Given that these microbes are involved in several aspects of host biology, it is not surprising that a number of studies have focused on the impact that these diverse microbial communities have on host health.

Gut microbes have been shown to contribute to the development and maturation of the immune system and studies have demonstrated that interactions with gut microbes can drive the maturation of immune cells (Thursby & Juge, 2017). Evidently, immune deficiencies have been observed in germ-free mice, as CD4+ T cell populations are deficient in comparison to conventional specific-pathogen-free mice (Thursby & Juge, 2017).

In addition, it is suggested that the gut microbiota also plays a role in non-communicable diseases. It has been hypothesized that these microbial communities can be associated with several diseases that include cancer, autoimmune disorders, and neurological disorders (Lynch & Pederson, 2016); where studies have deduced an association between distinct gut microbiota compositions and the increased risk in individuals in the development of diseases such as diabetes, asthma, and allergies (Clemente *et al*., 2012; Lynch & Pederson, 2016).

These gut symbionts are also a major source of short-chain fatty acids (SCFAs), as they can digest dietary fibres to produce these substrates (Chambers *et al*., 2018). Comprised primarily of butyrate, acetate, and propionate, these metabolites have significant biological interest, as they

confer a variety of benefits to the health of the host. Butyrate have been shown to be important for colonocyte health, as it can be taken up and metabolized for energy (Chambers *et al.,* 2018). Butyrate contributes to gastrointestinal health by maintaining gut barrier function (Chambers *et al*., 2018). Acetate has been suggested to promote satiety and reduce cholesterol (Louis & Flint, 2017). Lastly, there has been emerging evidence of propionate and its role in gluconeogenesis in the liver (Reichardt *et al.,* 2014).

1.3 Ecology of the gut microbiota

1.3.1 Establishment and assembly

A vertebrate fetus is considered sterile until microbes begin to colonize the offspring during and shortly after birth. The process of microbial seeding in babies are largely dependent on the method of delivery, being born either through the vaginal canal or by caesarean section (c-section) (Cunnington *et al.*, 2016). Birth method is a crucial determinant in the establishment of the microbiome, as babies born through c-section have been found to harbour a gut microbiota that closely resemble the microbial communities of the skin microbiota. In contrast, babies born naturally will be colonized largely by microbes found in the vaginal canal (Clemente *et al.*, 2012). Maternal gut strains colonize the infant gastrointestinal tract more successfully than strains from other sources (Ferretti *et al.*, 2018) and the infant gut microbiota is dominated by Bifidobacteria (Bäckhed *et al.*, 2015). Over time, the gut microbiota matures and its composition changes rapidly during the first post-natal years, expanding in diversity (Thursby & Juge, 2017). It has been suggested that Bifidobacteria play an important ecological role in the gut microbiota, driving succession in the gut microbial communities of infants. Bifidobacteria house a repertoire of genes that allow for the degradation of complex glycans and it has been suggested that

Bifidobacteria are capable of facilitating the colonization of other microbes (Lewis & Mills 2017; Turroni *et al.,* 2018). These microbes may be responsible for niche-modification in the gastrointestinal tract through the early-life of an infant through the release of growth substrates during the metabolism of complex carbohydrates, allowing other microbes to propagate (Milani *et al.* 2015). This rapid expansion in microbial diversity decreases once childhood is reached but, remains less diverse in comparison to adult communities (Lynch & Pedersen, 2016).

The gut microbiota stabilizes once adulthood is reached, possessing rich and diverse microbial communities composed of thousands of species (Lozupone *et al.,* 2012). Although the gut microbiota contains a vast number of microbes, most healthy adult microbial communities in the gastrointestinal tract are dominated by Bacteriodetes and Firmicutes (Lynch & Pedersen, 2016). However, even though it is dominated by the two phyla, inter-individual variations in the gut microbiota exist, and differences in species and at the strain-level are apparent (Lynch & Pedersen, 2016). Furthermore, adult microbiotas are fairly resilient. Perturbations in the gut microbiota are difficult to implement, as the niches and resources are occupied by a well-established rich and diverse microbial community (Walter *et al.,* 2018).

1.3.2 Factors that influence the gut microbiota

Millions of years of evolution have led to interdependency between host and microbes. As a result, it is not surprising that host actions, whether innate or extrinsic, can significantly affect the microbiota. Ultimately, it is important to evaluate the several exogenous and endogenous factors that contribute to the shaping of the bacterial communities harboured in the gastrointestinal tract.

1.3.2.1 Immunology

The host immune system is a constituent in influencing on the gut microbiota. In addition to the removal of invading pathogens, the immune system can regulate the intestinal microbiota through the use of antimicrobials and its innate functions. For example, the secretion of IgA by plasma cells can affect the composition of the microbiota by managing the expansion of anaerobic bacteria in the small intestine. This is done by limiting the exposure of the epithelial cell surfaces to bacteria in the gastrointestinal tract (Kawamoto *et al.*, 2012). Intestinal paneth cells are also capable of deploying antimicrobial peptides such as defensins, altering the gut microbial communities (Ostaff *et al.*, 2013).

1.3.2.2 Mucin glycoconjugates

Host mucus also play a role in influencing the composition of the gut microbiota. Mucus is continuously secreted and synthesized by the goblet cells, covering the gastrointestinal tract in a protective and lubricative layer. Interestingly, the mucosal lining in the human gastrointestinal tract is composed of an inner and outer layer. The inner layer is absent of microbes and acts as an effective barrier, preventing contact between the epithelium and bacteria. This is because it is constituted by densely packed proteoglycans that do not allow bacteria to penetrate (Sicard *et al.,*2017). The outer layer is loosely attached and is exposed to proteolytic activity of the gut microbiota, acting as a source of nutrients for bacteria (Sicard *et al.,* 2017). Additionally, although bacteria can adhere to the outer mucosal layer, the constant slough off makes it difficult for the formation of biofilms (Frese *et al.,* 2013). A component of mucus, mucin, act as an integral part in bacterial colonization of the gastrointestinal tract (Sicard *et al.*, 2017). Mucins are glycoproteins of which are synthesized with glycosyltransferases starting with 1 of 8 core

structures and further decorated with a plethora of oligosaccharides and are further outfitted with a diverse set of carbohydrates. These glycan moieties can include galactose, N-acetylglucosamine (GlcNAc), N-acetylgalactosamine (GalNAc), fucose, and sialic acid (Corfield, 2015; Tailford *et al.*, 2015; Turroni *et al.*, 2018). The variety of glycan moieties branched on the epitopes of mucins are a potential source of energy that can be utilized by bacteria in the gut and can be accessed through the act of glycan foraging.

Initially, mucinolytic activities were first strictly associated with pathogens, however this is no longer the case, as glycan foraging activities has now been observed in several commensal bacteria of the gut microbiota (Tailford *et al*., 2015). To date, only a small number of microbes from the gut microbiota have been empirically established to forage glycans from mucins; among them, and not limited to, are several species of *Bacteroides, Akkermansia, Bifidobacterium,* and *Ruminococcus* (Tailford *et al.*, 2015; Turroni *et al*., 2018). Bacteria that possess mucin degrading genes are capable of liberating and metabolizing the carbohydrates affixed to mucins, giving those able to exploit the resource a competitive advantage (Tailford *et al*., 2015). This was demonstrated in germ-free mice fed with a simple sugar diet. When inoculated in competition, *Bacteroides thetaiotaomicron* and a reciprocal mutant, lacking the polysaccharide utilization loci required for mucin *O*-glycan utilization, revealed that mucin degradation and utilization of the branched glycans provided an advantage for the colonization of the gastrointestinal tract (Martens *et al.*, 2008). In addition, the breakdown of mucin has downstream effects on the gut ecosystem, as these carbohydrates can be released into the lumen of the host gastrointestinal tract and utilized by other members of the gut microbiota (Belzer *et al*., 2017; Sicard *et al*., 2017; Tailford *et al.,* 2015).

Host glycosylation of mucins suggests a long-standing evolutionary trait in response to the colonization of microorganisms in the gut; acting as a robust selective mechanism for the colonization of microbes as a source of energy and as a mechanism for attachment (Sicard *et al*., 2017). A host evolutionary response is further suggested as germ-free mice possess shortened mucin glycans and have been observed to downregulate the fucosylation of mucins 28 days postnatal (Arike *et al.*, 2017). Interestingly, glycosylation of mucins in germ-free mice can be reconstituted in response to the inoculation of known mucin glycan foraging microbes into the gastrointestinal tract (Bry *et al.*, 1996; Umesaki *et al.*, 1981; Nanthakumar *et al.*, 2013).

1.3.2.3. Diet

A pivotal role is played by diet in determining the assembly of gut bacterial communities. By dictating the nutrients accessible to those microbes residing in the host, diet can act as a selective force for the gut ecosystem. Diet and its influence on the microbial communities of the gastrointestinal tract have been extensively studied (Gentile & Weir, 2018). Shifts in the microbiota due to diet can be observed and the consumption of specific substrates can enrich certain groups of microbes in the gut. For example, adults fed with 5 grams of galactooligosaccharides (GOS) were found to have a hundred-fold increase in the abundance of *Bifidobacterium*, this effect is further amplified when the diet was supplemented with 10 grams of GOS (Davis *et al.*, 2011). Additionally, a study examined genes encoding for the enzyme known as porphyranase, required for the breakdown of porphyran, is regularly found in the microbiomes of the Japanese populations, but absent in Western populations. Porphyran is a common polysaccharide of nori, of which is considered a Japanese staple (Hehemann *et al.*, 2010) .

Diet-driven changes can be observed early in human life, where the method of feeding in postnatal infants impact the composition of the gut microbiota substantially. Breastfeeding infants will ingest human milk oligosaccharides (HMOs), a substrate found abundantly in breast milk; these carbohydrates pass through undigested into the distal parts of the infant's gastrointestinal tract (Marcobal & Sonnenburg, 2012). This substrate offers little to no direct benefit to babies themselves, since infants do not possess the necessary glycoside hydrolases required to metabolize HMOs. Instead, these glycans are used to enrich the gut microbiota, serving as a selective substrate for the microbes that can utilize the complex carboxyhydrates (Marcobal & Sonnenburg 2012). Backhed et al. were able to observe the effects of breastfeeding on the gut microbiota by following a cohort of infants. The gut of breastfed newborns was dominated with *Bifidobacterium* and *Lactobacillus* and did not change until breast feeding ceased (Bäckhed *et al.*, 2015). In contrast, the composition of infants fed with formula were found to be drastically different. They found that although the gut microbial community is dominated by *Bifidobacterium,* it was significantly lower in formula fed babies and have a more diverse composition (Bezirtzoglou *et al.*, 2011; Bäckhed *et al.*, 2015). There has been an increasing amount of evidence that suggest that the gut microbiota effects early childhood development (Martínez *et al.*, 2018), thus, it has been suggested that supplementation of HMOs into formula may be used to help a microbiota develop more "naturally" in formula-fed infants (Marcobal & Sonnenburg 2012).

The consumption of dietary fibres have also been indicated to have an important effect on the richness and diversity of the gut microbiota (Gentile & Weir 2018). Dietary fibres are a major source of microbiota-accessible carbohydrates (MACs); similar in concept to HMOs, MACs offer little to no direct benefit to humans that ingest it, as they are indigestible by human-

derived enzymes; MACs pass by undigested into the distal gastrointestinal tract and act as a metabolically available source of carbon and energy for the residential microbes (Deehan & Walter, 2016; Sonnenburg *et al.*, 2016). Akin to HMOs, MACs have been found to significantly influence the gut microbiota. A study determined that a diet with reduced accessibility to MACs, much like modern post-industrialized diets, may be responsible for a decline in microbial diversity. This was observed in the gut microbiota across generations of humanized mice, as certain taxa were lost due to the lack of MACs in the diet. More concerningly, not only are certain members of the gut microbiota no longer inherited by the next generation of mice, reintroduction of MACs to mice after several generations of a low-MAC diet was not followed by the recovery of the lost taxa in the microbiota (Sonnenburg *et al.,* 2016). However, in the same study, they demonstrated that this repercussion can be alleviated by reintroducing the lost taxa to the younger generation of mice when fed in conjunction with MACs (Sonnenburg *et al*., 2016).

In addition to low dietary fibres, post-industrialized diets are characterized with a high fat content. An increase in dietary fats have also been noted to contribute to alterations in the gut microbiota and associated with the decrease in diversity of microbial communities (Murphy *et al.*, 2015). Mice fed a high-fat diet (HFD) were found to have a decreased population of Bacteroidetes and an increase in both Firmicutes and Proteobacteria (Hildebrandt *et al.,* 2009).

The ramifications of HFDs and low dietary fibres on the structure of the gut microbial communities in humans have been formulated from studies that compare the composition of the gut microbiota between industrialized and non-industrialized countries. Schnorr and colleagues investigated the gut microbial composition of Hadza hunter-gatherers from northwestern Tanzania and compared the microbiomes and diet to an urbanized Italian population (Schnorr *et*

al., 2014). Their findings showed the gut microbiota of the Hadza had higher abundance of *Prevotella* and *Treponema,* decreased *Bacteroides,* and were absent of *Bifidobacterium* (Schnorr *et al.*, 2014). Furthermore, comparison between the fecal microbiota of European children and children from a rural village in Burkina Faso, Africa, indicated diet-based differences in the microbial composition (De Filippo *et al.*, 2010). Upon analysis, the diversity of European children was lower than that of the rural African children and the European children's gut microbiota resembled that of a post-industrialized diet, dominated mainly by Firmicutes and Proteobacteria (De Filippo *et al.*, 2010). Both studies attributed their findings to the differences in diet, where the non-industrialized populations consisted mainly of plant-derived fibres and polysaccharides (De Filippo *et al.*, 2010).

The aim of these extensive studies was to examine address the growing concerns of non-communicable diseases (i.e. inflammatory bowel syndrome, allergies, colon cancer, and autoimmune diseases) that have become increasingly prevalent in post-industrialized countries but largely absent in non-industrialized countries. Evidence for the link between these diseases and the gut microbiota have emerged and suggests that the incorporation of a post-industrialized diet, rich in fats and sugars and lacking dietary fibres, are causing an imbalance in the intricately evolved gut ecosystem caused by the promotion of proinflammatory bacteria in the gut microbiome. These findings are apparent, the gut microbiome can be shaped by diet and the negligible amounts of dietary fibre consumed in modern diets, known as the fibre gap, are concerning and calls for strategies to incorporate high amounts of dietary fibres into the diet, or risk losing beneficial microbes from the gastrointestinal tract as a potentially long-lasting consequence (Deehan & Walter, 2016).

1.3.2.4 Modulation of the gut microbiota

The relationship between the microbiota and its influence on human health provides an enticing justification to actively modulate these communities. Antibiotics are often used as a modulating agent when it comes to infectious diseases caused by pathogenic microbes. However, the bactericidal and bacteriostatic effects of antibiotics can result in severe repercussions in the microbiota. Antibiotics of the broad-spectrum variety have dysbiotic consequences on the gut microbiota by decreasing richness and diversity, disrupting up to a third of the community (Dethlefsen *et al.*, 2008), shifting the composition of the bacterial communities after the perturbation and altering the structure of these communities in comparison to the prior stable state (Dethlefsen & Relman, 2011). In addition to this, post-treatment of antibiotics leaves the gastrointestinal tract susceptible to invasion. Antibiotics can unintentionally target and remove commensal members residing in the gastrointestinal tract, dampening pathogen exclusion, providing foreign microbes an opportunity to colonize the gut microbiota, and potentially resulting in the restructuring of the microbial communities. Vacancies in previously occupied niches of the gastrointestinal tract can be exploited by pathogenic microbes (Francino, 2015). An example of unwarranted complications from antibiotic usage is antibiotic-associated diarrhea (AAD), which is diarrhea experienced after treatment with antibiotics and can be caused by invasive or opportunistic bacterial infections (Bartlett, 2002)W. A well-known consequence of AAD are *Clostridum difficile* infections (CDI) and are prevalent in patients with compromised microbiotas (Seekatz & Young, 2014). Antibiotics have been demonstrated as the cause of this disease, as chronic CDI infections can be established in the gastrointestinal tract of mice after the use of clindamycin, a consequence resulting from the removal of indigenous members from the microbiota (Buffie *et al.*, 2012).

Another strategy to modulate the gut microbiota is the administration of live microbes. Although the induction of microbes into the gastrointestinal tract has spanned for thousands of years in human history, the concept of probiotics was only established decades ago (McFarland, 2015). The first documentation of introducing microorganisms into the gastrointestinal tract was through the ingestion of yogurt produced by lactic acid fermenting lactobacilli in the early 1900s (McFarland, 2015). Since then, a repertoire of strains consisting of *Lactobacillus, Bifidobacterium, Streptococcus, and Enterococcus* have been identified and are utilized regularly as probiotics (Pandey *et al.*, 2015; McFarland, 2015). Determined based on a set of properties, the criteria for probiotic strains include safety (i.e. isolated from the gastrointestinal tract of healthy humans or animals), functionality and survivability at the designated target site, and technological usability (i.e. viability and stability of the probiotic product) (McFarland, 2015). Probiotics can be applied to aid in digestion, synthesize and provide vitamins, and the prevent pathogens from colonization through competition. Probiotics can also be utilized as biotherapeutics, having been recommended to patients afflicted with AAD, including those who have contracted CDI (Issa & Moucari, 2014).

Consumption of prebiotics is another strategy to modulate the gut microbiota and are defined as "a nondigestible compound that, through its metabolization by microorganisms in the gut, modulates composition and/or activity of the gut microbiota, thus conferring a beneficial physiological effect on the host prebiotics" (Holscher, 2017). In contrast to probiotics, prebiotics are ingested to stimulate compositional and metabolic changes of an existing indigenous microbiota rather than the introduction of an exogenous microorganism. A variety of fructans and galactans are categorized as such, and among them the most commercially available are inulin, fructooligosaccharides (FOS), and GOS (Pandey *et al.*, 2015). Substrates such as the

aforementioned HMOs are not prebiotics *per se*, however, HMOs can successfully modulate the composition and metabolic activity of the microbiota with beneficial physiological outcomes in a similar manner as prebiotics. HMOs differ by performing beyond the scope of prebiotics. These compounds are also capable of blocking pathogens from colonizing an infant gut independent of their gut-modulating influence (Bode, 2009). As a result, it is suggested that compounds of this caliber should be referred to as having a 'prebiotic effect(s)' (Bode, 2009).

Probiotics and prebiotics can be integrated together to form synbiotics. Currently there are two recognized approaches to synbiotics. First being complementary synbiotics, where the selected probiotic and prebiotic will promote the health of the individual independent of one another with a compounded positive effect from the two variables. Although the prebiotic may indirectly benefit the probiotic, it is not the main intention (Markowiak & Śliżewska, 2017). The hybridization of the probiotic and prebiotic concept results in synergistic synbiotics. Similar to complementary synbiotics, a probiotic is chosen because of the beneficial health effects it may exert on an individual, however, the prebiotic is specifically chosen to enhance the fitness of the probiotic, either to improve the survivability and growth or the stimulate its activity while in the host (Markowiak & Śliżewska, 2017). Synergistic synbiotics are advantageous when compared to probiotics or prebiotics alone and complementary synbiotics. Not only does the prebiotic promote the metabolic activity of the designated probiotic microbe but, by alleviating competition with the native gut microbiota, synergistic synbiotics can also be used to address the responder/non-responder effect (Holscher, 2017). Due to differences in the gut composition and diet among individuals, these factors can make the beneficial effects of probiotics or prebiotics unpredictable, leaving only a limited number of subjects receiving the intended benefits. It is speculated that this may be as a result of either the absence of microbes essential for fermenting

a prebiotic or that the probiotic is outcompeted by the resident microorganisms (Holscher, 2017). Specifically tailoring the prebiotic for utilization by probiotics addresses both issues; this scheme ensures that the prebiotic is fermented and that the probiotic is given a competitive advantage with a secure source of nutrients, increasing the effectiveness of each component.

Modulation of the gut microbiota is a complex paradigm with several factors in need of consideration. However, models have been adapted in order to develop an understanding of the modulation process of probiotics. As such, the induction of a microorganism(s) into the gastrointestinal tract is comparable to a biological invasion; with certain barriers that must be bypassed in order to be successfully instated (Walter *et al.*, 2018). The establishment of a microbe into the microbiota can be perceived in four stages which involve the introduction, establishment, growth and persistence, and impact of the species (Elsas *et al.*, 2015; Walter *et al.*, 2018). First, the organism must be in an active state and a sufficient amount must be administered. This could be dose dependent, as increasing the frequency of exposure may increase the chances of engraftment. Secondly, it must overcome the habitat filters in the host (i.e. host immune response or low pH of stomach acid). Third, when a niche is occupied, it must be able to secure nutrients and become an active member in the microbiol community, overcoming negative symbiotic relationships (i.e. predation and competition). Lastly, it must be able to impact the gut ecosystem (Walter *et al.*, 2018). The desired outcome of a probiotic application can range anywhere from providing additional nutrients to the host or to be used for the mitigation of irritable bowel symptoms and, although not necessarily required, propagation would enable the microbe to persist and colonize the gastrointestinal tract, thereby prolonging the benefits inherited from the modulation (Walter *et al.*, 2018). This is especially important for

patients suffering from chronic gastrointestinal disorders, such as the persistence of fecal microbiota transplants from donors into patients suffering from CDI (Issa & Moucari, 2014).

This topic can be further broadened when examining the interplaying processes of gut modulation in more depth and the characteristics of the potential colonist. As mentioned before, dosage and an adequate number of microbes must be administered but, preselecting the traits for more favorable survival will increase the success of modulation. A candidate probiotic can be selected based on origin, as choosing microbes that are autochthonous to the host may extend persistence or improve the potential to colonize the gastrointestinal tract, and having an evolutionary association with the host may allow the microorganisms in question to competently adapt to specific target sites and compete more efficiently against the resident communities (Walter *et al.*, 2018). Exogenous and endogenous host related factors should also be assessed for successful modulation. As demonstrated earlier in this review, a plethora of conditions from the host influences the gut microbiome; including host genetics, physiology, immunology, and diet. Habitat filters of the host are constituted in order to select for organisms that are beneficial. This is most evident when the members of the gut microbiome are dominated by specific phyla (Thursby & Juge, 2017).

1.4 Interactions between members of the gut microbiota

The gut microbiota can influence its own dynamics; as the gastrointestinal tract of a host and the microbial communities housed resemble a thriving ecosystem. These microbes can interact with one another and form symbiotic relationships that range from mutualistic to antagonistic associations that consequently shape these assemblages (Moran, 2006).

1.4.1 Competition

Interactions between members of the gut microbiota can lower the fitness of one another. Known as competition, these interactions can be either exploitative or interference. Exploitative competition is an indirect interaction and is usually defined by microbes competing for a common resource or niche (Coyte & Rakoff-Nahoum, 2019). Genomic analyses can be used as a tool to predict exploitative competition between gut bacterial. For instance, members that share or possess similar genetic content required for the ability to digest a carbohydrate may indicate competition for this resource in the gut. This can be also studied *in vitro* by directly culturing singles strains on a specific resource and analyzing the utilization of the substrate (Coyte & Rakoff-Nahoum, 2019).

In contrast to exploitative competition, interference competition directly affects other species and involves the application of contact-dependent mechanisms (Coyte & Rakoff-Nahoum, 2019). An example would be the deployment of the type VI secretion system. Bacteria that possess such apparatus can physically deliver toxins by injection to eliminate other members in the gut microbiota (Coyte & Rakoff-Nahoum, 2019). The secretion of antimicrobials and bacteriocins are also considered as mechanisms for interference competition (Coyte & Rakoff-Nahoum, 2019; Thursby & Juge, 2017).

1.4.2 Cooperation

Recently, there has been a surge of studies investigating the cooperative behaviour between microbes in the gut microbiota. This encompasses positive associations between gut members that span from commensal, where one species fitness is improved and the other is unaffected, or true cooperation, in which species have specifically evolved to benefit one another (Coyte & Rakoff-Nahoum, 2019). This can be facilitation of one species by another in the

gastrointestinal tract. An example would be the facilitation of *Lactobacillus taiwanensis* by *Lactobacillus reuteri* (Lin *et al.,* 2018). Alone, *L. taiwanensis* cannot effectively establish biofilms in germ-free mice. However, when mice were co-colonized with *L. reuteri* and *L. taiwenensis,* the cell density of the latter, and in some cases both species, increased suggesting synergistic interactions between the two symbionts (Lin *et al.* 2018). Cooperation between microbes can also be exemplified by the generation and distribution of 'public goods', which are metabolically expensive products that are released into the extracellular environment. An array of public goods exists and consist of autoinducers, siderophores, antibiotic-degrading enzymes, and matrix components for biofilm formation, all of which can be utilized by non-producing recipients of the local bacterial population for a fitness advantage (Morris *et al.*, 2013; Seth & Taga, 2014; Sonnenburg & Sonnenburg, 2014; Rakoff-Nahoum *et al.*, 2016; D'Souza *et al.*, 2018). Cross-feeding interactions have been shown to facilitate the colonization of microbes in the gastrointestinal tract. This was demonstrated by the metabolism of complex carbohydrates by *Bacteriodetes ovatus* releasing substrates that *Bacteriodetes vulgatus* can utilize for growth (Rakoff-Nahoum *et al.,* 2016).

1.4.3 Metabolic cross-feeding in the gut microbiota

Public goods can also exist in the exchange of metabolites. Microbes can beneficially affect one another through the provision of molecules from one microbe that constitute a nutrient for another, known as cross-feeding (Seth & Taga, 2014). D'Souza and colleges were able to categorize a series of microbial cross-feeding interactions based on two characteristics, the degree of reciprocity and the investment of the involved partners (D'Souza *et al.*, 2018). The degree of reciprocity is recognized as either unidirectional or bidirectional, determining which microbes are developing and/or receiving the metabolites. Another element is the investment of

the corresponding partners, which is gauged by the cost of synthesizing the molecules exchanged and are used to group the interactions as either by-product or cooperative cross-feeding. By-product cross-feeding is the exchange of metabolites involving a "selfish" producer at no extra cost of resources. In contrast, cooperative cross-feeding involves a partner(s) actively investing energy into producing metabolites that benefit the recipient(s) (D'Souza *et al.*, 2018).

Based on these two principles, five different types of cross-feeding paradigms can be described. Unidirectional by-product cross-feeding is the result of one of the partners releasing a metabolic by-product that is utilized by the other, the producer does not invest any extra cost nor receives anything in return. This is commonly recognized with the release of an end-product that is scavenged and metabolized by another cell. Bidirectional by-product cross-feeding in contrast has both members benefiting off each reciprocating partner's by-product, without any additional expenditure of energy by either participant. By-product reciprocity is a case where a cooperative act is undergone by a member, producing costly metabolites that can be utilized by the partner, the reciprocating partner then releases a by-product which the initial individual can then metabolize. The cross-feeding equivalent of an altruistic relationship is unidirectional cooperative cross-feeding; in this situation, the producer shares their public goods at a cost and receive nothing in return from the recipients. Lastly, there is bidirectional cooperative cross-feeding. This mutualistic interaction involves both members benefitting from each other through the exchange of energetically costly metabolites (D'Souza *et al.*, 2018).

An array of mechanisms exists for cross-feeding between partners and can be influenced by the lifestyle of the microbes (i.e. planktonic versus biofilm communities). Resources can be transferred through contact-independent methods, which revolves around releasing the substrates into the extracellular environment for recipient uptake. Contact-independent mechanisms include

passive diffusion through the membrane or active transportation out of the cell. The methods deployed are usually determined by the size of the molecule; smaller substrates, such as hydrogen or potassium atoms, vitamins, and amino acids are commonly diffused out of the membrane; and is commonly linked with the by-product cross-feeding scheme as a consequence of metabolic leakage (D'Souza *et al.*, 2018). Larger molecules or those particularly difficult to release due to charge or polarity characteristics (ex. siderophores, enzymes, and large polymers), require the investment of energy to be made available for the local population; ATP-binding cassette transports and vesicular-mediated transport, such as outer membrane vesicles and outer-inner membrane vesicles, have been suggested as active transport strategies (D'Souza *et al.*, 2018). Cross-feeding interactions can also be completed through contact-dependent manners, where a physical connection is established between each participating individual. Microbes can form links to one another through dedicated structures, namely vesicle chains and nanotubules, or through existing structures that can be adapted for transport, for example, flagella-like filaments. This can also be achieved simply through cell-cell contact, as cross-feeding can be initiated after direct membrane contact (D'Souza *et al.*, 2018).

Metabolic end-products that result from fermentation processes can be utilized by microbes. These interactions can be traced to resemble intricate metabolic networks between coexisting microbes and have been shown to play a key role in the formation of SCFAs (Engels *et al.*, 2016; Louis & Flint, 2017). The process of breaking down complex carbohydrates by communities of microbes in the gut is possible, a phenomenon known as classical syntrophy (Seth & Taga, 2014) and cross-feeding of intermediary metabolites such as lactate, succinate, and 1,2-propanediol (1,2-PDO) are key to the production of SCFAs (Louis & Flint, 2017). For example, Belzer et al. were able to demonstrate the metabolism of host mucus and the utilization

of released substrates from other gut commensal microbes (Belzer *et al.*, 2017). By-product reciprocal cross-feeding was observed in the break-down of mucin between *Akkermensia muciniphila* and *Eubacterium hallii*. Mucus was first deglycosylated and metabolized by *A. muciniphila*, resulting in the release of oligosaccharides and acetate, and used subsequently by *E. hallii* for growth, producing additional SCFAs in the form of propionate and producing vitamin B12 as end products. The vitamin B12 can be further utilized by *A. muciniphila,* stimulating further production of propionate (Belzer *et al.,* 2017).

1.5 *Lactobacillus reuteri* as a model to study ecology and evolution in the gut ecosystem

The Gram-positive bacterium, *Lactobacillus reuteri,* is considered autochthonous to the digestive tract of a number of vertebrate hosts and are a common gut symbiont of a large subset of animals such as rodents, pigs, chickens, and humans (Oh *et al.*, 2010; Frese *et al.*, 2011; Walter *et al.*, 2011). *L. reuteri* strains are fastidious and, under optimal conditions, have a replication doubling rate of less than one hour (Walter *et al.*, 2011) and, like other lactobacilli, the species requires easily fermentable sugars, vitamins, amino acids, and nucleotides (Walter *et al.*, 2011). Physiological and anatomical differences between humans and animals cause the bacterium to utilize different ecological strategies. In pigs, rodents, and chickens, *L. reuteri* form biofilms in the upper digestive tract of these hosts (Walter *et al.*, 2011). This is possible because some *L. reuteri* can adhere to the mucus-free stratified squamous epithelium that exists in the porcine par esophagus, rodent forestomach, and chicken crop (Walter *et al.*, 2011). Interestingly, population genetics and comparative genomics have shown that different *L. reuteri* strains form clades that correspond to their host origin with lineage-specific genes that reflect adaptations to the niche characteristics in the gastrointestinal tracts of specific hosts (Oh *et al.,* 2010; Frese *et*

al., 2011). The combination of genomic analysis and functional studies give insight into the evolutionary and ecological strategies of the gut symbiont. For instance, *L. reuteri* strains derived from the gut microbiota of animals with a squamous epithelium in the digestive tract have evolved with host-specific adaptations, possessing genes that are required for the formation of biofilms and cell wall adhesion (Frese *et al.,* 2011).

Genomic analysis of rodent *L. reuteri* strains have shown that it has evolved a series of functions congruent to the establishment within the rodent gastrointestinal tract (Fig. 1). These genes constitute colonization factors that include surface proteins for adhesion to epithelial surfaces, a secretory system for adhesion proteins to the cell surface, a urease cluster responsible for acid resistance, and two-component regulatory systems for quorum sensing (Fig. 1) (Frese *et al.,* 2011; Wilson *et al.,* 2014). The genes encoding these functions are nearly exclusively found in the genomes of rodent isolated *L. reuteri* (Frese *et al.,* 2011). Functional analysis using a combination of germ-free mice and non-functioning mutants determined the ecological importance of these genes. Frese *et al.* observed a complete absence of biofilm formation in mono-associated mice following the disruption of serine-rich repeated protein (SRRP) genes, large surface proteins required for the adhesion to the epithelial surfaces in *L. reuteri* (Frese *et al.,* 2013). The impairment of the SecA2-SecY2 secretory system, transport system for the SRRP like adhesion protein, indirectly impeded the formation of biofilms in the mouse forestomach, as the presence of the large surface proteins were significantly reduced on the cell surface (Frese *et al.,* 2013). *L. reuteri* possessing a non-functioning urease cluster, mutation in the urea hydrolase gene (*ureC*), were still able to establish biofilms in mono-associated mice (Frese *et al.,* 2013). However, the strain's competitiveness in the colonization of the mouse squamous epithelium was severely reduced. The UreC mutant was outcompeted and the total *L. reuteri* population

consisted of only a small fraction of the mutant strain when co-colonized with the wild-type strain in gnotobiotic mice (Wilson *et al.,* 2014). Mutations in the quorum sensing genes also negatively impacted the establishment of biofilms in the gut (Frese *et al.,* 2013).

In contrast, the human gastrointestinal tract does not possess stratified squamous epithelial cells and, as previously mentioned, have a luminal epithelium that is covered by mucus, making biofilm formation difficult. The ecological strategies of *L. reuteri* to establish itself in the human gut are not well understood, but it has been speculated that *L. reuteri* colonizes the lumen of the gastrointestinal tract in humans, which would require fast replication rates to avoid wash-out (Walter *et al.*, 2011). As a result, it is suggested that epithelial cell layers enriched with lactobacilli are non-existent and *L. reuteri* inhabit the distal portions of the intestinal tract instead (Frese *et al.* 2011). In addition, the genes responsible for the formation of biofilms and adhesion proteins commonly found in rodent strains are largely absent in *L. reuteri* isolated from humans (Frese *et al.,* 2011). Instead, the genes that confer the ability to synthesize cobalamin (vitamin B12), produce reuterin, and to utilize 1,2-PDO, an intermediary metabolite present in the gut of humans, are highly conserved in the majority of human derived *L. reuteri* and have been speculated to contribute to the colonization of the human gut (Fig. 1) (Frese *et al.,* 2011; Walter *et al.*, 2011). Moreover, mucus-binding proteins were also found in human isolated *L. reuteri,* suggesting an evolutionary adaptation to its host physiology (Mackenzie *et al.,* 2010).

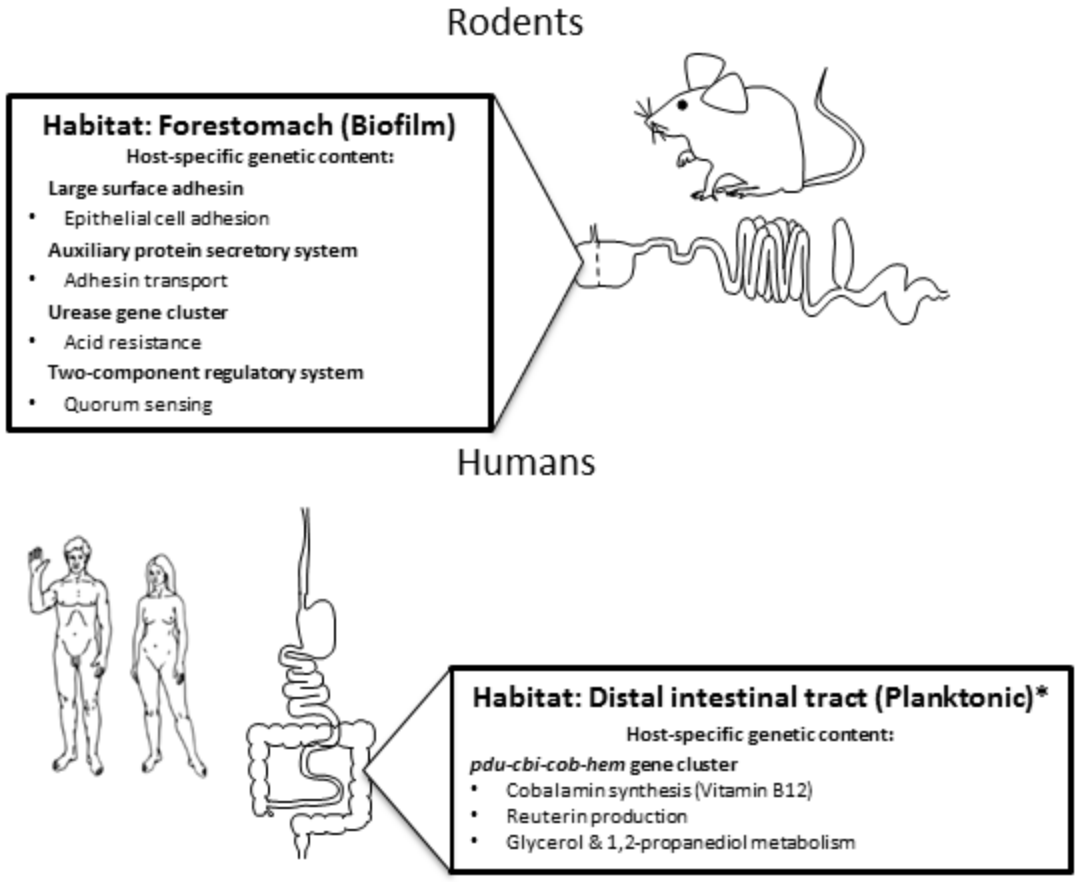

Figure 1. Host-specific gene content in rodent and human isolated *L. reuteri* strains. At a strain-level, *L. reuteri* lineages possess host-specific genomic content. Rodent derived strains possess genes required for the survival and establishment of biofilms in the forestomach. Human derived strains have been hypothesized to inhabit the distal portions of the gastrointestinal tract planktonically (*) and possess the *pdu-cbi-cob-hem* gene cluster which has been speculated to be an important colonization factor in humans. Figure designed in accordance to findings from Frese *et al.,* 2011 and Frese *et al*., 2013.

L. reuteri are known to utilize a variety of compounds as electron acceptors (i.e. fructose, glycerol, nitrate, and 1,2-PDO) as additional oxidizing agents to increase its metabolic efficiency (Gänzle, 2015). The utilization of an external electron acceptor allows *L. reuteri* to recycle reduced cofactors (i.e. NADH) and utilize the acetate pathway to generate additional ATP (Gänzle, 2015). The metabolism of glycerol and 1,2-PDO in *L. reuteri* is performed by the diol dehydratases encoded within a 58-gene *pdu* cluster (Sriramulu *et al.,* 2008). 1,2-PDO is disproportionated by the glycerol/diol dehydratase, PduCDE, to the intermediate form, propionaldehyde, allowing it to be further processed (Figure 1). Interestingly, it has been found that this enzyme is isofunctional as it is able to utilize both glycerol and 1,2-PDO (Sriramulu *et al.,* 2008). Following the conversion of 1,2-PDO, propionaldehyde is further reduced to propanol and oxidized to propionate by other Pdu enzymes for the recovery of NADH and to generate ATP, respectively (Fig. 2) (Sriramulu *et al.,* 2008; Gänzle, 2015). This process is performed in proteinaceous microcompartments in order to negate the toxic and harmful mutagenic effects of propionaldehyde on the cell (Sampson & Bobik, 2008). Additionally, during hexose metabolism the intermediary substrate, acetyl-phosphate, is produced and the utilization of 1,2-PDO as an electron acceptor provides an alternative metabolic route for acetyl-phosphate. In the absence of an external electron acceptor, acetyl-phosphate is reduced to ethanol in order to recover NADH. However, propionaldehyde can be used to recover NADH during the metabolism of 1,2-PDO (Fig. 2) (Gänzle, 2015). As a result, 1,2-PDO utilization allows acetyl-phosphate to be dephosphorylated to acetate by an acetate kinase to generate an additional ATP instead (Fig. 2) (Gänzle, 2015). The extended capacity in ATP production and cofactor regeneration through the metabolism of 1,2-PDO and glycerol enhances the growth rates of *L. reuteri.* Studies have demonstrated the increased growth rates conferred by glycerol and 1,2-PDO consumption in

human derived *L. reuteri* in food fermentations (Gänzle, 2015). Rapid growth of *L. reuteri* in sourdough was attributed by its ability to utilize glycerol as an external electron acceptor (Lin & Gänzle, 2014). This was confirmed by competition experiments, where the a wild-type *L. reuteri* strain was able to outcompete an isogenic mutant lacking the genes encoding the glycerol dehydratase (Lin & Gänzle, 2014). This phenotype was also observed when glycerol was added as an exogenous substrate during carbohydrate fermentation in liquid media (Talarico *et al.,* 1990). Utilization of 1,2-PDO also demonstrated enhanced growth in *L. reuteri* (Rattanaprasert *et al.*, 2014).

Growth substrates are abundantly available in the proximal digestive tract of rodents, pigs, and chickens. However, the acquisition of fermentable sugars in the distal intestinal tract of humans is challenging for the microbial residents, as much of it is absorbed by the small intestine and other enteric microbes that reside in the gut (Walter *et al.*, 2011). Because of this, the metabolism of 1,2-PDO by *L. reuteri* may potentially play a crucial role in the colonization of the human gut. Also, the conservation of the *pdu* cluster may suggest an evolutionary strategy for human-lineage *L. reuteri* strains (Frese *et al.,* 2011).

Both glycerol and 1,2-PDO can be found in the human gut. Glycerol is commonly used as a humectant, solvent, and sweetener in food products (Gänzle, 2015). Glycerol can also be found in the form of triglycerides, or dietary fat. Although some of it are absorbed as it passes through the digestive tract, trace amounts do reach the colon (Talarico *et al.,* 1990). 1,2-PDO is a common metabolite of enteric species and the result of anaerobic fermentation of deoxyhexose sugars, such as fucose and rhamnose, in the colon (Boronat & Aguilar, 1981). Furthermore, these two carbohydrates are common moieties of host glycoconjugates and plant fibres, respectively

(Gänzle, 2015). Potentially, this mechanism that causes increased growth rates in *L. reuteri* may be a contributing factor in the colonization of the human gut.

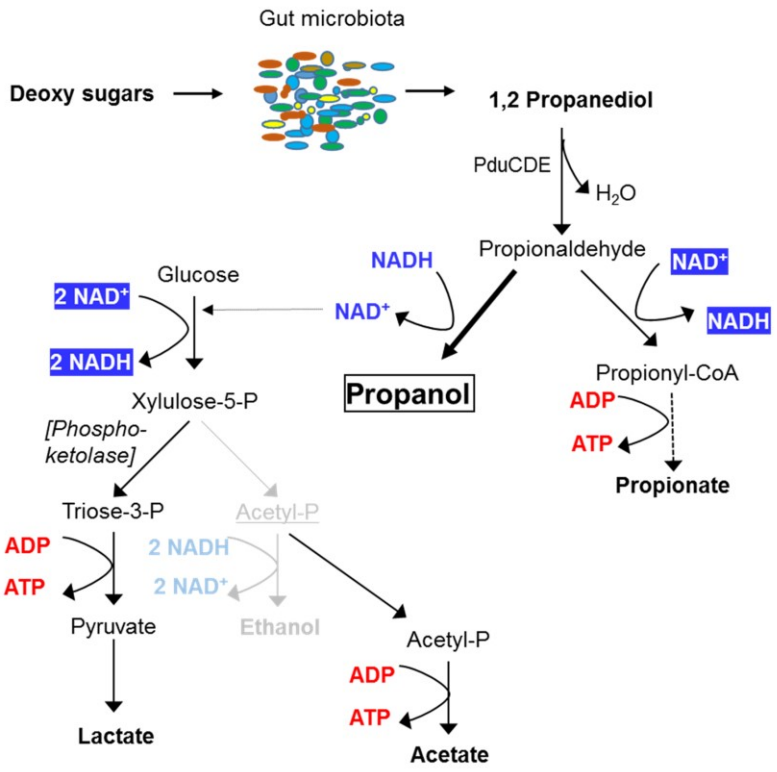

Figure 2. Graphical illustration of metabolic pathways for the metabolism of 1,2-PDO as an external electron acceptor during hexose fermentation. 1,2-PDO derived from the gut microbiota can be used to alleviate the use of acetyl-phosphate (Acetyl-P) as a hydrogen acceptor to regenerate NAD+ and produce additional ATP. Metabolic end products are printed in bold. Figure derived from Duar, 2017.

Although *L. reuteri* can be isolated from human feces, it appears that its prevalence has been declining over the past decades and are rarely detected in present day (Walter *et al.*, 2011). It has been speculated that changes in the modern lifestyle (i.e. diet, sanitation, dispersal, and antibiotics) may have displaced *L. reuteri* as a dominant member of the human gut microbiota (Walter *et al.,* 2011). A recent study observed the ecological performance of a mixture of *L. reuteri* strains that originated from different host-lineages in different vertebrate hosts (mice, chickens, pigs, and humans) (Duar *et al.,* 2017). *L. reuteri* strains from rodents and chickens were enriched when administered into germ-free mice and antibiotic-treated chicken hosts, respectively. Interestingly, *L. reuteri* from human lineages did not show enhanced fitness in the human gut when compared to other *L. reuteri* strains of different host origins, and all strains were transient and washed out within 5-7 days (Duar *et al.,* 2017). These findings support the hypothesis that *L. reuteri* derived from rodents and chickens have evolved specific adaptations to their respective host ecology, however, this conclusion remains ambiguous among strains from the human-lineage (Duar *et al.,* 2017). The inability of the *L. reuteri* strains that originate from humans to establish themselves in the gastrointestinal tract may in part be due to the conventional microbiota of the human subjects and that the niche maybe already be occupied (Duar *et al.,* 2017). Additionally, the human subjects in this study followed a modern lifestyle, which has already been speculated to affect the ecological success of *L. reuteri* (Duar *et al.,* 2017). This speculation is further reinforced by findings in a study that characterized and compared the gut microbiomes and lifestyles of Americans (post-industrialized) and Papua New Guineans (non-industrialized). Specifically, Martínez *et al.* discovered that *L. reuteri* are a dominant member of the gut microbiota in Papua New Guineans and are rarely detected in American individuals (Martínez *et al.,* 2015).

1.6 The role of *Lactobacillus reuteri* for host health

L. reuteri have been studied intensively and have been shown to contribute to the health of the host (Mu *et al.,* 2018). *L. reuteri* have been speculated to be a source of vitamins B9 and B12, as several strains are capable of synthesizing these molecules (Taranto *et al.*, 2003; Sriramulu *et al.*, 2008; Mu *et al.*, 2018) and are a source of acetate and propionate (Louis & Flint, 2017). This microbe is also able to regulate the immune system through the inhibition of proinflammatory cytokines or induction of regulatory T cells and have been shown to modulate the oral, gastrointestinal, and vaginal microbiota (Mu *et al.*, 2018).

In addition to this, studies have shown *L. reuteri* to be particularly effective against known pathogens of gastrointestinal tract related diseases (Mukai *et al.*, 2002; Walsham *et al.*, 2016; Spinler *et al.*, 2017; Mu *et al.*, 2018). *Helicobacter pylori* infections can be potentially treated with the administration of *L. reuteri*. Early infections can be inhibited by *L. reuteri* by limiting the colonization capacity of *H. pylori*. Adherence to host glycolipids, known to be associated with the gastrointestinal mucosa have been demonstrated to limit the binding of *H. pylori*, suggesting a reduction in bacterial load through competition (Mukai *et al.*, 2002). The production of antimicrobials by *L. reuteri* may also contribute to pathogen inhibition. Currently being investigated as a next-generation biotherapeutic for CDI, *L. reuteri* are intrinsically resistant to antibiotics used to combat CDI and the production of reuterin, a broad-spectrum antimicrobial produced from the fermentation of glycerol (Talarico *et al.*, 1990), make *L. reuteri* a promising candidate to combat *C. difficile* infections (Spinler *et al.,* 2017). Reuterin has been demonstrated to be effective against *C. difficile.* Minibioreactors with established human fecal communities were perturbed with antibiotics and subsequently infected with *C. difficile* and reuterin-dependent inhibition was observed in these communities with the addition of *L. reuteri*

fermenting glycerol. In contrast, this outcome was not distinguished in the reactors with *L. reuteri* absent of glycerol (Spinler *et al.,* 2017).

L. reuteri have also been linked to neurodevelopment. Mice offspring from mothers on maternal HFD (MHFD) displayed social impairments. In fact, germ-free mice that suffer from social deficits can be rescued by the fecal transplantation from the offspring of mice from maternal regular diets but, not from the offspring of those from MHFD (Buffington *et al.*, 2016) . Upon further analysis it was found that the offspring of the MHFD microbiota is diminished in *L. reuteri* and sociability could be significantly improved by the administration of *L. reuteri* in MHFD offspring; demonstrating the effect of *L. reuteri* and its promotion of oxytocin-mediated functions via the gut-brain axis (Buffington *et al.*, 2016).

It has become clear that *L. reuteri* contributes to a number of factors that involve health. Additionally, it has been shown that *L. reuteri* can be safely consumed at high doses (Mu *et al.,* 2018). Together, these circumstances make a compelling case to utilize this species as a probiotic.

1.7 Knowledge Gaps

The metabolism of 1,2-PDO has been demonstrated in several commensal bacterial species (i.e. *E. hallii* and *L. reuteri*) and is attributed to the possession of the *pdu* operon. In *L. reuteri,* the preservation of these genes among the human-lineages suggests that this mechanism constitutes an adaptation to the characteristics of the gastrointestinal tract in humans. Although the cross-feeding of 1,2-PDO among members of the gut microbiota have been interpreted through metabolic pathways of gut microbes and metagenomic analyses, the ecological

significance of this interaction has yet to be demonstrated empirically between non-pathogenic members of the gut microbiota.

Additionally, even though the metabolism of 1,2-PDO and the resulting enhanced growth benefits has been established in *L. reuteri,* it is unknown if the species engages in syntrophic interrelationships with other members of the gut microbiota that produce this intermediary metabolite, and if this interaction contributes to its ecological competitiveness.

1.8 Objectives and hypothesis

The goal of this project is to characterize the ecological importance of metabolic cross-feeding of 1,2-PDO between *L. reuteri* and commensal gut bacteria. It has long been speculated that the heavily conserved *pdu* cluster is ecologically relevant in *L. reuteri*, but this remains to be elucidated. In this thesis, it is hypothesized that *L. reuteri* can engage in syntrophic interactions with other gut commensal bacteria (*Bifidobacterium breve* and *Escherichia* coli) through the utilization of 1,2-PDO derived from the fermentation of deoxyhexose sugars (fucose and rhamnose) for a fitness advantage, and that the cross-feeding of 1,2-PDO, dependent on the *pdu* cluster, will be a critical factor in the colonization of *L. reuteri* in the gastrointestinal tract. Findings in this thesis contribute to a better apprehension of the ecological and evolutionary forces that shape the gut ecosystems.

This thesis will contribute to the understanding on the significance of cross-feeding and other cooperative interactions and how these microbial consortia function in the gastrointestinal microbiota (O'Connell *et al.*, 2018; Rakoff-Nahoum *et al.*, 2016). These findings are imperative to understanding the ecological and evolutionary forces that shape the gut ecosystems. Furthermore, by elucidating mutualistic associations between members of the gut microbiota, a

better understanding would have implications towards improving microbial-based gut modulation strategies, such as probiotics. Difficulties are often encountered in the modulations of the gut ecosystems as probiotics are often transient, which is in due part to the resilient nature of the gut microbiota, and resists engraftment (Walter *et al.,* 2018). It has been suggested that an ecological framework be considered for probiotic applications (Bindels *et al.,* 2015; Walter *et al.,* 2018). Mutualistic and facilitative associations between members of the gut microbiota can be used to produce more effective probiotic applications, potentially increasing long-term persistence, in which these findings can be utilized in the design of probiotic strain mixtures, synbiotic products, or for the personalization of applications.

2. Chapter 2 Materials and Methods

2.1 Bacterial strains and culture conditions

Bacterial strains used in this study are listed in Table 1. *L. reuteri* strains were grown in de Man, Rogosa, and Sharpe (MRS) medium (Difco) under anaerobic conditions (5% CO_2, 5% H_2, and 90% N_2). *B. bifidum* and *B. breve* strains were grown anaerobically in MRS medium supplemented with 0.05% L-cysteine. *E. coli* were grown in Luria-Bertani (LB) broth with agitation. All incubations were performed at 37 °C.

Table 1. Strains used in this study.

Species (strain code)	Origin	Relevant features	Reference
Lactobacillus reuteri			
ATCC PTA 6475	Breast Milk	1,2-PDO utilizer with complete *pdu* cluster	(Zhang *et al.*, 2018)
ATCC PTA 6475 Δ*pduCDE*	Isogenic mutant of PTA 6475	Glycerol/diol dehydratase (*pduCDE)* mutant	(Zhang *et al.*, 2018)
Escherichia coli			
MG1655	Lab-derived strain	L-rhamnose utilizer, 1,2-PDO producer	The Coli Genetic Stock Center (CGSC)
Bifidobacterium bifidum			
PRL2010	Infant stool	Mucin degrader, L-fucose producer	(Turroni *et al.*, 2010)
Bifidobacterium breve			
UCC2003	Infant stool	L-fucose utilizer, 1,2-PDO producer	(Mazé *et al.*, 2007) (Egan *et al.*, 2014)
UCC2003 Δ*fucP*	Isogenic mutant of UCC2003	L-fucose transporter (*fucP)* mutant	

2.2 Evaluation of the impact of 1,2-PDO on *L. reuteri* ATCC PTA 6475 growth

Overnight cultures of PTA 6475 and Δ*pduCDE* were inoculated at 1% into 15 ml of half-strength mMRS (Stolz *et al.*, 1995) containing 25mM glucose alone, 50mM of 1,2-PDO (Sigma-Aldrich) alone, or 25mM glucose plus 50mM of 1,2-PDO. Growth of cell cultures were

monitored based on optical density at 600 nm (OD_{600}) with a spectrophotometer every 3 hours over the span of 12 hours. 1 mL samples were collected for HPLC analysis every 3 hours. All experiments were performed in triplicate at 37 °C under anaerobic conditions.

2.3 *In vitro* production of 1,2-PDO and cross-feeding assay development

Pre-cultures of *B. breve* strains and *E. coli* were prepared as follows. Full-strength mMRS supplemented with 30mM cellobiose ± 30mM L-fucose were inoculated with 1% of overnight cultures of UCC2003 or Δ*fucP*. Full-strength mMRS containing 25mM of glucose or 30mM of L-rhamnose were inoculated with 1% of overnight *E. coli* MG1655 cultures. These fermentations were conducted under anaerobic conditions for 24 hours at 37 °C. Conditioned medias were prepared from pre-cultures as follows. Cells were removed from pre-cultures by centrifugation (5000 x g for 10 minutes) and the supernatant was collected. The supernatant was supplemented with half-strength mMRS (50% w/v mMRS dry reagents to supernatant) and 25mM glucose (dry reagent). These were further adjusted to pH 6.6 and filter sterilized (0.22 μm), stored at 4 °C and used within 48 hours. Conditioned medias were subsequently named as shown in Table 2. Conditioned medias were inoculated with *L. reuteri* strains (1% inoculation) and growth was monitored for 12 hours by measuring OD_{600} with a spectrophotometer. OD_{600} measurements and 1 mL samples for HPLC analysis were collected every 3 hours. All experiments were performed in triplicate under anaerobic conditions at 37 °C.

Table 2. Media used for *in vitro* cross-feeding experiments.

Fermenting Strain	Deoxyhexose Sugar	Other carbohydrates	Abbreviation	Purpose
E. coli MG1655	N/a	25 mM Glucose	EM (G)	Control for *L. reuteri* growth absent of 1,2-PDO production
	30 mM Rhamnose	N/a	EM (R)	Determine effect of 1,2-PDO produced from Rhamnose fermentation on *L. reuteri*
B. breve UCC2003	N/a	30 mM Cellobiose	BM (C)	Control for *L. reuteri* growth absent of 1,2-PDO production
	30 mM Fucose	30 mM Cellobiose	BM (CF)	Determine effect of 1,2-PDO produced from Fucose fermentation on *L. reuteri*
B. breve UCC2003 Δ*fucP*	N/a	30 mM Cellobiose	BΔM (C)	Control for *L. reuteri* growth absent of 1,2-PDO production
	30 mM Fucose	30 mM Cellobiose	BΔM (CF)	Control for *L. reuteri* growth absent of 1,2-PDO production

N/a: not applicable

2.4 Experiments in gnotobiotic mice

All animal experiments were performed with the approval of the Animal Care and Use Committee (ACUC) of the University of Alberta (AUP 00002764). Germ-free Swiss-Webster mice (6-16 weeks of age, male and female) were bred and maintained in the Health Sciences Laboratory Animals Services (HSLAS) Facility at the University of Alberta. Mice were randomly selected and moved from a flexible-film isolator and housed in sterile, individually ventilated, positive-pressured biocontainment cages for the duration of the experiments (IsoCage P Biocontainment; Tecniplast). To avoid confounding effect of glycerol, which is also utilized by the *pdu* cluster diol/glycerol dehydratase (Sriramulu *et al.*, 2008), an irradiated fat-free diet (34.4% glucose and 34.4% cornstarch; Teklad TD.180765) was used in order to minimize

possible interference from the hydrolysis of triglyceride fats. After transfer to the biocontainment cages, mice were fed with the new diet for 3 days before colonization with the bacteria.

To study syntropy of 1,2-PDO in the gastrointestinal tract, groups of mice (*n=5*), 2 to 3 mice per cage, were assigned to receive either *Bifidobacterium-L. reuteri* triple-species mixtures or *E. coli-L. reuteri* double-species mixtures (Table S1 & S2). To test for cross-feeding of 1,2-PDO produced from mucin derived fucose, mice were gavaged with *Bifidobacterium-L. reuteri* triple-species mixtures containing *B. bifidum* PRL2010*,* either UCC2003 or Δ*fucP*, and by association with either PTA 6475 or Δ*pduCDE* (Single *L. reuteri* strains), or both strains (*L. reuteri* strains in competition) (Table S1). To test for cross-feeding of dietary rhamnose, we gavaged mice with *E. coli-L. reuteri* double-species mixtures containing *E. coli* and PTA 6475 or Δ*pduCDE* (Single *L. reuteri* strains), or both (*L. reuteri* strains in competition) (Table S2). Rhamnose was provided in the drinking water at 2 % rhamnose w/v (Table S2). Each mouse was gavaged with 200 µL of the corresponding bacterial cell mixtures containing ~10^8 viable cells of each strain. Fecal pellets were collected from individual mice 1, 3, 5, and 7 days after inoculation and plated. Selective plating was used to enumerate bacterial cells in fecal samples as follows: Modified Rogosa plates were used to quantify *L. reuteri* strains (Duar *et al.*, 2017). PTA 6475 and Δ*pduCDE* were differentiated using a reuterin hydrazone detection assay (Anna Rosander *et al.*, 2008) . MacConkey agar (Difco) was used for quantifying *E. coli*. *Bifidobacterium* were selected using *Bifidobacterium* selective iodoacetate mupirocin (BSIM) agar as previously described (Lewis *et al.*, 2015). *B. bifidum* PRL2010 and *B. breve* strains were differentiated based on colony morphology.

2.5 Metabolite analysis of post-fermentation

1,2-PDO, propanol, propionate, acetate, and ethanol were measured using High Pressure Liquid Chromatography (HPLC). A BioRad Aminex HPX-87H column (300 mm x 7.8 mm) and a refractive index detector was used (HPLC-RI). Samples taken from fermentations were mixed with 70 % $HClO_4$ (0.0 05% v/v), stored at 4 °C overnight to precipitate proteins, centrifuged (18,800 x g for 5 minutes), filtered (0.22 µm), and stored at -20 °C before injection into HPLC. 10 µL were injected and eluted with 5 mM H_2SO_4 at a flow rate of 0.4 ml/min at 70 °C. 1,2-PDO, propanol, propionate, acetate, and ethanol were quantified using external standards.

2.6 Statistical analysis

Statistical significance between growth curves were determined by two-way analysis of variance (ANOVA) with Bonferroni multiple comparisons test ($\alpha = 0.05$).

Comparisons between PTA 6475 and Δ*pduCDE* CFUs recovered over the duration of the gnotobiotic mice experiments were performed by unpaired two-tailed Student's *t*-test. Tests were conducted between *L. reuteri* strains that were associated with (i) UCC2003 or (ii) Δ*fucP* in the *Bifidobacteria* triple-species experiments and *E. coli*-*L. reuteri* double-species experiments with rhamnose (iii) present or (iv) absent.

For the gnotobiotic mice experiments inoculated with the 'single *L. reuteri* strain*'* mixtures (Table S1 & 2), CFUs of PTA 6475 and Δ*pduCDE* recovered from mouse feces were used to produce normalized ratios. Ratios were generated using the formula (equation 1) below, where a_n is a CFU value for PTA 6475 - from a single mouse - used in the comparison, b_n is the CFU value of a Δ*pduCDE* from each mouse in the group*,* and n_b is the total population of mice inoculated with the mutant strain used in the experiment.

$$Normalized\ ratio = \frac{a_n}{\Sigma\left(\frac{(b_1 + b_2 + \cdots + b_n)}{n_b}\right)} \quad (1)$$

The formula was used to generate sets of ratios for the following comparisons from the 'Single *L. reuteri* strain' mice experiments: (i) UCC2003 vs Δ*fucP* (ii) *E. coli-L. reuteri* double-associated mice in the presence vs the absence of rhamnose. Statistical significance between the sets of ratios were determined by a non-parametric Mann-Whitney test (*P* value < *0.05*).

Fisher's exact test was used to determine statistical significance between *L. reuteri* population frequencies from mouse groups inoculated with '*L. reuteri* strains in competition' mixtures (*P* value < *0.05*). This was performed between groups of mice from either: (i) *Bifidobacterium-L. reuteri* triple-species associations including UCC2003 vs associations including Δ*fucP* (ii) *E. coli-L. reuteri* double-species associations with the mouse diet supplemented with rhamnose vs without rhamnose.

Statistical analyses were performed using GraphPad Prism 6.07.

3. Chapter 3 Results

3.1 Characterization of isogenic mutant and wild-type *L. reuteri* strains

We first confirmed the impact of 1,2-PDO on *L. reuteri* growth *in vitro* before performing cross-feeding experiments. The presence of 1,2-PDO in the media containing glucose significantly enhanced the density of *L. reuteri* ATCC PTA 6475 (referred to as PTA 6475), but not its *pduCDE* mutant (referred to as Δ*pduCDE*), as a higher OD_{600} was observed over temporal growth (Fig. 3A). In contrast, this phenotype was not observed in PTA 6475 in glucose alone or the growth of Δ*pduCDE* under any conditions (Fig. 3A). Interestingly, PTA 6475 grown on glucose alone had a slightly higher OD_{600} than Δ*pduCDE* (Fig. 3A). It is possible that this difference was observed because of residual glycerol in beef extract used in the procurement of mMRS, as glycerol can also be used as an external electron acceptor through the *pduCDE* genes. Importantly, neither PTA 6475 nor Δ*pduCDE* were able to use 1,2-PDO as the sole carbon source for growth (Fig. 3A). To confirm that the enhanced growth of PTA 6475 was due to 1,2-PDO metabolism, the metabolic end products were analyzed by HPLC. As shown in Figure 3B & C, growth of PTA 6475 but not Δ*pduCDE* led to a reduction of 1,2-PDO and an increase of the metabolic end-products propanol and propionate (Fig. 3B & C). Utilization of 1,2-PDO resulted in the production of acetate and decreased production of ethanol in PTA 6475 but not Δ*pduCDE* (Fig. 3D & E; Fig. S1A & B). Taken together, these results confirm that PTA 6475 is able to utilize 1,2-PDO through the *pduCDE* genes and that the reducing branch of the pathway, which regenerates electron acceptors formed in glucose metabolism, is preferred over the oxidizing branch which produced ATP and propionate.

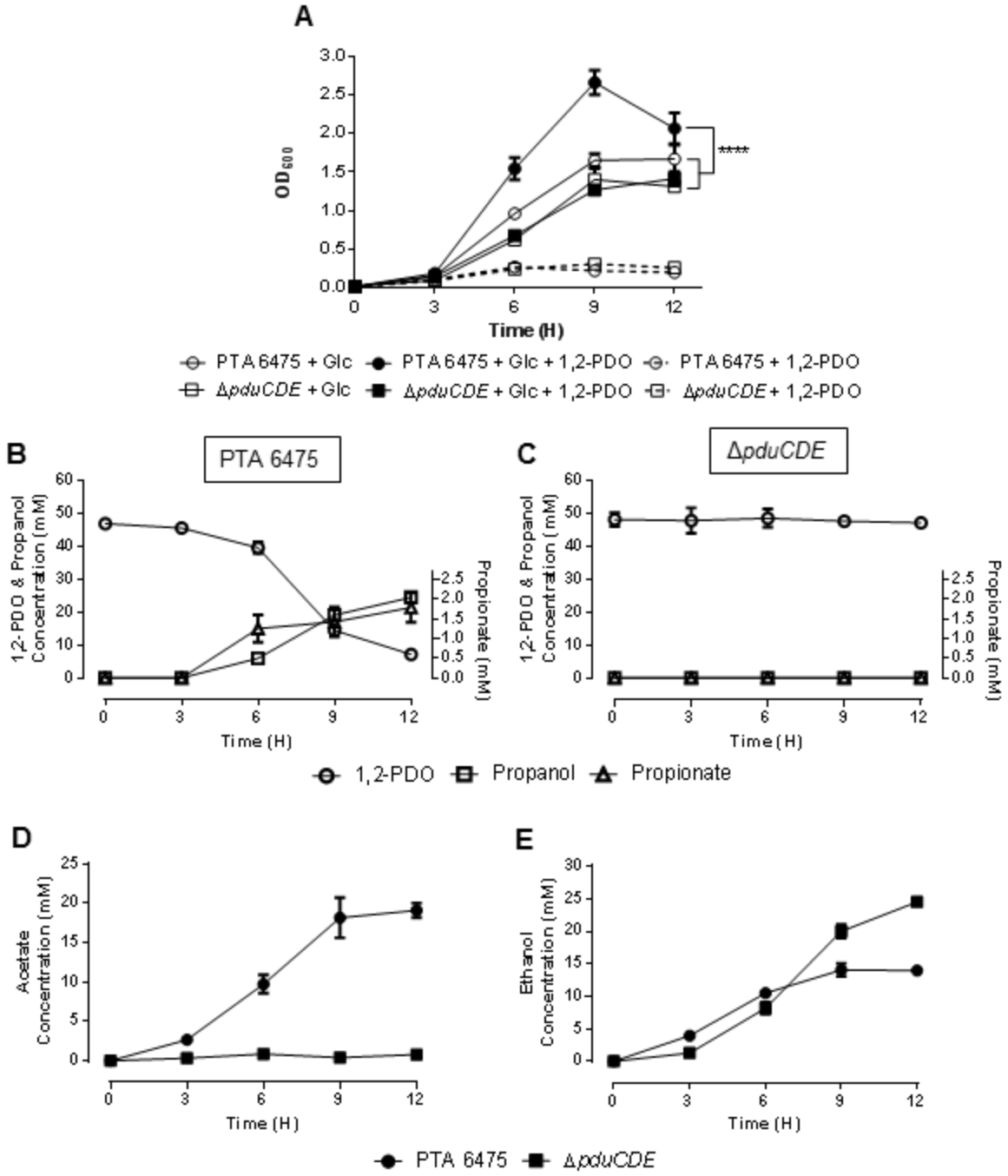

Figure 3. Impact of 1,2-PDO and metabolite formation in cultures of PTA 6475 and Δ*pduCDE*. (A) *L. reuteri* strains were grown in half-strength mMRS supplemented with either glucose (Glc; 25 mM), a combination of glucose and 1,2-PDO (50 mM), or 1,2-PDO alone. Asterisks indicate a significant difference (Two-way ANOVA; $p < 0.001$) in growth of PTA 6475 on glucose plus 1,2-PDO compared to the other conditions. (B-C) Utilization of 1,2-PDO and production of propanol and propionate by (B) PTA 6475 and (C) Δ*pduCDE* during growth on glucose in the presence of 1,2-PDO. (D-E) Production of (D) acetate and (E) ethanol by the two strains during growth on glucose in the presence of 1,2-PDO.

3.2 Gut symbiont derived 1,2-PDO enhance growth of *L. reuteri in vitro*

We developed an experimental system to study cross-feeding between gut bacteria that produce 1,2-PDO and *L. reuteri*. *Bifidobacterium breve* and *E. coli* were selected, which produce 1,2-PDO from fucose and rhamnose, respectively, substrates not utilized by *L. reuteri*. Since *in vitro* growth rates of *L. reuteri*, *B. breve*, and *E. coli* are different, cross-feeding was not studied in co-culture. Instead, *B. breve* and *E. coli* were first grown under optimal conditions on the specific substrates that result in the production of 1,2-PDO. Spent supernatant obtained from these fermentations were supplemented with glucose and half-strength mMRS (conditioned media; see Materials and Methods for details on media preparation) and used for analyzing the growth kinetics and metabolite production of the *L. reuteri* strains (Fig. 4A & B; Table 2). Conditioned media from an isogenic mutant of *B. breve* with a deletion in the L-fucose transporter (*fucP)* that is not able to metabolize fucose to produce 1,2-PDO was utilized as a control (Fig. 4A; Table 2).

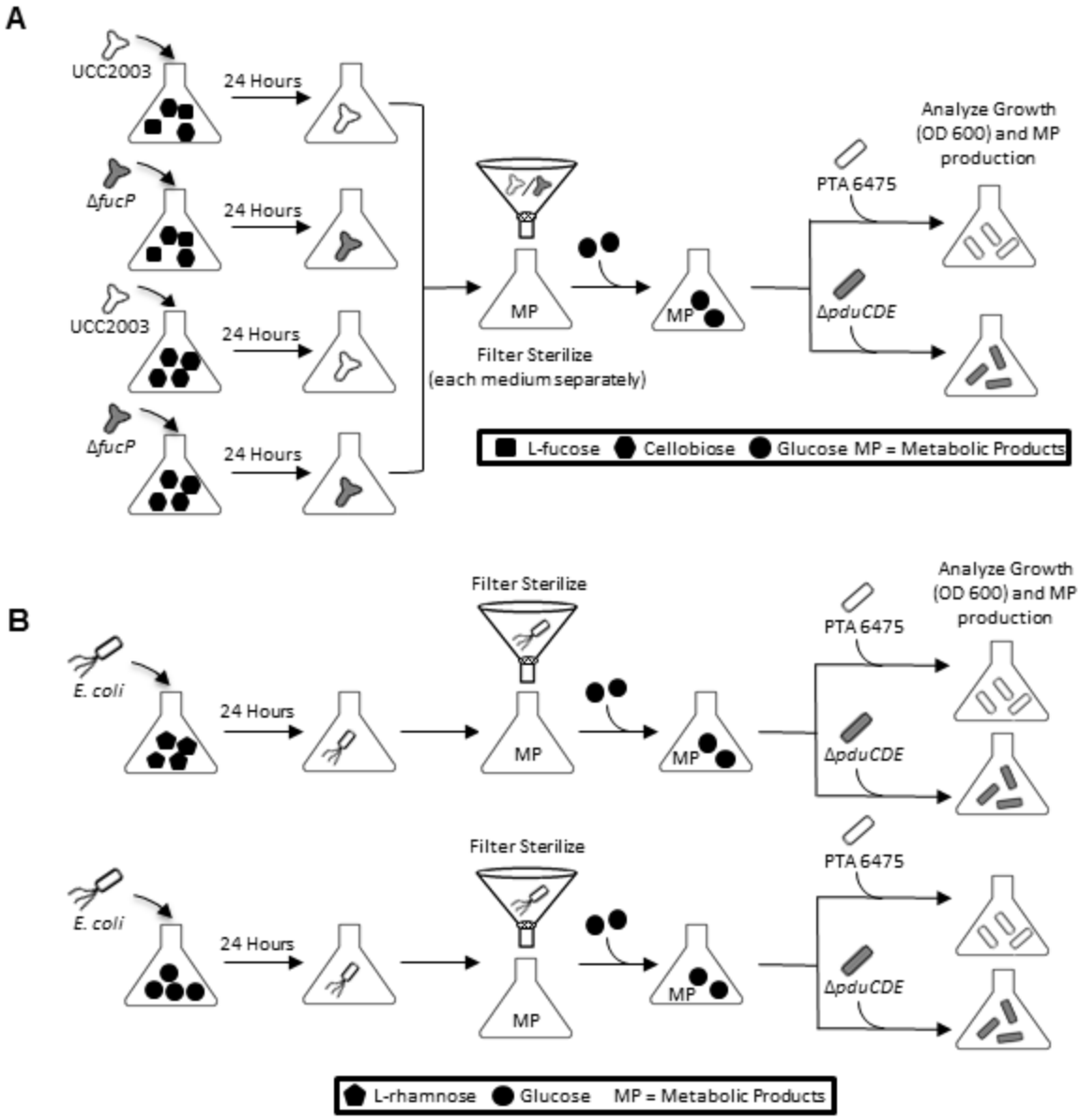

Figure 4. Experimental approach for *in vitro* studies of syntrophic interactions between *L. reuteri* and *B. breve* or *E. coli*. Schematic representation of experimental procedures outlining the production of conditioned media from (A) *B. breve* pre-culture fermentations of cellobiose and fucose, and (B) *E. coli* pre-culture fermentations of rhamnose or glucose, for subsequent culture of *L. reuteri* strains in the conditioned media.

B. breve UCC2003 (referred to as UCC2003) and its *fucP* mutant (referred to as Δ*fucP)* were grown in medium containing cellobiose and with or without fucose. UCC2003 does not grow on fucose as its sole carbohydrate source, yet co-utilizes the substrate and produces 1,2-PDO when supplemented with cellobiose (which is not metabolized by *L. reuteri*), and in this fucose/cellobiose-containing medium UCC2003 and Δ*fucP* reached similar growth density after 24 hours of growth (Fig. S2). As shown in Figure 5A, PTA 6475 but, not Δ*pduCDE*, reached a significantly higher OD_{600} between hours 6 and 12 when grown in conditioned media with supernatant of UCC2003 that fermented fucose and cellobiose (Fig. 5A). This difference was also observed when compared to *L. reuteri* strains cultured in conditioned media of UCC2003 absent of fucose in the pre-culture or in conditioned media of supernatant from Δ*fucP* (Fig. 5A & B). Otherwise, PTA 6475 and Δ*pduCDE* growth was indiscernible in the conditioned medias derived from previous *B. breve* fermentations (Fig. 5A & B). HLPC analysis confirmed the presence of 1,2-PDO solely in the conditioned media of UCC2003 grown with fucose (Fig. S3A), and showed that enhanced growth of *L. reuteri* was associated with conversion of 1,2-PDO to propanol (Fig. 5C), which was not detected in the cultures of Δ*pduCDE* (Fig. 5D). Propionate, acetate, and ethanol could not be determined due to interference of unknown compounds in the *L. reuteri* medium. In addition, we confirmed that fucose and cellobiose did not alter the growth kinetics of the *L. reuteri* strains through the *pduCDE* genes (Fig. S4A & B).

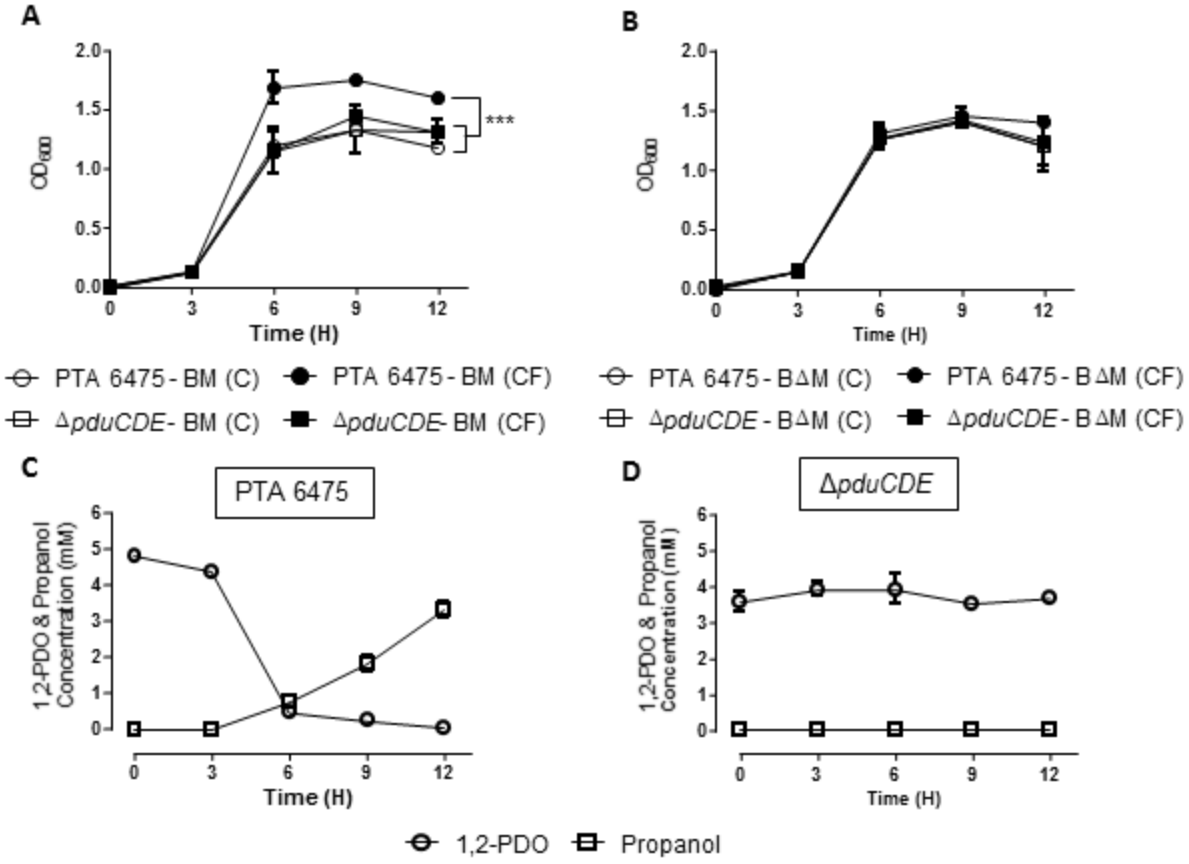

Figure 5. Growth and metabolites from PTA 6475 and Δ*pduCDE* in conditioned media of *B. breve* UCC2003 and its Δ*fucP* mutant grown with cellobiose alone or with the addition of fucose. Growth curves of (A) *L. reuteri* strains in UCC2003 conditioned media and (B) *L. reuteri* in Δ*fucP* conditioned media. Asterisks indicate a significant difference (Two-way ANOVA; $p < 0.001$) in growth of PTA 6475 grown in UCC2003 conditioned media that had fermented cellobiose and fucose together compared to the other conditions. (C-D) Utilization of 1,2-PDO and production of propanol in cultures of (C) PTA 6475 and (D) Δ*pduCDE* grown in the conditioned media of *B. breve* UCC2003 grown in the presence of fucose. Propionate, acetate, and ethanol could not be determined due to interference of unknown compounds in the *L. reuteri* medium. Abbreviations: BM, UCC2003 conditioned media; BΔM, Δ*fucP* conditioned media; (C), pre-culture fermentations of cellobiose only; (CF), pre-culture fermentations of cellobiose with added fucose (See Table 2 for more details about media used in the study).

Cross-feeding experiments revealed that *L. reuteri* could also benefit from 1,2-PDO produced from the fermentation of rhamnose by *E. coli* (Fig 4B; Fig. 6; Table 2). As shown in Figure 6A, both PTA 6475 and Δ*pduCDE* had similar growth profiles in the conditioned media from supernatant of *E. coli* grown in glucose (Fig. 6A). In contrast, PTA 6475 reached a

significantly higher OD_{600} at the 9th and 12th hour time point following inoculation into conditioned media from *E. coli* grown on rhamnose (Fig. 6A). Moreover, the growth of Δ*pduCDE* in conditioned media from the *E. coli* fermentations of glucose or rhamnose were similar, suggesting that Δ*pduCDE* is incapable of metabolizing 1,2-PDO produced from the fermentation of rhamnose as an electron acceptor (Fig. 6A; Fig. S3B). Importantly, growth experiments of *L. reuteri* strains in media with rhamnose with or without glucose confirmed that rhamnose could neither be used as a carbon source nor utilized through the *pdu* cluster to alter growth (Fig. S4C & D). Metabolite analysis revealed that only PTA 6475 could consume 1,2-PDO and produce propanol, propionate, and acetate when cultured in the conditioned media of *E. coli* supplied with rhamnose (Fig. 6B-D; Fig. S5). Ethanol could not be determined due to interference of an unknown compound in the *L. reuteri* medium.

Together, these findings confirm that PTA 6475 can utilize 1,2-PDO produced by *B. breve* and *E. coli* from the fermentation of deoxyhexose sugars to enhance its growth capabilities.

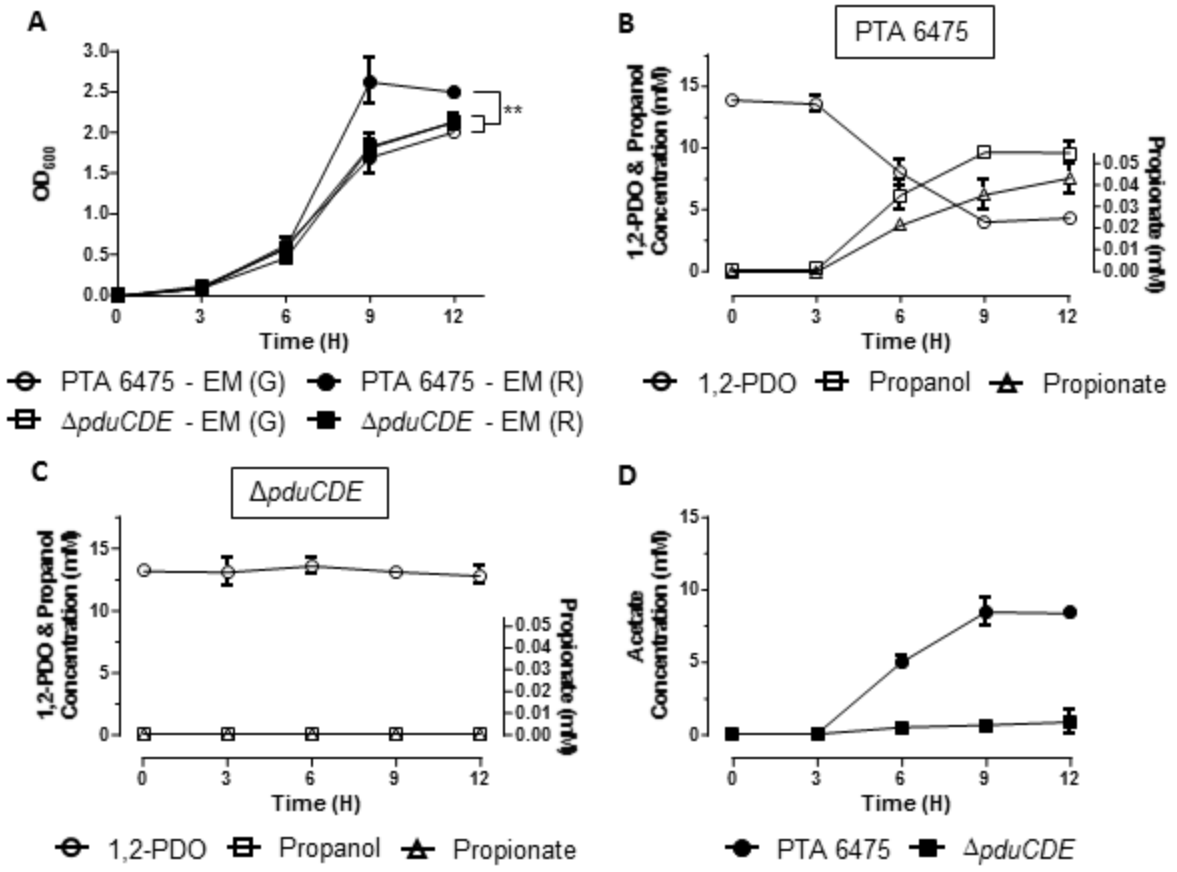

Figure 6. Growth and metabolites of PTA 6475 and Δ*pduCDE* in conditioned media of *E. coli* grown with glucose or rhamnose. (A) Growth curves of *L. reuteri* strains in *E. coli* conditioned media. Asterisks indicate a significant difference (Two-way ANOVA; $p < 0.01$) in growth of PTA 6475 grown in *E. coli* conditioned media that had fermented rhamnose compared to other conditions. (B-C) Utilization of 1,2-PDO, and production of propanol and propionate by (B) PTA 6475 and (C) Δ*pduCDE* grown in conditioned media from *E. coli* grown with rhamnose. (D) Comparison of acetate production by the two strains grown in conditioned media from *E. coli* grown with rhamnose. Ethanol could not be determined due to interference of an unknown compound in the *L. reuteri* medium. Abbreviations: EM, *E. coli* conditioned media; (G), fermentation of glucose by *E. coli*; (R), fermentation of rhamnose by *E. coli*. (See Table 2 for more details about media used in the study).

3.3 Importance of 1,2-PDO cross-feeding in the gastrointestinal tract

The ecological relevance of cross-feeding based on 1,2-PDO in the gastrointestinal tract was investigated with a series of colonization experiments in gnotobiotic mice (Table S1 & S2). As described earlier, *B. breve* produces 1,2-PDO from fucose. Host mucins are an intrinsic

source of fucose in the gastrointestinal tract, but *B. breve* does not possess glycosidases required for mucin degradation (Egan *et al.*, 2014). 1,2-PDO cross-feeding between *B. breve* and *L. reuteri* was therefore studied in a triple-species associated mouse in the presence of the mucinolytic bacteria, *B. bifidum*, capable of degrading mucin and releasing fucose but cannot utilize it nor produce 1,2-PDO (Fig. 7A; Table S1) (Egan *et al.*, 2014)). The three species formed stable populations through the duration of the single *L. reuteri* strain experiments, with *Bifidobacteria* species colonizing between ~10^8-10^9 CFU/g and *L. reuteri* strains between ~10^7-10^8 CFU/g (Fig. S6A-B & D-E; Fig. S7A). PTA 6475 formed higher populations than Δ*pduCDE* when bacterial mixtures contained UCC2003, but differences did not reach statistical significance due to high variation between mice (Fig. S7A). However, normalized ratios between the experiments containing single *L. reuteri* strains were generated (equation 1; see Materials and Methods for details on normalized ratios) and, as shown in Figure 8A, the ratio of PTA 6475 to Δ*pduCDE* was significantly higher in the presence of UCC2003 as compared to mice colonized with Δ*fucP* (Fig. 8A). Interestingly, although the ratio between PTA 6475 and Δ*pduCDE* was greater than 1 when the *L. reuteri* stains were co-colonized with wild-type *B. breve* UCC2003, it was substantially lower than 1 when *L. reuteri* was paired with Δ*fucP* (Fig. 7A). These finding suggests that the *pdu* cluster is a burden to the fitness of *L. reuteri* unless 1,2-PDO is provided by *B. breve*.

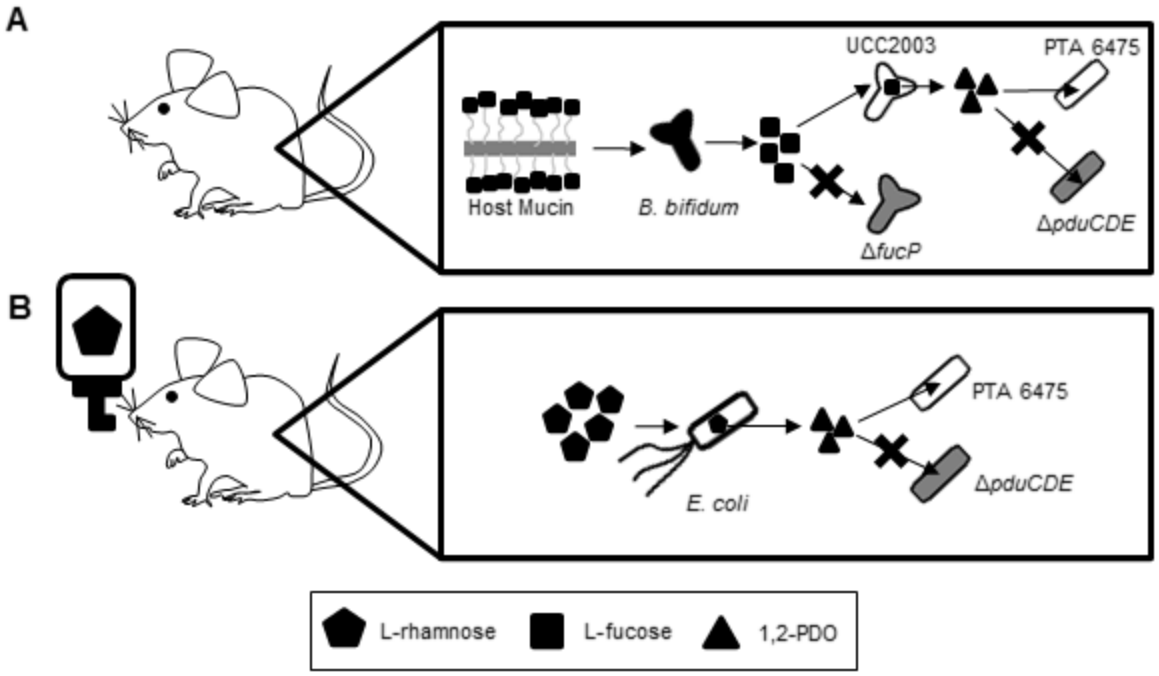

Figure 7. Graphical illustration of hypothesized trophic interactions of 1,2-PDO in gnotobiotic mice. (A) In triple-species associated gnotobiotic mice (colonized by *B. bifidum, B. breve, and L. reuteri*) *B. bifidum* liberates fucose - from degradation of host mucin - which is metabolized by *B. breve* UCC2003 producing 1,2-PDO, that is subsequently utilized by PTA 6475. (B) In dual-species (*E. coli* and *L. reuteri*) associated mice whose diet has been supplemented with rhamnose added through the drinking water, *E. coli* metabolizes rhamnose producing 1,2-PDO that is subsequently utilized by PTA 6475.

In a parallel set of experiments, we tested the importance of 1,2-PDO syntrophy in quadruple-colonized gnotobiotic mice that contained *B. bifidum*, UCC2003 or Δ*fucP*, and PTA 6475 and Δ*pduCDE* in direct competition (Table S1). Similar to the single *L. reuteri* strain inoculations, the colonization of *Bifidobacteria* species were comparable among groups (~10^8-10^9 CFU/g). *L. reuteri* strains formed stable populations (~10^7-10^8 CFU/g), and as with the experiments using single strains of *L. reuteri,* counts of PTA 6475 were higher than the mutant when the inoculum included UCC2003, although differences did not reach significance (Fig. S6C & F; Fig. S7B). Significant differences were observed once the relative proportions of *L. reuteri* strains of the total *L. reuteri* population were analyzed, with PTA 6475 reaching significantly higher proportions (>75%) in mice also colonized with UCC2003 in comparison to

mice colonized with Δ*fucP* (Fig. 8B). Interestingly, the Δ*pduCDE* mutant reached around 75% in the absence of the 1,2-PDO producing *B. breve* strain (Fig. 8B), supporting the notion that the *pdu* cluster is a fitness burden to *L. reuteri,* yet becomes beneficial once 1,2-PDO is provided. Overall, these observations demonstrated that *B. breve* can provide 1,2-PDO as the result of a trophic chain from the degradation of mucin by *B. bifidum* that facilitates the colonization of *L. reuteri* in the gastrointestinal tract.

A set of dual-associated gnotobiotic mouse experiments were also conducted to test if the production of 1,2-PDO, from the metabolization of dietary rhamnose by *E. coli,* influences the fitness of *L. reuteri* in the gastrointestinal tract. Mice were gavaged with *E. coli* and PTA 6475 or Δ*pduCDE*, either on their own or in competition, and were provided with rhamnose in the drinking water (Fig. 7B; Table S2). Although stable populations of *E. coli* were reached in all mice, rhamnose led to a significant increase of the cell numbers of *E. coli* in the gut (~10^9 CFU/g in the absence of rhamnose and ~10^{10} CFU/g with rhamnose supplemented in the diet) (Fig. S8). Contrary to the findings with the bifidobacterial-containing mixtures, Δ*pduCDE* colonized with a higher cell density and outcompeted PTA 6475 in all the conditions tested (Fig. 8C & D). This was indicated by ratios that were less than 1 between PTA 6475 and Δ*pduCDE* in the inoculum containing a single *L. reuteri* strain (Fig. 8C) and the total populations of *L. reuteri* consisting of around 60% Δ*pduCDE* in direct competition (Fig. 8D). Interestingly, these results were similar in comparison to the ratios and total population abundance found in the triple-species *Bifidobacterium* experiments associated with Δ*fucP* (Fig. 8), again confirming the apparent fitness burden of the *pdu* cluster.

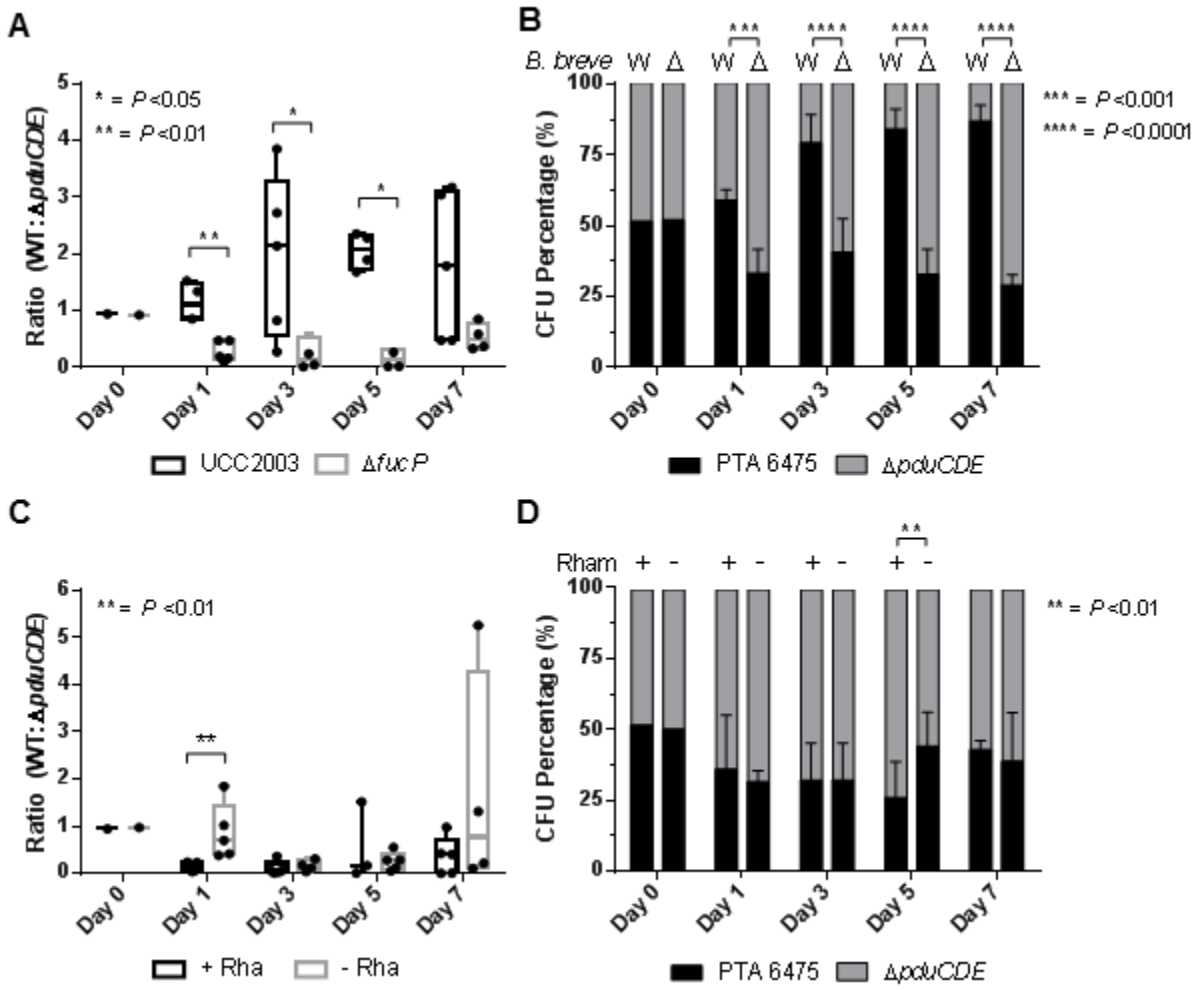

Figure 8. Populations of *L. reuteri* PTA 6475 and its Δ*pduCDE* mutant in the gastrointestinal tract of triple-species and double-species associated gnotobiotic mice. (A) Normalized ratios of PTA 6475 to Δ*pduCDE* obtained from *Bifidobacterium-L. reuteri* triple-species associated gnotobiotic mice in which colonization by PTA 6475 and Δ*pduCDE* was tested separately. (B) Percent of colony forming units for PTA 6475 and Δ*pduCDE* mutant as measured in triple-species associated gnotobiotic mice in which the two *L. reuteri* strains were tested in competition. "W" (wild-type) indicates mice colonized with *B. breve* UCC2003 and "Δ" indicates mice colonized with the Δ*fucP* mutant. (C) Normalized ratios of PTA 6475 to Δ*pduCDE* in *E. coli-L. reuteri* double-species associated gnotobiotic mice in which colonization of PTA 6475 and Δ*pduCDE* was tested separately. (D) Percent of colony forming units for PTA 6475 and Δ*pduCDE* mutant in double-species associated gnotobiotic mice in which the two *L. reuteri* strains were tested in competition. "+" indicates the presence of rhamnose (Rha) in the diet, while "-" indicates absence of rhamnose in the diet. Day 0 indicates the *L. reuteri* strain proportions in the respective strain mixture inoculums in each mouse experiment. Statistical significance for ratios and percent abundance (CFU) was determined using Mann-Whitney test and Fisher's exact test, respectively.

4. Chapter 4 Discussion and Conclusion

In the highly competitive ecosystem of the gastrointestinal tract, the ecological success of a bacterium depends to a large part on its ability to obtain resources to generate energy. In this study, we demonstrate that *L. reuteri* can engage in trophic interactions with bacteria that are common in the human gut and provide 1,2-PDO for *L. reuteri* to regenerate reduced metabolic cofactors. Using isogenic mutants in both the bacterium that produces 1,2-PDO and *L. reuteri*, we demonstrate that this syntrophy is in fact based on the metabolic intermediate. Our findings further established that the *pduCDE* genes constitute a fitness burden for *L. reuteri* in the gut unless 1,2-PDO is provided to make the cluster ecologically advantageous. Our findings therefore provide insight into both the ecological role and evolution of the *pdu* cluster in *L. reuteri*.

Our results demonstrate that *in vitro*, *L. reuteri* can obtain a growth advantage by cross-feeding from 1,2-PDO derived from the fermentation of fucose and rhamnose by *B. breve* and *E. coli*, respectively. These findings extend previous work showing that *pdu* cluster encoding *L. reuteri* strains grow at a faster rate and to a higher cell yield in the presence of glycerol (Sriramulu *et al.*, 2008; Rattanaprasert *et al.*, 2014). In Accordingly, the analysis of metabolic products during these experiments provided evidence that 1,2-PDO functions as an electron acceptor, allowing *L. reuteri* to use the acetyl-phosphate to generate an extra ATP via the acetate pathway (Gänzle, 2015) .

In the gastrointestinal tract, propionate is produced through one of three biochemical pathways: acrylate, succinate, and propanediol (Reichardt *et al.*, 2014). Our *in vitro* experiments demonstrate syntrophic production of propionate through the propanediol pathway. In addition,

we show that 1,2-PDO metabolism by *L. reuteri* may further contribute to SCFA formation in the gastrointestinal tract with the conversion of acetyl-phosphate to acetate (Gänzle, 2015). As a metabolic shift from the production of ethanol to acetate was observed while in the presence of an electron acceptor (Gänzle, 2015). This has important health implications, as intestinal propionate and acetate have been suggested to impact host physiology by contributing to gluconeogenesis in the liver, reducing cholesterol, and promoting satiety, respectively (Reichardt *et al.*, 2014; Louis & Flint, 2017).

Our work establishes the ecological relevance of the *pdu* cluster for the colonization of a gut symbiont in the gastrointestinal tract. In addition, our data suggest facilitation from metabolic cross-feeding as a result of the degradation of host mucin among commensal gut bacterium *in vivo.* Trophic interactions as a result of mucin degradation have previously been suggested to play a key role in the facilitation of bacterial species in the gut microbiota and have been demonstrated *in vitro* (Martens *et al.*, 2008; Turroni *et al.*, 2010; Turroni *et al.*, 2018) and with bacterial pathogens (i.e. *Salmonella* spp. and *C. difficile*) *in vivo* (Ng *et al.*, 2013; Faber *et al.*, 2017). Here, we provide a proof-of-concept through the production of 1,2-PDO from fucose released from host glycoproteins, conferring a fitness advantage to *L. reuteri* in the murine gut. Parallel experiments with a non-1,2-PDO-producing strain further validated these findings.

Although our experiments indicate 1,2-PDO syntrophy between bifidobacteria and *L. reuteri*, equivalent findings were not observed in mouse experiments with *L. reuteri* and *E. coli* despite provision of rhamnose through the drinking water and an enhanced *E. coli* population. This unexpected observation may be attributed to a phenomenon called carbon catabolite repression or the "all-or-none" effect in *E. coli*, in which a hierarchy-based regulatory system controls the sequential uptake of carbon sources (Kremling *et. al.,*2014). The diet provided for the mouse

experiments was highly saturated with glucose (34.4% w/w). *In vitro,* the presence of glucose, suppresses the uptake of other carbohydrates in *E. coli* (Aidelberg *et al.*, 2014), and we confirmed this repression with *E. coli* MG1655 (Fig. S3B). Hence, it is possible that the uptake and metabolization of rhamnose into 1,2-PDO was suppressed in the mice gut.

Interestingly, the mouse experiments revealed a clear fitness burden of the *pduCDE* genes in both the mouse experiments with *E. coli* and the *B. breve* Δ*fucP* mutant. Fitness trade-offs are well understood in antibiotic resistance bacteria, where antibiotic resistance genes lead to a reduction of growth (Basra *et al.*, 2018). Our findings indicate that genes that facilitate syntrophic interactions are also subjected to fitness trade-offs in that they are only beneficial when the metabolite is provided. Such trade-offs have also been shown in cross-feeding based on the exchange of carbohydrates. *Bacteroides ovatus* possesses an enzyme system dedicated to the digestion of polysaccharides that does not directly benefit itself, but rather cooperative members of the gut microbiota through reciprocal cross-feeding. This enzyme system is energetically unfavorable and in the absence of a reciprocating species, a mutant strain deleted of this enzyme system can outcompete the wild-type (Rakoff-Nahoum *et al.*, 2016). The fitness burden provides a potential explanation for the evolution of the *pdu* cluster in *L. reuteri* (Duar *et al.*, 2017), especially deletion of the *pdu* cluster from most rodent strains as bacteria providing 1,2-PDO are likely not present in the forestomach in significant numbers (Walter *et al.*, 2011; Frese *et al.*, 2011) . In the human proximal gut, 1,2-PDO is readily provided (Saxena *et al.*, 2010; Gänzle, 2015), which might explain why the *pdu* cluster is conserved among these strains. However, it has to be mentioned here that the *pdu* cluster also encodes for additional functions, such as the utilization of glycerol as an electron acceptor, production of cobalamin, and production of the

antimicrobial compound reuterin, which constitute additional factors in the evolution of this cluster (Frese *et al.*, 2011).

Our findings contribute to our understanding on the importance of cross-feeding and other mutualistic interactions in intestinal ecosystems that determine ecological performance of individual members and ultimately determine how communities function (Rakoff-Nahoum *et al.*, 2016; O'Connell *et al.*, 2018; Centanni *et al.*, 2018). Such information is vital in our understanding of the ecological and evolutionary forces that shape gut ecosystems. In addition, an understanding of mutualistic interactions has important implications as it can be translated to improved microbial-based gut modulation strategies (i.e. probiotics). A challenge encountered in the field of probiotics is that gut ecosystems are homeostatic, resilient to change, and thus difficult to modulate, and most probiotics do not persist (Maldonado-Gómez *et al.*, 2016; Walter *et al.*, 2018; Khalesi *et al.*, 2018; Zmora *et al.*, 2018). One solution to this problem is the adoption of an ecological framework for probiotic applications (Bindels *et al.*, 2015; Walter *et al.*, 2018). A consideration of the mutualistic and facilitative interactions between community members can be used in the design of probiotic strain mixtures or the personalization of probiotic applications with the goal to achieve a more successful long-term persistence of probiotic strains, which might be beneficial for certain applications. For example, syntrophy based on 1,2-PDO could be considered in generating probiotic products by pairing *L. reuteri* with *Bifidobacterium* species that release fucose from the degradation of host-derived substrates and convert it into 1,2-PDO (Egan *et al.*, 2014; Tailford *et al.*, 2015). Additionally, *Bifidobacteria* are more prevalent in the gastrointestinal tract of infants (Lewis *et al.*, 2015; Ferretti *et al.*, 2018) and are known to utilize HMOs, releasing fucose (Turroni *et al.*, 2010; O'Connell *et al.*, 2018; Bunesova *et al.*, 2018), allowing an effective synergistic combination with *L. reuteri.* Furthermore,

syntrophy of 1,2-PDO derived from gut symbionts and *S. enterica* serovar Typhimurium have been demonstrated, with an isogenic mutant, to play a role in promoting pathogen expansion in the gut (Faber *et al.*, 2017). *L. reuteri* could play a therapeutic role in excluding pathogenic *Salmonella* during gastroenteritis, by directly competing for the intermediary metabolite. Overall, this information can not only be used to formulate probiotic mixtures and synbiotic products, but potentially personalize probiotic applications based on the baseline microbiome (Maldonado-Gómez *et al.*, 2016).

5. Supplementary Materials

Table S1. Bifidobacterium-L. reuteri triple-species associations for gnotobiotic mouse experiments.

Inoculum	Single *L. reuteri* strains				*L. reuteri* strains in competition	
***B. bifidum* PRL2010**	+	+	+	+	+	+
***B. breve* UCC2003**	+	+	-	-	+	-
B. breve* UCC2003 Δ*fucP	-	-	+	+	-	+
***L. reuteri* ATCC PTA 6475**	+	-	+	-	+	+
L. reuteri* ATCC PTA 6475 Δ*pduCDE	-	+	-	+	+	+

+: present, -: absent

Table S2. E. coli-L. reuteri double-species associations for gnotobiotic mouse experiments.

Inoculum	Single *L. reuteri* strains				*L. reuteri* strains in competition	
Rhamnose	+	+	-	-	+	-
***E. coli* MG1655**	+	+	+	+	+	+
***L. reuteri* ATCC PTA 6475**	+	-	+	-	+	+
L. reuteri* ATCC PTA 6475 Δ*pduCDE	-	+	-	+	+	+

+: present, -: absent

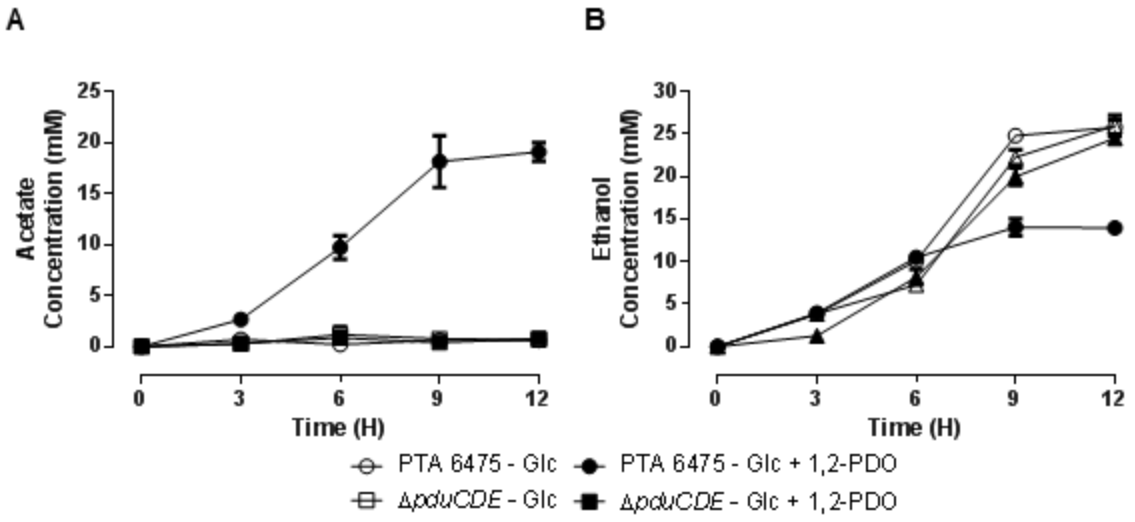

Figure S1. Acetate (A) and ethanol (B) production of PTA 6475 and Δ*pduCDE* during fermentation of glucose (Glc) in the presence or absence of 1,2-PDO.

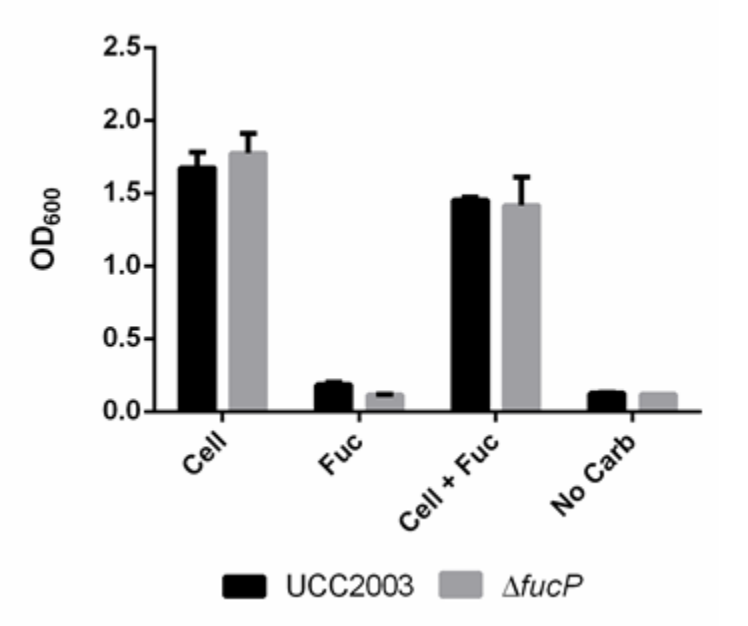

Figure S2. Growth of *B. breve* strains in mMRS supplemented with cellobiose (30 mM; Cell), fucose (30 mM; Fuc), cellobiose and fucose, or with no carbon source (No Carb) after 24 hours.

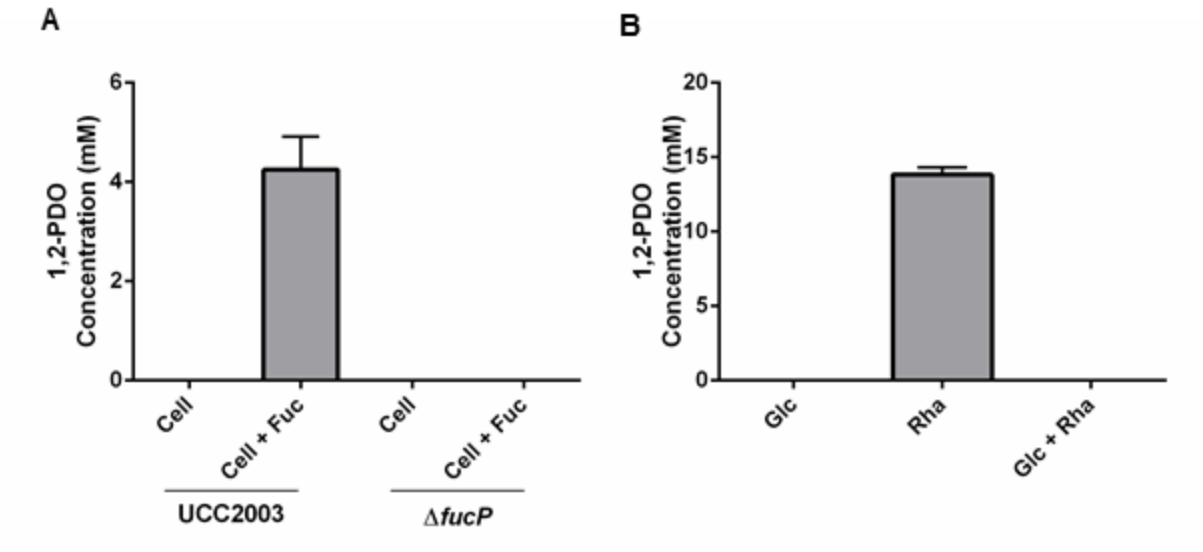

Figure S3. Total 1,2-PDO production by (A) UCC2003 and Δ*fucP* fermentation of cellobiose (Cell) and cellobiose plus fucose (Cell + Fuc) and from (B) *E. coli* fermentation of glucose (Glc), rhamnose (Rha), or glucose and rhamnose (Glc + Rha).

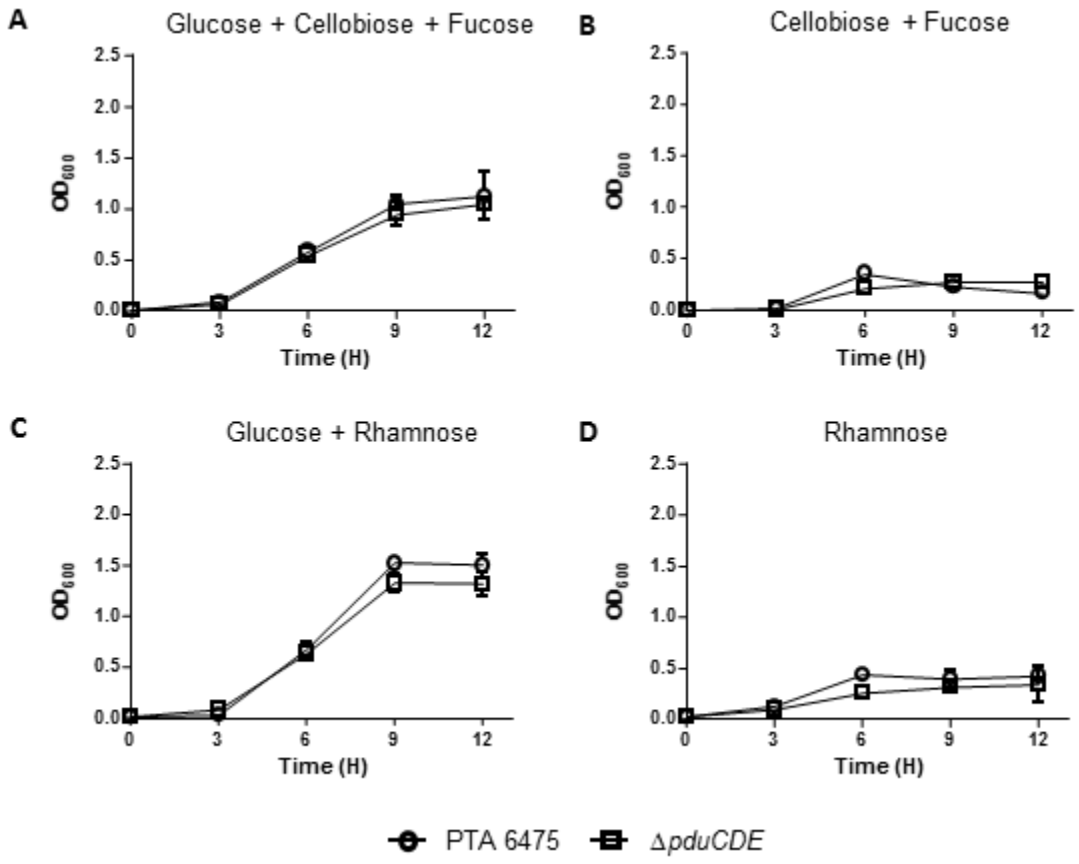

Figure S4. *L. reuteri* strains do not utilize (A & B) cellobiose, fucose, or (C & D) rhamnose as growth substrates or electron acceptors when cultured with glucose.

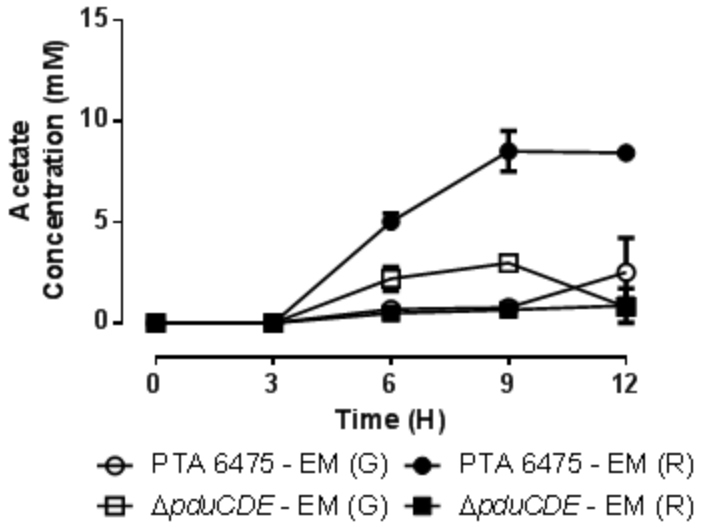

Figure S5. Acetate production of PTA 6475 and Δ*pduCDE* in the conditioned media of *E. coli* fermentation of glucose and rhamnose. In the symbol labels, *E. coli* conditioned media is abbreviated as EM, followed by fermentation of glucose by *E. coli* as indicated with (G), and rhamnose with (R) (See Table 1 for more details about media used in the study).

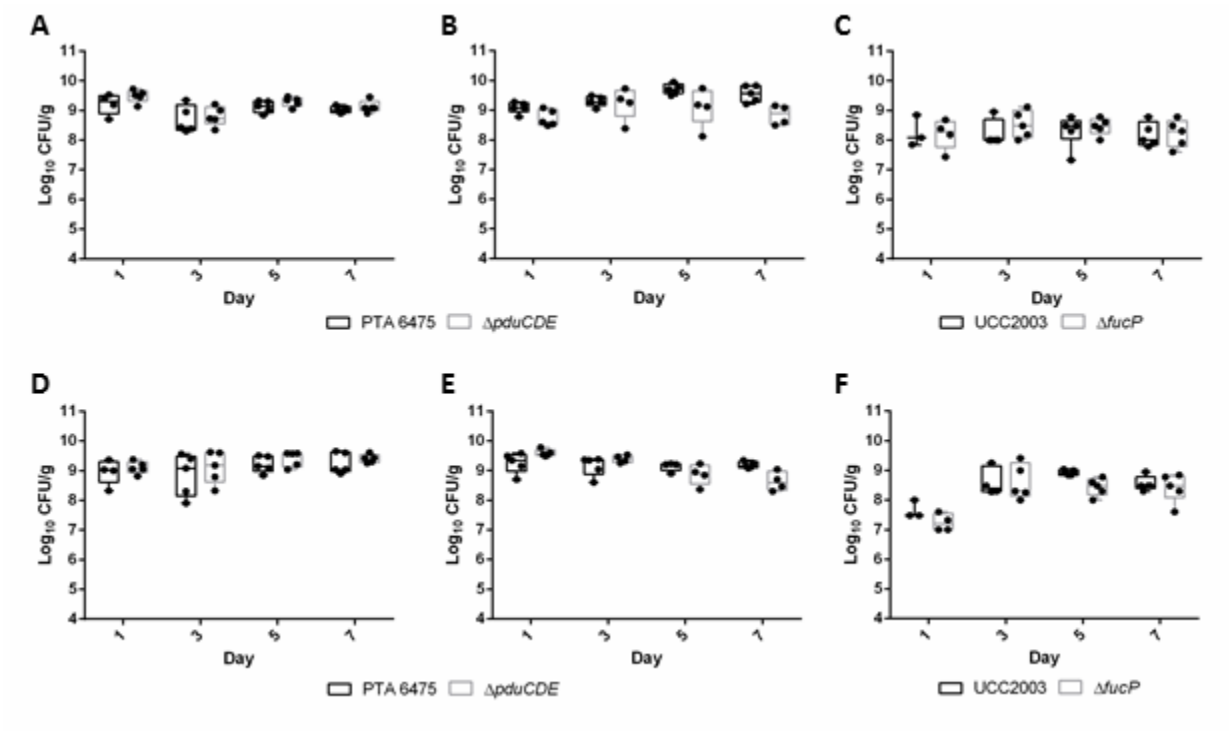

Figure S6. Quantification of *Bifidobacteria* from triple-species associated gnotobiotic mice experiments. (A to C) *B. bifidum* PRL2010 CFUs recovered from feces of gnotobiotic mice inoculated with a bacterial mixture containing (A) UCC2003 and PTA 6475 or Δ*pduCDE,* (B) Δ*fucP* and PTA 6475 or Δ*pduCDE,* and from mixtures containing either (C) UCC2003 or Δ*fucP* from *L. reuteri* competition mixtures. (D to F) *B. breve* CFUs recovered from feces of gnotobiotic mice. (D) UCC2003 and (E) Δ*fucP* CFUs from 'single *L. reuteri* strain' inoculations. (F) CFUs of UCC2003 and Δ*fucP* from *L. reuteri* in competition.

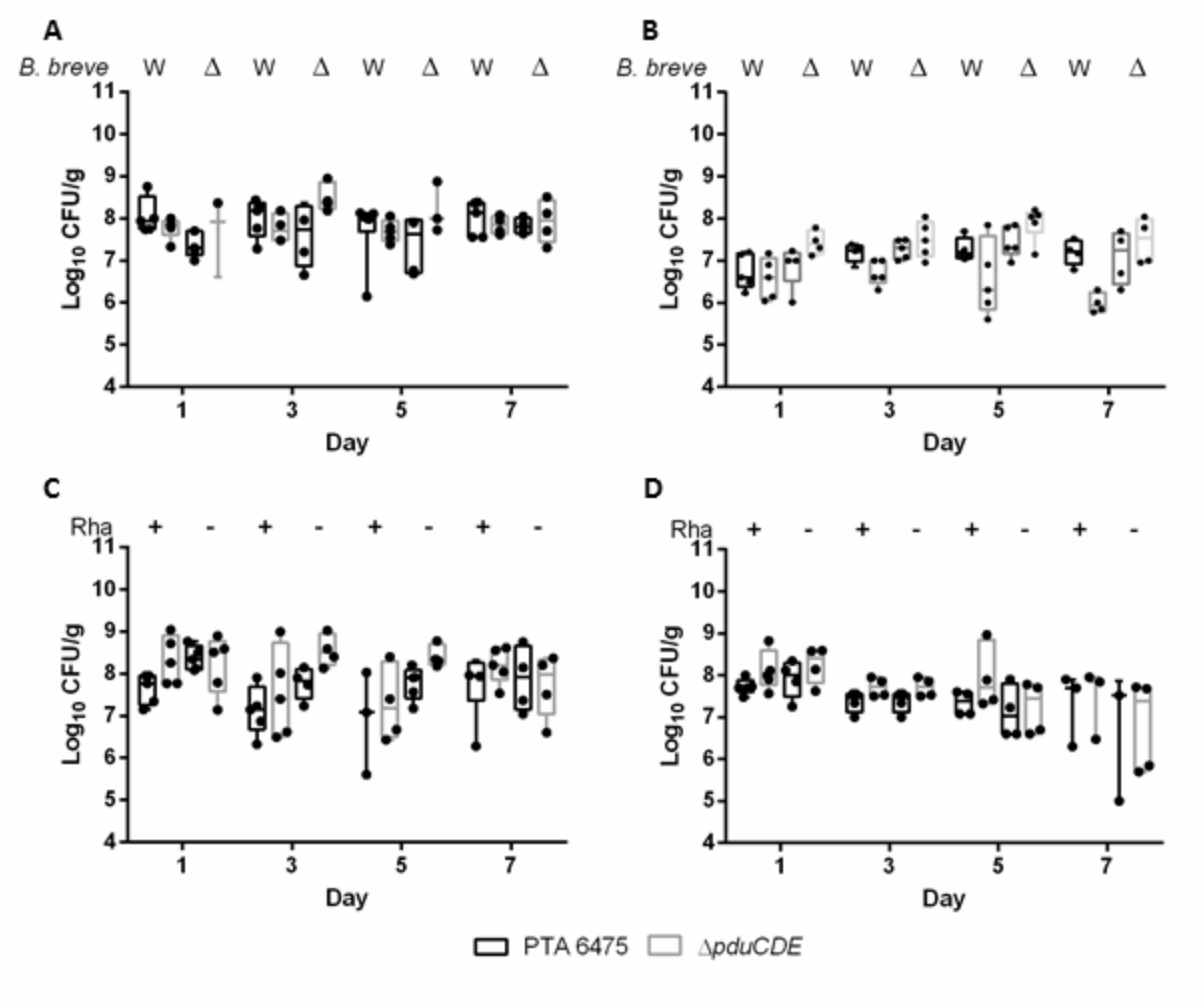

Figure S7. Quantification of *L. reuteri* from triple-species and double-species associated gnotobiotic mice experiments. (A-B) CFUs of *L. reuteri* recovered from feces of the *Bifidobacterium* triple-species gnotobiotic mice experiments containing either (A) single *L. reuteri* strains or (B) *L. reuteri* strains in competition. (C-D) CFUs of *L. reuteri* recovered from feces of *E. coli* double-species gnotobiotic mice experiments from either (C) single *L. reuteri* strains or (D) *L. reuteri* strains in competition.

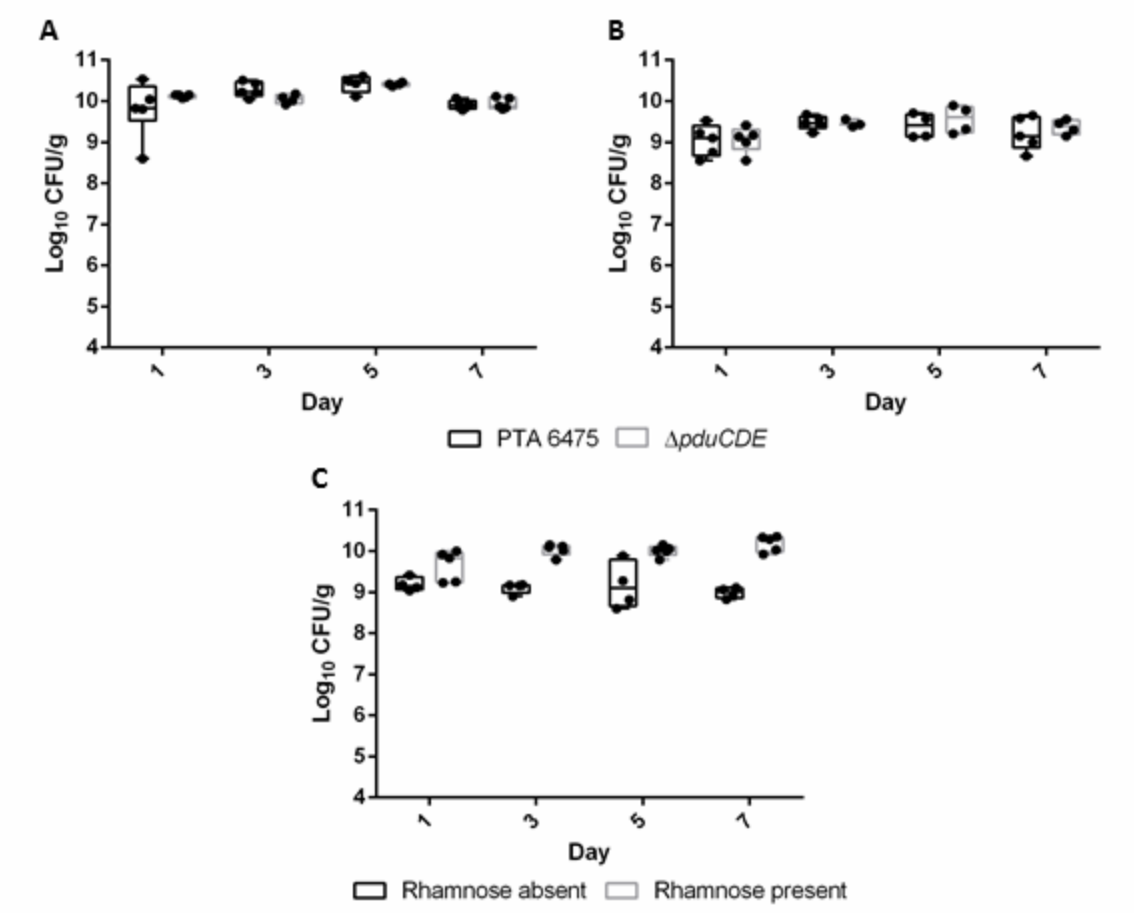

Figure S8. Quantification of *E. coli* from double-species associated gnotobiotic mice experiments. CFUs of *E. coli* recovered from 'single *L. reuteri* strain' bacterial mixtures in the (A) presence of rhamnose in the diet, (B) absence of rhamnose in the diet, and from (C) *L. reuteri* in competition where rhamnose was either supplemented into the mouse diet or not.

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