PONTIFICIA UNIVERSIDAD CATOLICA DE CHILE

ESCUELA DE INGENIERIA

BI-DIRECTIONAL CULTURES REVEAL METABOLIC CROSS-FEEDING BETWEEN GUT COMMENSALS

BELÉN ALEJANDRA HIRMAS OLIVARES

Thesis submitted to the Office of Research and Graduate Studies in partial fulfillment of the requirements for the Degree of Master of Science in Engineering

Advisor:

DANIEL GARRIDO CORTÉS

Santiago de Chile, Agosto 2021

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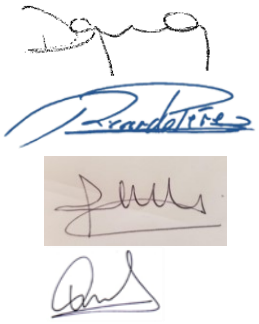

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To my family, thank you for all your support.

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RESUMEN

La microbiota intestinal humana se define por miles de interacciones, algunas caracterizadas por *cross-feeding* o intercambio de subproductos metabólicos. La inulina y el xilano son polisacáridos de la dieta que son fermentados por redes microbianas en el intestino grueso. La fermentación de estos resulta en diferentes perfiles de ácidos grasos de cadena corta, los cuales son beneficiosos para el huésped. En este trabajo se estudió las interacciones metabólicas entre dos microorganismos comensales del intestino, *Phocaeicola dorei* y *Lachnoclostridium symbiosum*, al usar inulina o xilano como fuente de carbono. Se hipotetiza que existen interacciones de *cross-feeding* entre ambas bacterias, donde *P. dorei* actúa como consumidor primario de carbohidratos complejos y produce metabolitos que pueden ser consumidos por *L. symbiosum* para producir butirato. El ensayo bidireccional mostró que *L. symbiosum* alcanza mayor crecimiento en presencia de *P. dorei*, mientras que el ensayo unidireccional mostró que es necesaria una interacción simultánea para que ocurra esta relación de comensalismo. Los experimentos de consumo de sustrato y de expresión diferencial de genes mostraron un aumento en el metabolismo de ambas bacterias en co-cultivo respecto al monocultivo, lo cual no necesariamente se relaciona con un aumento en el crecimiento, como es el caso de *P. dorei*. Las interacciones de *cross-feeding* fueron evidentes en inulina, ya que *P. dorei* degradó los polisacáridos a fructosa que luego *L. symbiosum* pudo consumir. El lactato y succinato también fueron alimentados de forma cruzada en cultivos con ambas fuentes de carbono, lo cual concuerda con simulaciones realizadas en nuestro laboratorio. *P. dorei* produjo propionato en medio estándar (mZMB) y en mZMB con xilano, mientras que ambas bacterias produjeron mayor concentración de acetato en xilano. La producción de butirato está directamente vinculada con el crecimiento de *L. symbiosum*, siendo mayor en co-cultivo respecto al monocultivo e independiente de la fuente de carbono utilizada. Estos resultados muestran un ejemplo interesante de interacciones metabólicas microbianas y su importancia sobre el potencial efecto beneficioso para el huésped.

Palabras Claves: Microbiota intestinal, *cross-feeding*, SCFA, prebióticos

ABSTRACT

The gut microbiota is defined by thousands of interactions, some of them characterized by cross-feeding or exchange of metabolic byproducts. Inulin and xylan are two major dietary polysaccharides that are fermented by microbial networks in the large intestine. The fermentation of these polysaccharides results in different profiles of short-chain fatty acids, which are beneficial for the host. We study the metabolic interactions between two gut commensals, *Phocaeicola dorei* and *Lachnoclostridium symbiosum*, using inulin or xylan as sole carbon source. We hypothesized that a cross-feeding interaction occurs between these bacteria, where *P. dorei* act as the primary fermenter of complex carbohydrates, releasing simpler metabolites that can be consumed by *L. symbiosum* to produce butyrate. Indeed, bidirectional assays showed that *L. symbiosum* reached a higher growth level when *P. dorei* is present in the culture, while unidirectional assays showed that simultaneous interactions are needed for this commensalism relationship to occur. Substrate consumption and gene expression analysis showed an enhanced metabolism of both bacteria in co-culture compared to monoculture, which did not necessarily relate with enhanced growth as in *P. dorei's* case. As *P. dorei* degraded polysaccharides to fructose that *L. symbiosum* could consume, cross-feeding interactions were evident in inulin. Lactate and succinate were also cross-fed in cultures with both carbon sources, which agrees with simulations ran in our laboratory. Propionate was only produced by *P. dorei* in standard media (mZMB) and mZMB with xylan, while both bacteria produced acetate at higher concentrations in xylan. Butyrate production seemed directly linked to *L. symbiosum*'s growth, being higher in co-culture than monoculture, and independent of the carbon source. These results detail a remarkable example of microbial metabolic interactions and their importance in the potential beneficial effects for the host.

Keywords: Intestinal microbiota, cross-feeding, SCFA, prebiotics

1. INTRODUCTION

The human intestine is an ecosystem that houses trillions of microorganisms such as bacteria, fungi, and archaea (Schluter & Foster, 2012). This ecosystem consists of different niches, the proximal region where the host absorbs dietary nutrients and the small and large intestine, colonized by microorganisms that degrade and consume the nutrients left by the host (Walter & Ley, 2011) (Figure 1-1).

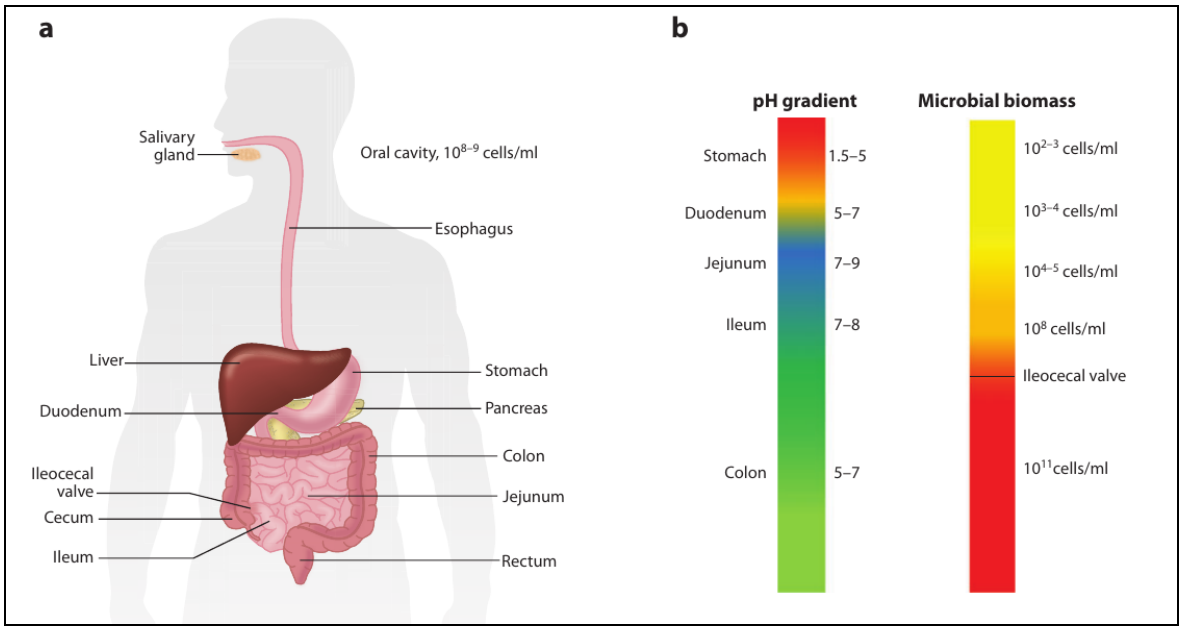


Figure 1-1. (a) Sections of the gastrointestinal track. (b) pH and microbial biomass of the different sections of the gastrointestinal track. The large intestine begins after the ileocecal valve and ends in the rectum. Source: Walter & Ley (2011).

Within bacteria, the most abundant phyla in the large intestine are Firmicutes, Bacteroidetes, Actinobacteria, Verrucomicrobia, and Proteobacteria (Almeida et al., 2021, Walter & Ley, 2011). The abundance and diversity of species play a major role in the stability of the community, directly impacting in the wealth of the host (Thursby & Juge, 2017).

1.1 The importance of the Human Gut microbiome

The human gut microbiome performs a series of beneficial actions for the host like the assimilation of complex dietary substrates, production of bioactive compounds, detoxification, immune response stimulation and providing a protection barrier against pathogens (Pérez-Cobas et al., 2013, Dethlefsen et al., 2008, Walker & Lawley, 2013). A robust microbiota can resist environmental disturbances, being able to maintain its composition or return to it. New studies have shown that functionality of the microbiota may be as important as composition, where the microbiota is able to maintain or recover its protein and metabolomic profile while changing its composition after a disturbance. These functional redundancy increases the robustness of the community, as different bacteria can play the same role (Moya & Ferrer, 2016).

An unstable microbiota is more prone to disrupt homeostasis in the face of high disturbances or environmental stress. This state of dysbiosis is often characterized by the loss of keystone species and overgrowth of opportunistic pathogens, altering the relative abundance of each phylum (Weiss & Hennet, 2017). Incrementing evidence shows that dysbiosis is associated with intestinal and immune diseases and even with metabolic and neurological disorders (Weiss & Hennet, 2017, Grochowska et al., 2019). Then, the promotion of a robust microbiota is essential to reduce the chances of diseases.

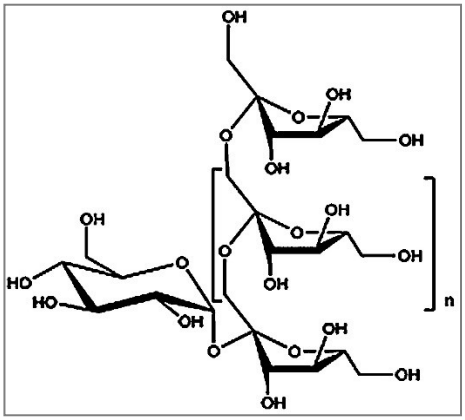
1.2 The effect of diet and complex carbohydrates

Since birth, the gut microbiota is regulated by many factors, dynamically modulating microbial abundances according to age and certain perturbations (Claesson et al., 2011). Its composition and activity are largely dictated by diet (Graf et al., 2015), especially by molecules that escape digestion in the small intestine, rendering them available for fermentation (Weiss & Hennet, 2017, Wu et al., 2011). Studies have shown that a drastic change in our diet can lead to a transient change in gut species in less than 5 days (Wu et al., 2011). This phenomenon is caused by the differential abilities of consumption of

each species, which give a comparative advantage to the ones that can utilize the diet's nutrients.

Dietary fibers are an example of this. As the host does not produce enzymes for their degradation, they pass undegraded to the large intestine (Pandey et al., 2015, Graf et al., 2015). Species of the gut microbiota have developed complex mechanisms to degrade fiber to smaller chains, which can be consumed by other species.

Fibers fermented by the gut microbiota include structurally diverse polysaccharides found mostly in plant cell walls. Plant inulin is a class of β – (2-1) linked fructosyl-fructose with a degree of polymerization < 200, typically between 10-60 (Shoaib et al., 2016, Hosseini et al., 2011) (Figure 1-2). Inulin is a well reported prebiotic, promoting the growth of *Bifidobacterium* species as observed by several *in vivo* studies (Costabile et al., 2010. Hiel et al., 2020, Shoaib et al, 2016). Several other species from *Bacteroides* and *Lactobacillus* genera have the ability to grow in inulin (Shoaib et al, 2016, Sheridan et al., 2016). Inulin also shapes the gut microbiota decreasing the numbers of undesirable microorganisms such as *Bilophila wadsworthia* (Vandeputte et al., 2017), and promoting the production of beneficial metabolites like butyrate and propionate as shown in an *in vitro* study in the simulator of human intestinal microbial ecosystem (SHIME) (Van de Wiele et al., 2004).



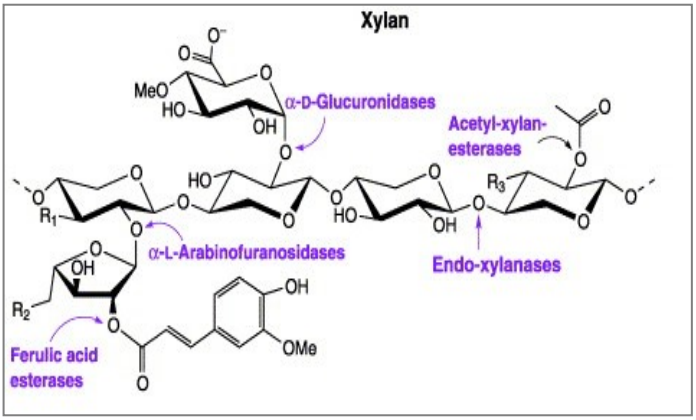


Figure 1-2. Inulin (left) and xylan (right) structures. The enzymes that break the different bonds in xylan structure are shown in purple. Source: (left) Shoaib et al. (2016) and (right) Shallom & Shoham (2003).

Other classes of fermentable fibers include arabinoxylans, pectins, resistant starch and xylan. Xylan is the second most abundant component in plant cell walls especially in grains and seeds (Naidu, Hlangothi & John, 2018). They are composed of linear chains of xylose from 10 to 100 monomers, with side chains composed of glucuronic acid, acetyl groups and arabinose depending on its botanic source (Koukiekolo et al, 2005, Naidu, Hlangothi & John, 2018) (Figure 1-2). The mechanisms for xylan degradation and fermentation have been well described in a few *Bacteroides* species such as *B. ovatus* (Dodd, Mackie & Cann, 2011, Larsbrink et al., 2014). *In vivo* studies have shown that the intake of xylo-oligosaccharides (XOS) by healthy volunteers has a beneficial response, incrementing the abundance of *Bifidobacterium* species and butyrate in fecal samples (Okazaki, Fujikawa, & Matsumoto, 1990, Lecerf et al., 2012). These results place xylan as a possible prebiotic, and it is already being used in the food industry to create functional products (Singh, Banerjee & Arora, 2015).

1.3 Microbial interactions

Gut microorganisms have evolved intricate mechanisms for interacting with each other, for example through competitive inhibition, or metabolic exchange of byproducts by cross-feeding (Stubbendieck, Vargas-Bautista & Straight, 2016). Both inulin and xylan appear to be fermented by complex networks of microorganisms, where some could act as primary fermenters accessing the complex linkages contained in these fibers. This process releases intermediate degradation products such as smaller carbohydrate chains, or intermediate metabolites such as lactate and succinate. These metabolites participate in cross-feeding interactions, being used by secondary fermenters resulting in fermentation of more oxidized end-products (Smith et al., 2019). The major metabolic

end-products of the microbiome are gases (H_2, CO_2) and short-chain fatty acids (SCFAs) (Musso, Gambino & Cassader, 2010, Graf et al., 2015), defined as *"carboxylic acids with aliphatic tails of 1–6 carbons"* such as acetate (C2), propionate (C3) and butyrate (C4) (Parada et al., 2019) (Figure 1-3). These are produced in an approximate ratio of 3:1:1 respectively (Cummings et al., 1987), reaching concentrations of 20-140 mM in the gut lumen (Singh, Vishwakarma & Singhal, 2018). SCFAs are an important energy source for the host, where butyrate is the main energy source for the colonocytes (Boesmans et al., 2018). In addition, SCFAs have been increasingly involved in physiological host responses. Butyrate is a key epigenetic regulator inhibiting histone deacetylases in colonocytes (Chriett et al., 2019), resulting in a suppression of inflammatory pathways and cytokines. This effect is additional to the binding of all three SCFAs to G-protein receptors GPR41 and GPR43 (Kim et al., 2013), which results also in anti-inflammatory responses.

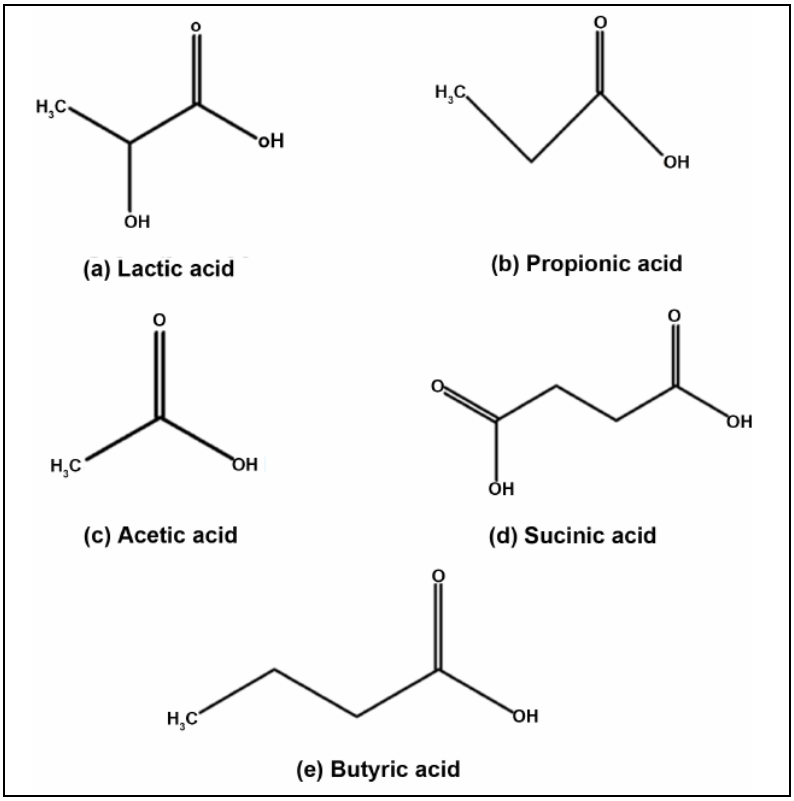


Figure 1-3. SCFAs molecular representation. Source: Singh, Vishwakarma & Singhal (2018).

Metabolic cross-feeding is a common interaction in the gut microbiota, at least *in vitro*. Main molecules exchanged are fermentation byproducts such as acetate, lactate and succinate. Major butyrate producing bacteria such as *Roseburia* sp., *Faecalibacterium prausnitzii* and *Eubacterium rectale* preferentially use these acids as carbon source for butyrate production (Louis & Flint, 2017). Other molecules participating in cross-feeding interactions are macromolecule degradation products. For example, polysaccharide remnants released usually by a *Bacteroides* species could in turn be used by another microorganism. Then, cross-feeding interactions are critical to understand and eventually predict the impact of different dietary fibers over SCFAs production.

1.4 State of the art and motivation

Previous studies have shown that two gut commensals, *Phocaeicola dorei* (previously assigned to the *Bacteroides* genus), and *Lachnoclostridium symbiosum*, appear to be keystone species for butyrate production within a 15-species synthetic consortium (Gutiérrez & Garrido, 2019). The supernatant of the co-culture of both microorganisms using xylan as carbon source showed an anti-inflammatory effect in epithelial cells, reducing inflammation caused by TNF-α (Thomson et al. 2018). Interestingly, this effect was not observed when the microorganisms were cultured in inulin. Other studies by our laboratory confirmed the anti-inflammatory effect of this supernatant in HT-29 cell line and showed that it also helped to protect the cellular barrier of CACO2 cells exposed to TcdB toxin (unpublished results).

Phocaeicola dorei (*P. dorei,* Pd) is a Gram-negative, rod-shaped, non-motile, non-spore-forming, strictly anaerobic bacterium (Figure 1-4). It is a common commensal of the gut microbiome isolated from human feces in 2006, capable of breaking down a wide variety of complex carbohydrates, including inulin and xylan (Bakir et al., 2006, Wexler

y Goodman, 2017, Thomson et al., 2018). Previous studies have shown a correlation between this bacterium and certain diseases such as type I diabetes (Davis-Richardson et al., 2014) and low abundance in coronary artery disease patients (Yoshida et al., 2018).

Lachnoclostridium symbiosum (*L. symbiosum*, Ls) is a Gram-negative, rod-shaped and strictly anaerobic sporulating bacteria (Figure 1-3). It belongs to the phylum *Firmicutes*, class *Lachnospiraceae* and is a butyrate producer. Firstly isolated in 1976 (Kaneuchi et al., 1976), this bacteria is mainly known for its butyrate production when cultured with a primary fermenter (Thomson et al., 2018, Gutiérrez & Garrido, 2019).

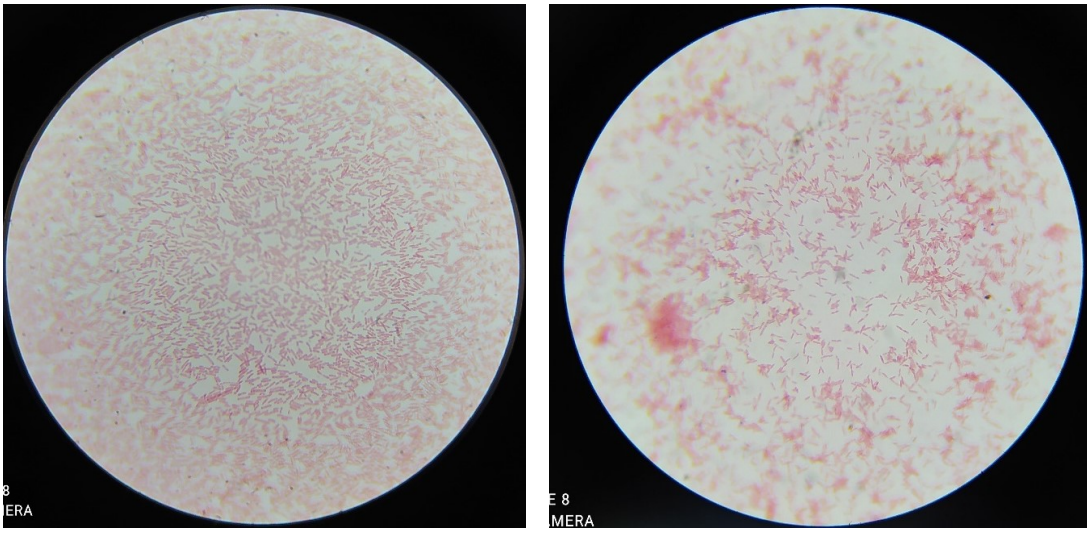


Figure 1-4. Gram stain of *P. dorei* (left) and *L. symbiosum* (right).

Preliminary results of our group have studied *in silico* the potential metabolic interactions between these microorganisms using Genome-Scale Metabolic Models (GSMs). These results suggested that succinate and lactate are major exchange molecules provided by *P. dorei* to *L. symbiosum* (unpublished results). However, there is no experimental evidence for their metabolic interactions *in vitro*. Using a combination of unidirectional and bi-directional cross-feeding assays, here we aimed to understand how these microorganisms exchange metabolites and adapt their metabolisms in presence of the other when cultured in inulin and xylan.

1.5 Hypothesis and Objectives

1.5.1 Hypothesis

Phocaeicola dorei acts as a primary fermenter of inulin and xylan, producing metabolites that are consumed by *Lachnoclostridium symbiosum*, stimulating its growth and butyrate production.

1.5.2 Objectives

The main objective of this study is to determine the metabolic interactions between *P. dorei* and *L. symbiosum* in cultures using inulin and xylan as carbon source. To achieve this, the specific objectives are:

1) To determine the growth and carbohydrate consumption of both microorganisms in monoculture and unidirectional and bidirectional co-cultures, using inulin and xylan as carbon source.
2) To compare the changes of gene expression in genes of interest between monocultures and co-cultures.
3) To evaluate the changes in SCFAs production associated to cross-feeding in co-cultures.

2. MATERIALS AND METHODS

2.1 Strains and culture media

Phocaeicola dorei DSM 17855 and *Lachnoclostridium symbiosum* WAL-14673 were obtained from BEI resources. For inoculums and initial tests, both microorganisms were cultured in Reinforced Clostridium Media (RCM, Becton-Dickinson, Franklin Lakes, NJ) supplemented with 0.5 g/L of L-cysteine (Loba Chemie, India). Incubations were performed at 37 °C for 48 h in an anaerobic jar (Anaerocult, Merck, Darmstadt, Germany) with anaerobic packs (Gaspak EM, Becton-Dickinson, Franklin Lakes, NJ, USA). For experiments, we used a modified version of ZMB medium (mZMB), optimized by Medina et al. (2017) to allow the growth of representative gut bacteria.

2.2 Monoculture and unidirectional assay

Monocultures were carried out in 96-well plates for consumption testing of different metabolites, such as inulin, xylan, succinate and lactate. For unidirectional assays, the microorganisms were reactivated for 48 h as mentioned before, after which were centrifuged at 5000 rpm and washed with mZMB media. Each bacterium was inoculated in tubes containing mZMB with 2% inulin or xylan and cultured for 48 hours in anaerobic conditions.

In parallel, inoculums were prepared in 4 mL of RCM with L-cysteine for 48 hours before inoculation. Two identical 96-well plates were prepared using the same mediums and were left to reduce in an anaerobic jar for 48 h at room temperature. The wells disposed for unidirectional assays were filled with 100 µL of ZMB, while the wells disposed for monocultures were filled with 200 µL of the specific medium.

Right before inoculation, the tubes containing mZMB with each carbon source and bacteria were centrifuged at 10.000 rpm for 5 min and the supernatant was filtered with filters of 0.22 µm pore size (Jet Biofil, China) (Figure 2-1). 100 µL of each supernatant was added to the wells of the 96-well plate that had 100 µL of mZMB. Immediately

after, the inoculums in RCM were centrifuged at 5000 rpm x 1 minute and resuspended in mZMB to a final OD of 1. Plates were inoculated with 5% v/v in biological triplicates for 24 and 48 h, and absorbance was measured at 600 nm in a Synergy H1 spectrophotometer (BioTek, Agilent Technologies). Gram stain was performed in all the stages of the experiment to check the purity of the cultures (Figure 1-4).

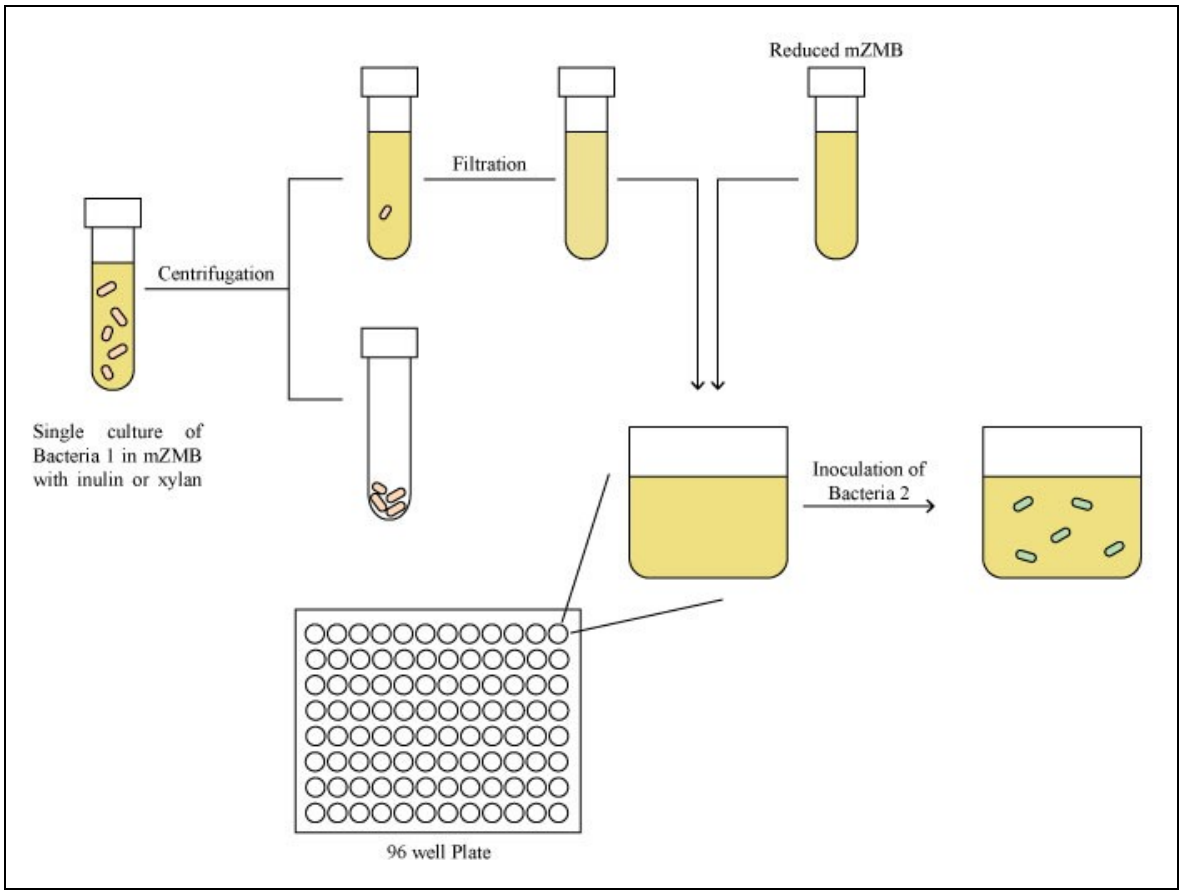


Figure 2-1. Set up of the unidirectional assay after inoculum activation in RCM.

2.3 Bi-directional assay

Both bacteria were cultured in Tissue Culture Plate Inserts (Jet Biofil, China), where *L. symbiosum* was always cultured in the upper insert and *P. dorei* in the lower well. The bacteria were separated by the permeable membrane of the insert (0.1 µm pore size), which allowed the passage of small carbohydrates and metabolites (Figure 2-2).

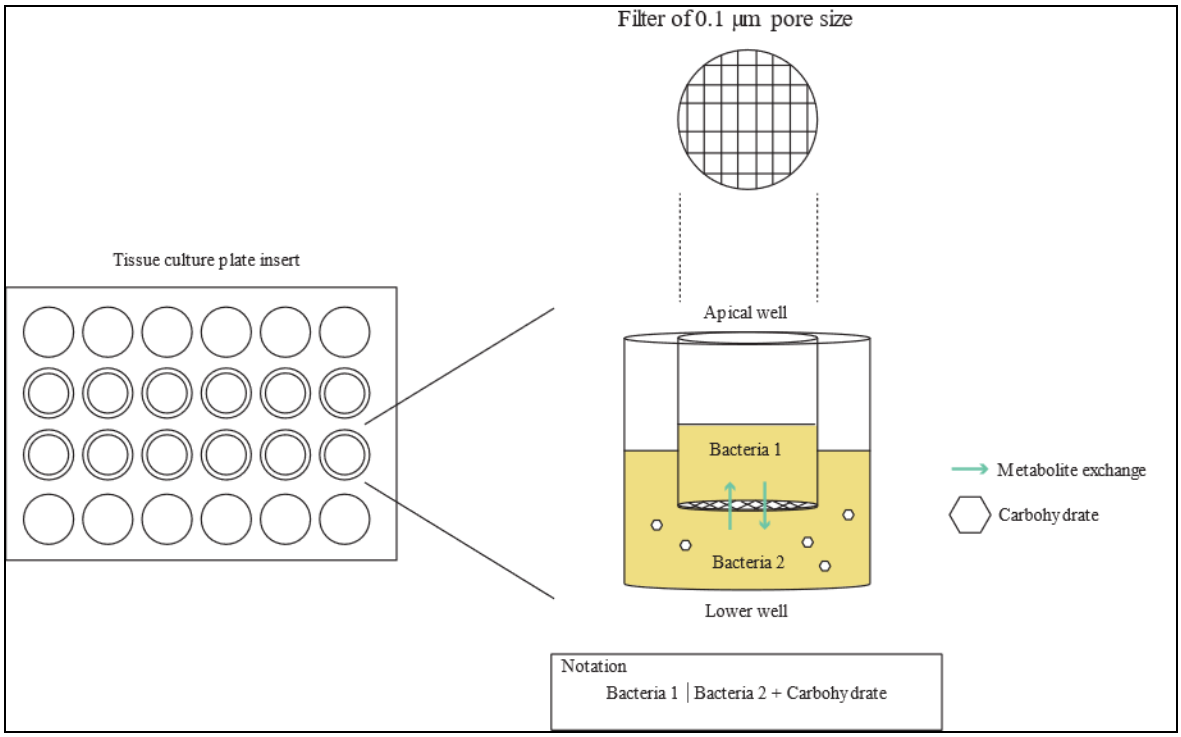


Figure 2-2. Diagram of the Tissue culture plate used for bidirectional assays. The notation used in all this study is explained in the rectangle: the bacterium in the upper insert goes left, the membrane is represented by " | " and the bacterium in the lower well goes right.

The cultures were performed in 250 µL and 1 mL of mZMB for the inserts and wells respectively. Eighteen conditions of mono and co-cultures were tested in duplicate, using 2% of lactose (Heyn, Santiago, Chile), inulin (Piping Rock, Ronkonkoma, NY) or xylan from birchwood (Sigma-Aldrich, St. Louis, MO, USA) as sole carbon source. Of

those, the conditions used in the posterior experiments are listed in table II-1, while the other conditions are listed in Appendix A. Monocultures were carried out in order to compare with the co-cultures under the same conditions.

Table II-1. Principal conditions tested in the bidirectional study. "-" represents the conditions where the lower well was without an insert.

Carbon source	Type of culture	Upper well (250 µL)	Lower well (1 mL)
None	Monoculture	*L. symbiosum* in mZMB	mZMB
	Monoculture	-	*P. dorei* in mZMB
	(-) Control	-	*m*ZMB
Inulin	Monoculture	*L. symbiosum* in mZMB	mZMB with inulin
	Monoculture	-	*P. dorei* in mZMB with inulin
	Co-culture	*L. symbiosum* in mZMB	*P. dorei* in mZMB with inulin
	(-) Control	-	*m*ZMB with inulin
Xylan	Monoculture	*L. symbiosum* in mZMB	mZMB with xylan
	Monoculture	-	*P. dorei* in mZMB with xylan
	Co-culture	*L. symbiosum* in mZMB	*P. dorei* in mZMB with xylan
	(-) Control	-	*m*ZMB with xylan
Lactose	Monoculture ((+) control)	*L. symbiosum* in mZMB with Lactose	mZMB
	Monoculture ((+) control)	-	*P. dorei* in mZMB with lactose
	(-) Control	-	*m*ZMB with lactose

For the experiment, bacteria were reactivated in RCM with L-cysteine for 48 h, after which were centrifuged at 5000 rpm for 1 minute and washed with mZMB without carbon source. Before inoculation, the plate was incubated in an anaerobic jar at room temperature for 48 h to reduce the medium. Inoculations were performed at 5% v/v and the plates were incubated in an anaerobic jar with anaerobic packs at 37 °C for 24 and 48 h. OD600 was measured at 0, 24 and 48 h by resuspending the content of each well or insert and transferring 200 µL to a 96 well plate. Measurements were carried out in a Synergy H1 spectrophotometer (BioTek, Agilent Technologies) and the cultures were transferred to Eppendorf tubes and centrifuged at 10.000 x *g* x 2 min. The supernatant was separated and both pellets and supernatants were stored at -80 °C until use.
To calculate the final optical density of each measurement, the mean of each basal medium without bacteria was subtracted to the cultures according to the carbon source.

2.4 Carbohydrate profile.

Thin layer chromatography (TLC) was performed in silica gel F 60 (Merck, Germany), using 1-butanol/ethanol/water 10:8:5 v/v as running buffer and 1% orcinol in 10% H_2SO_4 in ethanol as the revealing reagent (Arumugam et al., 2019). Two µL of each sample were used and the chromatogram was developed in one run and let dry. After pouring the detector and drying, the silica gel was heated at 100 °C until the samples were detectable in the gel.

2.5 SCFA quantification.

Acetic, butyric, lactic, propionic, and succinic acid of chosen supernatants were measured in a Lachrom liquid chromatograph (Merck-Hitachi), using a Aminex® HPX-87H Ion exclusion column (300mmx7.8mm, Bio-Rad) and an isocratic mobile phase with 5 mM H_2SO_4 (Mendoza et al., 2017). 30 µL of supernatant were injected at a flux of 0.45 mL/min, at 35°C for 35 minutes. Standard curves were created with nine

dilutions of 30 g/L to 0.155 g/L of each acid in HPLC grade water. Ten conditions plus a sample of mZMB at 48 h were tested in biological duplicates. Controls of the chromatograph and data analysis were done using Multi-HSM Manager software.

2.6 Gene expression

2.6.1 RNA extraction

RNA was extracted from the pellets of the samples used in HPLC analysis. Total genetic material was extracted using a modified version of the phenol/chloroform/isoamyl alcohol method (Medina et al., 2017) and its purity was checked by measuring the 260/280 absorbance ratio in a Tecan Infinite M200 Pro plate reader (Tecan Trading AG, Grödig, Austria). After extraction, samples were immediately treated with DNAse I (New England BioLabs) using the manufacturer's protocol, but with an incubation time of 15 minutes. Absorbance was measured and the samples were stored at -80 °C until use.

2.6.2 Reverse Transcription.

RNA was converted into cDNA with the AffinityScript qPCR cDNA Synthesis kit (Agilent Technologies, Texas), using the manufacturer's protocol and oligo-dT random primers. All samples of each microorganism were treated at the same time, including a negative control with free nuclease water and an RNAse block control for two of each bacterial samples. Final quality of cDNA was assessed by measuring the 260/280 absorbance ratio in a Tecan Infinite M200 Pro plate reader and absorbance curves from 230 nm to 300 nm were obtained in order to rule out contamination by unwanted chemicals. All cDNA samples were stored at -20°C until use.

2.6.3 Fold change gene expression.

Twenty pair of primers were designed with primer-BLAST (NIH) (9 and 11 genes for *L. symbiosum* and *P. dorei*, respectively), with melting temperatures around 60 °C and gene sizes of ~250 pb (Appendix B). Eight genes were chosen for *L. symbiosum*, related with amino acid degradation, fructan metabolism and butyrate production pathways. Ten genes were chosen for *P. dorei* related to SCFAs production and inulin and xylan consumption. 16S rRNA was used as a reference gene for both bacteria.

qPCR reactions used SensiFAST™ SYBR No-ROX (Meridian Bioscience, Ohio, USA) under the manufacturer's protocol with half of the reaction volume and 1 µL of cDNA. Amplification was performed in a StepOnePlus equipment (Applied Biosystems, USA) using 96-well optical plates MicroAmp Fast Optical (ThermoFisher, USA). Reactions were carried out in a 3-step cycling format, with an initial cycle at 95°C for 2 minutes and 40 cycles at 95°C for 5 seconds (denaturation), 60°C for 10 s (annealing) and 72°C for 10 s (extension). All conditions had two biological replicates with three technical replicates each. A standard curve was created by doing ten-fold dilutions of genomic DNA and a negative control was included for each pair of primers.

2.6.4 Data Analysis.

Ct values and efficiency of the reactions were obtained using the Agilent Aria 1.7 software. Data was checked manually and faulty curves (with no amplification or delayed amplification with respect to its technical replicates) were removed. An excel file was generated and analyzed using R. To obtain the relative gene expression we used the efficiency-corrected method (Pfaffl, 2001), given by the equation:

$$\text{Ratio} = E_{\text{target}}^{\Delta Ct\_\text{target}} / E_{\text{Reference}}^{\Delta Ct\_\text{reference}} \quad (1)$$

Where $\Delta Ct\_i = Ct_{\text{basal condition}} - Ct_i$ and $E = 10 - 1/\text{slope}$. The slope of each gene was obtained from the standard curve and considered equal to all samples for that gene.

Technical replicates were averaged, but the ratio was calculated independently for biological replicates and then averaged.

3. RESULTS

3.1 Unidirectional culture assays

We first evaluated the growth of each bacterium in different substrates to identify which conditions allow their growth. *P. dorei* showed good growth in inulin and lactose, but less in xylan (Figure 3-1). It had a basal growth in mZMB with no carbon source, suggesting it could consume proteins of the medium. The use of *L. symbiosum's* supernatant apparently did not benefit *P. dorei's* growth in xylan, as the growth curve is similar to the monoculture in the same carbon source (green curves). Similarly, *P. dorei* grew to a similar rate and OD with *L. symbiosum's* supernatant in inulin compared to its growth in mZMB with inulin, slightly decreasing at 48 h (blue curves).

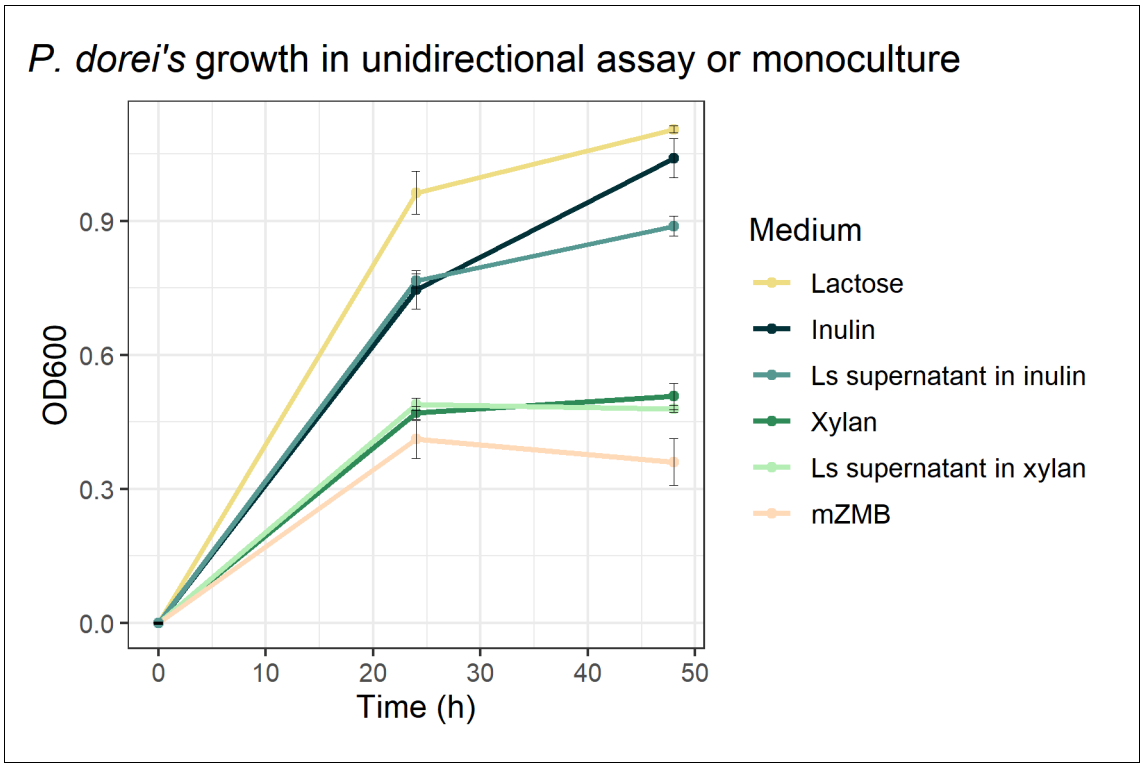


Figure 3-1. Growth curves of *P. dorei* growing in different carbon sources in unidirectional set-up or monoculture. Measurements at OD600 were taken at 0, 24

and 48 h. Each condition is in biological triplicate and error bars indicate standard deviation.

L. symbiosum was cultured in different metabolites, monosaccharides, disaccharides, and polysaccharides, in addition to the spent supernatant of *P. dorei* in unidirectional assays (Figure 3-2). As this bacterium is less studied and is not known for fermenting complex carbohydrates, we wanted to check if it could consume monosaccharides of inulin and xylan. To study if this bacterium could benefit of exchange metabolites, we also cultured it with lactate and succinate, which are produced by *P. dorei* as degradation products.

Similar to *P. dorei*, maximum growth of *L. symbiosum* occured in lactose (Figure 3-2). Interestingly, growth in fructose exceeded significantly the growth in inulin, suggesting that this bacteria could consume small degradation products from inulin. In contrast, growth in xylose and xylan were similar to basal growth in mZMB. This bacterium showed a medium to poor growth in inulin, as well as in the supernatant of *P. dorei* in xylan or inulin. Growth in lactate or succinate as sole carbon source was not significantly different to mZMB alone, indicating no apparent benefit for this bacterium (Figure 3-2).

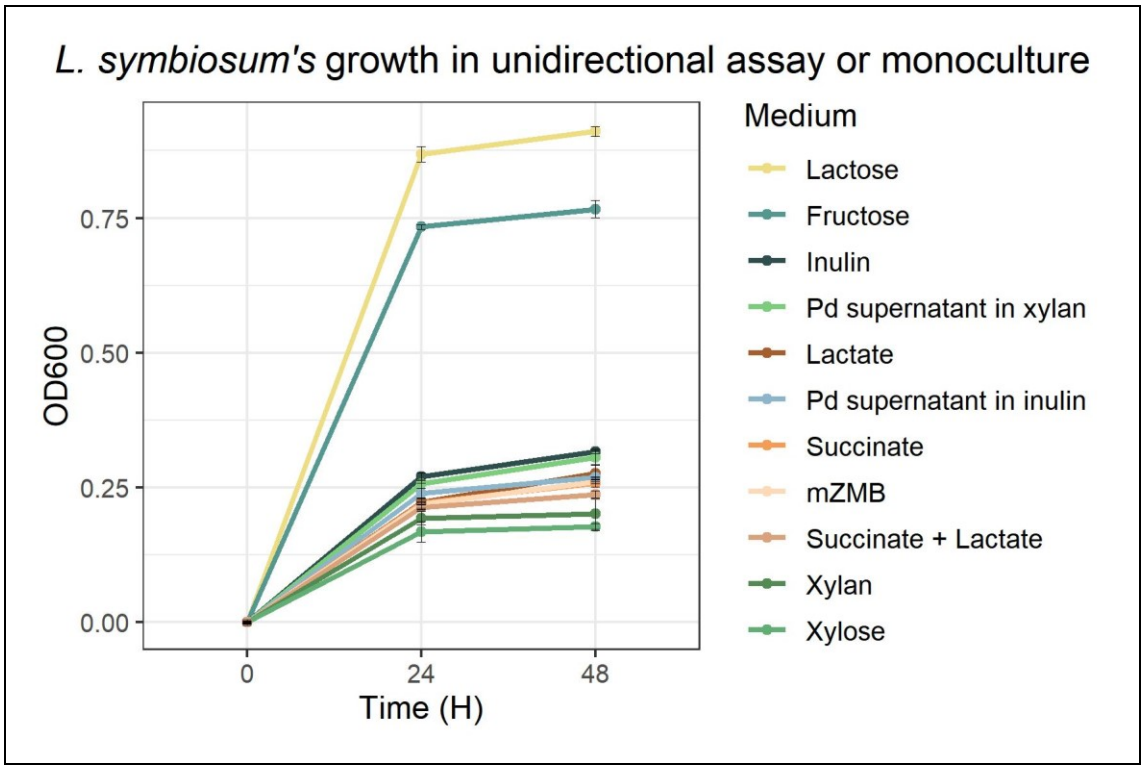


Figure 3-2. Growth curves of *L. symbiosum* growing in different carbon sources in unidirectional set-up or monoculture. Measurements at OD600 were taken at 0, 24 and 48 h. Each condition is in biological triplicate and error bars indicate standard deviation. For simplicity, the legend is arranged according to the order of the curves in the graph.

3.2 Bi-directional culture assays

We later studied the growth in co-culture bidirectional assays, which allow the simultaneous growth of two microorganisms in Tissue Culture Plate inserts. In this set-up, each well has an apical compartment inside, separated from a basal well by a 0.1 µm membrane filter (Figure 2-2). Microorganisms are cultured either in the basal or apical well, where the filter allows the exchange of small and medium-sized metabolites reaching chemical equilibrium. This simple system allows capturing the simultaneous influence of one microorganism on another´s without direct contact between them, and

eventually make a proper comparison with metabolic modeling predictions at steady state.

In monoculture, *P. dorei* reached high OD values in lactose and inulin, but a slower growth in xylan, probably due to its structural complexity that makes it harder to consume (Figure 3-3). As expected from the literature and previous works, this bacterium has the ability to consume inulin and xylan, growing more than in mZMB alone.

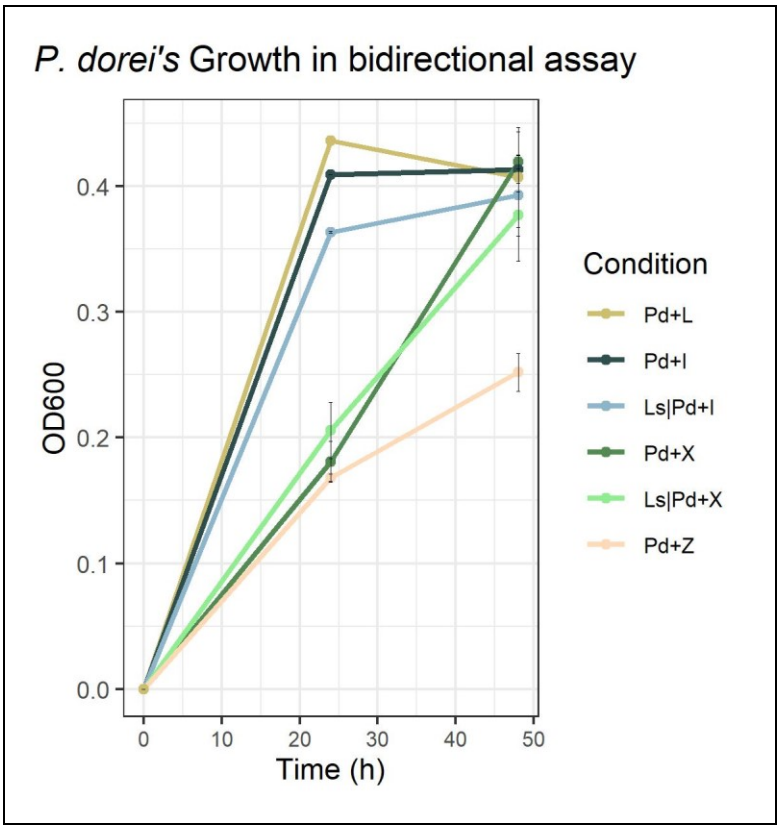


Figure 3-3 Growth curves of *P. dorei* (Pd) in different conditions in the bidirectional assay at 0, 24 and 48 h. Error bars indicate standard deviation. “L” = Lactose, “I” = Inulin, “X = xylan, “Z” = mZMB.

The presence of *L. symbiosum* in co-culture did not modify the maximum growth of *P. dorei* in inulin (p-value > 0.05), but slightly decreased it in xylan (p-value < 0.05) (Figure 3-4 and Figure 3-7). This suggest that *P. dorei* does not benefit from the interaction with *L. symbiosum*.

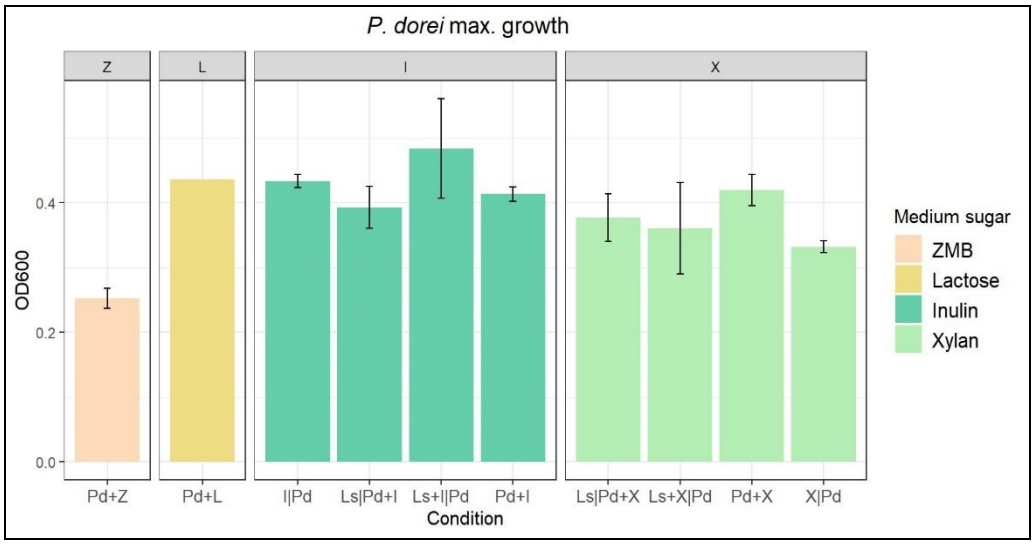


Figure 3-4. *P. dorei*'s maximum growth registered for each condition tested in the bidirectional assay. Graphs are divided and colored by carbon source. Error bars indicate standard deviation. "Lactose", "Inulin" and "Xylan" are mZMB with the carbon source.

L. symbiosum presented an important growth on medium with no carbon source (Figure 3-5), which correlates with the known ability of several clostridia to ferment amino acids (Amaretti et al., 2019). However, growth of *L. symbiosum* using inulin was higher than the basal when the carbohydrate was in the lower well (p < 0.05), indicating it can partially use this substrate for growth (Figure 3-6). Co-cultures showed that *L. symbiosum* increased its growth in the presence of *P. dorei*, both in xylan and in inulin (Figure 3-7). This could imply a one-way benefit from *P. dorei* to *L. symbiosum*.

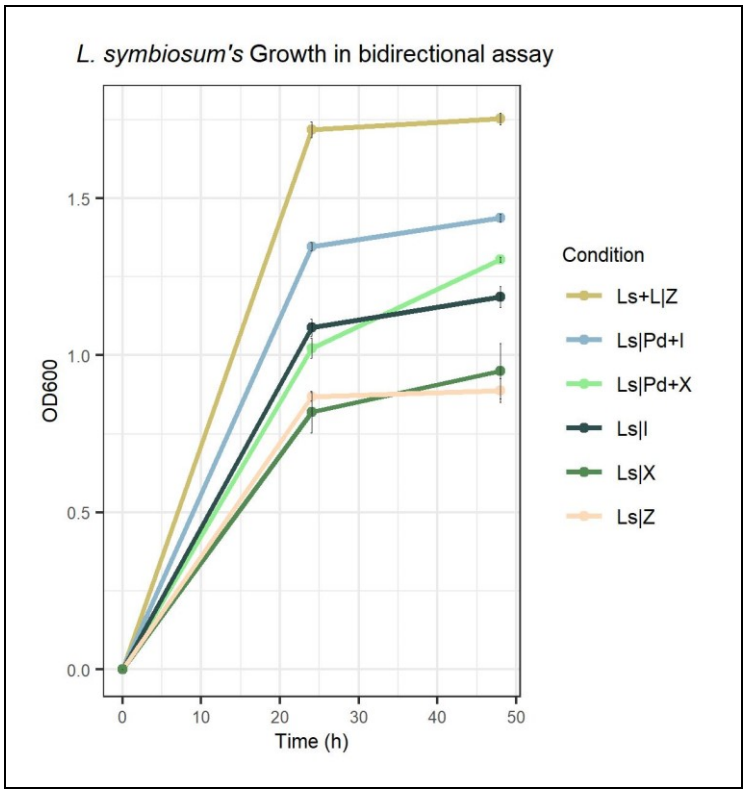


Figure 3-5. Growth curves of *L. symbiosum* (Ls) in different conditions in the bidirectional assay at 0, 24 and 48 hours. Error bars indicate standard deviation. "L" = Lactose, "I"= Inulin, "X = xylan, "Z" = mZMB.

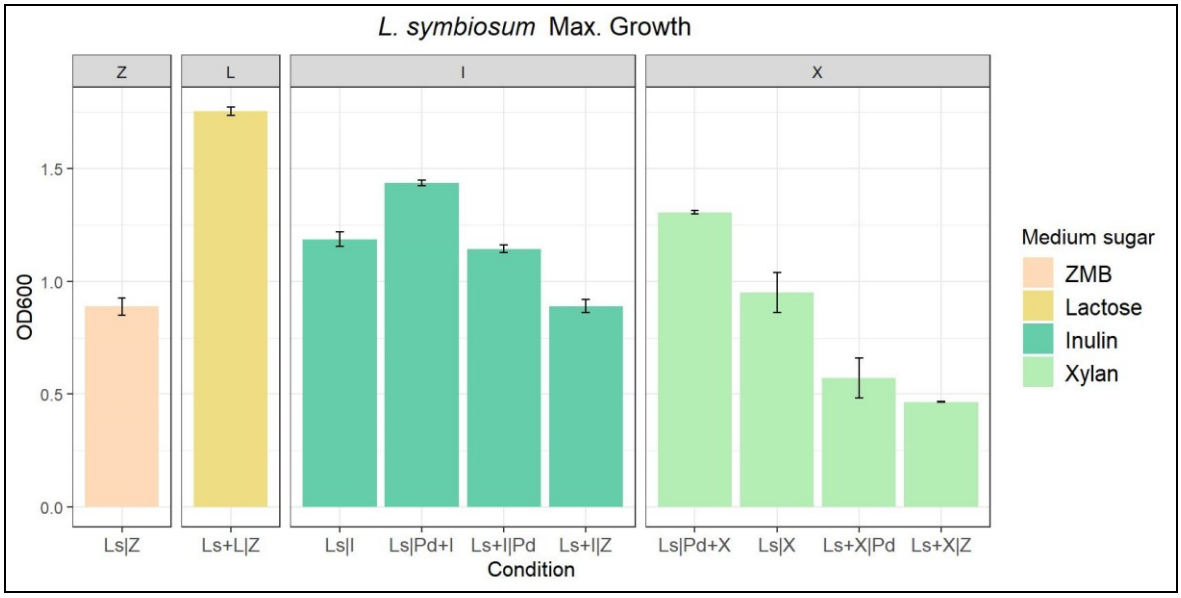


Figure 3-6. *L. symbiosum*'s maximum growth registered for each condition tested in the bidirectional assay. Graphs are divided and colored by carbon source. Error bars indicate standard deviation. "Lactose", "Inulin" and "Xylan" are mZMB with the carbon source.

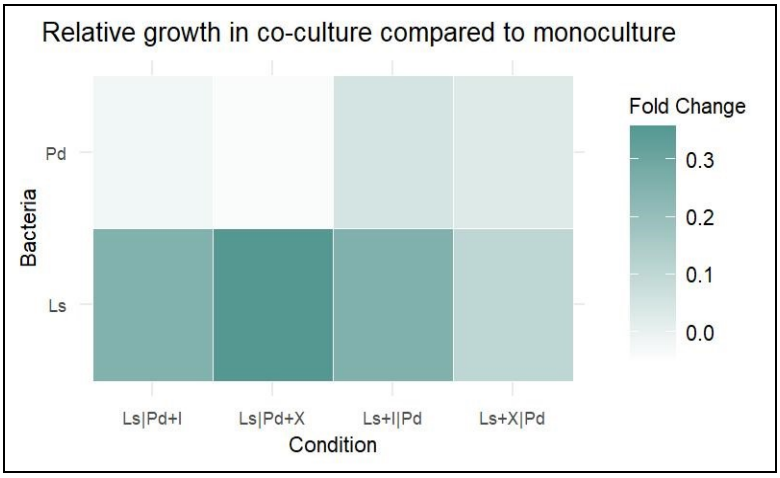


Figure 3-7. Relative growth in co-cultured with respect to monoculture for *P. dorei* (up, Y axis) and *L. symbiosum* (down, y axis) in the co-cultures tested in the bidirectional study. Darker color shows that the bacteria grow more in co-culture than in monoculture in that condition.

3.2 Substrate consumption

We screened the supernatants of bi-directional assays for carbohydrate consumption. The negative control of mZMB with inulin showed that this carbohydrate consists of multiple chain lengths of fructans (Figure 3-8). Indirectly, we observed similar concentrations and distributions of fructans in both upper and basal compartments, showing that inulin easily equilibrates in both sides of the membrane. Xylan showed a smaller concentration in the upper insert compared to the lower well, which means that the carbohydrate did not equilibrate through the membrane. This could be explained by

xylan length chain and complexity, whose size was probably bigger than the pore size of the membrane (Figure 3-9).

TLC plates for *P. dorei* showed a reduction in total inulin concentration, especially the intermediate and smaller fractions (Figure 3-8). No apparent consumption could be seen by *L. symbiosum* in the gel, but its monoculture in fructose suggests that it can consume small fructans in the medium. Their co-culture showed a significant reduction in inulin concentration, much more than each microorganism in monoculture. These results suggest an accelerated consumption of inulin in co-culture, reaching almost full consumption at 24 h.

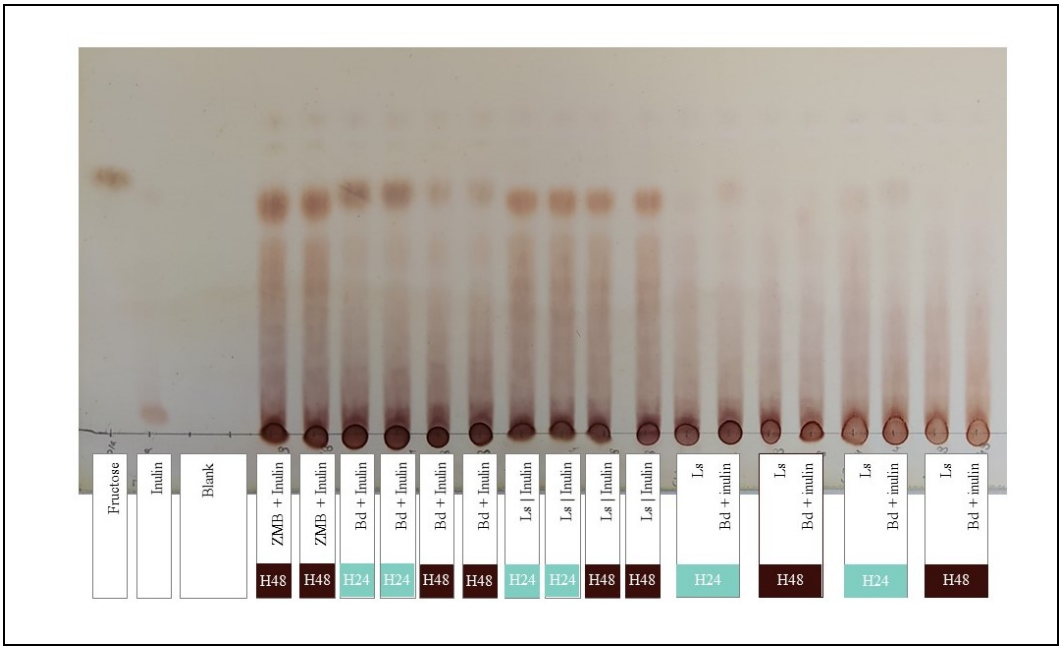


Figure 3-8. Thin layer chromatography of all the conditions in mZMB with inulin of the bidirectional assay. Each rectangle is one condition in the well of the bacteria (e.g., "Ls | Z" shows the insert with *L. symbiosum*), were the wide rectangles show first the insert and then the lower well. Samples of hour 24 are marked in light blue and samples of hour 48 are marked in brown, while standards are colorless.

In accordance with its growth, xylan utilization by *P. dorei* showed a partial consumption after 48 h (Figure 3-9). It can be observed that *P. dorei* in monoculture leaves several xylan degradation products, while *L. symbiosum* was not able to degrade xylan on itself nor consume xylose (Figure 3-2). The co-culture of both microorganisms showed a reduction in total carbohydrates in the medium, but no visible degradation products. This could be explained by a slower or lack of xylan degradation by *P. dorei* or a full consumption of the degradation products released by *P. dorei*.

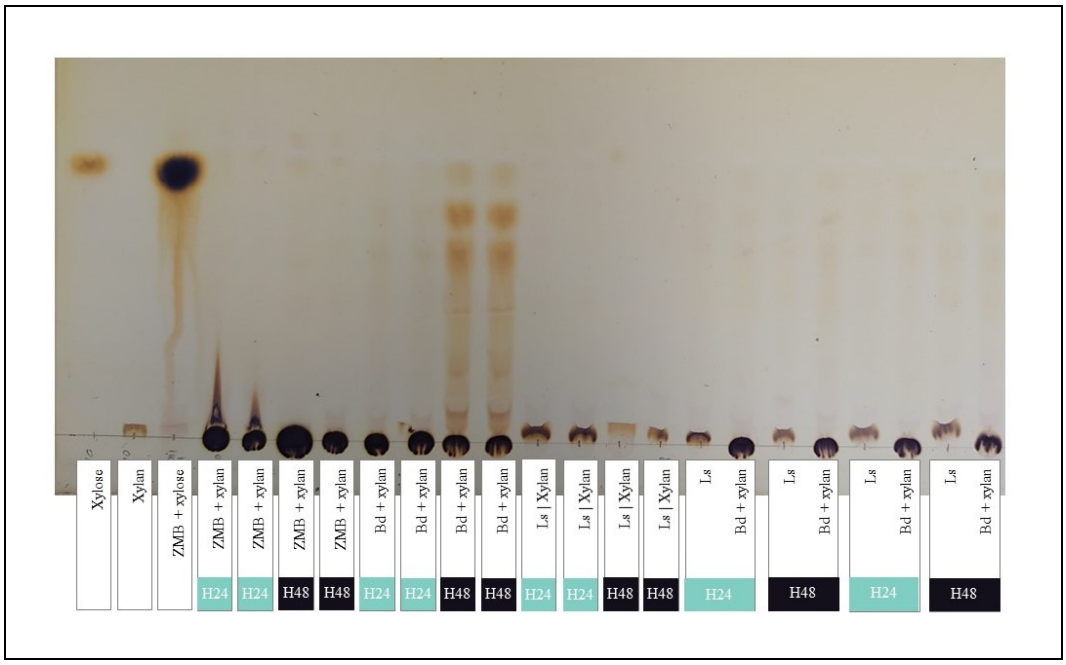


Figure 3-9. Thin layer chromatography of all the conditions in mZMB with xylan of the bidirectional assay. Each rectangle is one condition in the well of the bacteria (e.g., "Ls | Z" shows the insert with *L. symbiosum*), were the wide rectangles show first the insert and then the lower well. Samples of hour 24 are marked in light blue and samples of hour 48 are marked in dark blue, while standards are colorless.

3.3 SCFA bidirectional exchange

Both bacteria produced acetate in monocultures with no carbon source (Figure 3-10). However, acetate amounts were higher in cultures in xylan than in inulin. We observed an increase in acetate production in co-culture compared to single cultures, more in xylan than in inulin. This correlates with a higher combined activity compared to single cultures.

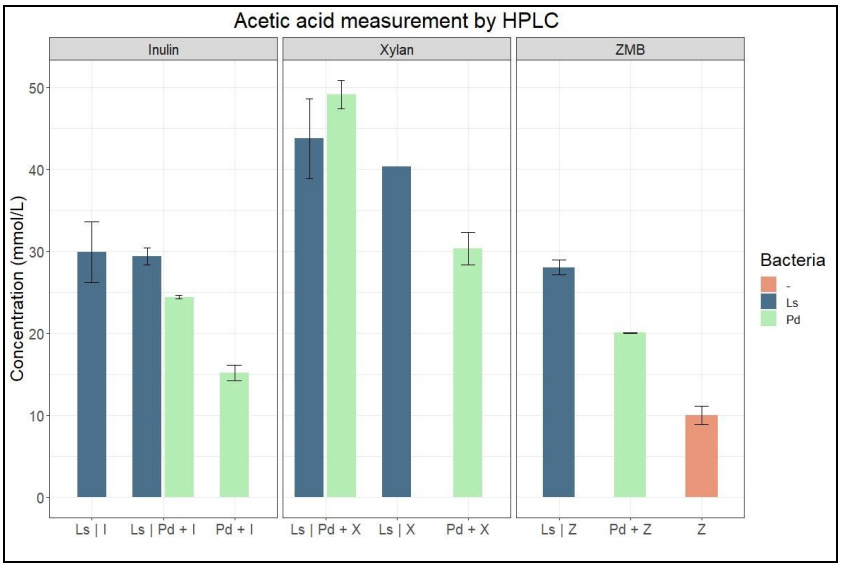


Figure 3-10. Acetic acid measured at 48 h, separated by medium. "Ls" = *L. symbiosum*, "Pd" = *P. dorei*, "I" = mZMB with Inulin, "X" = mZMB with xylan, "Z" = mZMB without carbon source.

Single cultures of *P. dorei* produced 17 mM of lactate in inulin and 7 mM lactate in xylan, while lactate was not produced by *L. symbiosum* (Figure 3-11). The presence of *L. symbiosum* caused a reduction of lactate in both compartments, suggesting consumption. This was estimated to be 15% in inulin and 80% in xylan. As *L. symbiosum* was able to use certain fructan fractions, it is possible that these are preferred

over lactate in the presence of inulin, explaining the smaller lactate consumption percentage (Figure 3-15).

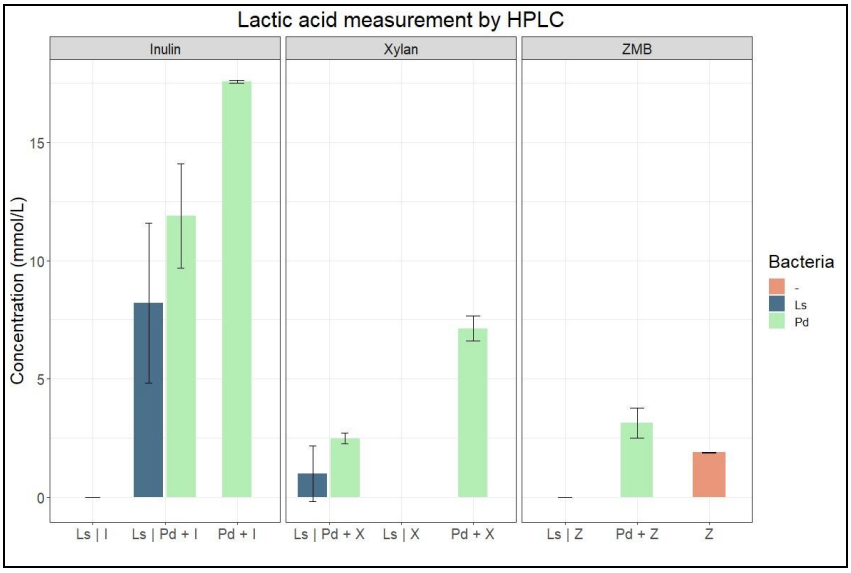


Figure 3-11. Lactic acid measured at 48 h, separated by medium. "Ls" = *L. symbiosum*, "Pd" = *P. dorei*, "I" = mZMB with Inulin, "X" = mZMB with xylan, "Z" = mZMB without carbon source.

Similar to lactate, only *P. dorei* produced succinate (Figure 3-12). Its concentrations were higher after growth in xylan than in inulin. A reduction in succinate was observed in xylan co-cultures and to a lesser extent in inulin, which suggests that succinate produced by *P. dorei* is cross-fed to *L. symbiosum*.

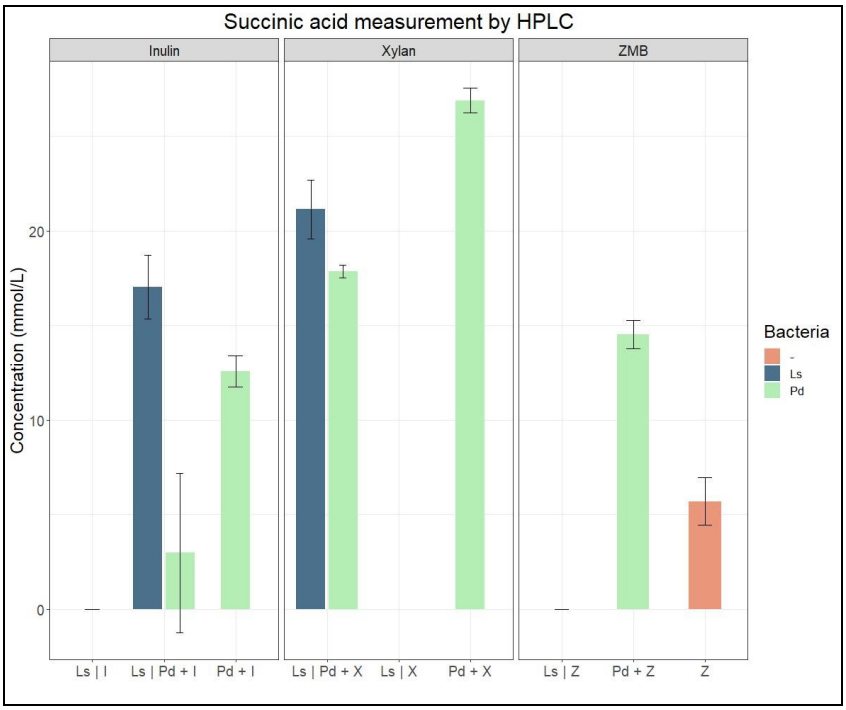


Figure 3-12. Succinic acid measured at 48 hours, separated by medium. "Ls" = *L. symbiosum*, "Pd" = *P. dorei*, "I" = mZMB with Inulin, "X" = mZMB with xylan, "Z" = mZMB without carbon source.

Propionate was only produced by *P. dorei* in blank media and in mZMB with xylan (Figure 3-13). Co-culture showed a smaller concentration of propionate with respect to the monoculture, but as no propionate is seen in *L. symbiosum*'s compartment, no interactions can be suggested regarding this SCFA.

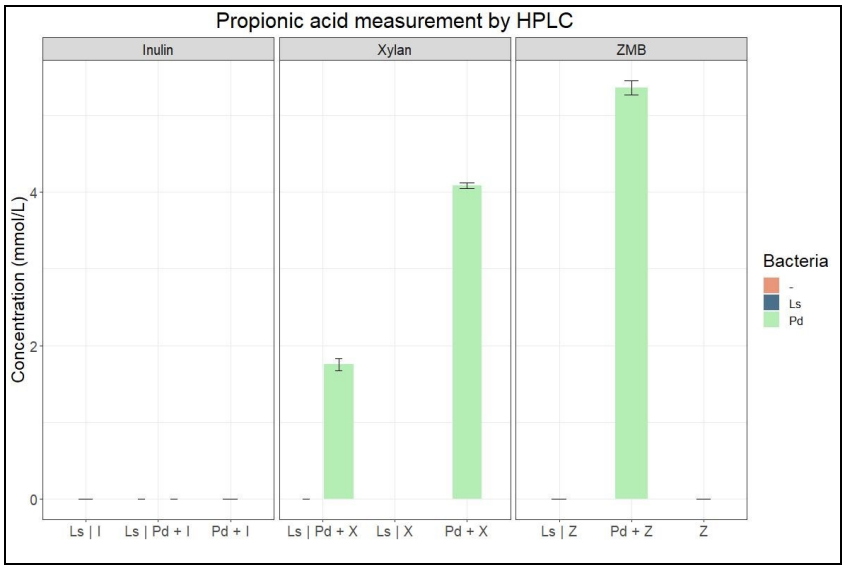


Figure 3-13. Propionic acid measured at 48 h in the bidirectional assay, separated by medium. "Ls" = *L. symbiosum*, "Pd" = *P. dorei*, "I" = mZMB with Inulin, "X" = mZMB with xylan, "Z" = mZMB without carbon source.

Finally, concentrations between 20 and 30 mM of butyrate were detected in *L. symbiosum* monocultures in any carbon source or control medium (Figure 3-14). *P. dorei* showed smaller amounts of this SCFA, invariable in all its monocultures. Butyrate concentrations in *L. symbiosum* wells were not different when *P. dorei* was present in the basal compartment, showing no cross-feeding interaction. In addition, these results indicate that butyrate production by *L. symbiosum* is independent of culture conditions, and that *P. dorei* does not modulate its production.

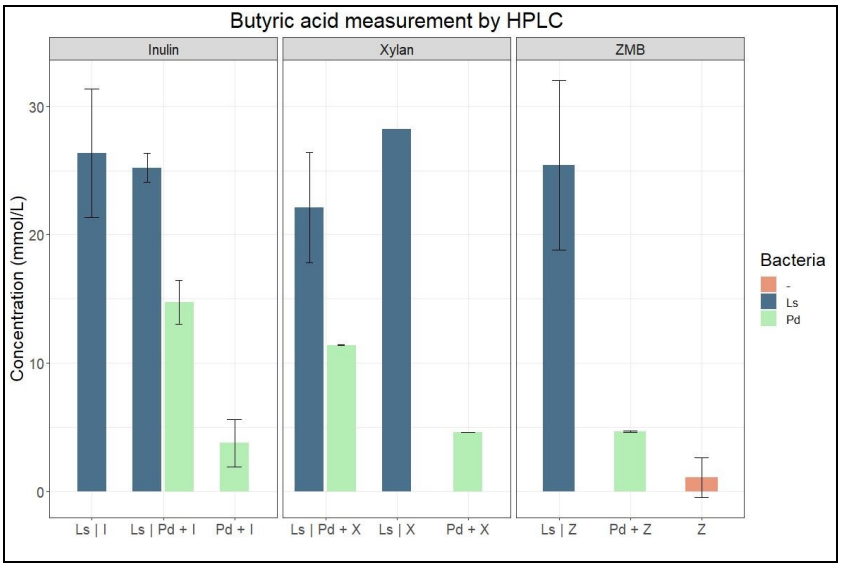


Figure 3-14. Butyric acid measured at 48 h, separated by medium. "Ls" = *L. symbiosum*, "Pd" = *P. dorei*, "I" = mZMB with Inulin, "X" = mZMB with xylan, "Z" = mZMB without carbon source.

Finally, we made a comparison of the quantity of SCFAs between the co-cultures in inulin and in xylan (Figure 3-15). The total amount of each metabolite was calculated by multiplying the concentration by the volume of the well (250 µL for the insert and 1000 µL for the lower well) and adding both values. The metabolic profiles differed by the carbon source, as acetate, propionate and succinate are higher in xylan, while butyrate and lactate are higher in inulin.

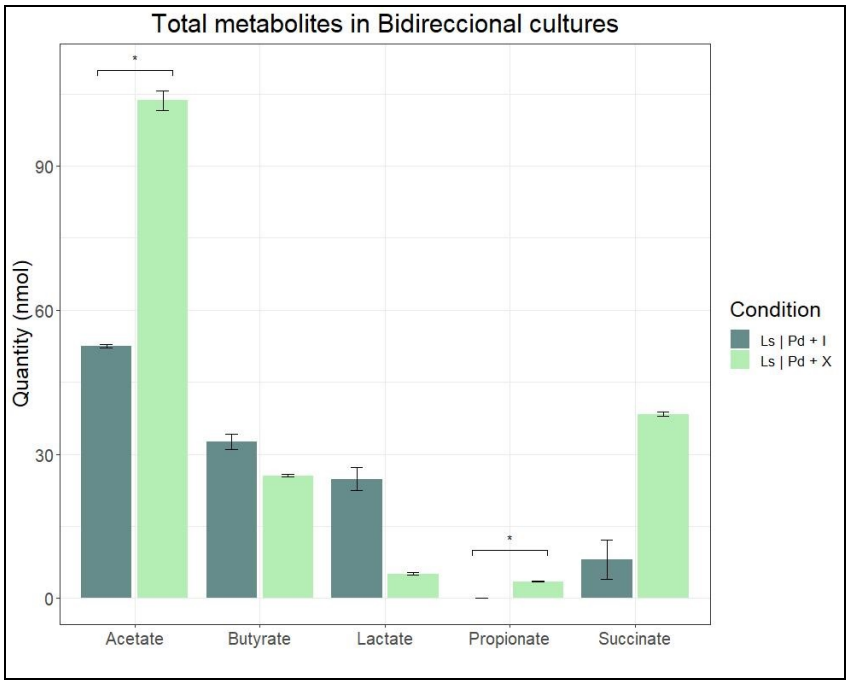


Figure 3-15. Total metabolites measured at 48 h in co-cultures in inulin (blue) and in xylan (green). * Indicates that the difference is significant with p-value < 0.05 (t-student). "Ls" = *L. symbiosum*, "Pd" = *P. dorei*, "I" = mZMB with Inulin, "X" = mZMB with xylan.

3.4 Changes in gene expression

We finally studied the relative changes in the expression of genes related with the production and degradation of the metabolites studied, whose functions are briefly described in Appendix F. We used two different basal conditions to calculate the fold change: comparing the monocultures in inulin or xylan with the monoculture in mZMB alone (Appendix G), and the co-cultures with the monoculture in mZMB with the same carbon source (Figures 3-16 and 3-17). This approach allowed us to study the effect of incorporating a carbon source and then the effect of culturing with the other bacteria.

Regarding carbohydrate-utilization genes, the monoculture of *P. dorei* in inulin induced a levanase, which associates with the degradation observed in the TLC. However, its

expression in co-culture showed a reduction with respect to monoculture, leading to an increase in relative expression of another fructofuranosidase (Figure 3-16). As both enzymes serve similar functions, one possible explanation is that *P. dorei* induces different glycolytic enzymes in mono and co-culture.

P. dorei showed the induction of putative xylan-utilization genes in monoculture in xylan (Figure 3-16). Interestingly, the expression of these genes increased several fold in the presence of *L. symbiosum*. These observations support an increased consumption of xylan in co-culture, which could explain the absence of xylan-degradation products in co-culture.

Putative genes participating in SCFA production by *P. dorei* appeared to be more expressed in co-cultures and especially in xylan (Figure 3-16). This correlates with the higher acetate production observed. According to HPLC results, propionate was only produced in xylan, which correlates with the overexpression of propionyl-CoA carboxylase in this substrate. Finally, a gene encoding a fumarate reductase was more expressed in xylan than in inulin in *P. dorei*, which was consistent with higher succinate production in these conditions (Figure 3-15).

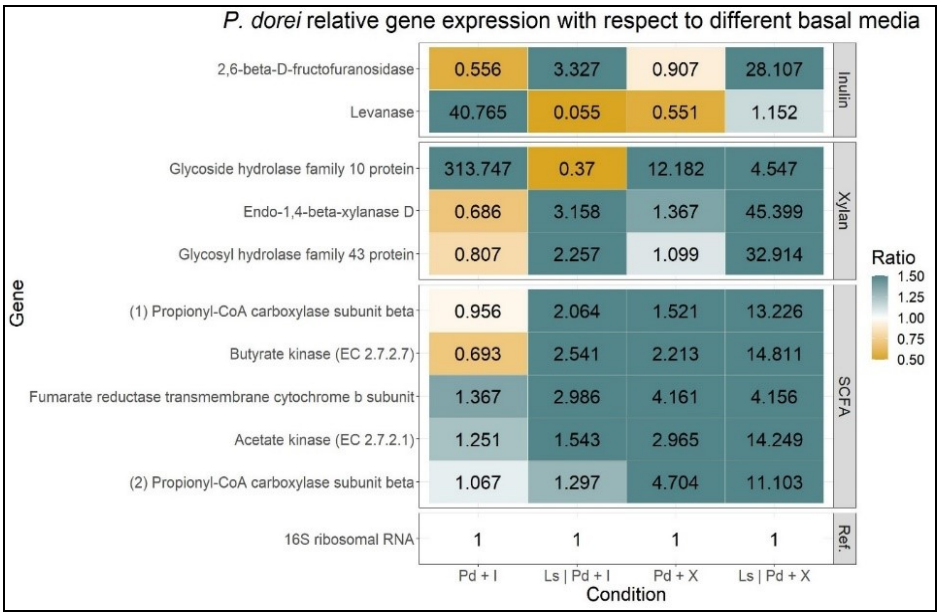


Figure 3-16. Relative expression of genes of interest of *P. dorei*. Each column is one culture condition, and the rows represent the genes measured. Right labels indicate the pathways at which belongs each gene, while the number and color represent the ratio of expression calculated with equation (1). Ratios over 1.5 and under 0.5 are colored as the limits of the ratio's legend, indicating overexpression or repression. "Pd + I" = *P. dorei* in mZMB with inulin, "Ls | Pd + I" = co-culture of *L. symbiosum* with *P. dorei* in mZMB with inulin, "Pd + X" = *P. dorei* in mZMB with xylan, "Ls | Pd + X" = co-culture of *L. symbiosum* with *P. dorei* in mZMB with xylan.

In general, all genes studied in *L. symbiosum* were overexpressed in the co-culture in inulin. Genes related to Fructan metabolism were not the exception, and its overexpression in co-culture in inulin could be due to the increment of fructose in the medium as result of *P. dorei's* degradation of inulin. These results support the higher inulin degradation activity observed in co-culture compared to single cultures.

Genes participating in butyrate synthesis in *L. symbiosum* were induced in inulin co-culture with *P. dorei*, but also in monocultures in xylan (Figure 3-17). Crotonase is part of the acetyl-CoA pathway, while the other three genes belong to the 4-aminobutyrate pathway (Appendix F). Both pathways seem to be active in inulin co-culture and xylan monoculture, while xylan co-culture showed a repression in these genes.

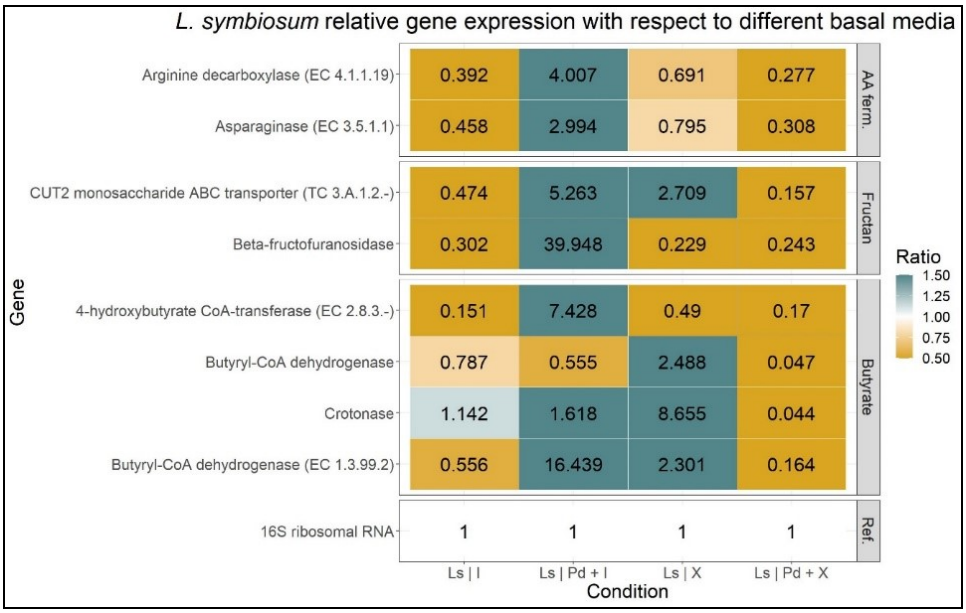


Figure 3-17. Relative expression of genes of interest of *L. symbiosum*. Each column is one culture condition, and the rows represent the genes measured. Right labels indicate the pathways at which belongs each gene, while the number and color represent the ratio of expression calculated with equation 1. Ratios over 1.5 and under 0.5 are colored as the limits of the ratio's legend. "Ls | I" = *L. symbiosum* in mZMB with inulin, "Ls | Pd + I" = co-culture of *L. symbiosum* with *P. dorei* in mZMB with inulin, "Ls | X" = *L. symbiosum* in mZMB with xylan, "Ls | Pd + X" = co-culture of *L. symbiosum* with *P. dorei* in mZMB with xylan.

4. DISCUSSION

Here we studied the metabolic interactions between two gut commensal microorganisms, *P. dorei* and *L. symbiosum*. Results obtained in this work correlated well with predictions from GSMs (unpublished results), which showed a cross-feeding of lactate and succinate. Both bacteria showed substantial changes in growth, substrate consumption, gene expression and SCFAs production with respect to their co-culture or inulin/xylan utilization.

4.1 Monocultures in unidirectional and bidirectional assays

Uni and bidirectional assays were useful to preliminary determine which carbon sources or metabolites enhanced growth of the microorganisms. Monocultures in inulin and xylan showed the same pattern of growth for both bacteria in the unidirectional and bidirectional study, which strengthen the results obtained by these assays.

Monocultures of *L. symbiosum* showed no apparent benefit of none of the substrates used with respect to the growth in mZMB alone, except for lactose and fructose. Carbohydrate consumption by *L. symbiosum* partially disagreed with the study of Kaneuchi et al. (1976) (Appendix H), as our strain consumed lactose but did not consume xylose. The little effect of lactate and succinate as sole carbon source on *L. symbiosum*'s growth could be explained by the need of a continuous supply of these metabolites supporting its growth. In addition, there could be other metabolites beyond lactate and succinate that support the beneficial effect of *P. dorei* on *L. symbiosum*.

In culture tissue plate inserts, *P. dorei* had a smaller average growth than *L. symbiosum*, even when this bacterium tends to grow faster in tubes. This could be due to dilution differences, as *P. dorei* was always cultured in 1 mL in the lower well and *L. symbiosum* in 250 µL in the upper compartment.

4.2 Differences between unidirectional and bidirectional assays

When studying the growth in co-culture with respect to monoculture, the results obtained in both set-ups were different. The bidirectional assay showed a beneficial interaction from *P. dorei* to *L. symbiosum*, while the unidirectional assay showed no apparent benefit. This could be due to the experimental differences between these assays, which probably represent different biological processes. Unidirectional assays provide all metabolites in a spent media to another microorganism, which could contain a plethora of metabolites of interest but could also be inhibitory. Other consideration is that the supernatant was mixed with new mZMB with no carbon source (1:1 V/V) before inoculation, which was done to warranty the presence of essential compounds. However, this also meant that the supernatant was diluted, so the metabolic profile and possible exchange metabolites were less available that in the original supernatant. Finally, as the supernatant was centrifuged and filtered to avoid crossed contamination, some metabolites could get lost in the process. All these details of the experimental set-up affect in the result obtained and must be taken in consideration parallel to the microorganism's biology.

Bidirectional assays instead provide a platform where both microorganisms grow and interact at the same time, which is more realistic and closer to their natural environment. It showed that these microorganisms engage in an interaction of commensalism, where *L. symbiosum* obtains a benefit in its fitness from *P. dorei* metabolites, but the latter is rather unaffected. As *P. dorei* is known for being able to consume complex carbohydrates, it acted as primary degrader of the dietary fiber, releasing monosaccharides and metabolites that could be used by *L. symbiosum*.

The plates used in bidirectional assays provide a separation between bacteria that allows for multiple metabolites exchange to reach chemical equilibrium. This set-up inherently modified the volumes of the wells after 48 hours of culture, so chemical concentrations equate in both sides of the membrane. This would affect OD measurements under these conditions, since wells with smaller volumes could have higher ODs due to concentration.

4.3 Correlations between growth, metabolism, and gene expression

We observed that *P. dorei* was able to vigorously utilize inulin and xylan, supporting the growth of *L. symbiosum* in co-culture. As *L. symbiosum* cannot break up inulin but rather consumes the smaller chains in the medium, results here show it benefits from fructose produced by *P. dorei* crossing through the membrane. This result was supported by the qPCR overexpression of beta-fructofuranosidases in both species co-culture. In contrast, *L. symbiosum* has not the machinery for xylan nor xylose fermentation, compared to *P. dorei* which released extensive xylan-degradation products.

Interestingly, co-culturing both microorganisms in both substrates apparently resulted in an accelerated substrate consumption. One explanation can be the competition for resources between both bacteria that enhances the consumption pathways at which each bacterium is efficient. For example, as *L. symbiosum* consumes proteins, *P. dorei* must enhance the consumption of the metabolites or substrates at which its more efficient to utilize, as complex carbohydrates. The higher degradation of complex carbohydrates leaves smaller chains, which can be consumed by *P. dorei* and *L. symbiosum* or by *P. dorei* alone, leading to a faster depletion of the carbon source.

While other studies have provided examples of increased substrate consumption in co-culture, here we show these interactions are contact-independent, indicating they rely exclusively on metabolic exchange.

Xylan TLC plates showed no degradation products in co-culture, even when the bidirectional assay shows a similar growth of *P. dorei* with respect to monoculture. As said before, this could indicate that xylan was not degraded in the co-culture or, in contrast, was completely depleted by enhanced activity of both microorganisms. However, unidirectional monoculture showed that *L. symbiosum* did not grow better in xylan or xylose with respect to mZMB alone, so it probably cannot use this carbon source. As *P. dorei* did not grew more in xylan, it was unlikely that this bacterium depleted the carbon source, especially since it is a complex fiber. However, gene expression showed an overexpression in xylan degrading enzymes in co-culture which supports the hypothesis of a greater consumption. Then, it is necessary to quantify total

carbohydrates in the supernatant in order to elucidate if xylan was consumed or not in this condition.

The metabolic profile was dependent of the different conditions, where the best example is propionate production in mZMB and mZMB with xylan. Several *Bacteroides* species have been reported to produce propionate while degrading proteins (Louis & Flint, 2017, Morrison & Preston, 2016). Then, the decrease in propionate production when there is carbon source could be due to a preference in carbohydrate consumption over amino acids degradation.

Propionate is a beneficial metabolite for the host, contributing to gluconeogenesis, appetite regulation, cholesterol reduction and lipids metabolism (Morrison & Preston, 2016). Propionate-producing consortia have also shown to support the mitochondrial membrane potential after disruption with clindamycin (El Hage et al., 2019). Then, its production in xylan presents an advantage over inulin co-culture.

Finally, there were some aspects at which this methodology was unconclusive. For example, gene expression of amino acids' degradation by *L. symbiosum* gets overexpressed in inulin co-culture and repressed in the other conditions. A repression in amino acids consumption is strange because *L. symbiosum* should consume them in all conditions, especially in xylan as it cannot consume the carbon source. As only two genes were considered in this analysis, it was probably not representative of all the amino acids degradation. Then, it is necessary to extend the variety of genes measured to include the pathways of degradation of other amino acids and quantify total amino acids that remain in the supernatants.

4.4 Consequences of SCFAs production and cross-feeding interactions

HPLC results of lactate and succinate suggested a possible cross-feeding interaction from *P. dorei* to *L. symbiosum* in xylan. Previous studies have documented that some species from *Lachnospiraceae* can consume lactate and acetate to produce butyrate (Louis & Flint, 2017).

Another finding of interest is the different metabolic profiles obtained after inulin or xylan fermentation by *P. dorei* in co-cultures (Figure 3-15). In accordance with previous studies (Appendix E), inulin fermentation resulted in higher concentrations of lactate, but xylan utilization showed an enrichment in acetate and succinate. Acetate production and exchange has been studied in xylanolytic and butyrate producing bacteria (Chassard & Bernalier-Donadille, 2006), mainly as a degradation product of xylan. As both of our microorganisms showed acetate production in monoculture, it can be hypothesized that none of them consumes it under these conditions.

Butyrate production was constant within these conditions, showing a certain independence of the carbon source used and of *P. dorei*'s presence. These results are consistent with unpublished studies of our laboratory but differs from the study of Thomson et al. (2018), where butyrate is produced in higher amounts in xylan than in inulin (Appendix E). *L. symbiosum*'s genome have the 4-aminobutyrate/succinate, glutamate and acetyl-Coa pathways for butyrate production (Vital, Howe & Tiedje, 2014), which gives flexibility to produce butyrate from different carbohydrates, amino acids and metabolites (Appendix I).

As indicated before, propionate was solely produced after xylan fermentation, and this observation was validated with gene expression of *P. dorei* Propionyl CoA carboxyases. These metabolic differences are possibly explained by the chemical nature of both fibers (fructose vs xylose), which are metabolized by different microbial pathways. Therefore, inulin and xylan differentially support cross-feeding interactions that could influence different bacterial populations in the microbiota.

5. CONCLUSIONS

The human gut microbiota is an important community that affects the host in multiple ways. Its interactions with the host are explained in part by the production of metabolites derived from the diet, which play essential roles in the metabolism, immune homeostasis, and physiology. Then, the study of bacterial production of beneficial metabolites is important to understand the inner workings of this community and to develop new approaches for promoting its stability.

In this study we focused on the interaction between two commensal bacteria of the gut microbiota, *P. dorei* and *L. symbiosum*. These bacteria presented a commensalism interaction where *L. symbiosum* obtained a benefit from different metabolites released by *P. dorei*. Both bacteria increased their metabolism in presence of the other, probably due to interactions of competition for resources and cooperation by the production of useful byproducts. Interdependency was more evident in inulin, where both microorganisms partially used inulin fractions. While this is only one of thousands of potential interactions, this data is one example of how dietary polysaccharides influence microbial interactions resulting in different abundances and metabolic profiles. These data could be useful in modeling approaches such as GSMs to improve their predictive capabilities, as well as in the development of interventions aiming to increase butyrate levels in subjects undergoing microbiome dysbiosis.

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APPENDIX

APPENDIX A: SECONDARY CONDITIONS TESTED IN THE BIDIRECTIONAL ASSAY

Table A-1: Conditions tested in the bidirectional assay where the carbon source is always in the upper insert. "-" represents conditions that does not use an apical well.

Carbon source	Type of culture	Upper well (250 µL)	Lower well (1 mL)
Inulin	Monoculture	*L. symbiosum* in mZMB with inulin	mZMB
	Monoculture	mZMB with inulin	*P. dorei* in mZMB
	Co-culture	*L. symbiosum* in mZMB with inulin	*P. dorei* in mZMB
Xylan	Monoculture	*L. symbiosum* in mZMB with xylan	mZMB
	Monoculture	mZMB with xylan	*P. dorei* in mZMB
	Co-culture	*L. symbiosum* in mZMB with xylan	*P. dorei* in mZMB
None	Monoculture	-	*L. symbiosum* in RCM
	Monoculture	-	*P. dorei* in RCM
	(-) Control	-	RCM

APPENDIX B: PRIMER DESIGN FOR GENES IN QPCR

Table B-1: General information of the primers designed for fold change measurement by qPCR.

Bacteria	Direction	Product	Pathway	Tm(c)	Sequence	Gene Size (bp)
Ls	F	arginine decarboxylase (EC 4.1.1.19)	AA Fermentation	60.5	ATCCCCCTTGGTATGAGGCT	185
	R			60.5	TAGAAGTGGGTTCCGTGTGC	
	F	asparaginase (EC 3.5.1.1)	AA Fermentation	58.4	CGCTTGGCTTTGAGGCTTTT	167
	R			60.5	ACTGTCTTTCAGGACAGCCG	
	F	4-hydroxybutyrate CoA-transferase (EC 2.8.3.-)	Butyrate pathway	60.5	TCACCCAAATCAGCGGAGTC	196
	R			60.5	CCGTATTCCGTAACCGCGTA	
	F	butyryl-CoA dehydrogenase	Butyrate pathway	58.4	TAGGCGCTTTCGGCTTAACA	248
	R			60.5	TGTTCCGAAGGTGAATCCCG	
	F	Crotonase	Butyrate pathway	60.5	GGAAGTTGGCCTGGGAATCA	213
	R			60.5	GGAGCATTCTTCGCAATGCC	
	F	butyryl-CoA dehydrogenase (EC 1.3.99.2)	Butyrate pathway	62.5	CTCATCGGACTTCCCTACGC	154
	R			60.5	CATTGGCAATACTTCCGCCG	
	F	monosaccharide ABC transporter substrate-binding protein, CUT2 family (TC 3.A.1.2.-)	Fructan Metabolism	58.4	AATGGCATTCACGATGCTGG	160
	R			60.5	CTTGTAAGATGCCGCTTCGG	
	F	beta-fructofuranosidase	Fructan Metabolism	59.4	ATGTTCACTTCCAGCAGACGA	278
	R			61.3	CGGCTGGTAAATCGGTCCAAA	
	F	16S ribosomal RNA	Reference Gene	58.4	AAAGAGAAGCAAGACCGCGA	274
	R			62.5	CACCTTCCGATACGGCTACC	
Bd	F	2,6-beta-D-fructofuranosidase	Inulin	60.5	GGCGGCTGCATTGAGTTATG	223
	R			60.5	AGGGTCCAGCTCTATCAGCA	
	F	levanase	Inulin	60.5	ATGAGCCCGAACAGAAGTGG	274
	R			60.5	CGATGTCCGTCAAAATCGCC	
	F	propionyl-CoA carboxylase subunit beta	SCFA	58.4	GTTTCGCACGTTTCAACGGA	234
	R			60.5	CGCCGTAAGCATACAGCAAC	
	F	butyrate kinase (EC 2.7.2.7)	SCFA	62.5	CGATCCCGAAGTGGTAGACG	174
	R			60.5	CCTCCCAGATGCACCACAAT	
	F	fumarate reductase transmembrane cytochrome b subunit	SCFA	58.4	TCTGACGTTTCACATGGCGA	206
	R			60.5	CGTTCACTGCATAGCGTTCG	
	F	acetate kinase (EC 2.7.2.1)	SCFA	60.5	CCGGCTAACCTGAAAGGTGT	227
	R			58.4	TTCGGATCAACGCCAAGGAA	
	F	propionyl-CoA carboxylase subunit beta	SCFA	60.5	CGAGAAACAACACGCCCAAG	272
	R			60.5	CCAGTTGTTGCACCTTCACG	
	F	glycoside hydrolase family 10 protein	Xylan	60.5	CATTGCCGGATTAATGGCCG	266
	R			60.5	CAAAACGAACCAACCGGTCC	
	F	endo-1,4-beta-xylanase D	Xylan	56.4	ATGCGGCAGCGAATCAAAAA	241
	R			60.5	TTGAAAGGTCCTTCTGGGCG	
	F	glycosyl hydrolase family 43 protein	Xylan	60.5	CAATGCCTTCCCTGGGTTCT	222
	R			58.4	TTGCAGGTAGCCAAACCGAT	
	F	16S ribosomal RNA	Reference	60.5	AGGATGACTGCCCTATGGGT	288
	R			58.4	AGCATTTCACCGCTACACCA	

APPENDIX C: ABSORBANCE VALUES OF THE MEDIUMS WITHOUT BACTERIA OF UNIDIRECTIONAL AND BIDIRECTIONAL ASSAYS

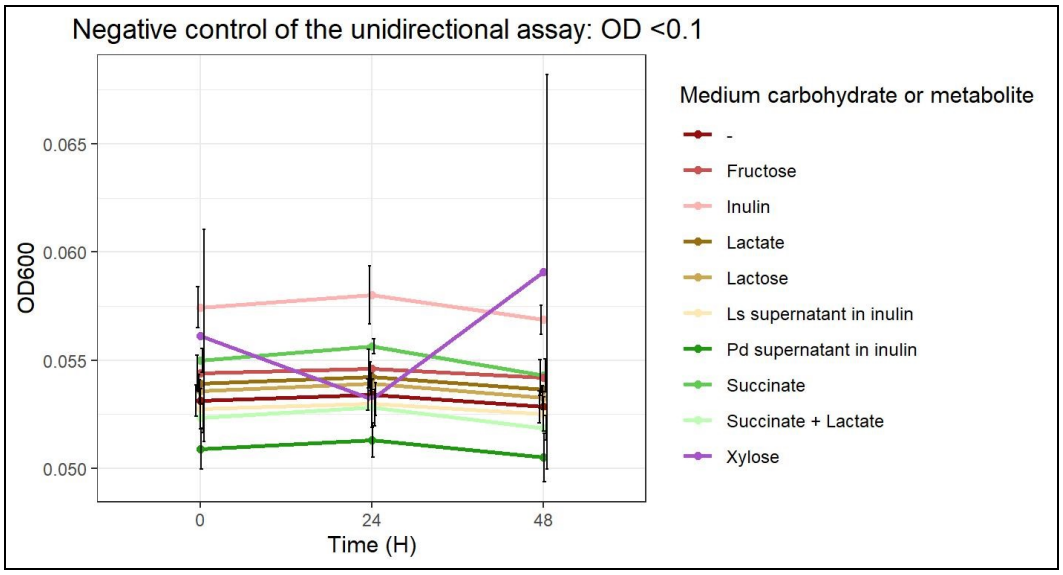

Figure C-1: Absorbance of the mediums used in the unidirectional assay which OD600 were below 0.1. Error bars indicate standard deviation. "-" = mZMB without carbon source, "Ls" = *L. symbiosum*, "Pd" = *P. dorei*.

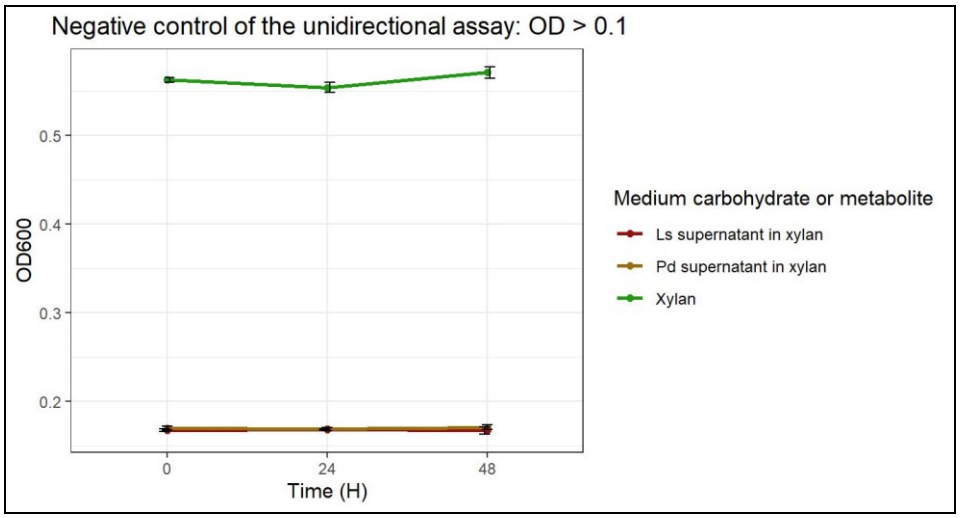

Figure C-2: Absorbance of the mediums used in the unidirectional assay which OD600 were above 0.1. Error bars indicate standard deviation. “Ls” = *L. symbiosum*, “Pd” = *P. dorei*.

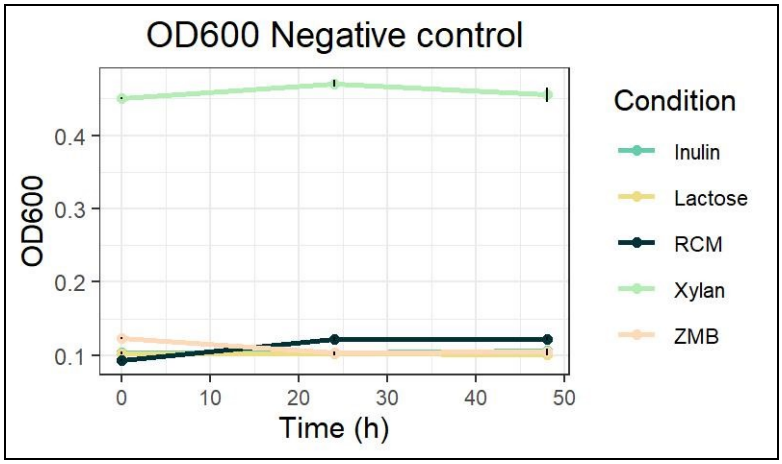

Figure C-3: Absorbance of the mediums used in the bidirectional assay. Error bars indicate standard deviation.

The first observation is that xylan intervenes in the absorbance measurement, while inulin had the same basal absorption than mZMB with no carbon source (Figures A-2.2 and A-2.3). This effect was reduced by subtracting the OD600 of each medium to each condition that had the same carbon source. However, this represents a source of error in the case of bidirectional cultures in xylan because it does not consider the transfer of xylan from one compartment to the other nor the consumption by *P. dorei*. These two actions decrease the interference of xylan in the OD600 of the well, so the subtraction of mZMB with 2% xylan can cause an underestimation of the bacterial growth.

APPENDIX D. GROWTH CURVES COMPARING CARBOHYDRATE POSITION ACROSS THE MEMBRANE IN THE BIDIRECTIONAL ASSAY

To evaluate if the carbon source could cross through the membrane, we cultured both microorganisms with the carbon source in the same and in the opposite compartment. *L. symbiosum* had an important decrease when the carbohydrate was in its compartment compared to when it was in the other well (Figure C-2), indicating inhibition by substrate. *P. dorei* tended to grow slightly more when the carbohydrate was in its well (Figure C-1), but in both cases grew to a higher OD that the basal in mZMB. This suggested that the carbohydrate can pass through the membrane, which was later confirmed with TLC.

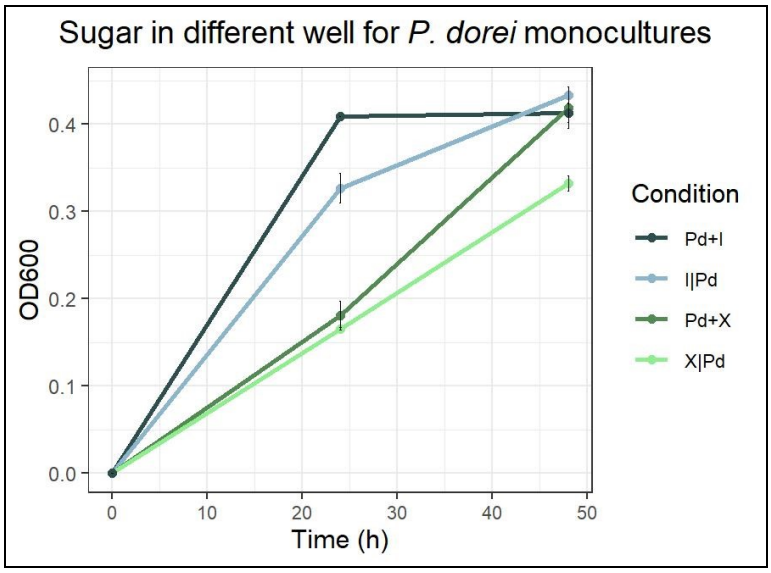

Figure D-1: Absorbance of the monocultures of *P. dorei* when inulin and xylan are in the apical or in the lower well. Error bars indicate standard deviation. "I" = mZMB with inulin, "X" = mZMB with xylan, "Pd" = *P. dorei*.

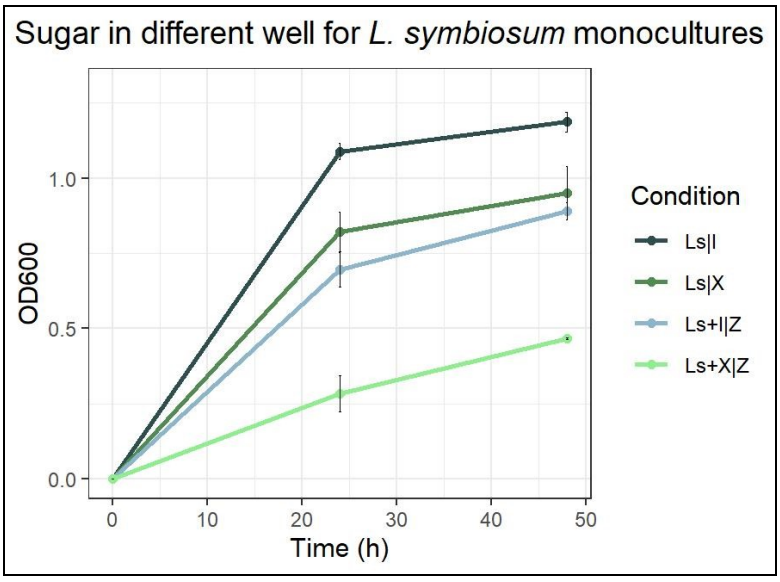

Figure D-2: Absorbance of the monocultures of *L. symbiosum* when inulin and xylan are in the apical or in the lower well. Error bars indicate standard deviation. "I" = mZMB with inulin, "X" = mZMB with xylan, "Ls" = *L. symbiosum*.

APPENDIX E: SCFA PRODUCTION IN CO-CULTURES OF *P. DOREI* AND *L. SYMBIOSUM* IN XYLAN AND INULIN

Table E-1: Production of acetate, lactate, propionate and butyrate in co-cultures of *P. dorei* and *L. symbiosum* reported in bibliography. All samples are from co-cultures in bioreactors in mZMB with xylan or inulin, measured with HPLC as in this study.

Carbon Source	Time (h)	Acetate (mM)	Lactate (mM)	Propionate (mM)	Butyrate (mM)	Source
Xylan	-	35.8	17.7	0.5	20.5	Unpublished
Xylan	-	55.6	50.1	5.5	15.6	Unpublished
Xylan	24	~ 30	~ 17	-	~ 105	Thomson et al., 2018
Xylan	48	~ 25	~ 3	-	~ 115	Thomson et al., 2018
Inulin	-	48	17.4	1.1	28.2	Unpublished
Inulin	-	50.1	62.5	0	3.8	Unpublished
Inulin	24	~ 22	~ 20	-	~ 78	Thomson et al., 2018
Inulin	48	~20	~ 15	-	~ 65	Thomson et al., 2018

Table E-2: Production of SCFA by *L. symbiosum* experimentally observed in PYFG medium in the study of Kaneuchi et al., (1976). "++" = major product, (+) = moderate product, "-" = not produced.

Metabolite	Production in PYFG medium
Acetic acid	++
Propionic acid	-
Butyric acid	++
Lactic acid	(+)
Succinic acid	-

APPENDIX F: BRIEF EXPLANATION OF THE FUNCTION OF GENES MEASURED BY QPCR.

Table F-1: Genes function in *P. dorei* metabolism. Source: KEGG PATHWAY database.

Gene	Function	E.C. number
2,6-beta-D-fructofuranosidase	Inulin and levan degradation to fructose.	3.2.1.80
Levanase	Hydrolysis of fructans at their beta -2,1 (inulin) and beta -2,6 (levan) bonds.	3.2.1.80
Glycoside hydrolase family 10 protein	Endo-xylanase function. Hydrolyze beta-1,4 bonds.	3.2.1.8
Endo-1,4-beta-xylanase D	Endo-xylanase function. Hydrolyze beta-1,4 bonds.	3.2.1.8
glycosyl hydrolase family 43 protein	Endo-xylanase function. Hydrolyze beta-1,4 bonds.	3.2.1.8
(1) Propionyl-CoA carboxylase subunit beta	Reversible enzyme of the propionate degradation pathway. Converts Propionyl-CoA to (S)-2-Methyl-Malonyl-CoA.	6.4.1.3
(2) Propionyl-CoA carboxylase subunit beta	Reversible enzyme of the propionate degradation pathway. Converts Propionyl-CoA to (S)-2-Methyl-Malonyl-CoA.	6.4.1.3
Butyrate kinase	Reversible enzyme of the butyrate production pathway. Converts Butanoate to Butanoyl-P	2.7.2.7
Fumarate reductase transmembrane cytochrome b subunit	Reversible enzyme of the Krebs cycle that converts fumarate to succinate.	1.3.5.4/ 1.3.5.1
Acetate kinase	Reversible enzyme that phosphorylates acetate	2.7.2.1

	to Acetyl-P, which then is converted to pyruvate.	
16S ribosomal RNA	Reference.	

Table F-2: Genes function in *L. symbiosum* metabolism. Source: KEGG PATHWAY database.

Gene	Function	E.C. number
Arginine decarboxylase	Aminoacids metabolism. Converts Arginine to Agmatine	4.1.1.19
Asparaginase	Aminoacids metabolism. Converts L-Asparagine to L-Aspartate	3.5.1.1
CUT2 monosaccharide ABC transporter	Ribose and D-xylose tranporter RbsB subunit.	TC 3.A.1.2.-
Beta-fructofuranosidase	Starch metabolism. Converts sucrose to Beta-D-fructose	3.2.1.26
4-hydroxybutyrate CoA-transferase	Reversible enzyme of the butyrate production pathway. Converts 4-Hydorxy-butanoate to 4-Hydroxy-butanoyl-coA	2.8.3.-
Butyryl-CoA dehydrogenase	Enzyme of the butyrate production pathway. Converts Butanoyl-CoA to Crotonoyl-CoA	1.3.8.1
Crotonase	Reversible enzyme of the butyrate production pathway. Converts (S)-3-Hydroxybutanoyl-CoA to Crotonoyl-CoA.	4.2.1.17
Butyryl-CoA dehydrogenase	Enzyme of the butyrate production pathway. Converts Butanoyl-CoA to Crotonoyl-CoA	1.3.99.2
16S ribosomal RNA	Reference.	

APPENDIX G: FOLD CHANGE EXPRESSION OF THE GENES WITH RESPECT TO MONOCULTURE IN MZMB.

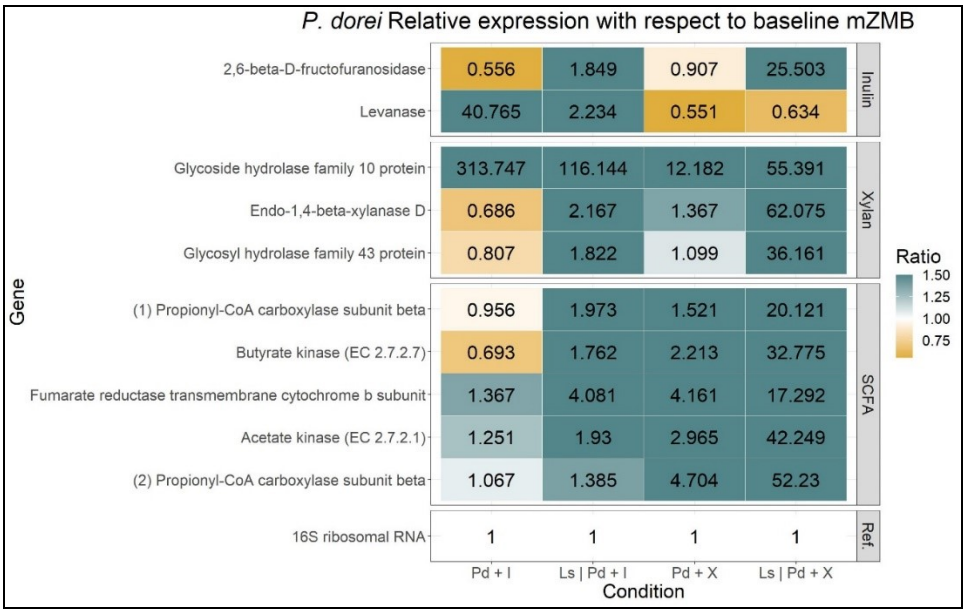

Figure G-1: Relative expression of genes of interest of *P. dorei* with respect to the expression in mZMB monoculture. Each column is one culture condition, and the rows represent the genes measured. Right label indicates the pathways at which belongs each gene, while the number and color represent the ratio of expression calculated with equation 1. Ratios over 1.5 and under 0.5 are colored as the limits of the ratio's legend. "Pd + I" = *P. dorei* in mZMB with inulin, "Ls | Pd + I" = co-culture of *L. symbiosum* with *P. dorei* in mZMB with inulin, "Pd + X" = *P. dorei* in mZMB with xylan, "Ls | Pd + X" = co-culture of *L. symbiosum* with *P. dorei* in mZMB with xylan.

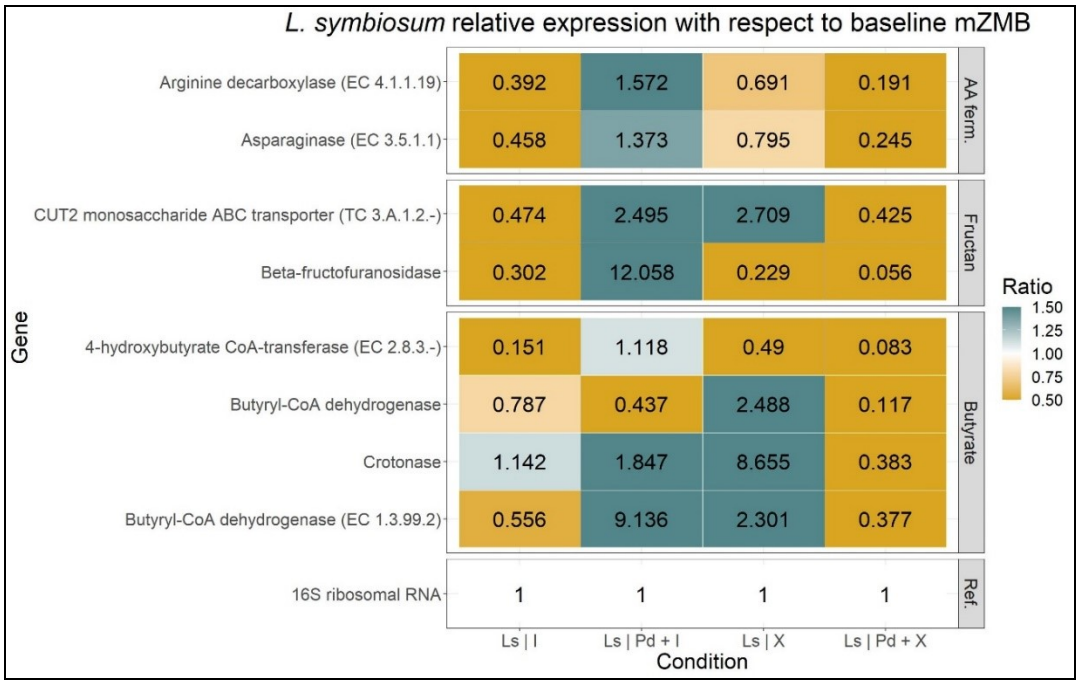

Figure G-2: Relative expression of genes of interest of *L. symbiosum* with respect to the expression in mZMB monoculture. Each column is one culture condition, and the rows represent the genes measured. Right label indicates the pathways at which belongs each gene, while the number and color represent the ratio of expression calculated with equation 1. Ratios over 1.5 and under 0.5 are colored as the limits of the ratio's legend. "Ls | I" = *L. symbiosum* in mZMB with inulin, "Ls | Pd + I" = co-culture of *L. symbiosum* with *P. dorei* in mZMB with inulin, "Ls | X" = *L. symbiosum* in mZMB with xylan, "Ls | Pd + X" = co-culture of *L. symbiosum* with *P. dorei* in mZMB with xylan.

APPENDIX H: SUBSTRATE CONSUMPTION OF *L. SYMBIOSUM* OBSERVED IN PREVIOUS STUDIES

Table H-1: Consumption of carbohydrates by *L. symbiosum* experimentally observed in the study of Kaneuchi et al., (1976). "+" = consumed, "-" = not consumed

Compound	Consumption
Arabinose	+
Xylose	+
Ribose	-
Glucose	+
Fructose	+
Lactose	-
Inulin	-

APPENDIX I: BUTYRATE PRODUCTION PATHWAYS BY INTESTINAL BACTERIA

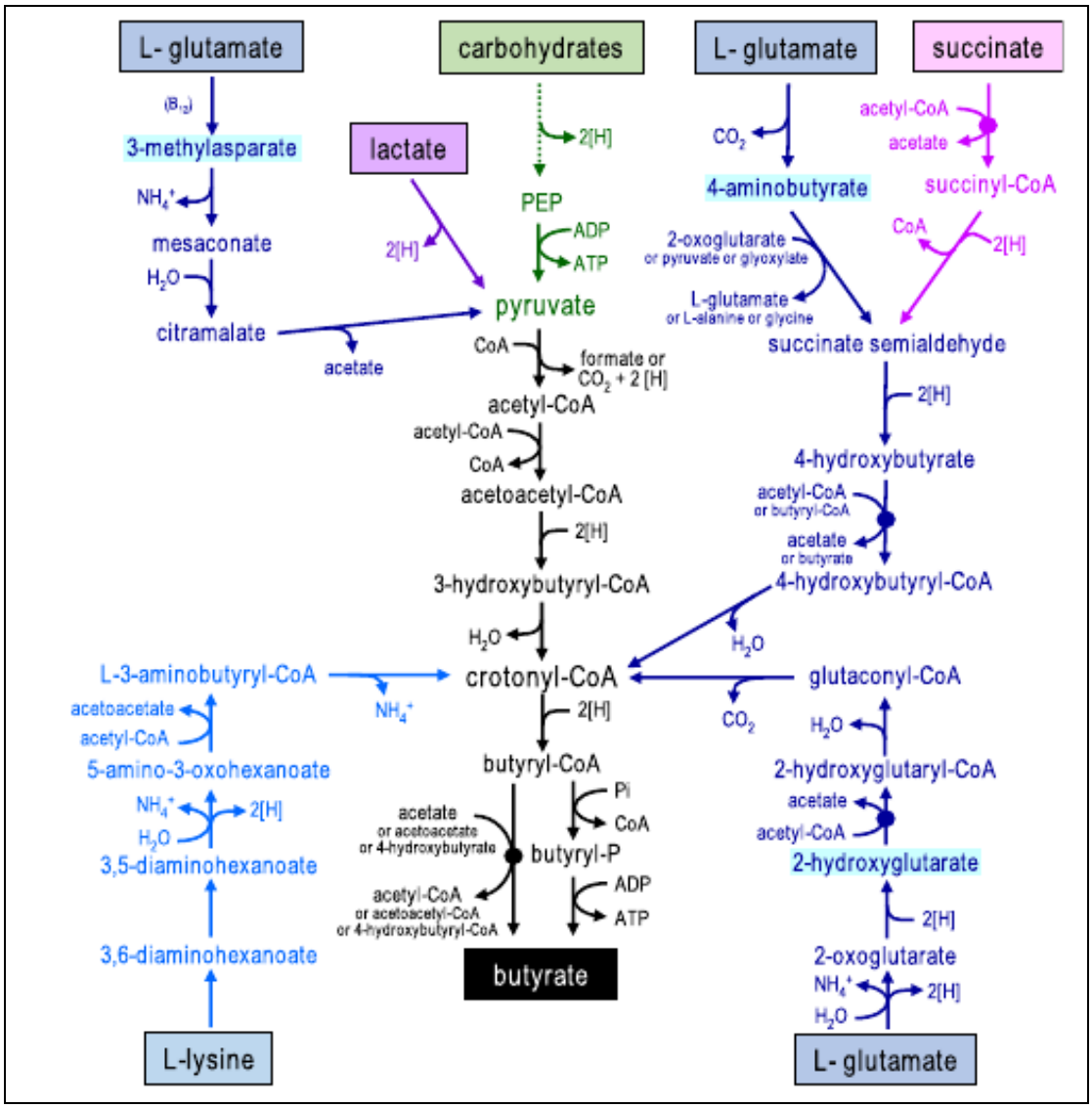

Figure I-1: All pathways know for butyrate production by intestinal bacteria. Each pathway is represented by a color. Source: Louis & Flint, 2017.