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## Lignocellulose-degrading microbial consortia

Cortes Tolalpa, Larisa

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# Chapter 7

## *General discussion*

Larisa Cortés Tolalpa

## ***The selection of lignocellulose biomass (LCB)-degrading microbial consortia and synergism***

Decomposing microbial communities are capable of degrading almost every compound on Earth, from natural compounds such as lignocellulose (Lynd et al. 2002) to persistent organic pollutants, such as polychlorinated biphenyl and polycyclic aromatic hydrocarbons (Edwards and Kjellerup 2013). One of the forms in which microbial communities are used for practical purposes is through of specialized microbial consortia, which are capable to efficiently perform specific tasks.

The selection of conditions is crucial for obtaining a stable, functional and efficient consortium. The goal of my thesis was to assess the biodegradation of lignocellulosic plant waste. The use of such waste is still in its infancy, as the main impediment for its application is its recalcitrant nature due to its heterogeneous and complex composition (Himmel et al. 2007). My focus was on the structuring of microbial consortia and the underlying driving forces, after which I moved on to study the potential synergisms that occur in such consortia.

### ***Why focus on salt-tolerant consortia?***

The mandatory use of pretreatments, which help to open the closed structure of lignocellulose, is at the origin of the formation of salts. Currently, some of the most popular pretreatments are the application of acid or alkaline solutions (Talebnia et al. 2010) which are followed by a necessary neutralization step. During the latter step, a considerable amount of salt is formed before moving on to the crucial fermentation processes. To avoid inhibition of these , the salt has to be removed, which incurs technological and financial efforts (Sun et al. 2016). Focusing on these issues, I selected microbial consortia capable to degrade lignocellulose under saline conditions.

### ***Importance of the substrate***

Previous studies have shown that different types of substrates resulted in different enzymatic responses and modification in the community structure of degrading bacteria consortia (Irwin et al. 2003). In **Chapter 2**, I addressed the following question: What would be the influence of different lignocellulose sources, on the selection of degrader consortia from the same source and on their

degradation capacity. I selected the degrader consortia using a batch sequential enrichment method by applying forest soil as an inoculum and wheat straw (WS), switch grass (SG) and maize (MZ) as carbon sources. It was hypothesized that, due to the overall similarities in composition of the substrates, the final selected consortia would have the same microbial composition. Differently from what was expected, the final selected consortia presented different (bacterial and fungal) community compositions. Diverse bacterial and fungal species were found depending on the substrate type. Despite the differences, I also found similarities between the final consortia. That is, the bacterial species *Sphingobacterium kitahiroshimense*, *Enterobacter amnigenus*, *Raoultella terrigena*, *Pseudomonas putida* and *Stenotrophomonas rhizophila* and the fungal strains *Coniochaeta ligniaria* and *Acremonium* sp. were isolated from all treatments. These organisms may be considered to constitute “generalist degraders”, capable of consuming LCB from diverse plant wastes. In terms of functionality, the consortia consumed differently the components of the substrates. Considering the degradation of the three main components of LCB (cellulose, hemicellulose and lignin), the consortia from SG were the most efficient, followed by the MZ-derived, WS-derived and WS-derived consortia at pH 9. According to the complete data set, I inferred that the consortia were strongly influenced by the substrate type, followed by the pH. The differences in community composition and degradation capability of the selected consortia cannot be explained in terms of the compositional ratio between the substrates (WS, SG and MZ) because they were roughly similar. However, the results may be explained by the differences in the sugar composition and the interconnection between the moieties that form the substrate. It is possible, therefore, that these factors are the main drivers of the consortial structures. In accordance with the present results, other studies have also demonstrated that the composition of biomass substrates not only influences the community composition of biomass-deconstructing bacterial consortia (Gladden et al. 2012; Poszytek et al. 2017) but also the glycoside hydrolase activities (Irwin et al. 2003).

### ***Importance of the inoculum***

Wheat straw is a key waste lignocellulose substrate in the world (Saleem Khan and Mubeen 2012; Patni et al. 2013). In **Chapter 3**, I addressed the following question; how may the inoculum source influence the composition and the functioning of the selected degrading consortia? In particular, I speculated that due to microbial functional redundancy, the selected consortia might be highly diverse. As an alternative, I hypothesized that, given that “Everything is everywhere”, quite similar consortia might emerge when using inocula coming from different sources.

I deliberately selected three divergent habitats namely forest soil, decaying wood and canal sediment. After the sequential enrichment using wheat straw as a sole carbon source, the data clearly indicated that the three microbial sources yielded phylogenetically-different but functionally-similar enriched consortia. Final consortia were different in terms of microbial composition, where the sediment-derived consortia appeared to be the most different compared with consortia derived from soil and wood, based on sequencing data analysis. Analysis of the most enriched bacterial members showed that *Sphingobacterium multivorum* and *Acinetobacter* sp. were present in all consortia. *Citrobacter freundii*, *Flavobacterium* sp. and *Asticcacaulis benevestitus* were shared between the soil- and wood-derived consortia, *Chryseobacterium* sp. and *Pseudomonas* sp. were shared between the wood- and sediment-derived consortia and finally *Klebsiella variicola* was shared between the consortia derived from soil and sediment (Table 1). Interestingly, the three consortia consumed the components of the substrate to a similar extent. However, when the consortia were analysed at the enzymatic level, they differed. Each set of consortia clearly presented specific enzymatic profiles, resulting from the different sets of organisms and potentially different secreted enzymes working on the substrate. In general, the wood- and soil-derived consortia had higher enzymatic activities than the sediment-derived ones. However, the soil-derived consortia presented the highest  $\beta$ -xylosidases activities, whereas wood-derived ones exhibited higher glucosidase activities. Both had similar  $\beta$ -cellobiohydrolase,  $\beta$ -galactosidase and  $\beta$ -mannosidase activities. The inoculum source apparently influenced the stability of final consortia more than the resultant activity. Inocula from soil and wood incited consortia adapted better to the experimental setting and both consortia reached stability faster than sediment-derived ones, as revealed by moving window analysis. These results may be explained by the fact that samples of decaying tree and forest soil, used as inocula, were mostly aerobic, whereas the sediment sample came from a largely anoxic environment. Therefore, the conditions used in the enrichment process resembled the original ones of wood and soil-derived consortia. These results are supported by data from Poszytek et al. (2017) who observed that, in the initial enrichment step, more adapted inoculum significantly influence the adaptation of microbial community structure of maize silage. Also, they indicated that the selection process caused changes in the bacterial population by substrate input, whereas the sample origin was relatively unimportant. In turn, de Vrieze et al. (2015) also showed the importance of selection of appropriate inoculum in obtaining efficient consortia for the establishment of a long term stable degradation process.

## *Halotolerant consortia*

In accordance with previous studies from our group (Jimenez et al. 2016), I examined the development of specialized microbial consortia capable of reaching the most recalcitrant part of LCB substrate. In addition, I added a high-salt condition to the system in order to mimic industrial conditions following acid or alkaline pretreatment and neutralization (Mathabatha 2010; Yu et al. 2016). In **Chapter 4**, I hypothesized that a soil adapted to high salinity, used as the inoculum source, would yield efficient LCB-degrader consortia, capable to work under saline conditions. The inoculum from salt-marsh soil indeed yielded unique enriched consortia that efficiently degraded wheat straw under high salinity, with the consortia consuming preferably the hemicellulose part of the substrate. The resultant consortia were very different from those found in previous enrichments described in **Chapters 2** and **3**. Only the bacterial species *Pseudomonas putida* and *Flavobacterium beibuense*, highly abundant, were shared with previous consortia.

Apparently, the presence of salt in the system enhanced the prevalence of bacteria over fungi. The main bacterial species were related to those found in marine settings, i.e. many members of the *Rhodobacteraceae* (*Albirhodobacter marinus*, *Oceanicola antarcticus*), *Halomonadaceae* (*Halomonas alkaliphila* and *Halomonas meridiana*) and *Photobacterium halotolerans* from the *Vibrionaceae*. I hypothesized that the consortia would be strongly affected if a highly recalcitrant substrate would be used. Thus, in the first part of the enrichment I adapted the consortia by growing on wheat straw, whereas in the second part I used pre-digested wheat straw (recovered from previous transfers). The consortia selected on the latter substrate degraded more lignin than those selected on fresh substrate. Hence, microbial communities that were selected exclusively on fresh substrate may not efficiently reach the recalcitrant part of the substrate. Furthermore, in the resultant consortia, bacteria dominated the degradation of highly recalcitrant substrate, as only bacterial species were enriched. The most abundant degraders were associated with the *Flavobacteriaceae*, with the species *Joostella marina* and *Flavobacterium beibuense*, followed by *Algoriphagus ratkowskyi*, *Pseudomonas sabulinigri* and *Halomonas meridiana*; fungal communities were severely affected as they decreased in density. Moreover, it was impossible to recover fungal isolates from the second part of the enrichment, being *Sarocladium strictum* the only strain recovered from fresh substrate.

The scarcity of fungi in the consortia could be explained by the experimental conditions used: 1) Longer duplication time than bacteria, 2) Stronger nutritional demand (depletion of nitrogen) (Meidute et al. 2008) and 3) Sub-optimal pH for fungal growth (Matthies et al. 1997).

The halotolerant consortia had phylogenetic compositions that were different from the other selected consortia. For example, typical marine families, i.e. *Rhodobacteraceae*, *Erythrobacteraceae* or *Microbacteriaceae* appeared as abundant in the halotolerant consortia. These findings provide support for the key relevance of selective conditions as drivers of the microbial consortia.

Remarkably, regardless of the inoculum source, substrate type or salt concentration, members of *Pseudomonadaceae* and *Flavobacteriaceae* were enriched across all consortia, specifically the species *Pseudomonas* and *Flavobacterium*. These organisms may be described as truly “generalist”, as they are probably highly adaptable to diverse conditions or environments. *Flavobacterium* species have been isolated from soil, sediment and marine/saline environments, and they are typically associated with decomposition of complex polysaccharides (Lambiase 2014). *Pseudomonas* species have been “accused” to be cheaters in the selected consortia; however, they may have a relevant role in the decomposition of recalcitrant regions of the lignocellulose substrate, as they might be able to degrade residual hemicellulose linked to lignin structures. Diverse genomic studies have shown their potential capacity for lignin degradation (Beckham et al. 2016). For instance, Ravi et al. (2017) found *Pseudomonas monteilli* and *Pseudomonas plecoglossicida* to be enriched in matured vegetal compost, being able to degrade a large amount of lignin-related compounds.

Table 1. The most abundant strains in selected microbial consortia in this study.

Source	Conditions		Species		Parameter		Reference
	Salt condition	LCB	Fungi	Bacteria	Family		
4SO	Regular	WS	<i>Coniochaeta ligniaria</i>	<i>Enterobacter amnigenus</i>	Enterobacteriaceae	Chapter 2	
			<i>Acremonium</i> sp.	<i>Klebsiella terrigena</i>			
				<i>Raoultella terrigena</i>			
4SO	Regular	WS		<i>Stenotrophomonas rhizophila</i>	Xanthomonadaceae	Chapter 3	
				<i>Pseudomonas putida</i>	Pseudomonadaceae		
				<i>Sphingobacterium</i> sp.	Sphingobacteriaceae		
				<i>Citrobacter freundii</i>	Enterobacteriaceae		
				<i>Sphingobacterium multivorum</i>	Sphingobacteriaceae		
				<i>Acinetobacter calcoaceticus</i>	Moraxellaceae		
				<i>Flavobacterium ginsengisoli</i>	Flavobacteriaceae		
4W	Regular	WS		<i>Citrobacter freundii</i>	Enterobacteriaceae	Chapter 3	
				<i>Sphingobacterium multivorum</i>			
				<i>Acinetobacter johnsonii</i>			
				<i>Flavobacterium ginsengisoli</i>			
				<i>Pseudocercospora humuli</i>			Moraxellaceae
				<i>Arthrographis kalrae</i>			
				<i>Lecythophora</i> sp.			
4SE	Regular	WS		<i>Exophiala capensis</i>	Flavobacteriaceae	Chapter 3	
				<i>Herpotrichiellaceae</i> sp.			
				<i>Rhodotorula mucilaginosa</i>			
				<i>Coniochaeta ligniaria</i>			
				<i>Penicillium citrinum</i>			
4SE	Regular	WS		<i>Plectosphaerella cucumerina</i>	Paenibacillaceae	Chapter 3	
				<i>Paenibacillus oceanisediminis</i>			
				<i>Klebsiella varicola</i>			
				<i>Aeromonas hydrophila</i>			
				<i>Sphingobacterium multivorum</i>			
	<i>Pseudomonas azotoformans</i>	Pseudomonadaceae					
	<i>Asticcacaulis benevestitus</i>		Caulobacteraceae				



<sup>a</sup> SO	Regular	MZ	<i>Coniochaeta ligniaria</i> <i>Acremonium</i> sp.	<i>*Sanguibacter inulinus</i> <i>*Comamonas jiangtzensis</i>	<i>Sanguibacteraceae</i> <i>Comamonadaceae</i>	Chapter 2
<sup>a</sup> SO	Regular	SG	<i>Coniochaeta ligniaria</i> <i>Acremonium</i> sp.	<i>*Paenibacillus xylinexedens</i> <i>*Delftia tsuruhatensis</i>	<i>Paenibacillaceae</i> <i>Comamonadaceae</i>	Chapter 2
<sup>b</sup> SSM	High	Fresh WS	<i>Sarocladium strictum</i>	<i>Albiflathobacter marinus</i> <i>Oceanicola antarcticus</i> <i>Paracoccus seriniphilus</i> <i>Pseudorhodobacter incheonensis</i> <i>Altererythrobacter indicus</i> <i>Erythrobacter gaetbuli</i> <i>Microbacterium oleivorans</i> <i>Micrococcus yunnanensis</i> <i>Arthrobacter nicotianae</i> <i>Bacillus oleronius</i> <i>Demequina aestuarii</i> <i>Halomonas alkaliphila</i> <i>Photobacterium halotolerans</i> <i>Sanguibacter inulinus</i> <i>Staphylococcus capitis</i> <i>Joostella marina</i> <i>Flavobacterium beibuense</i>	<i>Rhodobacteraceae</i>  <i>Erythrobacteraceae</i> <i>Microbacteriaceae</i> <i>Micrococcaceae</i> <i>Bacillaceae</i> <i>Demequinaceae</i> <i>Halomonadaceae</i> <i>Vibrionaceae</i> <i>Sanguibacteraceae</i> <i>Staphylococcaceae</i> <i>Flavobacteriaceae</i>	Chapter 4
<sup>b</sup> SSM	High	Pre-digested WS	None recovered	<i>Algoriphagus ratkowskyi</i> <i>Pseudomonas sabulinigri</i> <i>Halomonas meridiana</i>	<i>Cyclobacteriaceae</i> <i>Pseudomonadaceae</i> <i>Halomonadaceae</i>	Chapter 4

<sup>a</sup>The bacterial group in **SO-WS** plus the strains. **a**. Species based on isolation. **b**. Species based on sequencing. Microbial source: **SO**, forest soil; **SE**, sediment from canal; **W**, decaying tree; **SSM**, salt marsh soil. Substrate: **WS**, wheat straw; **SG**, switchgrass; **MZ**, maize also known as corn stover.

## *Synergism, microbial interactions and genomics*

Microbial interactions are inherent to the establishment of any microbial community (West et al. 2007). Previous studies have also noted the importance of interactions in the stabilization and functioning of microbial consortia (Pandhal and Noirel 2014; Jagmann and Philipp 2014; Ghosh et al. 2016; Jiménez et al. 2017). This is especially important in the development of lignocellulose degrader consortia (Zuroff and Curtis 2012). According to Deng and Wang (2016), the complexity of lignocellulose-type compounds favors synergistic relationships within degrader consortia, while reducing antagonism. Given the complexity of wheat straw, in **Chapter 5** I addressed the question whether synergistic interactions could be found between members of the selected consortia. First, I screened the most abundant bacterial and fungal isolates from the wood- and soil-derived consortia (**Chapter 3**); those were able to grow on wheat straw as a sole carbon source and did not present antagonistic interactions when co-cultured on wheat straw. In one key pair, the structure of the substrate influenced the interaction between the strains. These presented synergistic growth and synergistic enzymatic activity only when growing in co-culture on wheat straw. In detail, *Sphingobacterium multivorum* w15 and *Citrobacter freundii* so4 constituted the most synergistic minimal consortium. Co-cultures on glucose and synthetic lignocellulose (CMC, xylan and lignin; semi-recalcitrant) demonstrated that the cooperation was directly linked to the complexity of the substrate. Simply speaking, they did not exhibit synergism when growing on glucose. The interaction between *S. multivorum* w15 and *C. freundii* so4 was apparently bidirectional, as each strain presented an increase in the growth when its culture received the supernatant of the other strain grown in wheat straw, but not when the latter was grown on glucose. The results provided support for the hypothesis that synergistic interactions between the degrader strains are based on their complementation with respect to degradation and metabolism, such as caused by complementary lytic enzymes and/or diverse metabolic intermediates.

Further analysis was required to understand the synergistic relationship between the degraders *C. freundii* so4 and *S. multivorum* w15. Either species, or phylogenetically-close strains from the *Enterobacteriaceae* and *Sphingobacteriaceae*, respectively, were found to consistently be very abundant in the selected lignocellulose degrader consortia (**Chapter 2** and **Chapter 3**). Jiménez et al. (2014) had already reported similar data. Genome analyses can provide information on the functional potential of microorganisms and so the understanding of the biochemical mechanisms of plant polysaccharide degradation can be fostered (Koeck et al. 2014).

In **Chapter 6**, I proposed the use of the synergistic pair *C. freundii* so4 and *S. multivorum* w15 as a minimum model lignocellulose degradation consortium. Thus, I performed a general physiological characterization, as well as, a full description of the genomes of *C. freundii* so4 and *S. multivorum* w15. Important differences were found at the physiological and genomic levels between the synergistic strains.

Within the confines of the experiment, *S. multivorum* w15 clearly had the capacity to grow on the polysaccharides inulin, pectin, laminarin, dextrin and related compounds ( $\alpha$ -,  $\beta$ - and  $\gamma$  cyclodextrin) as single carbon sources. It also exhibited a preference of consumption of oligo-saccharides like melezitose (a trisaccharide found in honeydew) and stachyose (a tetra-saccharide found in legume seeds). This, contrary to *C. freundii* so4, which grew preferably on amino acids and carboxylic acids, where laminarin was the only polysaccharide that *C. freundii* so4 was able to use as a sole carbon source. The genomic analysis further placed a focus on LCB hydrolytic capacities. However, to be able to explain the positive relationship, it was necessary to check other differences in the metabolisms of the two organisms. In the first place, *S. multivorum* w15 appeared to be specialized in consumption and degradation of polysaccharides. At genomic level, it possessed a varied arsenal of genes encoding degradative enzymes that presumably are specialized in the degradation of complex carbohydrates. I particularly focused on the degradation of hemicelluloses, it was found 367 genes associated with carbohydrate enzyme active (CAZy) family enzymes, where 193 encoded for glycoside hydrolases (GHs) and 50 for carbon binding modules (CBMs). Remarkably, *S. multivorum* w15 possesses 22 genes encoding proteins from glycoside hydrolase family GH43. This family includes enzymes with activities of glucosidases, arabinofuranosidases, xylosidases and glucosaminidases. Moreover, it is one of the two CAZy families implicated in the degradation of arabinoxylan, the most abundant component of wheat straw (Abot et al. 2016). *S. multivorum* w15 had a low investment in fermentative pathways as compared to *C. freundii* so4. On the other hand, *C. freundii* so4 apparently was not a versatile specialist in polysaccharide degradation, as it only exhibited 137 genes for CAZy family enzymes, of which 61 were GHs and 12 were CBMs. *C. freundii* so4 showed a preference for degradation of intermediates of cellulose, as it had a larger number of genes for these (than w15), mainly from CAZy families GH1 and GH13, which are involved in cellulose and pectin degradation. The most common enzymes in family GH1 are  $\beta$ -glucosidases and  $\beta$ -galactosidases, and those in GH13 (the major GH family acting on substrates containing  $\alpha$ -glucoside linkages)

were hydrolases, transglycosidases and isomerases (CAZyedia Consortium 2017). Phenotypic analyses confirmed the activity of enzymes from GH1 and GH13 as *C. freundii* so4 showed the preference of consumption of intermediaries of cellulose degradation such as cellobiose, maltose and melibiose, respectively; strain so4 was also found to use the oligosaccharide raffinose, which is present in a wide variety of plants, as a sole carbon source. *C. freundii* so4 presented a large investment in amino acid metabolism, pyruvate and propanoate metabolisms, which matched the phenotypic characterization.

The results of this thesis provided a basic framework for determining the potential contribution of *C. freundii* so4 and *S. multivorum* w15 in wheat straw degradation, based on their metabolic differences. Diverse authors have highlighted the importance of genomic studies for determining the potential of degradation of polysaccharides in bacteria, next to the identification of CAZy families relevant in biomass deconstruction (Berlemont and Martiny 2015). Notoriously, the most abundant genes for CAZy enzymes in the genome of *S. multivorum* w15 encoded proteins of families GH2, GH20, GH29, GH43, GH78, GH92 and GH109. Interestingly, López-Mondéjar et al. (2016a) found exactly the same families as the most abundant ones in the genome of *Pedobacter* O48, a bacterium highly abundant in forest litter that also presented high lignocellulolytic enzymatic activity. Moreover, Jiménez et al. (2015a) found that CAZy families GH2, GH43, GH92 and GH95 were enriched in two LCB degrader consortia, where the genes were most likely affiliated to the genomes of *Sphingobacterium*, *Bacteroides*, *Flavobacterium* and *Pedobacter* spp. This finding suggests that these enzyme-families have important roles in the degradation of plant biomass in specialized degrader bacteria. Family GH43 has emerged as a relevant CAZy family in the degradation of hemicellulose. For instance, glycosyl hydrolases belonging to family GH43 were synthesized only when the degrader consortia grew in wheat straw and xylan (Jiménez et al. 2015b). This was revealing, as it pointed to a unique role of this CAZy family in the degradation of such types of complex polysaccharides and specifically decomposition of hemicellulose component.

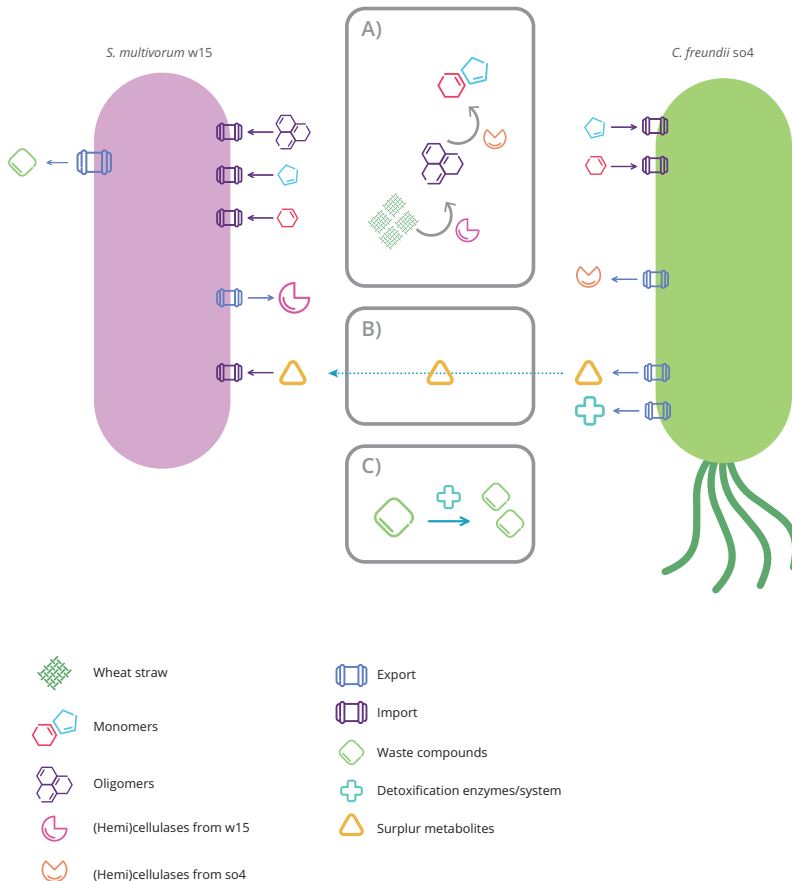
Genomics coupled with transcriptomics and/or proteomics can be used as an analytical tool that underpins a mechanistic model (López-Mondéjar et al. 2016b). Phenotypic and genomic information led me to propose that the cooperative interactions between the two strains may be described as either a **cross feeding interaction** or a cooperation based on metabolic exchange (Cavaliere et al. 2017). The latter occurs when a species degrades a primary energy source and produces an intermediate compound, which could also be a by-product,

that then is used by the second species (Germerodt et al. 2016). In the case of *C. freundii* so4 and *S. multivorum* w15, the synergistic relationship may come about as a result of a complementary hydrolytic battery of secreted enzymes with different activities that might allow the strains attacks on larger extensions of the complex substrate, where *C. freundii* so4 may be more focused on cellulose and *S. multivorum* w15 on hemicellulose. Hence, there may be different roles in the degradation system, with *S. multivorum* w15 acting as a *primary degrader*, while *C. freundii* so4 may have a more *secondary degrader* role. In addition, this refers to a second hypothesis, it may serve as a “cleaner” and a metabolite producer. In the following lines I describe the hypotheses for explaining the roles of *S. multivorum* w15 and *C. freundii* so4 in the presumed interaction when growing on wheat straw as the sole carbon source.

### ***Model of the interaction***

A) *S. multivorum* w15 may be acting as ***primary degrader*** attacking the substrate by the synthesis of a large amount of hydrolytic, debranching and auxiliary enzymes that break complex pieces of the wheat straw, for instance enzymes from the CAZy family GH43 (involve in hemicellulose degradation in LCB). The enzyme activity of the strain w15 releases oligomers that may activate the expression of diverse glycoside hydrolytic enzymes in *C. freundii* so4, which are different from those produced by the strain w15. Then, the strain so4 may act as a ***secondary degrader*** by synthesizing and exporting extra (hemi)cellulases in the culture that transform the intermediaries into monomers. The complementary in the enzymatic activity of *C. freundii* so4 may favour the releasing of monomers that both strains can consume easily and enhancing the cellular growth. The metabolic differences between the two strains avoid competition for the same resources, for instance *S. multivorum* w15 probably utilises di-saccharides and oli-saccharides for growing, as it presented a preference in the consumption of compounds as sucrose, turanose, gentibiose or even stachyose (a tetra-saccharide found in seed of legumes). While, *C. freundii* so4 presented a marked preference in the consumption of simpler carbon source, mainly monosaccharides such as mannose, galactose and arabinose (Figure 1). *C. freundii* so4 may also contribute to the system by avoiding the limiting rate of hydrolytic enzymes as the activities of these are inhibited by accumulation of final product. Therefore, by reduction of intermediate compounds, generated by the lytic armoury of the strain w15, *C. freundii* so4 may promote the continue activity of the enzymes in the culture, that favours the degradation of substrate as, what will be reflected in increasing cell growth.

- B) Additional to the complementary role in the degradation process, *C. freundii* so4 could also have a key role in the synergistic system by excreting surplus metabolites that *S. multivorum* w15 can use but not produce, for example amino acids and derived compounds.
- C) Moreover, according to genomic data *C. freundii* so4 might participate in detoxification of the system by reduction of waste compounds produced by microbial growth, such as intermediary compounds derived from cellulose and hemicellulose degradation. Its genome presented an important investment in oxidative stress response and detoxification. In particular genes for glutathione metabolism and glutathione transcriptional regulation of formaldehyde detoxification (FrmR) and for very diverse oxidoreductases were found the latter may detoxify compounds such as phenolics (Karigar and Rao 2011).



**Figure 1** Proposal model of microbial interaction. The description of the model is in the text.

### ***Cooperative interactions are triggered by the complexity of the carbon source***

The complete degradation of complex wheat straw moieties may require the participation of very diverse organisms (Bayer et al. 2013). In **Chapter 2** I observed how the composition of lignocellulose substrates affects the microbial structure of degrader consortia, while in **Chapter 3** I found that applying the same lignocellulose substrate led to the the selection of a microbial “core”, which was presented in all consortia, consisting of members of four families: *Sphingobacteriaceae*, *Flavobacteriaceae*, *Moraxellaceae* and *Enterobacteriaceae*. Then in **Chapter 4**, I also found that exposition to recalcitrant bonds strongly affected the structure of the microbial communities in the degrader consortia. These collective results lead me to propose that the bonds linking the moieties within the substrate exert a significant selective force on the structure of microbial consortia. Moreover, in **Chapter 5** I observed how the degree of complexity of the substrate affects the interaction between collaborating strains. The synergistic strains only presented cooperative interaction when they were grown together on substrates with complex structure such as wheat straw or synthetic wheat straw, whereas cooperation dwindled away, when grown together on glucose. These data constitute strong indicators for the tenet that the complexity of the chemical configuration across the compounds of LCB (structural complexity) modulates the level cooperation between the strains. With the available information I gathered so far, it is not possible to describe the mechanism of the positive interactions in the system. To make further progress in this area, it is necessary to apply complementary analyses, such as transcriptomic or proteomic studies. However, I posit here that the interactions in halotolerant consortia growing on pre-digested wheat straw (**Chapter 4**) will be tighter between the members as it requires more specialized metabolic capacities to be able to deal with the highly recalcitrant substrate. The results of sequencing showed how the change between fresh and pre-digested substrate importantly affected the microbial composition of the final consortia by significantly reducing the diversity of the consortia.

## Conclusions

- LCB type is the main factor that drives the community structure of degrader consortia. Both the complexity of the structure and the bonds will be the key selectors of the microbial community emerging from the inoculum by shaping the community structure and inherently the interactions within the selected consortia.
- Members of the families *Enterobacteriaceae*, *Xanthomonadaceae*, *Pseudomonadaceae* and *Sphingobacteriaceae* are thought to constitute generalist degraders in the selected consortia. Their diverse metabolic capacities may have favored their enrichment as well as the cooperative interactions between them. Fungal species were very dependent on the inoculum source, except for *Coniochaeta ligniaria* and *Penicillium* sp., which were found across all consortia, indicating an important role in degradation process.
- Genomic plasticity within complex natural microbiomes allows these to adapt and achieve desired functionalities in most systems. The inherent functional redundancy within microbiomes is another key facet of microbial adaptive processes. The habitats selected as inoculum sources likely presented microbial communities with the full potential for obtaining consortia capable of efficiently degrading LCB. And, clearly, the abiotic conditions at the source (such as oxygen availability) strongly influence the composition of the final consortia.
- Given the fact that the subsequent transfers on wheat straw led to the selection of members of just four bacterial families, we posit that 1) Substrate type and not inoculum source was the main driver of the selection of degrading consortia and 2) Particular members of these families had the desirable metabolic compatibility for growth on wheat straw in liquid and aerobic conditions.
- In contrast, species of *Flavobacteriaceae*, *Cyclobacteriaceae*, *Pseudomonadaceae* and *Halomonadaceae* formed a core of specialized organisms that together are capable of degrading highly recalcitrant wheat straw under high salt concentration. Remarkably, fungi very likely did not play major roles in the degradation of lignocellulose under these conditions.



- The selection of conditions for obtaining specialized microbial consortia have to be well thought out, as the different lignocellulose moieties as well as the type of bonds linking them drive the selection and the interaction between the microbial members.
- *Citrobacter freundii* so4 and *Sphingobacterium multivorum* w15 were found to be enriched in lignocellulose degrader consortia, as explained by their complementary roles in the degradation process, due to their different metabolic capacities that allow them to consume and grow in different types of recalcitrant lignocellulose biomass. Cross-feeding is posited to be the most likely mechanisms that explain the interaction between the degrader strains. *S. multivorum* w15 has a genome that is well adapted to hemicellulose degradation. It may thus act as the *primary degrader*, whereas *C. freundii* so4 may be acting as a *secondary degrader* by consuming and transforming intermediaries of lignocellulose degradation process.

### ***Applications and future perspectives***

In **Chapter 2**, I discussed the importance of selection of lignocellulose substrate for obtaining efficient LCB-degrading consortia. However, this study did not deeply analyze the final consortia as it was based only on PCR-DGGE and isolated strains. Thus, to fully understand the composition of those LCB-grown consortia, I propose to examine the composition by 16S rRNA gene sequencing, as well as by characterization of enzymatic activities.

#### ***Increasing consortia complexity:***

In **Chapter 5**, I tested bi- and tri-cultures formed of the most abundant species in the consortia obtained in **Chapter 3**, and assessed their synergistic behaviour. However, in these analyses, I refrained from comparing the degradation efficiencies to those of complete consortia. Thus, I propose the study of consortia resembling the bacterial “core” found in **Chapter 3**. These are formed by at least four organisms, each one representative of the aforementioned most enriched families. Such cultures could be the most efficient lignocellulose degrader consortia, keeping the simplicity necessary for further characterization.

Considering that the halotolerant consortia was active on highly recalcitrant substrate, I hypothesize that these members of the consortia would present

stronger metabolic dependencies than those obtained in fresh substrate. I suggest the study of the interactions between the five most abundant organisms in the consortia, *Joostella marina*, *Flavobacterium beibuense*, *Algoriphagus ratkowskyi*, *Pseudomonas sabulinigri* and *Halomonas meridiana*. I propose to study the strains in an experiment similar to **Chapter 5**, by co-culturing them in different combinations on wheat straw; and to determine the degradation potential of the substrate, as these five strains could represent very efficient and minimal halotolerant degrader consortia.

### ***Understanding bipartite interactions:***

I propose a deeper exploration of the data of the genomes of *C. freundii* so4 and *S. multivorum* w15, in order to elucidate the interaction between them. Concretely, a detailed revision of the carbohydrate transport could be performed, e.g. lignocellulose derivatives, focusing on ABC-type transporters, PTS-mediated transport and cationic symporters. For answering the hypothesis about the possible participation of *C. freundii* so4 in the detoxification of the culture, I propose an extensive analysis of the genome of strain so4 looking for potential detoxifying genes that may have been overlooked or not described yet (Mukhopadhyay et al. 2012).

### ***Mechanisms driving synergism:***

To fully understand the mechanism behind the synergistic relationship between *C. freundii* so4 and *S. multivorum* w15, I propose a global transcriptomic analysis of the strains, growing in mono- and co-culture on wheat straw. Comparing the transcription profiles between monocultures and co-cultures it may be possible to identify genes from degradation and general metabolic systems express by the strains when growing together on a recalcitrant carbon source. This analysis could help to confirm or reject the some hypotheses proposed in this thesis. The analysis must include samples at different incubation time-point, as the transcription is a very dynamic process. Additionally, it is possible to have extra transcriptional experiment of the strains growing in co-culture on synthetic wheat straw and compare these results with those from co-culture on wheat straw. This comparison could help to identify key genes exclusively activated by the presence of complex linking bonds and it would lead to the identification of enzyme-system involve in the degradation of recalcitrant regions of the substrate. An alternative is to do this experiment with the minimal consortia formed by the suggested four strains: *Citrobacter freundii*, *Sphingobacterium multivorum*, *Acinetobacter* sp. and

*Flavobacterium ginsengisoli*. Moreover, as a sideline, I recommend the study of the genome of the *Flavobacterium ginsengisoli* strain, as *Flavobacteriaceae* was one of the two families found in all degrading consortia, including the halotolerant one.

### ***Recalcitrant substrates:***

By monitoring the structure of the consortia built in **Chapter 2** and **Chapter 3** - fed always with fresh substrate - I observed that after transfer six (to ten) there were no significant changes in these. Thus, if we really aim to address the problem of recalcitrance of lignocellulose substrates, we need to reach recalcitrant areas of the substrate components (crystalline cellulose, lignin, etc.), as well as the bonds (between hemicellulose and lignin). Thus, observational studies of adhering cells as well as enzymes to the substrate constitute a promising way forward.

### ***The potential use of halotolerant consortia and enzymes:***

Halotolerant bacteria strains such as *Halomonas* spp. isolate from **Chapter 4** may represent a key source of extracellular enzymes capable to degrade LCB under saline condition. Many members of the *Halomonadaceae* family have already been used for the production of (halostable) cellulases, amylases, xylanases, proteases and lipases (Mathabatha 2010). For instance, *Halomonas* sp. PS47 can produce halostable cellulases that work on wheat bran (Shivanand et al. 2013). Moreover, such cellulases have been successfully applied for saccharification of lignocellulose biomass pretreated with ILs (Gunny et al. 2014). However, further research is certainly necessary in this area, before halotolerant consortia or strains, or their enzymes, can be applied for bioconversion of lignocellulose waste biomass.

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