Lignocellulose-degrading microbial consortia

Importance of synergistic interactions

Larisa Cortés Tolalpa

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“We must believe in ourselves or no one else will believe in us; we must match our aspirations with competence, courage and determination to succeed”

Rosalyn Yalon

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Chapter 1

General introduction

Larisa Cortés Tolalpa

Lignocellulose biomass composition

During the past century, world energy consumption has mostly depended on the utilization of fossil fuels, which has provoked negative changes in our climate and increased the emission of greenhouse gases in the atmosphere. An important emerging trend in the 21th century is the switch from non-renewable fossil resources to renewable ones for the production of biofuels and other valuable compounds. The use of lignocellulose materials has emerged as an attractive and sustainable source of carbon for this. Recycling of carbonaceous materials is also important considering the actual scarcity of arable land. Khoo et al (2016) indeed support the use of lignocellulosic biomass (LCB), for example for the production of biofuels, as it can reduce the dependency on fossil fuels and contribute to climate change mitigation.

LCB is the most abundant source of reduced carbon on earth, being wheat (*Triticum aestivum*) the major LCB source. Wheat is a major food crop in the world, next to rice and maize. Around 75% of the total agricultural residues are derived from these three crops at global level (Xie and Peng 2011). Other lignocellulose residues include maize, sugar cane bagasse, switch grass, decaying wood and miscanthus, next to most of the waste produced by the food industry, see Table 1 (Dyk and Pletschke 2012; Dyk et al. 2013).

Table 1. **Sources of lignocellulose biomass and compositional percentages.**

Sourcet	Classification	Type	Cellulose (%)	Hemicellu-lose (%)	Lignin (%)	Ash (%)
Agriculture	Crop residues	Wheat straw	37–41	27–32	13–15	11-14
		Maize	38-40	6.1-28	7-21	3.6.7
		Rice straw	28-36	23–28	12–14	14–20
		Barley straw	31-45	5–20	-	-
		Cotton stalk	80–95	27–38	14–19	2-7
		Sugarcane bagasse	32–48	19–24	23–32	1.5–5
		Sorghum Straw	32–35	24–27	15–21	-
	Herbaceous energy crops	Switchgrass	31-35	24-28	17-23	-
		Miscanthus giganteus	37–45	19–25	17–21	1–3
		Grasses	25-40	25-30	15-20	-
Industry	Forest	Poplar (hardwood)	4–55	24–40	18–25	1–4
		Pine (softwood)	25–42	21–30	18–26	0.3–2
City	Food waste	Nut shell	25-30	25-30	30-40	-

Information adapted from the following references (Wei et al. 2009; Kumar et al. 2015; Ravindran and Jaiswal 2016; Cai et al. 2017)

Generally speaking, lignocellulose biomass consists of about 30-44% cellulose, 23-50% hemicellulose and 7.7-15% lignin (Figure 1). The remaining fraction in the LCB is made of pectin, proteins, ash, salt minerals and silica Table 1 (Ravindran and Jaiswal 2016; Cai et al. 2017). Below I discuss each of them separately.

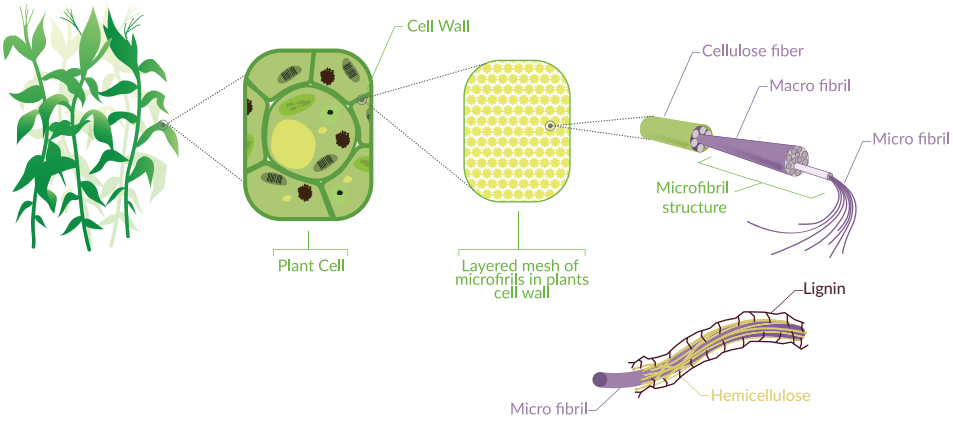

Figure 1. Schematic representation of lignocellulose distribution in plant material and composition.

Cellulose. Cellulose consists of linear chains of glucose molecules linked by β-1-4 glycosidic bonds. There are two types of cellulose structures, i.e. amorphous and crystalline. The amorphous form is soluble and is easily digested by enzymes, while the crystalline structure is formed by cellulose chains that are strongly linked by hydrogen bonds, forming microfibrils. This type of cellulose is very recalcitrant to degradation and solubilization (Figure 1) (Ravindran and Jaiswal 2016; Cai et al. 2017).

Hemicellulose. Hemicellulose has a more variable configuration than cellulose. It is a heterogeneous polymer that is composed of short polysaccharide chains, such as xylan, mannan, galactan and arabinan. In these, the monomers are mainly five monosaccharides: D-xylose, L-arabinose (pentoses), D-galactose, D-mannose and D-glucose (hexoses). Xylan, the most abundant molecule, is formed by β-D-xylopyranosyl residues linked by β-1,4-glycosidic bonds. Its most abundant form is heteroxylan, which is comprised of xylose residues in the backbone, also carrying acetate, arabinose and glucose residues (Dyk and Pletschke 2012).

Lignin. Lignin is an aromatic polymer formed by lignols within a three-dimensional structure. Its chemical arrangement provides the rigid structure of plants. The three lignol monomers are hydroxyphenyl alcohol, coniferyl alcohol and synapyl alcohol (Ravindran and Jaiswal 2016; Khoo et al. 2016).

In the LCB, hemicelluloses and cellulose are linked by hydrogen bonds, whereas both moieties are linked to lignin by covalent bonds (Ravindran and Jaiswal 2016; Khoo, Ee, and Isoni 2016), forming a highly complex and heterogeneous structure.

Benefits of lignocellulose degradation and factors affecting its bioconversion

It has been estimated that, globally, LCB in agricultural waste amounts to about $1.5x10^{11}$ ton per year (Guerriero et al. 2016). The utilization of this biomass for production purposes can be considered as an environmentally-friendly process that can mitigate greenhouse gas emission. In the past, studies have mainly focused on the conversion of cellulose to simpler monomers for subsequent transformation to, for instance, ethanol. There are increasing research efforts focused on the bioconversion of hemicellulose, which means the production and subsequent utilization of pentose and hexose molecules (Gírio et al. 2010; Ji et al. 2011). Overall, the production of diverse commodities by industrial applications of compounds derived from the hemicelluse, cellulose and also lignin parts of lignocellulose biomass is widely expanding (Figure 2) (Guerriero et al. 2016).

In spite of the great promise of the utilization of LCB in waste, it is still necessary to develop methods to overcome its inherent recalcitrant nature. Lignocellulose materials, from an evolutionary point of view, have evolved to a complex chemical structure in order to resist microbial degradation or animal assault. At the molecular level, the recalcitrance of LCB is related to the following: The lignin content, the degree of crystallinity of cellulose, the polymerisation degree of the polysaccharides and the available surface area of the biomass (d'Errico et al. 2015; Sun et al. 2016). Recently, it was found that the degree of ester linkage between the lignin and the carbohydrate moieties of the LCB also influence its degradability (Rabemanolontsoa and Saka 2016). The methods used to reduce the recalcitrant nature of LCB can be divided in pre-treatments or enzymatic hydrolyses.

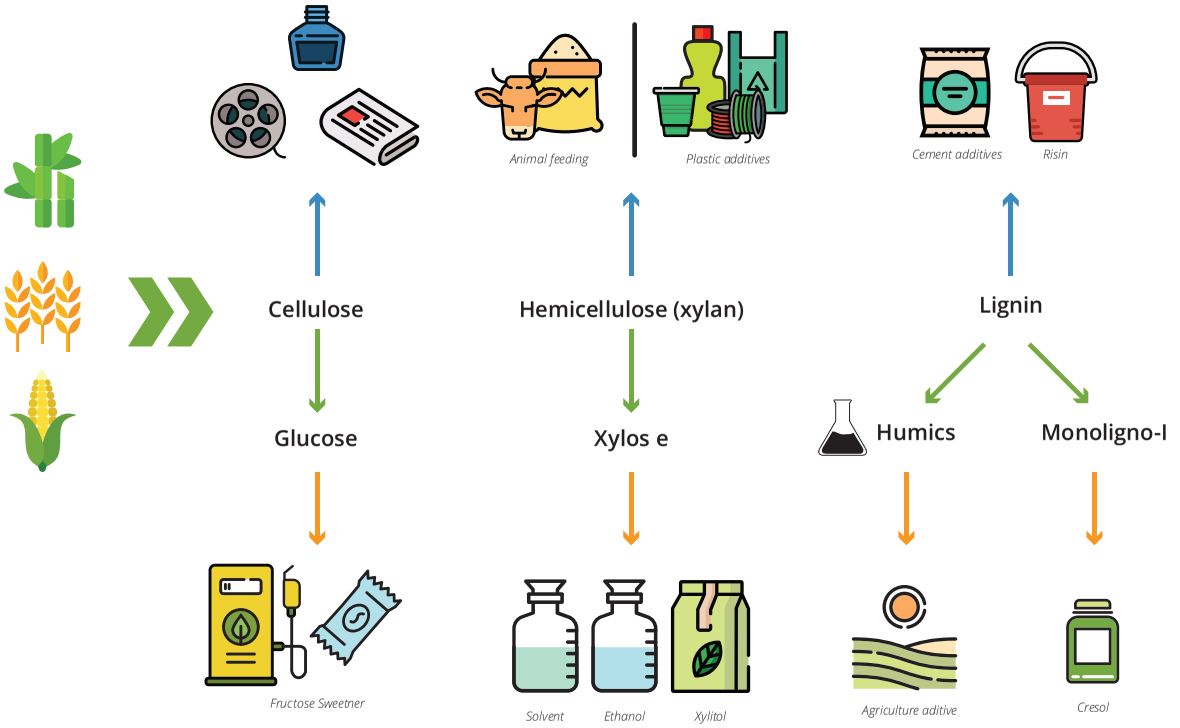

Figure 2. Products that can be derived from the main components of lignocellulose substrate, like sugar cane, wheat straw and maize. Products from the main component (*blue arrows)* and those obtained from the biotransformation of the monomers (*orange arrows*). Cellulose can be transformed into pharmaceutical, cosmetic, printing ink and paper products. Xylan can serve as a source for plastic additives, strengthening agents in paper and textile printings. Lignin can be used as a cement additive, and in the production of resins and flocculants. The monomer of cellulose, glucose, can be transformed into biofuel, whereas xylose, from hemicellulose, can produce furfural (a polymer precursor or solvent), xylitol or ethanol. Monomers of lignin such as humic acids are used in agriculture; monolignols are used as precursors of cresol, catechols, resorcinols, quinones, vanillin and guaiacols. Figure made based on Guerriero et al. (2016).

Pretreatments

Usually, LCB is not easily accessible for enzymes or microorganisms. Thus, the biomass needs to be mechanically, biologically or physicochemically pretreated to make the cellulose and hemicellulose moieties accessible for efficient enzymatic depolymerisation (Talebnia et al. 2010). Ideally, an effective pretreatment process should adhere to five criteria: 1) Increasing sugar yield and minimizing sugar loss; 2) Maximize exposure contact surface by reducing level of intertwining and particle size, 3) Maximize conversion rate by increasing hydrolysis rate; 4) Reduce the production of inhibitory compounds that affect the downstream process, and finally 5) Reduce cost by reducing energy consumption. Figure 3 shows the classification of selected major pretreatment strategies.

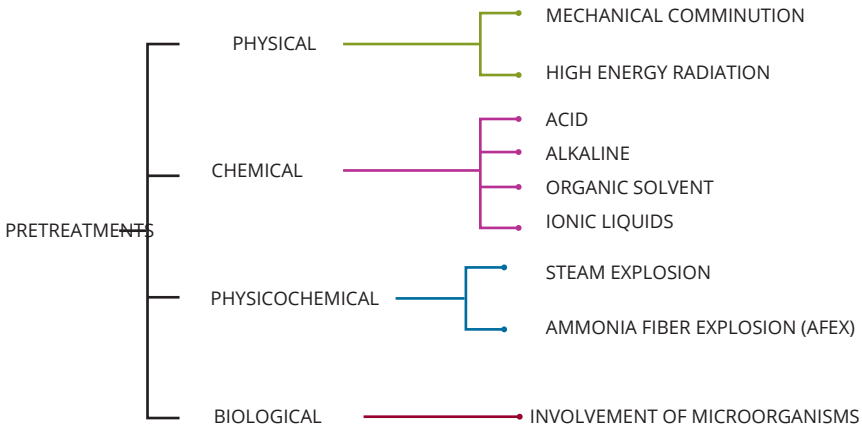

Figure 3 Classification of pretreatment processes. These are generally classified into physical, physicochemical, chemical and biological processes. The processes can also be combined, for example mechanical followed by thermal treatment, in order to achieve higher sugar release efficiencies, low toxicant production and low energy consumption.

Physical pretreatments are aimed at increasing the accessible surface area of LCB by reduction of the particle size and pores and disrupting the regular structure. It includes chipping, grinding and milling. Alternatively, possible physical pretreatment are pyrolysis, gamma radiation, microwave treatment, infrared heating and sonication (Kumar et al. 2015).

Chemical pretreatments involve the use of diluted acid or alkali, oxidation agents and organic solvents and ionic liquids (ILs). The most commonly applied methods are acid and alkaline hydrolysis. Acid hydrolysis is applied on raw LCB to improve downstream enzymatic hydrolysis, while alkaline treatment increases

the digestibility of the polysaccharides and facilitates enzymatic attack, as it increases porosity and incites an enhanced surface area. In both cases the main disadvantage is the formation of inhibitory compounds, next to high costs of waste disposal and a large environmental footprint (Sun et al. 2016) (Table 2).

Physicochemical processes use a combination of pretreatments such as hydrothermal treatment, under which liquid hot water (LHW) and steam explosion (SE), with chemical treatment, e.g. ammonia fiber explosion (AFEX) (Sun et al. 2016). **Steam explosion** is one of the most expensive pretreatments, nevertheless, it is one of the most effective and it is especially applied on lignocellulose waste materials. This method uses high-pressure saturated steam at between 0.69 and 4.83 MPa with temperatures of 160 - 260°C for several seconds to a few minutes (Jönsson and Martin 2016). **Ammonia fiber explosion (AFEX)** is an alkaline thermal pre-treatment, where the lignocellulose biomass is exposed to liquid ammonia at high temperature and pressure for a short period, which is followed by a rapid pressure release.

Biological pretreatments are environmentally friendly and economical alternatives for the disruption of the lignocellulose complex matrix. Differently from physical and chemical pretreatments, biological pretreatments are done under mild conditions of pressure and temperature. Various organisms have been used in biological pretreatments, mainly white rot fungi, that attack both cellulose and lignin, next to brown rot fungi that attack only cellulose (Sindhu and Pandey 2016). Two examples illustrate the effect of this type of pretreatments over the plant biomass. In one case a fungal consortia was used on raw maize, the result was the reduction of the 43% of the lignin content, thus increased the hydrolysis rate in seven folds (Song et al. 2013). While, in other example, *Punctualaria* sp. TUFC20056 was applied on bamboo culms achieving the reduction of lignin in 50% (Suhara et al. 2012). Because no chemicals are used, there is no need for recycling or recovering chemicals, there is no release of toxic compounds to the environment and energy is saved. On the other hand, biological pretreatments have a very low rate of hydrolysis resulting in a time-consuming process (Vasco-Correa et al. 2016).

At the present time, pretreatments are mandatory for utilization of lignocellulose biomass, but the main disadvantages are the increase in the production cost, as well as the generation of toxic compounds that not only interfere with downstream bioprocess, but also negatively affect the environment (Sun et al. 2016).

In Table 2 I show a review of different types of pretreatments, as well as advantages and disadvantages of each. Nowadays, the development and application of effective pretreatment to lignocellulose biomass is clearly urgent. Thus, efforts should go into developing an efficient method that breaks up the substrate and does not inhibit microbial activity as well as enzymatic degradation of the substrate. Such a method may be substrate- and even climate-specific.

Enzymatic hydrolysis

The hydrolysis of lignocellulose, because of its complexity, requires numerous enzymes with different specificities working in a synergistic manner. The variation in structure between substrates from different sources and the effect of different types of pretreatments further increase the complexity of developing a standard method. In Figures 4-6 I show an overview of the types of enzymes that are required to degrade complex lignocellulose substrates. In a generic sense, microorganisms produce two types of enzyme systems for lignocellulose degradation: (1) freely released enzyme systems, which are mostly produced by many aerobic bacteria and fungi, and (2) multi-enzyme complexes named cellulosomes, which are mostly found in anaerobic bacteria such as *Clostridium* (Dyk and Pletschke 2012; Bayer et al. 2013)

Cellulose degradation

To degrade cellulose, three major groups of enzymes are required to work synergistically: endoglucanases, exoglucanases (cellobiohydrolases) and β-glucosidases (Figure 4). Endoglucanases (endo-β-(1,4)-glucan hydrolases) are characterized by their hydrolysis of internal β-(1,4)-glucosidic linkages. These enzymes attack low-crystallinity regions of the cellulose fibers. Exoglucanases (exo-β-(1-4)-glucanases) remove the cellobiose units from the free chain ends. They have a preference for attacking longer chain substrates than β-glucosidases. In addition, β-glucosidases hydrolyse cellobioses and other short-chain β-1,4-oligosaccharides, realising glucose monomers. Most β-glucosidases are active on a range of β-dimers of glucose (Kumar et al. 2008; Gupta and Verma 2015).

Hemicellulose degradation

Due to its more complicated composition compared to cellulose, hemicellulose requires a larger number of different enzymes to be hydrolyzed effectively. Enzymes involve in the degradation of hemicellulose can be divided into depolymerising enzymes (cleaving the backbone) and those that remove substituents (Dyk and Pletschke 2012).

Table 2. Physical, chemical and biological pretreatments used on lignocellulose biomass.

Type	Pretreatment	Advantage	Disadvantage	By-product formation
I.Physical				
Mechanical size reduction	•Chipping (10-30 mm) •Grinding •Milling (0.2-2mm)	•Increase accessible surface area •Reduce crystallinity	•High energy consumption, problems at industrial scale	•No inhibitor formation
	•Gamma irradiation by gamma		•No removal of lignin and hemicellulose	•No inhibitor formation
II.Chemical				
Acid hydrolysis	Diluted acid: H_2SO_4, HCl, H_3PO_4 and HNO_3	•Reduction hemicellulose content •Hydrolysis of hemicellulose to monosaccharides	•Problems for recovery the chemical	•Aliphatic carboxylic acids Furans phenolic compounds
Alkaline	•Diluted NaOH (complemented H_2O_2) •$Ca(OH)_2$	•Reduces lignin content	•Pollution •Costly recover chemical	•Acetic acid, dicarboxylic acid phenolic compounds
Organic solvent	•Methanol, ethanol, acetone, ethylene glycol and their mixture	•Almost total hydrolysis of hemicellulose •Separation and recovery of high quality lignin	•High price of organic solvents	•Specific to the solvent used
Ionic liquids	•Organic solvent, liquid at room temperature	•Reduce crystallinity efficiently	•High cost ILs •High cost of washing the salt	•Inhibition of enzymes by high salt concentration
III.Physico-chemical				
Steam explosion	•Hot water •High pressure	•Solubilize hemicellulose •Affect lignin structure •Cost effective	•High equipment cost	•Acetic acid •Minor amount of furans •Aldehydes
Ammonia fiber explosion (AFEX)	•Liquid ammonia (NH_4OH) •High pressure and temperature	•Cellulose swell •Increase accessible surface area	•High equipment cost •Ammonia cost	•No inhibitor formation
IV.Biological	•Brown, white and soft fungi	•Degrade lignin and hemicellulose	•Long incubation period	•Possible consumption of carbon source

With information from the following references Talebnia et al. (2010); Kumar et al. (2015); Ravindran and Jaiswal (2016); Sun et al. (2016); Jönsson and Martin (2016).

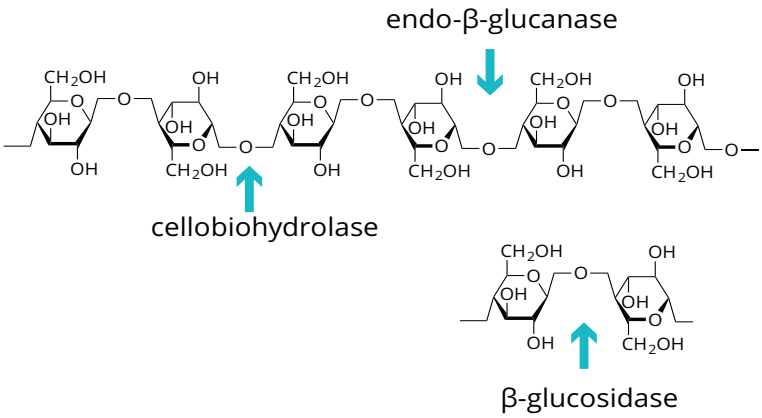

Figure 4 Cellulose degradation. Molecular structure of cellulose and sites of action of the most common endoglucanase, cellobiohydrolase and β-glucosidase.

Xylan is the most abundant component in the hemicellulose part of wheat straw. For its degradation it is necessary the action of endo-xylanases (endo-1,4-β-xylanases), which cleave the xylan backbone into shorter oligosaccharides is required, followed by the activity of β-xylosidase, which cleaves short xylo-oligosaccharides into xylose. For the degradation of glucomannan, the action of endo-mannanase and β-mannosidase is needed. Generally, xylans and mannans have several different substituents linked to the main backbone, such as arabinose, acetyl, galactose and glucose groups. Some of the key enzymes involved in hemicellulose degradation are α-L-arabinofuranosidases, α-glucuronidases, α-galactosidases, feruroyl esterases, acetyl xylanesterases and acetylmannan esterases (Gupta and Verma 2015) (Figure 5).

Enzymes with hemicellulolytic activity are generally classified into glycosyl hydrolase (GH) families GH2, GH10, GH11, GH16, GH26, GH30, GH31, GH39, GH42 and GH43. Among these families, family GH43 is particularly interesting. This is one of the largest families of GHs in the carbohydrate active enzymes (CAZy) database, having 4555 members. The family comprises a range of debranching enzymes that may act in the degradation of hemicellulose, particularly arabinoxylans. Moreover, an action on pectin is supposed. Family GH43 has emerged as an important CAZy enzyme family for biodegradation of complex substrates such as biopolymers.

Evidence for the importance of GH43 family comes from genomics studies of

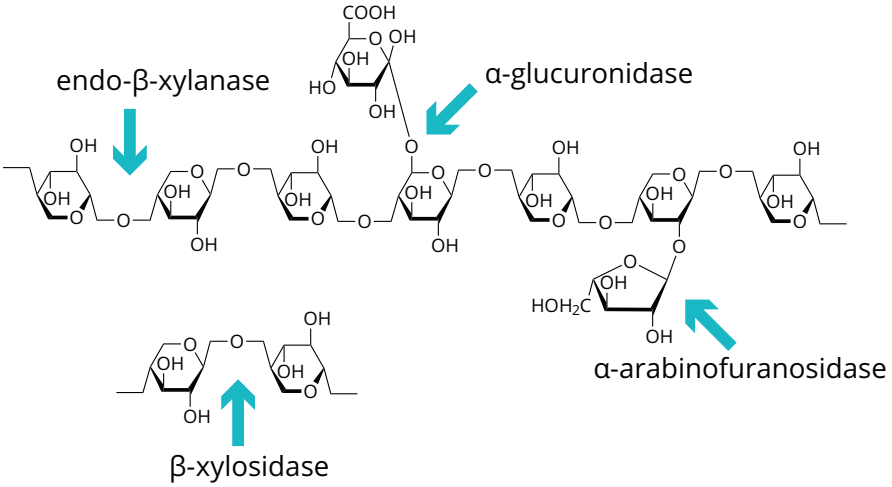

Figure 5 Hemicellulose degradation. Molecular structure of hemicellulose and sites of action of the most common endoglucanase, endo-β-xylanase, α-glucoronidase, β-xylosidases and α-arabinofuranosides. Other enzymes needed in degradation of hemicellulose are acetyl xylan esterase, endo-mannanase, β-mannosidase, ferulic acid esterase, α-galactosidase and p-coumaric acid esterase.

LCB degrading microorganisms (Bayer et al. 2013) as well as the human gut microbiome (El et al. 2013), where enzymes of the GH43 family were identified as one of the most abundant CAZy enzymes present. In addition, in a metagenome study of a soil-derived degrader consortia bred with wheat straw showed a significant enriched of the families GH43, next to GH2, GH92 and GH95 (Jiménez et al. 2015a). Moreover, a proteomic study has shown that GH43 family proteins were synthesized exclusively when the degrader bacteria grew on wheat straw, but not on cellulose or other less complex carbon sources (López-Mondéjar et al. 2016). In the same line, a metasecretome analysis, from three different microbial consortia fed with wheat straw, xylose and xylan, revealed that only the wheat straw selected-consortia synthesized glycosyl hydrolases belonging to family GH43 (Jiménez et al. 2015b). This was revealing, as it pointed to a unique role of this CAZy family in the degradation of such type of complex polysaccharides and specifically related with the decomposition of hemicellulose. In the light of these results, the study of GH43 has recently become fundamental in our understanding of lignocellulose degradation context.

Lignin degradation

The degradation of lignin is catalysed by two main classes of enzymes: Peroxidases (lignin and manganese) and laccases. Working together, these enzymes lead to the complete degradation of lignin. Lignin peroxidase is a heme-containing glycoprotein, which requires hydrogen peroxide as the oxidant. This enzyme degrades non-phenolic lignin units (Figure 6).

Manganese peroxidases act on phenolic and non-phenolic lignin units through lipid peroxidation reactions. They oxidize Mn^{2+} to Mn^{3+} which then oxidizes phenol rings to phenoxy radicals, leading to decomposition of the compound. On the other hand, laccases catalyse the oxidation of phenolic units, phenolic compounds and aromatic amines to radicals. The lytic activity of laccases can be increased by the addition of 3-hydroxyanthranilic acid, 2,2 P-azino-bis (3-ethylthiazoline-6-sulfonate) which will act as a redox mediator (Sindhu and Pandey 2016).

Accessory enzymes

There are indications that many other enzymes contribute to lignocellulose degradation in ways that are not yet clearly understood. These so-called accessory enzymes act on less abundant linkages found in lignocellulosic biomass and include carbon binding modules (CBM), arabinases, lyases, pectinases, galactanases, several types of esterases and polysaccharide monooxygenases (PMOs) (Dyk et al. 2013). Among these auxiliary enzymes, the most relevant groups are CBM, PMOs and glucuronyl esterase family-15 class (CE15).

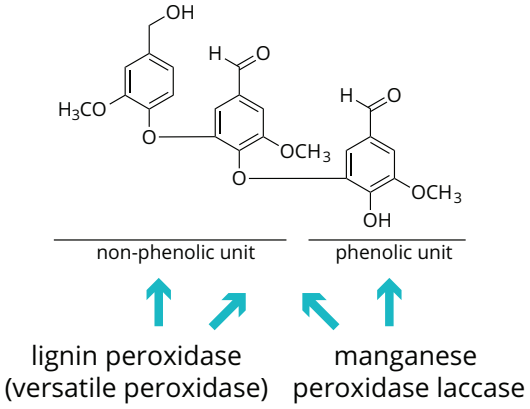

Figure 6 Lignin degradation. Lignino-lytic enzymes and their selectively action on lignin components. Lignin peroxidase can directly oxidize both phenolic and non-phenolic lignin units. Laccase and manganese peroxidase can directly oxi-dize phenolic lignin units but requires the help of a mediator to act on non-phenolic units. Versatile peroxidase can either act like lignin peroxidase or manganese per-oxidase.

CBM are autonomously folding and functioning protein domain that lack catalytic activity but are able to bind to carbohydrate chains through their active site (Brumm 2013). CBM are important because they allow the hydrolytic enzymes to remain bound to the substrate while they perform their hydrolytic activity (Cantarel et al. 2009). CBM confer high selectivity in the binding, being able to target different substrate forms depending on different structural characteristics (Dyk and Pletschke 2012).

PMOs are enzymes that enhance the degradation of recalcitrant biopolymers by hydrolytic enzymes. They work synergistically with glycoside hydrolases (GHs) in the degradation of cellulose and chitin. PMOs enable a rather unusual depolymerisation of crystalline cellulose through an oxidative mechanism (Beeson et al. 2015). PMOs are intriguing enzymes, not only because they oxidize C-H bonds but also because their chemistry does not require separation of the polysaccharide chain from the crystalline matrix backbone for bond cleavage. The PMOs are very recent additions to the known polysaccharide degradation machinery, as their oxidative chemistry was reported for the first time in 2010 (Vaaje-Kolstad et al. 2010).

CE15 are accessory enzymes that present an interesting function: They break down bonds between hemicellulose and lignin, which is one of the factors of the recalcitrance of the lignocellulose biomass, hence the CE15 represent an incredible potential for industrial lignocellulose degradation processes. Thus type of enzymes hydrolyse the ester bond between lignin alcohol and the 4-O-methyl-D-glucuronic acid side chain of xylan in plant cell walls (Sunner et al. 2015). The first CE15 enzyme was purified from the fungus *Schizophyllum commune* (Špániková and Biely 2006) and the majority of CE15 studies derive from saprotrophic fungi despite the fact that many bacterial species have genes encoding homologues of fungal enzymes (De Santi et al. 2016). The presence of these genes represent an opportunity to investigate the CE15 family enzymes and their potential participation in lignocellulose degradation.

Microbial communities and ecology-based biotechnology

Microbial communities dominate almost every habitat on the planet. From oceans to soil, plants and animal/human bodies, systems are populated with diverse microbial communities that play very important roles in global processes. These range from the key life supporting biogeochemical cycles, to processes involved in plant/animal/human health. The systems on Earth thus offer a rich reservoir of

microbial functions that can be harnessed to serve human needs, such as in the environmentally-friendly biotechnological approaches. As described by Jimenez et al. (2016) and Maruthamuthu et al. (2016) the application of selected microbial consortia, over single strains, for such biotechnological purposes presents important advantages.

One of them is that microbial consortia are able to perform complex tasks that single strains struggle to perform in, for example, lignocellulose degradation. The reason is that the deconstruction of complex substrates requires many different types of enzymes and diverse chemical reactions, all at the same time or at short time intervals, in the same system. Moreover, division of labor (Jimenez et al 2017), resilience to environmental fluctuations (resistance in variation substrate composition) and resistance to microbial invasion play major roles (Pandhal and Noirel 2014; Mallon et al. 2015).

Ecology-based biotechnology

Usually, biotechnological processes that produce compounds and/or enzymes employ pure cultures of microorganisms that have been industrially bred for efficiency of growth and production. However, the use of single strains has clear limitations with respect to versatility. Hence, an alternative approach is offered by the application of ecology-based biotechnology (eco-biotechnology). Eco-biotechnology is the combination of the principles of microbial ecology with the purposes of industrial biotechnology. Hence, it is based on the application of ecological principles for the construction of cultures that are optimized for the production of metabolites and/or enzymes. The fundaments of this approach lie in the natural selection – via competition – leading to optimized efficiency of growth and production, rather than on genetic or metabolic engineering. In this, selective pressure for the desired metabolism is applied to an initially diverse microbial inoculum by choosing the substrate and operating conditions. Thus, the ecosystem conditions ' reign organisms' evolution towards an optimized process, via the Darwinian 'survival-of-the-fittest' principle (Johnson et al. 2009). Here, in the light of the complexity of the LCB, survival of the fittest may encompass the fittest collaborating group rather than single organism.

Thus, agricultural waste residues, for example wheat, grass or sugarcane are posited to be better degraded by consortia rather than pure cultures. Moreover, the use of mixed cultures may overcome problems of substrate specificity, product inhibition and low yields. Eco-biotechnology has already been used for

the optimal production of cellulases applying a mix culture of *Bacillus aerius*, *Bacillus anthracis*, *Cellvibrio japonicus* and *Klebsiella pneumoniae* to a mix of lignocellulose substrate (palm oil and rice straw) (Oke et al. 2016). Moita et al. (2014) demonstrated the feasibility of production of polyhydroxyalkanoates by applying a microbial consortia and using glycerol, a waste substrate without any pretreatment, as a sole carbon source. The results allow the development of an more efficient production process.

Habitat biasing and enrichment cultures

Enriched microbial consortia have already been employed in LCB degradation, and good efficiencies have been achieved (Wongwilaiwalin et al. 2010). In addition to the number of enzymes produce and greater microbial stability, a key advantage of microbial consortia lies on the fact that strain isolation by using conventional plating, which represents a complicated and time-consuming process, is avoided. Moreover, given that only a small fraction of existing microbes in complex habitats like soil and sediment can be cultured, the isolation of LCB degrading microbes is not always successful. Some of the strategies used to develop microbial consortia rely on habitat biasing and enrichment cultures.

Enrichment cultures – also called habitat biasing (Ekkers et al. 2012) – is a strategy in which a deliberate bias is introduced in an environmental sample to modulate the microbial community structure in a particular direction (*in situ* or *ex situ*). For instance, adding a selective medium will result in an enrichment of a target microbial community that is able to use the specific substrate present in the medium. This will increase the required functions in the selected microbiome, and thus the genes or operons of interest (Cretoiu et al. 2012; Jiménez et al. 2014). Haruta et al. (2002) obtained a stable and complex lignocellulolytic microbial consortium from successive enrichment cultures on rice straw compost. The consortium had a high activity on various cellulosic materials, including rice straw, paper and cotton. Along the same line, Wongwilaiwalin et al. (2010) presented an analysis of a structurally stable lignocellulose degrading microbial consortium together with the characterization of its lignocellulolytic enzyme systems. The consortium was deemed to be applicable for biomass degradation and conversion in the biotechnological industry. In our group, Jimenez et al. (2014), by using dilution stimulation-approach, obtained a microbial consortia capable to consume torrefied wheat straw as single carbon source, as well as, grow in presence of 5-hydroxymethylfurfural.

There are several approaches to produce LCB-grown enrichment cultures, and the selection of the enrichment technique depends on the specific purpose and conditions of the experiment. Some approaches and examples can be found in Table 3. Lee et al. (2013) provide an in-depth discussion of the enrichment approaches, their challenges and further work.

Table 3. Different enrichment approaches yielding different microbial consortia.

Enrichment	Approach	Example	Reference
Dilution-to-extinction	The original cultures is diluted until the desire activity is eliminated	Cellulose conversions to hydrogen	Wang et al. (2010)
Concentration-to-extinction	Increase of the concentration of the particular compound for selection of desired functional strains.	Degradation of contaminant	Chen et al. (2010)
Toxicity-to-extinction	A high concentration of a compound is added as a selective force, which will cause a long lag period until adaptation of the microbial community	Obtaining minimal functional consortium from activated sludge, the resulted consortium was able to remove individually meta-, para-, and ortho-cresol	Ho et al. (2010)
Heat-to-extinction	The selective pressure is the temperature.	Obtain a functional consortium from soil from hydrogen production	Zhang et al. (2008)
Hydrodynamics-to-extinction	Hydrodynamic selection pressure is applied through step-wised decrease of the hydraulic retention (HRT).	The microbial community had not varied significantly with the acclimation time but was highly affected to the HRT	Zhang et al. (2006)
Dilution-to-stimulation	Dilution the inoculum in succession, avoiding lose the function by checking along the enrichment process.	Selection of (hemi)cellulolytic bacteria. Lignocellulose degradation	Ho et al. (2012), Jiménez et al. (2014)

Dilution-to-stimulation approach

In this approach, an inoculum is introduced into an appropriate substrate in fresh medium. Following incubation, aliquots are sequentially transferred to new fresh medium with the substrate. Thus, in each transfer presumably the most efficient members of the consortium would be selected. The final culture would contain the most functional organisms while non-functional ones are presumably eliminated or reduced. The result would be an optimized consortium of reduced diversity capable to efficiently consume the substrate (Ho et al. 2012; Lee et al. 2013).

To use this type of enrichment technique, two main criteria have to be fulfilled. First, the original inoculum should be highly effective with respect to the target function to incorporate sufficient strains of interest. Second, the key functional organisms should be enriched to high densities and the constituent cells should be in a well-dispersed state so that serial dilution can preserve them, whilst removing non-functional strains.

The main advantage of the dilution approach is the reduction of the complexity of the community and the increased abundance of microorganisms capable to utilize the substrate of interest. Thus, analyses of the population composition and metabolic pathways can be carried out with a dramatic impact on the time length of the experiment. Moreover, this approach also lead to microbial communities that are highly efficient and stable – demonstrated by their tolerance to being sub-cultured several times in medium with and without cellulosic material (Haruta et al. 2002). Table 4 shows examples of microbial consortia able to degrade lignocellulose biomass. This high efficiency and stability is a reflection of the interactions between microbial members of the consortium.

Microbial interactions

Microbes in nature live in large communities, in these, their behavior is characterized by complex interactions with abiotic factors (environment) and biotic ones (other organisms). These interactions can be intraspecific, involving individuals of the same species, and/or interspecific, i.e. between individuals of different species (Ghosh et al. 2016). Dependency between the members within the community (or interdependency) can be large and dominating a system. A presumably large interdependency of microorganisms might be reflected in the fact that, very often, less than 1% of microbial life can be cultivated in the laboratory, presumably due to many bacteria depending on others for growth. (Pandhal and Noirel 2014).

Table 4. Examples of lignocellulose degrader microbial consortia, enriched from a variety of sources and substrate.

Source	Substrate	Final microbial composition	Condition	Reference
Feces mixtures	Filter paper, printing paper, cotton, and rice straw	*Clostridium* sp., uncultured *Betaproteobacterium*, *Brevibacillus* sp., *Pseudoxanthamonas* sp.	Facultative anoxic	Haruta et al. (2002)
Soil	Raw maize powder and filter paper	*Ralstonia* sp., *Clostridium* sp., uncultured *Firmicutes*, *Propionibacterium acnes*, uncultured *Betaproteobacterium*, *Pantoea* sp.	Facultative anoxic	Feng et al. (2011)
Bagasse compost	Bagasse, rice straw, and corn stover	8 major groups including *Clostridium* sp., *Thermoanaerobacterium* sp., *Rhodocyclaceae* sp.	Facultative anoxic	Wongwilaiwalin et al. (2010)
Variety of lignocellulosic substrates	Bermuda grass	*Pseudoxanthomonas byssovorax*, *Microbacterium oxydans*, *Bacillus* sp., *Ochrobactrum anthropi*, *Klebsiella trevisanii*	Oxic	Okeke and Lu (2011)
Forest soil	Wheat straw/wheat straw torrified	*Species* from *Raoultella*/*Klebsiella*, *Kluyvera*, *Citrobacter*, *Enterobacter*, *Pseudomonas putida*, *Acinetobacter calcoaceticus*, *Flavobacterium johnsoniae* and *Arthrobacter intermedia*	Oxic	Jiménez et al. (2014)
Forest soil	Wheat straw, corn stover, switch grass	*Sphingobacterium kitahiroshimense*, *Raoultella terrigena*, *Pseudomonas putida*, *Stenotrophomonas rhizophila*, *Coniochaeta ligniaria* and *Acremonium* sp. were present in at least three treatments.	Oxic	Brossi et al. (2015)
Soil, decaying tree and sediment	Wheat straw	*Sphingobacterium multivorum*, *Citrobacter freundii*, *Acinetobacter calcoaceticus*.	Oxic	Cortes-Tolalpa et al. (2016)

Types of interactions

Microbes present two main basic types of interactions: Cooperative (positive) and non-cooperative (negative). Cooperative interactions include mutualism and commensalism. In mutualism, both organisms benefit from the interaction. There are two types of mutualism, i.e. obligatory and facultative mutualism. In facultative mutualism, although both organisms benefit from the interaction, they can exist in separate pure culture (Jagmann and Philipp 2014). In obligatory mutualism, the organisms involved are not able to perform a specific action when separated from the interaction partner. In commensalism, an organism benefits from the interaction with another one, while the other one is neither positively nor negatively affected. For example, in biodegradation commensals may feed on compounds that are released by the indifferent partner organism (Germerodt et al. 2016).

In non-cooperative interactions, parasitism, amensalism and predation are included. A parasitic interaction is established if the recipient (the 'beneficiary') forces the producer to release a metabolite that it can use (Jagmann and Philipp 2014). In predation, an organism (the prey) is consumed by another one (the predator). For example, ciliates feeding on bacteria (Mitri and Foster 2013). In competition, two or more organisms occupy the same niche, which means that both are able to consume the same resource (Dolinšek et al. 2016) and so – according to ecological theory – one ultimately outcompetes the other one (Gause 1934). Competition for resources is probably the most frequent interaction in the microbial world, and it has strong consequences for enrichment or habitat biasing approaches.

However, in a generic sense, all of the aforementioned interactions may play roles in nature and they generally occur together in any ecosystem, among prokaryotic and eukaryotic organisms coexisting there. In mechanistic terms, interactions in the microbial world may occur by the transfer of molecular (and/or genetic) compounds or information. Examples are seen in the exchange of metabolites, metabolite conversion, signaling compounds, resulting in chemotaxis. Moreover, genetic exchanges can also take place (Ghosh et al. 2016). The exchange of compounds is key in microbial communication, in particular quorum sensing (QS), which enables a population to collectively regulate the gene expression in response to host and/or environmental signals, produced by the same or even by different species. Examples of microbial communication like QS are exchanges of secondary metabolites, production of siderophores, biofilm formation and cellular transduction signaling (Mesquita Braga et al. 2016).

Interactions in selected microbial consortia

As established in the previous sections, the use of microbial consortia for biotechnological purposes represents advantages over the classical use of monocultures. To be able to explore these advantages, it is important to consider the interactions between the microorganisms in question.

However, identification of specific interactions between microbes in a complex consortium is a difficult task, as it is highly likely that different interactions happen simultaneously (Pandhal and Noirel 2014). Application of synthetic microbial consortia could be a solution to this problem, as such consortia are usually composed of few well-identified organisms. Sometimes, those species do not co-inhabit the same environment, but their metabolic activities may be combined (Jagmann and Philipp 2014). Determination of nutrient utilization, metabolite production, exchange of metabolites, production of signal molecules, spatial distribution, genome sequence and population dynamics are fundamental to enhance our understanding of the interactions. The knowledge could be applied to extend the stability of the consortia and improve the process efficiency (Ghosh et al. 2016).

Microbial consortia are ideal for the conversion of LCB substrates by providing the complex metabolic functions necessary for efficient polymerization. In previous work, microbial consortia capable to efficiently consume wheat straw have been produced (Jimenez et al. 2014). In these studies, bacteria members of the final specialized communities were isolated and identified, like *Raoultella/Klebsiella, Enterobacter amnigenus, Arthrobacter intermedia, Citrobacter*, *Pseudomonas putida*, as well as the fungi species *Penicillium citrinum* and *Coniochaeta ligniaria*. However, although their metabolic degradation capacity was tested, the main drivers of the selection remain unknown. Also, we do not know how efficient the process was in the end and it is likely that the efficiency was low, leading to the accumulation of remaining parts of hemicellulose and lignin.

Despite the large number of studies focusing on developing microbial consortia for LCB degradation, a few questions remain unsolved, and answering them might be key to improving LCB degradation. First, a key question pertaining is related to the paradigm of Martinus Beijerinck (1922): 'Everything is everywhere and the environment selects' – i.e. to what extent different inocula when bred on the same substrate under similar conditions will yield a similar final consortium make-up? Furthermore, would the outcome of such enrichment be driven by phylogenetic or functional attributes? In other words, will similar or highly

different collaborating organisms appear in final consortia bred from different inocula on the basis of highly similar enrichment conditions? Alternatively, if the same inoculum is used to develop consortia on different LCB are the selective forces acting on the same inoculum be different across the suite of LCB materials – i.e. will the microbial consortia bred be similar? Second, what would happen with the composition of consortia if we add an extra stress factor like high salinity or increased recalcitrance substrate? Lastly, the complexity of the lignocellulose substrates makes synergistic actions of the degraders indispensable, thus the key question relates to understanding the mechanisms driving microbial interaction during LCB degradation.

Aim of this thesis

The aim of this thesis is to generate a better understanding of the key driving forces in the selection of specialized lignocellulolytic microbial consortia. Moreover, I aimed to gain fundamental knowledge about microbial cooperative interactions in the LCB degradation process. Such information is indispensable for the design, optimization and future application of degrader synthetic consortia.

General research questions of this thesis

- Do different lignocellulose sources (wheat straw, switch grass and maize) influence the composition of the degrader consortia as well as the degradation capacity? (Chapter 2)
- How does microbial source influence the final lignocellulose degrading consortia? (chapter 3)
- What organisms are key to the degradation process and how do they function? (Chapters 2 and 3)
- Which is the influence of high salinity levels on lignocellulose degrader consortia? Is it possible to obtain a viable degrader consortium using a highly recalcitrant substrate (pre-digested wheat straw) as the single carbon and energy source? How will the complexity of the substrate influence the composition of the final consortia? (Chapter 4)
- Is there any cooperative relationship between selected abundant lignocellulose degraders from the consortia? Is the cooperative interaction depending of the compositional structure of the carbon source? (Chapter 5)
- What main genome features can be highlighted to illustrate the cooperative behaviour of microbial degraders? (Chapter 6)

General hypotheses underlying this thesis

1. Given the overall similarity in the composition of the lignocellulose substrates, consortia built on different substrates but using the same microbial inoculum will present a very similar microbial composition.
2. Different microbial sources used as inocula on the same LCB substrate will, under highly similar conditions, generate phylogenetically-different but functionally similar enriched consortia, due to microbial functional redundancy.
3. In order to develop salt tolerant LCB degrader consortia, salt marsh soil can be used as inoculum source.
4. Substrate made highly recalcitrant to degradation by pre-digestion will allow the selection of highly-specialized microbial consortia that are capable of transforming the most recalcitrant part of the substrate into utilizable resources.
5. Given its spatial complexity, wheat straw promotes cooperative relationships between microbial degrader strains. Such cooperation is built on exchanges of compounds or enzymes.
6. The analysis of the genomes of two synergistic degrader strains provides fundamental knowledge that fosters our understanding of the cooperative relationships in degrader consortia. Such interactions are observed exclusively in organisms growing on complex carbon sources.

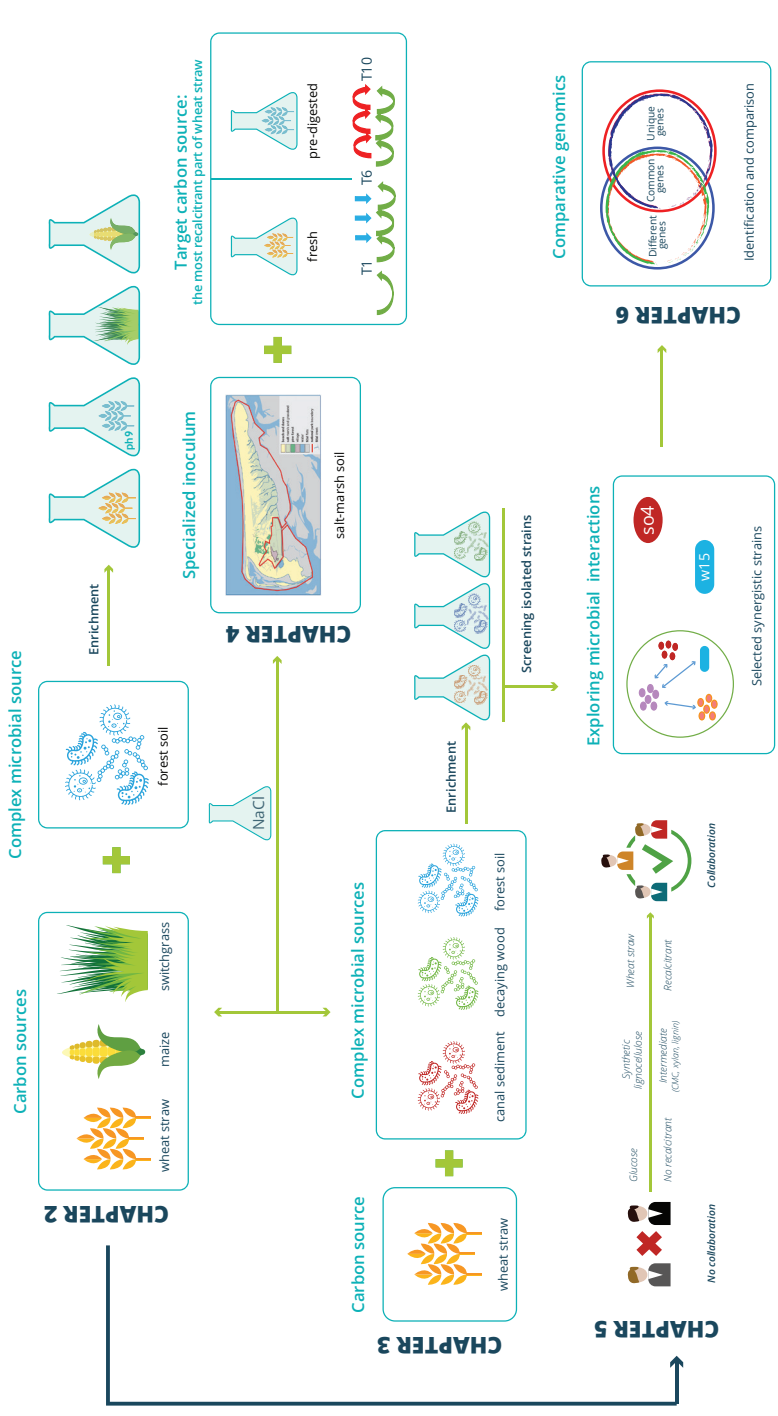

Figure 7 Work flow of this thesis. **The diagram shows the interconnection between the chapters in this thesis.** Each chapter corresponds to one general hypothesis.

Thesis outline

Chapter 2 explores the effect of the use of different lignocellulose substrates (wheat straw, switch grass and maize) and the same inoculum source (forest soil) on the selection of enriched LCB degrading microbial consortia. To determine whether the consortia is phylogenetically or functionally similar, I characterized the structure and composition of selected consortia obtained across the enrichment by using 16S rRNA and ITS based PCR-DGGE; I also identified potential lignocellulose degrading strains in the final bacterial consortia and determined the degradation potential of the consortia using infrared fourier-transform infrared spectroscopy (FT-IR). Microbial strains were recovered, identified and tested for potential lytic activity. A group of bacteria were presented in three of the four enrichments, they were *Sphingobacterium kitahiroshimense*, *Enterobacter amnigenus*, *Raoultella terrigena*, *Pseudomonas putida* and *Stenotrophomonas rhizophila,* as well as the fungi strains *Coniochaeta ligniaria* and *Acremonium* sp. All the strains in the core presented CMC-ase and xylanase activity. The data revealed that the substrate type importantly determine the final composition of the consortia.

Chapter 3 explores the importance of the inoculum source (forest soil, canal sediment and decaying wood) in the selection of microbial degrader consortia. I addressed this question by verifying (1) the structures of the lignocellulose degrading consortia produced after ten sequential enrichments on raw wheat straw, next to (2) the degradation potential of the substrate by these microbial consortia. Across the enrichments, the selected consortia were characterized by analyses of the structure of bacterial and fungal communities by using 16S rRNA gene and ITS based PCR-DGGE, respectively. I also evaluated the bacterial community composition of the final consortia by using 16S ribosomal RNA amplicon sequencing. Similarly, to **Chapter 2**, microbial strains were recovered, identified and tested for potential lytic activity. Identification of the most abundant members of the community reveled a bacteria core (common between the three final consortia) which was composed by species of *Sphingobacterium*, *Citrobacter, Acinetobacter* and *Flavobacterium* or *Chryseobacterium*. The fungal strains were consortia-derived specific, however, as the **Chapter 2**, *Coniochaeta ligniaria* and *Acremonium* sp. were also found. Determination of lignocellulose degradation by the microbial consortia revealed that hemicellulose was the most consumed part of the substrate. I further obtained isolates of the most abundant and potential most relevant organisms in the consortia, i.e. *Sphingobacterium*

multivorum, *Flavobacterium ginsengisoli, Chryseobacterium taihuense*, which lytic activities were remarkably high. The data revealed that the final composition was strongly influence by the initial inoculum.

Taking into account the importance of the substrate composition (**Chapter 2**) and the relevance on inoculum selection (**Chapter 3**) in the selection of a grader microbial consortia, in **Chapter 4** I explored the potential of using salt-marsh soil as inoculum for the production of microbial consortia capable of using wheat straw (as the sole carbon source) under highly saline conditions. Furthermore, I studied the possibility of increasing the recalcitrance of the substrate and how this increment in recalcitrance affects the composition of the microbial consortia. This was achieved by feeding fresh substrate to the consortia in the first part of the enrichment (transfers 1-6), whereas from transfers 7 onwards, I replaced fresh by pre-digested substrate, i.e. substrate that was previously degraded by the consortia. Briefly, the pre-digest substrate caused a notorious shift in the bacteria composition with a more strike effect on the fungi communities. Analysis of degradation capacity of the microbial consortia showed that enriched consortia bred with pre-digested substrate could degrade better cellulose and lignin than those selected in fresh substrate. Key cultivable degrader bacteria and fungi were also recovered, identified and tested for lytic activity. The most dominant bacteria in the consortia were *Joostella marina*, *Flavobacterium beibuense*, *Algoriphagus ratkowskyi*, *Pseudomonas putida* and *Halomonas meridiana*. The selected final consortia are a potential source of hydrolytic enzymes specialized on recalcitrance lignocellulose substrate and capable to work under saline condition.

Considering the potential role of microbial interactions in the degradation of complex polysaccharides, in **Chapter 5** I explored the collaboration capacity of selected microbial degrader strains recovered in chapter 3. First, the strains were screening for their ability to growth on wheat straw as a single carbon source as well as the production of hydrolytic enzymes. Then, using minimal synthetic consortia with selected degrader strains, I examined their interactivity on carbon sources with different levels of "recalcitrance". Monocultures and co-cultures were tested for growth and secretion of lytic enzymes, when growing in: (1) the simplest carbon source (glucose), (2) synthetic lignocellulose substrate (carboxymethyl cellulose, xylan-beechwood and lignin) and (3) wheat straw. Recalcitrance of the substrate was positively related to the microbial interactions, indicating that recalcitrance increases the cooperative relationship between the microbial species.

The synthetic community developed in **Chapter 5** showing the highest level of synergistic interaction was used, in **Chapter 6**, as model the study of microbial interactions triggered by the complexity of the substrate. In order to understand the functional complements of the two collaborating bacterial species, I sequenced the genomes of *Sphingobacterium multivorum* w15 and *Citrobacter freundii* so4. I then compared the genomes by focusing on the lignocellulolytic arsenal across these two strains. I joined the genome analyses and the physiological data and used them propose a possible mechanism for lignocellulose degradation in the context of this collaborative pair, which is influenced by the complexity of carbon source.

Chapter 7 discusses the findings of this thesis and explores the avenues to future work.

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Chapter 2

Soil-derived microbial consortia enriched with different plant biomass reveal distinct players acting in lignocellulose degradation

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Abstract

Here, we investigated how different plant biomass, and—for one substrate—pH, drive the composition of degrader microbial consortia. We bred such consortia from forest soil, incubated along nine aerobic sequential batch enrichments with wheat straw (WS1, pH 7.2; WS2, pH 9.0), switchgrass (SG, pH 7.2), and corn stover (CS, pH 7.2) as carbon sources. Lignocellulosic compounds (lignin, cellulose and xylan) were best degraded in treatment SG, followed by CS, WS1 and WS2. In terms of composition, the consortia became relatively stable after transfers 4 to 6, as evidenced by PCR-DGGE profiles obtained from each consortium DNA. The final consortia differed by ~40 % (bacteria) and ~60 % (fungi) across treatments. A 'core' community represented by 5/16 (bacteria) and 3/14 (fungi) bands was discerned, next to a variable part. The composition of the final microbial consortia was strongly driven by the substrate, as taxonomically diverse consortia appeared in the different substrate treatments, but not in the (WS) different pH one. Biodegradative strains affiliated to *Sphingobacterium kitahiroshimense, Raoultella terrigena, Pseudomonas putida, Stenotrophomonas rhizophila* (bacteria), *Coniochaeta ligniaria and Acremonium* sp. (fungi) were recovered in at least three treatments, whereas strains affiliated to *Delftia tsuruhatensis, Paenibacillus xylanexedens, Sanguibacter inulus* and *Comamonas jiangduensis* were treatment-specific.

Introduction

Wheat straw (WS), corn stover (CS), and switchgrass (SG) constitute excellent sources of lignocellulose with high potential for the production of useful compounds such as biofuel, polyolefin-based plastics and lactic acid. Lignocellulose is mainly composed of cellulose, hemicellulose, lignin and pectin (Demain et al. 2005; Mabee et al. 2005). Its composition in plant matter can vary according to plant type, and even within plant species (Amarasekara 2013), which affects its bioconversion (Hames et al. 2003; Hamelinck et al. 2005). Thus, one may surmise that WS, CS, and SG substrates potentially require diverse specialized combinations of microorganisms for its deconstruction (Christian et al. 2001; Sun et al. 2005; Energy 2006; Mani et al. 2006). It is currently accepted that proper biodegradation of lignocellulosic substrates requires a complex set of enzymes. Thus, peroxidases, laccases, endoglucanases, exoglucanases, β-glucosidases, fucosidases and xylanases (Wong et al. 1988; Lynd et al. 2002; Zhang and Lynd 2004), among other enzymes, may be required in different and fluctuating amounts and proportions. Moreover, cultures from pure isolates have often demonstrated unsatisfactory biodegradation rates (Koullas et al. 1992; Tae and Lee 2005). Hence, recent work has focused on plant biomass degradation by microbial consortia on the premise that the expected diversity of the microbially – secreted enzymes will result in efficient degradation rates (Schwarz 2001). The microbial groups involved may even be interdependent, with each one exerting distinct functions, the sum of which is synergistic for the process (Lee et al. 2013). And, as a result, the microbial consortia may also better withstand physiological fluctuations. Examining the microbial consortia bred on lignocellulosic plant biomass is useful for (1) understanding (2) designing, and (3) testing superior biodegradative agents (Zuroff and Curtis 2012).

To produce such superior microbial consortia, the dilution-to-stimulation approach, which uses sequential batch enrichments on the same substrate, is indicated (Ho et al. 2012), as it allows to establish stable microbial consortia with desirable biodegradation properties (Lu et al. 2005; Chen et al. 2009; Jiménez et al. 2014). Effective consortia can be readily derived from a source community from forest soil (Deangelis et al. 2011; Jiménez et al. 2014). However, in the light of the richness of lignocellulose biodegradative capacities in forest soil, it is important to assess to what extent the choice of lignocellulosic substrates (e.g., WS, CS, or SG) directs the assembly of efficient degrading microbial

consortia. Here, we hypothesized that, given the overall similarity in substrate composition, microbial consortia with largely similar structures will be produced from one source community in a sequential batch dilution-to-stimulation approach. However, an alternative hypothesis postulates that such communities are bound to be different in the light of the—possibly subtle— differences in substrate composition. Following these two divergent lines of reasoning, the main objectives of this study were (1) to produce effective microbial consortia on the aforementioned three substrates and (2) to test whether these diverse substrates (next to variation in pH for the WS treatment) drive the establishment of different (or similar) microbial lignocellulose-degrading consortia.

Materials and methods

Plant biomass preparation

We collected approximately 3 kg of each plant biomass—i.e., wheat straw, switchgrass and corn stover—in local farms in Groningen, The Netherlands. Each plant biomass raw material was transported to the laboratory (<24 h) at room temperature (20°C) for further processing. The raw material was air-dried at 50°C for 24 h before grinding using a hammer mill, yielding pieces <1 mm.

The experimental design encompassed three different treatments with respect to the plant biomass used (a proxy for different carbon sources), next to one (with WS), in which pH was varied as follows: wheat straw (WS1), switchgrass (SG), and corn stover (CS)—all maintained at pH 7.2, and wheat straw (WS2) under a pH 9.0. All treatments were performed in triplicate flasks *(n*=3*)*, and all flasks were kept under the same conditions along the whole experiment to avoid bias.

Serial batch enrichment cultures using the dilution-to-stimulation approach

Ten randomly taken soil samples of 10 g were collected from a forest soil (0 to 10 cm depth) in Groningen, the Netherlands (53.41 N; 6.90 E) in September, 2013 (before leaf abscission).

These samples were mixed to produce one representative soil sample to be used as the source inoculum for all treatments. The soil sample was transported to the laboratory at room temperature (20°C) for further processing (<24 h). Cell suspensions were prepared by adding 10g of the mixed soil to an Erlenmeyer flask (250 mL) containing 10 g of sterile gravel in 90 mL of 0.9 % saline (NaCl).

The flask was shaken for 20 min at 250 rpm, and 3 mL of cell suspension was then sampled and frozen (−20°C) for total DNA extraction. Moreover, aliquots (150 µL) of the cell suspension were added to triplicate flasks containing 15 mL of mineral salt medium (MSM), pH 7.2 for treatments WS1, SG, and CS and pH 9.0 for treatment WS2, with 1 % of the respective lignocellulosic substrates; all flasks were supplemented with 15 µL of standard trace element and vitamin solution. For a detailed description of this method, see Jiménez et al. (2014). Subsequently, flasks were incubated at 28°C with shaking at 150 rpm. Two controls, i.e., one without substrate and one without microbial source (for all substrates) were also set up. Cultures were monitored for growth at regular times, and once the systems reached high cell density (10^7–10^8 cells mL^{-1}) (between 5 and 6 days), aliquots (15 µL) were transferred to 15 mL of fresh medium (lignocellulose source in MSM supplemented with vitamins and trace elements) thus giving a dilution of 10^{-3}. This procedure was repeated nine times, giving in total nine enrichments. Cell counts were obtained by microscopy using a *Bürke-Turk* chamber (Blaubrand®) according to a standard protocol. The quantification was done directly after the transfer and at the end of growth in each transfer. The pH values of all treatments were regularly monitored and revealed to be largely stable along the incubation period.

Finally, samples were taken from the consortia, at the end of growth in each transfer, being one 2-mL aliquot (from each flask *n*=3) for DNA extraction and another 1-mL aliquot stored in glycerol (25 %) at −80°C.

Total DNA extraction and quantitative PCR

DNA extractions from the consortia were performed using 2 mL of each sample. The UltraClean Microbial DNA Isolation kit (MoBio® Laboratories Inc., Carlsbad, USA) was used according to the manufacturer's instructions. Bacterial and fungal counts were obtained by quantifying, respectively, the bacterial 16S rRNA gene (regions V5–V6) and the fungal first internal transcribed spacer (ITS1) region by quantitative PCR (qPCR) using 5 ng of extracted consortial DNA as the template and the primer sets 16SFP/16SRP and 5.8S/ITS1 (Pereira e Silva et al. 2012). Standard curves were constructed using serial dilutions of plasmids (1 to 8 log copies μL^{-1}) that contained cloned bacterial 16S rRNA gene and ITS1 fragments from *Serratia plymuthica* (KF495530) and *Coniochaeta ligniaria* (KF285995), respectively. Absolute quantification was carried out in three replicates on an ABI Prism 7300 Cycler (Applied Biosystem, Lohne, Germany). The bacterial and fungal abundances in the different samples were expressed as target gene copy numbers per milliliter. Statistical comparisons between the means were performed using one-way *ANOVA* (Tukey's test).

Substrate weight loss

At the end of transfers 6 and 9, the residual solid matter in the cultures was washed and dried as described in Du et al. (2015), after which the weight of the residual matter was measured and compared to a reference control treatment without the inoculum. The percentage of weight loss was defined as the ratio of the weight loss compared to the initial weight (%) as calculated by the following formula:

Substrate weight loss $(\%) = [(a - b) / c] \times 100$; where: a= residual control substrate weight; b= residual substrate weight; c= total substrate weight. Statistical comparisons of the samples' substrate weight losses were performed using one-way *ANOVA* of the means per treatment (Tukey's test).

Lignocellulosic composition of substrates and degradation rate

In order to determine the composition of each substrate and the degradation rate of their lignocellulosic components, we used fourier transformed infrared (FTIR) spectroscopy (Adapa et al. 2011). To do so, for all used substrates (i.e., WS, SG, and CS), we quantified the percentages of lignin, cellulose and hemicellulose (i.e., xylan from birchwood as the proxy) content before and after incubation (transfer nine). Prior to quantification, the material from the triplicates of each treatment (WS1, WS2, SG, and CS) was individually dried at 50°C for 24 h. Standard curves were determined using mixed components (i.e., lignin, cellulose and xylan) in eight different proportions (Table S1); this resulted in reference spectra and validation of the prediction of the lignocellulosic components. The compounds were measured using an FTIR spectrum machine (Thermo Fisher Scientific). The data were preprocessed using Savitzky–Golay differentiation (second derivative; polynomial order 2 and 31-point curve employed for each correction) in order to fit a polynomial regression to each successive curve segment. This generated smoothed curves (Savitzky and Golay 1964), followed a standard normal variate (SNV) to transform centers and scales of each individual spectrum (Dhanoa et al. 1994). After preprocessing, spectrum analyses were conducted, creating a partial least squares regression model using the standard curve, including an FTIR wavelength from 800 to 1800 cm^{-1} (FitzPatrick et al. 2012; Krasznai et al. 2012). The predictive model displayed R^2 values of 0.95, 0.96 and 0.97 for lignin, cellulose and hemicellulose, respectively. All quantitative values are expressed in percentages of each compound (lignin, hemicellulose, and cellulose) presented in each substrate. Data analyses were performed using the "Unscrambler" software

(CAMO Software, 2011).

Finally, degradation rates were determined, expressed as the ratio of the percentage of each component in the substrate after incubation compared to that before incubation as follows: Degradation rate (%) = $[(a - b) / a] \times 100$; where a= percentage of component in the substrate before incubation and b= percentage of component in the substrate after incubation. Statistical comparisons of the mean' degradation rates were performed using one-way *ANOVA* (Tukey's test).

PCR-DGGE analysis

Bacterial and fungal community structures were assessed by PCR-denaturing gradient gel electrophoresis (PCR-DGGE) of the total consortium DNA along transfers 1, 4, 6 and 9 (T1, T4, T6, and T9) in all treatments. Thus, PCR-DGGE enabled the evaluation of consortial development and stability during the enrichment process as well as the identification shifts among the final consortium profiles. The microbial consortia were considered to be stable when the community structures (for bacteria or fungi) presented a similar pattern along at least three sequential transfers. In order to provide taxonomic information of specific bands found in our DGGE patterns, we performed a co-migration analysis. Briefly, 16S rRNA gene sequences were amplified for key selected consortium strains (see later) using DGGE primers, after which the resulting amplicons were run in parallel with the consortium amplicons. Bands that co-migrated with consortium bands were considered to presumptively identify organisms in the consortium patterns.

DGGE was performed in the Ingeny Phor-U System (Ingeny International, Goes, The Netherlands). PCR was performed with primers F968-GC clamp and R1401.1b for the bacterial 16S rRNA gene. For fungal communities, primers EF4/ITS4 were used in the first PCR, which was followed by a second amplification with the primers ITS1f-GC/ITS2. Primer sequences, PCR mixtures and cycling conditions were used as previously described (Pereira e Silva et al. 2012). The DGGE was performed in 6% *(w/v)* polyacrylamide gels with 45–65 and 20–50% denaturant gradients for bacterial and fungal communities, respectively (100% is defined as 7 M urea with 40% deionized formamide). Electrophoresis was carried out at 100 V for 16 h at 60°C, and the gels were stained for 30 min in 0.5 % TAE buffer with SYBR gold (final concentration of 0.5 μg L^{-1}) (Invitrogen, Breda, The Netherlands). Images were taken using Imagemaster VDS (Amersham Biosciences, Buckinghamshire, UK). Fingerprinting results were analyzed using the GelCompar software (Applied Maths, Sint- Martens Latem, Belgium). The quantity of bands for each treatment

was considered as a proxy roughly reporting on phylotype richness. We avoided quantifying band intensities since it may introduce bias into the analyses according to differences obtained in DNA templates and/or PCR efficiencies. Thus, presence/absence of band patterns were converted to Jaccard dissimilarity matrices for non-metric multidimensional scaling (nMDS) followed by the analysis of similarities (ANOSIM) statistical analysis using Primer6 (PrimerE, Ivybridge, UK). The global *R* values, generated by ANOSIM, can range from −1 to 1; objects that are more dissimilar between groups than within groups are indicated by an *R* greater than 0; an *R* value of 0 indicates that the null hypothesis of no difference is true (Flores-Mireles et al. 2007).

Isolation and identification of bacterial and fungal strains

Bacterial and fungal isolates were obtained from transfer 9 of all treatments on R2A agar (BD Difco®, Detroit, USA) and potato dextrose agar (PDA) (Duchefa Biochemie BV, Haarlem, the Netherlands), respectively. Serial dilutions were performed in MSM, and 100 µL of dilutions 10^{-5} to 10^{-8} were spread on the surface of each medium. Bacterial and fungal colonies with different morphologies were subsequently subcultured (aerobically) to purity. Totals of 11, 8, 9 and 8 bacterial and 4, 3, 3 and 3 fungal strains were thus isolated from treatments WS1, WS2, SC and CS, respectively. Bacterial isolates were preserved at 4°C (on solid R2A medium) and −80°C (liquid R2A medium in glycerol 25%), while fungal ones were cut from the solid medium (25 mm^2 squares), after which they were preserved in distilled water at room temperature (Castellani method). The UltraClean Microbial DNA Isolation kit (MoBio® Laboratories Inc., Carlsbad, USA) was used according to the manufacturer's instructions for genomic DNA extractions. The bacterial 16S rRNA genes were PCR amplified using 5 ng of DNA and primers B8F and 1406R according to Taketani et al. (2010). For fungal strains, we amplified the partial 18S rRNA gene using primers EF4 and ITS4 according to Jiménez et al. (2014). PCR products were sequenced by Sanger technology (LGC Genomics, Germany) using the 1406R primer (for bacteria) and ITS4 primer (for fungi). All resulting chromatograms were analyzed for quality using the Lucy algorithm (RDP website; http://rdp.cme.msu.edu/). In this, quality trimming by removing bases with low scores was applied. The level of minimum requirement was 400 bp with quality above 20 (phred score—one error per 100 bases read). Taxonomic assignment of the sequences was done using BLAST-N against the National Center for Biotechnology Information (NCBI) database (http://blast.ncbi.nlm.nih.gov/Blast.cgi). Sequences are publicly available in the GenBank database under accession numbers KR935800 to KR935847.

Screening of strains for (hemi)cellulolytic activity

Screenings for (hemi)cellulolytic activity were done in mineral medium agar (MMA) (0.2% $NaNO_3$; 0.1% K_2HPO_4; 0.05% $MgSO_4$; 0.05% KCl; 1% of vitamin solution; 1.5% agar). We evaluated the growth (negative, weak and positive) of the strains in the presence of 0.2% glucose (positive control), 0.2% carboxymethyl cellulose (CMC; Sigma-Aldrich) to analyze cellulase activity and 0.2% xylan from beechwood (Sigma-Aldrich) to analyze hemicellulase (xylanase) activity. A drop (15 µL) of bacterial culture grown overnight (100 rpm at 25°C) was introduced on to agar plate. Fungal strains (agar plugs of $25mm^2$) were placed in the center of the agar plate. All assays were performed in duplicate using as a negative control MMA without a carbon source. The plates were incubated at 28°C for 36h and, after evaluation of growth, they were flooded with Gram iodine (Kasana et al. 2008) for the detection of CMC-ase and xylanase activity. We screened a total of 36 bacterial and 13 fungal strains. CMC and xylan degradation was indicated by detection of clearing zones (haloes) around the colonies. A cut-off value of more than 2.0 mm was considered as a positive result.

Results

Bacterial and fungal abundances along the sequential batch transfers

In all batches of all treatments, the initial population sizes revolved around ~10^5 bacterial cells mL^{-1}, and these increased to ~10^8 bacterial cells mL^{-1} during incubation. Invariably, the cell densities increased rapidly to ~10^7 to ~10^8 over the first 3 days of incubation, indicating the occurrence of a phase of rapid growth, which was followed by a slower increase to the final cell densities. This pattern was consistently observed across treatments and transfers. No growth was observed in the control treatments (i.e., no substrate with inoculum and no inoculum with substrates). Overall, the qPCR measurements revealed the copy numbers of the bacterial 16S rRNA gene to vary from 5.05 ±1.17x 10^8 mL^{-1} (CS) (mean±SD) to 9.22 ±0.21x10^8 mL^{-1} (WS1) after growth, whereas these were 1000-fold lower at the onset of each growth step. Thus, for all treatments, the bacterial densities reached rather similar maximum levels from similar initial levels (Fig. 1A). In contrast, the abundances of fungal propagules (after growth at each step) showed larger variation across transfers and treatments ranging from 6.94 ±3.84x10^5 (WS2) to 8.18 ±5.30x10^7 ITS1 copies mL^{-1} (WS1) (Fig. 1B). Remarkably, significantly higher numbers of fungal propagules were observed in the WS than in the SG and CS samples (*ANOVA*, P<0.05).

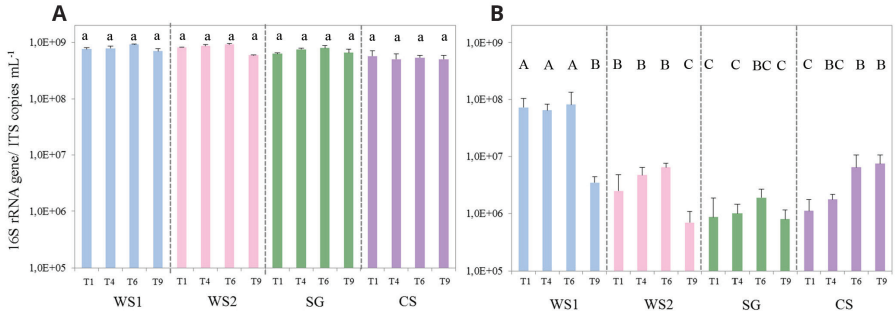


Figure 1 Copy numbers (y axis) of **(A)** ***Bacterial*** 16S rRNA gene and **(B)** ***Fungal*** ITS region across transfers **1, 4, 6 and 9 for all treatments.** Transfers and treatments are indicated on the x axis. Error bars represent the standard deviation of the means of three independent replicates. ***Different lowercase letters (a)*** refer to differences among the 16S rRNA gene abundances within treatments and ***uppercase letters (A-C)*** to differences among ITS1 region abundances across treatments (*ANOVA*, P<0.05). Abbreviations: ***WS1*** - wheat straw pH 7.2, ***WS2*** - wheat straw pH9.0, ***SG*** - switchgrass pH 7.2, ***CS*** - corn stover pH 7.2

Substrate weight loss

We evaluated substrate weight loss after microbial consortium development on the different plant biomass along transfers 6 (T6) and 9 (T9) through gravimetric determination of dry substrate. Following T6, substrate weight losses were minimally 36.05±0.04% (WS2) and maximally 42.06±0.06% (CS). These increased significantly at T9 when values of 42.04±0.06% (WS2, minimum) and 48.04±0.04% (CS, maximum) were found. The values were significantly different (*ANOVA*, P<0.01, Fig. 2).

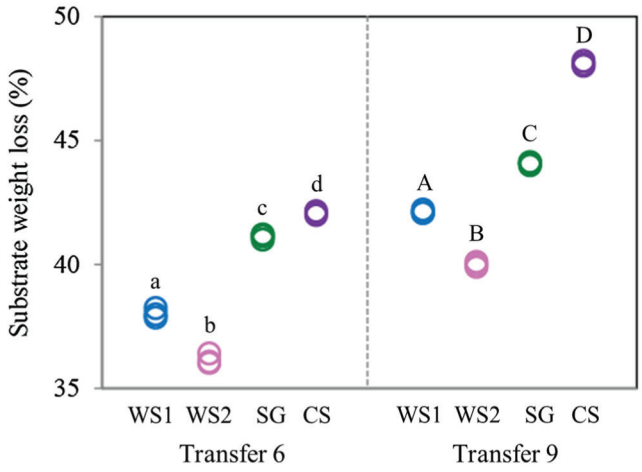


Figure 2 Substrate weight loss (%) of different substrates in the transfers 6 and 9. Different lowercase letters *(a-d)* refer to differences among treatments in T6 and uppercase ones *(A-D)* to differences among treatments, at T9 (*ANOVA*, P<0.01). Abbreviations: *WS1* - wheat straw pH 7.2, *WS2* - wheat straw pH 9.0, *SG* - switchgrass pH 7.2, *CS* - corn stover pH 7.2

Lignocellulosic composition of WS, CS and SG substrates and degradation rate

The composition of all plant matter in terms of lignin, cellulose and hemicellulose (xylan) was measured for all substrates. Moreover, we measured these parameters before and after consortial growth in transfer 9, allowing calculation of the degradation rate of these components (Table 1; Fig. 3). WS, CS and SG differed within limits with respect to the presence of the main measured components lignin, cellulose, and hemicellulose (Table 1). In terms of degradation by the T9 consortium, we found the highest lignin degradation rate in treatment

SG (39.32±4.04 %), whereas the highest degradation rate of cellulose occurred in treatment WS1 (51.92±0.41 %) and of hemicellulose in CS (62.79±4.69 %). Moreover, considering the total degradation of lignocellulosic components (i.e., lignin+cellulose+xylan), SG turned out to be the most efficiently degraded substrate (47.67±2.33 %), followed by CS (43.81±1.53 %), WS1 (43.40±0.69 %) and WS2 (38.60±2.29 %).

Table 1. Lignocellulosic composition (lignin, cellulose, and hemicellulose) of substrates*

Substrate	Lignin (%)	Cellulose (%)	Hemicellulose (%)
WS[a]	22.2±0.8	45.5±1.3	31.3±0.9
WS1[b]	18.0±1.0	21.9±0.6	16.1±0.7
WS2[b]	18.4±0.5	24.2±0.8	18.1±1.6
SG[a]	22.3±0.9	45.9±1.5	24.0±1.0
SG[b]	13.5±1.0	23.8±1.0	10.9±1.1
CS[a]	25.2±0.8	40.3±1.7	30.3±0.2
CS[b]	17.4±0.6	25.2±0.3	11.2±1.0

Abbreviations: ***WS1*** - wheat straw pH 7.2, ***WS2*** - wheat straw pH 9.0, ***SG*** - switchgrass pH 7.2, ***CS*** - corn stover pH 7.2
[a] Substrate before incubation
[b] Substrate after incubation
*Average and standard deviation of three replicates

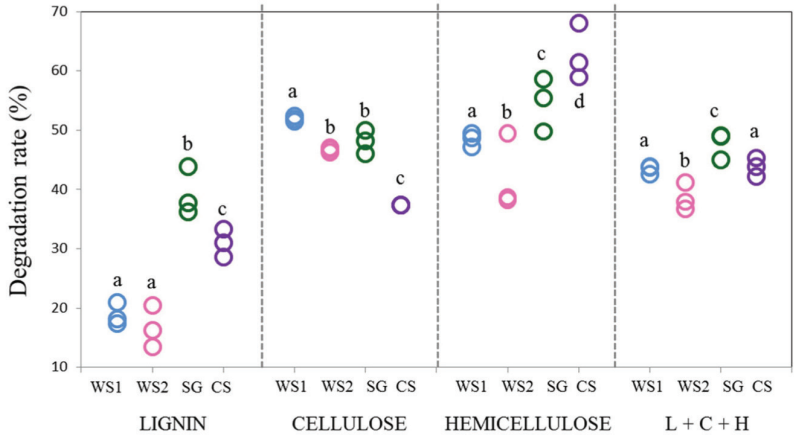


Figure 3 Degradation rates of lignocellulosic components of substrates in transfer 9. *Different letters (a-d)* refer to differences among the means of treatments (*ANOVA*, P<0.01). Abbreviations: ***L*** - lignin, ***C*** - cellulose, ***H*** - hemicellulose, ***WS1*** - wheat straw pH 7.2, ***WS2*** - wheat straw pH 9.0, ***SG*** - switchgrass pH 7.2, ***CS*** - corn stover pH 7.2

Analysis of microbial consortium structures by PCR-DGGE

Using total consortium DNA, bacterial 16S rRNA gene and ITS region-based PCR-DGGE analyses were used to evaluate the evolution of the community structures across the sequential batches per treatment (Fig. 3 and 4). The source inoculum contained a "cloud" of bands, estimated to encompass at least 60 bands in the bacterial fingerprints and >45 bands in the fungal ones. The data further showed that the triplicates of each treatment in each transfer consistently depicted similar communities per treatment, with reduced richness as compared to the source inoculum. This was true for both the bacterial and fungal communities.

For treatments WS1, WS2 and CS, the bacterial community fingerprints showed highest numbers of bands (here taken as proxies for the richness of dominant organisms) in the initial transfer, with decreases afterwards (from initially 13, 13 and 11 to finally 10, 11 and 8 bands, respectively) (Fig. 4 a, b, d). On the other hand, band numbers increased in the SG consortia from initially 8 to finally 13 bands along the transfers (Fig. 4 c). Stability in the community compositions was observed after transfer 6 in WS1, SG and CS and after transfer 4 in WS2 (Fig. 4 A–D). Fungal richness revealed a trend that was similar to that observed for bacterial richness along the transfers. In transfer 1, WS1, WS2, SG and CS showed 23, 23, 10 and 15 bands respectively, which declined to respectively 7, 6, 5 and 7 bands in transfer 9. This trend was consistent across the replicates (Fig. 4 e–h). The fungal community structures reached stability after transfer 6 in WS1 and SG and after transfer 4 for WS2 and CS (Fig. 4 E–H).

The T9 PCR-DGGE profiles were then compared across the treatments (Fig. 5). Cluster analysis of these profiles revealed ~40 and ~60% of differences across treatments for the bacterial and fungal consortia, respectively (Supplementary Fig. S1). Clearly, substrate type, next to pH for the treatments using WS, drove the bacterial community structures (Fig. 5A), these being partially variable and partially stable. Thus, a common core, consisting of five bands, was observed across all treatments (i.e., B1, B5, B6, B7 and B8; Fig. 4 (a)). Next to this core, another band was found to be common between treatments WS1 and WS2 (B3; Fig. 5 (a)) and yet another one between treatments SG and CS (B4; Fig. 5 (a)). Using co-migration analyses, we found that the core consortium bands B1, B5, B6, B7 and B8 were similar to those from the strains (see later) affiliated with *Sphingobacterium kitahiroshimense*, *Enterobacter amnigenus*, *Raoultella terrigena*, *Pseudomonas putida* and *Stenotrophomonas rhizophila*, respectively (Fig. 5a). Band

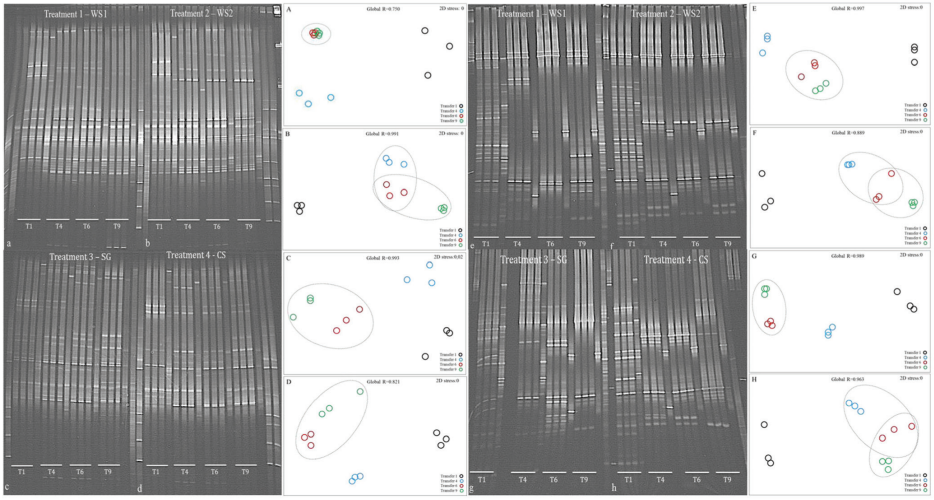


Figure 4 Community fingerprints (PCR-DGGE) of bacterial and fungal communities along transfers 1, 4, 6 and 9 on different substrates.(a) *WS1,* **(b)** *WS2,* **(c)** *SG* and **(d)** *CS*, for *bacterial communities* and **(e)** *WS1,* **(f)** *WS2,* **(g)** *SG* and **(h)** *CS*, for *fungal communities. A, B, C* and *D* represent nMDS and statistical analyses (*ANOSIM*; global R value) for bacterial communities in the different substrates and *E, F, G* and *H* for fungal communities in the different substrates. Abbreviations: ***WS1*** - wheat straw pH 7.2, ***WS2*** - wheat straw pH 9.0, ***SG*** - switchgrass pH 7.2, ***CS*** - corn stover pH 7.2

B2 was assigned to *Paenibacillus xylanexedens*, which was only present in the SG consortium. With respect to the fungal communities, substrate type also was a main factor driving the community structures. Treatments WS1 and WS2, which used the same substrate (wheat straw) under different pH values, incited similar fungal community structures (Fig. 5B). Three common bands, potentially reflecting the existence of a fungal core community, were observed in the final consortia across all treatments (F3, F4 and F5; Fig. 5b) next to a common one for treatments WS1 and WS2 (F1; Fig. 5 b) and another one for treatments SG and CS (F2; Fig. 5b).

Isolation of bacterial and fungal strains from enriched cultures

Totals of 36 bacterial and 13 fungal strains recovered from each treatment (Supplementary Fig. S2a, S2b) at T9 were presumptively identified by 16S rRNA gene (bacteria) and ITS1 (fungi) sequencing (Table 2). Specifically, 11, 8, 9 and 8 bacterial and 4, 3, 3 and 3 fungal isolates recovered from WS1, WS2, SG and CS respectively, were thus identified. The bacterial strains obtained from WS1 were affiliated (>99 % identity with NCBI database entries; number of strains indicated between parentheses) with *R. terrigena* (3), *S. kitahiroshimense* (3), *K. terrigen*a (1),

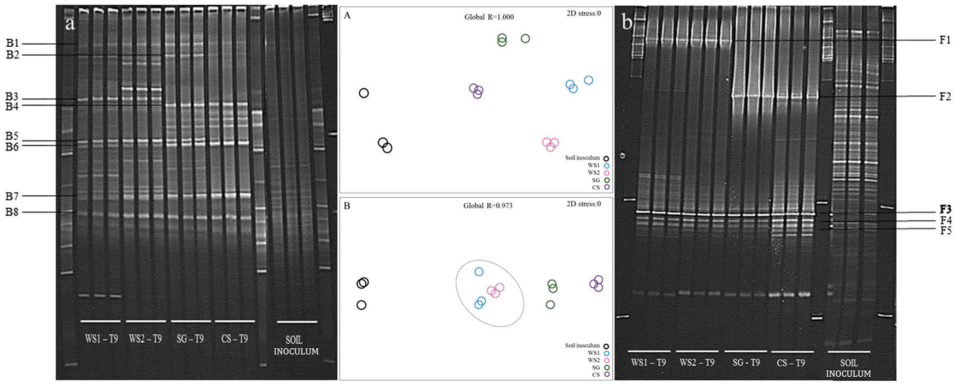


Figure 5 Community fingerprinting (PCR-DGGE) for (a) ***bacterial*** **and (b)** ***fungal*** **communities in the final consortia on different substrates and for the original soil inoculum.** ***A*** and ***B*** represent nMDS and statistical analyses (*ANOSIM*; global R value) for bacterial and fungal communities respectively. To details about B1 - B8 and F1 - F5, see text. Abbreviations: ***WS1*** - wheat straw pH 7.2, ***WS2*** - wheat straw pH 9.0, ***SG*** - switchgrass pH 7.2, ***CS*** - corn stover pH 7.2 T9 - transfer 9

P. putida (2), *S. rhizophila* (1), and *E. amnigenus* (1). Strains from WS2 were affiliated with *Pseudomonas putida* (2), *R. terrigena* (2), *S. kitahiroshimense* (2), and *S. rhizophila* (2). Treatment SG yielded strains affiliated with *S. kitahiroshimense* (2), *R. terrigena* (2), *E. amnigenus* (3), *P. xylanexedens* (1), and *D. tsuruhatensis* (1). Strains obtained from treatment CS were affiliated with *S. rhizophila* (2), *S. kitahiroshimense* (2), *P. putida* (1), *C. jiangduensis* (1), and *S. inulinus* (2). The isolated fungal strains for all treatments were affiliated (>95 % identity with NCBI database entries) with *C. ligniaria* and *Acremonium* sp. (Table 2; Fig. 6).

Bacterial and fungal (hemi)cellulolytic activities

In the light of their presumed dominance in the PCR-DGGE profiles, we tested the microorganisms affiliated with *S. kitahiroshimense*, *E. amnigenus*, *R. terrigena*, *P. putida* and *S. rhizophila*, next to *P. xylanexedens*, for their ability to deconstruct plant biomass. We thus tested (hemi)cellulolytic activity for these, next to other isolates (CMC-ase and xylanase). Twenty one bacterial strains derived from treatments WS1 (5/11), WS2 (6/8), SG (5/9), and CS (5/8), respectively, showed positive CMC-ase as well as xylanase activities. Indeed, the strains affiliated with *Sphingobacterium kitahiroshimense*, *R. terrigena*, *P. vranovensis*, *S. rhizophila* (bacteria), *C. lignaria*, and *Acremonium* sp. (fungi), presumably belonging to the microbial "cores", showed positive (hemi)cellulolytic activity. In addition, specialist isolates (*P. xylanexedens*, *S. inulus*, and *C. jiangduensis*) also showed CMC-ase and xylanase activity (Table 2 and Supplementary Fig. S2c; S2d).

Table 2. Taxonomic affiliation and enzymatic activity of bacterial and fungal isolates from transfers 9 of all treatments

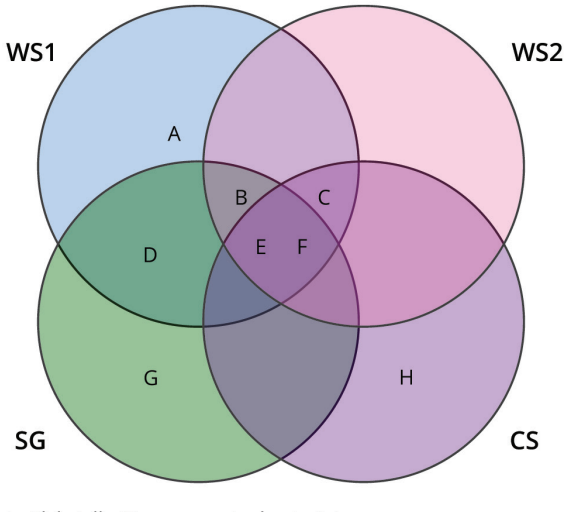
T	Isol	Identification	Cov %	Ident %	Access No.	Enz act
Bacteria						
WS1	1	*Raoultella terrigena* Rsh21 16S ribosomal RNA gene	100	100	KF796627.1	x
	2	*Sphingobacterium mizutani* Ht8-22 16S ribosomal RNA gene	99	99	JF899285.1	–
	3	*Raoultella terrigena* PSB15 16S ribosomal RNA gene	100	99	HQ242728.1	x
	4	*Klebsiella terrigena* 16S ribossonal RNA gene SW4 partial	100	99	Y17670.1	x
	5	*Sphingobacterium mizutani* Ht8-22 16S ribosomal RNA gene	100	99	JF899285.1	–
	6	*Sphingobacterium mizutani* Ht8-22 16S ribosomal RNA gene	99	99	JF899285.1	–
	7	*Pseudomonas putida* ATCC 17494 16S ribosomal RNA gene	100	100	AF094740.2	–
	8	*Pseudomonas putida* 214-D 16S ribosomal RNA gene, partial sequence	100	100	EF615008.1	–
	9	*Stenotrophomonas rhizophila* BG9 16S ribosomal RNA gene	100	100	KJ997741.1	x
	10	*Raoultella terrigena* PSB15 16S ribosomal RNA gene	100	99	HQ242728.1	x
	11	*Enterobacteram nigenus* h-14 16S ribosomalRNA gene	100	100	KC139434.1	x
WS2	12	*Pseudomonas putida* 214-D 16S ribosomal RNA gene, partial sequence	100	100	EF615008.1	-
	13	*Raoultella terrigena* PSB15 16S ribosomal RNA gene	100	100	HQ242728.1	x
	14	*Sphingobacterium kitahiroshimense* 10C 16S ribosomal RNA gene	100	99	NR_041636.1	x
	15	*Pseudomonas vranovensis* IBFC2012-27 16S ribosomal RNA gene	100	99	KC246044.1	x
	16	*Stenotrophomonas rhizophila* BG9 16S ribosomal RNA gene	100	100	KJ997741.1	x
	17	*Raoultella terrigena* RN16 16S ribosomal RNA gene	100	100	KC790281.1	-
	18	*Sphingobacterium kitahiroshimense* 10C 16S ribosomal RNA gene	100	99	NR_041636.1	x
	19	*Stenotrophomonas rhizophila* BG9 16S ribosomal RNA gene	100	100	KJ997741.1	x
SG	20	*Sphingobacterium kitahiroshimense* 10C 16S ribosomal RNA gene	100	99	NR_041636.1	x
	21	*Sphingobacterium kitahiroshimense* 10C 16S ribosomal RNA gene	100	99	NR_041636.1	x
	22	*Raoultella terrigena* Rsh21 16S ribosomal RNA gene	100	99	KF796627.1	x
	23	*Raoultella terrigena* Rsh21 16S ribosomal RNA gene	100	99	KF796627.1	x
	24	*Enterobacter amnigenus* h-14 16S ribosomal RNA gene	100	100	KC139434.1	-

		25	*Enterobacter amnigenus* h-14 16S ribosomal RNA gene	100	100	KC139434.1	-
		26	*Paenibacillus xylanexedens* JDG191 16S ribosomal RNA gene	82	99	JX035957.1	x
		27	*Delftia tsuruhatensis* LAM 29 16S ribosomal RNA gene	100	99	EU019989.1	-
		28	*Enterobacter amnigenus* h-14 16S ribosomal RNA gene	100	100	KC139434.1	-
	CS	29	*Sanguibacter inulinus* 16S ribosomal RNA: ST50 clone: NTS14	99	100	AB920571.1	x
		30	*Sphingobacterium faecium* Gen5 16S ribosomal RNA gene	100	99	KJ726588.1	-
		31	*Stenotrophomonas rhizophila* BG9 16S ribosomal RNA gene	100	100	KJ997741.1	x
		32	*Sphingobacterium anhuiense* CW 186 16S ribosomal RNA gene	100	99	NR_044477.1	-
		33	*Sanguibacter inulinus* 16S ribosomal RNA: ST50 clone: NTS14	100	100	AB920571.1	x
		34	*Pseudomonas alkylphenolia* KL28, complete genome	100	99	CP009048.1	-
		35	*Stenotrophomonas rhizophila* BG9 16S ribosomal RNA gene	100	100	KJ997741.1	x
Fungi							
	WS1	1	*Coniochaeta ligniaria* 2w1F 18S ribosomal RNA gene	93	99	KF285992.1	x
		2	*Coniochaeta ligniaria* 2w1F 18S ribosomal RNA gene	91	99	KF285992.1	x
		3	*Coniochaeta ligniaria* 2w1F 18S ribosomal RNA gene	91	99	KF285992.1	x
		4	*Acremonium* sp. 11665 DLW-2010 18S ribosomal RNA gene	98	96	GQ867783.1	x
		5	*Coniochaeta ligniaria* 2w1F 18S ribosomal RNA gene	92	99	KF285992.1	x
		6	*Acremonium* sp. 11665 DLW-2010 18S ribosomal RNA gene	98	96	GQ867783.1	x
		7	*Acremonium* sp. 11665 DLW-2010 18S ribosomal RNA gene	99	97	GQ867783.1	x
	SG	8	*Coniochaeta ligniaria* 2 t2.1 F 18S ribosomal RNA gene	94	96	KF285995.1	x
		9	*Coniochaeta ligniaria* 2w1F 18S ribosomal RNA gene	95	99	KF285992.1	x
		10	*Acremonium* sp. 11665 DLW-2010 18S ribosomal RNA gene	98	95	GQ867783.1	x
		11	*Coniochaeta ligniaria* 2w1F 18S ribosomal RNA gene	94	97	KF285992.1	x
		12	*Acremonium* sp. 11665 DLW-2010 18S ribosomal RNA gene	95	96	GQ867783.1	x
	CS	13	*Acremonium* sp. 11665 DLW-2010 18S ribosomal RNA gene	98	97	GQ867783.1	x
		36	*Comamonas jiangduensis* Amp3 16S ribosomal RNA gene	100	99	KJ726553.1	x

Abbreviations: ***WS1*** - wheat straw pH 7.2, ***WS2*** - wheat straw pH 9.0, ***SG*** - switchgrass pH 7.2, ***CS*** - corn stover pH 7.2, ***T***- treatment, ***Isol*** - isolate, ***Cov*** - coverage, ***Ident*** - identity, ***Access No***. access number, ***Enz. Act*** - enzymatic activity

Discussion

The development of efficient microbial consortia to deconstruct plant biomass is of great industrial interest. The biodegradation process involves a network of enzymatic transformations that requires timely production by microbial cells and



A: *Klebsiella (Gammaproteobacteria)*
B: *Raoultella (Gammaproteobacteria)*
C: Stenotrophomonas; Pseudomonas (Gammaproteobacteria)
D: Enterobacter (Gammaproteobacteria)
H: Sanguibacter (Actinobacteria);Comamonas (Betaproteobacteria)
E: Sphingobacterium (Bactereoides); Coniochaeta
F: Acremonium
G: Delftia (Betaproteobacteria); Paenibacillus (Firmicutes)

Figure 6 Venn diagram indicating unique and common bacterial and fungal strains across all treatments. Abbreviations: ***WS1*** - wheat straw pH 7.2, ***WS2*** - wheat straw pH9.0, ***SG*** - switchgrass pH 7.2, ***CS*** - corn stover pH 7.2.

extracellular availability. Moreover, stress conditions might be better endured by consortia than by single strains as a result of community interactions. In this study, different plant biomass sources were used to produce specific microbial consortia for lignocellulose degradation. The dilution-to-stimulation approach used worked well, as verified by observing the growth of bacterial cells in each step, which reached up to ~10^8 cells mL^{-1} after 5 to 6 days of incubation. Previous work from our lab (Jiménez et al. 2014)—using a similar approach to enrich lignocellulose degraders—observed that maximal cell densities of 10^7–10^8 cells mL^{-1} were reached after 6 to 8 days. However, lower temperatures and shaking conditions were used than the ones used in this study (i.e., 25°C and 100 rpm). Consistent with Jimenez et al. (2014), the fungal communities did not build up high densities in the enrichment systems (Fig. 1b), with ITS1 gene copy numbers remaining well below the bacterial 16S rRNA gene copy numbers. On all substrates, the microbial consortia, across all treatments, effected enhanced substrate weight loss from transfers 6 to 9, with values ranging from a minimum of about 36% (WS2, in transfer 6) up to around 48% (CS, in transfer 9). In addition, treatments SG and CS had higher values of substrate weight loss than treatments WS1 and WS2 (Fig. 2). Such weight loss data were roughly consistent with the overall FTIR-based data (Table 1). Thus, the plant biomass degradative microbial consortia were apparently "trained" to become more efficient in the degradation process over time. Xu et al. (2009), testing the weight loss of corn stover in a culture of white rot fungus *Irpex lacteus*, described a substrate weight loss of ~20 % after 40 days of incubation. Similarly, Baldrian et al. (2003) showed the weight loss of wheat straw during growth of *Pleurotus ostreatus* to be at the level of ~30% after 20 days of incubation. Thus, in spite of the fact that we do not provide a side-by-side comparison, we conclude that mixed microbial consortia (i.e., consisting of both bacterial and fungal partners) have potentially higher biodegradative performance than single-isolate cultures. The FTIR-based analyses showed treatment SG to have the highest lignin degradation rate (ca. 39%), while the highest rates for cellulose and hemicellulose were obtained in the WS1 (ca. 52%) and CS (ca. 63%) treatments, respectively (Fig. 3). Whereas several previous studies have addressed plant biomass degradation by breeding different microbial consortia (Chen et al. 2009; Wang et al. 2011; Hui et al. 2013; Jiménez et al. 2014), none has studied the influence of different lignocellulose substrates or different pH conditions as factors driving the enrichment of specific microbial consortia once the same microbial source is used as an inoculum. Here, we clearly show that substrate type, next to pH (for treatments using

WS), are major driver of the microbial consortia that are bred from one source inoculum. Such consortia were consistent across replicates yet were found to be composed of different members across treatments (Fig. 5a). Given the fact that the lignocellulose compositions of the three used substrates were roughly similar (Table 1) and taking on board the evidence that the rates of decomposition of these different compounds were different across the treatments (Table 1), we can discern a scientific basis for the divergent microbial consortia emerging in the different substrates. These lie either or both in the presumed differences in soluble carbohydrate and sugar compositions or in the intricate bonds and/or branching within and between the three substrates that make up the lignocellulose moieties of the three plants. However, with respect to the bacterial parts of the consortia, we detected a restricted "core" consortium across the treatments, next to a treatment-specific one. The apparent "core" was consistently composed of organisms affiliated with *Sphingobacterium kitahiroshimense*, *Raoultella terrigena*, *Pseudomonas putida* and *Stenotrophomonas rhizophila*. Interestingly, these genera were also found to become abundant in previously-bred microbial consortia using (un)treated wheat straw as the carbon source (Jiménez et al. 2014). Presumably, these consist of "generalists" that grow upon common target in the diverse plant biomasses.

Moreover, the fungal consortia also revealed stable structures that were different from each other across the treatments. On top of that, treatments WS1 and WS2 revealed the emergence of statistically similar fungal community structures (Fig. 5b), indicating a general lack of effect of pH conditions on these communities, in this case specifically for the WS treatment. We cannot easily explain this fact, as Jiménez et al. (2014) noticed that fungal community structures enriched with wheat straw and torrified wheat straw were very dissimilar from each other.

Furthermore, a suite of highly active isolates that likely represent members of the core microbiota was obtained, and their analysis yielded important observations. First, as activity detection included the observation of haloes, the produced enzymes were externally secreted by the cells. Secretion is a critical, yet overlooked, bottleneck in studies that aim at the establishment of efficient microbial degrader consortia. Our *Raoultella* and *Klebsiella* isolates (*Enterobacteriales*) showed extracellular (xylanolytic/cellulolytic) activities, suggesting that these bacteria have metabolic roles in plant polymer degradation. This finding is possibly congruent with studies that showed members of the *Enterobacteriales* abound in insect herbivore microbiomes (Suen et al. 2010; Aylward et al. 2012).

The finding of *Stenotrophomonas*—like organisms (part of the core consortia) showing (hemi)cellulolytic activity—corroborates data obtained by Qi et al. (2011). The latter study on lignocellulosic substrate bioconversion by yellow mealworm gut microbiomes produced a degrading microbial consortium that contained key *Stenotrophomonas* strains for the degradation of lignocellulosic material. Interestingly, bacterial strains retrieved from CS (affiliated with *Comamonas* and *Sanguibacter*) and SG (affiliated with *Paenibacillus*) also showed degrader activities, suggesting these are potentially active lignocellulose degraders. Wang et al. (2013) reported organisms affiliated with *Paenibacillus* to be key degraders of lignocellulosic substrates (from reeds), whereas Cook et al. (2007) found *Sanguibacter suarezii* to degrade CMC, starch, methylumbelliferyl (MUF)-xylopyranoside, MUF-arabinofuranoside and MUF-glucopyranoside. Finally, it is noteworthy that *Coniochaeta* - and *Acremonium* like fungi with CMC-ase and xylanase activities, were consistently found across all treatments. Thus, such organisms might have key roles in the core degradative consortium. The genus *Coniochaeta* encompasses filamentous fungi that are active in the degradation of decaying wood in soil and are probably involved in hemicellulose degradation (Bayer et al. 2013). Recently, *Plectosphaerella* (which is highly related to *Acremonium*) has been reported to utilize xylose and CMC, yielding lipids (Summerbell et al. 2011; Li et al. 2012). Thus, the production of lipids by such organisms— using lignocellulose as a substrate—may constitute a metabolic pathway to be explored in order to yield oil-rich compounds—a process with high economic competitiveness (Liu et al. 2013).

In summary, we developed four lignocellulose degrading microbial consortia from forest soil using three different plant substrates. Substrate type was found to be the major driver of the composition of the bacterial and fungal communities in the final consortia, as evidenced by PCR-DGGE community profiling along the enrichments. Moreover, a common core consortium of low richness was detected. Further understanding of the biotic interactions in the bred consortia will pave the way for the establishment of an efficient multispecies based process for lignocellulose degradation.

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Supplemental information

Table S1. Cellulose. hemicellulose and lignin mixtures used to obtain reference spectra. Values are expressed in %.

Mixtures	Lignin (%)	Cellulose (%)	Hemicellulose (%)
1	100[a]	0	0
2	50	25	25
3	25	50	25
4	25	25	50
5	75	25	0
6	25	75	0
7	0	25	75
8	0	75	75

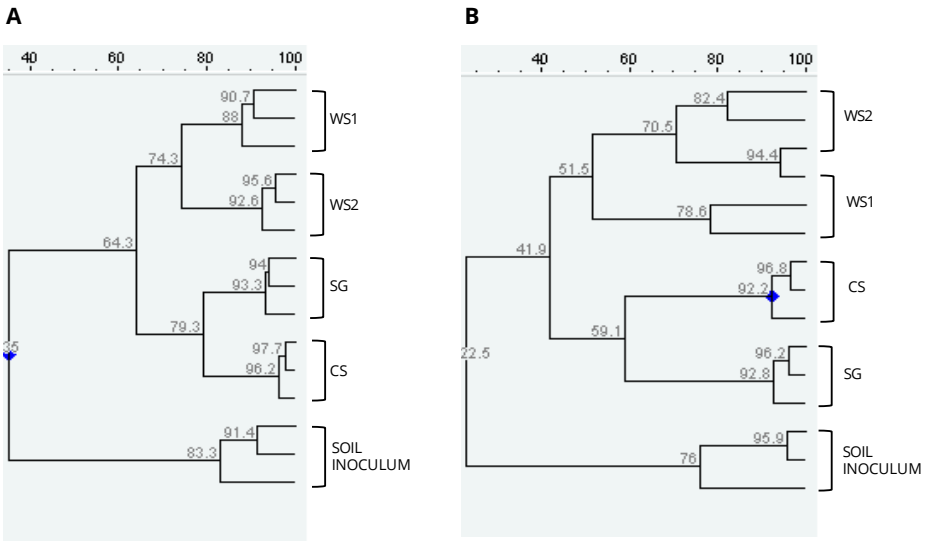

Figure S1 Cluster analysis of DGGE profiles from transfer 9, for all treatments and soil, targeting **(A)** *Bacterial 16S rRNA gene* and **(B)** *Fungal ITS region.*

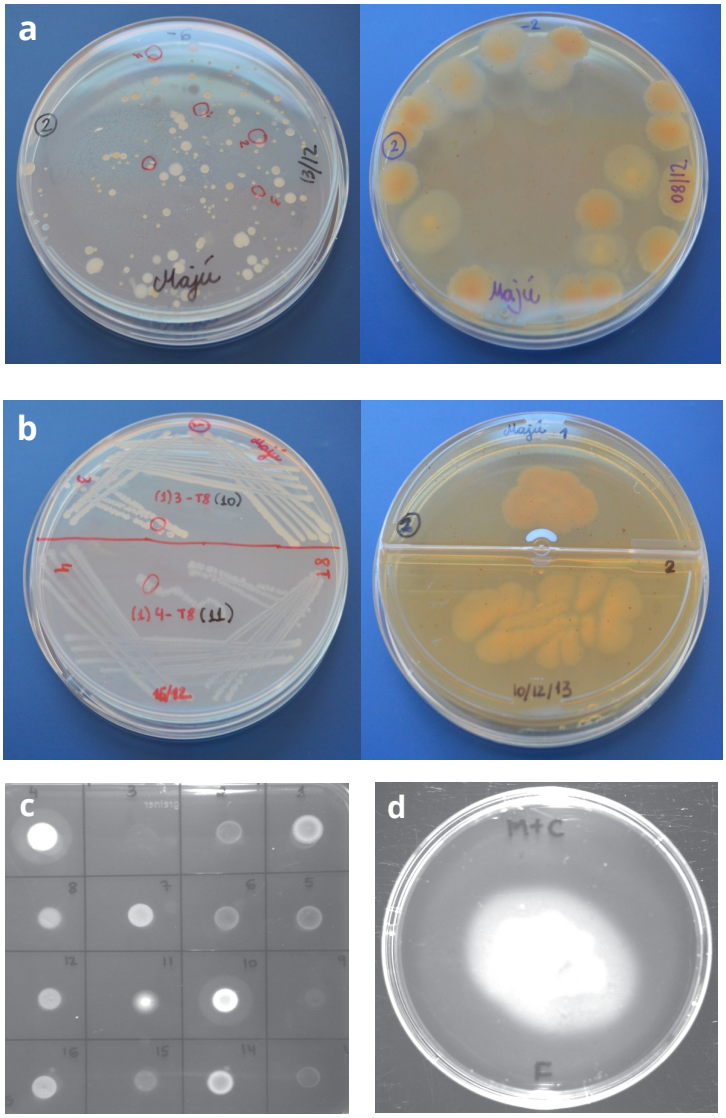

Figure S2 (a) *Isolation* and **(b)** *Purification of bacterial and fungal isolates* from the transfer 9 for all treatments and halo formation in **(c)** *Bacterial* and **(d)** *Fungal isolates* in the enzymatic test.

Chapter 3

The influence of microbial source in the development of microbial consortia capable to degrade lignocellulose substrate

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Abstract

Despite multiple research efforts, the current strategies for exploitation of lignocellulosic plant matter are still far from optimal, being hampered mostly by the difficulty of degrading the recalcitrant parts. An interesting approach is to use lignocellulose-degrading microbial communities by using different environmental sources of microbial inocula. However, it remains unclear whether the inoculum source matters for the degradation process. Here, we addressed this question by verifying the lignocellulose degradation potential of wheat (*Triticum aestivum*) straw by microbial consortia generated from three different microbial inoculum sources, i.e., forest soil, canal sediment and decaying wood. We selected these consortia through ten sequential-batch enrichments by dilution–to–stimulation using wheat straw as the sole carbon source. We monitored the changes in microbial composition and abundance, as well as their associated degradation capacity and enzymatic activities. Overall, the microbial consortia developed well on the substrate, with progressively decreasing net average generation times. Each final consortium encompassed bacterial/fungal communities that were distinct in composition but functionally similar, as they all revealed high substrate degradation activities. However, we did find significant differences in the metabolic diversities per consortium: in wood-derived consortia cellobiohydrolases prevailed, in soil-derived ones β-glucosidases, and in sediment-derived ones several activities. Isolates recovered from the consortia showed considerable metabolic diversities across the consortia. This confirmed that, although the overall lignocellulose degradation was similar, each consortium had a unique enzyme activity pattern. Clearly, inoculum source was the key determinant of the composition of the final microbial degrader consortia, yet with varying enzyme activities. Hence, in accord with Beyerinck's, "everything is everywhere, the environment selects" the source determines consortium composition.

Introduction

Lignocellulosic substrates such as wheat, grass and maize straws constitute important carbon and energy sources for microorganisms. In addition to diverse small molecules, cellulose and hemicellulose, both of which can be hydrolyzed to sugars for further biological or chemical utilization (Xu et al. 2013), and lignin constitute the major carbonaceous components in these substrates. Whereas high-value products such as biofuels and diverse building blocks for industrial products can be produced on the basis of the released compounds, the lignin moiety—a polymer of aromatic compounds like phenol—constitutes an important source material for the industrial production of adhesive resin and lignin gels. In plant tissue, the three moieties form a complex structure with intricate bonds, part of which is very difficult to breakdown. Thus, despite multiple research efforts, the current strategies for exploitation of lignocellulosic plant matter are still far from optimal, being hampered mostly by the challenge of degrading the recalcitrant parts of all three moieties.

In natural systems, lignocellulose degradation is carried out by multiple —coexisting—lignocellulolytic microorganisms. These include a range of fungi and bacteria capable of producing a variety of degrading enzymes. These microorganisms most likely establish synergistic relationships among each other and/or with other, non-cellulolytic, microbial species and these interactions are expected to enhance the substrate degradation rates (Lynd et al. 2002). For instance, in forest soils, fungal and bacterial communities play important roles; the former explore dead plant matter even at low moisture content of the soil and the latter may act as secondary responders (Lynd et al. 2002). Similarly, in decaying wood, fungi act together with bacteria, constituting the communities that collectively work on the substrate (Prewitt et al. 2014) whereas in sediment, we expect anaerobic cellulolytic bacteria, possibly concomitant with particular fungi, to be involved in the biopolymer degradation processes (Wei et al. 2009). Although cooperation between microbes seems to be the driving force behind lignocellulose degradation in natural habitats, in industry single strains are often used (Guerriero et al. 2015). Using microbial consortia instead of single strains for the biodegradation of lignocellulose allows one to take advantage of the microbial interactions, by making optimal use of their intricate regulatory systems, which may bypass problems of feedback regulation and metabolite repression that are often posed by single strains (Wongwilaiwalin et al. 2010). Additionally, this strategy may confer an optimal combination of enzyme production and

interaction of microbes. Enrichment culturing — also coined "habitat biasing" (Ekkers et al. 2012) — is a strategy in which a deliberate "bias" is introduced into an environmental sample in order to modulate the microbial community with respect to function (in situ or ex situ).

The selective media that are used enhance the prevalence of desired functions in a microbial community and so the genes and/or operons of interest, as was shown for chitin (Cretoiu et al. 2012) as well as hemicellulose degradation processes (Jiménez et al. 2013).

Microbial consortia with effective lignocellulose degradation capacity can be selected from different source materials (Wongwilaiwalin et al. 2010; Wang et al. 2011; Jiménez et al. 2013; Moraïs et al. 2014), leading to effective and structurally stable consortia that successfully degrade substrates even beyond the ones they were selected upon (Haruta et al. 2002). However, little is known about the differences that might arise when different microbial sources are used to breed such degrader consortia on the same substrate, i.e., whether microbial communities originating from different sources would converge to similar consortia when exposed to enrichment culturing. In that case, this convergence would provide evidence for the 100-year old Beijerinck adagium "Everything is everywhere and the environment selects".

Here, we investigated whether different source communities would generate similar lignocellulolytic microbial consortia when similar selection criteria are applied. Whether this adagium would be turned into reality presumably depends on (1) the nature of the source inocula and (2) the strength of the selective force applied. Thus, the aims of this study were (i) to determine the relevance of the microbial inoculum as the driver of the lignocellulose-degradative consortia produced after ten enrichment steps and (considering the high degree of functional redundancy often observed in microbial communities) (ii) to assess whether different source inocula result in similar degradation capacities. To this end, three different source inocula, i.e. microbiomes from forest soil, canal sediment and decaying wood, were used for serial-batch dilution-to-stimulation on severed wheat straw as the carbon- and energy-yielding substrate, in order to measure their effects on the final enriched consortia.

Materials and methods

Substrate preparation

Wheat straw used as the substrate was obtained from a local farm (Groningen, the Netherlands). It was air-dried (50°C) before cutting it into pieces of about 5 cm length. Then, the pieces were mixed with sterile distilled water and thoroughly ground, using a mill hammer, to pieces ≤1 mm in order to increase the surface to volume ratio. After this treatment, the wheat straw suspension was autoclaved at 121°C for 27 min before use. Sterility of the substrate was verified following plating on LB agar plates.

Selection of microbial consortia degrading wheat straw from three inoculum sources

The sources of the microbial communities used in this experiment were forest soil, decaying wood and canal sediment. Forest soil encompassed three randomly collected (53.41 N; 6.90 E) 10-g surface (0–10 cm) samples which were thoroughly mixed. The decaying wood was collected at the same site. A 20-cm decomposing tree branch (hardwood), with evident microbial growth on its surface, was used. In the laboratory, it was cut into small (<3 mm) pieces in sterile conditions. Sediment was collected from the bottom of an adjacent canal, using three random samples of about 10 g, which were thoroughly mixed. All samples were taken in February 2014 (winter season). Cell suspensions were prepared by adding 10 g of each of the microbial sources to 250 mL flasks containing 10 g of sterile gravel in 90 mL of mineral salt medium (MSM: 7 g/L Na_2HPO_4; 2 g/L K_2HPO_4; 1 g/L $(NH_4)_2SO_4$; 0.1 g/L $Ca(NO_3)_2$; 0.2 g/L $MgCl_2$, pH 7.2). All flasks were shaken for 30 min at 200 rpm (room temperature). To start the experiments, 250 µL of each cell suspension were added to triplicate 100-mL Erlenmeyer flasks containing 25 mL of MSM supplemented with 1% (w/v) sterilized wheat straw, 25 µL of vitamin solution (0.1 g Ca-pantothenate, 0.1 g cyanocobalamin, 0.1 g nicotinic acid, 0.1 g pyridoxal, 0.1 g riboflavin, 0.1 g thiamin, 0.01 g biotin, 0.1 g folic acid; H_2O 1 L) and 25 µl of trace metal solution (2.5 g/L EDTA; 1.5 g/L $FeSO_4$; 0.025 g/L $CoCl_2$; 0.025 g/L $ZnSO_4$; 0.015 g/L $MnCl_2$; 0.015 g/L $NaMoO_4$; 0.01 g/L $NiCl_2$; 0.02 g/L H_3BO_3; 0.005 g/L $CuCl_2$). All chemicals and reagents used in this work were of analytic molecular biology grade (Sigma-Aldrich, Darmstadt, Germany). All flasks were incubated at 28°C, with shaking at 200 rpm. The cultures were monitored by counting cells in a Bürker-Türk chamber at regular time intervals. At the start of the experiments,

around 5 log cells/mL were used. Once the systems had reached around 9 log cells/mL (and straw had visually been degraded), 25 µL of culture was transferred to 25 mL of fresh medium (dilution factor 10^{-3}). The procedure was repeated ten times, giving a total of ten sequential enrichment cultures. Following each transfer (T), part of the bred consortia was stored in 20% glycerol at −80°C. The consortia of the T1, T3, T6, and T10 flasks were used for all subsequent analyses, as detailed below. As controls, we used microbial sources in MSM without substrate (C1a, C1b, C1c) as well as MSM plus substrate without inoculum (C2a, C2b, C2c).

DNA extraction and quantitative PCR (q-PCR)

Aliquots (2 mL) of each selected culture were used for community DNA extraction using the "Power Soil" DNA extraction kit (inoculum sources) (MoBio® Laboratories Inc., Carslab, USA) or the "UltraClean" DNA Isolation Kit (each enriched consortium) (MoBio® Laboratories Inc., Carslab, USA). The instructions of the manufacturer were followed, except that the resuspension of the DNA from the inoculum sources was in 60 µL resuspension fluid. The 16S rRNA gene region V5-V6 (bacteria), as well as the ITS1 region (fungi), were amplified using 1 ng of community DNA as the template and primers 16SFP/16SRP and 5.8S/ITS1(Pereira e Silva et al. 2012), respectively. Standard curves were constructed using serial dilutions of cloned 16S rRNA gene and ITS1 fragments from *Serratia plymuthica* (KF495530) and *Coniochaeta ligniaria* (KF285995), respectively. Gene target quantification was performed, in triplicate, in an ABI Prism 7300 Cycler (Applied Biosystems, Lohne, Germany).

PCR-DGGE analysis

Total community DNA was used as the template for amplification of the partial 16S rRNA gene fragment using *Taq* DNA polymerase (Bioline, Lückenwalde, Germany) with primer F968 with a GC clamp attached to the 5' end and universal bacterial primer R1401.1b. For ITS1 amplification, primers EF4/ITS4 were used; this PCR was followed by a second amplification with primers ITS1f-GCITS2. Primer sequences, the reactions mixtures, and cycling conditions have been described (Pereira e Silva et al. 2012). The DGGE was performed in 6% (w/v) polyacrylamide gels with 45–65% and 20–50% denaturant gradients for bacterial and fungal communities, respectively (100% denaturant is defined as 7.0 M urea with 40% deionized formamide). Electrophoresis was carried out at 100 V and 75 mA, for 16 h at 60°C. The gels were subsequently stained for 40 min in 0.5% TAE buffer with SYBR gold (final concentration 0.5 µg/L) (Invitrogen, Breda, the Netherlands) (Fig.

S1 in the Supplementary Material). Gel images were digitized using Imagemaster VDS (Amersham Biosciences, Buckinghamshire, UK). The DGGE patterns were then transformed to a band-matching table using GelCompar II software (Applied Maths, Sint Martens Latem, Belgium).

Analysis of the three final consortia by sequencing of the 16S rRNA gene

Amplicons of 250 bp were generated on the basis of primers amplifying the V4-V5 of the 16S rRNA gene region. PCR amplifications were conducted in triplicate reactions for each of the 12 samples with the 515F/806R primer set (Table S1 in the Supplementary Material). PCR and sequencing were performed using a standard protocol (Caporaso et al. 2012). Illumina MiSeq sequencing was performing at Argonne National Laboratory (Illinois, USA). We processed the raw data using "quantitative insight into microbial ecology" (QIIME) software, version 1.91. The sequences were demultiplexed and quality-filtered using split_libraries_fastq.py default parameters (Bokulich et al. 2013). The derived sequences were then clustered into operational taxonomic units (OTU) using open-reference OTU picking against the Greengenes reference OTU database with a 97% similarity threshold (Rideout et al. 2014). Then, we performed quality filtering to discard OTUs present at very low abundance (<0.005 %) (Bokulich et al. 2013). An even sampling depth of 10,000 sequences per sample was used for assessing α- and β-diversity measures by using core_diversity_analyses.py.

Metrics for α-diversity were OTU richness (equivalent to species richness), Chao1 index (estimated species richness) and Faith's phylogenetic diversity (PD) index (phylogenetic relationship between OTUs). β-diversity analyses among the final consortia were performed using unweighted UniFrac distance matrix (Lozupone et al. 2011). Statistical analyses, i.e. matrix similarity and principal coordinate analysis (PCoA) and UniFrac were performed with the PREMIER 6 and PERMANOVA A+ software packages (Primer-E Ltd., Lutton, United Kingdom).

Substrate degradation analysis in the consortia.

After each growth step, the remaining particulate wheat straw was recovered from the microcosm flasks, after which this material was washed to remove microbial cells. The degradation rates of the components of the substrate, before and after incubation, were determined by Fourier-transformed infrared (FTIR) spectra (Adapa et al. 2011; Xu et al. 2013) and partial least

squares (PLS) regression. Spectra were obtained with a resolution of 4 cm^{-1} from Perkin Elmer Spectrometer FTIR (model UATR, version Two). Thirty-two scans were run per sample between 800 and 1800 cm^{-1} (Krasznai et al. 2012). Each sample (calibration and consortium samples) was analyzed in triplicate. Before PLS regression, all spectra were subjected to baseline correction and then corrected for physical effects by 2nd derivative Savitzky-Golay (FitzPatrick et al. 2012). A model was created on the basis of a calibration with standard mixtures, consisting of hemicellulose (proxy Beechwood xylan, ≥90%, Sigma Aldrich, Steinheim, Germany), cellulose (powder, D-516, Macherey-Nagel, Düren, Germany), and lignin (alkaline, Sigma Aldrich, Steinheim, Germany) in the proportion described in Table S2 in the Supplementary Material (Adapa et al. 2011). The model displayed R^2 values of 0.95, 0.97 and 0.99 for hemicellulose, cellulose and lignin, respectively. Correction and analysis of the spectra were conducted using Unscrambler X by CAMO software (FitzPatrick et al. 2012; Krasznai et al. 2012). All FTIR measurements were carried out on oven dried material (50°C, 24h). The degradation of hemicellulose components was calculated by subtracting the percentage of the residual substrate from the total percentage of each hemicellulose component before degradation. Degradation rate was calculated using the followed equation: $\frac{C_i - C_f}{C_i} x100$, where C_i is the total amount of compound before degradation and C_f is the residual component after degradation (Wang et al. 2011). One-way analysis of variance (*ANOVA*) followed by Tukey's HSD pairwise group comparisons was performed in IBM SPSS Statistics version 23 (SPSS, Illinois, USA).

Quantification of enzymatic activities related to (hemi) cellulose degradation in the consortia

Using the extracellular fractions (containing the "secretome") of the three final consortia (T10), the specific activities of β-xylosidases, β-galactosidases, β-mannosidases, cellobiohydrolases and β-glucosidases were measured. To do so, microbial cells and wheat substrate were harvested by centrifugation (5 min, 13,500 rpm; Thermo Fisher Scientific Centrifuge; Thermo Fisher, Waltham, USA), after which the supernatants were used directly in the tests. MUF-β-D-xylopyranoside,MUF-β-D-mannopyranoside, MUF-β-D-galactopyranoside, MUF-β-D-cellobioside, and MUF-β-D-glucopyranoside were used as the fluorogenic substrates (Sigma-Aldrich, Darmstadt, Germany). The reaction mixes consisted of 10 mM MUF-substrate in dimethyl sulfoxide, 15 µL McIlvaine buffer (pH 6.8) and 25 µL of supernatant. The reaction was incubated at 25°C for 45 min in the

dark, after which it was stopped by adding 150 µL of glycine–NAOH buffer (0.2 M, pH 10.4). Fluorescence was measured at an excitation wavelength of 365 nm and with emission at 445 nm. Enzymatic activities were determined from the fluorescence units using a standard calibration curve. We then determined total protein using the DC Protein Assay (BioRad, Hercules, USA) according to the manufacturer's instructions. The specific enzymatic activity was reported as the rate of MUF production (µM MUF per min per mg at 25°C, pH 6.8).

Identification and phylogenetic analysis of bacterial and fungal strains

From the three final degrader consortia, we isolated bacterial and fungal strains, using R2A (BD Difco®, Detroit, USA) and potato dextrose agar (PDA) (Duchefa Biochemie BV, Haarlem, The Netherlands), respectively. The isolation part can be found in Electronic supplementary material (ESM 1). For the identification of bacterial strains, primer 1406 was used (sequencing the 16S rRNA gene), whereas for fungal identification primer ITS4 was used (sequencing a partial region of the 18S rRNA gene) (Jiménez et al. 2013). After, the amplicons were sequenced by Sanger technology (LGC Genomics, Lückenwalde, Germany). All sequence chromatograms were analyzed for quality (Brossi et al. 2015). Taxonomic assignments of the sequences were done by using BLAST-N against the NCBI database (http://blast.stva.ncbi.nlm.nih.gov/Blast.cgi). We used the best BLAST hit affiliation for taxonomic assignment with a cut-off of 97% (identity) and 95% (coverage). Sequences are publicly available in the GenBank database under accession numbers KT265747 to KT265810 (Tables S3 and S4 in the Supplementary Material).

Matching bacterial strains with abundant OTUs

The recovered bacterial strains were linked to the OTUs based on sequence similarity and clustering. The almost-full-length 16S rRNA gene sequences from the strains were compared—in the specific V4 region—to sequences of the abundant OTUs using ClustalW. Phylogenetic analyses (*p*-distance) were conducted with MEGA v6 using Neighbor Joining. Evolutionary distances were computed using the Kimura-2 parameter method. The branch node strengths were tested with bootstrap analyses (1000 replications) (Fig. S2 in the Supplementary Material). Additionally, we also matched the presence of the bacterial strains in the final consortia by comparing their patterns to those observed with consortium PCR-DGGE (Fig. S3–S5 in the Supplementary Material).

Results

Effective wheat straw degrading microbial consortia produced from three different inoculum sources

For all inocula used, growth took place in each flask (28°C, with shaking), yielding well-developed microbial consortia at the end of each growth step. The three consortia were found to progressively raise their overall fitness (measured as average growth rate) along the transfers, as a progressive reduction of the incubation time necessary to reach maximal cell densities was recorded. Specifically, we found a significant increase in the growth rates of the final consortia (T10) as compared to those of the previous transfers (Fig. 1A). In contrast, the without-substrate negative control C1 revealed cell numbers that progressively decreased from 6 to 2 log cells/mL at T3, thereafter remaining below the detection limit (data not shown). The negative controls without added inoculum did not reveal the presence of any cells along the transfers.

The microscopic cell counts were corroborated by the 16S rRNA gene and ITS1 copy numbers (proxies for bacterial and fungal communities, respectively), measured in the T1, T3, T6 and T10 consortia (Fig. 1B, C). These results revealed that, at the end of each transfer, the three consortia reached maximal (bacterial) levels on the order of 9 log 16S rRNA gene copy numbers per mL, which was consistent with the aforementioned cell counts (Fig. 1B). Additionally, progressively lower numbers were detected in the (without-substrate) control flasks C1 from T1 to T3, revealing that any major growth was absent from the systems without added wheat straw (data not shown). Concerning fungal abundances, the numbers of ITS1 gene copies at the end of the first transfer, in all consortia, showed a marked reduction compared to those of the inoculum sources. However, along the transfers, these numbers increased slightly, reaching the maximal number in the last transfer. In detail, these numbers were 6.0 log ±0.1 (T1), 6.8 log ±0.4 (T10), and 5.1 log ±0.2 (T1) and 7.0 log ±0.1 (T10), for wood- and sediment-derived consortia, respectively. In the case of the soil-derived microbial consortium, a population size decrease occurred at T3 (5.8 log ±0.2), after which 7.4 log ±0.4 was reached at T10 (Fig. 1C).

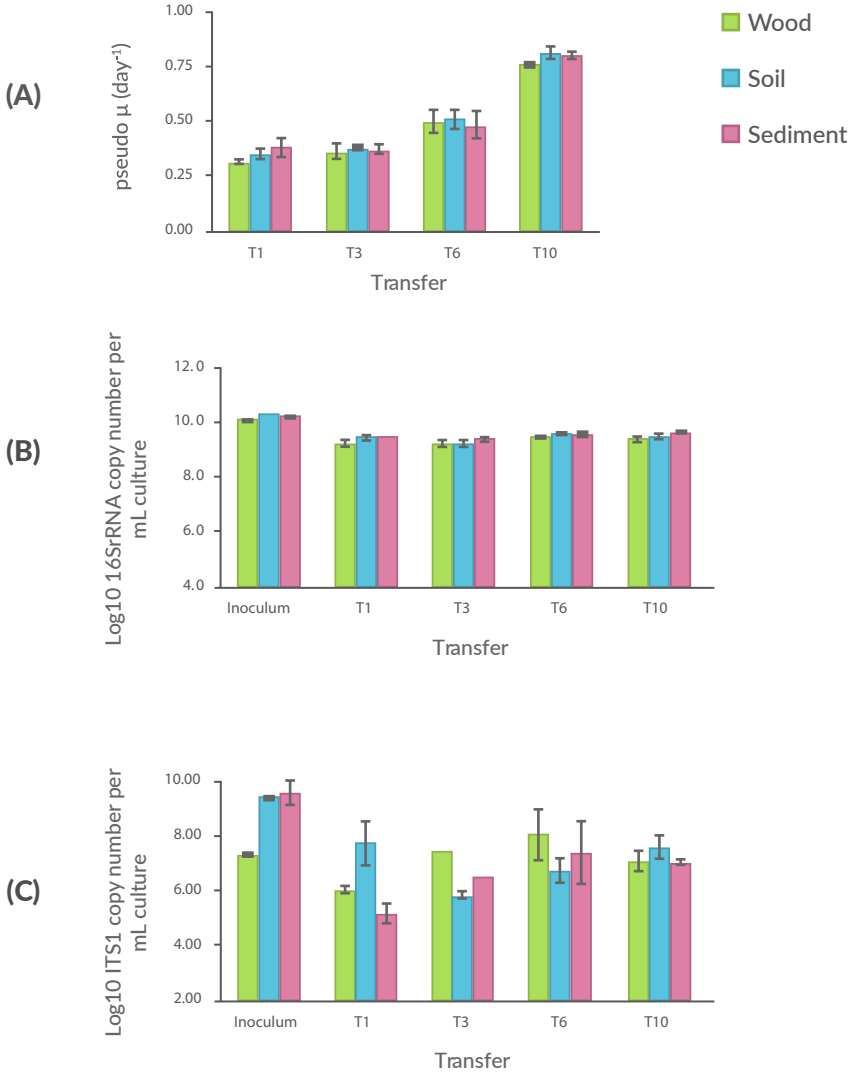


Figure 1 Sequential-batch selection of lignocellulose-degradative microbial consortia from three inoculum sources: decaying wood (*green*), forest soil (*blue*) and canal sediment (*red*). Bacterial and fungal growth rates increased along the transfers, which consisted of additions of inoculum (rate 1:1000) to each fresh medium. Data were collected after four transfers. The x-axis shows the transfer number, the y-axis represents **(A)** *pseudo μ*, rate of increase of bacterial cell, **(B)** *16S rRNA gene copy numbers*, **(C)** *ITS1 copy numbers* (both: log copies per mL) determined by qPCR. Bars refer to standard errors of the mean (n = 3). The three different microbial sources (Inoculum) were used as inocula for starting the enrichment process.

Analysis of the wheat straw degrading microbial consortia

Overall, the data clearly yielded evidence for the contention that inoculum source primarily determines the structure of the final effective consortia. The three microbial consortia were first analyzed by bacterial- and fungal-specific PCR-DGGE analyses, on the basis of the directly-extracted consortial DNA. The consortia revealed considerable changes in structure over time, as evidenced by reductions in the band numbers in the DGGE patterns for both the bacterial and fungal communities. The bacterial banding patterns were consistent between the triplicates per treatment, indicating reproducibility within the treatment in terms of consortium structure buildup. The fungal patterns, however, showed higher dissimilarity between treatments and transfers (Fig. S1 in the Supplementary Material).

Principal coordinate analysis of the bacterial (Fig. 2A) and fungal community patterns (Fig. 2B) based on DGGE profiles indicated (i) a clear clustering along inoculum source, (ii) separation of all patterns from the initial (inoculum) ones, and (iii) a progressive evolution with time, with persisting clustering along the microbial source. Moreover, PERMANOVA indicated the existence of significant differences between the consortia between the treatments for both bacteria ($P < 0.005$) (Fig. 2A) and fungi ($P < 0.005$) (Fig. 2B).

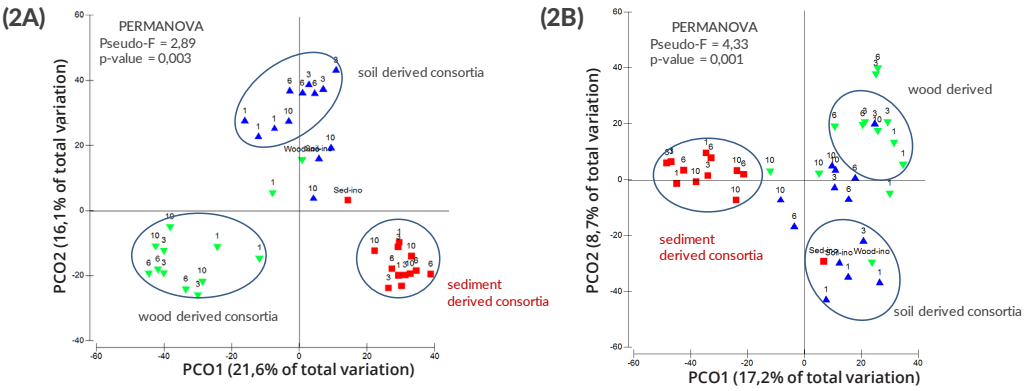


Figure 2 A, B Analyses of steps of the enrichment process and dynamics of the selected consortia. Principal coordinates analysis (PCoA) revealing well defined clusters along microbial inoculum, especially for the bacterial communities (the fungal communities were less clearly differentiated). **(A)** *Bacterial* and **(B)** *Fungal communities* obtained from the PCR-DGGE abundance data. The final communities (T10) for both bacteria and fungi are well grouped and differentiated from their respective source communities, indicating unique compositions depending on inoculum source.

Moving window analysis showed that the similarity between the bacterial community structures increased along the transfers. This was true for all consortia, i.e. those from wood, soil and sediment (Fig. 2C). Altogether, the data indicated a reduction in the diversity of the bacterial communities throughout the transfers. Specifically, according to the similarity percentage between the communities between T6 and T10 (higher similarity indicates greater stabilization), the consortia reached stabilization in the following order: wood-derived (81% ± 2), soil-derived (75% ± 13) and sediment-derived (50% ± 5) ones. The consortia derived from wood revealed only few changes in their structures at an intermediate time point (T6) compared with the other two consortia, whereas the soil-derived consortia reached stabilization between T6 and T10. In contrast, the sediment-derived consortia did not reach a plateau. Thus, stabilization was clearly achieved for the wood-derived but less so for the soil- and sediment-derived consortia.

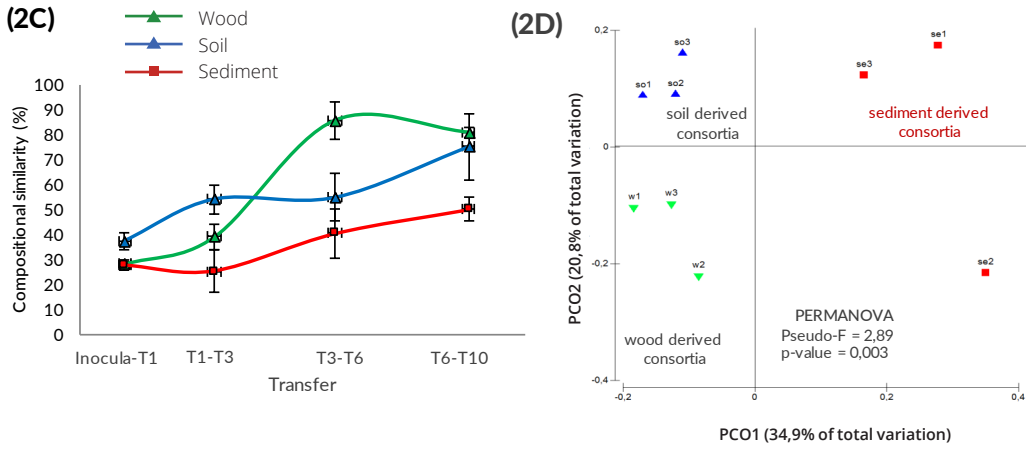


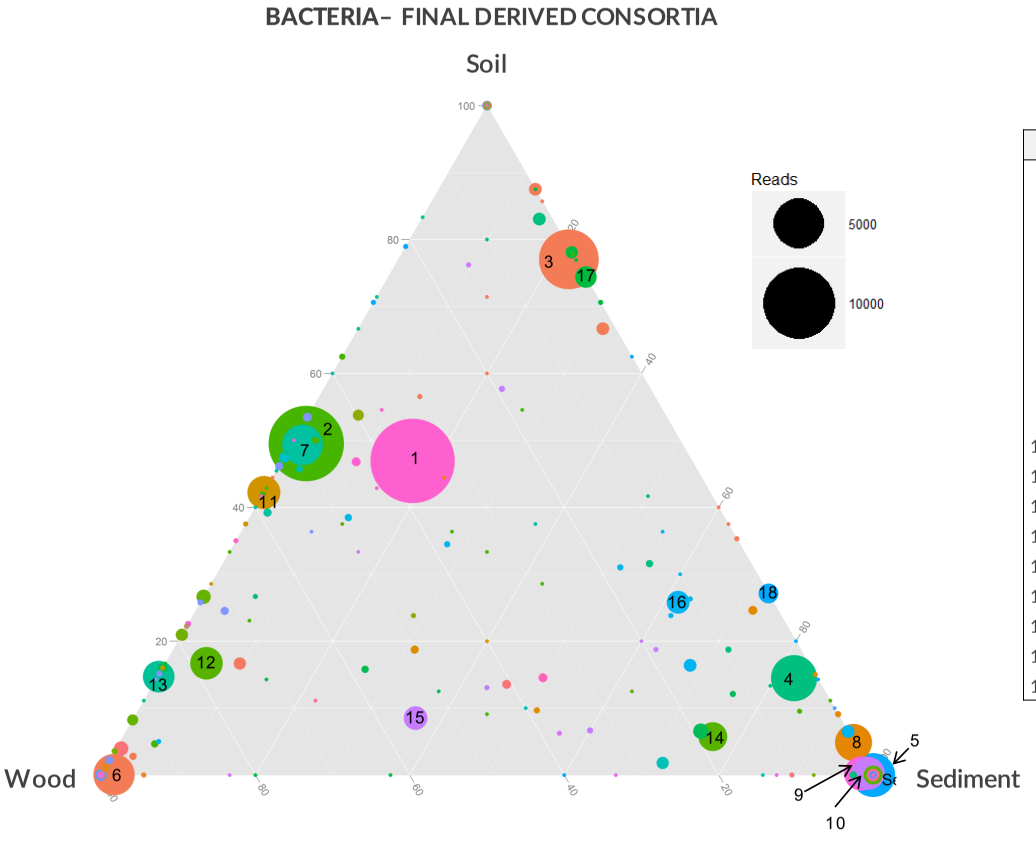
Figure 2 C, D Analyses of steps of the enrichment process and dynamics of the selected consortia. (2C) Moving window analysis *(MWA)*. Evaluation of the community divergence between two sequential transfers in the enrichment process, as measured by percentage of similarity. MWA showed how the communities evolve through the enrichment process. Consortia: wood-derived *(blue line)*, soil-derived *(green line)* and sediment-derived *(red line)*. **(2D)** Principal coordinates analysis *(PCoA)* of unweighted UniFrac distances for 16S rRNA gene sequencing data of final (T10) enrichment cultures (61-day incubation time) from decaying wood *(green triangle),* forest soil *(blue triangle)*, and canal sediment *(red square)* inocula. Ordination of bacterial communities showed strong separation with respect to community composition depending on the microbial source used as inoculum.

Detailed analysis of the bacterial consortia

Direct amplicon sequencing revealed that the bacterial richness values of the final consortia (wood, soil and sediment derived) were 241.7 ± 34.2, 227.7 ± 11.6, and 137.7 ± 19.7 OTUs, respectively, indicating that the final sediment-derived consortia were less rich than the other ones (*ANOVA*, $P < 0.05$). Regarding the bacterial community structures (β-diversities), PCoA of the unweighted UniFrac community distances showed that the final consortia (T10) were markedly different between the treatments (Fig. 2D). Moreover, PERMANOVA showed that the structure of the bacterial communities in these consortia was significantly affected by the inoculum source ($P < 0.005$). Specifically, more similar structures were found between the soil- and wood-derived consortia (0.42 ± 0.03), indicating that these two environments share comparable microbiomes. This was corroborated by the fact that the wood- (0.558 ± 0.042) and soil-derived consortia (0.558 ± 0.059) shared equal similarity to the sediment-derived ones. Delving into taxonomic affiliations (using OTUs with abundance >2%) revealed that members of three bacterial genera, i.e. *Sphingobacterium*, *Acinetobacter* and *Chryseobacterium*, constituted a "core"-type community that was present across all replicates of the three final consortia.

The relative abundance (%) of *Sphingobacterium* in the three consortia was 18.9 ± 1.8, 24.4 ± 4 and 16.6 ± 0.3, that of *Acinetobacter* was 14.7 ± 9.2, 22.2 ± 5.9 and 7.8 ± 7.8, and that of *Chryseobacterium* was 6.9 ± 7.9, 1.7 ± 0.7 and 7.9 ± 6.2, for the wood-, soil- and sediment-derived consortia, respectively (Fig. 3).

Each of the final consortia further revealed "unique" (only occurring in that type of consortium) microbiome members, i.e. the soil-derived consortia exclusively contained OTU 1024520, which was associated with *Comamonas testosteroni* (2.5 ± 0.2), the sediment-derived consortia had members of the genera *Paenibacillus* (OTU 1081222, *P. oceanisediminis*, 13.5 ± 9.6; OTU 1067651, *P. camelliae*, 2 ± 2.9), *Aeromonas* (OTU 839235, *A. hydrophila*; 9.1 ± 9.2), and *Ochrobactrum* (OTU 592636, *O. thiophenivorans*, 2.2 ± 3.2) (Table 1, Fig. 3). In the wood-derived consortia, we found high abundances of the genera *Pedobacter* (OTU 106847, *P. agri*, 0.4 ± 0.4), and *Taibaiella* (OTU 771274, *T. koreensis*, 1.2 ± 1.2); however, the unique OTUs were found in low relative abundances.



Bacterial composition		
1	*Sphingobacterium multivorum*	891031
2	*Citrobacter freundii*	528421
3	*Acinetobacter calcoaceticus*	988314
4	*Klebsiella variicola*	922761
5	*Paenibacillus oceanisediminis*	1081222
6	*Acinetobacter johnsonii*	4235445
7	*Flavobacterium succinicans*	1020921
8	*Aeromona hydrophila*	839235
9	*Sphingobacterium faecium*	824606
10	*Pseudomonas azotoformans*	646549
11	*Asticcacaulis benevestitus*	4300564
12	*Chryseobacterium taihuense*	1039092
13	*Flavobacterium lindanitolerans*	1145553
14	*Chryseobacterium taeanense*	1010721
15	*Pseudomonas putida*	829851
16	*Ochrobactrum thiophenivorans*	592636
17	*Comamonas testosteroni*	1024520
18	*Paenibacillus camelliae*	1067651

Figure 3 Differences in composition of the three final consortia bred from three different inocula. Ternary plot of OTU relative abundances. A common bacterial core is formed by members of the genera *Sphingobacterium*, *Acinetobacter* and *Chryseobacterium*. Resolution level of 800 reads.

Table 1. Most abundance OTU in final derived consortia from decaying wood, forest soil and canal sediment.

Consortia derived from	OTU	Taxonomic affiliation	Relative abundance (%)
Decaying wood	528421	*Citrobacter freundii*	19.3±5.1
	891031	*Sphingobacterium multivorum*	18±11
	4235445	*Acinetobacter johnsonii*	11.8±7.6
	1145553	*Flavobacterium lindanitolerans*	6.0±7.7
	1039092	*Chryseobacterium taihuense*	5.8±6.8
	1020921	*Flavobacterium ginsengisoli*	5.6±2.2
	4300564	*Asticcacaulis benevetitus*	4.4±3.1
	829851	*Pseudomonas putida*	2.2±1.1
Forest soil	891031	*Sphingobacterium multivorum*	23.4±3.7
	988314	*Acinetobacter calcoaceticus*	19.3±5.3
	528421	*Citrobacter freundii*	19.7±3.9
	1020921	*Flavobacterium ginsengisoli*	5.7±1.2
	4300564	*Asticcacaulis benevestitus*	3.2±4.5
	1024520	*Comamonas testosteroni*	2.4±0.2
	922761	*Klebsiella variicola*	2.1±1.5
Canal sediment	1081222	*Paenibacillus oceanisediminis*	13.5±9.6
	922761	*Klebsiella variicola*	12.3±6.8
	839235	*Aeromonas hydrophila*	9.1±9.2
	891031	*Sphingobacterium multivorum*	8.4±11.8
	824606	*Sphingobacterium faecium*	8.2±11.5
	646549	*Pseudomonas azotoformans*	7.6±10.6
	988314	*Acinetobacter calcoaceticus*	5.5±7.1
	1010721	*Chryseobacterium taeanense*	4.5±4
	746501	*Chryseobacterium taichungense*	2.5±2.2
	592636	*Ochrobactrum thiophenivorans*	2.2±3.2
	1067651	*Paenibacillus camelliae*	2±2

Similarity between the OTU 16S rRNA gene sequence and the taxonomic affiliation as in NCBI.

Substrate degradation patterns and enzymatic profiles of the final microbial consortia

The final consortia consumed the hemicellulose, cellulose, and lignin components of the substrate to grossly similar extents, as only small and insignificant differences were found between them (*ANOVA*, P > 0.05) (Fig. 4A). The variation levels prevented the drawing of strong conclusions with respect to the degradation efficacies. All final consortia were found to preferably consume the hemicellulose part of the substrate, which was more than 50% degraded. With respect to cellulose and lignin, the degradation rates were lower throughout. Interestingly, there was a trend in that the sediment-derived consortia had a subtle but non-significant lower hemicellulose and a higher cellulose degradation rate compared with the soil- and wood-derived consortia. The enzymatic profiling revealed that each of the three final consortia (T10) had a unique combination of specific enzymatic activities (Fig. 4B). Remarkably, the sediment-derived consortia showed a more even distribution of the activities, whereas the soil- and wood-derived consortia were dominated by β-glucosidases and cellobiohydrolases, respectively. Notably, β-xylosidase was the enzyme with the highest activity in all treatments. The high β-xylosidase activities corroborated the observation that the main degradation activity was on the hemicellulose part of the substrate.

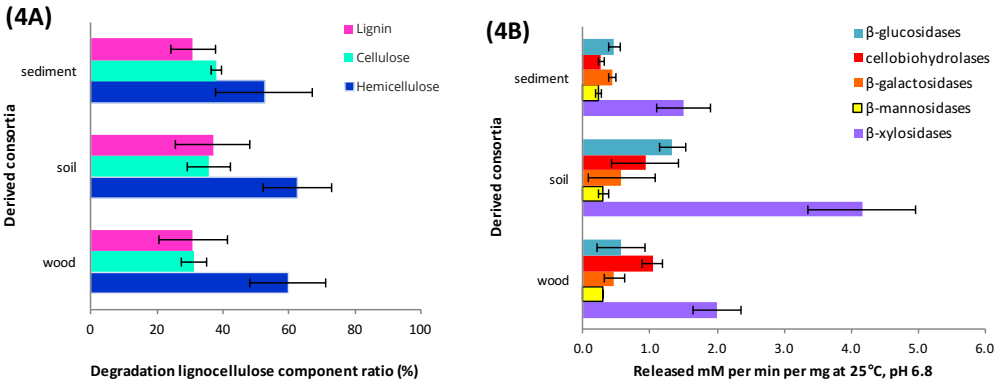


Figure 4 Degradation capacity profiles of **(A)** *Percentage of reduction* of hemicellulose, cellulose and lignin, after incubation with the different microbial consortia. **(B)** *Quantification of enzymatic activities* by methylumbelliferyl (MUF)-substrate, measured in the recovered culture supernatants.

Biodegradative bacterial and fungal strains from the wheat straw bred microbial consortia

In total, 90 bacterial strains were recovered from the three final consortia, 52 of which were identified on the basis of 16S rRNA gene sequencing. Using co-migration analysis, several DGGE bands were presumptively identified as being derived from several strains (Fig. S3–S5 in the Supplementary Material), indicating that these strains contributed to the dominant bacterial species present in the consortia. The strains were subsequently screened with respect to various enzymatic activities providing them with the ability to degrade lignocellulose.

This was indeed a widespread characteristic across the strains, as 70% showed enzymatic activity for at least two out of six enzymes tested (Table S3 and Fig. S6 in the Supplementary Material). By aligning the 16S rRNA gene sequence from the bacterial isolates with the OTUs obtained by sequencing the whole consortia (Table 2), we could verify that several strains that were highly abundant in the three consortia (according to the higher number of sequences for each specific OTU) presented key enzymatic activities (Table 2). These strains were *Sphingobacterium multivorum* soB22, wB15 and seB10, *Citrobacter freundii* soB4, *Lelliotia amnigena* soB12 and seB4, *Flavobacterium ginsengisoli* wB6 and soB9, *Chryseobacterium taihuense* wB4 and soB3, *Asticcacaulis benevestitus* wB3 and *Ochrobactrum thiophenivorans* soB16.

Moreover, from the three final consortia, we obtained several biodegradative strains showing α-D-glucosidase, β-D-glucosidase, β-D-galactosidase, and β-D-xylosidase activities, although none of these dominated in the consortia according to the OTU sequencing results (Table 2). These strains were affiliated to *Stenotrophomonas terrae* (wB16), *S. rhizophila* (seB11), and *Microbacterium foliorum* (wB9).

Interestingly, some closely-related strains (in some cases identified as the same species) isolated from the different consortia expressed different enzymatic activities. For instance, two *S. multivorum strains*, i.e. wB15 and soB22, *F. ginsengisoli* strains wB6 and soB8 and *C. taihuense* strains wB4 and soB3, recovered from the wood- and soil-derived consortia, respectively, revealed completely different enzymatic profiles (Table 2). These results indicated that each final consortium constitutes a unique community in which each member, even the same species, participates potentially with a strain-unique set of enzymes for the degradation of the lignocellulose.

Regarding the 40 fungal strains, partial ITS1 sequence analyses revealed that they belong to 11 different species. Testing the fungal strains for (hemi) cellulolytic activity in media with CMC, xylan and cellulose as the single carbon sources revealed extracellular enzyme activities in most of them.

Fungal strains from soil- and sediment- derived consortia presented the highest enzymatic activities, whereas only four strains isolated from wood had considerable activity in all the substrates. Moreover, two strains, wF4 and wF5 (associated with the taxa *Exophiala* and *Herpotrichiellaceae*, respectively), did not show any activity; the strains did not grow on glucose as a single carbon source (used as a positive control) (Table S4 and Fig. S7 in the Supplementary Material).

Table 2. Molecularly-identified organisms and strains in lignocellulolytic consortia bred from decaying wood, forest soil and canal sediment.

Class	OTU	Wood RA (%)	Soil RA (%)	Sediment RA (%)	Similarity (%)	Isolated bacteria straina (strain code)	A	B	C	D	E	F	Accession number
Sphingobacteria						*Sphingobacteriales*							
	OTU 891031 *S. multivorum*	18 ± 11	23.4 ± 3.6	8.4 ± 11.8	100	*S. multivorum (soB22)*	+	+	+	+	+	+	KT265757
					100	*S. multivorum (wB15)*		+	+	+			KT265760
	OTU 824606 *S.faecium*	< 2.0	0	8.2 ± 11.5	100	*S. faecium (seB10)*	+	+	+	+			KT265798
	OTU 528421 *C. freundii*	19.3 ± 5.2	19.7 ± 3.9	0		*Enterobacteriales*							
					100	*C. freundii (soB4)*				+			KT265771
Gammaproteobacteria					100	[b]*Lelliottia amnigena (soB12)*	+	+		+			KT265765
					100	*L. amnigena (seB4)*	+	+		+			KT265772
	OTU 922761 *K. varicola*	< 2.0	2.1 ± 1.5	12.3±6.8	100	[b]*Raoultella terrigena (wB13)*	+	+		+			KT265749
					100	*R. terrigena (soB20)*	+	+		+			KT265778
					100	*R. terrigena (seB9)*	+	+		+			KT265755
	OTU 569939 *S. terrae*	< 2.0	< 2.0	0	100	*S. terrae (wB16)*		+		+			KT265788
	OTU 544847 *S. rhizophila*	<2.0	<2.0	<2.0	100	*S. rhizophila (seB11)*	+						KT265763
						Flavobacteriale							
	OTU 1020921 *F. ginsengisoli*	5.6± 2.2	5.7 ± 1.2	< 2.0	100	*F. ginsengisoli (wB6)*	+			+			KT265776
					100	[c]*F. ginsengisoli .(soB8)*	+	+		+			KT265787
Flavobacteria					100	[d]*C.taihuense (wB4)*	+	+	+	+	+		KT265756
					100	[d]*C. taihuense (soB3)*	+						KT265758
	OTU 1039092 *C. taihuense*	5.8 ± 6.8	< 2.0	< 2.0		*Caulobacterales*							

Class	OTU	Wood RA (%)	Soil RA (%)	Sediment RA (%)	Similarity (%)	Isolated bacteria straina (strain code)	A	B	C	D	E	F	Accession number
Alphaproteobacteria	OTU 4300564 *A. benevestitus*	4.4 ± 3.1	3.2 ± 4.6	< 2.0	100	*A. benevestitus* (wB3)		+		+	+		KT265751
						Rhizobiales							
	OTU 592636 *O. thiophenivorans*	< 2.0	< 2.0	2.3 ± 3.2	100	*O. thiophenivorans* (soB16)	+	+	+		+		KT265790

Affiliation is taxonomic showed *Class*, *Order* and *Specie* level of the isolated bacterial strains. ***RA***, relative abundance. ***Similarity (%)***, related between the OTU sequence and the 16S rRNA from isolated recovered strains. Enzymatic activities: ***A = α-D-glucosidase*** ; ***B= α-D-glucosidase; C = α-D-mannosidase; D = β-D-galactosidase; E = β-D-xylosidase; F = α-L-fucosidase;***. **a**. Closest relative species. According to 16S ribosomal RNA gene sequence. **b**. Due to the high similarity in this family the multiple alignment of the analyses sequence region is the same. **c**. BLAST analysis of the strain soB8 identified as *Flavobacterium banpakuense*, however, multiple sequencing alignment indicated a perfect match with the OTU 1020921 affiliated to *Flavobacterium ginsengisoli* . **d**. BLAST analysis of the strain wB4 and soB3 identified as *Chrysobacterium hagamense*, however, multiple sequencing alignment indicated a perfect match with the OTU 1039092 affiliated to *Chrysobacterium taihuense* (Fig. S2).

Discussion

Microbial consortia have been proposed as a reliable and efficient alternative to single strains for lignocellulose degradation purposes (Jiménez et al. 2013; Brossi et al. 2015). When creating such consortia—usually achieved via dilution-to-stimulation approach—the source of the inoculum might determine the effectiveness of the final community. In this study, we addressed the question whether breeding different inocula on the same carbonaceous substrate, i.e. suspended severed wheat straw, would yield taxonomically and functionally similar microbial consortia. We used inocula from forest soil, decaying wood and canal sediment, and analyzed the nascent microbial consortia over time by cultivation-based as well as direct molecular approaches. Clearly, regarding the functioning of the consortia (i.e. degradation of, and growth on, wheat straw as carbon and energy source), high similarity was found.

The results thus touch upon two classical paradigms in microbial ecology, i.e. (1) Beyerinck's postulate "everything is everywhere" and (2) the functional redundancy across and within microbial communities. Overall, our data showed the three microbial consortia to be taxonomically quite different, with a small core community being detectable across them (at genus level). Thus, we cogitated that, within the confines of the experiment, microbial source rather than "environment" was the key driver of the composition of the final consortia, next to their intrinsic degradation and metabolic capacities.

Overall, in terms of lignocellulose degradation, such consortia revealed similar rates. Thus, different bacterial and fungal key players had likely been selected from the diverse pools of microorganisms, performing similar functions under the condition applied. In their local habitats, such communities are influenced by conditions like water availability, oxygen availability, redox potential, temperature and available nutrients (Wei et al. 2009; Montella et al. 2015). Thus, the dissimilarities between conditions reigning in the forest soil, decaying wood and canal sediment habitats, resulting in presumably widely divergent microbiomes, may be at the basis of the differences seen, even after ten 1:1000 transfers in wheat straw batch cultures. In other words, such historical contingencies were not overwhelmed, in taxonomical terms, by the selection applied.

Regarding their degradation capacity, each microbial consortium showed an overall similar degradation pattern (Fig. 4A) but different enzymatic activity profiles (Fig. 4B).

Thus, despite the overall functional redundancy regarding lignocellulose degradation, where the overall process rate was similar, the snapshot-like activity profiles differ. The degradation patterns in the final consortia were likely linked to the particular microbial compositions, as each organism likely contributed with different enzymes attacking the substrate (Table 2, Table S3 in the Supplementary Material). A remarkable finding was the fact that some bacterial strains, identified as the same or very closely related species, had completely different enzymatic palettes and that such differences were linked to the microbial source (Table 2).

Recently, Wongwilaiwalin et al. (2013) also compared the composition of bacterial consortia selected on the same substrate from different microbial inocula. The three consortia bred by them had similar composition at the phylum but were different at the genus level. Our findings stand in contrast to these, which may be attributed to differences in the enrichment conditions: whereas we used mesophilic temperature and mainly oxic conditions, they used high temperature, partial delignified substrate and anoxic conditions. Our findings, next to those of Wongwilaiwalin et al. (2013), showed the relevance of the inoculum, substrate selection and the culture condition for the final composition of the resulting consortia.

In spite of the fact that the three microbial consortia acted in a roughly similar overall manner on wheat straw (Fig. 4A), each revealed different sets of organisms and potentially different secreted enzymes working on the substrate. Wei et al. (2009) proposed different stages of increasing complexity in the microbial lignocellulose degradation process, where the degraders use a plethora of enzymes, in different combinations (Himmel et al. 2010; Moraïs et al. 2014). From the four major enzymatic realms that were invoked, i.e. free, cellbound, multifunctional and cellulosome-bound enzymes (Bayer et al. 2013), the first two classes are thought to play major roles in our systems. Although we expect such enzymes to be working synergistically, this remains to be tested.

We here propose that *S. multivorum* (OTU 891031) has an important contribution to the degradation process in both the wood- and soil-derived consortia, as it was present in high abundance and—albeit in isolation—showed high degradation potential (Table 2). Interestingly, in the sediment-derived consortia, next to *S. multivorum* (OTU 891031), two other strains likely were prominent contributors to the biodegradation process, i.e. *S. faecium* (OTU 824606) and *P. oceanisediminis* (OTU 1081222). The latter was the most abundant species; it has recently been reported as an important lignocellulose degrader (Liang et al. 2014).

Regarding the fungi, several previous studies have described the lignocellulose-biodegrading capacities of both *Ascomycota* (Guerriero et al. 2015) and *Basidiomycota* (Rytioja et al. 2014). For instance, *Trichoderma reesii* can produce a highly efficient set of enzymes for the degradation of cellulose (van den Brink and de Vries, 2011). In contrast, *Aspergillus* species produce mainly enzymes for pectin degradation (van den Brink and de Vries 2011; Guerriero et al. 2015). Although we predict the involvement of fungi in wheat straw degradation, it was difficult to define the relative contribution of these organisms within our final consortia. Also, the selection of fungi was found to be highly dependent on the inoculum source and on their capacities to thrive in liquid (shaking) cultures (Wongwilaiwalin et al. 2010; Jiménez et al. 2013; Simmons et al. 2014). However, we surmised that, in our consortia, fungal-secreted degrading enzymes may have worked in conjunction with the bacterially-released ones.

The results of this study add another piece of evidence to the within-species diversity issue. The Beyerinck "everything is everywhere" paradigm may be expanded with the addition: "but not everything that is dissimilar performs in dissimilar ways". Organisms that were shared across the microbial sources thus may have been involved in the degradation processes, but the overall process may have been supported by additional other organisms. Moreover, and rather surprisingly, taxonomically similar organisms may have been involved in different steps of the process, even within the species.

Accordingly, the efficiency of the degradation process is related to the physiological adaptation and ecological niches of some of the consortial members in their original environment. Additionally, our results indicated that functional redundancy acts upon different levels, as all final consortia presented the same function (ability to degrade the substrate) but the relative contribution of each enzyme to the overall degradation process was probably different.

This study revealed that inoculum source was the strongest driver of the composition of the wheat straw degrading consortia that were produced over ten sequential-batch enrichments. Conspicuous differences emerged between the three consortia, next to similarities, leading to the concept of a core bacterial community that was shared. In functional terms, mixtures of enzymes, with, collectively, grossly similar joint capacities, were probably produced. In future work, the consortial secretomes, next to those from individual strains, may be used as sources of enzymes in the quest to maximize the production of sugars from the complex wheat straw.

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Supplementary material

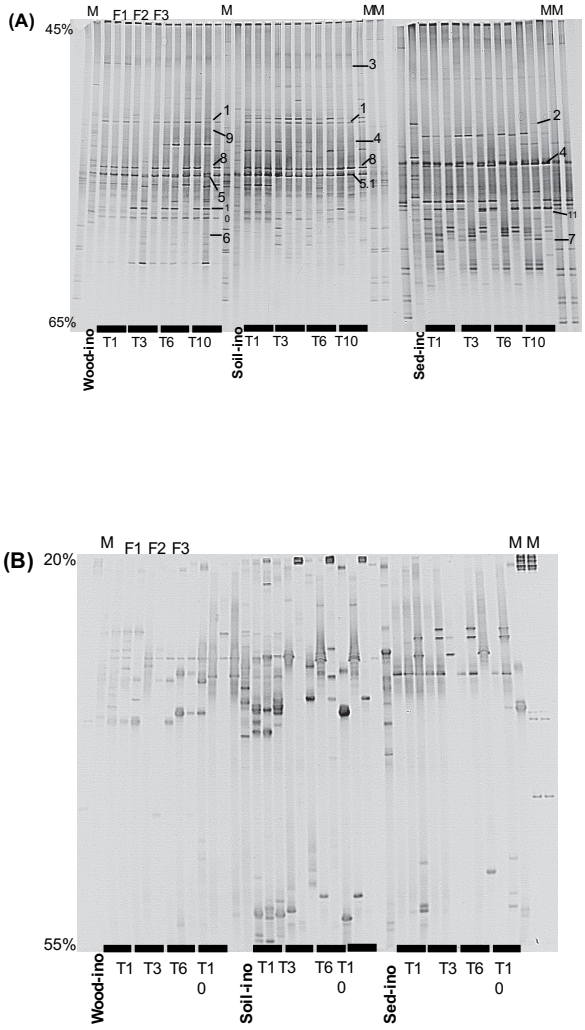


Figure S1 Analyses of steps of the enrichment process. PCR-DGGE analyses of **(A)** *Bacterial* and **(B)** *Fungal communities* at different transfer steps (T1, T3, T6 and T10). The DGGE patterns showed a reduction in the number of bands over experimental time for each of the three inocula. *M*: Marker.

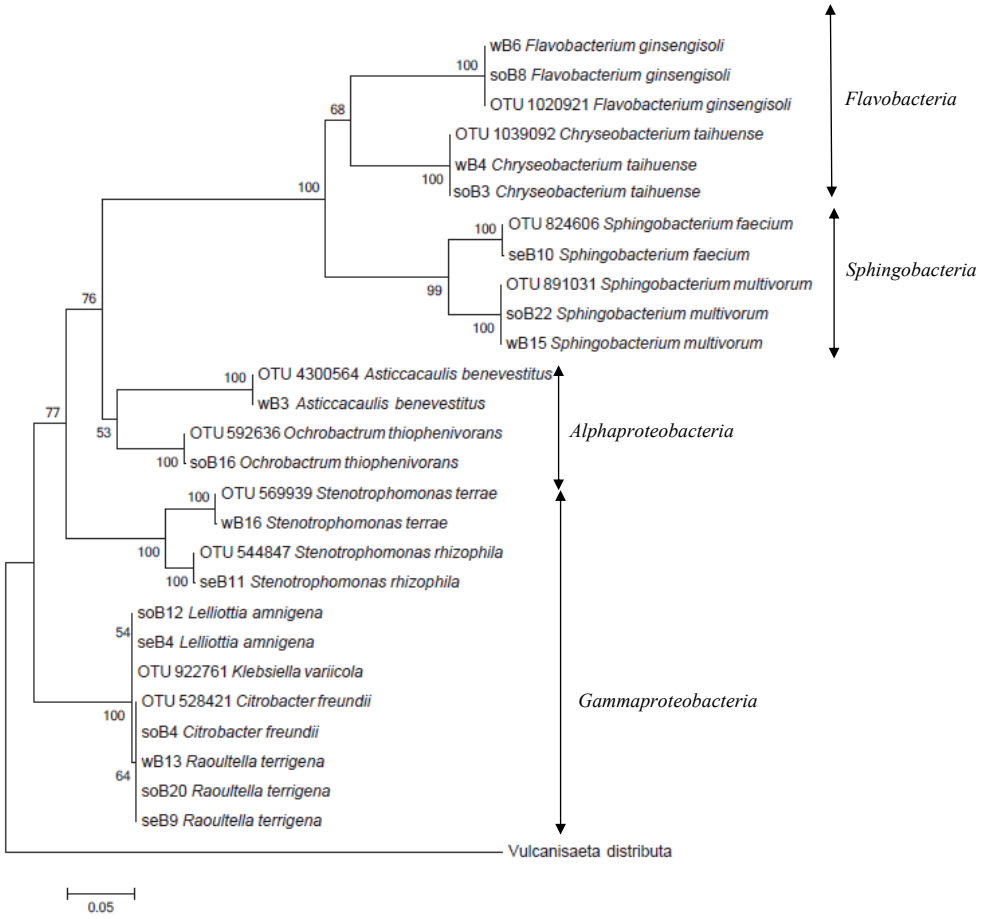
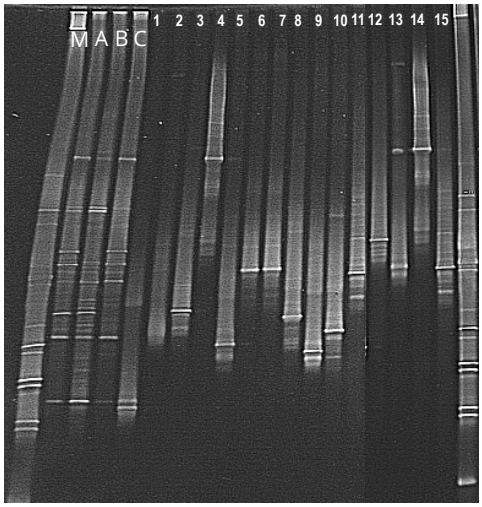
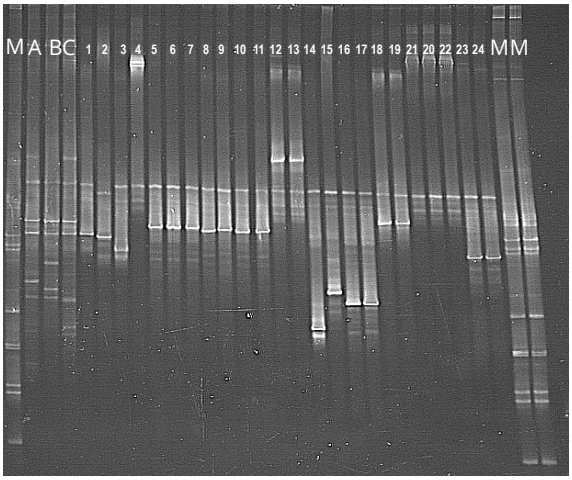


Figure S2 Neighbor Joining tree based on the comparison of 16S rRNA gene sequences from bacterial recovered strains and the most abundant OTUs in the final consortia from wood, soil and sediment inocula. Bootstrap values are expressed as percentages of 1000 replications. The scale bar estimates the number of substitutions per site. The name in the right part correspond to the taxonomic class.



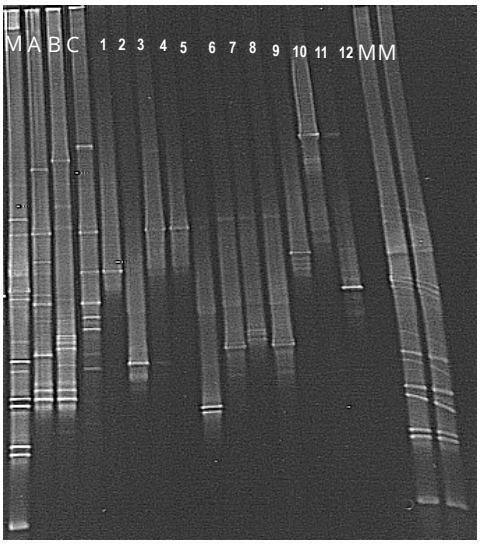
Line	Sample
A	Wood-consortia (T10), flask 1
B	Wood-consortia (T10), flask 2
C	Wood-consortia (T10), flask 3
1	*Achromobacter xylosoxidans*
2	*Acidovorax soli*
3	*Asticcacaulis benevestitus*
4	*Chryseobacterium taihuense*
5	*Delftia tsuruhatensis*
6	*Flavobacterium ginsengisoli*
7	*Flavobacterium ginsengisoli*
8	*Microbacterium gubbeenense*
9	*Microbacterium foliorum*
10	*Pseudomonas oryzihabitans*
11	*Stenotrophomonas terrae*
12	*Raoultella terrigena*
13	*Sphingobacterium multivorum*
14	*Sphingobacterium multivorum*
15	*Stenotrophomonas terrae*

Figure S3 Co-migration DGGE analysis of enriched *wood* derived consortia community (T10) and recovered bacteria strains.



Line	Sample
A	Soil-consortia transfer (T10), flask 1
B	Soil-consortia (T10), flask 2
C	Soil-consortia (T10), flask 3
1	*Acinetobacter johnsonii*
2	*Brevundimonas bullata*
3	*Chryseobacterium taihuense*
4	*Citrobacter freundii*
5	*Comamonas testosteroni*
6	*Comamonas testosteroni*
7	*Comamonas testosteroni*
8	*Flavobacterium banpakuense*
9	*Flavobacterium ginsengisoli*
10	*Flavobacterium ginsengisoli*
11	*Flavobacterium ginsengisoli*
12	*Lelliottia amnigena*
13	*Lelliottia amnigena*
14	*Microbacterium oxydans*
15	*Ochrobactrum thiophenivorans*
16	*Pseudomonas oryzihabitans*
17	*Pseudomonas putida*
18	*Raoultella terrigena*
19	*Raoultella terrigena*
20	*Sphingobacterium multivorum*
21	*Sphingobacterium multivorum*
22	*Sphingobacterium multivorum*
23	*Stenotrophomonas rhizophila*
24	*Stenotrophomonas rhizophila*

Figure S4 Co-migration DGGE analysis of enriched *soil* derived consortia community (T10) and recovered bacteria strains.



Line	Sample
A	Sediment-consortia (T10), flask 1
B	Sediment-consortia (T10), flask 2
C	Sediment-consortia (T10), flask 3
1	*Acinetobacter beijerinckii*
2	*Delftia tsuruhatensis*
3	*Lelliottia amnigena*
4	*Lelliottia amnigena*
5	*Oerskovia enterophila*
6	*Pseudomonas putida*
7	*Pseudomonas fluorescens*
8	*Pseudomonas putida*
9	*Raoultella terrigena*
10	*Sphingobacterium faecium*
11	*Stenotrophomonas rhizophila*
12	Negative control

Figure S5 Co-migration DGGE analysis of enriched *sediment* derived consortia community (T10) and recovered bacteria strains.

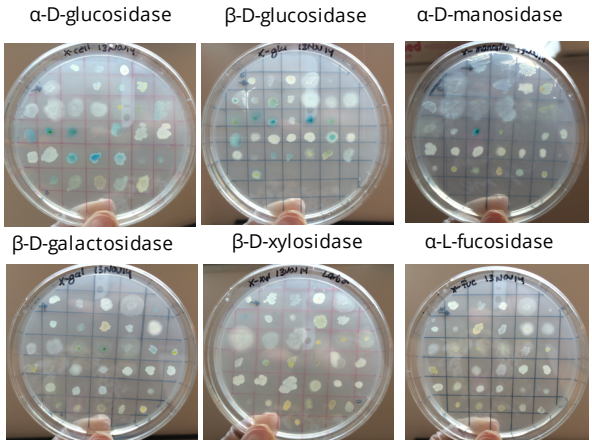


Figure S6 Enzymatic activity detection by chromogenic substrate, in active bacterial strains isolated from final wood, soil and sediment derived consortia.

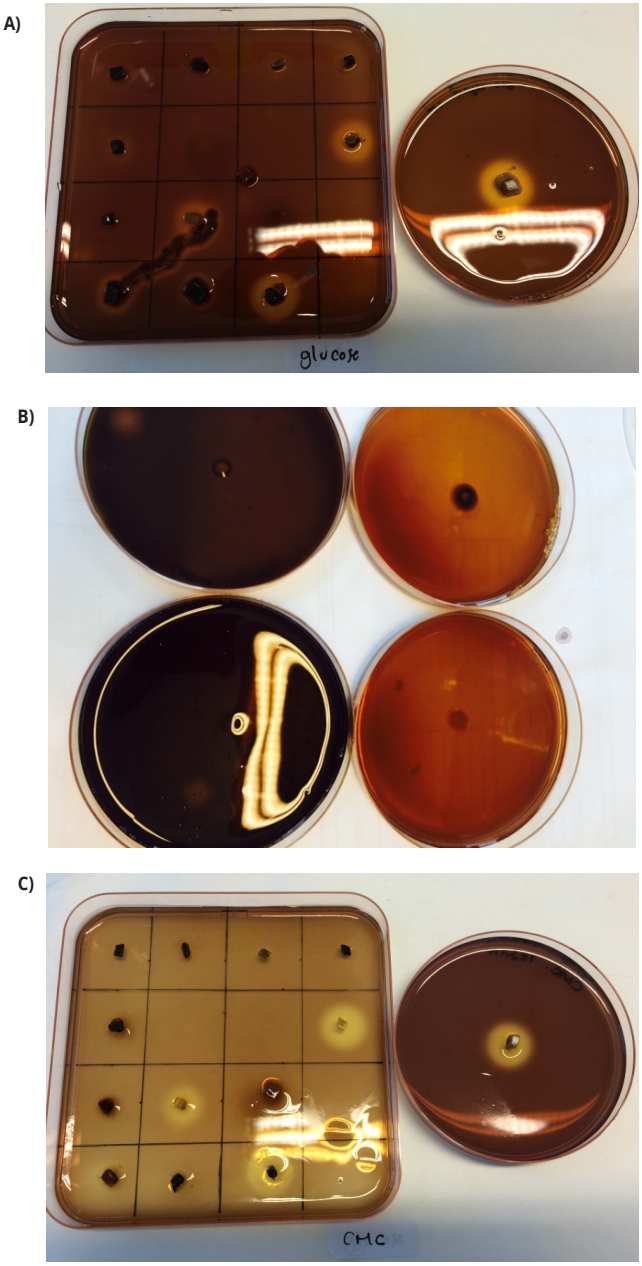


Figure S7 Enzymatic activity detection in CMC, xylan, and cellulose of fungal isolated from final wood, soil and sediment consortia.

TableS1. Primers for paired-end 16s community sequencing on the Illumina MiSeq platform. Bacteria primer 515F/806R. Forward primer 515F (GTGCCAGCMGCCGCGGTAA). Each reverse primer 806R sequence contains different barcode.

SampleID	Reverse Primer	BarcodeSequence	LinkerPrimerSequence	Source	Transfer	Order	Time day	Season	Description
W	806rcbc364	CACACAAAGTCA	GTGTGYCAGCMGCCGCGGTAA	wood	0	1	0	winter	Inoculum wood
10w1		ACTCTGTAATTA	GTGTGYCAGCMGCCGCGGTAA	wood	10	2	61	winter	w10_1
10w2		TCATGGCCTCCG	GTGTGYCAGCMGCCGCGGTAA	wood	10	3	61	winter	w10_2
10w3	806rcbc1443	CAATCATAGGTG	GTGTGYCAGCMGCCGCGGTAA	wood	10	4	61	winter	w10_3
So		GTCAGGTGCGGC	GTGTGYCAGCMGCCGCGGTAA	soil	0	5	0	winter	Inoculum soil
10so1	806rcbc1802	GTTGGACGAAGG	GTGTGYCAGCMGCCGCGGTAA	soil	10	6	61	winter	so10_1
10so2	806rcbc479	GTCACTCCGAAC	GTGTGYCAGCMGCCGCGGTAA	soil	10	7	61	winter	so10_2
10so3	806rcbc561	CGTTCTGGTGGT	GTGTGYCAGCMGCCGCGGTAA	sediment	10	8	61	winter	so10_3
Se	806rcbc1234	TTGAACAAGCCA	GTGTGYCAGCMGCCGCGGTAA	sediment	0	9	0	winter	Inoculum sediment
10se1	806rcbc1513	TAGTTCGGTGAC	GTGTGYCAGCMGCCGCGGTAA	sediment	10	10	61	winter	se10_1
10se2	806rcbc1916	TTAATGGATCGG	GTGTGYCAGCMGCCGCGGTAA	sediment	10	11	61	winter	se10_2
10se3	806rcbc1328	TCAAGTCCGCAC	GTGTGYCAGCMGCCGCGGTAA	sediment	10	12	61	winter	se10_3

Reference: Caporaso JG, Lauber CL, Walters WA, Berg Lyons D, Huntley J, Fierer N, Owens SM, Betley J, Fraser L, Bauer M, Gormley N, Gilbert JA, Smith G, Knight R (2012) Ultrahigh throughput microbial community analysis on the Illumina HiSeq and MiSeq platforms. ISME J 6:1621- 1624. doi: 10.1038/ismej.2012.8

Table S2. Cellulose, hemicellulose (xylan) and lignin mixtures used to obtain the prediction model.

Ternary mixtures	Lignin (%)	Cellulose (%)	Hemicellulose (%)
A	100	0	0
B	0	100	0
C	0	0	100
D	50	25	25
E	25	50	25
F	25	25	50
G	75	25	0
H	25	75	0
I	25	0	75
J	0	25	75
K	33	33	33
L	72	0	25
M	0	75	25

Table S3. Identification and enzymatic activities of bacterial strains isolated from final consortia: wood, soil and sediment derived consortia, obtained using three different inocula.

	Enzymatic activity						Taxonomy affiliation			
Code	**A**	**B**	**C**	**D**	**E**	**F**	**Closest relative**	**Cover (%)**	**Similarity (%)**	**Accession number**
wB1							*Achromobacter xylosoxidans*	99	99	KT265794
wB2							*Acidovorax soli*	99	99	KT265762
wB3		+		++	+++		*Asticcacaulis benevestitus*	98	98	KT265751
wB4	+	+	+	+			*Chryseobacterium taihuense*	98	99	KT265756
wB5		+		+	+++		*Delftia tsuruhatensis*	99	99	KT265782
wB6	+			+			*Flavobacterium ginsengisoli*	99	99	KT265792
wB7							*Flavobacterium ginsengisoli*	99	99	KT265754
wB8	++		++	+++	+++	++	*Microbacterium gubbeenense*	97	97	KT265752
wB9	+	+	++	++	++		*Microbacterium foliorum*	99	99	KT265781
wB10							*Pseudomonas putida*	97	99	KT265784
wB11							*Pseudomonas putida*	99	99	KT265776
wB12	++	+		++			*Raoultella terrigena*	99	98	KT265749
wB13	++	+		++			*Raoultella terrigena*	97	99	KT265761
wB14							*Sphingobacterium multivorum*	98	98	KT265760
wB15		++	+++	+			*Sphingobacterium multivorum*	99	97	KT265748
wB16		+		+			*Stenotrophomonas terrae*	99	99	KT265788
soB1							*Acinetobacter johnsonii strain*	98	99	KT265766
soB2	++						*Brevundimonas bullata*	95	99	KT265759
soB3	+						*Chryseobacterium taihuense*	99	98	KT265758
soB4				+++			*Citrobacter freundii*	99	99	KT265771

soB5							*Comamonas testosteroni*	96	99	KT265795
soB6							*Comamonas testosteroni*	99	99	KT265789
soB7							*Comamonas testosteroni*	99	99	KT265775
soB8	+						*Flavobacterium ginsengisoli*	100	98	KT265768
soB9				+			*Flavobacterium ginsengisoli*	91	99	KT265787
soB10	++			++			*Flavobacterium ginsengisoli*	100	99	KT265777
soB11	++						*Flavobacterium banpakuense*	96	99	KT265796
soB12	+++	+++		+			*Lelliottia amnigena*	100	99	KT265765
soB13							*Lelliottia amnigena*	99	99	KT265774
soB14	+++				+		*Microbacterium oxydans*	99	99	KT265770
soB15							*Mycobacterium septicum*	95	99	KT265753
soB16	+	+	+		+		*Ochrobactrum thiophenivorans*	95	99	KT265790
soB17							*Pseudomonas putida*	99	99	KT265767
soB18							*Pseudomonas oryzihabitan*	100	99	KT265793
soB19	++	+		++			*Raoultella terrigena*	96	98	KT265747
soB20	++	+		+			*Raoultella terrigena*	96	98	KT265778
soB21		++	+++	+	++		*Sphingobacterium multivorum*	100	97	KT265757
soB22	+	++	+++	+	++	+	*Sphingobacterium multivorum*	100	98	KT265750
soB23	++	++	+++	+	++		*Sphingobacterium multivorum*	100	98	KT265779
soB24	+++						*Stenotrophomonas rhizophila*	100	99	KT265769
soB25	++						*Stenotrophomonas rhizophila*	100	99	KT265763
seB1							*Acinetobacter beijerinckii*	98	99	KT265764
seB2							*Delftia tsuruhatensis*	96	99	KT265797
seB3	+			+++			*Lelliottia amnigena*	100	99	KT265773

seB4	+	+		+		*Lelliottia amnigena*	100	99	KT265772
seB5	+					*Oerskovia enterophila*	97	99	KT265785
seB6						*Pseudomonas putida*	100	99	KT265786
seB7	+				++	*Pseudomonas salomonii*	97	98	KT265791
seB8						*Pseudomonas putida*	97	99	KT265783
seB9	+	+		+		*Raoultella terrigena*	100	99	KT265755
seB10	+	+	+++	++		*Sphingobacterium faecium*	99	98	KT265798
seB11	+					*Stenotrophomonas rhizophila*	100	99	KT265780

Closest matches for 16SrRNA sequences of strains in each ERIC-PCR group isolated from wood, soil and sediment derived consortia enzymatic activities: ***A = α-D-glucosidase*** ; ***B= α-D-glucosidase; C = α-D-mannosidase; D = β-D -galactosidase; E = β-D-xylosidase; F = α-L-fucosidase***. Bacteria strains isolated from wood, soil and sediment derived consortia, wB, soB, sedB, respectively. Closest relative species according to 16S ribosomal RNA gene.

Table S4. Identification and degradation activities of fungal strains isolated from final consortia: wood, soil and sediment derived , obtained using three different inocula.

Code	From consortium	Identification (% Identity)*	Activity in glucose	Activity in CMC	Activity in xylan	Activity in cellulose	Accession number
wF1	wood	*Pseudocercospora humuli* (89%)	+	+	+	+	KT265799
wF2	wood	*Arthrographis kalrae* (96%)	+	+	+	+	KT265800
wF3	wood	*Lecythophora sp.* (92%)	++	+	++	+	KT265801
wF4	wood	*Exophiala capensis* (92%)					KT265802
wF5	wood	*Herpotrichiellaceae* sp. (91%)					KT265803
wF6	wood	*Rhodotorula mucilaginosa* (89%)	+	+	+	+	KT265804
soF1	soil	*Acremonium sp.* (97%)	+++	+++	+++	+++	KT265805
soF2	soil	*Mycosphaerella pyri* (90%)	+	+		+	KT265806
soB15	soil	*Mycobacterium septicum* (99%)	+++	+++	+++	+++	KT265753
sedF1	sediment	*Coniochaeta ligniaria* (96%)	+	+	+	+	KT265807
sedF3	sediment	*Penicillium citrinum* (99%)	+++	+++	+++	+++	KT265809
sedF4	sediment	*Plectosphaerella cucumerina* (95%)	+++	+++	+++	+++	KT265810

*Closest relative species- "best" hit partial 18S rRNA gene.

Chapter 4

High salinity tolerant consortia able to growth under a very recalcitrant substrate

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Abstract

The microbial degradation of plant-derived compounds under salinity stress remains largely underexplored. The pretreatment of lignocellulose material, which is often needed to improve the production of lignocellulose monomers, leads to high salt levels, generating a saline environment that raises technical considerations that influence subsequent downstream processes. Here, we constructed halotolerant lignocellulose degrading microbial consortia by enriching a salt marsh soil microbiome on a recalcitrant carbon and energy source, i.e., wheat straw. The consortia were obtained after six cycles of growth on fresh substrate (adaptation phase), which was followed by four cycles on pre-digested (highly-recalcitrant) substrate (stabilization phase). The data indicated that typical salt-tolerant bacteria made up a large part of the selected consortia. These were "trained" to progressively perform better on fresh substrate, but a shift was observed when highly recalcitrant substrate was used. The most dominant bacteria in the consortia were *Joostella marina*, *Flavobacterium beibuense*, *Algoriphagus ratkowskyi, Pseudomonas putida* and *Halomonas meridiana*. Interestingly, fungi were sparsely presented and negatively affected by the change in the substrate composition. *Sarocladium strictum* was the single fungal strain recovered at the end of the adaptation phase, whereas it was deselected by the presence of recalcitrant substrate. Consortia selected in the latter substrate presented higher cellulose and lignin degradation than consortia selected on fresh substrate, indicating a specialization in transforming the recalcitrant regions of the substrate. Moreover, our results indicate that bacteria have a prime role in the degradation of recalcitrant lignocellulose under saline conditions, as compared to fungi. The final consortia constitute an interesting source of lignocellulolytic haloenzymes that can be used to increase the efficiency of the degradation process, while decreasing the associated costs.

Introduction

Lignocellulosic plant biomass is the most abundant global carbon source. Aside its availability and low cost, its utilization can attenuate the conflict between food and energy crops (Kinet et al. 2015). However, the main obstacle in its widespread application is the high cost of the pretreatments, which are necessary to open the intricate polysaccharide structure. Such pretreatments enhance the accessibility of enzymatic attack (Talebnia et al. 2010) and decrease the proportion of crystalline cellulose and lignin content, the two main causes of the recalcitrance of lignocellulose. Overcoming this recalcitrance is fundamental for getting access to the polymers that yield sugar monomers, which can be transformed in valuable compounds such as sustainable biomaterials, biofuel, and biochemicals (Khoo et al. 2016).

In the past years, three different pretreatment processes have been proposed to improve the digestibility of lignocellulose materials. These aimed to foster (1) the degradation of hemicellulose, by acid or hot water treatment, (2) that of lignin, by alkaline pretreatment to break the lignin-carbohydrate linkage bond, and (3) the generic disruption of the matrix by thermal treatment (Brethauer and Studer 2015). Such pretreatments not only increase the global cost of the bioprocess but also generate diverse compounds that interfere with downstream processes (Jönsson and Martin 2016; Rabemanolontsoa and Saka 2016).

A promising new pretreatment method is based on the application of ionic liquids (ILs), organic salts ("green solvents") (Sun et al. 2016) that are liquid at room temperature. Using ILs, lignocellulose biomass is exposed to highly saline conditions that disrupt the rigid lignocellulose structure, leading to a considerable reduction in cristallinity and increased accessibility to enzymatic attack. However, when using acid/base treatment or ILs, subsequent enzymatic hydrolysis of the substrate can only be performed after several washing steps aiming at salt removal, as salt often inhibits enzymatic activity. The use of haloenzymes (or enzymes tolerant to high salinity) (Gunny et al. 2014) could represent a sound alternative strategy to increase the efficiency and reduce the cost of the bioprocess.

Dilution-to-stimulation has been used as a successful method to enrich microbial consortia capable of degrading plant biomass and their respective enzymes (Brossi et al. 2015; Maruthamuthu et al. 2016). These consortia have been obtained from a variety of sources (Cortes-Tolalpa et al. 2016) and are often

capable of degrading a range of lignocellulose materials (Okeke and Lu 2011; Brossi et al. 2015). For instance, we have shown that consortia obtained from different microbial sources naturally enriched in lignocellulose material quickly reach a stabilization phase (phase of relative stability of the consortium in terms of composition and activity) during the enrichment process (Cortes-Tolalpa et al. 2016). Although the various consortia did not differ in their final degradation potential, they reached this through different activities, as they differed in their enzymatic pools. Thus, the source of the inoculum used for the enrichment clearly influenced the final outcome and type of process. Despite the success of this approach, which leads to consortia capable of "attacking" or consuming the most labile part of the substrate, these consortia have been obtained under "low" salt concentrations. Given the importance of the microbial source, the development of such consortia using halotolerant microbes could provide an interesting perspective.

The aim of this study was to examine whether it is possible to obtain a halotolerant microbial consortium capable of degrading lignocellulose biomass (raw wheat straw) at high rate under high-salt conditions. For that, we used as inoculum the microbial community obtained from salt marsh soil from a the island of Schiermonnikoog, the Netherlands. This was previously found to be adapted to high-salt concentrations and to harbor key genes involved in lignocellulose degradation (Dini-Andreote et al. 2014; Wang et al. 2016). In addition, to generate consortia with high degradation potential under high-salt conditions, selection on pre-digested recalcitrant substrate was applied.

Methods

Culture media and lignocellulose substrate

For the experiment, we used a the mineral medium solution MMS (7 g/L $Na_2HPO_4 \cdot 2H_2O$; 2 g/L K_2HPO_4; 1 g/L $(NH_4)2SO_4$; 0.1 g/L Ca $(NO_3)_2 \cdot 4H_2O$; 0.2 g/L $MgCl_2 \cdot 6H_2O$ g/L, pH 7.2) (Cortes-Tolalpa et al. 2016), supplemented with 25 g per liter of NaCl. The medium was further supplemented with vitamin solution (0.1 g Ca-pantothenate, 0.1 g cyanocobalamine, 0.1 g nicotinic acid, 0.1 g pyridoxal, 0.1 g riboflavin, 0.1 g thiamin, 0.01 g biotin, 0.1 g folic acid; H_2O 1 L) and trace metal solution (2.5 g/L EDTA; 1.5 g/L $FeSO_4$; 0.025 g/L $CoCl_2$; 0.025 g/L $ZnSO_4$; 0.015 g/L $MnCl_2$; 0.015 g/L $NaMoO_4$; 0.01 g/L $NiCl_2$; 0.02 g/L H_3BO_3; 0.005 g/L $CuCl_2$). "Raw wheat straw" used as lignocellulose source, was air-dried (50°C) before cutting it into pieces of about 5 cm length and then the pieces were thoroughly ground,

using a mill hammer, to pieces ≤ 1 mm. No pre-treatment was performed (untreated raw substrate). Sterility of the substrate was verified following plating on trypticase soy agar (TSA) plates. All chemicals and reagents used in this work were of analytic molecular biology grade (Sigma-Aldrich, Darmstadt, Germany).

Sample collection

The source of the microbial community used in this experiment was soil from Schiermonnikoog island (53°29′ N 6°10' E), 10-g of surface soil (0–10 cm) representative of the 105-year old plot located at the end of the natural primary succession observed in this island (Wang et al. 2016), the soil samples were thoroughly mixed. These soils are characterized by pH varying from 7.4–7.6 and sodium concentration from 3541 ± 170 to 5188 ± 624 mg dm^{-3}, depending on the period of the year (Dini-Andreote et al. 2014). Cell suspension was prepared by adding 10 g of the soil to 250 mL flasks containing 10 g of sterile gravel in 90 mL of MMS. The suspension was shaken for 30 min at 200 rpm (room temperature).

Enriched consortia

To start the enrichment, 250 µL of the suspension was added to each of triplicate 100-ml Erlenmeyer flasks containing 25 mL of MMS supplemented with 1% (w/v) sterilized wheat straw, 25 µL of vitamin and 25 µl of trace metal solution. Flasks were incubated at 28°C, with shaking at 180 rpm. Cultures were monitored by counting cells in a Bürker-Türk chamber every day. Experiments started with around 5 log cells/mL. Once the systems had reached around 9 log cells/mL (and straw had visually been degraded), 25 µL of culture was transferred to 25 mL of fresh medium (dilution 10^{-3}). During the first part of the enrichment, from transfer one to six—the adaptation phase—we used fresh wheat straw. In the second part of the experiment, from transfer seven to ten—the stabilization phase—we used recalcitrant wheat straw. This consisted of the sterilized substrate recovered at the end of the adaptation phase (transfers five and six), partially consumed by microbial consortia, and therefore encompassing only the most recalcitrant structure of the substrate (Supplemental Fig. S1). Following each transfer (T), part of the bred consortia was stored in 20% glycerol at −80°C. The consortia of the T1, T3, T6, T7, and T10 flasks were used for all subsequent analyses, as detailed below. As controls, we used microbial sources in MMS without substrate (CA 1, 2, 3) as well as MMS plus substrate without inoculum (CB 1, 2, 3). Before starting the enrichment Erlenmeyer flasks containing 25 mL lignocellulose, media were autoclaved at 121°C for 27 min.

DNA extraction

One mL of selected cultures was used for community DNA extraction using the "Power Soil" DNA extraction kit (inoculum source) (MoBio® Laboratories Inc., Carlsbad, USA) and the UltraClean DNA Isolation Kit (each enriched consortium and isolates). The instructions of the manufacturer were followed, except that the resuspension of the DNA from the inoculum sources was in 60 µL resuspension fluid.

PCR followed by denaturing gradient gel electrophoresis (PCR-DGGE)

Total community DNA was used as the template for amplification of the partial 16S rRNA gene fragment by PCR with primers F968 with a GC clamp attached to the 5'-end and universal bacterial primer R1401.1b. For ITS1 amplification, primers EF4/ITS4 were used; this PCR was followed by a second amplification with primers ITS1f-GCITS2. Primer sequences, the reaction mixtures, and cycling conditions have been described (Brons and van Elsas 2008; Pereira e Silva et al. 2012). The DGGE was performed as reported by Cortes-Tolalpa et al. (2016). The DGGE patterns were then transformed to a band-matching table using GelCompar II software (Applied Maths, Sint Martens Latem, Belgium).

Quantitative PCR (q-PCR)

The 16S rRNA gene region V5-V6 (bacteria), as well as the ITS1 region (fungi), were amplified using 1 ng of community DNA as the template and primers 16SFP/16SRP and 5.8S/ITS1 (Pereira e Silva et al. 2012), respectively. Standard curves were constructed using serial dilutions of cloned 16S rRNA gene and ITS1 fragments from *Serratia plymuthica* (KF495530) and *Coniochaeta ligniaria* (KF285995), respectively. The gene target quantification was performed, in triplicate, in an ABI Prism 7300 Cycler (Applied Biosystem, Lohne, Germany).

Bacterial community sequencing and analyses

Amplicons of 250 bp were generated based on primers amplifying the V4-V5 of the 16S rRNA gene. PCR amplifications were conducted in triplicate reactions for each of the 18 samples with the 515F/806R primer set (Supplemental Table S1). PCR and sequencing were performed using a standard protocol (Caporaso et al. 2012). Illumina MiSeq sequencing was performed at GENEWIZ (South Plainfield, USA). We processed the raw data using the "quantitative insight into microbial ecology" (QIIME) software, version 1.91. The sequences were de-multiplexed and

quality-filtered using split_libraries_fastq.py default parameters (Bokulich et al. 2013). The derived sequences were then clustered into operational taxonomic units (OTUs) using open-reference OTU picking against the Greengenes reference OTU data base with a 97% similarity threshold (Rideout et al. 2014). Then, we performed quality–filtering to discard OTUs present at very low abundance (< 0.005%) of the total number of sequences (Bokulich et al. 2013). An even sampling depth of 20,000 sequences per sample was used for assessing α- and β- diversity measures. Metrics for α-diversity were Chao1 index (estimated species richness) and Shannon index (quantitative measure of species). β-diversity analyses among the final consortia were performed using unweighted UniFrac distance matrix. Matrix similarity, *PERMANOVA*, and principal coordinate analysis (PCA), were performed by using phyloseq (McMurdie and Holmes 2013). Differential OTU abundance was calculated using DESeq2 with phyloseq (Supplemental Fig. S2) (Love et al. 2014; Mcmurdie et al. 2014). The comparison was made between sequential transfers (inocula-T1, T1-T3, T3-T6, T6-T7, T7-T10) and between the two main phases, adaption and stabilization phase, respectively.

Isolation and identification of bacterial and fungi

From transfers 6 and 10, we isolated bacterial and fungal strains, using R2A (BD Difco®, Detroit, USA) and potato dextrose agar (PDA) (Duchefa Biochemie BV, Haarlem, The Netherlands), respectively. The isolation part can be found in Electronic supplemental material 1 (ESM 1). The primer pair U1406R and B8F was used for amplification of the 16S rRNA gene of bacterial strains, in the following PCR: initial denaturation at 95°C for 5 min; 35 cycles of 95°C for 1 min, 52°C for 30 s, 72°C for 2 min and final extension at 72°C for 7 min. For identification of fungal strains the primers EF4 and ITS4 were used for amplification of the ITS1 region of the 18S rRNA gene, according to the following PCR : initial denaturation at 95°C for 5 min; 34 cycles of 94°C for 30 s, 55°C for 30 s, 72°C for 1 min 30 s and final extension at 72°C for 5 min. The amplicons were sequenced by Sanger technology (LGC Genomics, Lückenwalde, Germany) and the sequence of the PCR product was further used for bacterial and fungal identification. Taxonomic assignments of the sequences were done using BLAST-N (http://blast.stva.ncbi.nlm.nih.gov/Blast.cgi). We used the best BLAST hit affiliation for taxonomic assignment with a cutoff of 97 and 95% of identity of bacteria and fungi, respectively, and 95% of coverage. Sequences are publicly available in the GenBank database under accession numbers MF619963 to MF620009 (Tables 3 and 4). The recovered strains have been deposited in the German Collection of Microorganisms and Cell Cultures (DSMZ, Braunschweig, Germany).

Matching bacterial strains with abundant OTUs

The recovered bacterial strains were linked to the OTUs based on sequence similarity. The almost-full-length 16S rRNA gene sequences from the strains were compared—in the specific V4-V5 region—to the sequences of the abundant OTUs using ClustalW. Phylogenetic analyses (pairwise distance) were conducted with MEGA v6 (Tamura et al. 2013) using Maximum Likelihood evolutionary distances that were computed using the Kimura-2 parameter method. The branch node strengths were tested with bootstrap analyses (1000 replications).

Screening of lignocellulolytic enzyme production in recovered bacterial strains

Cellulases and hemicellulases in bacterial strains were detected by model substrate coupled to chromogenic compounds. The compounds 5-bromo-4-chloro-3-indolyl α-D-glucopyranoside (X-glu), 5-bromo-4-chloro-3-indolyl β-D-cellobioside (X-cell), 5-bromo-4-chloro-3-indolyl α-D-mannopyranoside (X-man), 5-bromo-4-chloro-3-indolyl β-D-galactopyranoside (X-gal), 5-bromo-4-chloro-3-indolyl β-D-xylopyranoside (X-xyl), and 5-bromo-4-chloro-3-indolyl β-fucopyranoside (X-fuc) (Sigma-Aldrich, Darmstadt, Germany) were used to detect the production and activity of α-glucosidase, cellobiohydrolases, α-mannosidase, β-galactosidase, β-xylosidase, and α-fucosidase enzymatic activity, respectively (Cortes-Tolalpa et al. 2016). The strains were spread in duplicate on R2A plates containing 1 M NaCl and each one of the chromogenic compounds listed above. The plates were incubated for 48 h at 28°C. A positive enzymatic activity was observed as a blue colony growing on the plate.

Lignocellulose degradation by selected halotolerant consortia

The final microbial consortia from transfers 1, 3, 6, 7, and 10 were incubated with 1% (w/v) mulched wheat straw under the culture condition that was previously described. After incubation, the final remaining particulate wheat straw was recovered from the microcosm flasks; the substrate was washed to remove microbial cells and sieved to obtain the degraded particles.

The degradation rates of the components of the substrate, before and after incubation, were determined by Fourier-transformed infrared (FTIR) spectra (Adapa et al. 2011; Xu et al. 2013). All FTIR measurements were carried out on oven-dried material (50°C, 24 h). Thirty-two scans were run per sample; all spectra

between 800 and 1800 cm1 were used for the analyse (Krasznai et al. 2012). Each sample (calibration and consortium samples) was analyzed in triplicate. All spectra were subjected to baseline correction and then corrected for physical effects by second derivative Savitzky-Golay treatment (FitzPatrick et al. 2012). Correction and analysis using partial least squares (PLS) regression were conducted using Unscrambler X v.10 (CAMO, Woodbridge,USA). A mathematical model was created on the basis of a calibration with standard mixtures, consisting of hemicellulose (proxy beechwood xylan, ≥ 90%, Sigma-Aldrich, Steinheim, Germany), cellulose (powder, D-516, Macherey-Nagel, Düren, Germany) and lignin (alkaline, Sigma-Aldrich, Steinheim, Germany) in the proportion described in Supplemental Table S2 (Adapa et al. 2011). The model displayed R_2 values of 0.9876, 0.9889, and 0.9763 and a slope of 0.9788, 1.000, and 0.9987 for hemicellulose, cellulose, and lignin, respectively. These models were then used to infer the proportion of each component in the samples (FitzPatrick et al. 2012; Krasznai et al. 2012). Finally, the degradation of hemicellulose, cellulose, and lignin was estimated by subtracting the percentage of the residual substrate from the total percentage of each hemicellulose component before degradation. Degradation rate was calculated using the following equation: $\frac{C_i - C_f}{C_i} x100$ where C_i is the total amount of compound before degradation and C_f is the residual component after degradation (Wang et al. 2011).

Statistical analyses

One-way analysis of variance (*ANOVA*) followed by Tukey HSD pairwise group comparisons was performed in IBM SPSS Statistics version 24 (SPSS Inc., Chicago, USA).

Results

Halotolerant lignocellulolytic consortia are capable of degrading lignocellulose biomass under high-salt conditions

The microbial community from the salt marsh soil, used as the inoculum, was able to adapt to, and grow on, wheat straw as the single carbon and energy source and under saline conditions. Using microscopic counts, we found that, during the adaptation phase, from transfer one to six, the cultures exhibited a progressively increasing fitness, as indicated by an increasing specific growth rate over time. The average specific growth rate μ (h^{-1}; ± standard deviation; see Fig. 1A) increased from 0.22 h^{-1} (± 0.01) to 0.70 h^{-1} (± 0.03), from T1 to T6. In the stabilization phase, we observed an almost two fold reduction in the growth rate immediately after substrate change, which dropped from 0.70 h^{-1} (±0.03) to 0.38 h^{-1} (±0.02) (Fig. 1a, see T6 and T7), after which it remained constant until the end of the experiment (T10). The reduced apparent fitness of the consortia was thus related to the increased recalcitrance of the substrate.

The microscopic cell counts were corroborated by the 16S rRNA gene and ITS1 copy numbers determined by qPCR, which were used as proxies for bacterial (Fig. 1B) and fungal community density (Fig. 1C), respectively. At the end of each transfer in the adaptation phase, the consortia reached maximal bacterial levels of (log scale): 7.5 ± 1.3 (T1), 9.1 ± 0.002 (T3), and 9.2 ± 0.034 (T6) (average log 16S rRNA gene copies per mL ± standard deviation). In the stabilization phase, these values were similar: 9.2 ± 0.034 (T7) and 9.1± 0.02 (T10). The fungal abundances (measured by numbers of ITS1 gene copies) at the end of transfers 1, 3, 6, and 7 reached around (log scale) 6 per mL. However, we observed a significant reduction of ITS1 copies in the stabilization phase, from T7 to T10 (*t-test*, $P < 0.05$), indicating that under saline conditions, fungi were strongly deselected by the increase of substrate recalcitrance.

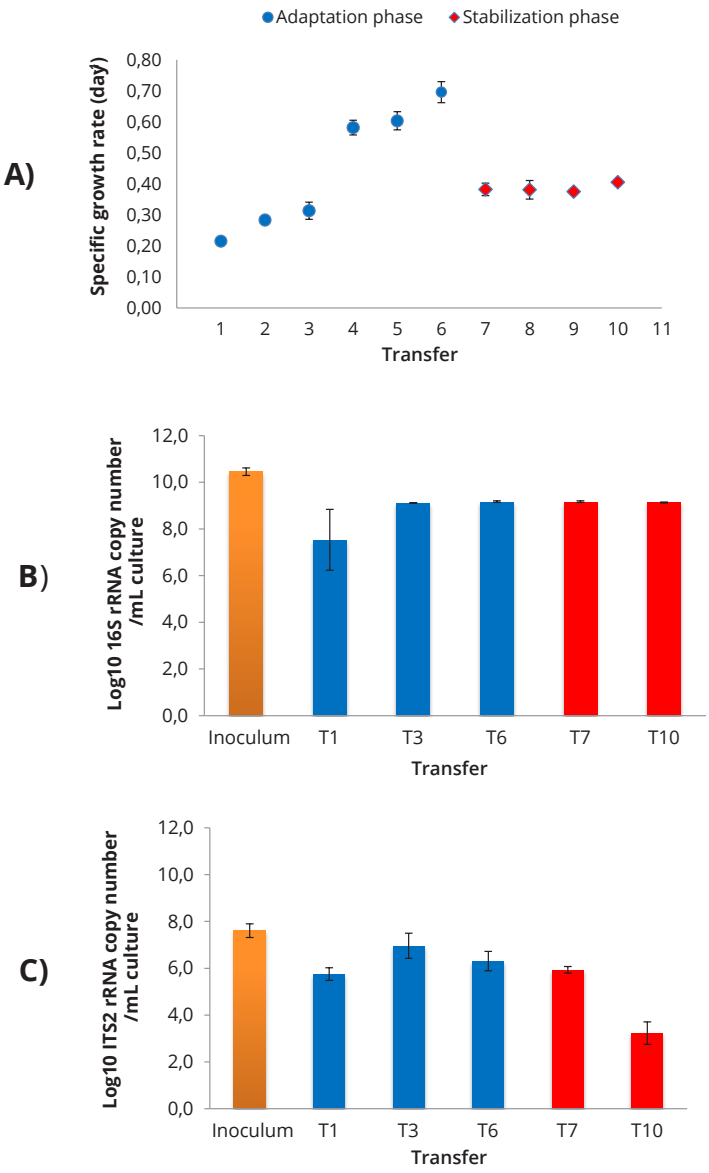

Figure 1 Microbial growth rates and abundances during the enrichment. (A) *Specific growth rate μ (day^{-1})* of microbial communities across the enrichment processes, as determined by microscopic cell counts.**(B)** *Bacterial abundances* during the enrichment (log copies per mL), as determined by qPCR targeting the 16S rRNA gene. **(C)** *Fungal abundances* during the enrichment (log copies per mL), as determined by qPCR targeting the ITS1 region. Yellow bars—original soil inoculum; blue circles and bars—adaptation phase using fresh lignocellulose substrate (transfer 1 to 6); red diamonds and bars—stabilization phase using pre-digested substrate (transfer 7 to 10). Bars refer to standard errors of the mean (n=3).

Shifts in bacterial and fungal community composition

The microbial consortia were first analyzed by bacterial- as well as fungal-specific PCR-DGGE to examine the overall changes in community composition in selected transfers. Multidimensional scaling (MDS) of the bacterial community composition indicated a clear separation between the inoculum and the enriched communities and revealed the existence of two different clusters, separated on the basis of growth on fresh (adaptation phase) versus recalcitrant substrate (stabilization phase) (*PERMANOVA*, $P < 0.05$, Supplemental Fig. S3; Supplemental Fig. S4).

In contrast, the fungal consortia did not reveal a strong clustering between adaptation and stabilization phases, although they were significantly different from each other (*PERMANOVA*, $P < 0.05$) (Supplemental Fig. S4). The change in fungal community composition in the stabilization phase was associated with a substantial reduction of the number of bands, confirming the previously described qPCR results, which indicated that, under the applied conditions, fungi are deselected and outcompeted by bacteria.

Degradation of wheat straw by the microbial consortia

All consortia were found to preferably consume the hemicellulose part of the substrate, which was up to 80% degraded (Fig. 2). None of the selected consortia presented significant differences in hemicellulose degradation (*ANOVA*, $P > 0.05$). Interestingly, the cellulose part of the wheat straw was degraded to a lower extent, i.e., slightly above 40% (Fig. 2). Comparisons between the consortia across time indicated there was no significant difference in the degradation of hemicellulose, cellulose, and lignin (*ANOVA*, $P>0.05$), except at T7 and T10, at which time points significant differences in the degradation of cellulose and lignin were found. The consortia at T10 degraded significantly more cellulose (64.2% ± 6.6) and lignin (61.4% ± 5.7) than those at T7 (cellulose 47%± 10.8 and lignin 47.8%± 6.6; *ANOVA*, $P < 0.05$) (Fig. 2). Comparing the two phases, the consortia from the stabilization phase were able to degrade significantly more lignin than those from the adaptation phase (*t-test*, $P < 0.05$).

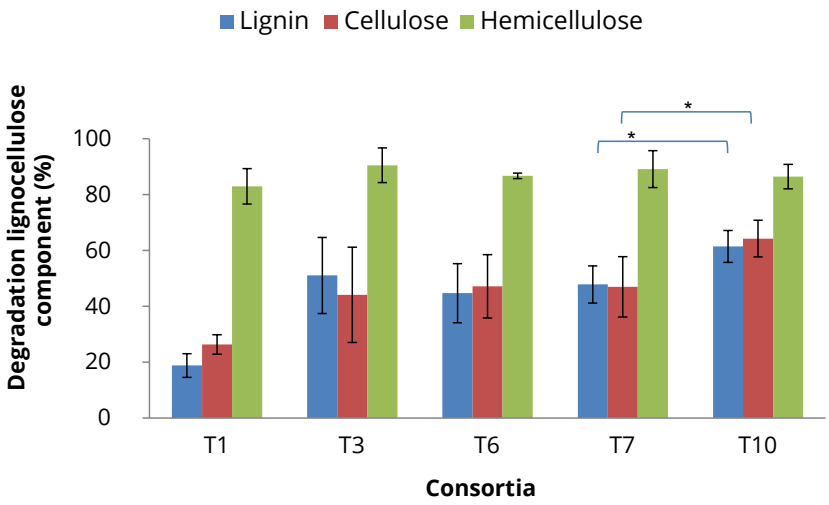

Figure 2 Lignocellulose degradation potential of the communities enriched during the experiment. Percentage reduction of ***hemicellulose, cellulose,*** and ***lignin*** contents of wheat straw ***(substrate)*** comparing with substrate recovered from an not uninoculated control. Explanation: 100% lignin, 100% cellulose, and 100% hemicellulose are equivalent at 18.3% of lignin, 42.5% of cellulose, and 32.5% of hemicellulose in the substrate respectively. Bars refer to standard errors of the mean (n = 3).

Communities structure of the degrading consortia, as determined by 16S rRNA gene-based sequencing

Direct amplicon sequencing performed on a selected number of transfers revealed grossly decreasing bacterial richness values along the transfers. Specifically, for the inocula and the T1, T3, T6, T7, and T10 consortia, the values were 4.84 ± 0.34, 3.49 ± 0.40, 3.40 ± 0.72, 3.14 ± 0.25, 3.41 ± 0.38, and 2.90 ± 0.27, respectively (log OTU number ± standard deviation). Moreover, significant differences in richness were found between the consortia in the adaptation and the stabilization phases, T1, T3 and T6 versus T7 and T10, respectively (*ANOVA*, $P < 0.05$).

Regarding the bacterial community structures (β-diversities), PCoA of the unweighted UniFrac community distances confirmed the previously described PCR-DGGE results. The data showed that the consortia selected on fresh substrate (adaptation phase, T1, T3, and T6) were markedly different from those selected on recalcitrant substrate (T7 and T10) (Fig. 3). *PERMANOVA* showed that, indeed, bacterial consortia were significantly different between the adaptation and stabilization phases, as driven by the change in the substrate ($P < 0.005$).

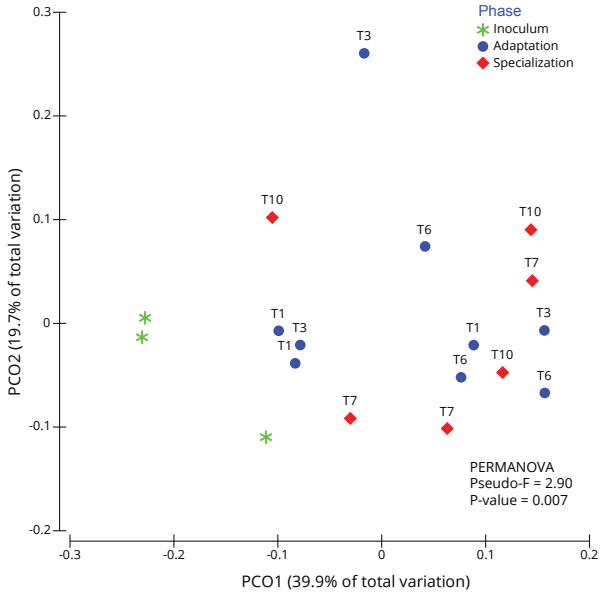

Figure 3 Shifts in bacterial community structure during the adaptation and stabilization phases of the experiment as derived from the 16S rRNA gene sequencing data (V4-V5 region). Principal coordinates analysis (PCoA) of unweighted UniFrac distances for 16S rRNA gene sequencing data of selected enrichment consortia (T1, T3, T6, T7, T10). Fresh substrate ***(blue circles),*** used substrate ***(red diamonds),*** inoculum ***(green asterisks).*** *PERMANOVA* indicated significant differences between the communities (P= 0.007, Pseudo-F = 2.90).

This indicated that a clear shift had occurred as a result of the transition from raw to recalcitrant substrate. The comparison of the bacterial consortia between the transfers showed that, in the adaptation phase, a large amount of OTUs was significantly affected by the enrichment, leading to a large turnover in community composition and positive selection of OTUs. In contrast, the turnover was lower in the stabilization phase,with relatively few OTUs being negatively affected by the confrontation with the recalcitrant substrate (T7) (Fig. 4). Comparison of the consortia at T7 and T10 (stabilization phase) revealed an increase of abundance of particular OTUs (Fig. 4). Thus, 19 OTUs were differentially selected in the adaptation phase (Table 1) and only five OTUs were positively affected by the change in the substrate during stabilization phase (Table 2). Four OTUs were present in both phases: OTU57506 (affiliated with *Halomonas alkaliphila*), OTU415 (affiliated with *Algoriphagus winogradskyi* or *ratkowskyi)*, OTU358 (*Joostella marina*), and OTU667 (*Flavobacterium beibuense*).

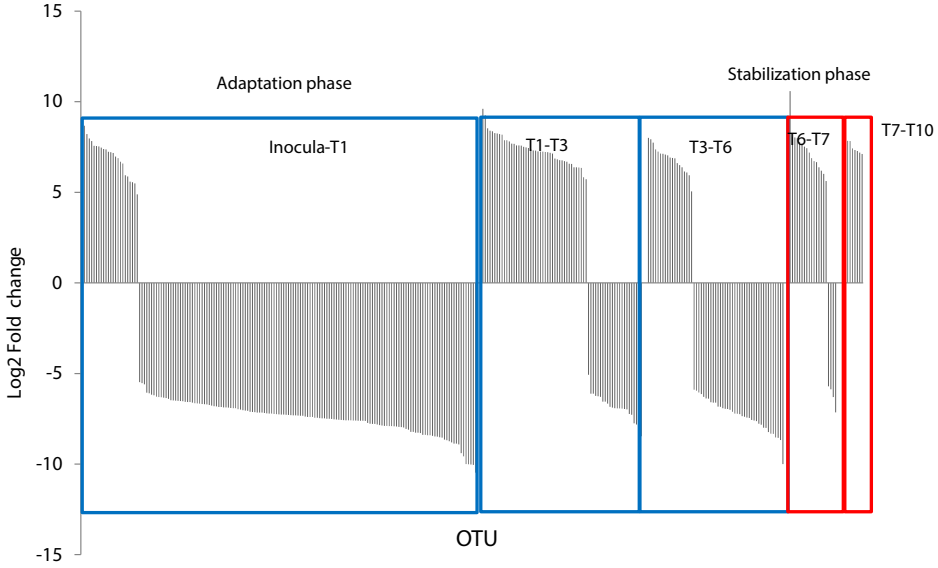

Figure 4 Number of OTUs (log2 fold change) that were positively and negatively influenced in the adaptation and stabilization phases of the experiment. DESeq2 function for phyloseq was used to obtain the statistically significant OTUs affected by the enrichment process and the change in substrate composition. Comparisons between selected transfers for adaptation phase included ***Inoculum vs T1, T1 vs T3,*** and ***T3 vs T6 (blue squares)***. In the stabilization phase, the comparison was made between ***T3 vs T6*** and ***T7 vs T10 (red squares).*** The adaptation phase shows an important reduction of numbers of OTUs, as indicated by a larger number of bars with negative values especially in the early and late transfers, whereas in the stabilization phase, we observed an increase in the number of OTUs selected—mostly OTUs with positive values were significantly different from one transfer to another.

Table 1. Abundant OTUs that were significantly enriched in the adaptation phase *(fresh substrate)*, as determined by 16S rRNA gene sequencing.

OTU	Taxonomic affiliation	*Identity (%)	Accession number reference*
OTU57506	*Halomonas meridiana*	99	DQ768627.1
OTU358	*Joostella marina*	99	KP706828.1
OTU667	*Flavobacterium beibuense*	99	KY819115.1
OTU496	*Flavobacterium suzhouense*	98	KM089833.1
OTU665	*Pseudomonas putida*	99	KM091714.1
OTU421	*Stenotrophomonas rhizophila*	99	MF381036.1
OTU176	*Paracoccus seriniphilus*	99	KX453219.1
OTU806	*Nitrosotalea* sp.	99	KJ540205.1
OTU659	*Altererythrobacter* sp.	99	KT325206.1
OTU850	*Halomonas alkaliphila*	99	MF928383.1
OTU49	*Proteinimicrobium ihbtica*	90	AM746627.1
OTU859	*Photobacterium halotolerans*	99	KT354559.1
OTU114263	*Devosia ginsengisoli*	99	KF013197.1
OTU93687	*Bacillus flexus*	99	MF319797.1
OTU253	*Halomonas taeanensis*	95	FJ444986.1
OTU71211	*Rhizomicrobium palustre*	97	NR_112186.1
OTU77552	*Halomonas variabilis*	99	KX351792.1
OTU158296	*Algoriphagus locisalis*	99	NR_115326.1
OTU66912	*Halomonas meridiana*	94	DQ768627.1

*****Similarity between the OTU sequence and that of the NCBI entry

Table 2. Abundant OTUs that were significantly enriched in the stabilization phase *(pre-digested substrate)*, as determined by 16S rRNA gene sequencing.

OTU	Taxonomic affiliation	*Identity (%)	Accession number reference*
OTU358	*Joostella marina*	99	KP706828.1
OTU667	*Flavobacterium beibuense*	99	KY819115.1
OTU415	*Algoriphagus ratkowskyi*	98	KM091714.1
OTU665	*Pseudomonas putida*	99	KM091714.1
OTU57506	*Halomonas meridiana*	99	DQ768627.1

*Similarity between the OTU sequence and that of the NCBI entry

Degradation of wheat straw by selected strains

In total, 47 bacterial strains were recovered from the consortia at T6 and T10. Most of the strains were isolated from both transfers, except for *Photobacterium halotolerans* A34, *Albirhodobacter marinus* C13, and *Paracoccus seriniphilus* C14, which were recovered only from the adaptation phase (T6) (Table 3). All were identified on the basis of 16S rRNA gene sequencing (Tables 3 and 4). Subsequently, bacterial strains were screened for the production of enzymes able to degrade X-glu, X-cell, X-gal, X-xyl, X-man and X-fuc (Tables 3 and 4). The data showed that such degradation potential was widespread across the strains. Of the 47 strains tested, only three did not show any enzymatic activity against the selected substrates. These were *Staphylococcus capitis* P1, *Bacillus oleronius* G13, and *Erythrobacter gaetbuli* G57. By aligning the 16S rRNA gene sequences recovered from the isolated bacteria with those of the OTUs obtained by direct sequencing (Fig. 5), we were able to pinpoint the strains that were highly abundant in the consortia (Tables 3 and 4). In the adaptation phase, nine strains were closely related to four enriched OTUs (Table 3). Those were affiliated with *Halomonas alkaliphila* (M10 and M11), *Photobacterium halotolerans* (A34, M14, M15, and M20), *Paracoccus seriniphilus* (C14 and M48), and *Altererythrobacter indicus* (P4, G10, and G19). In the stabilization phase, seven strains were closely related to four enriched OTUs (Table 4): *Halomonas meridiana* M11, *Algoriphagus winogradskyi* G63, *Jootella marina* (G54, G65, and ME32), and *Flavobacterium beibuense* (M35 and M44). Finally, we recovered two strains affiliated with *Pseudomonas sabulinigri* G20 and M7; however, these did not match the OTU665 (affiliated with *Pseudomonas putid*a) (Fig. 5).

Tables 3 and 4 show details of enzyme production by the strains. On the one hand, strains isolated from the adaptation phase yielded not only most of the tested hydrolytic activities, but also showed the highest activities. Remarkably, the strains affiliated with *Microbacterium oleivorans* (G37, G46) and *Devosia psychrophila* (G33-G35) revealed the production of five or even six hydrolytic enzymes (Table 3). On the other hand, strains isolated from the recalcitrant substrate were less versatile than those isolated from fresh substrate, as evidenced by the lower number of enzymatic activities (three out of six tested). Only the strains affiliated with *J. marina* (ME32, G54, and G65) presented the capacity to produce at least four hydrolytic enzymes with high activity.

Table 3. Enzymatic activity of the halotolerant lignocellulose degrading strains recovered from the adaptation phase and their associated OTUs, determined by comparing the complete 16S rRNA gene sequences from the strains with the partial sequences of the same gene obtained by amplicon sequencing. Enzymatic activities were determined by chromogenic essays using the substrates 5-bromo-4-chloro-3-indolyl α-D-glucopyranoside (X-glu), 5-bromo-4-chloro-3-indolyl β-D-cellobioside (X-cell), 5-bromo-4-chloro-3-indolyl α-D-mannopyranoside (X-man), 5-bromo-4-chloro-3-indolyl β-D-galactopyranoside (X-gal), 5-bromo-4-chloro-3-indolyl β-D-xylopyranoside (X-xyl), and 5-bromo-4-chloro-3-indolyl β-fucopyranoside (X-fuc)

Taxonomic affiliation				Enzymatic activity							
*Closest relative	Code	Cover (%)	Identity (%)	X-glu	X-cell	X-gal	X-xyl	X-man	X-fuc	Associated OTU	Accession number
Albirhodobacter marinus	C13	100	99	+++	−	−	++	++	−	−	MF619963
Altererythrobacter indicus	P4	100	99	+	−	+	−	−	−	OTU659	MF619965
Altererythrobacter indicus	G10	100	99	−	−	+++	+	−	−	OTU659	MF619966
Altererythrobacter indicus	G19	100	99	−	+++	+++	+++	−	−	OTU659	MF619967
Arthrobacter nicotianae	C6	100	99	+++	−	−	+++	+++	−	−	MF619969
Arthrobacter nicotianae	M16	100	99	+++	−	−	+++	+++	−	−	MF619968
Bacillus oleronius	G13	100	99	−	−	−	−	−	−	−	MF619970
Demequina aestuarii	G48	100	99	+++	+	−	−	−	−	−	MF619977
Demequina aestuarii	G52	99	99	+++	+	−	+++	−	−	−	MF619978
Demequina psychrophila	G33	100	98	+	+++	+++	+++	+	−	−	MF619971
Demequina psychrophila	G34	100	98	+++	+++	+++	+++	−	+++	−	MF619972
Demequina psychrophila	G35	100	98	+++	+++	+++	+++	−	+++	−	MF619973
Demequina psychrophila	G58	100	98	+++	+++	−	+++	−	+++	−	MF619975
Demequina psychrophila	G55	100	98	+++	+++	−	+	−	+	−	MF619974
Demequina psychrophila	G59	100	98	+++	+++	−	+	−	+++	−	MF619976
Erythrobacter gaetbuli	G57	100	96	−	−	−	−	−	−	−	MF619979
Halonomas alkaliphila	M10	100	99	+++	−	−	−	−	−	OTU850	MF619983
Halomonas alkaliphila	M11	100	99	+++	−	−	−	−	−	OTU850	MF619984
Microbacterium oleivorans	G37	99	99	+	+	+++	+	+++	+++	−	MF619992

Microbacterium oleivorans	G56	100	98	+	+	+	+	+	+	–	MF619991
Microbacterium oleivorans	G46	100	99	+++	+++	+++	+	+++	+++	–	MF619993
Micrococcus yunnanensis	G68	100	99	–	–	+	–	–	+++	–	MF619994
Oceanicola antarcticus	M45	100	97	–	+	–	–	–	–	–	MF619995
Paracoccus seriniphilus	C14	100	99	++	–	++	++	++	–	OTU176	MF619998
Paracoccus seriniphilus	M48	100	100	+++	–	+++	+++	+++	–	OTU176	MF619996
Paracoccus seriniphilus	G23	100	99	+++	–	+++	–	+++	–	OTU176	MF619997
Photobacterium halotolerans	A34	100	99	–	–	+++	–	–	–	OTU589	MF620002
Photobacterium halotolerans	M14	100	99	–	–	+++	–	–	–	OTU859	MF619999
Photobacterium halotolerans	M15	100	99	–	–	+++	–	–	–	OTU859	MF620000
Photobacterium halotolerans	M20	100	99	–	–	+++	–	–	–	–	MF620001
Pseudorhodobacter incheonensis	G11	100	99	+++	–	–	–	–	–	–	MF620006
Sanguibacter inulinus	G36	100	99	–	–	–	–	–	–	–	MF620007
Staphylococcus capitis	P1	100	99	–	–	–	–	–	–	–	MF620008
Staphylococcus epidermidis	EG46	100	99	+++	–	–	–	–	–	–	MF620009
Sarocladium strictum	HF1	84	96								MF621035

*Closest related species, according to the 16S ribosomal RNA gene sequence. Enzymatic activity: ***X-glu: glucosidases; X-cell: cellobiohydrolases; X-gal: galactosidases; X-xyl: xylosidases; X-man: mannosidases; X-fuc: fucosidases.*** Qualitative enzymatic activity detection: (–) no activity ; (+) low activity-light blue; (++) medium activity-medium blue; (+++) high activity- dark intense blue.

Table 4. Halotolerant lignocellulose degrader strains recovered from the stabilization phase. Potential degradation and matches to closest 16S rRNA gene sequences and associated OTUs. Enzymatic activities were determined by chromogenic essays using the substrates 5-bromo-4-chloro-3-indolyl α-D-glucopyranoside (X-glu), 5-bromo-4-chloro-3- indolyl β-D-cellobioside (X-cell), 5-bromo-4-chloro-3-indolyl α-D-mannopyranoside (X-man), 5-bromo-4-chloro-3-indolyl β-D-galactopyranoside (X-gal), 5-bromo-4-chloro-3-indolyl β-D-xylopyranoside (X-xyl) and 5-bromo-4-chloro-3-indolyl β- fucopyranoside (X-fuc).

Taxonomic affiliation	**Enzymatic activity**										
***Closest relative**	**Code**	**Cover (%)**	**Identity (%)**	**X-glu**	**X-cell**	**X-gal**	**X-xyl**	**X-man**	**X-fuc**	**Associated OTU**	**Accession number**
Algoriphagus winogradskyi / ratkowskyi	G63	100	99		+++	–	+++	–	–	OTU415	MF619964
Flavobacterium beibuense	M35	100	99	+++	–	–	–	–	–	OTU667	MF619980
Flavobacterium beibuense	M44	100	98	+++	–	–	–	–	–	OTU667	MF619981
Halomonas meridiana	G21	100	99	+++	–	–	–	–	–	OTU57506	MF619985
Halomonas neptunia	M8	100	99	+++	–	+++	–	–	–	–	MF619986
Halomonas venusta	M9	97	99	+++	+	–	–	–	–	–	MF619987
Joostella marina	G54	100	99	+++	–	+++	+	+++	–	OTU358	MF619989
Joostella marina	G65	100	99	+++	+++	+++	–	–	+++	OTU358	MF619990
Joostella marina	ME32	100	99	+++	–	+++	+++	+++	–	OTU358	MF619988
Pseudomonas sabulinigri	G20	100	99	+	–	–	–	–	–	–	MF620005

*Closest related species, according to the 16S ribosomal RNA gene sequence. Enzymatic activity: ***X-glu: glucosidases; X-cell: cellobiohydrolases; X-gal: galactosidases; X-xyl: xylosidases; X-man: mannosidases; X-fuc: fucosidases.*** Qualitative enzymatic activity detection: (−) no activity ; (+) low activity-light blue; (++) medium activity-medium blue; (+++) high activity- dark intense blue.

Fungal strains from the stabilization phase

As mentioned before, the change in the substrate had an important effect on the fungal community. Only one fungal strain was obtained from the adaptation phase (Table 3). It was affiliated with *Sarocladium strictum* HF1 and was obtained from all triplicate plates. It was, however, not possible to recover any fungal strain from the stabilization phase. Despite the bands observed in DGGE (based on the ITS1 region) in the stabilization phase, we observed a sharp decline in fungal abundance—as determined by qPCR targeting the same region (Fig. 1B)—at the end of the experiment (T10), which probably hindered isolation.

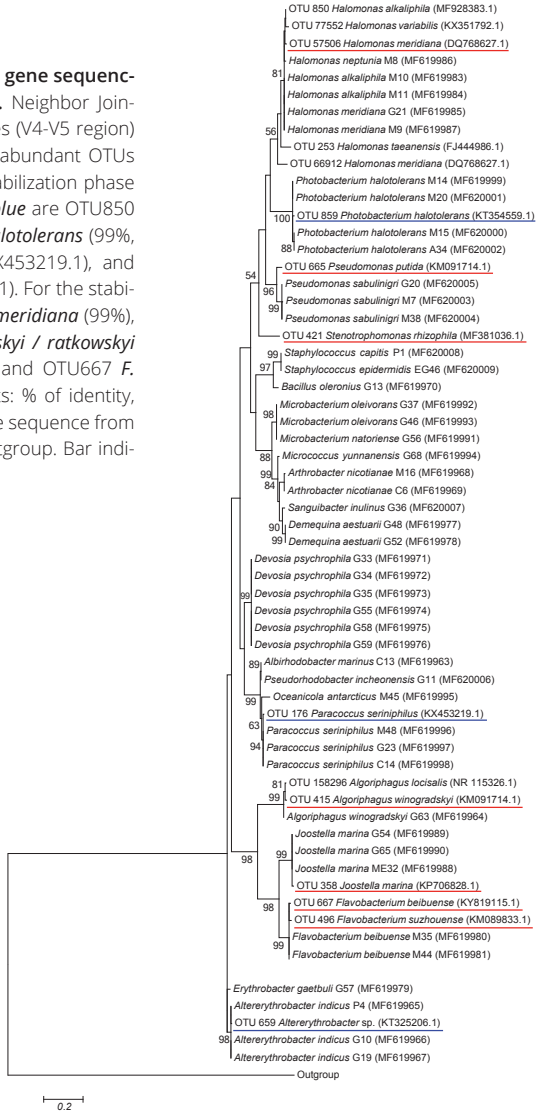

Figure 5. Phylogenetic affiliation of 16S rRNA gene sequences of isolated strains and sequenced OTUs. Neighbor Joining tree based on the 16S rRNA gene sequences (V4-V5 region) from bacterial strains and from the significant abundant OTUs at the end of the adaptation phase (T6) and stabilization phase (T10). For the adaptation phase, underlined in ***blue*** are OTU850 ***H. alkaliphila*** (99%, MF928383.1), OTU859 ***P. halotolerans*** (99%, KT354559.1), OTU176 ***P. seriniphilus*** (99%, KX453219.1), and OTU659, ***Altererythrobacter*** sp. (99%, KT325206.1). For the stabilization phase, underlined in ***red***, OTU57506 ***H. meridiana*** (99%), OTU665 ***P. putida*** (99%), OTU415 ***A. winogradskyi / ratkowskyi*** (98%), OTU358 ***J. marina*** (99%, DQ768627.1), and OTU667 ***F. beibuense*** (99%, KY819115.1). Between brackets: % of identity, reference accession number. The 16S rRNA gene sequence from ***Methanocaldococcus jannaschii*** was used as outgroup. Bar indicated divergence scale (0.2 = 20%).

Discussion

In this study, we produced and characterized microbial consortia—potential sources of lignocellulose degraders and their enzymes—that were capable of degrading wheat straw under high-salt concentrations, a condition often established by particular lignocellulose pretreatment steps. Thus, our selected halotolerant microbial consortia represented a clear prospect of lignocellulose degradation under saline conditions, as they may either be used to directly unlock lignocellulose biomass or to produce halotolerant lignocellulolytic enzymes. The latter application may eliminate the expensive washing steps, reducing costs.

Saline conditions favor bacterial over fungal degraders

Changes in wheat straw content can considerably affect the composition of microbial communities growing on it. Here, in particular, fungal densities decreased significantly in the stabilization phase (when recalcitrant substrate was used), hindering our ability to isolate fungal strains. It is generally believed that fungi are ubiquitous and capable of occupying virtually every ecological niche as a result of their ability to degrade a suite of organic compounds such as complex biological polymers. They may also play roles in degrading lignocellulose in marine environments (Richards et al. 2012), where the major factors affecting their diversities are salt concentration and temperature (Fuentes et al. 2015). For instance, it has been shown that several fungal strains recovered from mangrove systems are capable of growing on wood under high-salt conditions (Arfi et al. 2013). In our study, the only isolated fungal strain—*Sarocladium strictum*, previously known as *Acremonium strictum* (Summerbell et al. 2011)— is likely well adapted to saline environments, as it was previously isolated from a marine ecosystem (Fuentes et al. 2015).

Here it originated from a salt-marsh soil inoculum. It was,however, only recovered in the adaptation phase, declining in density (to below the detection limit) in the stabilization part of the experiment. Although we cannot pinpoint the exact reason for the observed decline in fungal density (considering that both temperature and salt concentration remained constant in our experiment), we argue that this reduction could be explained by nitrogen depletion in the recalcitrant substrate, consistent with the findings by Meidute et al. (2008). Thus, the impossibility to isolate fungal strains from the specialized consortia could be related to a very strong nutritional demand under the prevailing conditions, leading to a decline

in density that hindered isolation. Additionally, pH could be an important factor affecting the viability of the fungi in our system. During the cultivation, the pH decreased slightly from 7.2 to 6.8, which is higher than the optimal pH for fungal growth (between pH 2.2 and 6.5; Matthies et al. 1997). Moreover, the maintenance of the almost neutral pH along the incubation suggested a low production of organic acids (which indicates that massive fermentation did not occur).

The maintenance of prevailing aerobic conditions in the culture probably incited mostly oxidative phosphorylation processes. Thus, in the system (an agitated saline environment with a recalcitrant source of carbon and energy), bacteria probably had a main role in the degradation process. The dominance of bacteria over fungi in our halotolerant lignocellulose grown consortia is interesting, as previous studies, performed under non-saline conditions, suggested that fungal communities have a relevant participation in lignocellulose degradation, even working in liquid and agitated systems. For example, Brossi et al. (2015) found that *C. ligniaria* (strains WS1, WS2, SG8) had a significant role in the degradation of diverse lignocellulose feedstocks, while Jiménez et al. (2013) found the same organism (strain 2w1F) played a crucial role in the decomposition of wheat straw in presence of 5-hydroxymethylfurfural. In both cases, the dilution-to-stimulation approach was used for the selection of the degrader communities.

Substrate quality greatly impacts community composition

The findings in this study clearly indicate that substrate quality and composition direct the structure of microbial consortia (Simmons et al. 2014; Brossi et al. 2015), which developed to degrade either fresh (adaptation phase) or previously-degraded (recalcitrant) lignocellulose substrate (stabilization phase). Whereas the fresh substrate allowed the selection of a more generalist degrading community, composed of very specific bacterial and fungal strains, the recalcitrant substrate selected for more specialized, mostly bacterial, species. Interestingly, all replicates of the enrichment process gave fairly similar patterns, in terms of consortium development, both quantitatively (viz the bacterial and fungal abundance values) and with respect to the bacterial community structures, demonstrating the robustness of our findings. We thus posit here that a consistent selection of microorganisms with progressively higher abilities to grow (jointly) on the substrate had taken place. In the consortia, bacteria were quantitatively by far more important than fungi, and so we placed a greater focus on the bacterial part of the resulting consortia. This bacterial dominance was even exacerbated by the shift to a more recalcitrant substrate after T6.

Wheat straw degradation and potential involvement of identified strains

On the basis of all our data, we depict the degradation of wheat straw under saline conditions to proceed in a sequential manner, with different microbes being dominant in a spatiotemporally explicit form. The wheat straw, being recalcitrant, poses clear obstacles to degradation. The main hurdles are the presence of crystalline cellulose and the bonding between lignin and hemicellulose (shielding the latter component from access by key enzymes). We briefly discuss these issues in the paragraphs below.

Crystalline cellulose is highly recalcitrant to chemical and biological hydrolysis due to the strongly linked chains of cellodextrins. The decomposition of crystalline cellulose, for example filter paper, requires the production of specific cellulases. In our consortia, *Joostella marina* (OTU358) and *Flavobacterium beibuense* (OTU667) may have had a main role in cellulose degradation, as we observed increases in their abundances in the stabilization phase. Also, the consortia from this phase displayed higher cellulose degradation capacities than consortia from the adaptation phase. Both *J. marina* (OTU358) and *F. beibuense* (OTU667) belong to the *Flavobacteriaceae* (Bernardet et al. 2002). Members of this family have been isolated from soil, sediment and marine/saline environments, and they have been typically associated with decomposition of complex polysaccharides (Lambiase 2014). Some species in the family degrade soluble cellulose derivatives such as carboxymethyl-cellulose. However, since enzymes other than cellulases can degrade this compound, this does not demonstrate that these species are cellulolytic. *J. marina* probably had an important role in the degradation of recalcitrant regions of lignocellulose substrate, as it is capable to grow on complex hydrocarburic substrate (Rizzo et al. 2015). The organism is strictly aerobic and can grow in up to 15% NaCl, with glucose, arabinose, mannose, and cellobiose as single carbon and energy sources. Additionally, it has been reported to be positive for α-glucosidase, β-glucosidase, β-galactosidase, and α-mannosidase production (Stackebrandt et al. 2013). In our final consortia, *J. marina* could be associated with the degradation of the crystalline cellulose in the wheat straw. However, more studies are needed to demonstrate such cellulolytic capability. Currently, this characteristic is restricted to members of the *Cytophagaceae* family (Bernardet et al. 2002). Additionally, the *Flavobacterium* species found in this study (*F. beibuense* OTU667 and *Flavobacterium suzhouense* OTU496) may be only associated with the degradation of amorphous cellulose, which is readily digestible. These organisms can degrade soluble cellulose such as hydroxymethylcellulose and cellodextrin (Lambiase 2014).

Regarding lignin degradation or bond hydrolysis, the increasing abundance of *Pseudomonas* species (*P. putida* OTU665 and *P. sabulinigri* G20, M38, and M7) in the stabilization phase suggested a role for these organisms in the relevant transformation steps, such as the degradation of recalcitrant regions of the substrate like residual hemicellulose linked to lignin structures. *Pseudomonas* species stand out as having a great potential capacity for lignin degradation (Beckham et al. 2016). For instance, in a recent study, *Pseudomonas monteilli* and *Pseudomonas plecoglossicida* were enriched from mature vegetal compost. These organisms were found to degrade a large amount of lignin-related compounds (Ravi et al. 2017). In another study, Salvachúa et al. (2015) isolated *P. putida*, *Rhodococcus jostii*, and *Acinetobacter* sp. ADP1, all of which were able to depolymerize and catabolize high-molecular weight lignin (Salvachúa et al. 2015).

The most labile part of the substrate, hemicellulose, was probably mainly attacked by *Halomonas meridiana* (OTU 57506) and related species. Their decreased abundance in the stabilization phase could indicate that the hemicellulose part of the substrate was largely depleted. *H. meridiana* belongs to the class *Gammaproteobacteria*. It is a facultatively halotolerant organism capable of growth in NaCl concentrations between 0.1 and 32.5% (w/v). It is mostly found in marine environments (Octavia and Lan 2014). A recent study suggested that *H. meridiana* has great potential for biotechnology applications, as a producer of extracellular enzymes adapted to salinity (Yin et al. 2015).

Finally, *Algoriphagus winogradskyi/ratkowskyi* G63, belonging to the *Cytophagaceae*, could be involved in the degradation of both the hemicellulose and cellulose regions of the substrate. A genetic analysis of *Algoriphagus* sp. PR1 demonstrated its high capacity of polysaccharide degradation, as large numbers of genes encoding glycoside hydrolases, polysaccharide lyases, carbohydrate esterases, and glycosyltransferases were found (Alegado et al. 2011). Previous reports indicated that related strains cannot degrade filter paper (Lambiase 2014), however our strains were not yet tested for such activity.

Although the contribution of fungi to the degradation process seems to be restricted to the adaptation phase, previous reports have demonstrated the biotechnological application of *Sarocladium strictum*. Interestingly, this was our only isolated fungal strain, and one may envision a role for it in the production of cellulases direct from infested lignocellulose feedstock (Goldbeck et al. 2013). Also, a gene for gluco-oligosaccharide oxidase from this species has been

engineered (high catalytic activity and low substrate inhibition) for application in industrial plant polysaccharide degradation (Domon et al. 2013). Definitely, more studies are necessary on *S. strictum* to examine all its degradation capacities, although it might be restricted to conditions with high nutrient supply.

In conclusion, the construction of microbial consortia able to grow on wheat straw as a carbon and energy source under saline conditions offers access to salt-adapted or salt-tolerant enzymes (haloenzymes) that enable the development of processes under saline conditions. It is assumed that the selected organisms harbor the potential to naturally produce such salt-adapted enzymes, which are applicable in a bioprocess with raised NaCl levels. We propose that the key members of our consortia yield very interesting salt-tolerant enzymes for bioengineering, as follows: (1) *J. marina* (G54, G65, ME32): production of carbohydrate esterases, (2) *F. beibuense* (M35, M44): production of cellulases, (3) *P. sabulinigri* (G20, M38, M7): production of ligninases, and (4) *H. meridiana* (G21): production of hemicellulases. A key issue here is the precise combination of enzymes that is required to establish an efficient "saline bioprocess". Potentially, such an enzyme mixture is made on the basis of the organisms as described here.

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Supplemental information

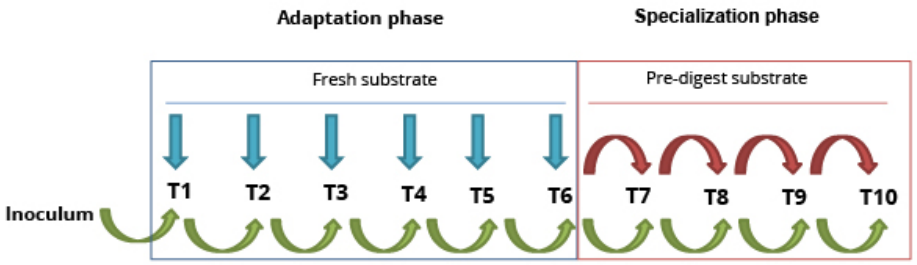

Figure S1 Schematic representation of the experimental approach, conducted in 2 phases, in which microbial communities from soils with high salt concentration were used as inoculum to select for halotolerant lignin- degrading microbial consortia. *Adaptation phase:* during the first 6 transfers was used fresh lignocellulose substrate *(blue arrow)*. *Stabilization phase:* from transfers 6 the substrate was recovered and used the sub-sequential transfer until T10 *(red arrow).*

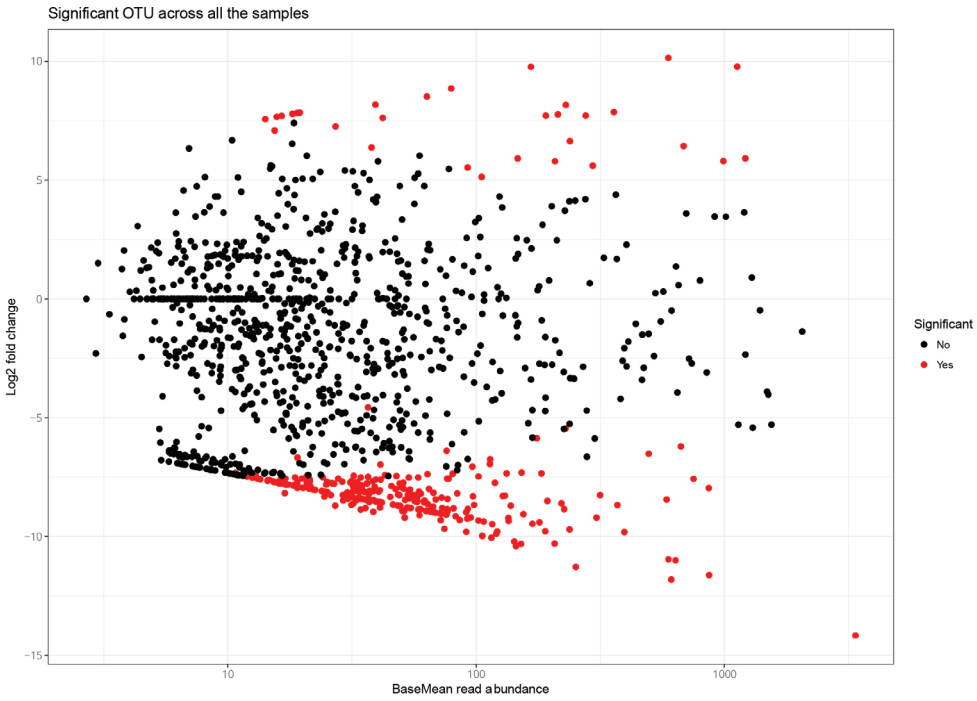

Figure S2 Identification of OTUs that changed significant in abundance (Y-axis: log2 fold change). DESeq2 function for ***phyloseq*** was used to obtain the statistically significant OTU affected by the enrichment process. OTUs with significant increase in ***red dots,*** OTU without significant increase in ***black dots.***

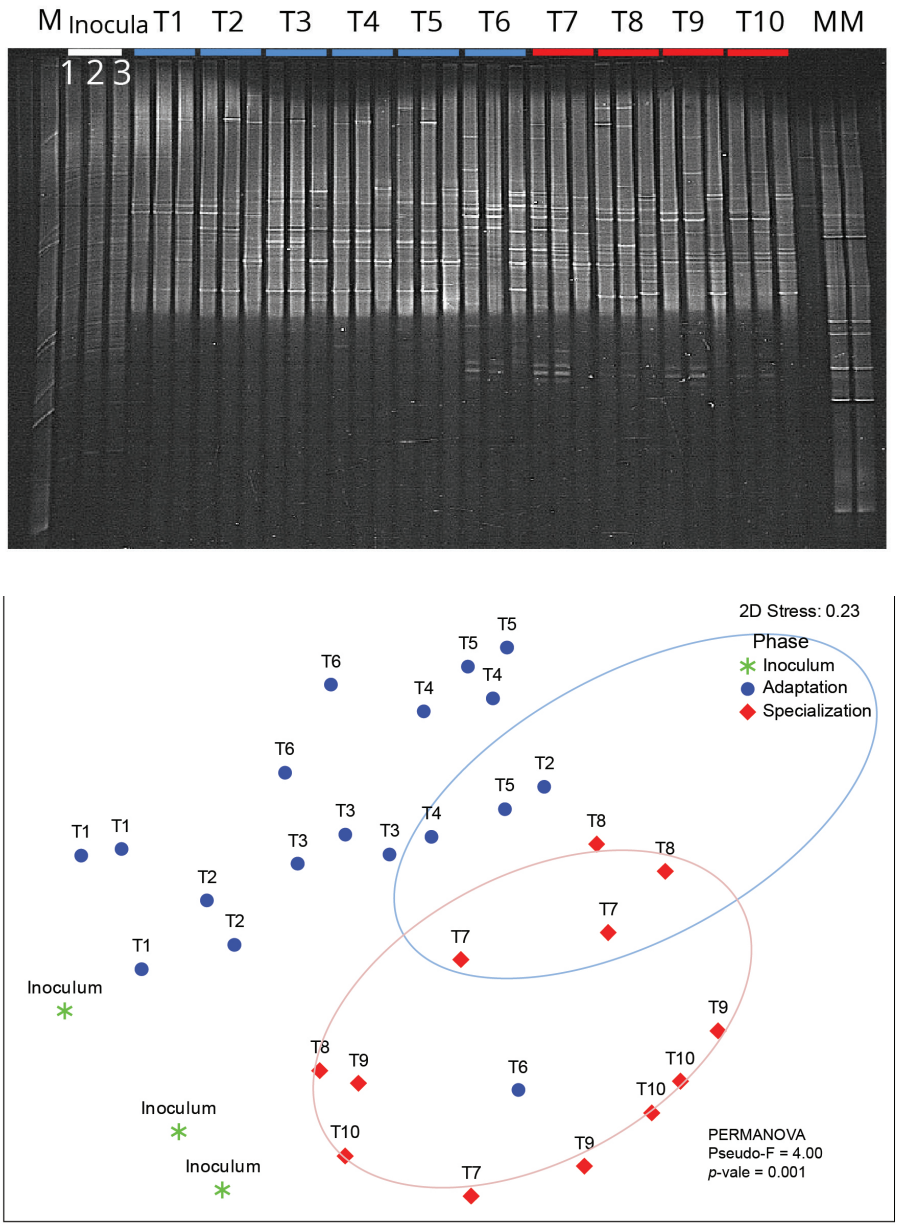

Figure S3 Multidimensional scaling (MDS) revealing shifts in bacterial community composition and well-defined clusters differentiating ***inoculum (green star),*** and the two enrichment phases: communities selected with ***fresh substrate (adaptation phase, blue circles)*** and those selected with ***pre-digested substrate (stabilization phase, red diamonds)***. MDS was constructed using data obtained from the PCR-DGGE targeting the 16S rRNA gene, using abundance data. *PERMANOVA* indicated significant difference between the communities (P= 0.001, Pseudo-F= 4).

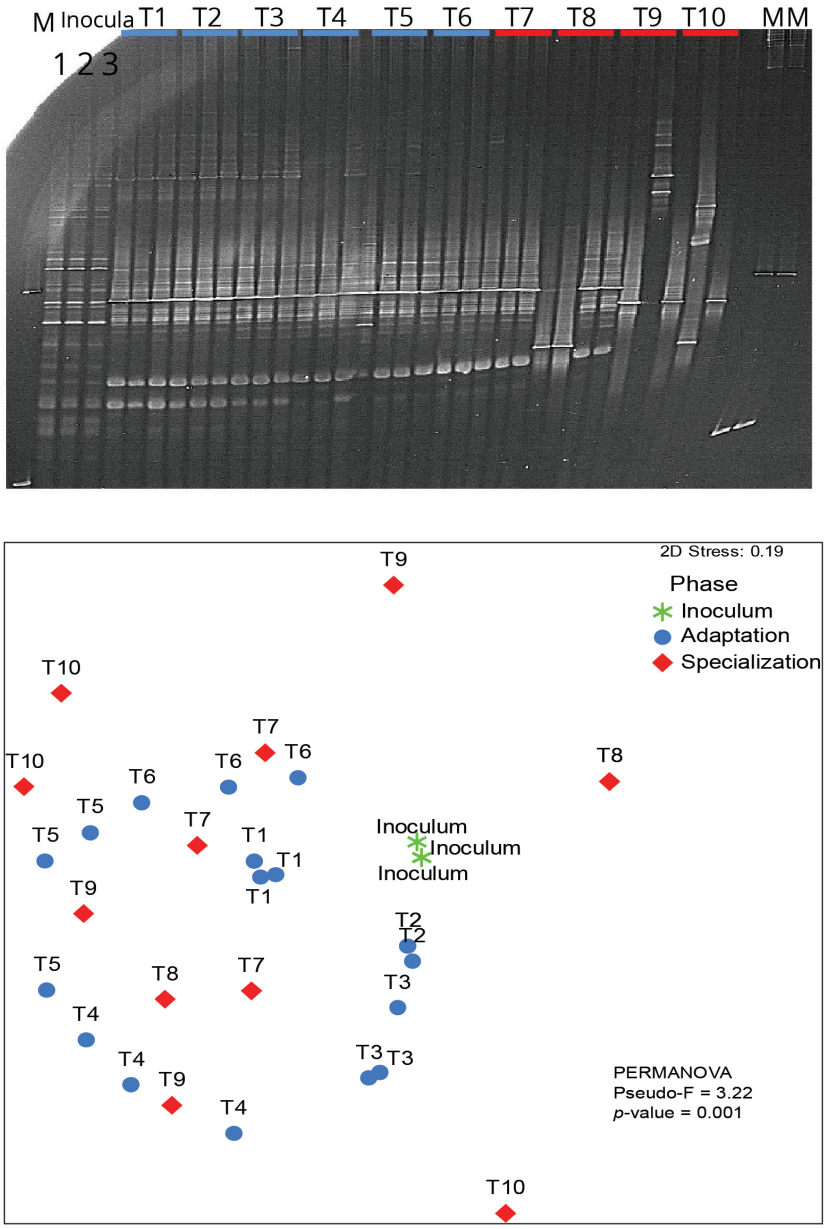

Figure S4 MDS revealing shifts in fungal community composition and well-defined clusters differentiating *inoculum (green star),* and the two enrichment phases: Communities selected with *fresh substrate (adaptation phase, blue circles)* and those selected with *pre-digested substrate (stabilization phase, red diamonds)*. MDS was constructed using data obtained from the PCR-DGGE targeting the 18S rRNA gene, using abundance data. PERMANOVA indicated significant difference between the communities (P= 0.001, Pseudo-F= 4).

Table S1. Primers used for paired-end 16S rRNA gene sequencing on the Illumina MiSeq platform (amplicon sequencing).

SampleID	Reverse primer	Forward primer	Source	Description
Inoculum_1	AGTACGCTATTGYCAGCMGCCGCGGTA	ACGCTCGACACCGYCAATTYMTTTRAGTTT	soil	Soil inoculum
Inoculum_2	ATAGAGTACTTGYCAGCMGCCGCGGTA	AGACGCACTCCCGYCAATTYMTTTRAGTTT	soil	Soil inoculum
Inoculum_3	TACAGATCGTTGYCAGCMGCCGCGGTA	TAGTATCAGCCCGYCAATTYMTTTRAGTTT	soil	Soil inoculum
T1_1	TAGTGTAGATTGYCAGCMGCCGCGGTA	TGATACGTCTCCGYCAATTYMTTTRAGTTT	soil	Transfer 1, flask 1
T1_2	TCTATACTATTGYCAGCMGCCGCGGTA	ATACGACGTACCGYCAATTYMTTTRAGTTT	soil	Transfer 1, flask 2
T1_3	CGCAGTACGATGYCAGCMGCCGCGGTA	ACATACGCGTCCGYCAATTYMTTTRAGTTT	soil	Transfer 1, flask 3
T3_1	CGTACAGTCATGYCAGCMGCCGCGGTA	ACTACTATGTCCGYCAATTYMTTTRAGTTT	soil	Transfer 3, flask 1
T3_2	CGTACTCAGATGYCAGCMGCCGCGGTA	ACTGTACAGTCCGYCAATTYMTTTRAGTTT	soil	Transfer 3, flask 2
T3_3	CTACGCTCTATGYCAGCMGCCGCGGTA	AGACTATACTCCGYCAATTYMTTTRAGTTT	soil	Transfer 3, flask 3
T6_1	TAGTCGCATATGYCAGCMGCCGCGGTA	ATAGAGTACTCCGYCAATTYMTTTRAGTTT	soil	Transfer 6, flask 1
T6_2	TGTCACACGATGYCAGCMGCCGCGGTA	TCGATCACGTCCGYCAATTYMTTTRAGTTT	soil	Transfer 6, flask 2
T6_3	TGTCGTCGCATGYCAGCMGCCGCGGTA	TCGCACTAGTCCGYCAATTYMTTTRAGTTT	soil	Transfer 6, flask 3
T7_1	ACACATACGCTGYCAGCMGCCGCGGTA	TCTAGCGACTCCGYCAATTYMTTTRAGTTT	soil	Transfer 7, flask 1
T7_2	ACATGACGACTGYCAGCMGCCGCGGTA	TGACGTATGTCCGYCAATTYMTTTRAGTTT	soil	Transfer 7, flask 2
T7_3	ACGTCTCATCTGYCAGCMGCCGCGGTA	ACAGTATATACCGYCAATTYMTTTRAGTTT	soil	Transfer 7, flask 3
T10_1	ACTCGCGCACTGYCAGCMGCCGCGGTA	ACTAGCAGTACCGYCAATTYMTTTRAGTTT	soil	Transfer 10, flask 1
T10_2	ATATAGTCGCTGYCAGCMGCCGCGGTA	CGCGTATACACCGYCAATTYMTTTRAGTTT	soil	Transfer 10, flask 2
T10_3	CACGTAGATCTGYCAGCMGCCGCGGTA	CGTACTCAGACCGYCAATTYMTTTRAGTTT	soil	Transfer 10, flask 3

Reference: Caporaso JG, Lauber CL, Walters WA, Berg-Lyons D, Huntley J, Fierer N, Owens SM, Betley J, Fraser L, Bauer M, Gormley N, Gilbert JA, Smith G, Knight R (2012) Ultra-high-throughput microbial community analysis on the Illumina HiSeq and MiSeq platforms. ISME J 6:1621–1624.

Table S2. Cellulose, hemicellulose (xylan) and lignin mixtures used to obtain the prediction model.

Ternary mixtures	Lignin (%)	Cellulose (%)	Hemicellulose (%)
A	100	0	0
B	0	100	0
C	0	0	100
D	50	25	25
E	25	50	25
F	25	25	50
G	75	25	0
H	25	75	0
I	25	0	75
J	0	25	75
K	33	33	33
L	72	0	25
M	0	75	25

ESM 1. Isolation of bacterial and fungal strains.

Serial dilutions were done in saline MSM (25 g/L NaCl) and 100 µL aliquots of the 10^{-1}- to 10^{-3} and 10^{-7} to 10^{-9} dilutions, for fungi and bacteria, respectively, were spread on the surface of each of the medium. Morphological differences of the colonies were used to select the isolates, which were streaked to purity and then preserved at -80°C (in LB broth with 20% glycerol). To obtain a presumptive identification, genomic DNA was produced by using the UltraClean® Microbial DNA isolation kit (MoBio®). For bacteria, we first de-replicated the isolates based on an ERIC-PCR using primers ERIC1R and ERIC2 (Versalovic et al. 1994; Puentes-Téllez and Elsas 2014). The ERIC-PCR cluster analyses were performed using GelCompar software. Bacterial 16S rRNA genes of representative strains for each ERIC group were amplified using 10 ng of DNA and primers B8F and U1406R (Taketani et al. 2010). For fungal strains (pretreated with liquid nitrogen), genomic DNA was obtained using UltraClean® Microbial DNA isolation kit (MoBio®).

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Chapter 5

Bacterial synergism in lignocellulose biomass degradation influence by complexity of carbon source

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Abstract

Lignocellulosic biomass (LCB) is an attractive source of carbon for the production of sugars and other chemicals. Due to its inherent complexity and heterogeneity, efficient biodegradation requires the actions of different types of hydrolytic enzymes. In nature, complex microbial communities that work efficiently and often synergistically accomplish degradation. Studying such synergisms in LCB degradation is fundamental for the establishment of an optimal biological degradation process. Here, we examine the wheat straw degradation potential of synthetic microbial consortia composed of bacteria and fungi. Growth of, and enzyme secretion by, monocultures of degrader strains were studied in aerobic cultures using wheat straw as the sole carbon and energy source. To investigate synergism, co-cultures were constructed from selected strains and their performance was tested in comparison with the respective monocultures. In monoculture, each organism – with a typical enzymatic profile – was found to mainly consume the cellulose part of the substrate. One strain, *Flavobacterium ginsengisoli* so9, displayed an extremely high degradation capacity, as measured by its secreted enzymes. Among 13 different co-cultures, five presented synergisms. These included four bacterial bicultures and one bacterial–fungal triculture. The highest level of synergism was found in a *Citrobacter freundii /Sphingobacterium multivorum* biculture, which revealed an 18.2-fold increase of the produced biomass. As compared to both monocultures, this bacterial pair showed significantly increased enzymatic activities, in particular of cellobiohydrolases, mannosidases, and xylosidases. Moreover, the synergism was unique to growth on wheat straw, as it was completely absent in glucose-grown bicultures. Spent supernatants of either of the two partners were found to stimulate the growth on wheat straw of the counterpart organism, in a directional manner. Thus, the basis of the LCB-specific synergism might lie in the specific release of compounds or agents by *S. multivorum* w15 that promote the activity of *C. freundii* so4 and vice versa.

Introduction

Millions of tons of agricultural waste are generated globally every year (Väisänen et al. 2016). Examples are wheat and maize straws, sugarcane bagasse and corn stover. Such lignocellulosic biomass (LCB) is useful as raw material for the production of value-added materials as well as fuels. LCB is composed of lignin, cellulose and hemicellulose, whereas pectin, proteins, small molecules, and minerals can also be present (Guerriero et al. 2016). The exact composition of LCB depends on factors such as plant cultivar type, plant age, local growth conditions, harvesting season and the quality of the soil used for cultivation. For instance, depending on cultivar, age and local conditions, wheat straw can contain 30–44% cellulose, 23–50% hemicellulose, and 7.7–15% lignin (Van Dyk and Pletschke, 2012). A clear impediment to the widespread use of wheat straw as raw material for value-added compounds is its relatively recalcitrant nature, which means it does not easily break down into its monomers. This recalcitrance is clearly caused by its complex chemical composition, and it relates to a major extent to the tight linkages between the lignin, hemicellulose, and cellulose parts. Moreover, the LCB physical structure, i.e., the degree of crystallinity and polymerization of cellulose and polysaccharide, is an important parameter that influences its degradability (Van Dyk and Pletschke, 2012; Bhattacharya et al. 2015).

As a reflectance of its inherent complexity, a large variety of organisms (producing diverse enzymes) is commonly needed to efficiently degrade LCB like into its monomer compounds. In nature, microbial communities commonly degrade it in a dynamic and time-dependent manner. The degraders are thus presumed to show dynamic responses to the substrate, reaching higher biomass when working together when than acting alone. This process is known as synergistic growth. Moreover, the degrading organisms may use enzymes with complementary activities (enzymatic synergism). Synergism in growth and that in enzymatic activity therefore reflect two processes that are often closely linked in microbial communities (Van Dyk and Pletschke, 2012; Cragg et al. 2015). We took these two definitions into our own work on microbial consortia, as proposed in the recent literature (Mitri and Foster, 2013; Deng and Wang, 2016). Given the fact that in natural systems synergism in LCB degradation processes is the rule rather than the exception, we surmised it is exacerbated in soil-derived microbial consortia selected on LCB.

What mechanisms are behind synergistic behavior in LCB degradation? According to classical knowledge and theory, microorganisms growing together on one substrate, when coexisting, most often divide labor, in a process called niche partitioning. Metabolic complementarity is the main process behind such niche partitioning, as revealed by the classical example of biofuel and hydrogen production through co-cultures of *Bacillus* and *Clostridium* on rice straw compost (Chang et al. 2008). So far, it has been relatively unknown to what extent complex substrates like LCB foster processes leading to coexistence. However, recently a co-culture of *Trichoderma reesei* and *Escherichia coli* growing on (pretreated) corn stover was found to be optimal in isobutanol production (Minty et al. 2013). The strategy was based on division of function between the two organisms. *T. reesei* secreted cellulolytic enzymes that transformed the LCB into soluble saccharides, whereas *E. coli* fermented these into isobutanol. Another recent study reported that, along the same lines, co-cultures of *Clostridium cellulovorans* (743B) and *Clostridium beijerinckii* (NCIMB 8152) also successfully produced butanol, under mesophilic conditions (Wang et al. 2015). These studies thus show the key importance of metabolic complementarity in LCB degradation, in which the cooperation between synergistic pairs is driven by exchanges of key metabolites, or by niche partitioning. However, we still do not understand the plethora of mechanisms, as well as the dynamism, that play roles in the microbial attack on the LCB wheat straw (Pandhal and Noirel, 2014; Dolinšek et al. 2016; Ghosh et al. 2016; Jia et al. 2016; Jiang et al. 2017). For instance, it remains unclear to what extent the composition/structure of the substrate affects the interactions between collaborating degraders. Moreover, the dynamism in the interactions and activities of collaborative organisms remains understudied.

In our previous work, a suite of microbial strains was isolated from three lignocellulolytic microbial consortia that had been selected by repeated growth on raw wheat straw as the single carbon and energy source. Most of the strains had shown promising lignocellulolytic capabilities (Cortes-Tolalpa et al. 2016). We here hypothesized that the wheat straw substrate, being complex and spatially structured, will promote 'division of labor,' and so cooperation, between some of the degrader strains. The aim of this study was, therefore, to uncover such synergisms and determine their potential. In this endeavor, we also addressed the potential mechanism behind the synergisms. The data showed that cooperative behavior was relatively 'common' in microbial consortia growing on wheat straw, but broke down when strain combinations were grown on simple substrates like glucose.

Materials and methods

Bacterial and fungal strains

The bacterial and fungal strains used in this study were isolated from three wheat-straw-grown microbial consortia that had originally been inoculated with forest soil, canal sediment and decaying wood derived microbiomes. Briefly, serial dilutions of extracts of the aforementioned biomes were prepared in solution (0.85%). Then, 100 mL aliquots of each dilution were spread onto the surface of R2A (BD Difco, Detroit, MI, United States) and potato dextrose agar (PDA) plates, to isolate fungi and bacteria, respectively. Morphological differences of the colonies were used in the selection procedure of the isolates, which were streaked to purity and then preserved at -80ºC (in LB broth with 20% glycerol and potato dextrose broth for bacteria and fungi, respectively). *Coniochaeta ligniaria* sedF1 reflected a dominant colony in the PDA plates, and so was thought to represent the main viable fungus (Cortes-Tolalpa et al. 2016).

Culture media

Three media, based on mineral medium (below) were used, on the basis of three different carbon sources. These were (1) "raw wheat straw" (1% w/v), (2) "synthetic recalcitrant biomass" (SRB) [0.3% carboxymethyl cellulose (CMC) (VWR, Leuven, Belgium), 0.5% xylan-beechwood (Sigma–Aldrich, Darmstadt, Germany) and 0.1% lignin (Sigma–Aldrich, St. Louis, MO, United States)] and (3) "glucose" (0.3%) (Merck, Darmstadt, Germany). The raw wheat straw was air-dried (50ºC) before cutting it into pieces of about 5 cm length. Then, the pieces were thoroughly ground, using a mill hammer, to pieces 1 mm. No pre-treatment was performed (untreated raw substrate). All carbon sources were taken up in mineral medium [7 g/L $Na_2HPO_42H_2O$; 2 g/L K_2HPO_4; 1 g/L $(NH_4)_2SO_4$; 0.1 g/L $Ca(NO_3)_24H_2O$; 0.2 g/L $MgCl_26H_2O$ g/L, pH 7.2] (Jiménez et al. 2013; de Lima Brossi et al. 2015; Cortes-Tolalpa et al. 2016) supplemented with vitamin solution (0.1 g Ca-pantothenate, 0.1 g cyanocobalamine, 0.1 g nicotinic acid, 0.1 g pyridoxal, 0.1 g riboflavin, 0.1 g thiamin, 0.01 g biotin, 0.1 g folic acid; H_2O 1 L) and trace metal solution (2.5 g/L EDTA; 1.5 g/L $FeSO_47H_2O$; 0.025 g/L $CoCl_2$; 0.025 g/L $ZnSO_47H_2O$;0.015 g/L $MnCl_2$; 0.015 g/L $NaMoO_42H_2O$; 0.01 g/L $NiCl_2$; 0.02 g/L H_3BO_3; 0.005 g/L $CuCl_2$). Sterility of the substrate was verified following plating on trypticase soy agar (TSA) plates. All chemicals and reagents used in this work were of analytical molecular biology grade (Sigma–Aldrich, Darmstadt, Germany). Erlenmeyer flasks containing 25 mL of the medium were autoclaved at 121ºC for 27 min before use.

Monocultures and co-cultures

Monoculture refers to the microbial strains growing alone in a flask. Co-culture refers to combined strains growing in a flask. Triplicates were used throughout. The selection of strains for the construction of the synthetic pairs was based on relative abundance, enzymatic activity and antagonism assay data, as reported earlier (Cortes-Tolalpa et al. 2016). After a first screening (Table 1), six bacterial and one fungal strain(s) were selected to examine the behavior in co-cultures. Thus 13 co-cultures were formed (Table 2).

Microbial culture and growth measurements

The mono- and co-cultures were grown in Erlenmeyer flasks (in triplicates). To prepare inocula, microbial strains were pre-grown on TSA plates at 28ºC for 48 h. Then a fresh colony of each strain was dissolved in sterile saline (0.85% NaCl). The fungal strain was first adapted to growth in liquid medium (potato dextrose broth) for 48 h. The optical density of the bacterial and fungal suspensions were then checked, after which they were adjusted to that representing a standard cell density of about 5 log cells per mL. The incubation conditions were 28ºC with shaking at 180 rpm. Microbial growth was measured at regular time points, i.e., every 24 h until 72 h. At each time point, 1 mL culture was harvested, cells were spun down (20 min, 13,300 rpm, 4ºC – Eppendorf centrifuge, Hamburg, Germany), and the supernatant was used for enzymatic activity analyses. Then, cells were resuspended in sterile saline and the resulting suspensions used for serial dilution plating on TSA. The inoculated plates were incubated at 28ºC for 24–48 h, after which the developed colonies were counted. Thus, growth was monitored by CFU counting following incubation. To determine the maximal growth rates of the cultures (μ, h^{-1}), the numbers of CFUs measured during the exponential growth phase were log-transformed and the slope of each growth curve was used. Flasks with culture medium without cells were used as negative controls (NCs).

Lignocellulolytic enzyme activity assays

The activities of four different enzymes were monitored at time points 0, 24, 48, and 72 h. Substrates for β-glucosidase (BG) (EC. 3.2.1.37), cellobiohydrolase (CBH) (EC. 3.2.1.91), β-mannosidase (BM) (3.2.1.25), and β-xylosidase (BX) (EC. 3.2.1.37) activities were used. The first two substrates report on the degradation of cellulose and the last two on that of the hemicellulose part of wheat straw. The activities were quantified on the basis of the (enzyme-specific) substrate label 4-methylumbelliferone (MUB): 4-MUB-β-glucosidase, 4-MUB-β-cellobiosidase,

4-MUB-β-mannosidase, and 4-MUB-β-xylosidase (Sigma–Aldrich, Darmstadt, Germany). The reaction mixtures consisted of 150 mL diluted supernatant (usually 1/4) in MOPS buffer (50 mM, pH 6.5; Sigma–Aldrich, Darmstadt, Germany) and 2 mM of MUB substrate in black 96-well plates. The reactions were incubated 1 h at 28°C in the dark, after which 30 µL of NaOH (1 M) was added. Fluorescence was measured at an excitation wavelength of 365 nm with emission at 445 nm. The enzymatic activities were then calculated from the fluorescence units using a standard calibration curve. Supernatant recovered from the NC was also tested, and thus served as the NC. The enzymatic activities are reported as the rate of MUB production (nmol MUB per h at 28°C, pH 6.8). All assays were done in triplicate.

Antagonistic interaction assays

Antagonistic interactions were tested with Burkholder's 'spot-on-lawn' method (Burkholder et al. 1966). Strains were confronted with each other in a set-up to obtain a full interaction matrix of all strains with each other. Lawns of each strain were created by mixing exponentially grown cultures (optical density 0.5 at 600 nm) with soft carboxymethyl cellulose (CMC)-xylan agar medium (CMC 0.2%, xylan 0.1%, yeast extract 0.05%, 1.5% agar) and pouring these onto the surface of LB agar plates. Following solidification, five microliters of overnight cultures of selected bacterial or fungal strains were added on top (Pérez-Gutiérrez et al. 2013; Aguirre-von-Wobeser et al. 2014). The plates were incubated for 48 h at 28°C, after which they were inspected for inhibition haloes around the growth of the test strains. The broad-spectrum antibiotic streptomycin was used as a control (data not shown).

Synergism

The degree of enzymatic synergism (DS) (Van Dyk et al. 2013) was calculated by dividing the observed enzymatic activity from each co-culture (secretome) by the sum of the individual activities of the secretome from the respective monocultures. Greater values of the calculated DS indicate a greater enzymatic synergism. Synergistic growth was defined as having occurred when the biomass developed in the co-culture was significantly (*t-test*, $P < 0.05$) higher than the sum of the biomasses achieved in the respective monocultures.

Induction experiment

Monocultures of strains *Sphingobacterium multivorum* w15 and *Citrobacter freundii* so4 were prepared as described above, using either raw wheat straw or glucose

as the carbon source. Supernatants were harvested and filtered (0.2-mm pore size filter). No viable cells were detected in the supernatants. For the induction of strain w15, 10% of the final volume of *C. freundii* so4 culture supernatant was added to the *S. multivorum* w15 culture. Moreover, *C. freundii* so4 was treated in the reciprocal way. For both cultures, the supernatants were added at the onset of the incubation. Triplicate treatments were used. The controls consisted of strains growing with the addition of 10% of the medium. The growth and enzymatic activities were then monitored over time and compared with their respective controls.

Statistical analyses

For the detection of differences in growth across the cultures, we used Student's *t-test*. Since the enzymatic activity data had a non-normal distribution, even after log transformation (x+1), we used the non-parametric Kruskal–Wallis test. Regression analyses between monoculture growth rates and enzymatic activities were performed in SPSS (data not shown). Data were considered to be significant at $P < 0.05$.

Results

Testing for potential antagonisms on CMC–xylan agar medium

The strains used in these tests are shown in Table 1. Testing for antagonism across all pairs of strains revealed that, under the conditions used, none of the bacterial strains exhibited antagonism to any of the other strains (data not shown). Considering the fungal strain *C. ligniaria* sedF1, we found no antagonistic effect of it on any of the bacterial strains.

Monocultures

Twenty-three among 51 bacterial strains obtained from the wheat straw microbial consortia (Table 1) were able to grow aerobically in monoculture in minimal medium with wheat straw as the sole source of carbon and energy. All of the 23 growth-positive bacterial cultures grew from a start density of around 5, to a final density of around 8 log cell/mL after 48–72 h. The strains revealed different specific growth rates, expressed as μ (h^{-1}) (Figure 1). The fungal strain *C. ligniaria* sedF1 also grew well. We decided to work further with these bacterial strains, omitting the 28 non-growers from this study. In addition, we included the fungal strain *C. ligniaria* sedF1 on the basis of the prevalence of this fungal species across all wheat straw grown enrichments.

Growth rates

The specific growth rates, expressed as μ (h^{-1}), of the 23 bacterial strains (Table 1), next to that of the single fungal strain can be seen in Figure 1. Three main groups were observed, typified by either high, intermediate or low growth rates. Eight strains fell in the high-growth-rate class [average μ = 0.13 h^{-1} (±0.0013)]. These were: *Ochrobactrum thiophenivorans* so16, *Comamonas testosteroni* so5, *Microbacterium foliorum* w9, *Delftia tsuruhatensis* w5, *Oerskovia enterophila* se5, *Stenotrophomonas rhizophila* so24, *Chryseobacterium taihuense* w4 and *Stenotrophomonas terrae* w16. The second group, composed of 12 strains, revealed intermediate growth rates [i.e., μ = 0.10 h^{-1} (±0.005)]. These were *Acinetobacter johnsonii* so1, *Lelliottia amnigena* so12, *S. multivorum* so22, *C. freundii* so4, *Achromobacter xylosoxidans* w1, *S. multivorum* se10, *Raoultella terrigena* w13, *Microbacterium gubbeenense* w8, *Acinetobacter beijerinckii* se1, *S. multivorum* w15, *Microbacterium oxydans* so14, and *Flavobacterium ginsengisoli* so9. The remaining two bacterial strains (as well as the fungus) grew slowly, with a μ of 0.08 ± 0.006 h^{-1}. These were *F. banpakuense* so11 and *C. taihuense* so3, next to *C. ligniaria* sedF1 (Table 1 and Figure 1A).

Degradation potential

We examined the production of extracellular β-glucosidases, cellobiohydrolases, β-mannosidases, and β-xylosidases in each of the monocultures. The data show that only five strains (*A. johnsonii* so1, *A. beijerinckii* se1, *C. testosteroni* so5, *O. enterophila* se5, and *D. tsuruhatensis* w5) did not yield any enzymatic activity on the four substrates (Figure 1B). For the remaining 18 bacterial and one fungal strain, specific suites of released enzymes were found (Figure 1B). For all enzymes, the total activities measured consistently increased over time, being maximal at 72 h (Figure 1). This indicated growth-related enzyme secretion across all these strains. However, none of the monocultures showed a clear relationship between enzymatic activity and growth rate (using regression analysis) (data not shown). For instance, *C. testosteroni* so5, *O. enterophila* se5, and *D. tsuruhatensis* w5 revealed high growth rates on the wheat straw, but they did not reveal activity on any of the enzyme substrates (Figure 1). On the other hand, the intermediate-growth-rate *F. ginsengisoli* so9 showed very high β-glucosidase, cellobiohydrolase, and β-xylosidase activities. In contrast, *O. thiophenivorans* so16 revealed the highest μ of all strains (0.13 h-1 ± 0.005), whereas it revealed only intermediate values of the four enzymatic activities (Figure 1B).

Tabla 1. Taxonomic affiliation of microbial strains used in this study.

	Taxonomy affiliation				
Strain	**Closest relative**	**Class**	**Family**	***Identity (%)**	**Accession number**
w4	*Chryseobacterium taihuense*	*Flavobacteria*	*Flavobacteriaceae*	99	KT265756
so3	*Chryseobacterium taihuense*	*Flavobacteria*	*Flavobacteriaceae*	98	KT265758
so4	*Citrobacter freundii*	*Gammaproteobacteria*	*Enterobacteriaceae*	99	KT265771
so22	*Sphingobacterium multivorum*	*Sphingobacteria*	*Sphingobacteriaceae*	98	KT265750
w15	*Sphingobacterium multivorum*	*Sphingobacteria*	*Sphingobacteriaceae*	97	KT265748
se10	*Sphingobacterium faecium*	*Sphingobacteria*	*Sphingobacteriaceae*	98	KT265798
w6	*Flavobacterium ginsengisoli*	*Flavobacteria*	*Flavobacteriaceae*	99	KT265792
so9	*Flavobacterium ginsengisoli*	*Flavobacteria*	*Flavobacteriaceae*	99	KT265787
so11	*Flavobacterium banpakuense*	*Flavobacteria*	*Flavobacteriaceae*	99	KT265796
so1	*Acinetobacter johnsonii*	*Gammaproteobacteria*	*Moraxellaceae*	99	KT265766
se1	*Acinetobacter beijerinckii*	*Gammaproteobacteria*	*Moraxellaceae*	99	KT265764
so5	*Comamonas testosteroni*	*Betaproteobacteria*	*Comamonadaceae*	99	KT265795
so16	*Ochrobactrum thiophenivorans*	*Alphaproteobacteria*	*Brucellaceae*	99	KT265790
se5	*Oerskovia enterophila*	*Actinobacteria*	*Cellulomonadaceae*	99	KT265785
so12	*Lelliottia amnigena*	*Gammaproteobacteria*	*Enterobacteriaceae*	99	KT265765
so14	*Microbacterium oxydans*	*Actinobacteria*	*Microbacteriaceae*	99	KT265770
so24	*Stenotrophomonas rhizophila*	*Gammaproteobacteria*	*Xanthomonadaceae*	99	KT265769
w1	*Achromobacter xylosoxidans*	*Betaproteobacteria*	*Alcaligenaceae*	99	KT265794
w5	*Delftia tsuruhatensis*	*Betaproteobacteria*	*Comamonadaceae*	99	KT265782
w8	*Microbacterium gubbeenense*	*Actinobacteria*	*Microbacteriaceae*	97	KT265752
w9	*Microbacterium foliorum*	*Actinobacteria*	*Microbacteriaceae*	99	KT265781
w13	*Raoultella terrigena*	*Proteobacteria*	*Enterobacteriaceae*	99	KT265761
w16	*Stenotrophomonas terrae*	*Gammaproteobacteria*	*Xanthomonadaceae*	99	KT265788
**sedF1	*Coniochaeta ligniaria*	*Sordariomycetes*	*Coniochaetaceae*	96	KT265807

*Closest relative species. According to 16S or 18S (**) ribosomal RNA gene sequence comparisons.

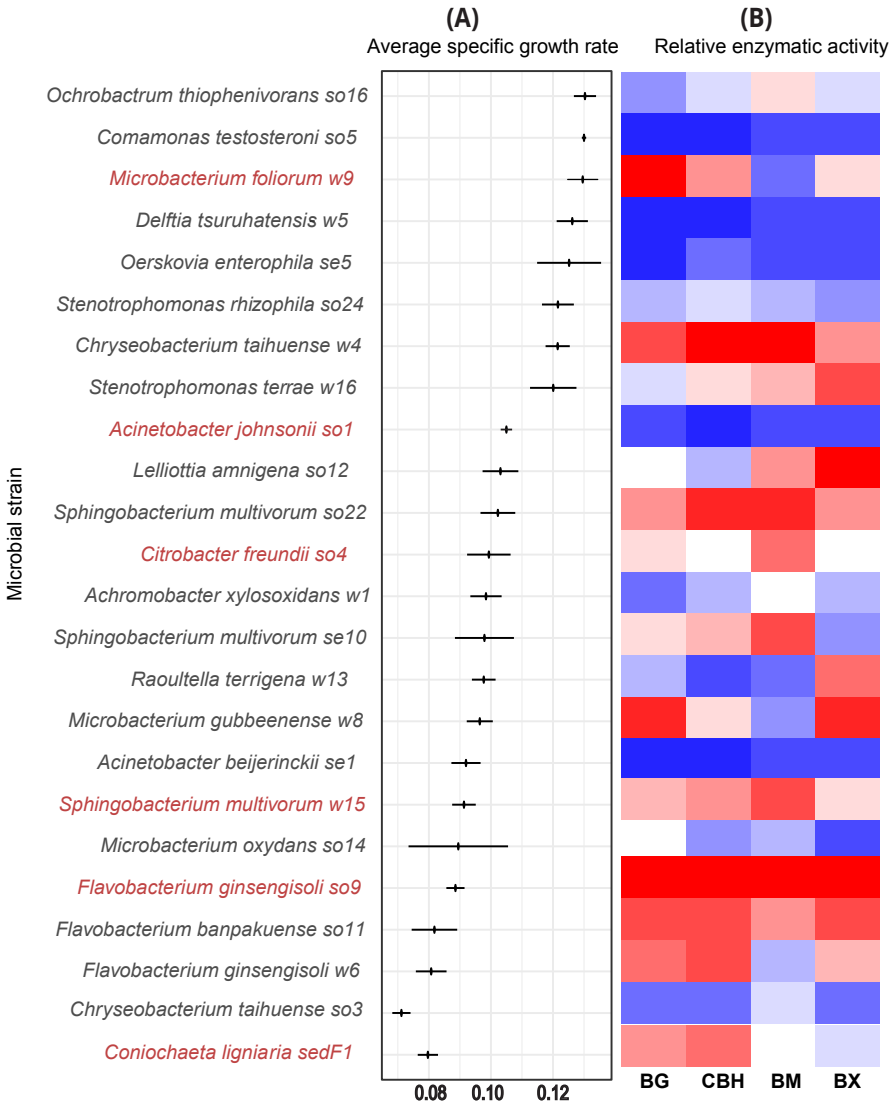

Figure 1 Screening of growth and degradation capacity of selected microbial strains. Microbial strains were isolated from three different enriched consortia. **(A)** The left panel shows *specific growth rates,* μ (h^{-1}), in decreasing order. Horizontal line represents standard deviation across triplicates. Selected strains are shown in *red*. **(B)** The right panel shows relative activity of four lignocellulolytic enzymes, *BG, β-glucosidase; CBH, cellobiohydrolase; BM, β-mannosidase;* and *BX, β-xylosidase.* The relative enzymatic activity is reported in nmol MUB released per h at 28°C, pH 6.8. Activity values are normalized by using log (x+1).

Co-cultures

Starting from the premise that bacteria, next to fungi, make up the major part of the wheat-straw-selected microbial consortia (Cortes-Tolalpa et al., 2016), we used educated guesses to assemble co-cultures with presumed collaborative substrate degradation activity. The co-cultures thus included a selection of highly performing or high-abundance bacteria, next to a dominant fungus (Table 2).

Table 2. Microbial composition of the co-cultures in this study.

		Taxonomy affiliation		
Co-culture	Strain code	Strain 1	Strain 2	Strain 3
A	so4, w15	*C. freundii* so4	*S. multivorum* w15	
B	so4, so22	*C. freundii* so4	*S. multivorum* so22	
C	so4, so1	*C. freundii* so4	*A. johnsonii* so1	
D	w15, so1	*S. multivorum* w15	*A. johnsonii* so1	
E	so9, so1	*F. ginsengisoli* so9	*A. johnsonii* so1	
F	so4, so9	*C. freundii* so4	*F. ginsengisoli* so9	
G	w15, so9	*S. multivorum* w15	*F. ginsengisoli* so9	
H	so4, w9	*C. freundii* so4	*M. foliorum* w9	
I	w15, w9	*S. multivorum* w15	*M. foliorum* w9	
J	so4, w15, so1	*C. freundii* so4	*S. multivorum* w15	*A. johnsonii* so1
K	so4, w15, so9	*C. freundii* so4	*S. multivorum* w15	*F. ginsengisoli* so9
L	so4, w15, sedF1	*C. freundii* so4	*S. multivorum* w15	*C. ligniaria* sedF1
M	so4, w15, w9	*C. freundii* so4	*S. multivorum* w15	*M. foliorum* w9

Bacterial strain selection

Combinations of strains were formed on the basis of (1) the abundance values of the respective bacterial types in the three source microbial consortia (Cortes-Tolalpa et al. 2016), (2) the performance of strains in the current tests of growth and enzymatic activity on wheat straw. Thus, the enzyme-active *C. freundii* so4, *S. multivorum* strains w15 and so22, and *A. johnsonii* so1 were selected (OTUs dominant in wood/soil derived wheat straw bred consortia, and *S. multivorum* also in the sediment-derived consortia) (Table 3). In addition, *F. ginsengisoli* so9 was also chosen because it revealed the highest enzymatic activities of all screened strains. Finally, *M. foliorum* w9, presented in low abundance, was included in the work because it revealed a high growth rate and maximal glucosidase activities when grown on wheat straw at all time points (Figure 1B).

Table 3. Relative abundance and growth of most abundant bacterial strains in the final consortia derived from decaying wood, forest soil, and canal sediment.

Selected bacteria strain	Relative abundance (%) in consortia derived from:*		
Affiliation/code	**Wood**	**Soil**	**Sediment**
C. freundii – so4	19.3 ± 5.1	19.7 ± 3.9	<2
S. multivorum – w15/so22	18 ± 11	23.4 ± 3.7	8.4 ± 1.8
A. johnsonii – so1	11.8 ± 7.6	<2	<2
F. gingengisoli – so9	5.6 ± 2.2	5.7 ± 1.2	<2
M. foliorum w9	<2	<2	<2

*Taken from Cortes-Tolalpa et al. (2016).

Fungal strain selection

The fungal strain *C. ligniaria* sedF1 (dominant in the sediment-derived wheat-straw-bred consortia) was selected (see Materials and Methods), as it revealed growth on lignocellulose, with considerable activity of β-glucosidases (1023.0 ± 9.4) and cellobiohydrolases (156.9 ± 0.4) in monoculture. Moreover, previous work had shown that this fungus may promote bacterial growth by removal of toxic compounds on torrified grass (Trifonova et al. 2009). This fungus has consistently been isolated from LCB grown microbial cultures, as reported in several recent studies (Jiménez et al. 2013; de Lima Brossi et al. 2015; Cortes-Tolalpa et al. 2016); it may itself have an important role in wheat straw degradation.

Growth in Co-cultures

Bacterial–bacterial bicultures

From the 13 co-cultures, four bicultures (A, C, D, and J) revealed synergistic growth, as evidenced by comparing the growth in the biculture to that in the monocultures of each of the strains.

Bicultures H, I, K, and M did not show synergistic growth (*t-test*, $P > 0.05$) (Supplementary Figure S1A), whereas bicultures B, E, F, and G exhibited a partial positive interaction. In the latter, only one of the strains in the pair benefited from being in the coculture (Supplementary Figure S1A). These were, for bicultures E, F, and G (in which the strong enzyme producer *F. ginsengisoli* so9 was involved): strains so1, so4, and w15, respectively. In the case of biculture B, both strains so4 and so22 revealed enhanced growth (as compared to the monoculture counterparts), although this was not significant (Supplementary Figure S1A).

The synergistic bicultures with highest gain in biomass were: A (*C. freundii* so4/*S. multivorum* w15), C (*C. freundii* so4/*A. johnsonii* so1), D (*S. multivorum* w15/*A. johnsonii* so1), and J (*C. freundii* so4/*S. multivorum* w15, *A. johnsonii* so1) (*t-test*, P < 0.05) (Figure 2A). Culture A revealed an increase of 18.2 (±0.3)-, C of 18.3 (±1.3)-, D of 20.5 (±0.6)-, and J of 15.3 (±2.4)-fold.

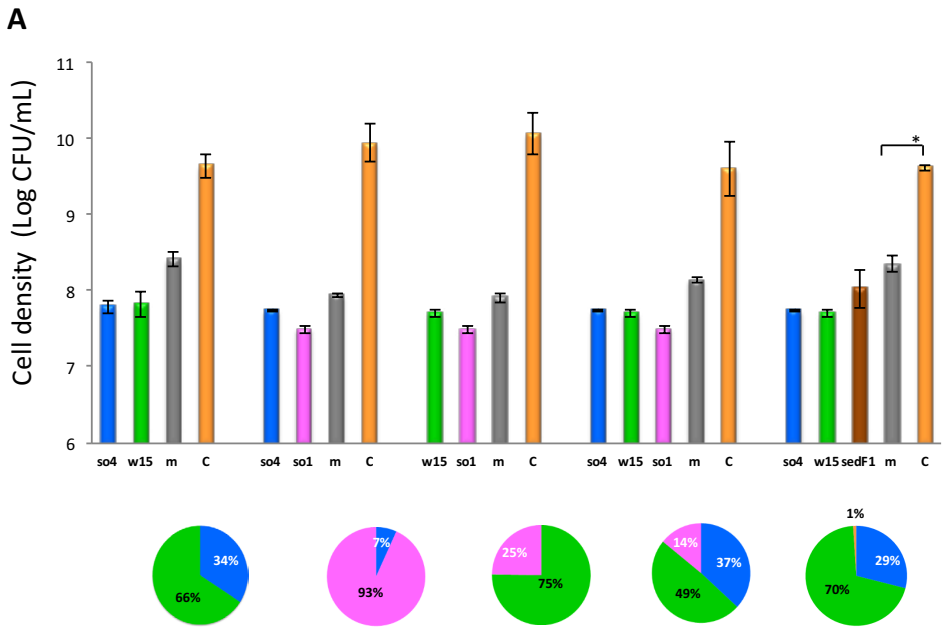

Figure 2A Characterization of synergistic co-cultures. (A) *Cell densities* (log CFU/mL) after 48 h. Significant differences between the sum of monocultures and co-cultures (*t-test*, P < 0.05) shown by *. Explanation: ***m, sum of monocultures (gray-m)***; and ***C, co-cultures (yellow-C);*** **so4**, *C. freundii*, **w15**, *S. multivorum*, **so1**, *A. johnsonii*, **sedF1**, *C. ligniaria*. In the pie chart, the proportions of the individual strains in the co-culture at the end of the culture are shown.

Bacterial-bacterial-fungal triculture

Only one bacterial-fungal triculture revealed synergistic growth (L). Triculture L, assembled by mixing *C. freundii* so4 *S. multivorum* w15 and *C. ligniaria* sedF1, revealed quite interesting results, as both bacterial strains exhibited synergistic growth in the presence of the fungus. In contrast, the fungus performed better in the monoculture (*t-test*, P<0.05). Thus, in the triculture *C. freundii* so4 showed a growth increase of 27.8 (±0.8) and *S. multivorum* of 28.2 (±1.5) fold, compared to the respective monocultures. In contrast, the fungal strain showed a decrease in growth (43.9 ± 2.7 fold), compared with its biomass in monoculture.

Degradation potential in co-cultures

In most of the co-cultures, the production of β-glucosidases, cellobiohydrolases, β-mannosidases, and β-xylosidases was stimulated in a mixture- and time-dependent manner. This indicated mutual effects of the strains in spurring the production and/or secretion of lignocellulolytic enzymes. In other words, the activities measured in the co-cultures exceeded those found in the corresponding monocultures (Figure 2B).

Along the duration of the experiments, co-cultures C, H, J, K, L (Table 2) did not show any synergistic enzymatic activity. In contrast, cultures E and F (Table 2) displayed very high enzymatic activities at the end of the incubation period (72 h). Thus, measured activities were: 10351 ± 635.2 (for BG), 2205 ± 174.9 (for CBH), 5181.2 ± 847.9 (BG), and 515.4 ± 107.9 (for CBH), respectively (relative enzymatic activities reported in nmol of MUB released per h at 28ºC, pH 6.8). The increased enzymatic activities were attributed to the presence of the high-enzyme producer *F. ginsengisoli* so9 across these cultures. Surprisingly, *F. ginsengisoli* so9 did not display any synergism in mixtures with other strains (Supplementary Figure S1B).

Among the five co-cultures that were synergistic for growth (Table 2), two did not show any synergistic enzymatic activities (C and J), whereas three others did (A, D, and L) (Figure 2B). In Figure 2B, we show the increase in enzymatic activities in the co-culture compared with the summed respective monocultures. For co-culture A, synergistic activities were found for cellobiohydrolases (DS_{CBH} = 15.3±0.5), β-mannosidases (DS_{BM} = 2.3±0.3), and β-xylosidases (DS_{BX} = 2.3 ± 0.5). Co-culture D exhibited exclusively (raised) cellobiohydrolase activities (DS_{CBH} = 17.4 ± 0.2). Concerning the two bacterial-fungal co-culture L, synergism in the activities of cellobiohydrolases (DS_{CBH} = 2.0 ± 0.2), β-mannosidases (DS_{BM} = 1.9 ± 0.1), and β-xylosidases (DS_{BX} = 2.2 ± 0.2) were found (Figure 2B). Overall, the most 'compatible' biculture, in terms of enzymatic activities, was the system composed of *C. freundii* so4 and *S. multivorum* w15 (A). This system was 'growth-synergistic,' next to "enzyme-synergistic." Interestingly, a clear commonality in co-cultures A, B, G, I, and M (which presented synergism in cellobiohydrolases, β-mannosidases, and β-xylosidases) was the presence of *S. multivorum* in the form of strains w15 or so22 (Supplementary Figure S1B).

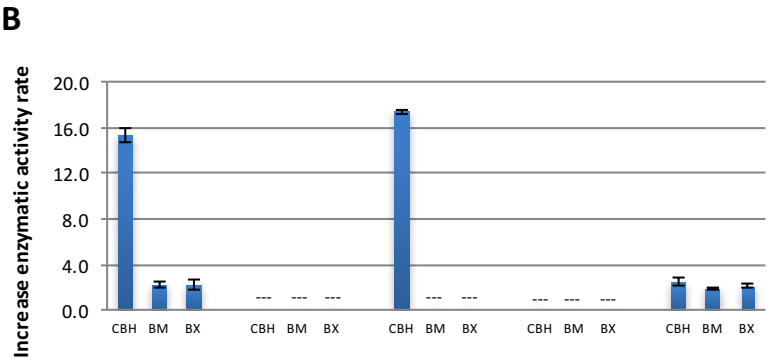

Figure 2B Characterization of synergistic co-cultures. (B) *Synergistic enzymatic activities* in the supernatant from synergistic co-cultures. Y-axis shows the increase (fold) in the enzymatic rate in the co-culture compared with that in the separate monocultures (summed). X-axis shows the respective enzymatic assay. ***CBH, cellobiohydrolase; BM, β-mannosidase; BX, β-xylosidade***. Only co-cultures ***A, D,*** and ***L*** presented synergistic enzymatic activities with the tested enzymes. Enzymatic activity data were based on nmol MUB produced per h at 28ºC, pH 6.8. Bars indicate standard deviations across triplicate systems. (-) indicate below detection.

Influence of the carbon source on collaboration between C. freundii so4 and S. multivorum w15

To investigate if the carbon source has an influence on the collaborative behavior within bicultures, we selected the aforementioned most synergistic one, composed of *C. freundii* so4 and *S. multivorum* w15. Growth experiments were set up, in mono- and bicultures, on carbon sources with increasing levels of complexity and degradability, namely (1) glucose, (2) SRB (CMC, xylan, and lignin), and (3) wheat straw. Overall, the data revealed a strong relationship between the substrate type (see Materials and Methods) and the level of collaborative interaction in the system (Figure 3). Interestingly, in the biculture grown on glucose, no synergistic relationship was found (Figure 3A). When the strains were grown on SRB, synergistic growth was only observed at the end of the incubation period, i.e., after 72 h (Figure 3B). In sharp contrast, significant synergistic growth (*t-test*, $P \leq 0.05$) along the incubation time was observed for the two strains growing together on the (raw) wheat straw (Figure 3C).

Specificity of collaborative/synergistic growth

In the bicultures growing in SRB, after 72 h, *C. freundii* so4 showed an increase in density of 24.6 fold (±1.4), while *S. multivorum* w15 showed an increment of 24.2 fold (±7.9). Notably, the monoculture of the latter strain revealed a long lag phase,

while *C. freundii* so4 did not reveal such a phenomenon (Figure 3B). Growing in biculture on raw wheat straw, after 24 h, *C. freundii* so4 presented an increase in density of 15.4 fold (±3.2), while *S. multivorum* w15 showed an increment of 19.4 fold ± 0.6 (Figure 3C). In contrast, there was no substantial fold increase in the bicultures grown on glucose for any of the two strains (Figure 3A). Hence, we posit that the level of recalcitrance of the substrate was congruent with the strength of the collaborative relationship between the two bacterial degraders.

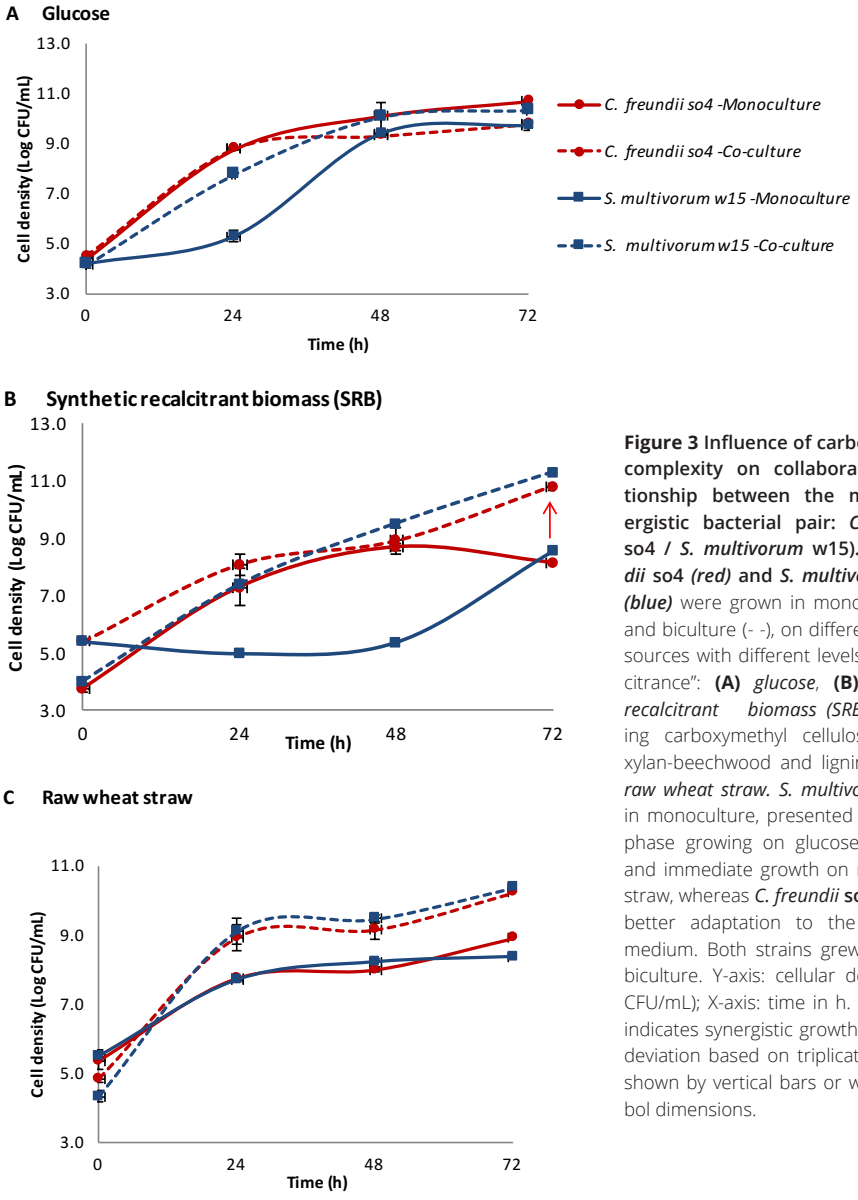

Figure 3 Influence of carbon source complexity on collaborative relationship between the most synergistic bacterial pair: ***C. freundii* so4 / *S. multivorum* w15**). ***C. freundii* so4 *(red)*** and ***S. multivorum* w15 *(blue)*** were grown in monoculture (-) and biculture (- -), on different carbon sources with different levels of "recalcitrance": **(A)** *glucose*, **(B)** *synthetic recalcitrant biomass (SRB,* containing carboxymethyl cellulose [CMC], xylan-beechwood and lignin) and **(C)** *raw wheat straw. S. multivorum* **w15**, in monoculture, presented a long lag phase growing on glucose and SRB and immediate growth on raw wheat straw, whereas ***C. freundii* so4** showed better adaptation to the synthetic medium. Both strains grew better in biculture. Y-axis: cellular density (log CFU/mL); X-axis: time in h. ***Red arrow*** indicates synergistic growth. Standard deviation based on triplicate systems shown by vertical bars or within symbol dimensions.

Degradation potential

C. freundii so4 and *S. multivorum* w15 growing in biculture in SRB did not exhibit synergistic enzymatic activity in the initial phases of the experiment. However, at the end of the incubation time (72 h), enzymatic synergism became apparent, as revealed by BG, CBH, BM, and BM assays. Specifically, the co-cultures displayed the following DS values: 6.4 (±3.9), 2.4 (±0.6), 4.8 (±2.6), 6.4 (5.7 ± 0.6), respectively (Supplementary Figures S2 A–D). Clearly, the enhanced cell densities at later stages of incubation drove the strains to synergism also at the enzymatic level.

Are released compounds at the basis of the synergism?

To explore the mechanism involved in the synergism, we selected the *C. freundii* so4/*S. multivorum* w15 pair. Monocultures of each strain were treated with freshly harvested supernatants of their partner strain, in two different conditions. In the first case, both supernatant donor strains had been grown on raw wheat straw and in the second case on glucose. The supernatants originating from growth in the two different media affected partner strains to very different extents (Figure 4). Both partners of the pair revealed significant (*t-test*, $P < 0.05$) growth enhancements when treated with supernatants from the partner organism grown in raw wheat straw. However, this was not the case for the cultures grown in glucose. Below, we provide details of the growth and enzymatic potential parameters.

Growth

Upon addition of the supernatant of the counterpart strain grown in raw wheat straw, *C. freundii* so4 (growing on raw wheat straw) exhibited a biomass increase of 27.9 fold (±0.7) (Figure 4A) as compared to the respective control monoculture. *S. multivorum* w15 revealed a similar 24.9 (±2.7) fold biomass increment following induction (Figure 4B). In contrast, when supernatants were used from bacterial donors grown in glucose, *S. multivorum* w15 (growing on raw wheat straw) presented a longer log phase and a growth reduction of 45-fold (±4.1) at 24 h. However, at the end of the experiment (72 h), the strain reached the same biomass as the control (growing on raw wheat straw) (Figure 4B). *C. freundii* so4 growing on raw wheat straw and induced by the counterpart strain supernatant (growing in glucose) showed a slight (3.7 ± 0.1 fold) increase in biomass at 48 h. However, this strain had the same biomass as the control one at the end of the incubation (27 h) (Figure 4A).

Degradation potential

Remarkably, the enzymatic activities in the cultures induced by supernatants of strains growing in glucose did not show significant differences from those

in the control (uninduced) cultures in both cases (Supplementary Figure S3). In contrast, the enzymatic activities in the two cultures that had been treated with supernatants from raw wheat straw grown partner strains revealed an important difference, in both directions. The monocultures growing on raw wheat straw, at time zero, did not show any enzymatic activity in the four assays (β-glucosidases, cellobiohydrolases, β-mannosidases, and β-xylosidases) (data not shown). In contrast, upon treatment with supernatants from the RWS-grown partner, high enzymatic activities were found in all assays of the resulting supernatants as from the start of the culture, as compared to the untreated control. The impact on the activity was the same for both strains (*C. freundii* so4, *S. multivorum* w15) (Supplementary Figure S3).

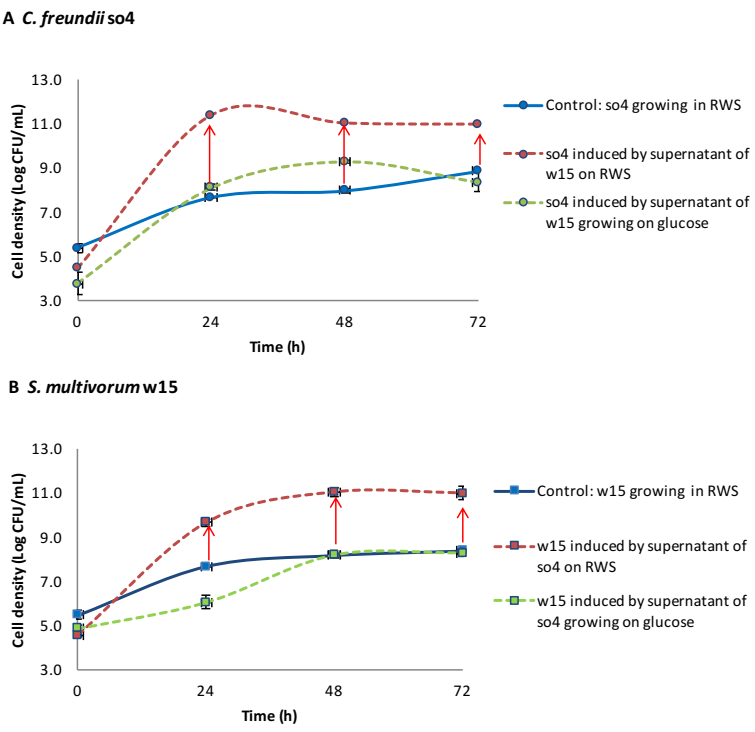

Figure 4 Induction experiment. Effect of supernatant from *C. freundii* so4 *(circle)*, growing on raw wheat straw (RWS) or glucose, on the growth of *S. multivorum* w15 *(square)* and vice versa. In **(A)** *C. freundii* **so4** is the recipient and *S. multivorum* **w15** is the donor grown in monoculture on raw wheat straw ***(red)*** or glucose ***(green)***; supernatant from ***RWS-grown*** strain w15 produced a significant increase (*t-test*, $P < 0.05$) in the growth of *C. freundii* **so4** (as compared to the control on ***RWS (blue)***). In **(B)** *S. multivorum* **w15** is the recipient and *C. freundii* so4 is the donor, growing monoculture on ***RWS (red)*** or glucose ***(green)***; supernatant from ***RWS-grown*** strain so4 produced a significant increase (*t-test*, $P < 0.05$) in the growth of *S. multivorum* **w15** (as compared to the control) on ***RWS (blue)***. Red arrow indicates synergistic growth. Standard deviation based on data from triplicate systems - (shown by vertical bars or within symbol dimensions).

The effect of the supernatants of donor *S. multivorum* w15 on *C. freundii* so4 was relatively constant, with somewhat increasing values along the culture time, compared with the control (Supplementary Figure S3). Thus, *S. multivorum* w15 presumably collaborates with *C. freundii* so4 by contributing diverse enzymatic activities. Conversely, *S. multivorum* w15 as a recipient of *C. freundii* so4 supernatant showed enhanced enzymatic activity only during the first 24 h of incubation. However, this did not impact the growth of w15, indicating that *C. freundii* so4 stimulates *S. multivorum* w15 temporarily by a mechanism different from enzymatic enhancement.

Discussion

The interest in using co-cultures or consortia in the LCB bioprocess industry has increased recently. For instance, microbial consortia have been proposed as key agents in the degradation of wheat straw (Jiménez et al. 2013; Ghosh et al. 2016; Jia et al. 2016). The underlying assumption was that they provide a perfect mix of diverse lignocellulolytic enzymes required to degrade the recalcitrant compounds in wheat straw. In particular, metabolic cooperation between microorganisms and synergistic action of secreted enzymes may allow for an efficient degradation process (Taha et al. 2015; Jiménez et al. 2017). In this study, we aimed at characterizing to what extent cooperation between individual populations from the microbial consortia affects lignocellulose degradation, by characterizing co-cultures (in comparison to monocultures) of lignocellulose degrading bacteria and fungi. The cultures were monitored through time, thus providing a dynamic view of both growth and enzyme activities. Our results clearly indicate that bacterial synergism does play a substantial role in subsets of organisms in such consortia and that the relationship between strains inhabiting the same system is dependent on the complexity of the carbon source.

Metabolic complementarity

Overall, a positive relationship was found between the abundance of particular degrading bacteria (in raw wheat straw derived consortia) and their capacity to grow on the substrate (Figure 1 and Supplementary Figure S1). This finding corroborated the conclusion that the enrichment process used indeed allowed the selection of strains with high LCB degradative capacity. We further addressed the ability of selected lignocellulose degrading strains to establish a [positive] relationship with each other, as suggested in an earlier study (Cortes-Tolalpa et

al. 2016). Synergistic interactions were indeed observed in five of 13 co-cultures, and metabolic complementarity of the component strains was invoked as the most likely mechanism involved. For instance, the most promising synergistic pair, composed of *C. freundii* so4 and *S. multivorum* w15 (biculture A) displayed superior growth in co-culture as compared to the respective monocultures, with synergistic activities of several hydrolytic enzymes (Figure 2A). *C. freundii* and *S. multivorum* differ widely in their metabolic properties. *C. freundii* is a member of the *Enterobacteriaceae*, a facultatively anaerobic family, with motility by flagella. It is able to grow on glycerol as well as citrate as sole carbon sources (Rosenberg et al. 2014a). *S. multivorum* belongs to the *Sphingobacteriaceae*. It is a strict aerobe, which does not possess flagellar motility. It is able to produce acid from a large variety of carbohydrates (including α-D-glucopyranoside and α-D-mannopyranoside) by oxidative processes. In fact, the organism is able to grow on p-hydroxy-butyrate as a single carbon source, but not on glycerol, like *C. freundii*. Moreover, *S. multivorum* is well known as a producer of extracellular enzymes, mainly xylosidases, proteases, and lipases (Rosenberg et al. 2014b). Both strains are capable of transforming cellobiose.

Division of labor

In our study, *S. multivorum* w15 probably contributes to cultures growing on wheat straw with efficient extracellular enzymes. In particular the release of different types of xylosidases seems to be a common feature among *S. multivorum* strains (Malfliet et al. 2013; Lian et al. 2016). Here, growing on raw wheat straw, *S. multivorum* w15 produced powerful cellobiohydrolases and β-xylosidases; such enzymes were not found with *C. freundii* so4 when grown under the same conditions (Figure 2A). We also found highly active β-xylosidases from *S. multivorum* strains w15 and so22, grown on wheat straw singly and in co-culture (Figure 2 and Supplementary Figure S1). Moreover, it has been indicated that *S. multivorum* has lignin-degradation potential, which suggests the organism may also play a role in the degradation of the lignin present in wheat straw (Taylor et al. 2012). Such key metabolic activities allow *S. multivorum* to establish positive interactions with *C. freundii* so4. On the other hand, *C. freundii* so4 showed excellent growth on glucose, as opposed to *S. multivorum* w15. However, strain w15 did grow well in the glucose bicultures, which indicates that *C. freundii* so4 exerted a positive metabolic effect on its counterpart strain (Figure 3). We hypothesized that it probably provides redox power and contributes to the degradation of oligosaccharides to simpler sugars. This might be stimulated

by its high motility, allowing it to explore the substrate. Furthermore, given the strict aerobic metabolism of *S. multivorum* w15, it is very likely that *C. freundii* so4 produces metabolic intermediates that *S. multivorum* w15 can consume, allowing it to reach higher cell densities in co-culture than in monoculture.

Furthermore, the observed growth stimulation of the *S. multivorum* w15 as well as the *C. freundii* so4 monocultures following treatment with the supernatant of the counterpart wheat-straw-grown strain further corroborates the contention that synergistic interactions take place when growing on wheat straw. We speculate that, in both cases, the recipient strain was capable of reaching increased cellular density after receiving, from the donor, a considerable number of secreted enzymes, next to (potentially) other compounds. With respect to the latter, signaling could be involved. This is corroborated by the fact that a quorum sensing system has been found in *C. freundii* (Rosenberg et al. 2014a; Wang and Zhou, 2015). Although we cannot precisely pinpoint the mechanisms that drive the interactions in our co-cultures, as well as the large increase of enzymatic activities observed in them (Figures 1, 2), the supernatant-induced growth stimuli (Figure 4) provide clear evidence for synergistic interactions. Moreover, the metabolic differences between the two strains suggest that they divide 'labor' in the transformation of the heterogeneous wheat straw, allowing their co-cultures to build up an enhanced biomass. Importantly, the synergism was only observed with supernatants harvested from cells growing on raw wheat straw, but not with those from glucose-grown cells, indicating the relevance of the chemical complexity of the substrate (see below).

Influence of the carbon source

The complexity of carbon sources can have a substantial influence on the metabolism of heterotrophic organisms (Deng and Wang, 2016). Klitgord and Segrè (2010), using flux balance analysis, found that different media formulations (based on carbon, nitrogen, sulfur, and phosphorus) affect the interactions between microorganisms (Klitgord and Segrè, 2010). In our study, the more complex the substrate was, the more synergistic the relationship between *C. freundii* so4 and *S. multivorum* w15 became. Thus, the emergence of synergism in subsets of the original wheat-straw-grown microbial consortia can be linked to the inherent heterogeneity of the substrate, suggesting that the complexity of the carbon source can strongly modify the relationship between degrader strains. Specifically, we hypothesized that the level of synergism between

bacteria involved in LCB degradation processes is related to the differential presence of bonds in substrates of different complexity. In the SLB, the three main components (cellulose, xylan, and lignin) were not tightly bound together in a matrix, such as was the case for the raw wheat straw. Thus, the finding that the collaborative bacterial pair showed synergism only at the end of the experiment is in line with this lower number of bonds (Figure 3B). Specifically, the presence of bonds between lignin and the complex carbohydrates cellulose and hemicellulose, or between them, may have been at the basis of the observed synergism. Such bonds determine to some extent the recalcitrance of the LCB (Du et al. 2014; Arnling Bååth et al. 2016). Notwithstanding our enhanced understanding of the bias of synergism and the link to recalcitrant bond numbers, further studies are necessary to understand this phenomenon in greater detail.

Overall, the data indicate that, when grown on raw wheat straw as the sole *C* and energy source, degradative strains first consume the labile parts of the substrate, after which they are in need to collaborate to access the remaining recalcitrant sources of carbon. We here posit that 'multipolymer' or 'peeling' synergism could be a model description of the mechanism involved in the synergism between *S. multivorum* w15 and *C. freundii* so4 on raw wheat straw. In this type of synergism, proposed by Selig et al. (2008) and Várnai et al. (2011), cellulose and hemicellulose are, at the same time, "peeled off" by enzymatic action, exposing new structures of the substrate to the hydrolytic enzymes that are or become available. For the complete hydrolysis of the raw wheat straw, different types of lignocellulolytic enzymes are probably required, in a temporally and spatially dynamic manner (Selig et al. 2008; Várnai et al. 2011).

Final remarks

Overall, this study reveals that, in LCB degradation processes, co-cultures of particular nature are superior to monocultures, as they allow division of labor in the metabolic processes that are required by the substrate. Clearly, microorganisms often lack some key metabolic pathways, which may be supplemented by others (Mikesková et al. 2012; Abreu and Taga, 2016; Ghosh et al. 2016). Thus, LCB degradation, in the end, may impose 'group selection' pressure on the process participants, in which 'group' is not defined by 'kin' but is rather determined by complementarity in a spatially- and temporally-explicit process. Our findings are consistent with recent data that show that co-cultures often present improved performance over corresponding monocultures. The mechanisms involved may

include enhanced substrate utilization, overcoming of nutritional limitations, reduction of the levels of cheaters/scavengers and achieving superior overall activity, conversion and enzymatic action (Feng et al. 2011; Okeke and Lu, 2011; Zuroff et al. 2013; Liao et al. 2015; Valdez-Vazquez et al. 2015).

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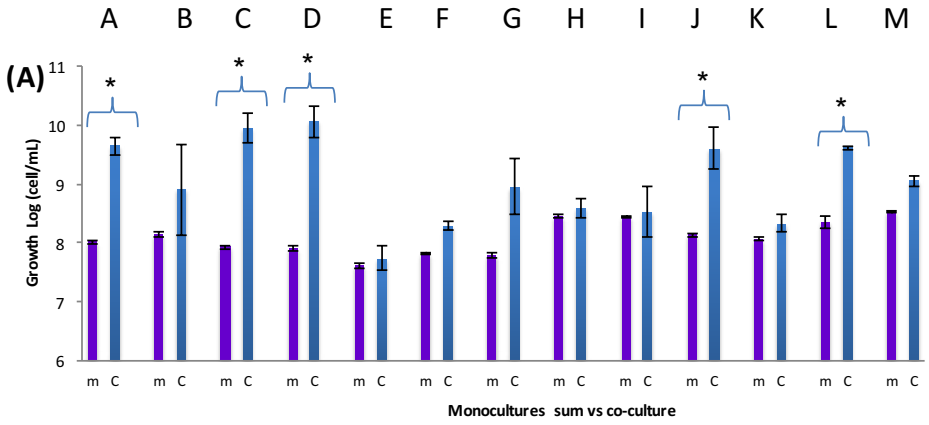
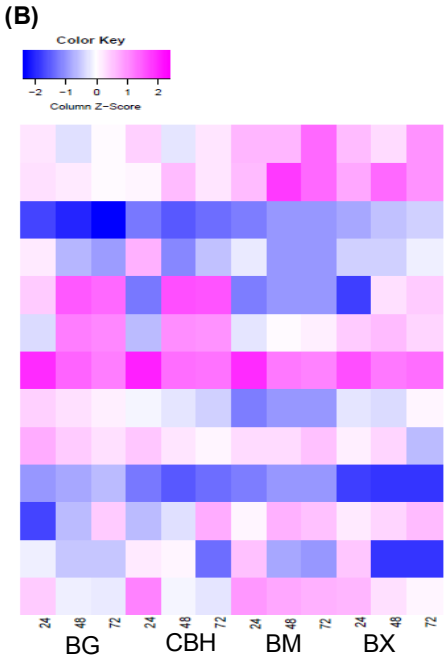
Supplementary





Co-culture -⟶Composition

A -- *C. freundii* so4, *S. multivorum* w15

B -- *C. freundii* so4, *S. multivorum* so22

C -- *C. freundii* so4, *A. johnsonii* so1

D -- *S. multivorum* w15, *A. johnsonii* so1

E -- *F. ginsengisoli* so9, *A. johnsonii* so1

F -- *C. freundii* so4, *F. ginsengisoli* so9

G -- *S. multivorum* w15, *F. ginsengisoli* so9

H -- *C. freundii* so4, *M. foliorum* w9

I -- *S. multivorum* w15, *M. foliorum* w9

J -- *C. freundii* so4, *S. multivorum* w15, *A. johnsonii* so1

K -- *C. freundii* so4, *S. multivorum* w15, *F. ginsengisoli* so9

L -- *C. freundii* so4, *S. multivorum* w15, *C. lignaria* sedF1

M -- *C. freundii* so4, *S. multivorum* w15, *M. foliorum* w9

Figure S1 In **(A)** is shown the sum of growth from ***monocultures (purple)*** and ***co-cultures (blue)***, significant differences between the sum of monocultures and co-culture, *t-test* (P<0.05). Standard deviation correspond to triplicate. In **(B)** heatmap that displayed co-culture average (from triplicates) and normalized ***enzymatic activities BG: β-glucosidases, CBH: cellobiohydrolases, BM: β-mannosidases*** and ***BX: β-xylosidases***, along the incubation time (24-72h), relative enzymatic activity reported in nmol MUB per h at 28°C, pH 6.8.

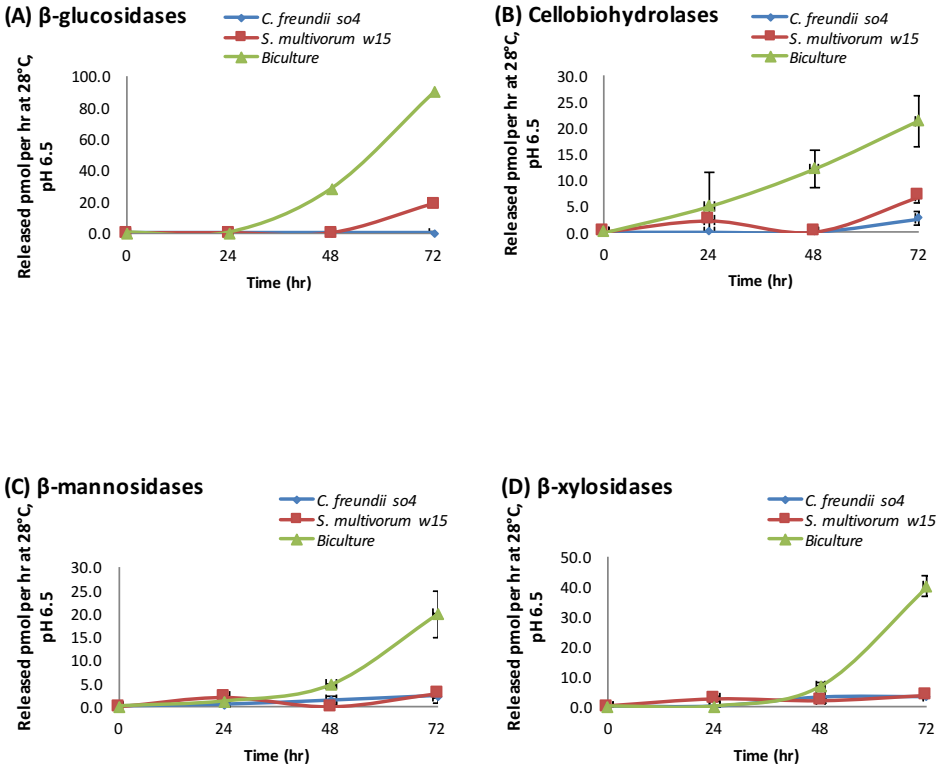


Figure S2 Enzymatic activities from *S. multivorum* w15 and *C. freundii* so4 growing in synthetic recalcitrant biomass. (A) *β-glucosidases,* **(B)** *cellobiohydrolases*, **(C)** ***β-mannosidases***, **(D)** ***β-xylosidases*** enzymatic activity from monocultures ***S. multivorum* w15** *(red)* and ***C. freundii* so4** *(blue)* and the ***co-culture* (w15, so4)** *(green)*. Standard deviation correspond to triplicate systems.

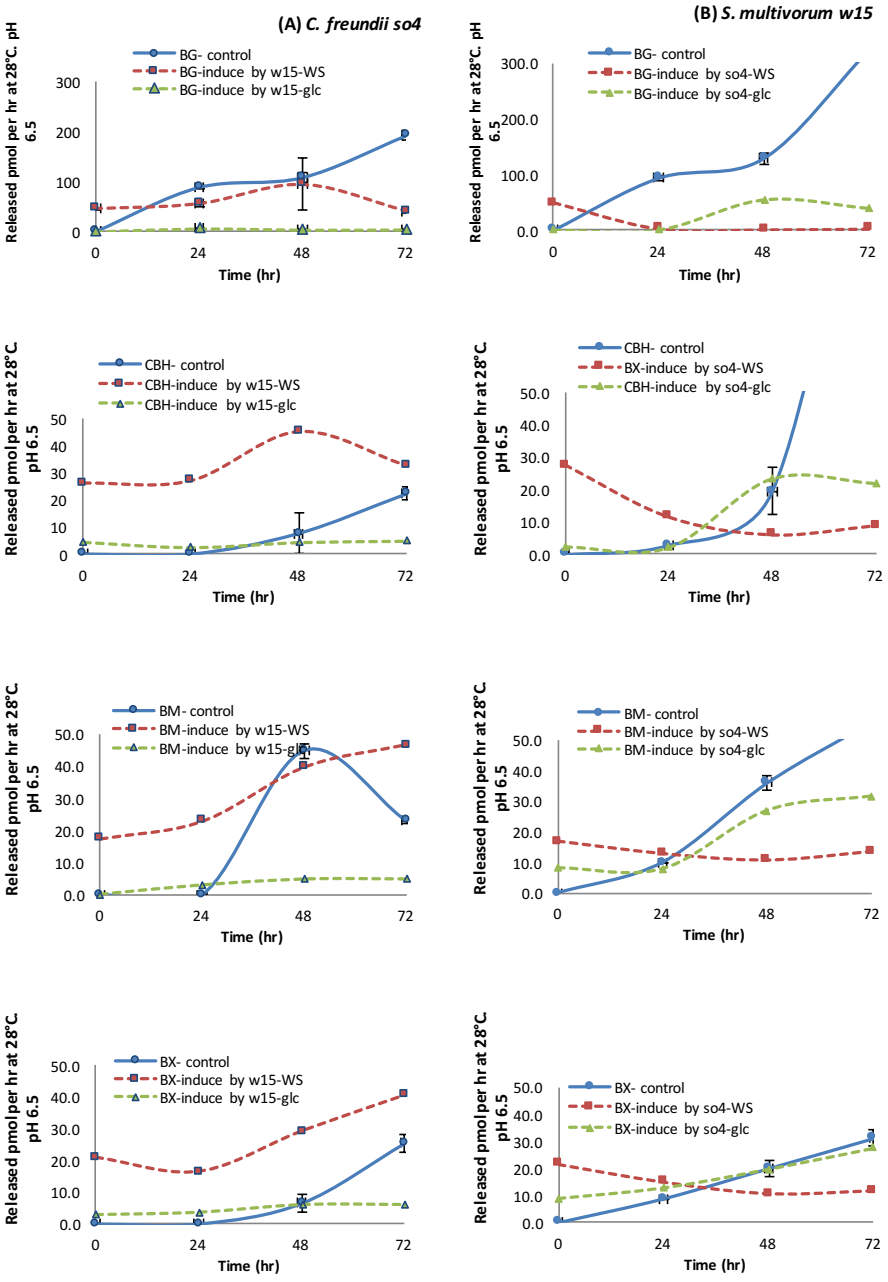


Figure S3 Enzymatic activities from induction experiment. β-glucosidases, cellobiohydrolases, β-mannosidases and β-xylosidases activities *(secretome)* from **(A)** *C. freundii* so4 *induced* by supernatant from *S. multivorum* **w15** and **(B)** *S. multivorum* **w15** *induced* by supernatant of *C. freundii* **so4**. The donor strains was grown on *glucose (green)*, grown on *RWS (red)*. In *blue* the each strains *grown on RWS as a control* Standard deviation correspond to triplicate systems.

Chapter 6

Comparative genome analysis of Citrobacter freundii so4 and Sphingobacterium multivorum w15: a minimal consortium model for synergistic interaction in lignocellulose degradation

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Joana Falcão Salles · Jan Dirk van Elsas

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Manuscript in preparation

Abstract

In previous work, the bacterial strains *Citrobacter freundii* so4 *and Sphingobacterium multivorum* w15 revealed a synergistic relationship when growing together on wheat straw (WS) as the single carbon and energy source. Here, we presented an analysis of the draft genome sequences of these two strains. The *C. freundii* so4 genome has 4,883,214 bp with a G+C content of 52.5%; 4,554 protein-encoding genes and 86 RNA genes. *S. multivorum* w15 has a genome of 6,678,278 bp, with a G+C content of 39.7%, 5,999 protein-encoding genes and 75 RNA genes. Only the *C. freundii* so4 genome revealed motility genes. Moreover, its predicted metabolic capacity favoured the consumption of amino acids and simple sugars, with laminarin as the sole exception. In contrast, the *S. multivorum* w15 genome revealed a capacity to consume complex polysaccharides, e.g. a preference to grow on intermediates of starch degradation. A large number of genes (367) were associated with CAZy family enzymes, 193 encoding glycosyl hydrolases (GHs)and 50 carbohydrate binding modules (CBMs). Remarkably, 22 genes were predicted to encode enzymes from glycoside hydrolase family GH43. Potentially implicated in the degradation of wheat straw. In contrast, the *C. freundii* so4 genome had 137 CAZy family genes, of which 61 encoded GHs and 12 CBMs. Thus *S. multivorum* w15 and *C. freundii* so4 had a complementary lytic armoury that allow them to attack different WS polymers, resulting in parallel and potentially complementary catabolism. We posit here that *S. multivorum* w15 acts as a secretor of hydrolytic enzymes that attack hemicellulose components while *C. freundii* so4 does so for the cellulose component. Moreover, it may enhance the growth by converting oligosaccharides to simpler ones. Moreover, *C. freundii* so4 could: 1) produce and excrete secondary metabolites that *S. multivorum* w15 can consume, and 2) detoxify the system by reduction of accumulated by-products. The positive interactions between these strains can be defined as cooperative cross-feeding.

Introduction

Agricultural waste such as wheat and maize straws and bagasse from sugar cane constitute lignocellulosic biomass (LCB) substrates composed of three main components: cellulose, hemicellulose and lignin. The proportion of these components is dependent on plant species, time and growth conditions to mention some (Sorek et al. 2014). LCB substrates represent promising alternatives to carbon sources for the production of useful compounds such as plastics or biodiesel (Guerriero et al. 2016). The utilization of waste materials is particularly relevant as mankind is threatened by the depletion of sources of energy and global warming due to the extensive use of petroleum based energy (Kumar et al. 2015).

The degradation of LCB not only requires a large variety of hydrolytic enzymes, i.e. cellulases, hemicellulases and ligninases (Himmel et al. 2007), but also, from each of these three enzymes groups, different types of enzymes with different cleavage specificities. For complete degradation, the (additional) action of carbohydrate binding modules (CBMs), that bind cellulose or hemicelluloses, and helper enzymes such as lytic polysaccharide monooxygenases (LPMOs), xylan esterases (CEs) and polysaccharide lyases (PLs) are necessary (Koeck et al. 2014).

Degradation of LCB is a complex process. In nature, it is only efficient if diverse microorganisms contribute, mainly bacteria and fungi (Cragg et al. 2015). These produce diverse lytic as well as auxiliary enzymes, which work in a synergistic manner (Lynd et al. 2002). Moreover, depending on the type of substrate, interactions within the degrader microbial communities emerge, that could be either positive or negative. We observed that the occurrence of such positive microbial interactions is influenced by the complexity of carbon source (Cortes-Tolalpa et al. 2017), which was in line with the finding that the presence of complex carbon sources stimulates synergistic and reduces antagonistic interactions (Deng and Wang 2016).

"Division of labour" (DOL) is one of the strategies used by microorganisms for dealing with complexity (Jiménez et al. 2017). This phenomenon is observed - for example - in a microbial food chain when it is necessary to consume complex organic compounds. There are examples of DOL in the cycles of carbon, as well as of sulfur and nitrogen (Falkowski et al. 2008). According to West and Cooper (2016), "division of labour" can be defined as the cooperation between individuals

that are each specialized in specific tasks. Some of the requirements for DOL are (1) presence of diverse phenotypes (individuals that perform different tasks), (2) cooperation (the tasks performed by one individual will benefit the other individual) and (3) the division of tasks favours adaptation to the environment (increasing the fitness of all individuals involved).

Degradation of LCB by microbial consortia is still not completely deciphered, particularly regarding the interactions that take place during the degradation process. Clearly, a better understanding of the process will improve the design and utilization of microbial consortia at industrial level (Song et al. 2014). There are valid attempts to design minimal consortia for this (Cortes-Tolalpa et al. 2017).

In a previous study, we described a collaborative relationship between two bacteria, identified as *Citrobacter freundii* so4 and *Sphingobacterium multivorum* w15. The strains were recovered from soil- and wood-derived consortia grown on wheat straw (Cortes-Tolalpa et al. 2016). The synergistic activity between these bacteria included hydrolytic enzyme activities and growth. The two strains presented synergism exclusively when grown on wheat straw or on synthetic recalcitrant biomass, but not when grown on glucose (Cortes-Tolalpa et al. 2017). Moreover, strains related to w15 and so4 have been found to be very abundant in consortia able to degrade diverse LCB substrates (Jiménez et al. 2014b; Brossi de Lima et al. 2015) suggesting their potential key roles in the degradation.

However, the genetic capabilities of both *C. freundii* so4 and *S. multivorum* w15 are as yet unknown. Hence, to foster our understanding of the mechanisms behind the synergism, it was necessary – as a first step - to gather key information from their genomes. Here, we hypothesized that the collaborative roles of the two species in LCB degradation can be understood from genome analyses. Hence, we sequenced, annotated and compared the genomes of *C. freundii* so4 and *S. multivorum* w15 to this end. We placed a particular focus on their LCB hydrolytic capacities.

Materials and methods

Strains and growth conditions

C. freundii so4 and *S. multivorum* w15 have been isolated from microbial consortia able to degrade raw wheat straw (Cortes-Tolalpa et al. 2016). Both strains were able to grow in monoculture using raw wheat straw as the sole carbon source (Cortes-Tolalpa et al. 2017). For routine purposes, strains were grown in Lenox medium (10g/L tryptone; 5 g/L, yeast extract, 5 g/L NaCl; Sigma-Aldrich, Darmstadt, Germany). The cultures were incubated overnight at 28 °C and 180 rpm.

Phenotype microarray testing

The Phenotype MicroArray assay was used (96-well GN2 and PM2A plates; Biolog Inc. , CA, USA) to test the catabolic capabilities of *C. freundii* so4 and *S. multivorum* w15. The arrays consisted of 190 carbon sources, encompassing alcohols, amides, amines, amino acids, carbohydrates, carboxylic acids, esters, fatty acids and polymers. Single colonies, of each strain, were picked from TSA plates on which they were subcultured, to produce cultures in Lenox medium which were incubated overnight, with shaking, at 28°C. A homogenous suspension of inoculum was made with IF-0a GN/GP inoculation fluid (72101) and diluted to 0.001 OD at 590 nm; in the case of the PM2A plate, the inocula were supplemented with 150μL of Biolog redox dye mix A (100X). The inoculum was kept for 2 h at room temperature and then 150 μL of the suspension was added into each well of the GN2 MicroPlate. The microplates were incubated at 28°C and read at 0, 6, 12, 24, 48, 72 and 84 hours with a microtiter plate reader at 590nm (Holmes et al. 1994). Analyses of the data were performed using the area under the (growth) curve (AUC) as the criterion (Kalai Chelvam et al. 2015).

DNA extraction

Total genomic DNA was extracted from the liquid and shaken cultures of the two strains by the use of the UltraClean DNA Isolation Kit (MoBio® Laboratories Inc., Carslab, USA), following the instructions of the manufacturer.

Genome sequencing and assembly

Whole-genome sequencing of *C. freundii* so4 and *S. multivorum* w15 was performed using the Illumina NextSeq 500 V2 platform by 150bp paired-end reads (LGC Genomics Gmbh, Berlin, Germany). Assembly and scaffolding of the

sequence data were performed using SPAdes 3.5.0, according to the workflow described by Nurk et al. (2013).

Genome annotation

Genome drafts were annotated by Rapid Annotation Subsystem Technology (RAST) (Aziz et al. 2008). For *C. freundii* so4, final assembly resulted in 46 contigs with an N50 of 282 822 bp. For *S. multivorum* w15, 90 contigs were obtained, and an N50 value of 133 589 bp.

Metabolic pathway comparison

First, it was identified the number of distinct reactions per metabolic pathway according to the enzyme commission number (EC number), EC numbers do not specify enzymes, but enzyme-catalyzed reactions. If different enzymes, for instance from different organisms, catalyze the same reaction, then they receive the same EC number. Then, taking the total distinct reactions per metabolic pathway as the 100 percent was calculated the percentage of distinctive EC according to the number EC found in each strain per pathway. Finally, the functionality of the pathway was confirmed by using the metabolic tool comparison in RAST. The notion of functioning is defined by having genes for all the functional roles that compose a variant of a subsystem or pathway (Overbeek et al. 2014).

Genome statistics

The predicted genes were translated and the resulting data used to probe the Pfam database (Finn et al. 2014) as well as the COG database through the MicroScope platform (Vallenet et al. 2017). Signal-P server 4.1 was used to predict signal peptide regions (Petersen et al. 2011). Transmembrane domains were identified using THMMH server 2.0 (Krogh et al. 2001). OrthoFinder was used to identify single-copy genes in the genomes (Emms and Kelly 2015). PlasmidFinder was used to look for plasmids (Carattoli et al. 2014).

Phylogenetic analysis

RNAmmer was used for identification of rRNA (Lagesen et al. 2007) . The 16S rRNA gene sequences (NODE_31_length_1713_cov_124.214_ID_61) of *C. freundii* so4 and (NODE_70_length_5327_cov_127.629_ID_139) of *S. multivorum* w15 were used for phylogenetic analyses. Closely-related 16S rRNA genes from type strains of *C. freundii* and *S. multivorum* were recovered from the SILVA ribosomal RNA database (Quast et al. 2013) and a phylogenetic tree was constructed using the

neighbor joining method. MEGA v 6.0 software was used to calculate pairwise P-distance values. Bootstrap analysis was performed with 1,000 repetitions.

Degradative enzymes

Predicted genes were translated and used to search in carbohydrate-active enzyme annotation (dbCAN 5) for identification of carbohydrate active enzymes (CAZy); the coverage value was above 0.5 with an e–value < $1e^{-18}$ (Yin et al. 2012). Classifications of the CAZy families were done manually using the CAZy (http://www.cazy.org/) as well as CAZypedia databases (www.cazypedia.org).

Accession numbers

The project has been deposited at DDBJ/ENA/GenBank under the accession numbers PHGU00000000 and PHGV00000000. The version described in this manuscript is PHGU00000000 and PHGV00000000. The strains used in this study have been deposited in the German Collection of Microorganisms and Cell Cultures (DMSZ, Braunschweig, Germany). *C. freundii* so4 is deposited under the number DSM 106340T; *S. multivorum* w15 is in the process for obtaining the accession number from DMSZ.

Results

Carbon utilization profile

***Citrobacter freundii* so4.** Overall, out of 190 carbon sources tested, *C. freundii* so4 was able to grow on 52 (Figure 1, Table S1), leaving a total of 138 substrates un-used. Only *C. freundii* so4 could grow in 30 different compounds as a single energy source that *S. multivorum* w15 could not use (Table S2). Strain so4 showed preference for consumption of simple carbon sources, eight amino acids (L-histidine, hydroxy-L-proline, D-alanine, L-alanine, D-serine, L-aspartic acid, L-alanyl-glycine), seven carboxylic acids (succinic, 5-keto-D-gluconic, D-glucuronic, D,L-lactic, D-galacturonic, D-gluconic and D-saccharic acid). In the same manner, the strain was able to grow in diverse carbohydrates, i.e. the sugar alcohols glycerol, D-sorbitol, D-mannitol and m-inositol, the monosaccharides D-arabinose and glucose-6-phosphate, the ketose dihydroxyacetone and the deoxy sugar L-fucose (Figure 1, Table S1).

***Sphingobacterium multivorum* w15.** Strain w15 grew on a total of 42 compounds (Figure 1, Table S1, leaving 148 substrates un-used). Only *S. multivorum* w15 was able to grew on 20 different compounds as a sole carbon source that *C. freundii* so4 could not consume. Interestingly, it preferably grew on di-saccharides (lactulose,

palatinose, sucrose, turanose, gentibiose), a trisaccharide found in honeydew (D-melezitose) and on stachyose, a tetra-saccharide found in seed of legumes. The strain presented the interesting capacity to grow on oligosaccharides derived from polymers, specifically starch (dextrin, α-, β- and γ-cyclodextrins), as well as pectin and inulin (a polysaccharide produced by plants like cichory), as the sole carbon source (Figure 1, Table S2).

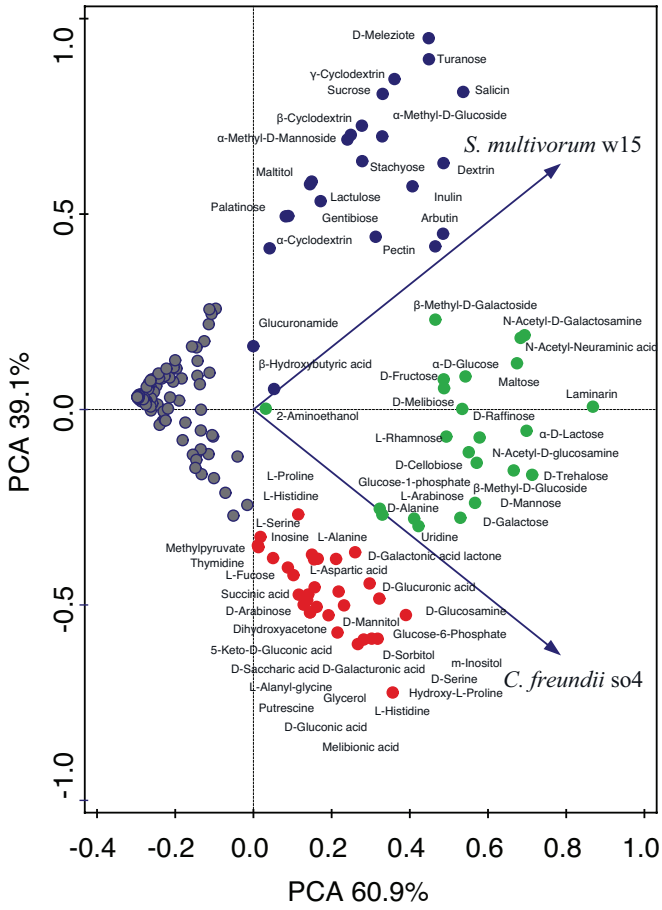

Figure 1 Principal component analysis showing the metabolic capacity of the strains *C. freundii* so4 and *S. multivorum* w15. The ability to consume the individual compounds as a single source of energy was tested using BIOLOG PM2A and GN2 plates. ***C. freundii* so4** exhibited the capacity to grow on 52 compounds ***(red and green)*** principally intermediate metabolites, mainly amino acid, organic acid, sugar acid and monosaccharides ***(red*** and ***green)***. In ***red*** are shown the compounds that were consumed only by ***C. freundii* so4**. ***S. multivorum* w15** grew in 42 compounds ***(blue*** and ***green)***, it presented preference for disaccharides, oligosaccharides and polymers. In ***blue*** are shown the compound that only ***S. multivorum* w15** was able to use as a single energy source. In ***green*** compounds than ***both strains*** were able to consume to different extents.

Compounds used by the two strains. *C. freundii* so4 and *S. multivorum* w15 were able to consume 22 compounds in similar rates. Nine of these 22 were monosaccharides: α-D-glucose, glucose-1-phosphate, D-fructose, D-mannose, D-galactose, L-arabinose, β-methyl-D-glucose, N-acetyl-D-glucosamine and β-methyl-D-galactoside. Five were disaccharides: maltose, D-melibiose, α-D-lactose, D-trehalose, D-cellobiose. Moreover, D-alanine was the only amino acid that both strains could consume as the single carbon source, whereas the polymer laminarin was also shared between them (Figure 1, Table S3).

Genome descriptions

The genome of *Citrobacter freundii* so4 was found to be 4, 883, 214 bp in length, having 52.5% G+C content on average. Of the total 4,703 predicted genes, 4,554 were protein-encoding genes, 585 were detected as genes encoding hypothetical proteins and 86 RNA genes. Of the latter, 11 encoded rRNA (nine 5S rRNA, one 23S rRNA and one 16S rRNA) and 75 tRNA. Three CRISPR repeat regions were found in this genome. No plasmids were found (Table 1).

Table 1. Genome statistics of *C. freundii* so4 and *S. multivorum* w15.

	C. freundii so4		*S. multivorum* w15	
Attribute	Value	% of total	Value	% of total
Genome size (bp)	4883214	100	6678278	100
Coding region (bp)	4323598	88.54	5967041	89.35
DNA G+C content (bp)	2565641	52.54	2655951	39.77
DNA scaffolds	46	-	90	-
Total genes	4703	100	6087	100
Protein-encoding genes	4554	96.83	5999	98.55
RNA genes:	86	1.83	75	1.23
rRNA	11		9	
tRNA	75		66	
Pseudogenes	149	3.17	88	1.45
Genes assigned to COGs	3915	83.25	3854	63.31
Genes assigned to Pfam domains	3970	84.41	2871	47.17
Genes codifying signal peptides	416	8.85	691	11.35
Genes coding for transmembrane helices	1106	23.52	1241	20.39
CRISPR repeats	1	-	3	-
Plasmids	-	-	-	-

-: not detected; rRNA: ribosomal RNA, tRNA: transfer RNA

The genome of *Sphingobacterium multivorum* w15 had a length of 6, 678, 278 bp, with a G+C content of 39.7% on average. Of the 6,087 predicted genes, 5,999 were predicted to encode proteins, 734 were detected as hypothetical proteins. There were 75 genes for RNAs, of which 9 rRNAs (seven 5S rRNA, one 23S rRNA and one 16S rRNA) and 66 tRNAs. Only one CRISPR repeat sequence was found. No plasmids were found.

The complete genome statistics of strains *C. freundii* so4 and *S. multivorum* w15 can be found in Table 1.

Taxonomic affiliations

Initial identification of strain so4 based on 16S rRNA gene sequencing showed 99% similarity of the full rRNA sequence with that of the type strain of *C. freundii,* DSM 30039 (Cortes-Tolalpa et al. 2016). Here, we extended this analysis by using the 16S rRNA gene found from the genome sequence and doing alignment with closely-related (type) strains. Figure 2A shows the resulting phylogenetic tree.

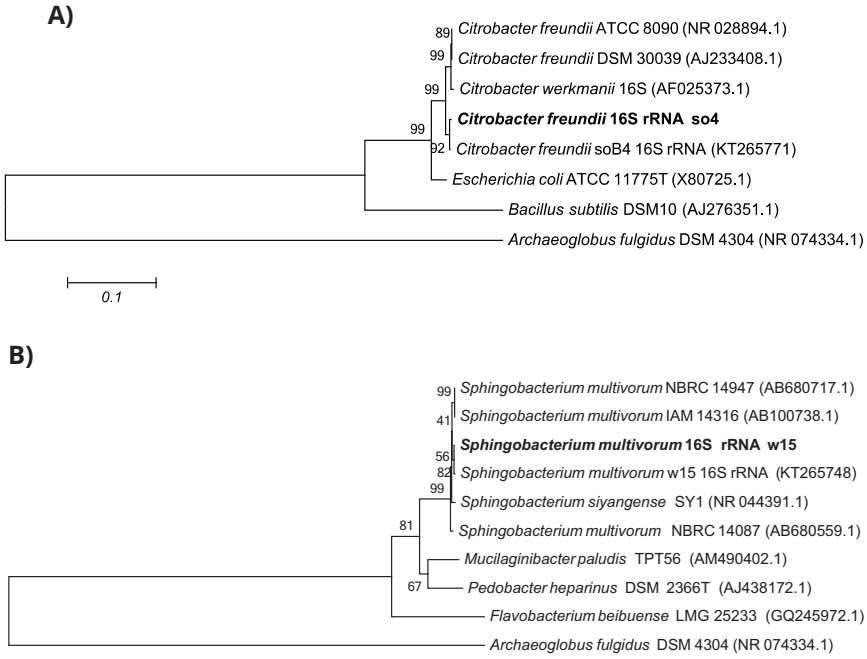

Figure 2 Neighbour-joining phylogenetic tree based on 16S rRNA gene sequences. The tree indicates the relationship between **A)** the isolate ***C. freundii* so4 B)** the isolated ***S. multivorum* w15** and other closely related type strains, isolates are in bold. Bootstrap values based on 1000 replications are listed as percentages at branching points. The sequence of ***A. fulgidus*** DSM 4304 was used as an outgroup. Accession numbers are given in parentheses. The bar scale shows 0.1 nucleotides substitutions per nucleotide position.

Summarizing, a very tightly-knit group of organisms appeared that includes strain so4, and uniquely includes all other *Citrobacter (freundii)* sequences (Figure 2A). Strain *S. multivorum* w15 was identified based on the only 16S rRNA gene sequence found. It clustered in a broad group of organisms that were all classified as *S. multivorum* or alike, being 99% similar to the type strain *S. multivorum* IAM 14316T. In the phylogeny tree, *S. multivorum* w15 is presented in bold. The tree includes the closest bacterial species to strain w15 that belong to the *Sphingobacteriaceae* family (Figure 2B).

Table 2. **Number of genes encoding proteins associated with general COG functional categories.**

	C. freundii so4		***S. multivorum w15***		
Code	**Value**	**% of total[a]**	**Value**	**% of total[a]**	**Description**
J	190	4,15	188	3,13	Translation, ribosomal structure and biogenesis
K	375	8,20	460	7,66	RNA processing and modification
A	1	0,02	-	-	Transcription
L	174	3,81	204	3,40	Replication, recombination and repair
B	-	-	1	0,02	Chromatin structure and dynamics
D	47	1,03	40	0,66	Cell cycle control, Cell division, chromosome partitioning
V	49	1,07	105	1,75	Defense mechanisms
T	218	4,77	312	5,20	Signal transduction mechanisms
M	259	5,67	333	5,55	Cell wall/membrane biogenesis
N	130	2,85	20	0,33	Cell motility
U	123	2,69	85	1,42	Intracellular trafficking and secretion
O	149	3,26	182	3,03	Posttranslational modification, protein turnover, chaperones
C	307	6,72	225	3,75	Energy production and conversion
G	413	9,04	355	5,91	Carbohydrate transport and metabolism
E	480	10,51	341	5,68	Amino acid transport and metabolism
F	85	1,86	73	1,22	Nucleotide transport and metabolism
H	165	3,61	147	2,45	Coenzyme transport and metabolism
I	134	2,93	147	2,45	Lipid transport and metabolism
P	345	7,55	425	7,08	Inorganic ion transport andmetabolism
Q	111	2,43	90	1,50	Secondary metabolites biosynthesis, transport and catabolism
R	559	12,24	638	10,62	General function prediction only
S	346	7,57	326	5,43	Function unknown

[a] Based on the total number of protein-encoding genes in the genome.

Assignment of translated genes to COG categories

Analysis of the genome of *C. freundii* so4 showed that a high number (83.25%) of the protein-encoding genes matched COG functional categories (Table 2). This left 16.75% of the genes unexplained by the COG categorization. With respect to the COG-based distinctions, large numbers of genes were found to be related with production and conversion of energy (307), transport and metabolism of amino acids (480) and of carbohydrates (413) (Table 2).

Analysis of the genome of *S. multivorum* w15 revealed that 63.31% of the protein-encoding genes were associated with COG functional categories, this leaving 36.69% unexplained. Interestingly, the COG-definable genome part exhibited large numbers of putative genes associated with defence and signal transduction mechanisms (105), as well as genes associated with transport of ions (425). These gene counts were higher than those found in the *C. freundii* so4 genome (Table 2).

General metabolism

According to the RAST assignments, the genome of *C. freundii* so4 had a larger number of genes encoding proteins associated with the metabolism of carbohydrates (in total 706). Of these, the largest part was involved in monosaccharide metabolism (184). Surprisingly, a majority was predicted to be involved in central carbon metabolism (138 genes), followed by di- and oligo-saccharide metabolism (86), fermentation (84) and sugar alcohol metabolism (83). Furthermore, the genome of *C. freundii* so4 revealed a major investment in amino acid metabolism (438 genes), cofactors/vitamins (314), metabolism of proteins (295), metabolism of RNA (248), cell wall (236), respiration (188), membrane transport (187), stress responses (175) and lipid metabolism (166) (Table S4). Interestingly, the *C. freundii* so4 genome exhibited a large number of genes associated with chemotaxis and motility (143); these genes were functional, as experimentally shown in the supplementary physiological characterization (SPC) (Figure 3, Table S2).

With respect to *S. multivorum* w15, its genome revealed a large number of putative genes encoding proteins associated with carbohydrate metabolism. In total, 451 genes were found in this category. Regarding carbohydrate degradation, the majority of the genes were associated with the transformation/utilization of monosaccharides (90), di- and oligo-saccharides (88), and polysaccharides (38). Further major investments, as evidenced by the numbers of genes on the

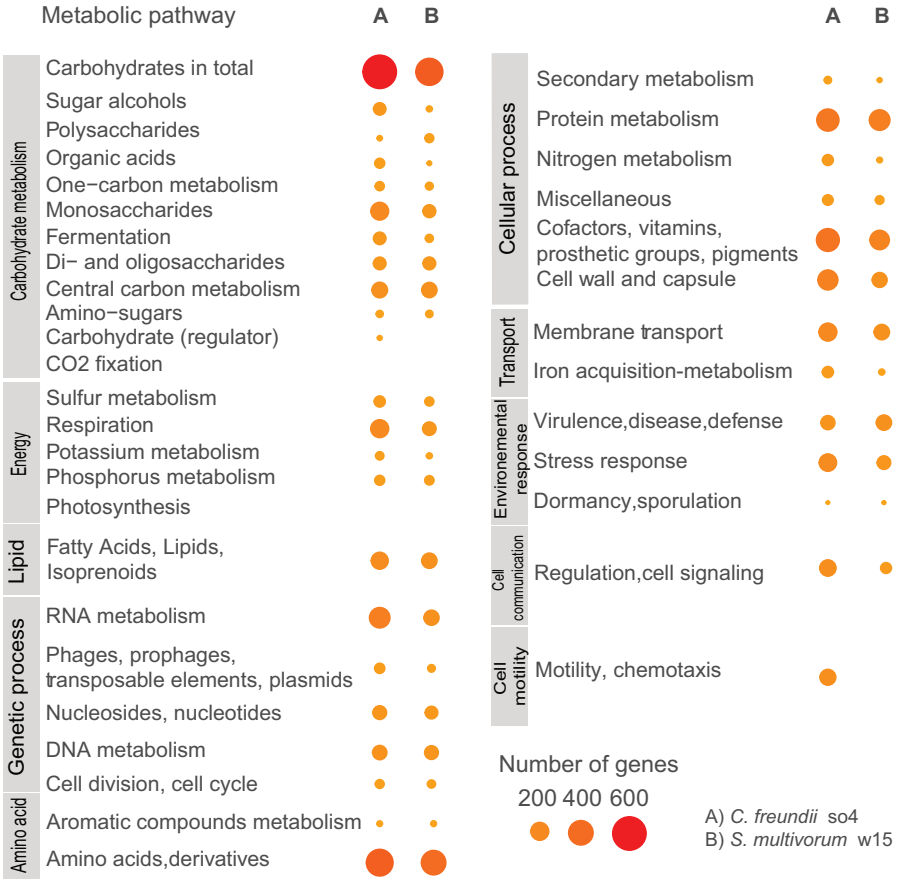

Figure 3 Predicted functional subsystems in *C. freundii* so4 and *S. multivorum* w15. Based on RAST results and on KEGG assignments. Size and color of circles indicates gene abundance.

genome, were in amino acid metabolism (364), metabolism of proteins (250), cofactors and vitamins (222), membrane transport (134), defence systems (132), lipid metabolisms (132), RNA metabolisms (129), cell wall (125) and DNA metabolisms (104). According to RAST annotation, *S. multivorum* w15 genome exhibited none genes associated with chemotaxis and motility and this was confirmed by phenotypic characterization (Figure 3, Table S4, SPC).

Comparison of metabolic pathways in *C. freundii* so4 and *S. multivorum* w15. With respect to the metabolic pathways that could be identified, both strains presented similar percentage in the number of enzyme-catalyzed reactions need for using basic pathways as citrate cycle (TCA cycle), glycolysis-gluconeogenesis and pentose phosphate pathway (PPP) (Table 3), which was

confirmed by a prediction functionality tool analysis from RAST. *C. freundii* so4, being a facultative anaerobe, was expected to reveal the presence of genes for the respective enzymes, as it showed a larger enzyme-catalyzed reactions for pyruvate, propanoate and ascorbate-aldarate metabolism (Table 3). Prediction analysis showed that only strain *C. freundii* so4 had active the glutathione pathway,

Table 3. Number of distictive enzymes observed in different metabolic pathways found in C. freundii so4 and S. multivorum w15. Values represent the percentages of genes/enzymes needed for a functional pathway.

Pathway	Distinct Ecs	*C. freundii* so4 (%)	*S. multivorum* w15 (%)
Citrate cycle (TCA cycle)	41	63	59
Glycolysis/Gluconeogenesis	22	58	49
Pentose phosphate pathway	37	62	57
Pyruvate metabolism	64	51	37
Propanoate metabolism	47	44	21
Pentose and glucoronate interconversions	56	45	39
Glutathione metabolism	40	42	25

Distinct reactions per metabolic pathway according to the enzyme commission number (EC number). Comparison based on KEGG database. EC numbers do not specify enzymes, but enzyme-catalyzed reactions. If different enzymes, for instance from different organisms, catalyze the same reaction, then they receive the same EC number.

which is in charge of the detoxification of formaldehydes, as well as seven reactions involve in propionate catabolism, those were malonate decarboxylase, malonate transcriptional regulator, malonyl CoA acyl carrier protein transacylase, phosphoribosyl-dephospho-CoA transferase, triphosphoribosyl-dephospho-CoA synthetase, propionate catabolism operon regulatory protein PrpR and propionate--CoA ligase.

Carbohydrate degradation

The genome of *C. freundii* so4 presented a high abundance of genes involved in the metabolism of monosaccharides (184), sugar alcohols (83), 1-carbon metabolism (41) and fermentation (84) (Figure 4). Interestingly, the genome uniquely exhibited eight genes associated with carbohydrate metabolism. Specifically, this pertains to one gene for a carbon storage regulator (*csrA*) and seven genes forming the carbohydrate utilization cluster Ydj, which encodes for a hypothetical aldolase (YdjI), uncharacterized sugar kinase (YdjH), hypothetical zinc-type alcohol dehydrogenase-like protein (YdjJ), putative oxidoreductase

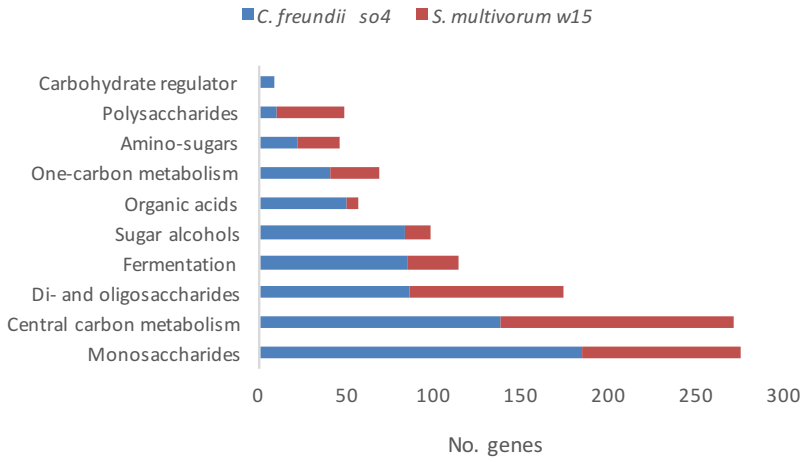

Figure 4 Number of genes encoding proteins of the carbohydrate subsystem in the genome of *C. freundii* so4 and *S. multivorum* w15.

(YdjL), putative transport protein (YdjK), hypothetical oxidoreductase (YdjG), putative HTH-type transcriptional regulator (YdjF). With respect to the genome of *S. multivorum* w15, it had 38 genes related to polysaccharide metabolism. It presented low numbers of genes involved in the metabolism of monosaccharides (90), fermentation (30), 1-carbon metabolism (28) and sugar alcohol and organic acid metabolism (14). With respect to the metabolism of di-saccharides (88) and amino sugars (24), the *S. multivorum* w15 investment in these metabolisms was similar to that of *C. freundii* so4 (Figure 4).

Analysis of lignocellulolytic potential in C. freundii so4 and S. multivorum w15

The genomes of both *C. freundii* so4 and *S. multivorum* w15 showed a plethora of genes predicted to encode proteins from several CAZy families, including those for GHs and CBMs. There were important differences between the two genomes in the total number of genes associated with lignocellulose degradation.

GH and CBM families. The genome of *C. freundii* so4 exhibited 137 predicted genes associated with CAZy family enzymes (Figure S1). Overall, it presented 61 genes coding for GHs and 12 for CBMs (Figure 5). Specifically, *C. freundii* so4 had genes for putative proteins from families CBM50 (chitin binding), CBM32 and CBM48 (binding pectin), and CBM34 (associated with starch attachment); the

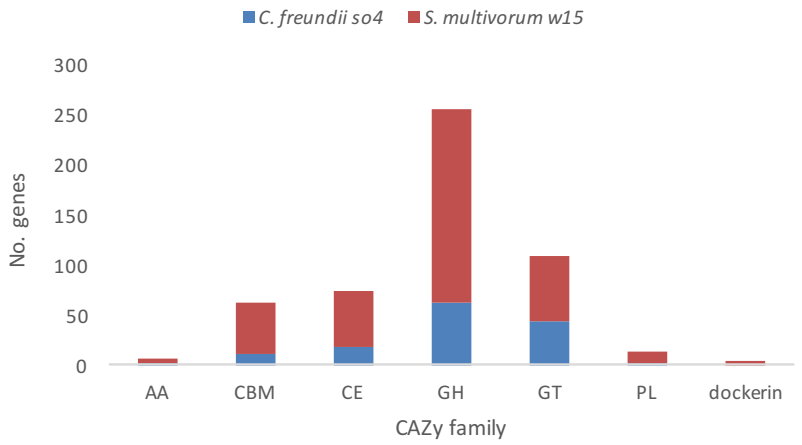

Figure 5 Predicted genes coding from CAZy families found in genome of *C. freundii* so4 and *S. multivorum* w15. *GH*, glycosyl hydrolases; *CBM*, carbohydrate binding modules; *AA*, auxiliary activity enzyme; *CE*, carbohydrate stereases; *GT*, glycosyltransferase; *PL*, polysaccharide lyases.

latter was exclusively presented in this strain (Figure 6).

The genome of *S. multivorum* w15 presented exhibited 386 predicted genes associated with CAZy family enzymes, most of which could be directly linked with lignocellulose degradation (Figure S1). Specifically, 193 genes encoding GHs and 50 encoding CBMs (Figure 5). Moreover, the genome of strain w15 had genes encoding proteins from 48 different GH families and from 16 different CBM families (Figure 6). Considering unique (CAZy and CBM) families, *S. multivorum* w15 had 62 types and *C. freundii* so4 only 20. When commonality was considered, we found the two strain's genomes to have 36 families in common (Figure 6).

Genes associated with hemicellulose degradation. The *C. freundii* so4 genome presented predicted genes encoding proteins from four families related to hemicellulose degradation, i.e. GH2, GH31, GH127 and GH43. These were also presented in the genome of *S. multivorum* w15 (Figure 6).

S. multivorum w15 – uniquely – exhibited predicted genes encoding proteins from seventeen GH families involved in the degradation of hemicellulose. These were: GH2, GH10, GH16, GH28, GH29, GH30, GH31, GH35, GH43, GH53, GH67, GH76, GH78, GH92, GH115, GH120 and GH127 (Figure 5). Where the most abundant were GH2 (19), GH29 (16), GH43 (22), GH92 (10). Moreover, we found two genes

encoding CBMs binding xylan: CBM9 and CBM13. The genome also harboured putative genes codifying CBMs capable to bind to cellulose, xylan, glucan and glucomannan, namely CBM4, CBM6, CBM16 and CBM44. Remarkably, CBM4 can bind to crystalline cellulose, a very recalcitrant part of the lignocellulose substrate (Figure 6). Surprisingly, *S. multivorum* w15 exhibited 22 predicted genes encoding proteins of family CBM32 and 3 of family CBM67 (both bind to pectin), as well as CBM48 (binding to: starch), CBM50 (chitin), CBM66 (fructan) and CBM61 (glycan).

Genes associated with cellulose degradation. *The C. freundii* so4 genome had only one putative gene encoding a protein from CAZy family GH5, which is associated with the degradation of crystalline cellulose (Figure 4). The strain also exhibited predicted genes encoding proteins from families GH3 (4) and GH1 (4). The latter were also presented in the genome of *S. multivorum* w15.

The genome of *S. multivorum* w15 uniquely presented predicted genes encoding enzymes of CAZy families GH51 and GH9 (associated with cellulose degradation). This in contrast to those for families GH1 and GH3 (6), which were also present in *C. freundii* so4. Moreover, *the S. multivorum* w15 genome exclusively revealed the presence of predicted genes encoding proteins from CBM8 and CBM30 families, which are associated with binding to cellulose.

***Genes for auxiliary enzymes*.** Predicted genes encoding enzymes from CAZy families CE1 (6), CE3 (1) and CE4 (3) were found in the genome of *C. freundii* so4 (Figure 5).

The genome of *S. multivorum* w15 presented predicted genes encoding enzymes from eight CE families and a high number of genes for enzymes of families CE1 (19), CE3(5) and CE4(6). Moreover, it uniquely had genes encoding proteins of families CE6, CE7, CE12, CE14 and CE15. Members of these protein groups have been associated with deacetylation of xylans and xylo-oligosaccharides. Also, family CE15 proteins may be responsible for breaking recalcitrant links between hemicellulose and lignin. The genome further had four genes encoding proteins of family AA3, which includes enzymatic activities of cellobiose dehydrogenase, dehydrogenase, glucose oxidoreductases, aryl-alcohol oxidase, alcohol (methanol) oxidase, and pyranose oxidoreductases. These enzymes support the action of glycoside hydrolases in lignocellulose degradation and protein structural analysis indicated that such enzymes could degrade and modify cellulose, hemicellulose and even lignin (Sützl et al. 2018). Moreover, one gene encoded a GH110 family enzyme (Figure 6); this family includes polysaccharide

depolymerases, that hydrolyse galactosyl-alpha-1,3-D-galactose linkages that are typically presented in complex substrate.

Genes for other CAZy family proteins. The genome of *C. freundii* so4 presented uniquely predicted genes encoding enzymes from CAZy families associated with starch degradation, i.e. GH4, GH37 and GH63. The genome also exhibited genes for family GH77 and GH88 enzymes, which was shared with the w15 genome (Figure 5). *S. multivorum* w15 exhibited the presence of a gene encoding a family GH110 protein (a polysaccharide depolymerase); genes encoding proteins of families associated with glycan degradation, i.e. GH18, GH20, GH89, GH116 and

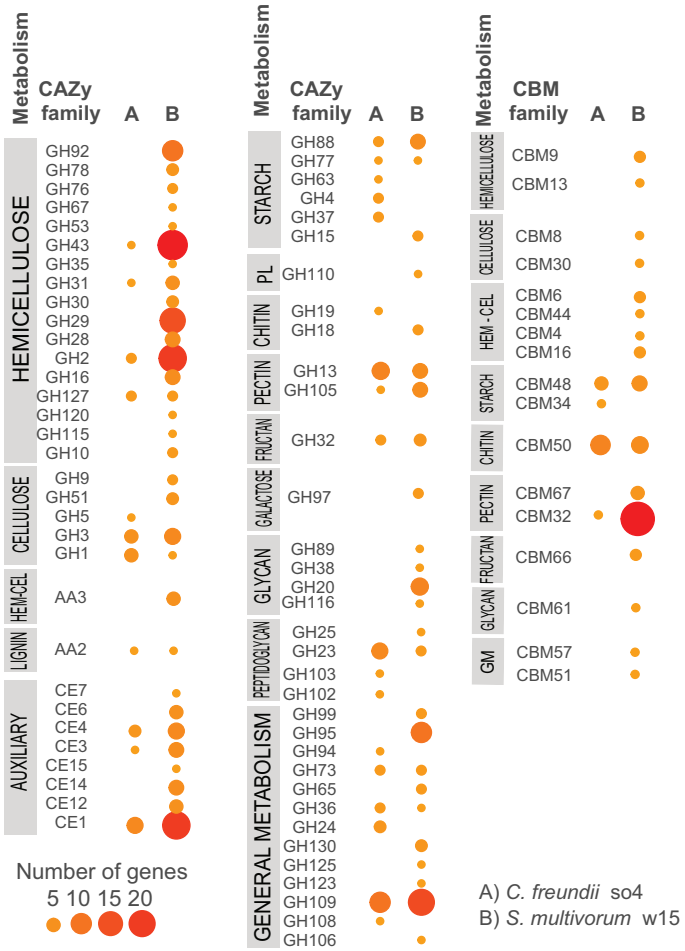

Figure 6 Predicted genes encoding proteins of different CAZy families in *C. freundi*i so4 and *S. multivorum* w15. *GH*, Glycosyl hydrolases; *CBM*, carbohydrate binding modules; *AA*, auxiliary activity; *CE*, carbohydrate esterases. Size and color of circles indicate number of genes.

GH38 as well as 17 genes from family GH109, which only presented the α-*N*-acetylgalactosaminidase (CAZypedia Consortium 2017) (Figure 6).

Overall, the genome of *S. multivorum* w15 appeared to enable the host organism to grow and survive by consuming complex carbohydrates, particularly hemicellulose related-structures. In contrast, the genome of *C. freundii* so4 revealed evidence for the tenet that it enables its host to survive by transforming and consuming simpler carbon sources.

In both genomes, we found genes from family AA2 peroxidase, at one copy each, which are involved in lignin degradation. Members of lytic cellulose mono oxygenases (AA10 family) were not found in any of the two genomes. Moreover, genes encoding proteins from CAZy families involved in the degradation of pectin, i.e. GH13, GH105 and CBM32, were found. However, only *S. multivorum* w15 was able to grow on pectin as the single carbon source. CAZy family GH13 is composed of enzymes that degrade the oligosaccharides stachyose and raffinose, present in a wide variety of plants (CAZypedia Consortium 2017), however, *C. freundii* so4 was not capable to grow on stachyose, and only grew on raffinose as a single carbon source.

Discussion

C. freundii so4 and *S. multivorum* form part of a core set of bacteria that are highly abundant in LCB degrader consortia, indicating their key role in lignocellulose degradation (Jiménez et al. 2014a; Brossi de Lima et al. 2015; Cortes-Tolalpa et al. 2016). Both strains can grow singly on wheat straw as the sole carbon source. However, when they are growing together on this substrate, they presented a synergistic relationship (Cortes-Tolalpa et al. 2017). Clearly, knowledge of their genomic features will advance our knowledge with respect to the mechanisms behind this synergism. The findings of this study clearly showed that metabolic differences and diverse polysaccharide degradation armoury lie at the basis of their cooperation. When growing together on LCB, these organisms may combine their degrader metabolic capacities which may allow them to consume the substrate in a more efficient way than either one of them alone. Hereunder, we explored the differences found in the metabolic palette of the two strains.

Proposed complementary roles of C. freundii so4 and S. multivorum w15 in wheat straw degradation

Our analyses indicated that differences in catabolism between *S. multivorum* w15 and *C. freundii* so4 growing on LCB, may lead to avoidance of competition for the same nutritional source (Figure 3, Table 3). Overall, the genomic data and the carbon consumption profiles indicated that *C. freundii* so4 has a metabolism more tuned to the transformation of simple carbon sources such as amino acids and metabolic intermediates of glycolysis and the TCA using these pathways for generation of energy. Moreover, a capacity of mixed acid fermentation was presented, which allows *C. freundii* so4 to grow in limiting oxygen concentration. In contrast, *S. multivorum* w15 (which is a strict aerobic organism), presented a strong preference for the utilization of more complex carbohydrates such as derivatives of dextrin. The organism probably makes more use of the pentose interconversion pathway, as it appears to have considerably diminished its investment in the pyruvate, ascorbate and propionate pathways.

With respect to lignocellulose degradation, *C. freundii* so4 prefered the consumption of (intermediary) sugars, products of cellulose hydrolysis and disaccharides with beta-glycosidic bonds, such as cellobiose (glucose β (1⟶ 4) glucose) or lactose (β-D-galactosepyranosyl-D-glucopyranose). In contrast, *S. multivorum* w15 showed a facility for the utilization of carbohydrate with a α bonds such as (glucose α(1⟶ 4) glucose), melibiose (D-gal-α 1 ⟶ 6 D-glucose) and γ-cyclodextrin.

Another example of how the strains may complement each other with respect to their metabolism is the following: *C. freundii* so4 is highly versatile in its capacity to spatially explore a substrate like WS, as it can swim to look for locally available resources, whereas *S. multivorum* w15 cannot. This forces the latter organism to produce the plethora of extracellular enzymes that are directly and locally required for digestion of unavailable substrate and acquiring the resulting smaller molecules. It is possible that *C. freundii* so4 – given its ability to move, can reach sites where nutrients become available, taking these up.

Roles of the strains in the system

Cooperation based on metabolic exchange occurs when a species uses metabolites produced by another species as sources of energy or building blocks for cell structures (Cavaliere et al. 2017). It is also known by the term cross-feeding. A key example is given by one strain degrading a primary energy source and producing a

compound (which could also be a by-product) that is then used by a second strain (Helling, Adams et al, 1980-ies; Germerodt et al. 2016). We propose that the two strains studied here, *C. freundii* so4 and *S. multivorum* w15, exhibited a cooperative cross-feeding interaction. While *S. multivorum* w15 worked in degradation of hemicellulose structures of substrate, *C. freundii* so4 had other functions in the system as transforming oligo-intermediaries, their consumption allowed the strain to grow an eventually produce secondary metabolites the *S. multivorum* w15 could use; as well as, *C. freundii* so4 could contribute in the detoxification of the culture. Three hypotheses might explain the positive relationship between the two strains**:** 1) complementary degradation capacity, 2) production and excretion of secondary metabolites and 3) stress response based mutualism. Hereunder, we briefly address these three scenario's.

Complementary degradation capacity

In the light of the very diverse composition and complex structure of wheat straw, no single organism can produce all enzymes required for its complete degradation including activities of hydrolyses, debranching and auxiliary. Given the fact that the capacities to produce and secrete such enzymes are presented across different members of degradative consortia, multiple species from the latter are required.

Thus, whereas each strain can efficiently hydrolyse different parts of the substrate, their combination is required. We posit here that *S. multivorum* w15 serves as the *primary* degrader, contributing with the production and releasing of a large variety of hydrolytic enzymes. Clearly, the organism invests large parts of its genome to degradation of xylan and xylose, which are main components of the hemicellulose part of the substrate. As evidenced on the basis of the genome analyses, strain w15 may use mainly proteins of CAZy families GH29 and GH43. The family GH29 proteins may be exo-acting α-fucosidases, which participate in glycan degradation (CAZypedia Consortium 2017). While, the main activities reported for CAZy family GH43 enzymes are α-L-arabinofuranosidases, endo-α-L-arabinanases, β-D-xylosidases and galactosidases. A significant number of enzymes in this family show both α-L-arabinofuranosidases and β-D-xylosidases activity, using aryl-glycosides as substrates (CAZypedia Consortium 2017). Moreover, family GH43 enzymes are also implicated in the degradation of arabinoxylan, the most abundant hemicellulose component of wheat straw (Abot et al. 2016) (Mewis et al. 2016). On another notice, *S. multivorum* w15 may also

employ enzymes from CAZy family GH2, which encompasses β-galactosidases, β-glucuronidases, β-mannosidases and exo-β-glucosaminidases. The finding of genes for CBM32-type proteins may indicate a capacity for uptake of monosaccharides and short oligosaccharides (CAZypedia Consortium 2017). Furthermore, the finding of genes for carbohydrate esterease family 1 (CE1) proteins was revealing, as CE1 is one the biggest and most diverse CE families. Family CE1 include the enzymes acetyl xylan esterases, feruloyl esterases, and carboxyl esterases that carry out the deacetylation of xylan and oligosaccharides. This could accelerate the degradation of polysaccharides facilitating the access of glycoside hydrolases to the substrate (Nakamura et al. 2017).

On the other hand, most available evidence points to a role for *C. freundii* so4 as a *consumer* of carbonaceous molecules, transforming smaller substrate fragments, that are produced by the action of *S. multivorum* w15, into even simpler ones. *C. freundii* so4 may be contributing with extracellular cellobiohydrolases that transform cellobiose into glucose monomers, which both strains can easily consume. *C. freundii* so4 also may provide lytic enzymes (GH1 family) that are different from those of *S. multivorum* w15 (from glycoside families GH5, GH3 and GH1). The most common enzymes in family GH1 are β-glucosidases and β-galactosidases, next to β-mannosidases, β-D-fucosidases and β-glucuronidases (CAZypedia Consortium 2017) as well as family GH13, which is the major glycoside hydrolase family acting on substrates containing α-glucoside linkages. GH13 contains hydrolases, transglycosidases and isomerases activities (CAZypedia Consortium 2017).

In cross feeding interactions is constantly observed that intermediary byproducts of the degradations inhibited the processing of degradation (Harvey et al. 2014). An intriguing hypothesis is that *C. freundii* so4 may be contributing to the system by reducing the limitation of hydrolytic enzyme activities, by processing metabolites intermediaries of cellulose degradation as cellobiose, as such activities may be subjected to inhibition by accumulation of final product. Thus, by reduction of (sugar) products of *S. multivorum* lytic activity, *C. freundii* so4 may promote the activity of such enzymes in the biculture.

Production and excretion of metabolites

Additional to complementary role in the degradation process, *C. freundii* so4 may be having an important contribution to the degradative system by producing and

excreting secondary metabolites that *S. multivorum* w15 can use, but not produce, for example amino acid and derivatives. Such metabolites that (temporarily) cannot be transformed, may be required to be transported out of the cell.

Stress response modulation

The catabolism of WS by the two strains may produce metabolites intermediary that accumulate in the cell and then are expelled to the culture medium than may be toxic to microbial cell, which can reduce growth rate and enzyme productions e.g. phenolic compounds, aldehydes and furan derivatives (Malherbe and Cloete 2002; Ling et al. 2014). Our genomic analyses, in particular the finding of 1) regulon oxidative stress response regulators SoxS and SoxR, 2) the genes of glutathione metabolism and glutathione transcriptional regulator of formaldehyde detoxification operon (FrmR), 3) nitrosative stress, specifically the fumarate and nitrate reduction regulatory protein and 4) the very diverse oxidoreductases can detoxify xenobiotics, such as phenolic and can efficiently oxidize inorganic compounds using oxygen as the final electron acceptor (Karigar and Rao 2011), in *C. freundii* so4 (but not in *S. multivorum* w15) are supportive of the tenet that strain *so4* is helping in the detoxification of the system and oxidative stress, as levels of accumulated waste compounds are reduced in the culture.

The differences in the metabolism between *C. freundii* so4 and *S. multivorum* w15 make these two organisms complementary in WS degradation, giving them different roles in the system. Whereas *S. multivorum* w15 may have the main role in degradation, in particular releasing hemicellulose hydrolytic enzymes, *C. freundii* so4 may be contributing with detoxification of the system, transforming sub-products of the degradation and providing intermediate metabolites that *S. multivorum* w15 cannot synthesize. In this way, both strains benefit from the joint activities, yielding improved growth on a very recalcitrant carbon source.

Perspectives

Our study provides a starting point for an improved understanding of cooperative degrader consortia. We also identified target genes, e.g. from families GH2, GH29, GH43, GH109, CBM32, CE3, CE4, CE14 and CE15 for further analyses. Given the fact that genomics studies are limited to assessments of the presence or absence of genes, it is indispensable to perform transcription analyses in future studies. Thus, in order to reveal the mechanism behind the cooperation of the synergistic degrader strains in our synthetic consortium, we propose that expression analysis of the system is performed, comparing the global expression patterns of the

monocultures against that of the co-culture growing on raw wheat straw. Based on our analyses, we suggest focussing on the expression of CBM32 and genes encoding enzymes from family GH43. Expression and synthesis of proteins of the latter family have been detected uniquely when strains or consortia were grow on wheat straw, xylan and xylose (Jiménez et al. 2015; López-Mondéjar et al. 2016).

Future studies may also address the effect of the structure of the compound on the cooperative relationship between the strains, as we previously found that more recalcitrant structures have key effects on synergistic behaviour (Cortes-Tolalpa et al. 2017). Clearly, enzymes involved in attack on recalcitrant regions in lignocellulose need to be studied, such as members of CAZy families CE3, CE4, CE14 and CE15. Only few studies have addressed this family of enzymes in bacteria, despite the fact that many bacterial species have genes encoding homologues of fungal enzymes (De Santi et al. 2016).

At the metabolic level, it is necessary to confirm the participation of *C. freundii* so4 in the system by verification of expression of metabolic pathways for the synthesis of metabolites and elimination of toxic compounds. The knowledge generated from transcriptomic analysis can be used for activating the expression and modulating the synthesis of enzyme cocktails that include hydrolytic, debranching and auxiliary enzymes for lignocellulose treatment.

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Supplementary Tables and Figures

Table S1. Selective compounds consumed by *C. freundii* so4.

Compound	ID KEGG	Carbon source	Type
Putrescine	C00134	Amine	Ester
L-Serine	C00065	Amino acid	Amino acid
D-Serine	C00740	Amino acid	Amino acid
Hydroxy-L-Proline	C01015	Amino acid	Amino acid
L-Alanyl-glycine		Amino acid	Amino acid
L-Proline	C00148	Amino acid	Amino acid
L-Histidine	C00135	Amino acid	Amino acid
L-Alanine	C00041	Amino acid	Amino acid
L-Aspartic acid	C00049	Amino acid	Amino acid
D-Glucosamine	C00329	Carbohydrate	Amino sugar
Dihydroxyacetone	C00184	Carbohydrate	Ketoses
Glycerol	C00116	Carbohydrate	Sugar alcohol
D-Sorbitol	C00794	Carbohydrate	Sugar alcohol
D-Mannitol	C00392	Carbohydrate	Sugar alcohol
m-Inositol	C00137	Carbohydrate	Sugar alcohol
D-Arabinose	C00216	Carbohydrate	Monosaccharide
Glucose-6-Phosphate	C00092	Carbohydrate	Monosaccharide
L-Fucose	C01019	Carbohydrate	Deoxy sugar
Succinic acid	C00042	Carboxylic acid	Carboxylic acid
5-Keto-D-Gluconic acid	C01062	Carboxylic acid	Carboxylic acid
D-Glucuronic acid	C00191	Carboxylic acid	Carboxylic acid
D,L-Lactic acid	C01432(L)	Carboxylic acid	Carboxylic acid
D-Galacturonic acid	C00333	Carboxylic acid	Acid sugar
D-Gluconic acid	C00257	Carboxylic acid	Acid sugar
D-Saccharic acid	C00818	Carboxylic acid	Acid sugar
Methylpyruvate		Ester	Ester
D-Galactonic acid lactone	C03383	Ester	Ester
Inosine	C00294	Nucleic acid	Nucleoside
Thymidine	C00214	Nucleic acid	Nucleoside

Table S2. Selective compounds consumed by *S. multivorum* w15

Compound	ID KEGG	Carbon source	Type
Glucuronamide	D01791	Amide	Amide
D-Melezitose	C08243	Carbohydrate	Trisaccharide
Stachyose	C01613	Carbohydrate	Tetrasaccharide
Salicin	C01451	Carbohydrate	Monosaccharide
Lactulose	C07064	Carbohydrate	Disaccharide
Palatinose	C01742	Carbohydrate	Disaccharide
Sucrose	C00089	Carbohydrate	Disaccharide
Turanose	G03588/C19636	Carbohydrate	Disaccharide
Gentibiose	C08240	Carbohydrate	Disaccharide
α-Methyl-D-Glucoside		Carbohydrate	Derived sugar
α-Methyl-D-Mannoside		Carbohydrate	Derived sugar
Maltitol	G00275	Carbohydrate	Disaccharide
Arbutin	C06186	Carbohydrate	Derived sugar
β-Hydroxybutyric acid	C01089	Carboxylic acid	Carboxylic acid
Inulin	G04981	Polymer	Polysaccharide
Pectin	C00714/ G10591	Polymer	Polysaccharide
Dextrin	C00721	Polymer	Oligosaccharide
α-Cyclodextrin	C00973	Polymer	Oligosaccharide
β-Cyclodextrin	C13183	Polymer	Oligosaccharide
γ-Cyclodextrin	C13183	Polymer	Oligosaccharide

Compounds highlighted are related with lignocellulose degradation.

Table S3. Compound consumed by both strains, *S. multivorum* w15 and *C. freundii* so4.

Compound	ID KEGG	Carbon source	Type
2-aminoethanol	C00189	Alcohol	Organic acid
D-Alanine	C00133	Amino acid	Amino acid
D-Raffinose	C00492	Carbohydrate	Trisaccharide
Glucose-1-phosphate	C00103	Carbohydrate	Monosaccharide
β-Methyl-D-Galactoside	C03619	Carbohydrate	Monosaccharide
α-D-Glucose	C00267	Carbohydrate	Monosaccharide
D-Fructose	C00095	Carbohydrate	Monosaccharide
N-Acetyl-D-Glucosamine	C00140	Carbohydrate	Monosaccharide
D-Mannose	C00159	Carbohydrate	Monosaccharide
D-Galactose	C00124	Carbohydrate	Monosaccharide
L-Arabinose	C00259	Carbohydrate	Monosaccharide
β-Methyl-D-Glucose		Carbohydrate	Derived sugar
Maltose	C00208	Carbohydrate	Disaccharide
D-Melibiose	C05402	Carbohydrate	Disaccharide
α-D-Lactose	C00984	Carbohydrate	Disaccharide
D-Trehalose	C01083	Carbohydrate	Disaccharide
D-Cellobiose	C00185	Carbohydrate	Disaccharide
L-Rhamnose	C00507	Carbohydrate	Deoxy sugar
N-Acetyl-D-Galactosamine	C01132	Carbohydrate	Amino sugar
N-Acetyl-Neuraminic acid	C00270	Carbohydrate	Amino sugar
Uridine	C00299	Nucleic acid	Nucleoside
Laminarin	C00771	Polymer	Polysaccharide

Compounds highlighted are related with lignocellulose degradation.

Table S4. Number of genes in the functional subsystems according to RAST assignation.

Function subsystems	*C. freundii* so4	*S. multivorum* w15
Carbohydrates (total)	706	451
CO_2 fixation	0	0
Respiration	188	100
Sulfur metabolism	65	40
Phosphorus metabolism	50	43
Potassium metabolism	33	14
Photosynthesis	0	0
Fatty acids, lipids and isoprenoids	166	132
Phages, prophages, transposable elements, plasmids	53	26
Nucleosides and nucleotides	104	86
DNA Metabolism	114	104
RNA Metabolism	248	129
Cell division and cell cycle	38	31
Amino Acids and Derivatives	438	364
Metabolism of Aromatic Compounds	12	13
Secondary Metabolism	24	8
Protein Metabolism	295	250
Nitrogen Metabolism	62	12
Miscellaneous	57	36
Cofactors, vitamins, prosthetic groups, pigments	314	222
Cell wall and capsule	236	125
Membrane transport	187	134
Iron acquisition and metabolism	65	15
Virulence, disease and defense	110	132
Stress response	175	101
Dormancy and sporulation	3	4
Regulation and cell signaling	152	61
Motility and chemotaxis	143	0

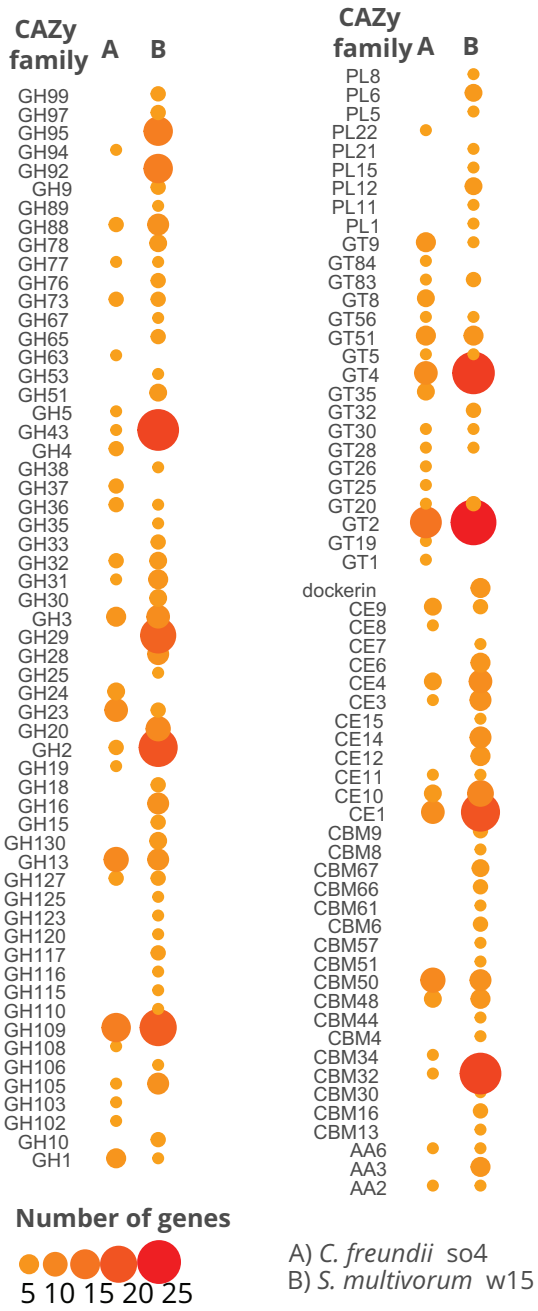

Figure S1 Total predicted genes belonged to CAZy families in **(A)** *C. freundii* **so4** and **(B)** *S. multivorum* **w15**. Size and color of circles indicates number of genes. ***GH***, Glycosyl hydrolases; ***CBM***, carbohydrate binding modules, ***AA***, auxiliary activity enzyme; ***PL***, polysaccharide lyase; ***GT***, glycosyltransferase; and ***CE***, carbohydrate estereases.

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 infered 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 β-xylosidases activities, whereas wood-derived ones exhibited higher glucosidase activities. Both had similar β-cellobiohydrolase, β-galactosidase and β-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.

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

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

*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 (α-, β- and γ 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 posses 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 β-glucosidases and β-galactosidases, and those in GH13 (the major GH family acting on substrates containing α-glucoside linkages)

were hydrolases, transglycosidases and isomerases (CAZypedia 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 synthetizing and exporting extra (hemi)cellulases in the culture that transform the intermediaries into monomers. The complementary in the enzymatic activity of *C. freundi* 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).

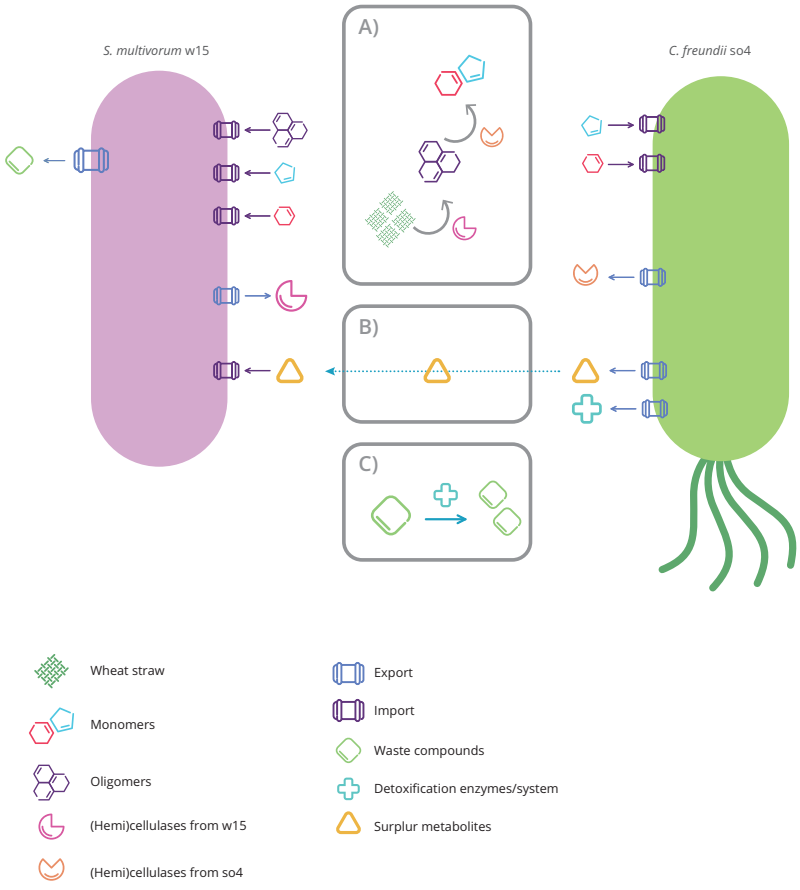

Figure 1 Proposal model of microbial interaction. The description of the model is in the text.

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 more 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 posible 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 detoxificating 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 exclusivelly 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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Supplementary Chapter

Physiological characterization of C. freundii s04 and S. multivorum w15

Larisa Cortés Tolalpa

Introduction

The genus *Citrobacter* was for first time assigned by Werkman and Gillen (1932). This is a polyphyletic genus that belong to the phylum *Proteobacteria*. They have been recovered from very different environment such as water, sewage and soil (Octavia and Lan 2014). By other hand, the genus *Sphingobacterium* belongs to the phylum *Bacteroidetes*, this phylum was created by Eiko Yabuuchi et al. (1983). *Sphingobacterium* group shows a particular biochemical profile, where the cell wall contains high quantities of sphingophospholipids. Currently, the *Sphingobacterium* genus includes up to 22 species. They have been isolated from several habitats such as diverse soil and compost (Lambiase 2014). *S. multivorum* comes from *multus*, many; and *-vorum*, devouring; *multivorum*, intended to mean "produces acid from many carbohydrates" (Taylor et al. 2012).

Materiales and methods

Physiological characterization

Staining Gram, transmission electron microscopy, motility assay, determination of optimal rage of temperature, pH and salinity

Staining Gram

The gram stained slides were prepared by taking a single colony and a drop of saline solution, the mix was spread on the slide and fixed with the flame. Gram Staining Kit (Sigma-Aldrich, Darmstadt, Germany) was used for the staining, the conventional method was applied, first the application and incubation of the solution and then rinsing with water the order was the following: 1) oxalate crystal violet solution, incubated 1 min; 2) iodine solution, incubated 1 min; 3) decolorizer solution (alcohol/acetona) 20s and 4) safranin solution, incubated 1 min. The slides were observed in an optical microscope 40x and the pictures were taken using 100x objective using immersion oil.

Transmission electron microscopy

Bacterial cells were incubated overnight in liquid media at 28 °C at 180 rpm and then 1 μL of the culture was deposited onto carbon copper grid and negatively stained with 2% (w/v) uranyl acetate for 1 min. Cell were observed under a Phillips CM120 electron microscopy (FEI Tecnai G2 Polara, Oregon, USA).

Phenotypic test

For identification and confirmation of the two gram negative strains, *C. freundii* so4 and *S. multivorum* w15, was used the GN2 MicroPlate (Gram negative identification). GN2 performs 95 discrete tests simultaneously and provides the "metabolic fingerprint". The principle of the BIOLOG MicroPlate test is based on reduction of the redox dye tetrazolium violet by metabolically-active bacterial cells. First, single colonies, of each strain, were chosen from the, sub-cultured on TSA plates, and incubated overnight at 28°C. A homogenous suspension of inoculum was made with GN-GP inoculation fluid (72101) and diluted to 0.001 OD at 590 nm. The inoculum was starved for 2 h at room temperature and then 150 µL of the suspension were added into each well of the GN2 MicroPlate. The microplates were incubated at 28°C and read at 0, 6, 24, 48, 72 and 168 hours with a microtiter plate reader at 590nm (Miller and Rhoden 1991; Holmes et al. 1994).

Motility assay

It was assessed by growing the bacteria strains on Motility Test Medium (10g/L pancreatin digest of casein, 3g/L NaCl, 4 g/L meat extract and 4 g/L agar) with triphenyltetrazolium chloride (TCC: 0.5 g/L, Sigma-Aldrich, Darmstadt, Germany). Tetrazolium salt is colourless; as the microorganism grows the dye is reduced to an insoluble red pigment. Motile organisms produce a pink colour that diffuses from the stab line. Organisms that are non-motile produce a red pigment that is confined to the stab line (Kelly and Fulton 1953).

Determination of optimal rage of temperature, pH and salinity

Optimal temperature of growth of *C. freundii so4* and *S. multivorum* w15 was detected by growing the strains on Lennox media (Sigma-Aldrich, Darmstadt, Germany) and monitoring the growth at different temperature (4, 20, 30, 50 and 80 °C). The pH range was verified by growing the bacteria strains on Lennox medium at different pH (3, 4, 5, 7, 9 10), while salt tolerance was tested by growing bacteria strains on Lennox medium at 0, 0.25, 0.5, 1, 4.5, 5, 10 and 20% of NaCl (Sigma-Aldrich, Darmstadt, Germany).

Results of physiological characterization of C. freundii so4

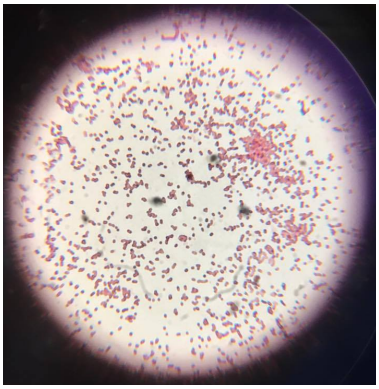
C. freundii so4 presented gram negative staining and rod shape (Figure S1). The pictures taken of *C. freundii* so4 by electron microscope exhibited the presence of flagella; the results in the soft agar an extended range oxidation of TCC (indicating the displacement in the agar) confirmed the motility capacity of the strain (Figure S2). Strain so4 exhibited to be mesophilic, because it was able to grow in the range temperature from 20°C to 40°C and it did not present growth at 45°C, 60°C and 80°C (Figure S3), while the optimal temperature of growth was around 30°C. In the case of pH tolerance, *C. freundii* so4 was able to grow between a pH of four and nine, being the optimal pH at seven (Figure S4). In the salt tolerance resistance, strains so4 was able to grow from 0 to 1% of NaCl (Figure S5).

Results of physiological characterization of S. multivorum w15

S. multivorum w15 presented gram-negative with a bacilli shape (Figure S1). The images of *S. multivorum w15* by electron microscope showed that the strains tended to stay aggregated, even growing in liquid medium. In soft agar presented no motility and oxidation of TCC was mainly done in the surface of the inoculation point (Figure S2). This strain showed mesophilic range of temperature, it was able to grow from 20°C to 30°C, being the optimal at 28 °C (Figure S3), while it did not present growth at 40°C, 45°C, 60°C and 80°C. In the case of pH tolerance, *C. freundii* so4 was able to grow between a pH of five and nine, being the optimal pH at seven (Figure S4). This strain could tolerate until 1% of NaCl (Figure S5).

Figures

A) *C. freundii* so4



B) *S. multivorum* w15

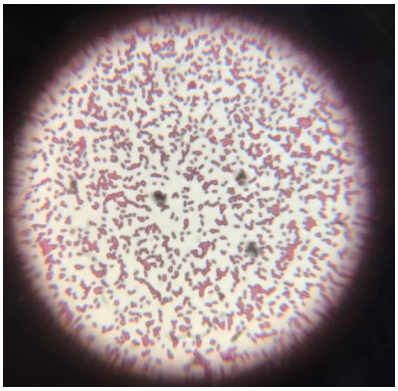
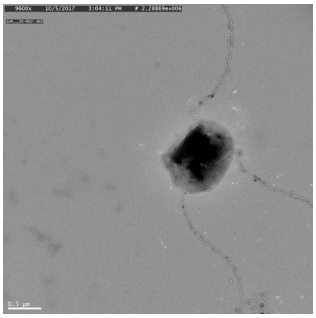
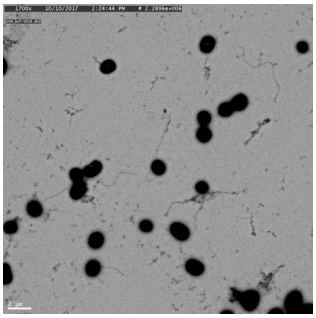


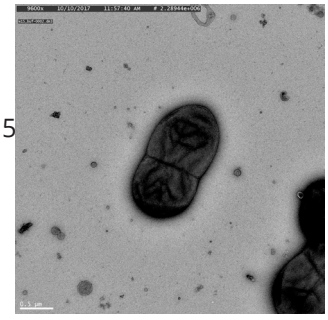
Figure S1 Tincion Gram.

(A) *C. freundii* so4





(B) *S. multivorum* w15



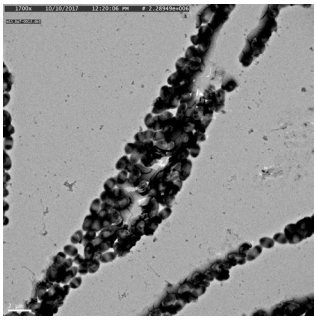


Figure S1 Electron, micrograph, of degrader bacteria strains in **A)** *C. freundii* **so4**, where is observed the production of flagella and **B)** *S. multivorum* **w15**, which did not present flagella. The picture were taken with FEI, Tecnai, G2, Polara, electronic, microscope.

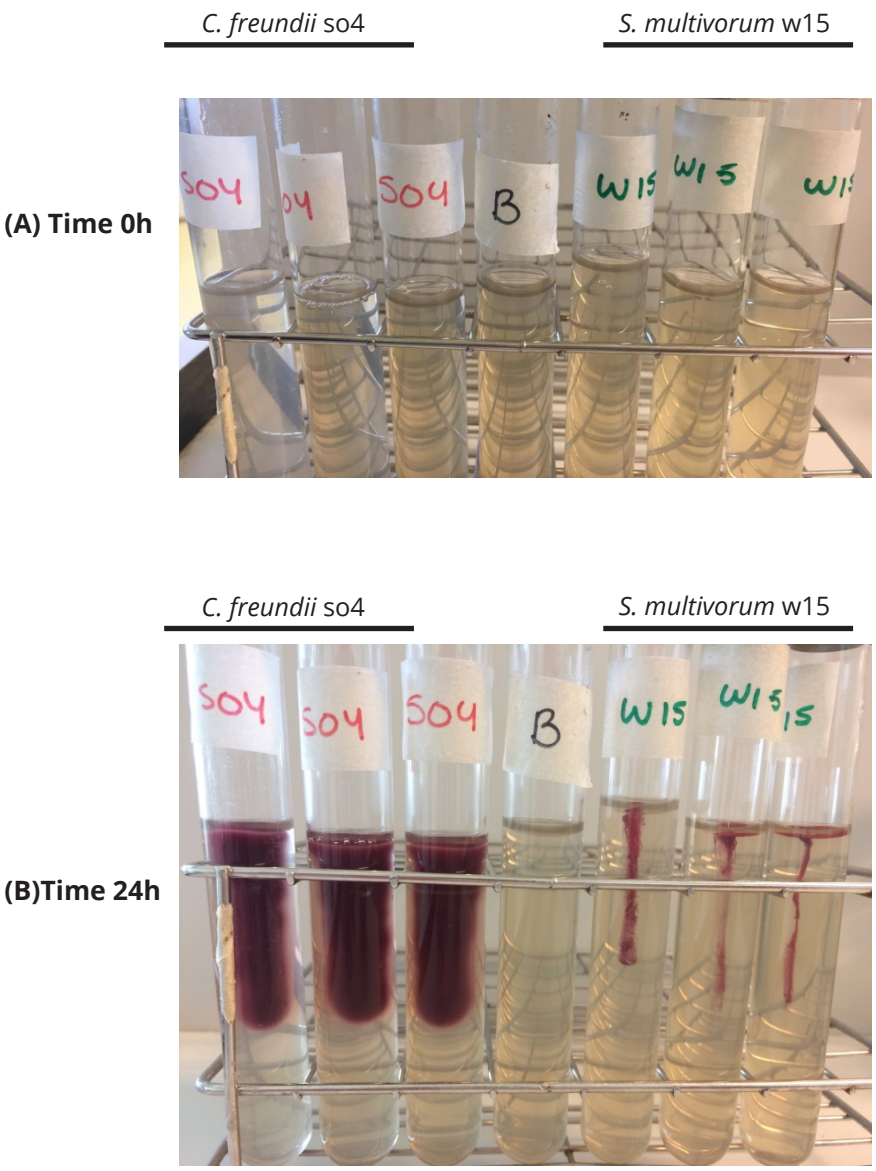


Figure S2 Motility assay. (A) Image shows inoculation point time; **(B)** Bacterial strains were incubated aerobically for 24 hours at 28ºC. ***S. multivorum*** **w15** did not exhibited motility capacity, while ***C. freundii*** **so4** presented motility determined by the red coloration due to the oxidation of the triphenyltetrazolium chloride (TCC: 0.5 g/L).

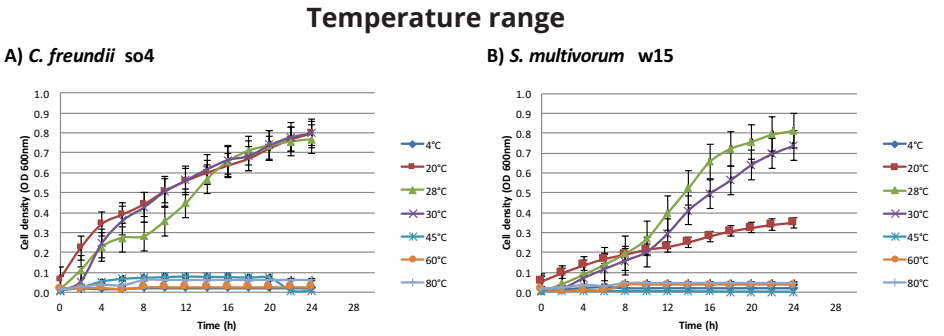


Figure S3 Growth response of **(A)** *C. freundii* **so4** and **(B)** *S. multivorum* **w15** at temperature range between 4 to 80°C.

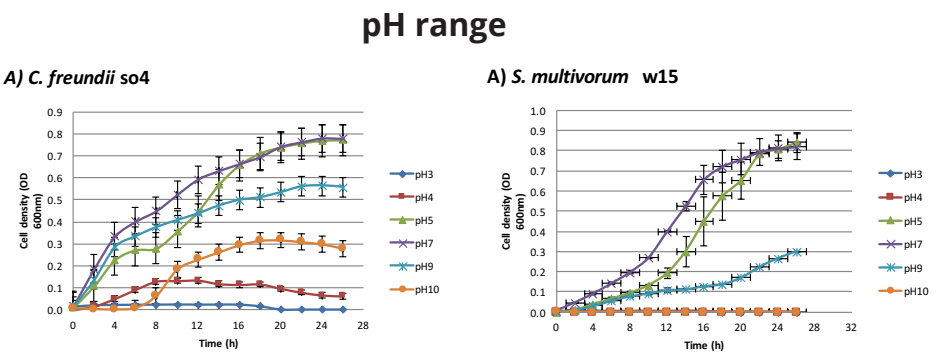


Figure S4 Growth response of **(A)** *C. freundii* **so4** and **(B)** *S. multivorum* **w15** at pH range between pH 3 and pH 10.

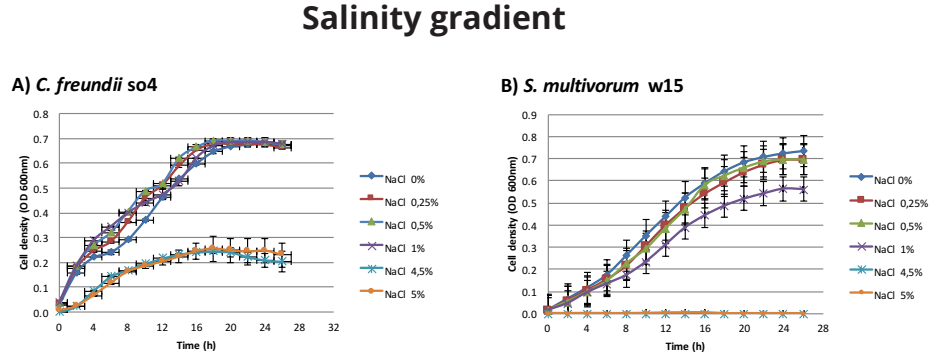


Figure S5 Growth response of **(A)** *C. freundii* so4 and **(B)** *S. multivorum* **w15** at salinity range between 0 and 5% of NaCl.

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Samenvatting

De uitputting van van olie afgeleide energie en de opwarming van de aarde openen de deur naar nieuwe ecologische manieren om energie en producten te verkrijgen. Lignocellulosesubstraten (LCB), zoals tarwestro (WS1), schakelgras (SG), maïs (CS) en suikerriet, vertegenwoordigen uitstekende en goedkope bronnen van koolstof die in waardevolle (energie) verbindingen kunnen worden omgezet. Niettemin is hun toepassing op grote schaal nog steeds beperkt.

Dit proefschrift opent met een inleiding, waarin ik een overzicht geef van de complexiteit van de structuur en samenstelling van LCB en de behoefte aan zeer divers arsenaal aan enzymen dat nodig is voor volledige afbraak. Dit arsenaal zal niet alleen cellulases, hemicellulases en ligninases bevatten, maar ook hulpenzymen. Dit in een poging om de factoren die van invloed zijn op de LCB bioconversie en de grotere uitdagingen te begrijpen, alsmede de toepassing van LCB op industriële schaal. Daarnaast beschrijf ik het mogelijke gebruik van microbiële consortia, in een eco-biotechnologische benadering, voor de afbraak van het inherent recalcitrante LCB. Tenslotte beschrijf ik de relevantie van de interacties binnen geselecteerde afbraakconsortia. Microbiële interacties sturen de stabiliteit en functionaliteit van microbiële gemeenschappen. Het is fundamenteel om grip te hebben op de interacties in de geselecteerde microbiële consortia teneinde een beter ontwerp van LCB-degrader consortia en hun toepassing in de industrie mogelijk te maken.

Vervolgens wordt in hoofdstuk 2 het effect onderzocht van het gebruik van WS1, SG, CS, en tarwestro bij pH 9,0 (WS2), met bosbodem als enig inoculum, voor de selectie van LCB-afbrekende microbiële consortia in sequentiele batchcultures. De uiteindelijke consortia werden fylogenetisch en functioneel onderzocht. PCR-DGGE analyse gaf aan dat de bacteriegemeenschappen stabiliteit bereikten na overdracht 6 in WS1, SG en CS en na overdracht 4 in WS2. Voor schimmelgemeenschappen werd stabiliteit gevonden na overdracht 6 in WS1 en SG en na overdracht 4 voor WS2 en CS. Substraattype, naast pH, was een sleuteldriver - voor de behandelingen met WS – van de bacteriële gemeenschapsstructuren. We vonden een “kern” van stammen in de laatste vier microbiële consortia gevormd door *Sphingobacterium* kitahiroshimense, *Enterobacter amnigenus*, *Raoultella terrigena*, *Pseudomonas putida* en *Stenotrophomonas rhizophila*, evenals de schimmels Coniochaeta ligniaria en *Acremonium* sp. Alle stammen in de kern

vertoonden CMC-ase- en xylanase-activiteiten. Het LCB-degradatiepotentieel van de geselecteerde consortia werd bepaald met Fourier-transform infrared-spectroscopie (FT-IR). De resultaten lieten zien dat substraattype de uiteindelijke samenstelling van de consortia bepaalt; diverse LCB-substraten veroorzaakten een combinatie van consortia, zelfs van een gewone inoculumbron.

In hoofdstuk 3 onderzocht ik het belang van de inoculumbron bij de selectie van microbiële afbraakconsortia door drie verschillende inocula uit bosbodem, kanaalbezinksel en rottend hout en het LCB-tarwestro als enige koolstofbron toe te passen bij de selectie van LCB-afbrekende microbieel consortia. De structuren van de bacteriële en schimmelgemeenschappen van de uiteindelijke geselecteerde consortia vertoonden een duidelijke clustering langs de inoculumbron en significante verschillen tussen de consortia werden gevonden.

Van hout afkomstige consortia bereikten als eerste stabiliteit, gevolgd door van de bodem afgeleide organismen, waarvan sediment-consortia nooit een stabilisatie van meer dan 50% bereikte. Nauwkeuriger karakterisering van de uiteindelijke samenstelling van de bacteriële gemeenschap, door 16S rRNA gen amplicon sequencing, toonde aan dat de meest voorkomende leden van de gemeenschap een bacteriële kern vormden, gemeenschappelijk tussen de drie uiteindelijke consortia. Deze kern werd gevormd door de soorten *Sphingobacterium*, *Citrobacter*, *Acinetobacter* en *Flavobacterium* of *Chryseobacterium*. Ook schimmelstammen waren specifiek van het consortium en (hoofdstuk 2) *Coniochaeta ligniaria* en *Acremonium* sp. werden geidentificeerd als sleutelorganismen hierin. Al deze organismen vertoonden hoge LCB transformerende activiteiten. De uiteindelijke consortia verbruikten hemicellulose-, cellulose- en ligninecomponentsubstraat op in grote lijnen vergelijkbare manier. Niettemin presenteerde elk van hen een uniek enzymatisch profiel. Samenvattend lieten de gegevens zien dat de uiteindelijke samenstelling en stabiliteit sterk werden beïnvloed door het initiële inoculum.

Vervolgens onderzocht ik in hoofdstuk 4 het potentieel van het gebruik van zoutmoerasbodem als inoculum voor de productie van microbiële consortia die in staat zijn om tarwestro te gebruiken onder zoute omstandigheden. Dit teneinde industriële condities na te bootsen. Verder heb ik onderzocht hoe het groeisubstraat van invloed was op de samenstelling van de microbiële consortia. Om dit te doen, in het eerste deel van de verrijking, voedde ik de consortia met vers substraat, terwijl ik in het tweede deel van de verrijking

voorverteerd substraat gebruikte. Het voorverteerde substraat veroorzaakte een dramatische verschuiving van de bacteriesamenstelling met een opvallend effect op de schimmelgemeenschappen. Het verse substraat had meer generalistische microbiële consortia, terwijl het voorverteerde meer gespecialiseerde microbiële consortia selecteerde die beter in staat waren om cellulose en lignine af te breken dan de eerdergenoemde. Ik identificeerde de lytische activiteit in de belangrijkste cultiveerbare degrader bacteriën en schimmels, waarbij de meest dominante bacteriën in de consortia *Joostella marina*, *Flavobacterium beibuense*, *Algoriphagus ratkowskyi*, *Pseudomonas putida* en *Halomonas meridiana* waren. Deze consortia zijn een potentiële bron van hydrolytische enzymen die zijn gespecialiseerd op recalcitrante lignocellulose en die in staat zijn onder zoute omstandigheden te werken.

Daarna, in hoofdstuk 5, heb ik de samenwerkingscapaciteit van geselecteerde microbiële afbraakstammen onderzocht (die in hoofdstuk 3 zijn gevonden). Tevens heb ik onderzocht hoe deze positieve interactie afhankelijk was van de complexiteit van de koolstofbron. Van stammen die domineerden in de geselecteerde consortia werd het vermogen om op tarwestro te groeien getest, evenals de productie van hydrolytische enzymen. Vervolgens creëerde ik minimale synthetische consortia met geselecteerde stammen en onderzocht ik hun interactiviteit op tarwestro. Het meest synergistische paar werd gevormd door *Citrobacter freundii* so4 en *Sphingobacterium multivorum* w15. Voor het onderzoeken of stimulus op bidirectionele wijze gebeurde, paste ik een inductie-experiment toe, waaruit bleek dat de stammen wederzijdse invloeden op elkaar uitoefenen. De stimulus werd toegeschreven aan verbindingen die zich in de supernatants van elk van hen bevonden. Tot slot toonde ik aan dat de mate van interactie samenhing met de complexiteit van de koolstofbron, aan de hand van in monoculturen en co-culturen in koolstofbronnen met verschillende niveaus van “recalcitrantie”: glucose, synthetisch lignocellulose substraat (CMC, xylan, lignine) en WS. Recalcitrantie van het substraat gaf een synergistische groei en enzymatische activiteit van uitgescheiden lytische enzymen, en derhalve verhoogde recalcitrantie de coöperatieve relatie tussen de microbiële soorten.

In hoofdstuk 6 heb ik de genomen van *Sphingobacterium multivorum* w15 en *Citrobacter freundii* so4 gesequenced in een poging om de functionele complementen van de twee samenwerkende bacteriesoorten te begrijpen. De vergelijking tussen de genomen gaf aan dat de stammen complementaire afbraakcapaciteit vertoonden, evenals verschillende metabolismebehoeften,

waardoor ze waarschijnlijk verschillende soorten afbraakenzymen produceerden en niet konden concurreren om dezelfde voedingsbronnen. Derhalve poneer ik dat positieve samenwerking tussen de stammen berust op kruiselingse voeding of samenwerking op basis van uitwisseling van metabolieten. Het genoom van *S. multivorum* w15 vertoonde 22 genen uit familie glycosylhydrolase 43, evenals 45 genen uit de familie van koolhydraatesterases; beide families zijn relevant geworden in afbraak van hemicellulose en recalcitrante bindingen in LCB.

Tenslotte, in hoofdstuk 7, heb ik de verschillende soorten consortia herbeschouwd. Samenvattend, ik heb speciaal aandacht besteed aan de geïdentificeerde microbiële stammen in de verrijkte consortia. Alle consortia verkregen onder niet-saline condities bevatten een kern van bacteriën gevormd uit leden van de families *Enterobacteriaceae*, *Xanthomonadaceae*, *Pseudomonadaceae* en *Sphingobacteriaceae*. Ondertussen was in de halotolerante consortia een kern aanwezig gevormd door *Flavobacteriaceae*, *Cyclobacteriaceae*, *Pseudomonadaceae* en *Halomonadaceae*. Opmerkelijk is dat alleen *Pseudomonas* sp. en *Flavobacterium* sp. alomtegenwoordig waren. Vervolgens, in een poging om de samenwerkingsrelatie tussen *S. multivorum* w15 en *C. freundii* so4, de meest synergistische stammen, te begrijpen, analyseerde ik de genomische en de fysiologische gegevens en gebruikte ik ze om een mogelijk mechanisme voor lignocelluloseafbraak voor te stellen voor dit samenwerkingspaar; in het model zou *S. multivorum* w15 kunnen werken als primaire afbreker en *C. freundii* so4 als secundaire afbreker. Daarnaast waren de productie en uitscheiding van secundaire metabolieten en modulatie van een stressrespons mogelijk andere belangrijke mechanismen.

Summary

The depletion of oil reserves as well as global warming open the door for the possibility to develop ecologically-sustainable ways to obtain energy and products. Lignocellulose biomass (LCB) such as wheat straw (WS1), switch grass (SG), maize (corn stover - CS) and sugar cane represent excellent and cheap sources of carbon that can be transformed into valuable (energy) compounds. Nevertheless, their application at large scale is still limited to date.

This thesis opens with an introduction, where I gave an overview of the complexity of the structure and composition of LCBs and the need for very diverse enzymes to achieve their complete breakdown. This includes not only cellulases, hemicellulases and ligninases, but also the mandatory participation of auxiliary enzymes. There is a need to explain and understand the factors that affect LCB bioconversion in order to tackle larger challenges involved in the processing of LCB at industrial scale. In the Introduction, I stressed the potential of microbial consortia (in an eco-biotechnological approach) for decomposition of LCB so as to deal with its inherent recalcitrant nature. Finally, I described the relevance of key interactions within such degrader consortia. Microbial interactions drive and shape the structure, stability and functionality of microbial communities. It is fundamental to understand the interactions within selected microbial consortia to allow the design of optimized LCB-degrader consortia for application in industry.

The next Chapter, 2, explores the effect of the use of WS1, SG, CS, wheat straw at pH 9.0 (WS2), and forest soil as inocula for the selection of LCB-degrading microbial consortia in a sequential batch approach. The final enriched consortia were studied phylogenetically and functionally. PCR-DGGE analyses indicated that the bacterial communities reached stability after transfer 6 in WS1, SG and CS and after transfer 4 in WS2. For fungal communities, stability was reached after transfers 6 in WS1 and SG and after transfers 4 for WS2 and CS. We found substrate type, next to pH, to drive the bacterial community structures for the treatments using WS. A “core” set of strains was found in the final four microbial consortia; the core set was formed by *Sphingobacterium kitahiroshimense*, *Enterobacter amnigenus*, *Raoultella terrigena*, *Pseudomonas putida* and *Stenotrophomonas rhizophila,* next to the fungi *Coniochaeta ligniaria* and *Acremonium* sp. All strains in the core presented CMC-ase and xylanase activities. The LCB (wheat straw)

degradation potential of the selected consortia was determined by using Fourier-transform infrared spectroscopy (FT-IR). The data revealed that substrate type determines the final structure of the consortia, and so diverse LCB substrates drive consortia apart, even from a common inoculum source.

In Chapter 3 I explored the importance of the inoculum source in the selection of LCB degrader consortia by applying three different inocula (from forest soil, canal sediment and decaying wood) and wheat straw as the sole carbon source in the selection of LCB degrading microbial consortia. The bacterial and fungal community structures in the final consortia clustered along inoculum source, with significant differences between the different consortia. Wood-derived consortia were the first to reach stability, followed by the soil-derived ones, with sediment-derived ones never reaching stabilization above 50% similarity. More precise characterization of the final bacterial community structures, by 16S rRNA gene amplicon sequencing, showed that the most abundant members of the community formed a bacterial core, which was common between the three final consortia. This core was formed by the genera *Sphingobacterium*, *Citrobacter, Acinetobacter* and *Flavobacterium* or *Chryseobacterium*. The fungal genera were consortium-specific; as in Chapter 2, *Coniochaeta ligniaria* and *Acremonium* sp. were found. All organisms presented high LCB transforming activities. The final consortia consumed hemicellulose, cellulose and lignin components to grossly similar extents. Nevertheless, each consortium revealed a unique enzymatic profile. Thus, the final consortium structure and stability were strongly influenced by the initial inoculum source.

In subsequent work (chapter 4), I explored the potential of salt-marsh soil to serve as the inoculum for the production of microbial consortia capable of using wheat straw under highly saline conditions; this mimicked realistic industrial conditions. Furthermore, I studied how an increase of the recalcitrance of the substrate affects the consortial structures. To do this, in the first part of the enrichment, I fed the consortia with fresh substrate whereas in the second part of the enrichment, I replaced fresh by pre-digested substrate. Pre-digested substrate caused a dramatic shift in the bacterial community structures, and also had a striking effect on the fungal communities. The fresh substrate selected a more generalist microbial community, while the predigested WS selected a more specialized microbial community that was better capable to degrade cellulose and lignin than the former one. I identified, and tested for lytic activity, key cultivable bacteria and fungi; the most dominant degrader bacteria in the consortia were

Joostella marina, *Flavobacterium beibuense*, *Algoriphagus ratkowskyi*, *Pseudomonas putida* and *Halomonas meridiana*. The final consortia constitute a potential source of hydrolytic enzymes specialized on recalcitrant lignocellulose substrate and capable to work under saline conditions.

In a next research effort, I explored the collaborative capacity of microbial degrader strains isolated from the consortia grown under non-saline conditions in relation to the complexity of the carbon source. First, I selected the abundant strains in the consortia, and screened these for their ability to grow singly on wheat straw as well as produce LCB hydrolytic enzymes. Then, I created minimal synthetic consortia with selected degrader strains and examined their interactivity on wheat straw. The most synergistic pair was formed by *Citrobacter freundii* so4 and *Sphingobacterium multivorum* w15. To assess the directionality of the synergism, I applied a reciprocal induction experiment, and showed that the two strains exert mutual influences on each other. The stimulus was attributed to compounds contained in the respective strain supernatants. Finally, I demonstrated that the positive interaction was triggered by the complexity of the carbon source, as it was largely absent from cultures grown in glucose and synthetic lignocellulose substrate (CMC, xylan, lignin), versus strongly present in those in WS. The WS substrate probably triggered synergistic growth and activity of secreted lytic enzymes. Overall, I concluded that recalcitrance increases the cooperative relationship between the microbial species.

In subsequent work (chapter 6), I sequenced the genomes of *Sphingobacterium multivorum* w15 and *Citrobacter freundii* so4 so as to understand the functional complements of the two collaborating bacterial species. Comparison of the two genomes indicated that the strains had complementary LCB degradative capacity as well as different metabolic needs, which probably allowed them to contribute with different types of degradation enzymes and to not compete for the same nutritional resources. Then, I posited that the positive cooperation between the strains came about as a result of cross-feeding or cooperation based on metabolite exchanges. Interestingly, I noticed that the genome of *S. multivorum* w15 exhibits 22 genes from glycosyl hydrolase family 43, as well as 45 genes from a family of carbohydrate esterases; both families have become relevant in the degradation of hemicellulose and recalcitrant bonds in the LCB, respectively.

Finally, the findings of the previous chapters were summarized and placed in a broader perspective, placing special emphasis on the microbial degrader

strains found over all enriched consortia (chapter 7). All consortia at regular salt concentration revealed the presence of a core set of bacteria, consisting of members of the families *Enterobacteriaceae*, *Xanthomonadaceae*, *Pseudomonadaceae* and *Sphingobacteriaceae*. In contrast, the halotolerant consortia were formed by *Flavobacteriaceae*, *Cyclobacteriaceae*, *Pseudomonadaceae* and *Halomonadaceae*. Remarkably, only *Pseudomonas* sp. and *Flavobacterium* sp. were present in all selected consortia. Then, in an effort to understand the cooperative relationship between *S. multivorum* w15 and *C. freundii* so4 (the most synergistic strains), I joined the genome analyses and the physiological data and used them to propose a possible mechanism for lignocellulose degradation within this collaborative pair. Briefly, it is possible that *S. multivorum* w15 is acting as the primary degrader and *C. freundii* so4 as a secondary degrader. In addition to the production and excretion of secondary metabolites and a contribution with stress response modulation.

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About the author

Larisa Cortes Tolalpa was born on the 19th of April 1985 in the small Mexico City and grew up in the *quartier* of Iztapalapa. At the age of 16 she was accepted in CECyT 6 "Miguel Othón de Mendizabal" aka "Voca 6", a specialized high school in biological sciences from National Polytechnique Institute (IPN). In this place she had her first encounter with the world of biology, chemistry and physics, feeling in love of laboratories science. In spite of undergoing two major spine surgeries, she concluded her high school on time and graduated as a chemical laboratory technician. In 2003, she started her bachelor studies in UPIBI-IPN, but she had to stop due to her third and final spine surgery. One year later she returned to study the Biotechnology Engineering major. She finished her studies in 2008, specialized in bioprocess, and for obtaining her final degree she designed a new pilot plant for the pharmaceutical company FERMIC. In 2009 she is accepted in the program of master in Biochemical Science in the Biotechnology Institute of UNAM – Cuernavaca. She worked in the lab of Dr. Bolivar Zapata, where the main research line is the metabolic engineering pathways of *Escherichia coli*. In this group, she worked in the transcriptomic analysis of the production of shikimic acid in 1L fermentor, specializing in biochemistry and microbial physiology. In September 2013, Larisa arrived in the Netherlands to start her PhD studies in the cluster of Microbial Ecology at the University of Groningen. She worked in a project base in eco-biotechnology, focused in the development and study of microbial consortia for the degradation of lignocellulose substrates for the production of valuable compounds. She is currently looking for new projects and she expects to contribute with her beloved Mexico with all the things she has learnt living abroad.

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