Top-down enrichment guides in formation of synthetic microbial consortia for biomass degradation

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Natural and Synthetic Anaerobic Consortia Accelerate Lignocellulose Deconstruction

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Abstract

Consortia-based approaches are a promising avenue towards efficient bioprocessing. However, many complex microbial interactions dictate community dynamics and stability. The rumen of large herbivores harbors a network of biomass-degrading fungi and bacteria, as well as archaea and protozoa that work together to degrade lignocellulose, yet the microbial interactions responsible for consortia stability and activity remain uncharacterized. In this work, we demonstrate a novel enrichment-based isolation method selecting for a minimal biomass-degrading community containing anaerobic fungi, methanogenic archaea, and bacteria. The enriched culture displayed an increase of up to 2.1 times the growth rate and 1.9 times the fermentation gas produced by the isolated fungus from the enriched culture alone. Metagenomic sequencing revealed functional compartmentalization of the community spread across anaerobic fungi (*Piromyces*), bacteria (*Sphaerochaeta*), and methanogens (*Methanosphaera* and *Methanocorpusculum*). The minimal consortium enabled more complete degradation of biomass, including

hemicelluloses like xylan and pectin and a wider range of sugar utilization like xylose and galacturonate. Complementing the “top-down” enrichment analysis, a synthetic rumen system was formed from the “bottom-up” with isolated fungi (*Piromyces finnis* or *Neocallimastix californiae*) and methanogens (*Methanobacterium bryantii*) where only the hydrogenotrophic connection was preserved between members. These synthetic consortia also showed improved growth rate and degradation compared to fungi alone, yet lack the temporal stability seen in native consortia, remaining in culture together for fewer than 10 transfers on average. Taken together, these two complementary approaches both resulted in productive microbial consortia with faster growth rates and wider substrate uptake than mono-cultured fungi.

Keywords

Microbial Consortia, Anaerobic Fungi, Lignocellulose, Metagenomics, Biomass

Introduction

Artificial, or synthetic, consortia hold the potential to revolutionize bioprocessing, because of their increased efficiency by distributing difficult processes across the individual members.[1] Consortia are currently utilized in some processes, like anaerobic digestion, where a complex mixture of microbes convert waste into synthesis gas. Typically, these complex communities are isolated or formed from nature, resulting in mixtures of undefined microbes where the members provide some functional or metabolic benefit to the community. However, when consortia are formed by combining individual microbes from the "bottom-up", wieldy microbes compete for the same resources often resulting in the dominance of one microbe that outperforms the others. Identifying key factors that both connect and stabilize the consortium is critical to overcome current limitations inherent in using microbial communities for bioprocessing.

Diverse microbial communities from nature participate in many different types of interactions, ranging from favorable to benign and even unfavorable, which can be leveraged to enhance the productivity of synthetic consortia.[2] These interactions help regulate community dynamics, resulting in stable macroscopic traits. Syntrophy is one possible interaction that helps tie together microorganisms that have a mutual benefit.[3] Syntrophy occurs when one microbe utilizes metabolites produced by a second microbe, resulting in an interwoven and dependent metabolism[4]. Obligate syntrophy has further benefits, where the removal of metabolic products allows utilization of substrates by a second microbe that they are unable to catabolize in isolation[4].

One route to identify mechanisms that regulate community formation, diversity, and stability is to investigate "minimal" native consortia, whereby key microbial players have

been enriched from a complex ecosystem. An ecosystem of key biotech relevance is the hindgut of large herbivores, where biomass is routinely degraded and anaerobic gut fungi are primarily responsible for degradation[5]. Though gut fungi have recently been characterized in isolation[5, 6], such growth does not accurately mimic their native environment. Alongside anaerobic fungi, archaea, bacteria, and protozoa are found in herbivore hindguts. Among these, anaerobic fungi have been shown to interact closely with methane producing archaea (methanogens)[7]. Methanogens siphon hydrogen and other metabolites from the fungi, allowing the fungi to more efficiently produce energy by increasing the flux through their hydrogenosomes[7]. In addition to greatly increasing biomass degradation, fungi-methanogen co-cultures are capable of directly converting crude biomass into methane[7]. Early and recent characterization of fungi-methanogen co-cultures[7-13] focused mainly on macroscopic effects of co-culture. Separate studies have considered native consortia – isolated fungi with natively associated methanogens, and *in vitro* consortia – separately isolated anaerobic fungi and methanogens. No previous studies have made direct comparisons between the two types of cultures, however, so the difference in effectiveness between the two types of cultures are not known.

In this study, we utilize "top-down" isolation to inform "bottom-up" reconstruction of a biomass-degrading microbial consortium. First, using next generation sequencing techniques, we identify the interactions between microbes in a minimal consortium isolated from nature. We then demonstrate that the mechanisms identified in the native community can be used to design a synthetic rumen consortium that greatly exceeds the performance of isolated members. The imparted robustness and stability are crucial to developing consortia capable of routine use in waste-to-chemical production.

Results and Discussion

Selective Enrichment Enables Isolation of a Stable Native Consortium

In order to capture a consortium representative of the rumen microbiome, a selective enrichment process was developed to isolate a minimal system (Figure 1). Methanogens and anaerobic fungi were successfully enriched for, as determined by periodic sampling of methane in the headspace of cultures (Figure S1) and methanogen-specific 16S and fungal ITS primers (Table S1). A rough estimate of diversity was calculated by cloning the ITS or 16S amplicons into vectors and selecting individual colonies for Sanger Sequencing. The ITS sequences were highly similar, with only a few slight differences likely due to the difficulty of sequencing ITS regions from anaerobic fungi due to extremely high AT content[14]. The closest cultured match to the enriched fungus was a *Piromyces* isolate, but with only 86% identity matching a cultured isolate across the entire ITS and 5.8S region. The sequences clustered distinctly from other fungal genera (Figure S2a), grouping into a clade with an uncultured isolate. Microscopy revealed the formation of monocentric sporangia (Figure S3) similar to the genus *Piromyces*. Methanogen-specific primers amplified two distinct sequences most similar to *Methanosphaera* and *Methanocorpusculum* isolates. The phylogenetic identity (Figure S2b) of the two methanogens was somewhat surprising, as the rumen and intestinal tracts of herbivores are often dominated by *Methanobrevibacter* methanogens[15]. However, it is likely that specific fungal-methanogen interactions led to isolation of the *Methanosphaera* and *Methanocorpusculum* over the more abundant *Methanobrevibacter*.

Metagenomic sequencing was performed to characterize the prokaryotic component of the consortium, but was not done for the eukaryotic population due to the complexity in sequencing fungal genomes.[6, 16] DNA was isolated from the prokaryotic culture after one generation of growth to capture abundances as close to the native community as possible. A summary of the metagenomic sequencing is detailed in Table S2. An initial search for 16S sequences revealed the two methanogens previously detected by methanogen primers, but also a third unique sequence, most similar to a *Sphaerochaeta* genome. The phylogenetic placement of each 16S sequence is shown in Figure S3. The assembled contigs were binned and checked for completion. Each bin was at least 93% complete with less than 2% contamination (Figure S4), which qualifies each as a high-quality draft according to the Minimum Information about a Metagenome Assembled Genome[17].

Native Rumen Consortium Accelerates Biomass Degradation

To compare the growth characteristics of the enriched consortium versus individual microbial constituents, a novel separation scheme was designed to separate the fungal component of the consortium from the prokaryotic part, as shown in Figure 1. While methanogens are resistant to penicillin and streptomycin, they are susceptible to chloramphenicol treatment. As such, pure fungal cultures were obtained by treatment with chloramphenicol. Similarly, fungi require a carbohydrate substrate, and removing that substrate source selected against fungal growth. The culture of fungi, bacteria and methanogens was tested for growth on a wide variety of substrates ranging from simple sugars to complex lignocellulose, and compared to each individual monoculture. Because of

the unique growth habits of anaerobic fungi, total gas production was measured as a proxy for growth, as in previous work[18].

The net specific growth rate across a variety of substrates, calculated from fermentation gas production, is shown in Figure 2A. The mixed culture outperformed either monoculture significantly ($p<0.05$) across all substrates except for switch grass. The growth rate of the consortium was up to 2.1 times faster than the isolated fungus alone. The amount of fermentation gas produced was enhanced as well (Figure 2B), where the consortium produced up to 1.9 times more gas than the isolated fungus. Finally, the degradation of substrates was dramatically enhanced, as shown by the visible degradation of filter paper in Figure 2C. The consortium fully disrupted the paper within 68 hours of inoculation, up to 40% faster than the fungus alone. Of the more complex substrates (reed canary grass, alfalfa stem, corn stover, and switch grass) the growth rate was greatest on reed canary grass for both the consortium and isolated fungus. Since the community was isolated using reed canary grass as the substrate, it is likely that the community members were tuned for optimal growth on reed canary grass. Interestingly, there were some substrates, such as xylan and pectin, on which the fungal monoculture had minimal growth, whereas the mixed culture produced significant growth and fermentation gas. No significant growth was demonstrated by the isolated methanogen/bacteria part on any substrate.

Metagenomic Analysis Suggests that Syntrophy and Compartmentalized Metabolism Drive Stability

Metagenomic sequencing of the consortium revealed a tightly interwoven community metabolism, despite some redundant capabilities (Figure 3). Syntrophy occurs between the methanogens and fungi, as well as the methanogens and the bacteria via hydrogen exchange and assimilation. However, this is just one part of the compartmentalization observed. From previous work[5, 6], it is known that the anaerobic fungi are enriched in biomass degrading enzymes, but these enzymes are primarily exo- and endo- cellulases. They contain some enzymes for degradation of smaller cellodextran fragments, yet they also have transporters capable of taking up these longer cellulose fragments[19]. In the enriched consortium, the fungi act as the primary degraders of the plant biomass, taking up primarily glucose. When grown in isolation, there are excess sugars present[20], which allows for the presence of "sugar cheaters" often seen in biomass-degrading consortia[21].

Analysis of the *Sphaerochaeta* genome bin (Supplementary Database 1) revealed very few enzymes that act on crystalline cellulose, however, the analysis showed many enzymes that further degrade the small fragments released by the fungal enzymes. The *Sphaerochaeta* member also contained many enzymes for pectin degradation, which explains the enhanced growth on pectin seen by the mixed culture (Figure 2). The role of *Spirochaetes* in pectin degradation was recently hypothesized from a moose rumen metagenomic survey[22], lending further evidence to its role in the rumen consortium. Furthermore, analysis of the transporters and metabolism in *Sphaerochaeta* showed that it is capable of taking up and utilizing a wide array of sugars like arabinose, mannose, galactose, xylose, and galacturonate (Supplementary Database 2).

The two methanogens had similar roles in the consortium, as both act as the terminal electron acceptors in the anaerobic community. However, analysis of the

Methanocorpusculum member revealed a pathway for formate utilization, allowing for growth on either H_2/CO_2 or formate. *Methanosphaera* have previously been shown to require a combination of H_2 and methanol for growth, however the genes were present for growth on H_2/CO_2 as previously shown for a different *Methanosphaera* species[23]. Methanol is released during the degradation of pectin and other hemicelluloses[24, 25], which could explain the role of the *Methanosphaera*. Previous analyses have demonstrated the importance of compartmentalized nitrogen metabolism[3], however our analysis revealed that compartmentalized carbon metabolism is likely the main factor for stability in the enriched community.

Since the enriched consortium was continuously passaged every 3-4 days, we used 16S and ITS metagenomic profiling 5 months after shotgun metagenomics sequencing to assay stability of consortium membership. The relative abundance of methanogens and bacteria was calculated from the metagenomics data by counting the number of reads aligned to each genome bin. As shown in Figure 4, the composition of the consortium and the ratio amongst the prokaryotic members was relatively stable across both time points sampled. In addition, the ITS sampling confirmed that the chloramphenicol-treated fungal monoculture had the same ITS sequence as the enriched culture (Table S1), suggesting that chloramphenicol treatment had no effect on the fungus present. Important to note, however, was that the rumen fluid in the culture medium produced a background 16S signal, even though clarified rumen fluid was used. The analysis, therefore, only contained sequences significantly enriched compared to the background uncultured medium control, and it is possible the signal of very low abundance members was masked by the background DNA from clarified rumen fluid.

"Synthetic Rumen" Systems Drive Enhanced Fermentation & New Growth Phenotypes

Given the compartmentalization revealed in the minimal rumen consortium, we formed synthetic consortia composed of microbes with similar metabolic dependencies and tested for biomass-degrading activity and stability in batch culture. *Piromyces finnis* and *Neocallimastix californiae* are well-studied members of the *Neocallimastigomycota* with high-quality genomes[6], and both were paired separately with methanogen *Methanobacterium bryantii* in culture. As shown in Figure 5, the synthetic co-cultures showed much greater total fermentation gas production (2.1x for *P. finnis* and 1.8x for *N. californiae*) compared to monocultures of fungi alone on simple biopolymers like Avicel and xylan. However, the synthetic co-cultures produced less total fermentation gas from reed canary grass than the native consortium, and did not show an improvement over the individual fungi alone. Despite the decreased gas production on reed canary grass, it is likely that the co-cultures metabolized more total carbon than the isolated fungi alone. Methanogenesis by *M. bryantii* consumes 5 moles of gas ($4H_2 + 1CO_2$) for every 1 mole of methane produced, and methane was at a concentration of ~10% at the end of growth (Figure 5B), suggesting that the total gas produced by the fungi in co-culture was greater than that of either fungus alone.

Interestingly, although *P. finnis* has the necessary genes for metabolism of xylose (and xylan)[6], it is incapable of utilizing it as the sole growth source under medium conditions used in this study (Figure 5C). However, the synthetic *P. finnis*-*M. bryantii* co-culture grew almost as well on xylan as on the glucose-based Avicel. The reason for the recovered phenotype of xylan utilization is unknown, however it is an important observation due to the high

proportion of xylose in biomass. Xylose utilization by members of the *Piromyces* genus has received attention in recent years as they possess a xylose isomerase instead of xylose reductase/xylitol dehydrogenase typical of other fungi[26]. The xylose isomerase pathway is more commonly found in bacteria, and might have arisen through horizontal gene transfer as many of the cellulases of anaerobic fungi have[6], which could explain the difficulty in metabolizing pure xylose or xylan. Another hypothesis is related to the availability of required metal cofactors for the xylose isomerase. The xylose isomerase from closely-related *Piromyces* sp. E2 was shown to operate optimally with a Mn^{2+} cofactor in place of other bivalent metal ions[27, 28]. *M. bryantii* requires several bivalent cofactors[23] and therefore likely affects the availability of these molecules to *P. finnis* in co-culture, either making Mn^{2+} more available or depleting the availability of Fe^{2+} or Mg^{2+}.

One major limitation of the synthetic co-cultures was stability. Although the synthetic rumen systems of *M. bryantii* paired with either *P. finnis* or *N. californiae* were cultivatable for several generations, they never reached the >2 years of stability achieved by the native community, often losing the associated methanogens within a month (10 culture transfers) of formation. Methanogens typically grow much more slowly than anaerobic fungi[20, 29], such that the paired methanogens may be lost due to dilution from consecutive culture transfer. Alternatively, the oxygen sensitivity of methanogens might lead to culture instability through brief oxygen exposure during transfer, largely avoided through potential oxygen scrubbing of the *Sphaerochaeta* bacterium in the native consortium. The stability seen from the synthetic co-cultures is still an improvement over consortia formed between competing microbes, where competition for resources leads to consortia instability. Importantly, the co-culture requires no genetic engineering like methods such as synthetic signaling[30] or

engineered auxotrophy[1] and therefore was applied effectively to microbes lacking genetic tools.

Conclusions

In this work, we have compared two unique methods for consortium formation – "top-down" enrichment of native communities and "bottom-up" formation of synthetic communities. In both cases, the microbial communities achieved increased fermentation gas compared to the biomass-degrading action of the anaerobic fungal component, and they were capable of utilizing a wider range of substrates. While both top-down and bottom-up methods resulted in consortia that produced more fermentation gas than isolated fungi alone, the top-down approach resulted in a more stable and more productive consortium. This is likely because the microbes present co-evolved in nature and naturally developed mechanisms for stability like syntrophy and compartmentalized carbon metabolism. Starting from a rich pool of microbes in the fecal material of a horse, the top-down approach enriched a mixture of microbes capable of effectively utilizing unpretreated biomass. The methanogens present in the native community are lower abundance members of the rumen, which suggests that other factors selected for their presence in culture over more abundant methanogens. These factors likely contributed to the increased stability of the native community (>2 years) compared to separately isolated methanogen and fungi in the synthetic rumen system (~1 month).

There are some limitations of the top-down approach, however. It is dependent on the starting microbial pool and the selective factors used to enrich for the community. The growth substrate utilized likely impacted the enriched community, and even though

anaerobic fungi are among the most robust organisms at degrading biomass, they can be outcompeted during the initial isolation unless antibiotics are present, limiting the total microbial diversity that can be captured. The approach worked well for this application, where the main goal was the utilization of biomass, however the bottom-up approach is more favorable for specific chemical production, or for drop-in modules to create a wider range of products. Future work includes determining the difference in community composition enriched for by changing the selective pressures like growth substrate or antibiotic treatment.

In summary, our work displays a direct comparison between natural and synthetic anaerobic consortia for lignocellulosic biomass degradation. The top-down approach resulted in a more productive microbial community and identified compartmentalized carbon metabolism as the main mechanism enabling productivity and stability. The bottom-up approach allowed for inclusion of microbes with desired properties - in this case sequenced genomes and well-characterized phenotypes. Taken together, these two complementary approaches represent unique paths towards implementing microbial consortia for bioconversion of lignocellulosic biomass.

Methods

Growth Medium, Isolation, and Microbial Cultivation

Fungi and consortia were grown in anaerobic fungal Medium C as previously described[5], supplemented with penicillin (1000 U/mL), streptomycin (1000 U/mL), nickel sulfate (0.2 µg/mL), and sodium 2-mercaptoethanesulfonate (40 µg/mL). Cultures were grown on 1% (w/v) reed canary grass unless otherwise stated. Avicel (Sigma Aldrich), Xylan (from corn stover, TCI Chemicals, Portland, OR), Pectin (from citrus fruits, MP biomedicals), Reed canary grass, corn stover, switchgrass, and alfalfa stems (all grass obtained from USDA-ARS Research Center, Madison, WI) were added prior to autoclaving media at 1% (w/v). Glucose and cellobiose were dissolved in water and sterile-filtered then added to media post autoclaving at 0.5% (w/v). Methanogens were cultured as previously described[23], in M2 Medium with 80%/20% H_2/CO_2 headspace, supplemented with methanol (1% v/v). All cultures were grown at 39°C without shaking.

Equine fecal materials were collected from the UCSB West Campus Stables, suspended in Medium C, serially diluted, and inoculated into Medium C with reed canary grass supplemented with penicillin, streptomycin, nickel sulfate, and coenzyme M (PS+). Cultures positive for fungal growth were passaged in consecutive batch culture every 3 to 4 days in Medium C supplemented with PS+. The presence of methanogens was verified by periodic determination of methane in the headspace of cultures (Figure S1). In addition, methanogen-specific 16S[31] and fungal ITS[32] primers were used to confirm the presence of both members (Table S1). A rough estimate of diversity was achieved by cloning the ITS or 16S amplicons into vectors and selecting 10 individual colonies for Sanger Sequencing.

Metagenomic Library Preparation and Sequencing

To isolate genomic DNA, cultures were grown in 40 mL of media in 60 mL Wheaton serum bottles until stationary phase (OD_{600} ~ 0.2–0.5) and then harvested by centrifugation for 30 min at 10,000×g at 4 °C. Cell pellets were resuspended in 0.5 mL TE Buffer (10 mM Tris, 1 mM EDTA, pH 8.0). Sodium dodecyl sulfate was added to a final concentration of 0.5%, proteinase K (New England BioLabs, Ipswitch, MA) was added to 100 µg/mL, and RNaseA (MoBio Laboratories, Carlsbad, CA) was added to 100 µg/mL. The mixture was incubated at 37 °C for 1 h. NaCl was added to 0.5 M, and 0.5 mL of phenol:chloroform:isoamyl alcohol (25:24:1) was added. The solution was mixed and then centrifuged at 13,000×g for 10 min at 4 °C. The aqueous phase was transferred to a new tube and 0.6 mL of isopropyl alcohol was added. The mixture was incubated at −20 °C for ~16 h and then centrifuged at 13,000×g for 5 min at 4 °C. The pellet was washed with 70% ethanol, centrifuged at 13,000×g for 5 min at 4 °C, and finally resuspended in 10 mM Tris buffer pH 8.0 and stored at −20 °C [33].

Genomic DNA (gDNA) was prepared for high throughput sequencing (HTS) using the TruSeq DNA PCR-Free library prep kit supplied by Illumina, Inc. (San Diego, CA). Briefly, purified gDNA were first fragmented using a Covaris (Woburn, Massachusetts) M220 Focused Ultrasonicator, followed by end repairs, size selection (~330 bp), end adenylation and paired-end adapters ligation using the kit. Prepped libraries were then quantified using Qubit (Life Technologies, Carlsbad, CA) and TapeStation (Agilent, Santa Clara, CA), before pooling. HTS was performed with an Illumina NextSeq500 sequencer using a 150 cycle, mid output kit (2x75 paired-end).

330

331 *Metagenomic Binning and Analysis*

332 Metagenomic reads were assembled using Megahit v1.1.2 [34]. Assembled contigs were binned
333 using MetaBAT v2.12.1 [35] and CONCOCT v0.4.1 [36], with BLAST used to manually curate
334 unbinned contigs. Binned genomes were annotated with the Department of Energy Systems
335 Biology Knowledgebase (KBase, http://kbase.us) automated pipeline. Genomic features
336 including ORFs, large repeat regions, rRNAs, CRISPRs, and tRNAs were identified and
337 annotated with the Rapid Annotations using Subsystems Technology toolkit (RASTtk)[37].
338 These gene annotations were combined with biochemical information from the Kyoto
339 Encyclopedia of Genes and Genomes (KEGG)[38] to reconstruct the metabolism of each genome
340 bin. Genome completion was determined utilizing CheckM v1.0.7 [39]. Metagenomic
341 abundance for each bin was calculated by mapping reads to the full assembly using Bowtie2
342 v2.3.2 [40]. Transporters were classified using the Transporter Classification DataBase[41],
343 downloaded on January 15, 2015. Results were filtered to only include hits that covered 70%
344 of both query and subject with an E-value less than 10^{-3}. Hits were then manually curated to
345 classify the most likely sugar specificity. CAZymes were predicted using dbCAN[42] v4 accessed
346 January 29, 2016.

347

348 *16S & ITS Profiling*

349 For ribosomal profiling, cultures were grown for 4 days and harvested by centrifugation at
350 3220 x g for 20 minutes at 4°C. DNA was extracted using the FastDNA SPIN kit for soil (MP
351 Biomedicals, Santa Ana, CA, USA) according to the instructions. 16S Primers were designed
352 using the Ribosomal Database Project[43] targeting the V5 region. ITS primers were as

previously described[16]. Primers had overhangs compatible with Nextera XT primers (P5 for forward and P7 for reverse). The sequences of all primers used are in Table S1. Amplification was performed in 50 μL reactions composed of 1 μL of extracted DNA, 10 μL of 5X Phusion GC Buffer, 1 μL of 10 mM dNTPs, 2.5 μL of 10 μM forward primer, 2.5 μL of 10 μM reverse primer, 0.5 μL of Phusion DNA Polymerase (New England BioLabs, Ipswitch, MA), and 32.5 μL of DNase-free H_2O. Amplification occurred with an initial 30 second denaturation at 98°C; followed by 30 cycles of 10 seconds at 98°C, 30 seconds at 57°C, and 30 seconds at 72°C; a final extension of 5 minutes at 72°C; and a hold at 4°C. Prepped libraries were then quantified using Qubit (Life Technologies, Carlsbad, CA) and TapeStation (Agilent, Santa Clara, CA), before pooling. HTS was performed with an Illumina NextSeq500 sequencer using a 150 cycle, mid output kit (2x75 paired-end). Both 16S and ITS reads were analyzed using QIIME[44] version 1.9.1. OTUs were picked using UCLUST[45] version 1.2.22q. The Greengenes[46] database version 13.8 was used to classify 16S reads, and the UNITE[47] database version 7 was used to classify ITS reads.

Methane Detection

Methane concentration was measured with a Shimadzu GC 14A equipped with an N-octane on Res-Sil C column at 50°C with N_2 carrier gas. Methane standards were run prior to sample analysis and results used to generate a standard curve. Standards were 10, 500, 2500, 10,000 (1%), 25,000 (2.5%), and 100,000 (10%) ppm methane. Range was typically 10^1, with 10^2 for high methane samples. Methane ppm for each test sample was found by comparing against the standard curve.

Data Availability

Raw reads from whole genome metagenomic sequencing and from amplicon metagenomic sequencing have been deposited under the accession number PRJNA471522.

Supporting Information.

Table S1: Measured 16S and ITS sequences

Table S2: Metagenomic sequencing statistics

Table S3: Binned genome statistics

Figure S1: Methane Production over time

Figure S2: Phylogeny of Consortia Members

Figure S3: Morphological features of *Piromyces sp.* H1B2

Figure S4: Genome Completion by CheckM

Supplemental Database 1: CAZyme analysis of metagenomes

Supplemental Database 2: Transporter analysis of metagenomes

Abbreviations

ITS – Internal Transcribed Spacer, CAZyme – Carbohydrate Active enzyme

Author Contributions

SPG, JAS, JKH, and MKT carried out enrichment experiments of the native consortium. SPG and JKH performed the sequencing, and SPG conducted the bioinformatic analyses. SPG compared growth measurements of the native and synthetic consortia. SPG, JAS, MKT, DLV, and MAO planned the experiments. SPG and MAO wrote the manuscript.

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411

Figure 1: Complementary methods to establish biomass-degrading microbial consortia. In "Top-Down" isolation, native communities are enriched by selective antibiotic treatment and consecutive culture. Individual constituents of the native culture can be separated by either removing the sugar source for the fungi selecting for methanogens and bacteria, or by treatment with chloramphenicol selecting for fungi. In "Bottom-Up" reconstruction, separately isolated methanogens and fungi are combined in a culture, filling the roles observed from the native consortium.

Figure 2: Native consortium exhibits faster growth and faster substrate degradation than fungus isolated from consortium. (A, B) Across all conditions tested, the native consortium displayed a much greater growth rate (A), up to 2.1 times the isolated fungal part alone. The total amount of fermentation gas produced was also greatly increased (B), up to 1.9 times the amount of the fungus alone. The improvement was much greater on the simple sugars like glucose and cellobiose than on the more complex grasses such as reed canary grass. Interestingly, minimal growth on pectin or xylan was determined for the fungi alone, whereas the consortium easily consumed them. No growth was determined on any substrate for the isolated methanogen part. Error bars represent the standard deviation of three biological replicates. Significance was tested using the Student's t-test, NS represents not significant, * represents $p < 0.05$, ** represents $p < 0.01$ (C) The enhanced growth and degradation of substrates by the native consortium can be easily seen when grown on filter paper. The consortium rapidly degraded the filter paper, while the fungi alone were just beginning to degrade it at 68 hours of growth.

Figure 3: Metagenomic analysis of the native consortium reveals compartmentalized substrate degradation and metabolism. An overview of substrate metabolism of each member is displayed. The fungi are primary degraders of biopolymers, degrading the long chains of cellulose and hemicellulose. They consume primarily glucose and do not consume many of the other five and six carbon sugars. The bacterium contains genes to degrade shorter cellulose and hemicellulose fragments, as well as genes to metabolize sugars left behind by the fungi. The *Methanosphaera* consumes methanol liberated from pectin degradation as well as H_2. Finally, the *Methanocorpusculum* consumes H_2, CO_2, and formate.

Figure 4: Community abundance remains stable despite continuous growth and culture transfer. Relative ratios of the prokaryotic members are displayed, determined from whole genome metagenomics 14 months after isolation and marker gene metagenomics 19 months after isolation. As displayed, the consortium demonstrated remarkable stability, with the relative ratios remaining essentially the same across two measured time points. This reflects the overall stability of the culture despite continuous culture and batch transfer.

Figure 5: Synthetic rumen systems produce much more total fermentation gas than isolated fungi on simple substrates. (A) Total accumulated pressure for cultures of *Piromyces finnis*, and *Neocallimastix californiae* paired with *Methanobacterium bryantii* and the native consortium are displayed for growth on Reed canary grass, Avicel, and Xylan. On avicel, the synthetic rumen system greatly outperformed the fungus alone, producing roughly double the amount of gas for both the synthetic and native consortia. (B) The

maximum concentration of methane measured from growth on Reed canary grass is shown for the native consortium and the two synthetic rumen systems. On reed canary grass, accumulated pressure is similar for both the isolated fungus and synthetic rumen system (Figure 5A), although the final concentration of methane in the headspace was >10% suggesting that the fungi produced more gas in the synthetic rumen system. (C) The synthetic rumen system of *P. finnis* paired with *M. bryantii* was able to utilize a wider range of substrates including xylan, whereas *P. finnis* alone demonstrated no growth on xylan.

Figure 1

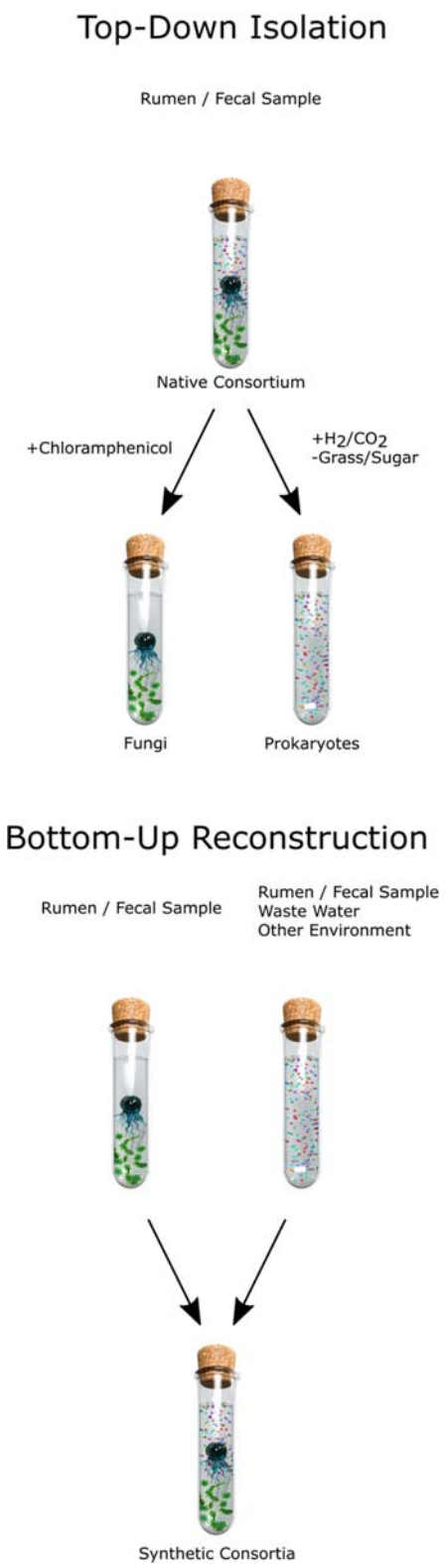

Top-Down Isolation
Rumen / Fecal Sample
Native Consortium
+Chloramphenicol
+H2/CO2
-Grass/Sugar
Fungi
Prokaryotes
Bottom-Up Reconstruction
Rumen / Fecal Sample
Rumen / Fecal Sample
Waste Water
Other Environment
Synthetic Consortia

Figure 2

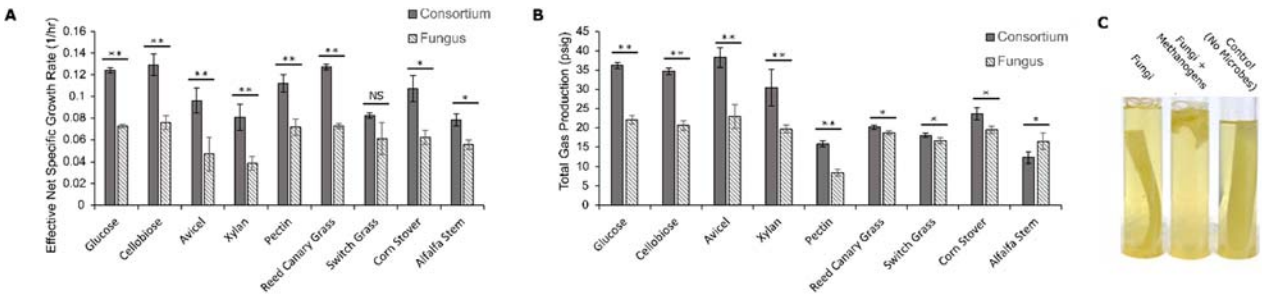

A
Effective Net Specific Growth Rate (1/hr)
Consortium
Fungus
Glucose
Cellobiose
Avicel
Xylan
Pectin
Reed Canary Grass
Switch Grass
Corn Stover
Alfalfa Stem
NS
B
Total Gas Production (psig)
C
Fungi
Fungi + Methanogens
Control (No Microbes)

Figure 3

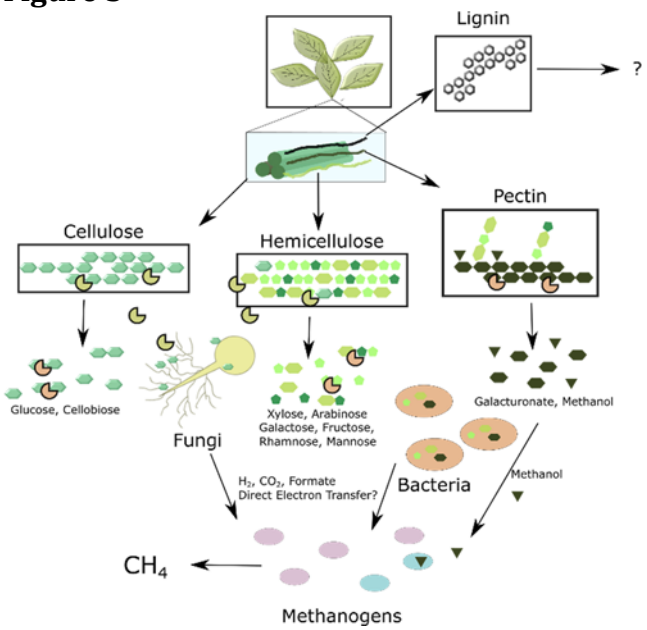

Lignin
?
Cellulose
Hemicellulose
Pectin
Glucose, Cellobiose
Xylose, Arabinose
Galactose, Fructose,
Rhamnose, Mannose
Galacturonate, Methanol
Fungi
H_2, CO_2, Formate
Direct Electron Transfer?
Bacteria
Methanol
CH_4
Methanogens

Figure 4

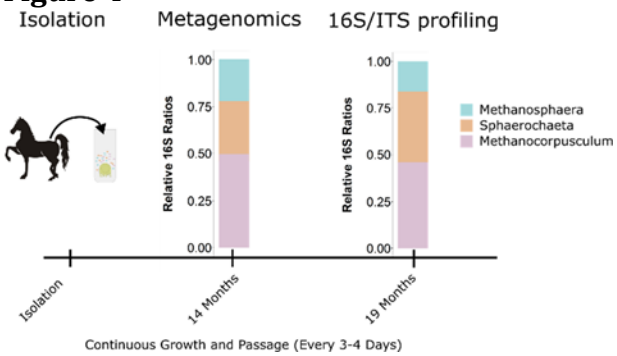
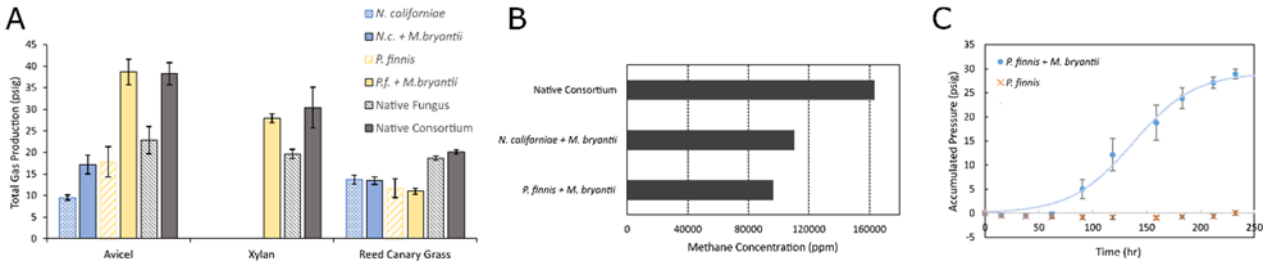

Isolation
Metagenomics
16S/ITS profiling
Relative 16S Ratios
1.00
0.75
0.50
0.25
0.00
Methanosphaera
Sphaerochaeta
Methanocorpusculum
Isolation
14 Months
19 Months
Continuous Growth and Passage (Every 3-4 Days)

Figure 5


A
N. californiae
N.c. + M.bryantii
P. finnis
P.f. + M.bryantii
Native Fungus
Native Consortium
Total Gas Production (psig)
0
5
10
15
20
25
30
35
40
45
Avicel
Xylan
Reed Canary Grass
B
Native Consortium
N. californiae + M. bryantii
P. finnis + M. bryantii
0
40000
80000
120000
160000
Methane Concentration (ppm)
C
P. finnis + M. bryantii
P. finnis
Accumulated Pressure (psig)
-5
0
5
10
15
20
25
30
35
0
50
100
150
200
250
Time (hr)

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Supporting Information

Table S1: Primers and Measured 16S and ITS sequences

Sanger Sequencing Methanogen-specific 16S primers	Met86F: GCTCAGTAACACGTGG Met1340R: CGGTGTGTGCAAGGAG
Methanosphaera sp H1B2 Sanger 16S sequence	GCTCAGTAACACGTGGATAACTTACCCTTAGGACTGGGATAACTCTGGGAAACTGGGGATAATACCGGATAT TAGGCTATGCCTGGAATGGTTTGCCTTTGAAACGTATTTTTCGCCTAAGGATAGATCTGCGGCTGATTAGGTC GTTGGTGGGGTAATGGCCCACCAAGCCTATGATCGGTACGGGTTGTGAGAGCAAGAGCCCGGAGATGGAAC CTGAGACAAGGTTCCAGACCCTACGGGGTGCAGCAGGCGCGAAACCTCCACAATGTACGAAAGTGCGATGG GGGAATCCCAAGTGTTATTCTTAACAGAATAGCTTTTCATTAGTGTAAAAAGCTTTTAGAATAAGAGCTGGGC AAGACCGGTGCCAGCCGCCGCGGTAACACCGGCAGCTCGAGTGGTAGCTGTTTTTATTGGGCCTAAAGCGTT CGTAGCCGGTTTGATTAGTCTTTGGTGAAAGCTTGTAGCTTAACTATAAGAATTGCTGGAGATACTATCAGAC TTGAAGTCGGGAGAGGTTAGAGGTACTACCGGGGTAGGGGTGAAATCCTATAATCCTGGGAGGACCACCTG TGGCGAAAGCGTCTAACTAGAACGATCTTGACGGTGAGTAACGAAAGCCAGGGGCGCGAACCGGATTAGAT ACCCGGGTAGTCCTGGCCGTAAACGATGTGGACTTGGTGTTGGAATGGCTTCGAGTTGTTCCAGTGCCGAAG GGAAGCTGTTAAGTCCACCGCCTGGGAAGTACGGTCGCAAGACTGAAACTTAAAGGAATTGGCGGGGGAGC ACCACAACGCGTGGAGCCTGCGGTTTAATTGGATTCAACGCCGGACATCTCACCAGGAGCGACAGCAGAATG ATAATCAGGTTGATGACCTTATTTGACTAGCTGAGAGGAGGTGCATGGCCGCCGTCAGCTCGTACTGTGAAG CGTCCTGTTAAGTCAGGCAACGAGCGAGACCCACGCCCTTAGTTACCAGCTTAATCTTCGGATTGATGGGCAC ACTAAGGGGACCGCCAGTGATAAACTGGAGGAAGGAGTGGACGACGGTAGGTCCGTATGCCCCGAATCTCC TGGGCTACACGCGGGCTACAATGGCTATAGCAATGGGTTTCTTCACTGAAAAGTGGTGATAATCTCCTAAATA TAGTCTTAGTTCGGATTGAGGGCTGTAACTCGCCCTCATGAAGCTGGAATGCGTAGTAATCGCATGTCACAAC CGTGCGGTGAATACGTCCCTGCTCCTTGCACACACCG
Methanocorpusculum sp H1B2 Sanger 16S sequence	GCTCAGTAACACGTGGTTAATCTGCCCTTGGGTGGAGGATACTCCCGGGAAACTGGGGCTAATACTCCATAG TGAATGCATGCTGGAATGCTGCATTCTCGAAAGATTCATCGCCCAAGGATGAGACTGCGTCCGATTAGGTCG TTGGTGGGGTAACGGCCCACCAAGCCTTTTATCGGTACGGGTTGTGGGAGCAAGATCCCGGAGATGGATTCT GAGACATGAATCCAGGCCCTACGGGGCGCAGCAGGCGCGCAAACTTTACAATGCGAGCAATCGTGATAAGG AAACCCTGAGTGCCTGTCGATGCAGGCTGTTCATATGTTTAAATTGCATGTGAAGAAAGGGCAGGGCAAGAC CGGTGCCAGCCGCCGCGGTAATACCGGCTGCTCGAGTGATGGCCACTATTACTGGGTTTAAAGCGTCCGTAG CTTGACTGTTAGGTCTCTTGGGAAATCTTTTGGCTTAACCAAAAGGCGTCTAAGAGATACCGGCATTCTTGGA ACTGGGAGAGGTAAGCCGTACTTCGGGGGTAGGAGTGAAATCTTGTAATCCTCGAGGGACGACCTATGGCG AAGGCAGCTTACCAGAACAGCTTCGACAGTGAGGGACGAAAGCTGGGGGAGCAAACGGGATTAGATACCCC GGTAGTCCCAGCCGTAAACAATGTGCGTTAGGTGTGTCGGTTACCACGCGTAACTGATGCGCCGAAGAGAAA TCGTGAAACGCACCACCTGGGAAGTACGGTCGCAAGGCTGAAACTTAAAGGAATTGGCGGGGGAGCACCAC AACAGGTGGAGCCTGCGGTTTAATTGGATTCAACGCCGGACATCTCACCGGATAAGACAGCTGAATGATTGT CAATCTGAAGGTTTTACATGACTAGCTGAGAGGAGGTGCATGGCCGTCGTCAGTTCGTACTGTGAAGCATCC TGTTAAGTCAGGCAACGAGCGAGACCCACGCCAACAATTGCCAGCAGCATCTCCGGATGGCTGGGGACATTG TTGGGACCGCCTCTGCTAAAGGGGAGGAAGGAATGGGCAACGGTAGGTCAGCATGCCCCGAATTATCCGGG CTACACGCGGGCTACAATGGCCGGGACAATGGGTAACGACACCGAAAGGTGCAGTCAATCTCCTAACCCCGG CCTTAGTTAGGATTGCGGGTTGCAACTCACCCGCATGAATCTGGAATCTGTAGTAATCGCGTTTCACTATAGC GCGGTGAATACGTCCCTGCTCCTTGCACACACCG
Sanger Sequencing ITS region primers	JB205: GGAAGTAAAAGTCGTAACAAGG JB206: TCCTCCGCTTATTAATATGC
Piromyces sp H1B2 representative ITS sequence	GGAAGTAAAAGTCGTAACAAGGTTTCCGTAGGTGAACCTGCGGAAGGATCATTAATAAATTTTTTTTTGAAAT AAAAAAAAAAATAATAATCATCCTACCCTTTGTGAATTTGTTTTGTTGAATAATTTTTTAAAATTATTTATTGTC TATCCAAGTTGGAAAGTTGTAAAAGACTCGAAGACTTGGAGCGGCATAAATAAATTTAAAGACTTTTTTCTAA AATTAAACTTTTTGTATTCATTTGTCTAAAATAATTTTTATAATTTATAAAAACAACTTTTGACAATGGATCTCTT GGTTCTCGCAACGATGAAGAACGCAGCAAAATGCGATAAGTAATGTGAATTGCAGAATACGTGAATCATCGA ATCTTCGAACGCATATTGCACTTTTTTAGTTTACTAAAAAAGTATGTCTGTTTGAGTATCAGTAAAATATTCTCA TAAAATTTTTAATTTTATGGTTATGAGTGTTTTACAACGATAAACAAGTTGTTTCACTTTAAATTGAATTTTTGA AGAAATTCTTTAATTCAAATTAAATTTGGATAAAATTTTCCCGTAAAAAGGAATAACTATACAAGTTTAATAAG AATTTGGATTTCTTTTATAAAAGGAAATTTATTAAATAACTTGATCTCAAATCAGATAAGAGTACCCGCTGAAC TTAAGCATATTAATAAGCGGAGGA
Illumina sequencing 16S amplicon primers	P5-783F: tcgtcggcagcgtcagatgtgtataagagacagGGWTTAGAWACCCBDGTAGTCC P7-894R: gtctcgtgggctcggagatgtgtataagagacagCGTACTYCCCAGGYGG
Illumina sequencing ITS amplicon primers	P5-ITS1: tcgtcggcagcgtcagatgtgtataagagacagTCCTACCCTTTGTGAATTTG P7-ITS4: gtctcgtgggctcggagatgtgtataagagacagCTGCGTTCTTCATCGTTGCG

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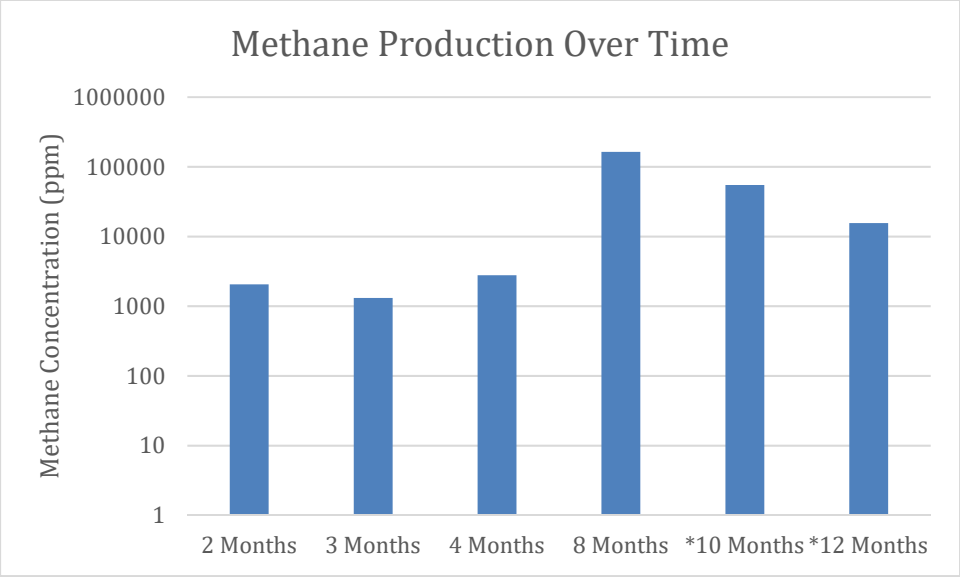
648 Table S2: Metagenomic sequencing statistics

Assembly	Whole Metagenome	*Methanosphaera* Bin	*Methanocorpusculum* Bin	*Sphaerochaeta* Bin
# contigs (>500 bp)	203	27	67	65
Total Length (bp)	6659799	1779166	2045527	2880050
GC %	48.26	29.68	53.35	55.74
N50	28	4	10	14
L50 (bp)	74369	128533	75586	77038

649

650 Figure S1: Methane Production over time



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652 Figure S2: Phylogeny of Consortia Members

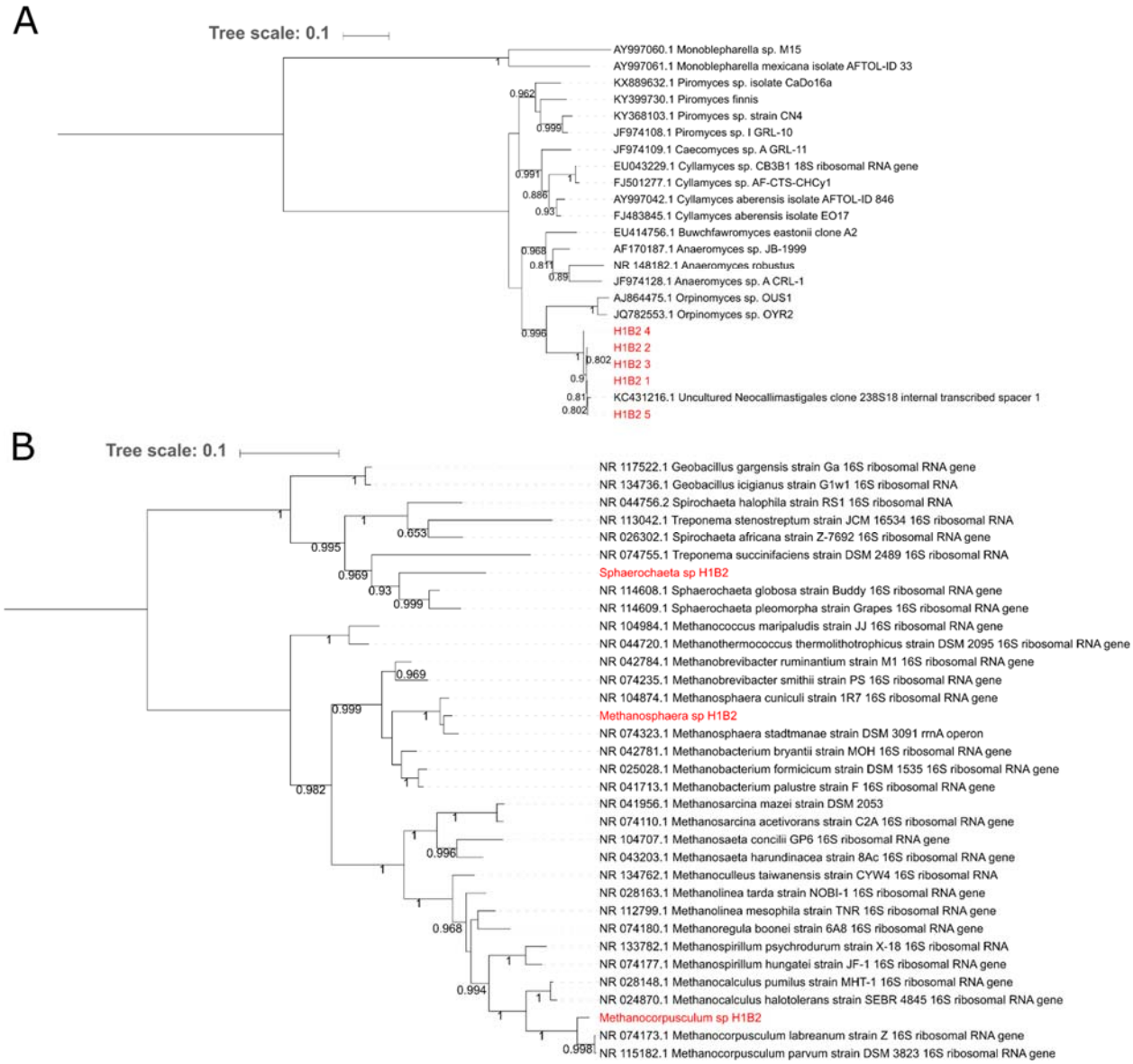


Figure S3: Morphological features of *Piromyces sp.* H1B2

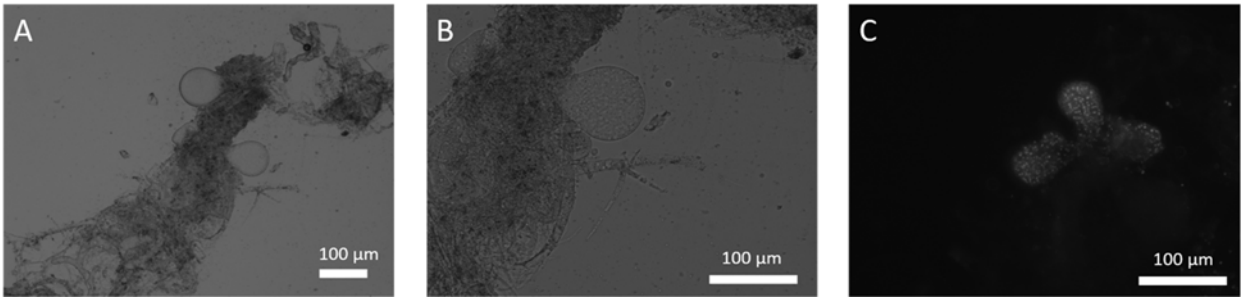
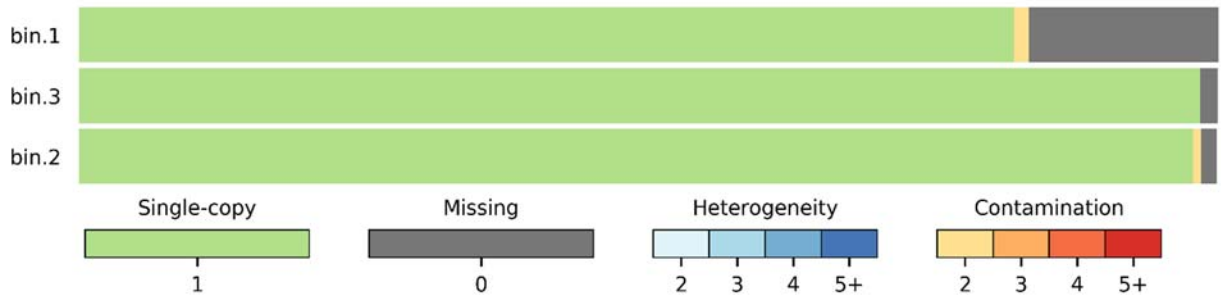


Figure S4: Genome Completion as Determined by CheckM


bin.1
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Supporting Information: Top-down Enrichment Guides in Formation of Synthetic Microbial Consortia for Biomass Degradation

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Discussion S1: *Meta-analysis of Published Literature Related to Methanogen-Fungus Co-Cultivation*

Metabolite exchanges occur between methanogenic archaea and anaerobic fungi in our datasets and others (cited in Table S4 below). Many previously published studies suggest that fungus-methanogen interactions accelerates the deconstruction of plant biomass, resulting in increased fermentation gas production. To address this hypothesis, we analyzed literature purporting to demonstrate increased substrate consumption in co-cultures of anaerobic fungi and methanogens relative to fungal monocultures, and investigated the data from these studies to determine whether suggested outcomes were statistically significant (Bauchop and Mountfort 1981; Mountfort et al. 1982; Marvin-Sikkema et al. 1990; Teunissen et al. 1991, 1992). Overall, we found that previous reports of increased deconstruction by co-culture were limited to qualitative assessments of substrate degradation rates.

Data were extracted from previous publications that concluded the presence of methanogenic archaea increased substrate deconstruction by anaerobic fungi and tested for statistical differences in rate of cellulose deconstruction using analysis of co-variance (ANCOVA) in the same manner that we examined our own deconstruction experiments presented here (Table S4). Contrary to concluding statements made in the publications themselves, we found no statistically supported evidence that co-cultivation with methanogens increased the rate of substrate deconstruction by these cultures ($p=0.18$-0.99, Table S3). Given the revised interpretation of the data, results agree that a fungal-methanogen syntrophy does not necessarily increase rates of substrate deconstruction.

Table S1: Primers used and 16S and ITS sequences from enriched consortia

Sanger Sequencing Methanogen-specific 16S primers	Met86F: GCTCAGTAACACGTGG Met1340R: CGGTGTGTGCAAGGAG
Methanosphaera sp H1B2 Sanger 16S sequence	GCTCAGTAACACGTGGATAACTTACCCTTAGGACTGGGATAACTCTGGGAAACTGGGGATAATACCGGATAT TAGGCTATGCCTGGAATGGTTTGCCTTTGAAACGTATTTTTCGCCTAAGGATAGATCTGCGGCTGATTAGGTC GTTGGTGGGGTAATGGCCCACCAAGCCTATGATCGGTACGGGTTGTGAGAGCAAGAGCCCGGAGATGGAAC CTGAGACAAGGTTCCAGACCCTACGGGGTGCAGCAGGCGCGAAACCTCCACAATGTACGAAAGTGCGATGG GGGAATCCCAAGTGTTATTCTTAACAGAATAGCTTTTCATTAGTGTAAAAAGCTTTTAGAATAAGAGCTGGGC AAGACCGGTGCCAGCCGCCGCGGTAACACCGGCAGCTCGAGTGGTAGCTGTTTTTATTGGGCCTAAAGCGTT CGTAGCCGGTTTGATTAGTCTTTGGTGAAAGCTTGTAGCTTAACTATAAGAATTGCTGGAGATACTATCAGAC TTGAAGTCGGGAGAGGTTAGAGGTACTACCGGGGTAGGGGTGAAATCCTATAATCCTGGGAGGACCACCTG TGGCGAAAGCGTCTAACTAGAACGATCTTGACGGTGAGTAACGAAAGCCAGGGGCGCGAACCGGATTAGAT ACCCGGGTAGTCCTGGCCGTAAACGATGTGGACTTGGTGTTGGAATGGCTTCGAGTTGTTCCAGTGCCGAAG GGAAGCTGTTAAGTCCACCGCCTGGGAAGTACGGTCGCAAGACTGAAACTTAAAGGAATTGGCGGGGGAGC ACCACAACGCGTGGAGCCTGCGGTTTAATTGGATTCAACGCCGGACATCTCACCAGGAGCGACAGCAGAATG ATAATCAGGTTGATGACCTTATTTGACTAGCTGAGAGGAGGTGCATGGCCGCCGTCAGCTCGTACTGTGAAG CGTCCTGTTAAGTCAGGCAACGAGCGAGACCCACGCCCTTAGTTACCAGCTTAATCTTCGGATTGATGGGCAC ACTAAGGGGACCGCCAGTGATAAACTGGAGGAAGGAGTGGACGACGGTAGGTCCGTATGCCCCGAATCTCC TGGGCTACACGCGGGCTACAATGGCTATAGCAATGGGTTTCTTCACTGAAAAGTGGTGATAATCTCCTAAATA TAGTCTTAGTTCGGATTGAGGGCTGTAACTCGCCCTCATGAAGCTGGAATGCGTAGTAATCGCATGTCACAAC CGTGCGGTGAATACGTCCCTGCTCCTTGCACACACCG
Methanocorpusculum sp H1B2 Sanger 16S sequence	GCTCAGTAACACGTGGTTAATCTGCCCTTGGGTGGAGGATACTCCCGGGAAACTGGGGCTAATACTCCATAG TGAATGCATGCTGGAATGCTGCATTCTCGAAAGATTCATCGCCCAAGGATGAGACTGCGTCCGATTAGGTCG TTGGTGGGGTAACGGCCCACCAAGCCTTTTATCGGTACGGGTTGTGGGAGCAAGATCCCGGAGATGGATTCT GAGACATGAATCCAGGCCCTACGGGGCGCAGCAGGCGCGCAAACTTTACAATGCGAGCAATCGTGATAAGG AAACCCTGAGTGCCTGTCGATGCAGGCTGTTCATATGTTTAAATTGCATGTGAAGAAAGGGCAGGGCAAGAC CGGTGCCAGCCGCCGCGGTAATACCGGCTGCTCGAGTGATGGCCACTATTACTGGGTTTAAAGCGTCCGTAG CTTGACTGTTAGGTCTCTTGGGAAATCTTTTGGCTTAACCAAAAGGCGTCTAAGAGATACCGGCATTCTTGGA ACTGGGAGAGGTAAGCCGTACTTCGGGGGTAGGAGTGAAATCTTGTAATCCTCGAGGGACGACCTATGGCG AAGGCAGCTTACCAGAACAGCTTCGACAGTGAGGGACGAAAGCTGGGGGAGCAAACGGGATTAGATACCCC GGTAGTCCCAGCCGTAAACAATGTGCGTTAGGTGTGTCGGTTACCACGCGTAACTGATGCGCCGAAGAGAAA TCGTGAAACGCACCACCTGGGAAGTACGGTCGCAAGGCTGAAACTTAAAGGAATTGGCGGGGGAGCACCAC AACAGGTGGAGCCTGCGGTTTAATTGGATTCAACGCCGGACATCTCACCGGATAAGACAGCTGAATGATTGT CAATCTGAAGGTTTTACATGACTAGCTGAGAGGAGGTGCATGGCCGTCGTCAGTTCGTACTGTGAAGCATCC TGTTAAGTCAGGCAACGAGCGAGACCCACGCCAACAATTGCCAGCAGCATCTCCGGATGGCTGGGGACATTG TTGGGACCGCCTCTGCTAAAGGGGAGGAAGGAATGGGCAACGGTAGGTCAGCATGCCCCGAATTATCCGGG CTACACGCGGGCTACAATGGCCGGGACAATGGGTAACGACACCGAAAGGTGCAGTCAATCTCCTAACCCCGG CCTTAGTTAGGATTGCGGGTTGCAACTCACCCGCATGAATCTGGAATCTGTAGTAATCGCGTTTCACTATAGC GCGGTGAATACGTCCCTGCTCCTTGCACACACCG
Sanger Sequencing ITS region primers	JB205: GGAAGTAAAAGTCGTAACAAGG JB206: TCCTCCGCTTATTAATATGC
Piromyces sp H1B2 representative ITS sequence	GGAAGTAAAAGTCGTAACAAGGTTTCCGTAGGTGAACCTGCGGAAGGATCATTAATAAATTTTTTTTTGAAAT AAAAAAAAAAATAATAATCATCCTACCCTTTGTGAATTTGTTTTGTTGAATAATTTTTTAAAATTATTTATTGTC TATCCAAGTTGGAAAGTTGTAAAAGACTCGAAGACTTGGAGCGGCATAAATAAATTTAAAGACTTTTTTCTAA AATTAAACTTTTTGTATTCATTTGTCTAAAATAATTTTTATAATTTATAAAAACAACTTTTGACAATGGATCTCTT GGTTCTCGCAACGATGAAGAACGCAGCAAAATGCGATAAGTAATGTGAATTGCAGAATACGTGAATCATCGA ATCTTCGAACGCATATTGCACTTTTTTAGTTTACTAAAAAAGTATGTCTGTTTGAGTATCAGTAAAATATTCTCA TAAAATTTTTAATTTTATGGTTATGAGTGTTTTACAACGATAAACAAGTTGTTTCACTTTAAATTGAATTTTTGA AGAAATTCTTTAATTCAAATTAAATTTGGATAAAATTTTCCCGTAAAAAGGAATAACTATACAAGTTTAATAAG AATTTGGATTTCTTTTATAAAAGGAAATTTATTAAATAACTTGATCTCAAATCAGATAAGAGTACCCGCTGAAC TTAAGCATATTAATAAGCGGAGGA
Illumina sequencing 16S amplicon primers	P5-783F: tcgtcggcagcgtcagatgtgtataagagacagGGWTTAGAWACCCBDGTAGTCC P7-894R: gtctcgtgggctcggagatgtgtataagagacagCGTACTYCCCAGGYGG
Illumina sequencing ITS amplicon primers	P5-ITS1: tcgtcggcagcgtcagatgtgtataagagacagTCCTACCCTTTGTGAATTTG P7-ITS4: gtctcgtgggctcggagatgtgtataagagacagCTGCGTTCTTCATCGTTGCG

Table S2: Metagenomic sequencing statistics of the prokaryotic strains sequenced from a naturally-enriched microbial consortium.

Assembly	**Whole Metagenome**	***Methanosphaera* Bin**	***Methanocorpusculum* Bin**	***Sphaerochaeta* Bin**
# contigs (>500 bp)	203	27	67	65
Total Length (bp)	6659799	1779166	2045527	2880050
GC %	48.26	29.68	53.35	55.74
N50	28	4	10	14
L50 (bp)	74369	128533	75586	77038

Table S3: Supernatant metabolites measured after 10 days of fungal or co-culture growth on two representative substrates. The top panel depicts metabolites following growth on Whatman filter paper (WP), and the bottom panel shows the same data for growth supported on reed canary grass (RCG). Reported errors represent standard deviations of biological replicates (n=3).

WP	Pressure (psig)	Hydrogen (%)	Methane (%)	Reducing sugars (g/L)	Formate (g/L)	Acetate (g/L)	Lactate (g/L)
N. californiae	10.2 ± 0.4	14.6 ± 2.6	0.0 ± 0.0	6.7 ± 2.9	0.9 ± 0.0	0.8 ± 0.1	0.8 ± 0.1
N.c. + M. bryantii	6.2 ± 0.3	0.0 ± 0.0	4.1 ± 0.1	5.6 ± 0.7	0.0 ± 0.0	1.0 ± 0.2	0.1 ± 0.0
A. robustus	4.5 ± 0.4	8.8 ± 0.7	0.0 ± 0.0	0.1 ± 0.1	0.5 ± 0.0	2.5 ± 0.3	0.0 ± 0.0
A.r. + M. bryantii	6.6 ± 0.4	0.0 ± 0.0	12.0 ± 1.7	0.0 ± 0.0	0.0 ± 0.0	1.2 ± 0.2	0.0 ± 0.0

RCG	Pressure (psig)	Hydrogen (%)	Methane (%)	Reducing sugars (g/L)	Formate (g/L)	Acetate (g/L)	Lactate (g/L)
N. californiae	10.4 ± 0.7	13.6 ± 2.1	0.0 ± 0.0	0.5 ± 0.0	0.7 ± 0.1	1.0 ± 0.2	1.0 ± 0.2
N.c. + M. bryantii	10.0 ± 0.2	0.0 ± 0.0	4.3 ± 0.2	0.6 ± 0.0	0.0 ± 0.0	1.5 ± 0.1	0.5 ± 0.2
A. robustus	6.7 ± 0.1	13.3 ± 2.4	0.0 ± 0.0	0.0 ± 0.0	0.7 ± 0.1	1.0 ± 0.0	0.0 ± 0.0
A.r. + M. bryantii	7.9 ± 0.2	0.0 ± 0.0	12.7 ± 3.0	0.1 ± 0.0	0.0 ± 0.0	1.2 ± 0.2	0.0 ± 0.0

Table S4: Extraction and re-analysis of published literature shows that fungal-methanogen co-cultivation does not result in increased substrate solubilization rate compared to fungal monoculture**.** The data purporting to show that methanogens increase the rate of substrate solubilization was analyzed using the same ANCOVA analysis as was used for the experimental data depicted in Figure 5. In each case, it was found that the rate of substrate solubilization difference between fungal monocultures co-cultures was not statistically significant ($p>0.05$). This finding is in agreement with the results presented in Figure 5 in the main text between *N. c.* and *M. b.*, and *A. r*. and *M. b.*, that also found no significant change.

Source Citation(s)	Organisms	ANCOVA p-value(s)	Notes
The Rumen and its Microbes, Hungate 1966	N/A	N/A	Origin of hypothesis
Bauchop and Mountfort *Applied and Environmental Microbiology*, 1981	Unidentified fungus + *M. barkeri* and *Methanobrevibacter* sp. RA1	p=0.1269	
Mountfort et al. *Applied and Environmental Microbiology*, 1982	Organisms not identified	p=0.9155	
Marvin-Sikkema et al. *Applied and Environmental Microbiology*, 1990	*Neocallimastix* sp. L2 + *M. bryantii, M. smithii,* or *M. aboriphilus*	p=0.1977	
Teunissen et al. *Archives of Microbiology*, 1991 & 1992	Several *Neocallimastix* and *Piromyces* + *M. formicicium*	p=0.32, 0.62, 0.18, 0.53	Data in 2 separate papers; 4 different comparisons
Li et al. 2017 *Journal of Basic Microbiology*, 2017	*Piromyces* sp. F1 + *M. thaueri*	p=0.99	Author scorrectly note no enhanced degradation by coculture
This work	*Neocallimastix californiae* + *M. bryantii*	p=0.3554	
This work	*Neocallimastix* sp. S3 + *M. bryantii*	p=0.1844	
This work	*Anaeromyces robustus* + *M. bryantii*	p=0.1831	

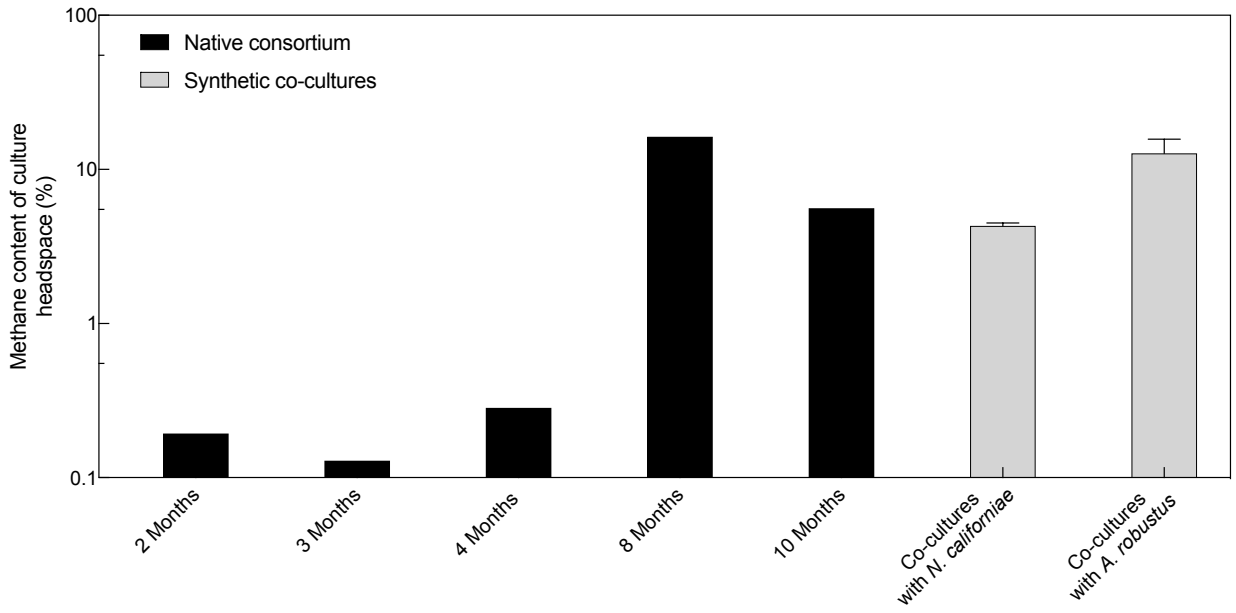

Figure S1: Methane production increases in an enriched native community over time and is comparable to methane produced by synthetic pairings. Headspace methane concentrations were periodically measured via gas chromatography (GC) during consecutive batch culture of the enriched native consortium and during subsequent experiments with indicated synthetic co-cultures. Methane production by the native consortium was low at initial enrichment, but eventually reached the levels seen in synthetic co-culture experiments. For the native consortium, bars represent single measurements of the culture headspace for methane percentage, but for synthetic pairings bars represent the means of biological triplicates and error bars represent the standard deviation.

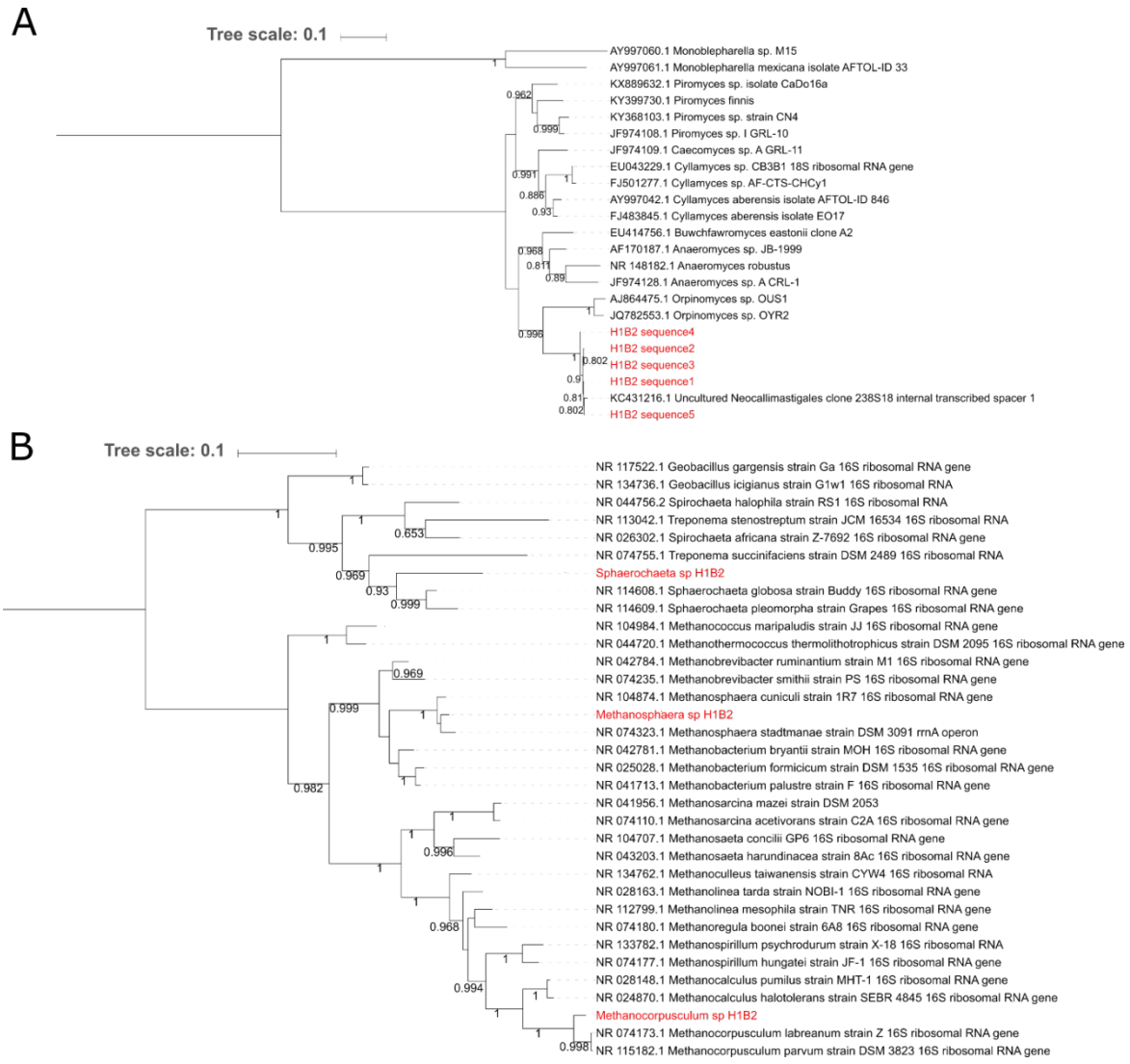

Figure S2: Phylogenetic placement of the microbial consortium members enriched from horse feces. A) ITS sequence alignment reveals that the fungus (H1B2 sequence 1-5) clusters distinctly from other sequenced fungal isolates. Five different Sanger sequencing runs were used due to the difficulties associated with the high AT% in the anaerobic fungal ITS regions B) 16S alignment of the three distinct sequences reveals two methanogens (*Methanocorpusculum* and *Methanosphaera*, denoted as *Methanosphaera* sp. H1B2 and *Methanocorpusculum* sp. H1B2) and one bacterium (*Sphaerochaeta*, denoted *Sphaerochaeta* sp. H1B2).

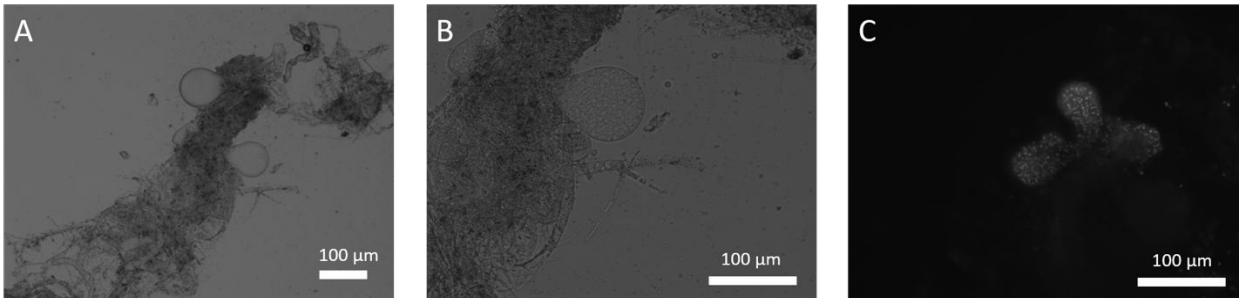

Figure S3: Light microscopy of the top-down enriched anaerobic consortium reveal a *Piromyces*-like fungus. Light microscopy of the fungal isolate (A and B) revealed abundant tapering rhizoids, multinucleated sporangia, and absence of nuclei in rhizoids with DAPI staining (C), which are generic characteristics for *Piromyces*.

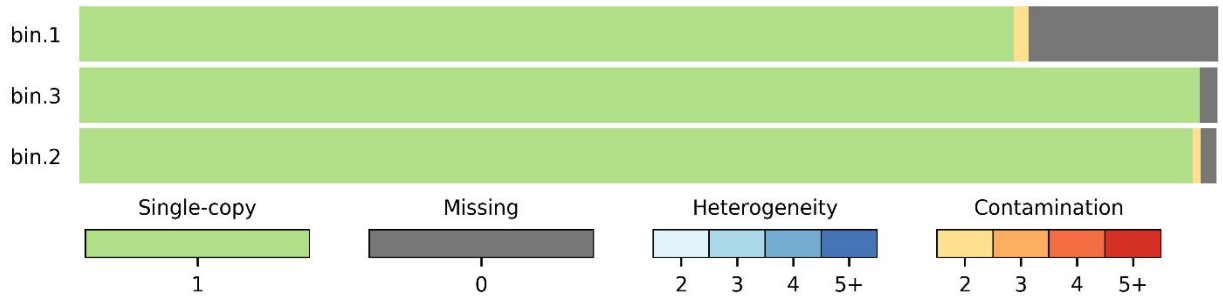

Figure S4: Metagenome assembled genome completion is high and contamination is low as determined by CheckM. The assembled metagenome was binned into distinct genome bins based on read coverage and tetranucleotide frequency using Metabat and CONCOCT. The completeness and quality of each bin was determined using the single copy gene sets employed through the CheckM method. Each bin achieved better than 90% completion with limited (< 2%) contamination and heterogeneity, qualifying as high quality Metagenome Assembled Genomes.

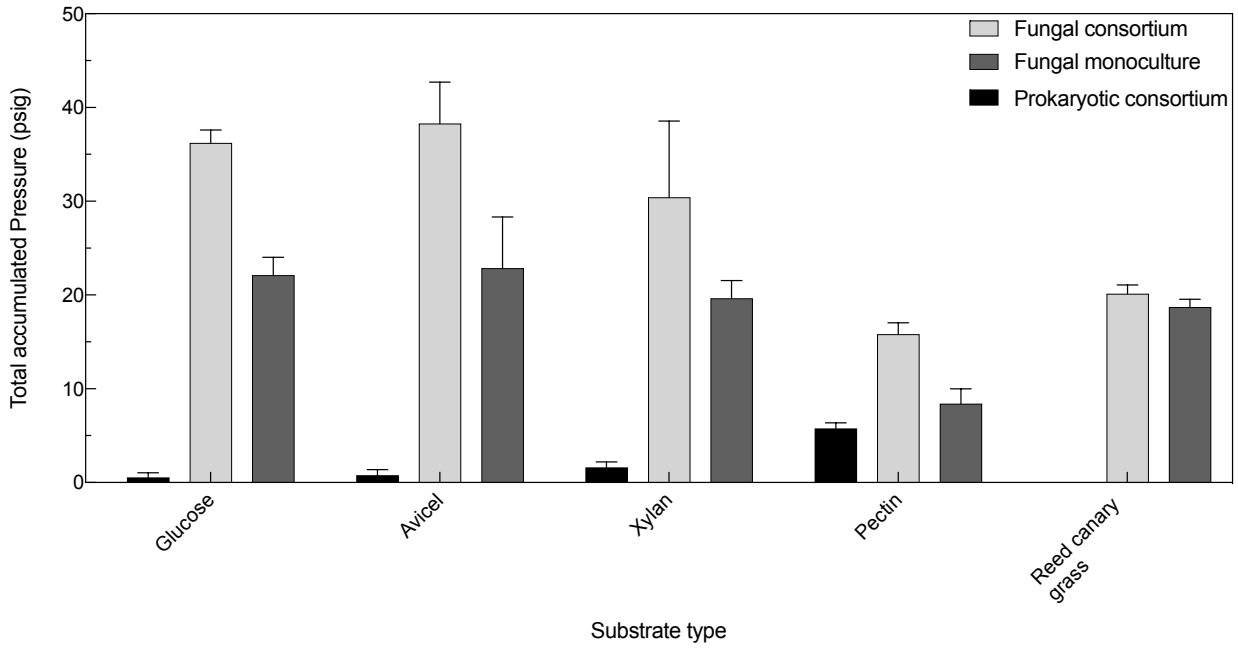

Figure S5: Total fermentation gas production by the enriched natural microbial consortia, the prokaryotic component of the consortium, and the fungal component of the consortium. Accumulation of total fermentation gases were tracked for 10 days in triplicate cultures that were vented daily, supported on the indicated substrates. Pressure accumulated was measured using the pressure transducer technique. Error bars represent the standard error of the mean (n=3).

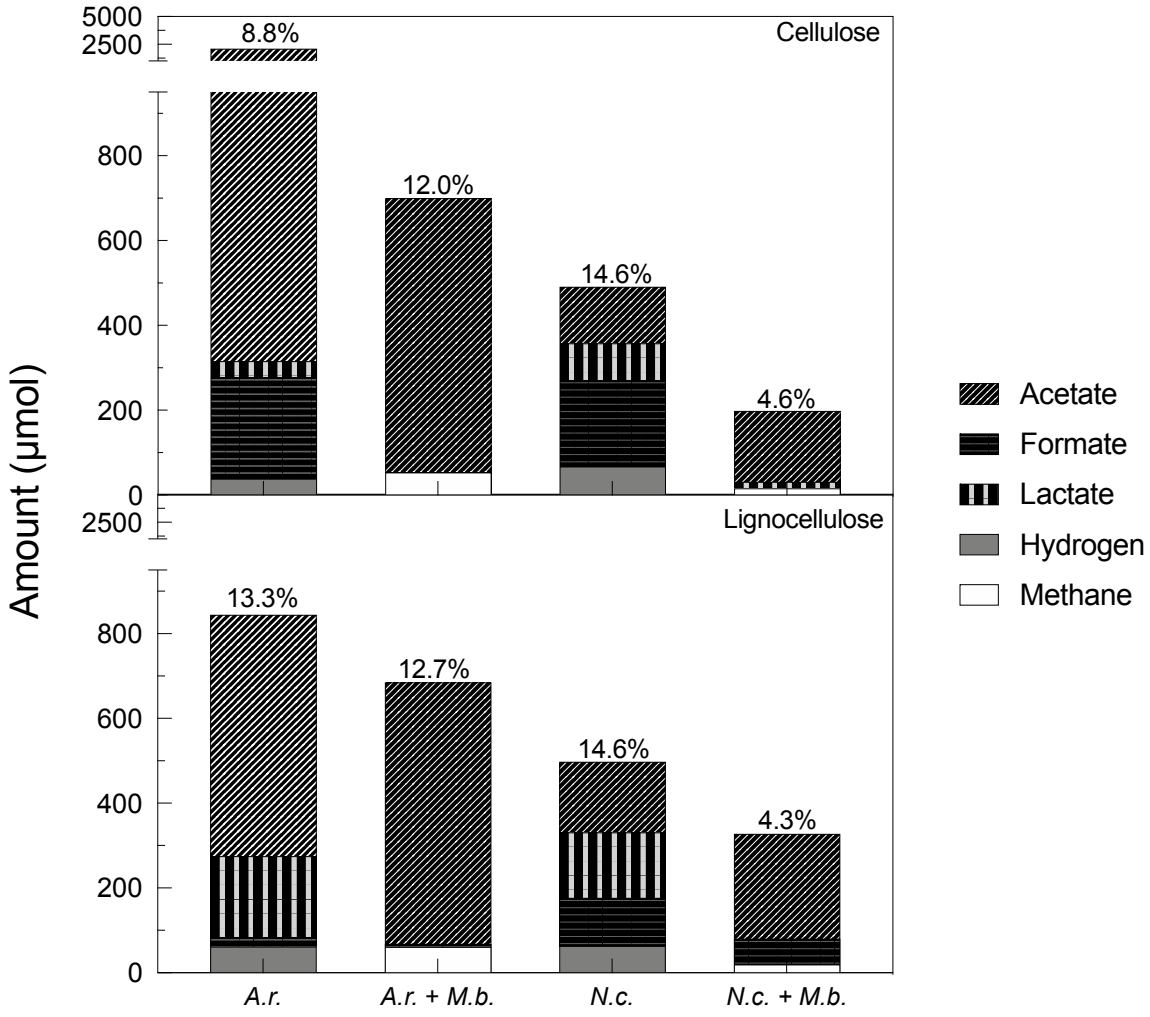

Figure S6: The presence of the methanogenic archaeon *Methanobrevibacter bryantii* (*M.b.*) alters the end-point metabolite profile of anaerobic fungi grown on both cellulosic and lignocellulosic substrates. Data were collected for two strains of anaerobic fungi, *N. californiae* (*N.c.*) and *A. robustus* (*A.r.*) in both the presence and absence of the methanogen *M. bryantii (M.b.)*. The bar displayed for each metabolite is the result of averaging three biological replicates. Standard deviations are not shown for clarity; however, values obtained from liquid chromatography have standard deviations less than 10% of the mean, and values obtained from gas chromatography have standard deviations

less than 17% of the mean (Table S3). Percentages indicated at the top of each bar-stack refer to the mean percentage of headspace gas that was measured as hydrogen in monocultures or as methane in co-cultures (n=3).

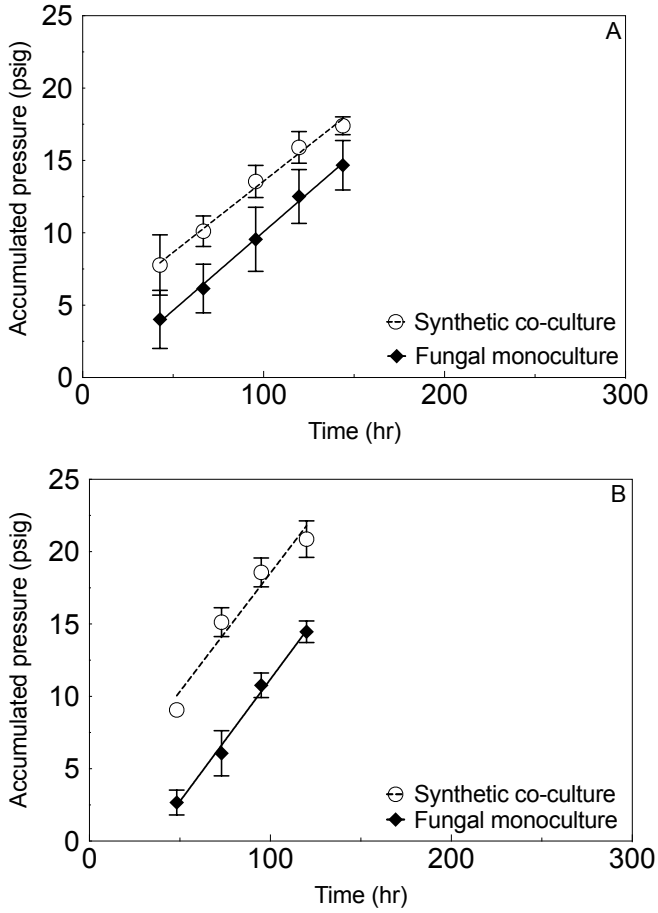

Figure S7: Comparison of gas production rates of fungal monocultures with gas production rates of synthetic co-cultures of anaerobic fungi and methanogens: Two anaerobic fungi were grown in isolation and paired with the methanogen *Methanobrevibacter bryantii* (*M.b.*) while total fermentation gas production was tracked using the pressure transducer technique. Data and linear regression models for *Neocallimastix californiae* (*N.c.*) and *M.b.* are shown in panel A while data and regression models for *Anaeromyces robustus* (*A.r.*) and *M.b.* are shown in panel B. Linear regressions were calculated for the steady state portion of culture growth which excluded lag and stationary phases. Slopes of linear regressions (*N.c.*=0.11±0.00 psig/hr, *N.c.*+*M.b.*=0.10±0.00 psig/hr, *A.r.*=0.17±0.01 psig/hr, *A.r.*+*M.b.*=0.16±0.03 psig/hr) were compared using

ANCOVA which determined that there was no statistically significant difference in the rate of fermentation gas production ($p>0.05$)

Supplemental Database 2: Transporter analysis of metagenomes

Number of high quality sugar transporters per MAG by predicted substrate

Predicted substrate	MAG= Sphaerochaeta_H1B2	Methanosphaera_H1B2
Maltose or maltooligosaccaride	5	0
Mannose	2	0
Glucose or galactose	2	0
Lipopolysaccride	1	0
Alginic acid	1	0
Xylan	1	0
N-acetyl glucosamine	0	1
Glycosyl groups	0	0
Total sugar transporters	12	1

Methanocorpusculum_H1B2
0
0
0
0
0
0
0
2
2