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1 **Co-cultivation of the anaerobic fungus *Caecomyces churrovis* with *Methanobacterium bryantii***
2 **enhances transcription of carbohydrate binding modules**

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33 **Abstract:**

34 Anaerobic fungi and methanogenic archaea are two classes of microorganisms found in the rumen
35 microbiome that metabolically interact during lignocellulose breakdown. Here, stable synthetic co-cultures
36 of the anaerobic fungus *Caecomyces churrovis* and the methanogen *Methanobacterium bryantii* (not native
37 to the rumen) were formed, demonstrating that microbes from different environments can be paired based
38 on metabolic ties. Transcriptional and metabolic changes induced by methanogen co-culture were evaluated
39 in *C. churrovis* across a variety of substrates to identify mechanisms that impact biomass breakdown and
40 sugar uptake. A high-quality genome of *C. churrovis* was obtained and annotated, which is the first
41 sequenced genome of a non-rhizoid forming anaerobic fungus. *C. churrovis* possess an abundance of
42 CAZymes and carbohydrate binding modules and, in agreement with previous studies of early-diverging
43 fungal lineages, N6-methyldeoxyadenine (6mA) was associated with transcriptionally active genes. Co-
44 culture with the methanogen increased overall transcription of CAZymes, carbohydrate binding modules,
45 and dockerin domains in co-cultures grown on both lignocellulose and cellulose and caused upregulation
46 of genes coding associated enzymatic machinery including carbohydrate binding modules in family 18 and
47 dockerin domains across multiple growth substrates relative to *C. churrovis* monoculture. Two other fungal
48 strains grown on a reed canary grass substrate in co-culture with the same methanogen also exhibited high
49 log2fold change values for upregulation of genes encoding carbohydrate binding modules in families 1 and
50 18. Transcriptional upregulation indicated that co-culture of the *C. churrovis* strain with a methanogen may
51 enhance pyruvate formate lyase (PFL) function for growth on xylan and fructose and production of
52 bottleneck enzymes in sugar utilization pathways, further supporting the hypothesis that co-culture with a
53 methanogen may enhance certain fungal metabolic functions. Upregulation of CBM18 may play a role in
54 fungal-methanogen physical associations and fungal cell wall development and remodeling.

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56 **Introduction**

57 Anaerobic fungi are efficient degraders of recalcitrant lignocellulosic biomass that are found in the
58 guts of herbivores. The high number of CAZymes (carbohydrate active enzymes) that anaerobic fungi
59 produce has driven efforts to collect genomic and transcriptomic data for a variety of emerging anaerobic
60 fungal species [1–5]. Gut fungi function within a community of biomass-degrading bacteria, protozoa, and
61 methanogenic archaea linked by complex metabolic interactions and functional redundancy.[6] Isolating
62 individual members of these natural consortia is one approach to develop a more detailed understanding of
63 microbial interactions, which can then be used to design optimized consortia for biotechnological
64 applications to break down lignocellulose-rich waste. These microbes can be selected through "top-down"
65 enrichment techniques such as serial cultivation or antibiotic treatment to isolate syntrophic pairs of fungi
66 and methanogens from naturally-occurring consortia. Alternatively, communities can be formed using
67 "bottom up" methods mixing separate axenic cultures of these microbes to create synthetic pairings linked
68 by metabolic dependency.[6–8]

69 Fungal-methanogen co-cultures have been extensively studied due to the mutually beneficial
70 relationship between the two organisms resulting from their complementary metabolism. – fungi produce
71 hydrogen (H_2) as an unwelcome byproduct of their own metabolism, which methanogens use in the
72 biosynthesis and release of methane [8–14] Many previous studies report that co-cultivation of anaerobic fungi
73 with methanogens can enhance biomass breakdown, but the metabolic mechanisms responsible for this
74 outcome are unclear and not uniformly reproducible.[15–19] For example, a recent study concluded that the
75 removal of fungal metabolites by methanogens does not increase the rate of gas production or the rate of
76 substrate deconstruction by a synthetic community of fungi and methanogens relative to fungal
77 monocultures.[8] It has also been hypothesized that co-cultivation of fungi and methanogens results in
78 increased sugar utilization and flux through the fungal hydrogenosome through increased transport and
79 carbon conversion.[14,20] Additionally, we recently reported that *M. bryantii* enhances the transcription of
80 genes encoding ABC transporters, MFS transporters and G-protein coupled receptors (GPCRs) in the
81 fungus *Anaeromyces robustus*, indicating that co-cultivation may increase the rate of sugar utilization

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82 through the increased expression of sugar transporters.[9] Although many studies have been conducted to
83 determine how co-cultivation with methanogens affects fungal metabolism and biomass breakdown, none
84 have characterized transcriptional and metabolic outcomes across a variety of relevant substrates, which is
85 critical to detangling competing effects of substrate response.[9,10]

86 Here, we present the first genome of an anaerobic non-rhizoid forming fungus of the *Caecomyces*
87 genus, and further examine its transcriptional response to the presence of methanogens in multiple synthetic
88 co-cultures supported on lignocellulose, hemicellulose, cellulose, and sugars. *Caecomyces churrovis* lacks
89 the extensive rhizoid network formed by other previously sequenced anaerobic gut fungi to aid in biomass
90 breakdown. Improvements in long-read sequencing technologies enabled assembly and annotation of
91 CAZymes and associated cellular machinery despite the complex fungal physiology, unknown ploidy, AT-
92 content, and repeat-richness. By combining RNA-seq with growth and chemical data, we determine how
93 the fungus responds to co-cultivation with a non-native methanogen in synthetic co-culture. While other
94 studies have examined global transcriptomic response and CAZyme regulation in anaerobic fungi cultivated
95 with methanogens on a single substrate, none to date have explored regulation across a range of substrates
96 or differences occurring in transcriptional regulation between multiple fungal strains on the same
97 substrate.[9,10] Through a combination of genomic, transcriptomic, and metabolomic data we found that the
98 *Caecomyces churrovis* genome possesses an abundance of both CAZymes and carbohydrate binding
99 modules as shown in Figure 1. Co-culture of *C. churrovis* with a non-native methanogen enhances
100 transcription of gene sets associated with fungal substrate binding and fungal-methanogen interactions such
101 as carbohydrate binding modules in families 1 and 18, pyruvate formate lyase (PFL) function in the cytosol
102 or possibly the hydrogenosome, and enzymes that are potential bottlenecks for sugar utilization in fungi
103 across multiple substrates. Overall, understanding how methanogen co-culture influences the fibrolytic and
104 metabolic behavior of anaerobic fungi aids in the design of new strategies for conversion of lignocellulose
105 to fermentable sugars and value-added products, as well as the genetic mechanisms that underpin fungal-
106 methanogen interactions.
107

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108 **Methods**

109 **Growing and harvesting cultures for RNA extractions.**

110 Anaerobic serum bottles containing 80 mL of modified medium C ("MC-") with 0.8 mL 100 × vitamin
111 solution and 0.8 g reed canary grass were inoculated with cultures of *C. churrovis* and *M. bryantii*: 1.0 mL
112 of *C. churrovis* or a combination of 1.0 mL of *C. churrovis* and 1.0 mL of *M. bryantii* (routine cultures
113 were cultivated as described previously by Swift, et al.).[9] The fungal and methanogen co-cultures and fungal
114 monocultures were grown anaerobically at 39°C in Hungate tubes filled with 9.0 mL of autoclaved modified
115 medium C[21] ("MC-"), containing 1.25 g/L yeast extract, 5 g/L Bacto™ Casitone, and 7.5 vol% clarified
116 rumen fluid, with either 0.1 g of milled reed canary grass, 0.1 g Avicel, 0.1 g Xylan, 0.5 ml of a 0.1 g/ml
117 sterile filtered glucose stock solution, or 0.1 g/ml of a sterile filtered fructose stock solution as the growth
118 substrate, and supplemented with vitamin solution post-autoclaving.[22] Pressure production was used as a
119 proxy for fungal growth, as described previously.[23] Daily pressure measurements were taken using a probe
120 pressure transducer to determine when the cultures reached the mid-log growth phase, based upon previous
121 pressure growth curves measured to stationary phase growth. Upon reaching mid-log growth phase, cultures
122 were harvested and stored for later RNA extraction. After sampling the headspace gas of the culture to
123 determine end-point methane and hydrogen concentrations for monocultures and co-cultures, a volume of
124 1.2 ml of the culture supernatant was pipetted off of the top of the culture and stored at -20ºC for later
125 HPLC analysis. The remainder of the culture was transferred to a 15 ml falcon tube and spun down at
126 10,000 g and 4ºC for 6 minutes. The remaining supernatant was then decanted or pipetted off depending
127 upon the integrity of the remaining cell pellet and replaced with 1 ml of RNA-later and mixed by pipetting.
128 Samples were then stored at -80C until extraction.

129 **Measuring Hydrogen and Methane Production.**

130 End-point methane and hydrogen measurements for both monocultures and co-cultures were taken from
131 the headspace of the culture tubes before harvesting the cultures. Daily measurements and sampling were
132 performed to monitor the growth of the co-cultures and monocultures in the following order. First the

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133 pressure in each sample was measured using a pressure transducer,[24] and the headspace composition was
134 measured on a gas chromatograph (GC)-pulsed, discharge helium ionization detector (Thermo Fisher
135 Scientific TRACE 1300).[25] Finally, the headspace pressure of the sample was vented return the headspace
136 to atmospheric pressure. The total moles of headspace gas were calculated using the ideal gas law. Gas
137 concentrations for H_2 and methane were calculated using an external standard calibration method. The gas
138 concentration could then be multiplied by the number of moles present both before and after the pressure
139 sampling in order to determine the moles of H_2 or methane produced. It was assumed that the amount of
140 gas dissolved in the liquid media was negligible for these calculations.

141 **HPLC Analysis**

142 Levels of volatile fatty acids present in the supernatant of both co-cultures and monocultures were measured
143 using an Agilent1260 Infinity HPLC (Agilent). Samples were prepared by acidifying to 5 mM using sulfuric
144 acid and subsequently incubating at room temperature for 5 minutes. Samples were then centrifuged for 5
145 minutes at 21,000g. The supernatant was syringe filtered into an HPLC vial (Eppendorf FA-45-24-11) using
146 a 0.22 µm PVDF filter. Samples were analyzed on an Agilent 1260 Infinity high-performance liquid
147 chromatography system (HPLC, Agilent, Santa Clara, CA) equipped with an auto-sampler unit (1260 ALS).
148 Separation of formate, acetate, and lactate was achieved with a Bio-Rad Aminex® 87H Ion Exclusion
149 Column for organic acids (Part No. 1250140, Bio-Rad, Hercules, CA) with a mobile phase of 5 mM sulfuric
150 acid. In-house standards were prepared with MC- blank culture medium as a base and sodium formate (ACS
151 Grade, Fisher Chemical S648500), sodium acetate (ACS Grade, Fisher Chemical S210500), L-lactic acid
152 sodium (99%, extra pure, Acros Organics 439220100) at VFA concentrations of 0.1 and 1 g/L.

153 **Genome Sequencing and Annotation of Anaerobic Fungus *Caecomyces churrovis***

154 The *Caecomyces churrovis* genome was sequenced using the PacBio sequencing platform. >10kb
155 fragments were size selected using Blue Pippin Size Selection, then 10 ug of genomic DNA was sheared to
156 >10kb fragments using Covaris g-Tubes. The sheared DNA was treated with exonuclease to remove single-
157 stranded ends and DNA damage repair mix followed by end repair and ligation of blunt adapters using

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158 SMRTbell Template Prep Kit 1.0 (Pacific Biosciences). The library was purified with AMPure PB beads
159 and size selected with BluePippin (Sage Science) at >10 kb cutoff size. PacBio Sequencing primer was then
160 annealed to the SMRTbell template library and sequencing polymerase was bound to them using Sequel
161 Binding kit 2.0. The prepared SMRTbell template libraries were then sequenced on a Pacific Biosystems'
162 Sequel sequencer using v3 sequencing primer, 1M v2 SMRT cells, and Version 2.0 sequencing chemistry
163 with 6 hour & 10 hour movie run times. 6mA modifications were detected using the PacBio SMRT analysis
164 platform (pb_basemods package; smrtanalysis version: smrtlink/8.0.0.80529). 6mA modifications were
165 then filtered and methylated genes were identified following the methods described in Mondo et al., 2017.[26]
166 The assembly was completed with Falcon (https://github.com/PacificBiosciences/FALCON) which
167 generates better assemblies than competing methods likely due to an improvement in isolation of high
168 molecular weight DNA and sequencing larger DNA fragments.[1] While annotating fungal genomes present
169 a challenge due to the lack of anaerobic fungal gene content in existing databases, the genome was annotated
170 using the JGI Annotation Pipeline, which employs a variety of gene modelers to discover genes. In addition
171 to homology-based modelers, ab-initio gene discovery tools and RNAseq based methods were used for
172 annotation. Models were determined to be allelic if they were located in regions on smaller scaffolds that
173 were > 95% identical at the nucleic acid level and > 50% of the smaller scaffold was covered by these
174 regions.

175 **Extracting RNA from Experimental Samples**

176 Samples were removed from storage at -80C and thawed on ice. After thawing, samples were spun down
177 for 6 minutes at 4ºC and 10,000 g and RNA later was removed. Cells were lysed for the reed canary grass
178 and Avicel cultures using bead beating for 1 minute in 30 second intervals. Total RNA was extracted using
179 the RNeasy Mini kit (QIAGEN) following the protocol for "Purification of Total RNA from Plant Cells
180 and Tissues and Filamentous Fungi" including an on-column DNAse digest. An Agilent TapeStation was
181 used to determine the quality of the sequenced RNA and Qubit High Sensitivity RNA Assay was used to
182 determine concentrations.

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183 **RNA Sequencing and Data Analysis**

184 Stranded RNASeq library(s) were created and quantified by qPCR for both monoculture and co-culture
185 samples. Stranded cDNA libraries were generated using the Illumina Truseq Stranded mRNA Library Prep
186 kit. mRNA was purified from 1 ug of total RNA using magnetic beads containing poly-T oligos. mRNA
187 was fragmented and reversed transcribed using random hexamers and SSII (Invitrogen) followed by second
188 strand synthesis. The fragmented cDNA was treated with end-pair, A-tailing, adapter ligation, and 8 cycles
189 of PCR. The prepared library was quantified using KAPA Biosystems' next-generation sequencing library
190 qPCR kit and run on a Roche LightCycler 480 real-time PCR instrument. The quantified library was then
191 prepared for sequencing on the Illumina HiSeq sequencing platform utilizing a TruSeq paired-end cluster
192 kit, v4. Sequencing of the flow cell was performed on the Illumina HiSeq 2500 sequencer using HiSeq
193 TruSeq SBS sequencing kits, v4, following a 2x150 indexed run recipe. Sequencing was performed using
194 an Illumina® Novaseq. The filtered reads from each library were aligned to the *Caecomyces churrovis*
195 genome using HISAT2 version 2.1.0.[27] Strand-specific coverage was generated using deepTools v3.1.[28]
196 Raw gene counts were generated using featureCounts, with only primary hits assigned to the reverse strand
197 were included in the raw gene counts.[29] Raw gene counts were used to evaluate the level of correlation
198 between biological replicates using Pearson's correlation and determine which replicates would be used in
199 the DGE analysis. DESeq2 (version 1.18.1)[30] was subsequently used to determine which genes were
200 differentially expressed between pairs of conditions. The parameters used to call a gene DE between
201 conditions were p-value < 0.05 and a log2fold change greater than 2. This log2fold change cutoff is more
202 stringent than the typical cutoff used in previous studies to account for variation in undefined rumen fluid
203 components across different batches of media. Raw gene counts, not normalized counts, were used for DGE
204 analysis since DESeq2 uses its own internal normalization. Subsequent analysis was done using the filtered
205 model gene catalog for *C. churrovis* provided for download on the Mycocosm website.

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209

210 **Results and Discussion**

211 ***The Caecomyces churrovis genome encodes an abundance of CAZymes and carbohydrate binding***
212 ***modules***

213 Anaerobic fungi are emerging platforms for hydrolysis of crude lignocellulose, as they produce
214 powerful CAZymes and mechanically associate with and often penetrate plant cell walls.[5,31,32] The first high
215 quality genome of a non-rhizoid forming anaerobic fungus from the *Caecomyces* genera was sequenced
216 with PacBio SMRT sequencing using high molecular weight DNA fragments, a method that is critical to
217 high-quality genome assemblies for anaerobic fungi.[33–35] Previously, we assembled a *de novo* transcriptome
218 of *C. churrovis* by pooling RNA from batch cultures grown on glucose, cellobiose, cellulose, and reed
219 canary grass, obtaining an inclusive set of expressed genes for these substrates.[5] The acquisition of the *C.*
220 *churrovi*s genome now enables more detailed investigation of genetic regulatory mechanisms, splicing,
221 ploidy, and comparative genomics that cannot be accomplished with a sole transcriptome. Based on genome
222 sequencing, 15,009 genes were annotated/identified, compared to the predicted 33,437 genes based on the
223 sequenced transcriptome (predicted by taking into account the number of transcripts less isoforms); this
224 difference in gene number prediction between transcriptomes and genomes is consistent across anaerobic
225 fungi and likely reflective of ploidy.[35,36] This discrepancy is largely explained by our observation that this
226 strain of *Caecomyces* is likely a diploid (or dikaryon), as we detected ~10k gene models on smaller scaffolds
227 in regions that were >95% identical to regions on larger scaffolds. These scaffolds were designated as
228 secondary scaffolds and these secondary models/alleles were not included in further analyses. Table 1
229 depicts genomic features for high-resolution sequenced anaerobic fungi, as reported by the JGI MycoCosm
230 pipeline.[37]

231 As noted in Table 1, the *C. churrovis* genome is GC depleted on the same order of magnitude as
232 the other sequenced anaerobic fungal strains. Such extreme codon biases have made it challenging to
233 heterologously express and evaluate the function of anaerobic fungal genes (like CAZymes) in model
234 systems.[38–40] Homopolymeric runs of amino acids are found in the *C. churrovis* genomes, which are

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235 common in the CAZyme machinery of anaerobic fungi, and could serve as glycosylation sites that prevent
236 proteolytic cleavage.[41] Collectively, the function of such features need to be better characterized if gut
237 fungal CAZymes from strains such as *C. churrovis* are to be heterologously produced in a model
238 organism.[41]

239 Anaerobic gut fungi possess an abundance of CAZymes with diverse functions, and are
240 particularly rich in hemicellulases (especially glycosyl hydrolase 10 family) and polysaccharide
241 deacetylases.[32] Some CAZymes are anchored by non-catalytic fungal dockerin domains (NCDDs) to
242 cohesin domains on large scaffoldin proteins to form enzymatic complexes called fungal cellulosomes.[35]
243 The high-resolution genome presented here enabled a Hidden Markov Model (HMM) analysis of *C.*
244 *churrovis* genome, which annotated 36 genes as fungal scaffoldins, compared to the 38 transcripts predicted
245 based on tblastn alignment of the previously sequenced transcriptome. The quantity of predicted proteins
246 identified as cellulases, hemicellulases, and other accessory enzymes along with the total number of
247 CAZymes for each of the 6 sequenced fungal strains are listed in Supplementary Table 1. Fewer total
248 CAZymes in the above categories were identified using predicted proteins found in the sequenced genome
249 (338) than were identified by counting the number of transcripts in the sequenced transcriptome (512),
250 which did not take ploidy into account. The highest abundance accessory enzymes identified in the genome
251 were pectin lyases (15.7% of all CAZymes), in contrast to the transcriptome, in which carbohydrate
252 esterases containing SGNH (defined by four invariant residues – serine, glycine, asparagine, and histidine)
253 hydrolase domains were identified as the most abundant (Supplementary Table 1).[42,43] However, the *C.*
254 *churrovis* genome also contains the smallest number of polysaccharide lyase domains (PLs) of any of the
255 6 fungal genomes characterized (Fig. 1 and Supplementary Table 1).

256 Proteins containing non-catalytic fungal dockerin domains (NCDDs) were also identified and
257 found to be relatively consistent across strains, in agreement with what was observed for transcript counts
258 (Table 1). However, in contrast to the observation that *C. churrovis* NCDD containing transcripts
259 represented only 15% of all CAZyme transcripts in comparison to 27.9-31.4% for the three other fungal

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260 strains examined, the number of NCDD containing proteins represented 30.8% of all CAZyme proteins for
261 *C. churrovis*, similar to the other three fungal strains (Table 1). This suggests that while *C. churrovis* may
262 place greater emphasis on secreted un-complexed, free enzymes to attack plant biomass and release
263 fermentable sugars compared to rhizoid- forming anaerobic fungi based on previously collected
264 transcriptional data, its genome still contains a proportion of NCDD containing proteins similar to that
265 observed in the genomes of rhizoid-forming anaerobic fungal genera. *C. churrovis* also has the second
266 highest number of carbohydrate binding module domains (CBMs) compared to five other high-quality
267 anaerobic fungal genomes (Figure 1). Further analysis revealed that of these genes, *C. churrovis* also
268 possessed the highest number of CBM family 18 domains among anaerobic fungi sequenced to date
269 (Supplementary Fig. 1).

270 It was previously reported that N6-methyldeoxyadenine (6mA) is associated with transcriptionally
271 active genes in early-diverging fungal lineages in a study using single-molecule long-read sequencing to
272 determine which adenines were methylated.[26] Of the 6,692 genes that were methylated when the *C.*
273 *churrovis* genome was sequenced, 4,063 had KOG annotations, 1,002 had KEGG annotations, 3,450 had
274 GO annotations, and 407 were annotated as CAZymes. Almost 1% of all adenines are methylated, and 93%
275 of modifications are at AT dinucleotides, as shown in Supplementary Figure 2A. Very few symmetric runs
276 were present, consistent with avoidance of TAT/ATA reported previously.[26] Modifications are primarily at
277 the start of genes, specifically ramping up in presence at the start of transcription (Supplementary Figure
278 2B). 6mA was rare in repetitive regions of the genome (Supplementary Figure 2C) and a large proportion
279 of total 6mA was restricted to genic space (Supplementary Figure 2D).

280 These results agree with the trends observed for other anaerobic fungal species, further serving to
281 identify 6mA as a widespread epigenetic mark in early-diverging fungi that is associated with
282 transcriptionally active genes.[26] Note that only ~6% of methylated genes in the genome are annotated as
283 CAZymes, indicating that these genes are not always highly transcribed, but rather the majority of
284 CAZymes are transcribed as needed in response to external stimuli, such as co-culture, growth substrate,

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285 etc. Nevertheless, association of gene expression with adenine methylation is necessary to understand and
286 develop transformation techniques, which has proven difficult in anaerobic fungi and other non-model
287 eukaryotic systems to date.[44,45] Accounting for methylated adenine cluster (MAC) positioning and other
288 epigenetic features could help achieve the methylation required to sufficiently overexpress target genes,
289 such as the CAZymes involved in applications requiring biomass breakdown in both fungal monoculture
290 and in anaerobic biomass-degrading consortia.[26]

291 ***Synthetic co-cultures of C. churrovis with methanogen M. bryantii produce methane***

292 Establishing synthetic co-cultures of anaerobic fungi with methanogens is a valuable tool to probe
293 the impact of co-culture on plant biomass breakdown, substrate uptake, and growth of the individual
294 microbes.[8] Once plant biomass has been broken down into its constituent sugars by fungal CAZymes, they
295 are catabolized by the fungi and other organisms in the native rumen environment.[46] Sugars consumed by
296 the fungi undergo glycolysis in the fungal cytoplasm, and the resulting malate and pyruvate are taken up
297 by the fungal hydrogenosome, where they are converted to H_2 and formate via hydrogenase and pyruvate
298 formate lyase, respectively.[2,47,48] The hydrogen and formate produced are then exported and available to
299 neighboring methanogens, which assimilate these products and ultimately generate methane.[33] As such, the
300 metabolic exchange between anaerobic fungi and methanogens benefits both microbes, since it is
301 hypothesized that fungal metabolic end products such as H_2 and formate may inhibit fungal growth and
302 function if allowed to accumulate, while the methanogens are provided with their required growth
303 substrates.[49]

304 Figure 2A summarizes the design of this experiment. Cumulative pressure was measured daily (as
305 a proxy for microbial growth) in order to determine when mid-log growth phase had been reached, at which
306 time the cultures were harvested for RNA extraction as shown in Figure 2B and C.[8] Gas chromatography
307 was used to determine the concentration of methane and hydrogen in the headspace gas of synthetic co-
308 cultures and fungal monocultures on each substrate prior to harvest for RNA extraction at mid-log growth
309 phase. No significant amount of hydrogen was detected in the co-cultures, and no methane was detected in

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310 the fungal monocultures, in agreement with *M. bryantii*'s H_2/CO_2 requirement for methane production[50], as
311 shown in Supplementary Figure 3. The absence of hydrogen in the co-cultures indicates that stable pairings
312 of the fungus and methanogen were formed on all substrates (Fig. 2D), which is consistent with previous
313 observations for the *N. californiae* and *A. robustus* anaerobic fungal strains paired with the same
314 methanogen and grown on cellulose and lignocellulosic reed canary grass.[8,9] Subsequently, transcriptional
315 regulation coupled with HPLC analysis was used to determine the impact of co-cultivation on fungal sugar
316 utilization, hydrogenosome function, secondary metabolite production, and membrane protein regulation
317 in stable, non-native fungal-methanogen co-cultures.

318 ***Co-culture with a methanogen enhances production of fungal Carbohydrate Binding Modules and***
319 ***fungal dockerins across multiple substrates***

320 Changes in the transcriptional regulation of anaerobic fungi when challenged by different substrates
321 indicates how the fungal CAZyme repertoire and fungal metabolism are adjusted in response to an altered
322 environment. Often, waste streams containing biomass in industrial settings can vary in composition,
323 potentially affecting bioreactor function through shifts in community composition and metabolic
324 function.[51,52] Examining these changes using RNA-seq reveals how variations in the composition of
325 undefined growth substrates impacts biomass breakdown and product generation. Differential regulation of
326 CAZymes and associated enzymatic machinery was examined for *C. churrovis* co-cultivated with *M.*
327 *bryantii* and was compared to *C. churrovis* fungal monocultures, both grown on Avicel®, reed canary grass,
328 glucose, fructose, and xylan. A proportionally greater number of genes annotated as CAZymes and
329 enzymatic machinery was upregulated in fungal-methanogen co-cultures relative to fungal monocultures
330 than were downregulated on lignocellulose- and hemicellulose-rich substrates, reed canary grass and
331 Avicel®. The opposite was true for co-cultures grown on substrates rich in soluble sugars, glucose, fructose
332 and xylan as shown in the Supplementary Figure 4A. The total number of genes upregulated or
333 downregulated for individual CBM, GH, CE, and GT families are shown in Supplemental Figure 4B-E.

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334 However, the majority of the number of top ten genes in these categories upregulated in fungal-
335 methanogen co-culture relative to fungal monoculture on all substrates were annotated as either CBM 18
336 family proteins or fungal dockerin domains, the majority of which were associated with genes of unknown
337 function. Figure 3 shows the top ten upregulated fungal genes annotated as CAZymes or associated
338 enzymatic machinery in co-cultures of the anaerobic fungus *C. churrovis* and the methanogen *M. bryantii*
339 relative to monocultures of *C. churrovis* grown on multiple substrates. The CBM family with the most
340 abundant number of genes in the sequenced genome, CBM 18, was consistently the gene classification with
341 the greatest log2fold change of any CAZyme orenzymatic machinery on all substrates in fungal-
342 methanogen co-cultures relative to fungal monocultures. Furthermore, the same CBM 18 gene
343 (*Caecomyces churrovis* protein ID 407913) had the greatest log2fold change in fungal-methanogen co-
344 cultures relative to fungal monocultures on reed canary grass, glucose, and fructose substrates. CBM family
345 18 modules contain approximately 40 amino acid residues and include members with functions linked to
346 modules with chitinase activity or which are lectins.[53,54] The modules may therefore either be attached to
347 chitinase catalytic domains or in non-catalytic proteins in isolation or as multiple repeats. These
348 carbohydrate-binding proteins possess diversity in ligand specificity and the ability to maintain enzymes in
349 proximity of the substrate, increasing enzyme concentration and potentially leading to more rapid
350 degradation of polysaccharides. These features make these proteins excellent candidates for use in
351 biotechnological applications designed for biomass breakdown.[55,56]

352 The observation that CAZymes, fungal dockerins, and other biomass degrading machinery are
353 upregulated in all co-cultures, even those grown on glucose is in agreement with previous studies conducted
354 for fungal-methanogen co-cultures on reed canary grass and glucose at mid-log growth stage.[9,10] Since the
355 majority of the top ten genes upregulated on all substrates were annotated as either CBM 18 family proteins
356 or fungal dockerin domains, this strongly suggests that co-culture with the methanogen *M. bryantii* results
357 in the transcriptional upregulation of enzymatic machinery associated with biomass degradation. Although
358 no transcriptional upregulation of scaffoldin-encoding genes was initially detected in this study, likely due

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359 to the more stringent log2fold change cutoff used to determine significant upregulation, Gene Set
360 Enrichment Analysis (GSEA) of the entire set of upregulated genes revealed that upregulated scaffoldins
361 are significantly enriched in co-cultures grown on Avicel® and reed canary grass.[57,58] These results agree
362 with the finding by Swift, et. al that transcription of fungal cellulosome components increases in co-
363 culture.[59] Another possibility is that the production of CBM18 transcripts is not related to plant biomass
364 breakdown but instead to interactions between the fungus and methanogen since differential expression is
365 observed across all conditions, including growth on glucose. Many of the dockerin domains not attached to
366 CAZymes contain a CotH kinase protein domain. Previous work showed that approximately 20% of DDPs
367 identified in five previously sequenced anaerobic fungi belonged to spore coat protein CotH and were also
368 present in bacterial cellulosomes.[2] These dockerin domain proteins belonging to spore coat protein CotH
369 have been speculated to be involved in plant cell wall binding, although this remains to be experimentally
370 validated.[60]

371 The top ten most highly upregulated genes according to log2fold change annotated as CAZymes,
372 CBMs, or fungal dockerins in co-cultures of *C. churrovis* with *M. bryantii* grown on reed canary grass were
373 compared to those upregulated in co-cultures of the same methanogen, *M. bryantii*, with fungal strains *A.*
374 *robustus* (previously published) and *N. californiae,* grown on the same substrate.[9] Of these genes, those
375 falling in categories common to all three strains, which included genes annotated as CBMs, dockerins, and
376 dockerin-fused CAZymes are included in Figure 4. The number of genes regulated in CBM, GT, PL, CE,
377 and GH families in the three fungal strains in co-culture versus fungal monoculture on reed canary grass
378 substrate are shown in Supplementary Figure 5. The most highly upregulated gene for each strain was a
379 CBM family 18 protein for both the *N. californiae* strain and the *C. churrovis* strain and a Carbohydrate
380 Esterase (family 1) protein for the *A. robustus* strain. For each strain, at least three of the top ten genes were
381 fungal dockerin domains, fused to CAZymes or genes of other function. This comparison suggests that co-
382 cultivation with a methanogen likely encourages substrate channeling between synergistic enzymes for both
383 rhizoid-forming fungal strains (*A. robustus* and *N. californiae*) and non-rhizoid-forming fungi (*C.*

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384 *churrovis*).[9,35] Previously, it was suggested that a smaller proportion of CAZyme transcripts containing
385 dockerin domains in the transcriptome of *C. churrovis* indicated a greater dependence on free enzymes
386 compared to rhizoid-forming gut fungal genera.[5] Nevertheless, with comparative transcriptomic data,
387 upregulation of these non-catalytic modules and CBMs are clearly observed when *C. churrovis* is cultured
388 with *M. bryantii*. This could indicate that anaerobic fungi, regardless of their usual mode of biomass
389 deconstruction, will respond to the presence of other microbes by increasing binding to fibrous substrates.
390 This would allow them more direct access to sugars released during biomass breakdown, which might
391 otherwise be consumed by other microbes.

392

393 ***Fungal co-culture with a methanogen may enhance PFL function and production of bottleneck***
394 ***enzymes in sugar pathways***

395 Transcriptional regulation coupled with HPLC analysis was used to determine the impact of
396 methanogen co-cultivation on fungal sugar utilization, genes potentially associated with hydrogenosome
397 function, secondary metabolite production, and membrane protein regulation in stable, non-native fungal-
398 methanogen co-cultures. Previous studies of fungal-methanogen co-cultures described increased sugar
399 utilization in co-culture.[14,61] As such, we hypothesized that genes encoding enzymes involved in sugar
400 catabolism would be upregulated in *C. churrovis* and *M. bryantii* co-cultures relative to fungal
401 monocultures. While some enzymes within these pathways showed changes for each substrate, no co-
402 culture condition resulted in uniform up or downregulation of all enzymes within a given sugar pathway,
403 as shown in Supplementary Figure 6. The enzymes that were upregulated in fungal-methanogen co-culture
404 relative to fungal monoculture on the same substrate may represent bottlenecks in these catabolic pathways.
405 We suspected that sugar utilization in co-cultures could also be increased through upregulation of sugar
406 transporters in the co-culture condition. We instead observe that in the presence of Avicel® and xylan, *M.*
407 *bryantii* induces transcriptional upregulation of genes that appear to encode proteins homologous to
408 prokaryotic Substrate Binding Proteins (SBPs), as well as Class C G-Protein Coupled Receptors (GPCRs)
409 as seen in Supplemental Table 2.[62–64] While the function of these protein domains and receptors remains

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410 unknown, we speculate that they may be involved in the increased binding of sugar polymers in the presence
411 of the methanogen; or in establishing physical interactions between the methanogens and fungi.[65]

412 A previous study showed that anaerobic fungal genomes encode a wide array of biosynthetic
413 enzymes of natural products including secondary metabolites - small, bioactive molecules known to
414 mediate a variety of interactions between microorganisms.[66–69] The majority of these genes were not
415 significantly differentially expressed between co-culture and monoculture conditions on the various
416 substrates in this study. However, two of these fungal genes were highly upregulated in co-culture (*p*-
417 adjusted <0.01). The first is a non-ribosomal peptide synthetase (NRPS)-like gene (protein Id 604712),
418 which was upregulated eight-fold during growth on fructose and on Avicel®. The second, a polyketide
419 synthase (PKS; protein Id 402343) was four-fold upregulated in co-culture compared to monoculture during
420 growth on xylan and reed canary grass, suggesting that some fungal secondary metabolites may mediate
421 the interaction between *C. churrovis* and *M. bryantii*, depending on the specific substrate. Co-culture
422 interaction may be most notable on Avicel® and xylan substrates, as both transporters and secondary
423 metabolite biosynthesis genes were upregulated in co-culture for both of these substrates.

424 Based on previous studies noting an increase in metabolites produced by the ATP-generating fungal
425 hydrogenosome during co-culture with methanogens, we hypothesized that genes associated with
426 hydrogenosomal function would be upregulated in methanogen co-culture.[70,71] A list of genes associated
427 with the fungal hydrogenosome of the *C. churrovis* strain was constructed based on homology with known
428 hydrogenosome components, shown in Supplemental Table 3. FASTA sequences from known
429 hydrogenosomal components identified in the fungal strain *Neocallimastix lanati*[4] were aligned to filtered
430 model proteins of *C. churrovis* using the blastp alignment program in MycoCosm. One or more genes
431 within the *C. churrovis* genome aligned to all listed hydrogenosomal enzymes found in *N. lanati*.
432 Regulation of these genes in co-culture compared to monoculture was examined for each substrate. As
433 shown in Supplemental Table 3, 21 genes were homologous to both pyruvate formate lyases (PFLs) that
434 were identified in the *N. lanati* genome.[4] This enzyme reversibly converts pyruvate and CoA into acetyl-

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435 CoA and formate, which plays a central role in anaerobic glucose fermentation.[72] It has been shown that
436 this enzyme is functional in hydrogenosomes of the anaerobic fungal species *Piromyces* sp. E2 and
437 *Neocallimastix* sp. L2.[73] The most notable upregulation of PFLs was observed in cultures grown on xylan
438 and fructose, where 15 of the 21 PFL genes identified by homology were upregulated in co-cultures
439 compared to monocultures grown on xylan and two genes identified by homology were upregulated in co-
440 cultures compared to monocultures grown on fructose as shown in Supplementary Table 3. Five additional
441 genes annotated as PFLs (or formate C acetyltransferases) according to Enzyme Commission (EC) number
442 rather than homology to the *N. lanati* genome were upregulated on xylan and one additional gene was
443 upregulated on fructose. One of these genes (Protein ID 428490) was upregulated in co-culture on all
444 substrates examined except reed canary grass. A previous study examining transcriptional regulation of co-
445 cultures of the native fungus-methanogen pairing *Pecoramyces* sp. F1 with the methanogen
446 *Methanobrevibacter thaueri* versus monoculture of the fungus grown on glucose did not detect a difference
447 in expression levels of PFL genes (although upregulation was detected at the protein level).[10]

448 Although we hypothesized that genes associated with the hydrogenosome would be
449 transcriptionally upregulated in the co-culture relative to the fungal monocultures based on the metabolic
450 data collected in previous work, transcriptional upregulation of genes associated with hydrogenosomal
451 function is limited, with the exception of pyruvate formate lyases in co-cultures grown on xylan and
452 fructose. It is important to note that further studies are needed to confirm that this transcriptional
453 upregulation of PFLs is associated specifically with the hydrogenosome, as PFLs function in both the
454 cytosol and the hydrogenosome. However, as a complement to the transcriptional information regarding
455 metabolic function in this study, end point metabolites present in the supernatant were measured using
456 HPLC upon harvest of the co-cultures and monocultures (Figure 5). Increases in the amount of acetate
457 produced in co-culture and the absence of significant amounts of ethanol and lactate indicate that some of
458 these genes may potentially be associated with hydrogenosome function for cultures grown on fructose,
459 since pyruvate can either be converted to lactate or ethanol by PFLs functioning in the cytosol or converted
460 to acetate by PFLs functioning within the hydrogenosome. Ethanol was also absent in cultures grown on

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461 xylan, although higher levels of lactate were observed in co-culture in addition to higher levels of acetate,
462 indicating that both cytosolic and hydrogenosomal PFLs may be upregulated in co-culture. GSEAPreranked
463 analysis also indicated that upregulated genes were enriched in pathways associated with pyruvate
464 metabolism and glycolysis for co-cultures grown on xylan, in agreement with the observed upregulation of
465 PFLs.[57,58]

466 While analysis of the end-point metabolites of *A. robustus* paired with *M. bryantii* in previous
467 work did not indicate a statistically significant difference in the level of formate in co-culture versus
468 monoculture, formate was absent in the *C. churrovis* and *M. bryantii* co-culture samples but present in
469 fungal monocultures.[9] Earlier studies concluded that this type strain of *M. bryantii* (DSM 863 M.o.H.) was
470 unable to produce methane from formate in pure culture.[74,75] The discovery of a formate transporter and
471 several copies of formate dehydrogenase genes upon sequencing the methanogen's genome has suggested
472 the possibility of growth on formate.[50] The observed upregulation of PFL genes and the absence of formate
473 in co-cultures in the current study provides evidence that this strain of *M. bryantii* can utilize formate under
474 certain conditions. A similar phenomenon has been observed for co-cultivation of a formate-producing
475 *Piromyces* fungal species and the natively associated methanogen *Methanobrevibacter thaueri*, a
476 methanogen that has been shown incapable of growth on formate.[20,76] It is possible that cultivating these
477 methanogens under the conditions required for co-culture with rumen anaerobic fungi stimulate formate
478 utilization by inducing function of the formate transporter and formate dehydrogenase discovered upon
479 sequencing the genome.[50]

480

481 **Conclusions**

482 Here, we have sequenced the first high-quality genome of a non-rhizoidal fungus, *Caecomyces churrovis*,
483 revealing an abundance of diverse CAZymes and the highest number of CBM family 18 domains among
484 anaerobic fungi sequenced to date. We found that co-cultivation of the *C. churrovis* fungal strain with the
485 non-native methanogen *M. bryantii* enhanced production of transcripts containing these chitin-binding
486 CBM 18 domains across a variety of substrates. Upregulation of CBMs and dockerin domains in fungal-

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487 methanogen co-culture with the same non-native methanogen relative to fungal monoculture on a
488 lignocellulose-rich substrate was also observed for two other previously sequenced fungal strains, *A.*
489 *robustus* and *N. californiae*. We hypothesize that the function of CBMs belonging to family 18 may not be
490 directly related to plant biomass breakdown but instead to interactions between the fungus and methanogen
491 since upregulation of transcripts containing these domains is observed across multiple cultivation
492 conditions, including both cellulose and lignocellulose-rich substrates as well as soluble sugars.
493 Upregulation of genes associated with sugar pathways and the functioning of the hydrogenosome for *C.*
494 *churrovis* and *M. bryantii* co-cultures relative to fungal monocultures of *C. churrovis* also suggests that co-
495 culture with a methanogen may enhance pyruvate formate lyase (PFL) function under certain cultivation
496 conditions and production of key enzymes in sugar utilization pathways. Overall, these observations
497 enhance our understanding of the mechanistic interactions between anaerobic fungi and associated
498 methanogens, which aids in our ability to design synthetic biomass-degrading microbial consortia.

499

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1 **Table 1:** Overview of sequenced anaerobic fungal genome features and statistics[2–4]

	Caecomyces churrovis	***Anaeromyces robustus***	***Neocallimastix californiae***	***Neocallimastix lanati***	***Piromyces finnis***	***Pecoramyces ruminantium***
Genome size (Mbp)	165.50	71.69	193.03	200.97	56.46	100.95
No. scaffolds	7737	1035	1801	970	232	32574
% GC content	19	16	22	18	21	17
Scaffold L50 (Mbp)	0.03	0.14	0.44	1.03	0.75	0.00
No. of gene models	15,009	12,832	20,219	25,350	10,992	18936
Gene % CAZymes	7.51%	6.73%	7.23%	7.05	6.45	5.67
No. of DDPs*	400	276	422	586	227	318
No. of scaffoldins	36	26	55	93	14	83
No. of diploid gene pairs	10972	147	1154	497	146	ND

2 *DDP=dockerin domain proteins.

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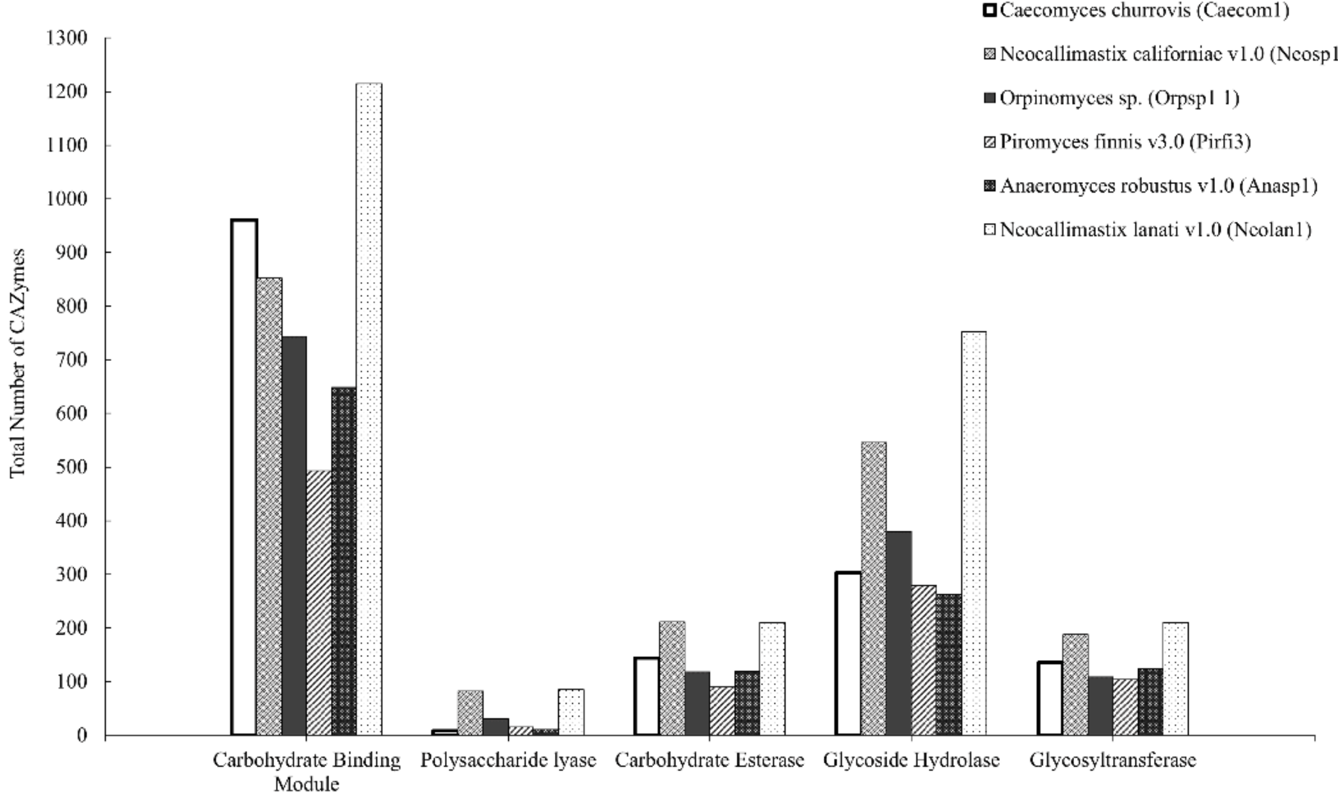

8

9 **Figure 1. Number of different types of CAZyme domains in six sequenced anaerobic fungi.** *C.*
10 *churrovis* has the highest number of domains annotated as carbohydrate-binding modules compared to
11 most other sequenced anaerobic fungi. Annotation data for these strains can be found at
12 https://mycocosm.jgi.doe.gov.
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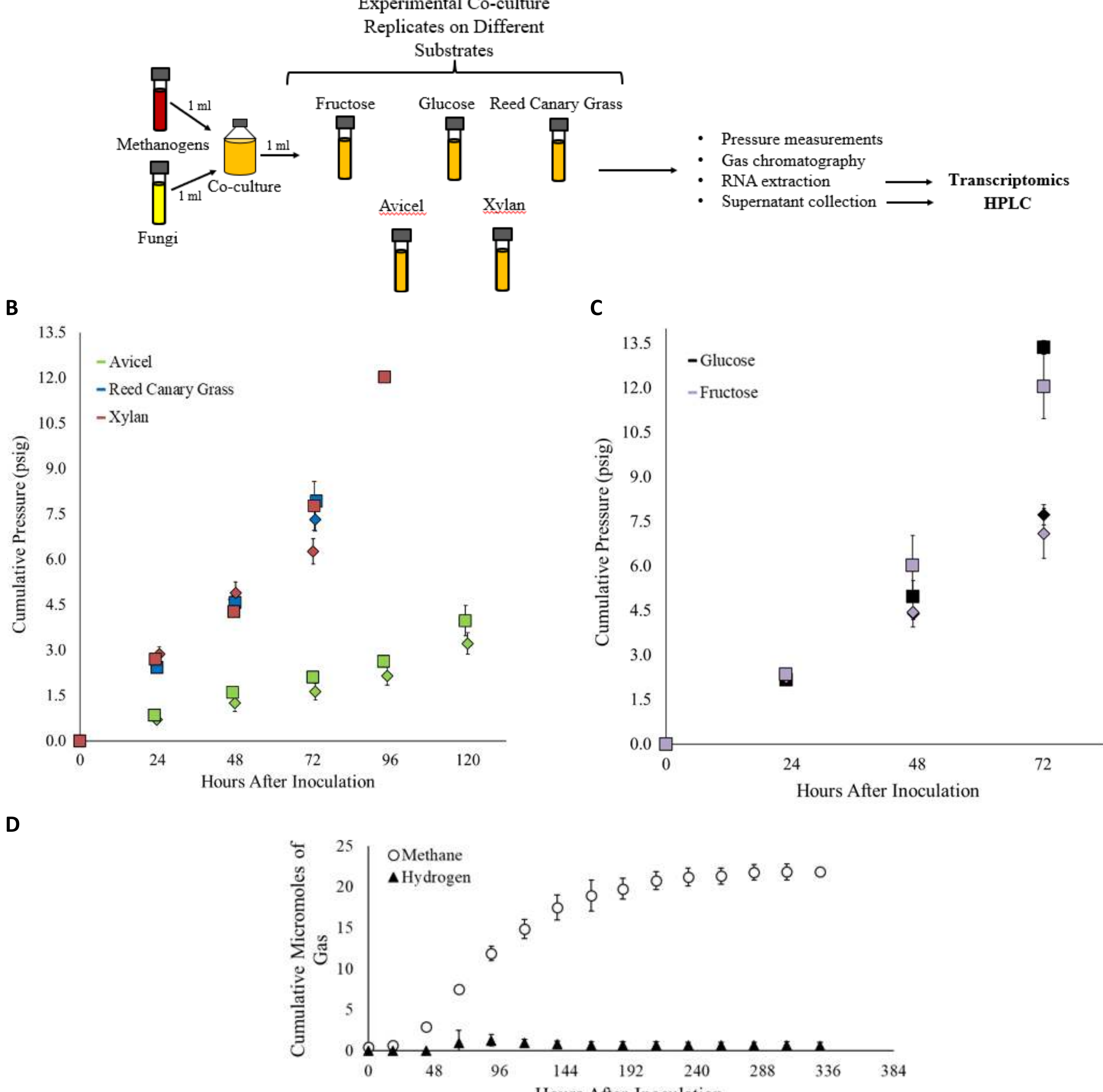

Figure 2. Monocultures and co-cultures were harvested at mid-log growth phase as determined by cumulative pressure. (A) Schematic of the experimental process of cultivating and harvesting co-cultures. A similar process was followed for cultivating and harvesting monocultures, except seed culture was inoculated with 1ml of fungus only.(B and C) Cultures were harvested at pre-determined pressure ranges indicative of the mid-log growth stage for each culturing condition. Cumulative pressure (psig) is plotted versus hours after inoculation for co-cultures and monocultures grown on biomass and components of biomass - reed canary grass, Avicel, and Xylan – in Figure B. Cumulative pressure (psig) is plotted versus hours after inoculation for co-cultures and monocultures grown on soluble sugars – glucose and fructose – in Figure C. Pressure readings for co-cultures are indicated by squares and pressure readings for monocultures are indicated by diamonds. Each substrate is color coded according to the key on the plot. Cultures were harvested at the mid-log growth phase, as indicated by the final pressure time point for each sample. (D) Longterm methane and hydrogen data produced by co-cultures of the anaerobic fungus *C. churrovis* and the methanogen *M. bryantii* on a reed canary grass substrate. Cultures were grown in a complex media formulation, in contrast to cultures harvested for RNA extraction which were grown on MC-. Low levels of accumulated hydrogen indicates stable co-culture over the course of fungal growth.

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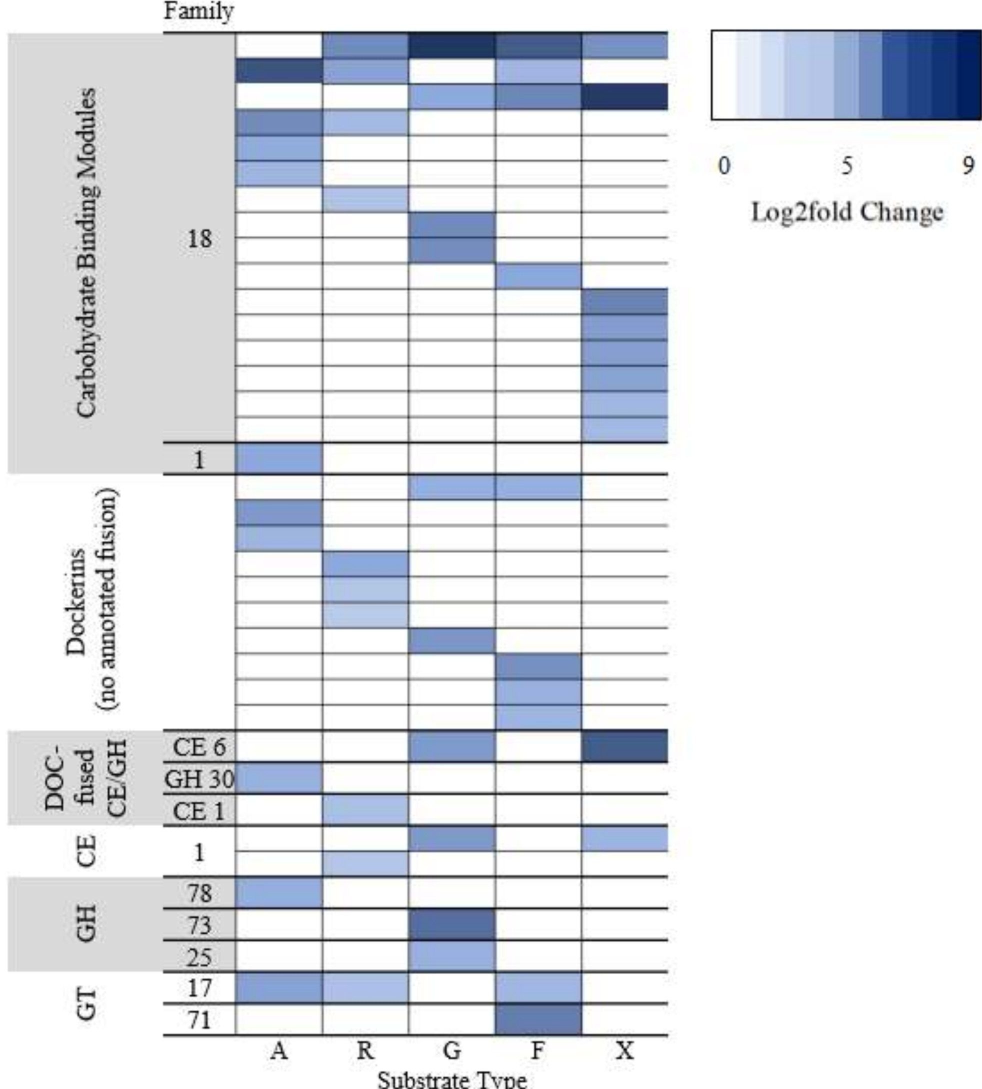

Figure 3. Plot of the top ten upregulated fungal genes annotated as CAZymes or associated enzymatic machinery in co-cultures of the anaerobic fungus *C. churrovis* and the methanogen *M. bryantii* relative to fungal monocultures of *C. churrovis* grown on multiple substrates. Co-cultures of the anaerobic fungus and the methanogen and fungal monocultures were grown on Avicel (A), Reed Canary Grass (R), glucose (G), fructose (F), and Xylan (X). Differential expression of fungal genes in co-cultures relative to fungal monocultures was determined using DESEQ2. The ten genes with the highest log2fold change in expression in co-culture relative to fungal monoculture are shown in the plot above for each substrate and organized into the following classifications: carbohydrate binding modules, dockerins, dockerin-fused carbohydrate esterase or glycoside hydrolases (DOC-fused CE/GH), carbohydrate esterases (CE), glycoside hydrolases (GH), and glycosyltransferases. Protein IDs are listed for each gene. CBMs were highly upregulated, indicating that there may be an increase in enzymatic machinery that aids in anchoring CAZymes to substrates in co-culture, even when grown on soluble sugars. A table containing a list of these genes and the associated log2fold change can be found in Supplementary Table 2.

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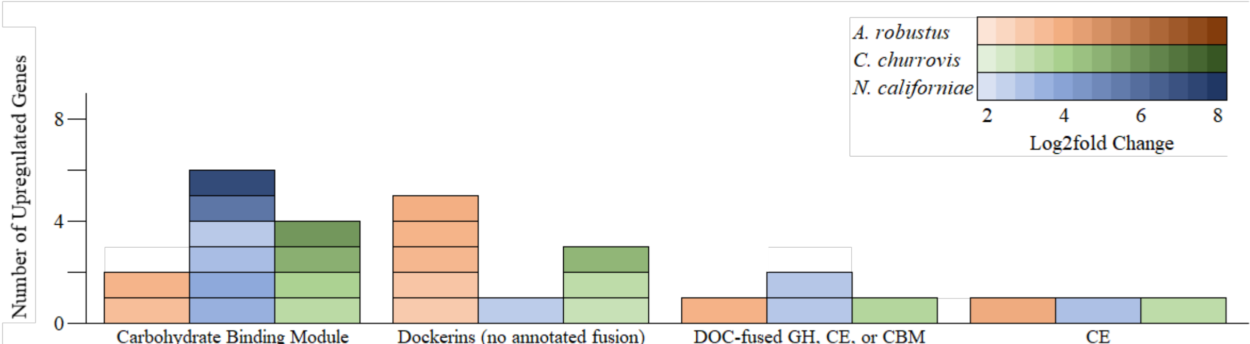

Figure 4. Plot of the top upregulated fungal genes annotated as CAZymes or associated enzymatic machinery in co-cultures of three different fungal strains paired with the same non-native methanogen, *Methanobacterium bryantii* relative to fungal monocultures grown on a reed canary grass substrate. Three different strains of anaerobic fungi, *Anaeromyces robustus*, *Neocallimastix californiae*, and *Caecomyces churrovis* were used to form separate co-cultures with *M. bryantii* and grown on a reed canary grass substrate along with monocultures of each fungus on the same substrate. Differential expression of fungal genes in co-cultures relative to fungal monocultures was determined using DESEQ2. The ten genes with the highest log2fold change in expression in co-culture relative to fungal monoculture are shown in the plot above, with the exception of genes that were not in a category with upregulated genes shared between all three strains (which included one upregulated glycoside hydrolase gene for *A. robustus* and one upregulated glycosyltransferase gene for *C. churrovis*). Genes were organized into the following classifications: carbohydrate binding modules (CBM), DOC (dockerins), dockerin-fused carbohydrate esterase, glycoside hydrolases or carbohydrate binding modules (DOC-fused GH/CE/CBM), and carbohydrate esterases (CE). A table containing a list of these genes and the associated log2fold change can be found in Supplemental Table 3.

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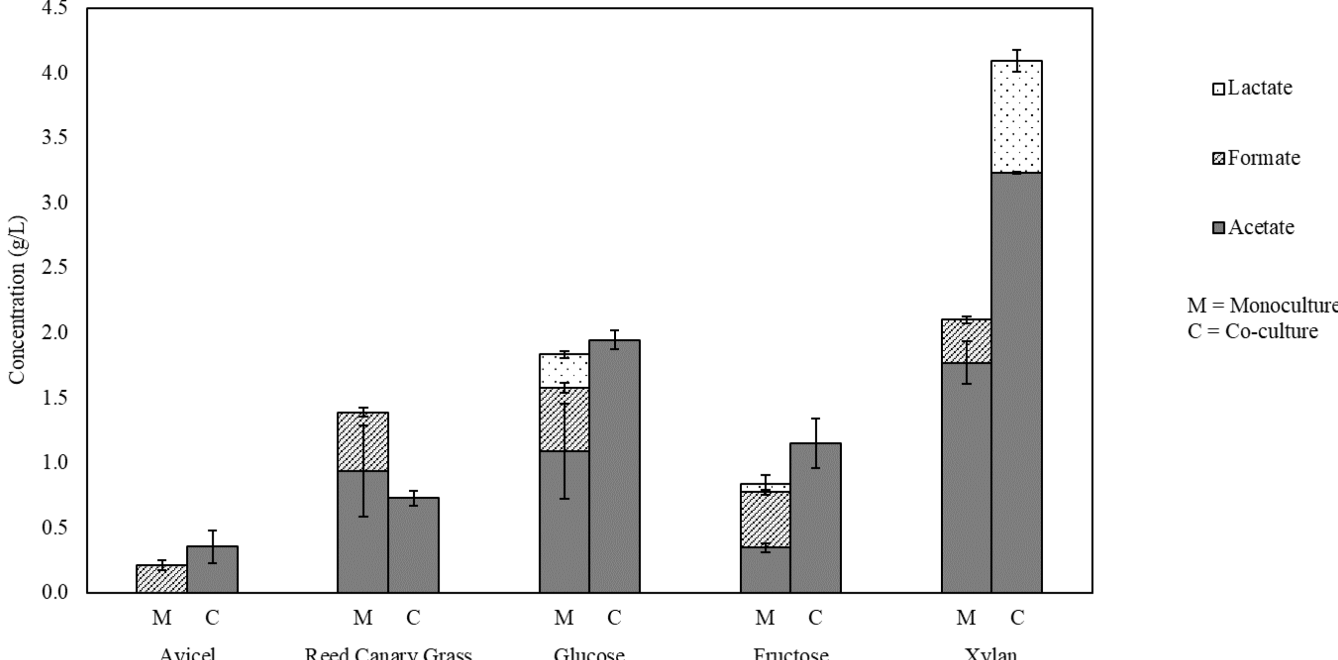

Figure 5. Accumulated metabolites for co-cultures of *C. churrovis* paired with *M. bryantii* versus monocultures of *C. churrovis* upon harvest. HPLC data is shown for co-culture and monoculture grown on each substrate. No formate was observed in co-culture on any substrate, suggesting that *M. bryantii* is capable of metabolizing formate. Trace amounts of ethanol were present in the cultures but fell below the 0.1 g/L limit of detection. This, in conjunction with increased levels of acetate in co-culture, indicates that some of the PFLs upregulated in co-culture in xylan and fructose may be functioning within the hydrogensome.

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