Characterizing Mixed Culture Beer

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Abstract

The increasing demand for novel and unique flavors in the craft beer industry has led to a rise in the use of Mixed Culture Fermentation (MCF). These cultures house complex microbial biomes that exhibit extraordinary metabolic diversity. Because of this, MCFs can produce beverages with unique profiles likely as a result of the biochemistry associated with their high heterogeneity. Using high-throughput DNA sequencing and bioinformatics it is possible to quantify the diverse conglomerate of microorganisms found in these cultures. There is potential to couple this information with metabolomics and transcriptomics to better understand, control and build cultures with desired characteristics. Such characteristics may include metabolites with pleasant aromas or flavors, pH adjustment, tolerance to environmental changes or fermentation speed.

We analyzed 6 mixed cultures by using high-throughput 16S rRNA gene sequencing and the DADA2 (Divisive Amplicon Denoising Algorithm 2) open source bioinformatic software package to identify unique sequences of genetic code to elucidate the makeup of the microbial communities. Our study revealed 122 different genera of Bacteria, 3 genera of Eucarya, and one genus of Archaea across the analyzed beer cultures. *Lactobacillus acetotolerans* was found to be the dominating strain of Bacteria, while the dominant eukaryotes were *Brettanomyces custersianus* and *Brettanomyces bruxellensis* yeasts. Only one genus of Archaea, *Methanobrevibacter sp.*, was found.

Introduction

Working with Golden's own CODA Brewing Company, we had the opportunity to quantify and more fully understand, the microbial communities of several cultures that have been mixed, created, and cared for by owner and head brewer, Luke Smith. Maintaining these types of cultures is an artform that has been practiced by brewers large and small throughout human history. Juxtaposed to the purebred predictable cultures that many commercial brewers use, MCFs can be finicky, temperamental, and hard to predict. Similar studies have been conducted on spontaneously fermented and mixed culture beers [1, 2, 3], but every fermentation is unique and may house niche routes of metabolism that produce novel beers. To learn how to better control and hand curate these cultures, we must first understand what species reside within them, and what biochemistry and metabolic potential they bring to the table, or in this case, what they bring to the pint glass.

The six cultures were named Bootleg Biology, Cantillon, Sleepy Head Sour '15, and Coda House Dregs numbers 1, 2, and 3. Each of these cultures has been curated by Smith over multiple years. Bootleg Biology refers to a yeast propagation company out of Nashville, Tennessee that focuses on providing tools for harvesting wild microbes for food and beverage fermentation, and the culture is a mixture of Bootleg Biology cultures. Cantillon refers to a Belgian brewery famous for its spontaneous fermented beers. This culture is a collection of different Cantillon dregs. Dregs refers to the sediment at the bottom of bottle fermented beers. This sediment is rich in the microbes that produced the beverage and can be harvested and regrown to be pitched into new batches of beer. The House Dregs refer to dregs gathered from Smith's own beers. These cultures are kept in small glass containers with airlocks to allow gas to escape and are occasionally fed sterilized wort, nutrient rich sugar water, to keep them happy and healthy. The art of blending different microbial communities like these is key to creating unique brews that may be unlike any others available. This blending also leads to large shifts in the microbial communities depending on the original diversity and nutrient content of each original culture. This inherently leads to the uncertainty of what each culture holds.

Methods

Sampling

Purpose: In this step we collected small liquid samples from each Coda culture. The cultures were selected by Smith based upon house favorites. The sample was placed directly into a liquid that preserves all the genetic information. This gives a genetic "snap-shot" of what is in the culture at that precise moment in time.

Procedure: After homogenizing the mixtures by swirling culture containers, 250 L of each culture was sampled and added to 750 µL of DNA/RNA shield (Zymo Research, Irvine, CA, USA) in a 2ml tube.

DNA Extraction and Sequencing

Purpose: Here we isolate the DNA from our collected sample. We then target a specific part of the DNA which will tell us what it belongs to and copy it many times over with the polymerase chain reaction. We then take these copies and "read" them to identify what's in there. The more copies present of certain "read" the more abundant that organism is in our sample.

Procedure: The ZymoBIOMICS™ DNA Miniprep Kit (Zymo Research, Irvine, CA, USA) was used per manufacturer's instructions to extract DNA from collected samples. Eluted DNA was then amplified using polymerase chain reaction (PCR) targeting the 16s rRNA gene of Bacteria and Archaea with 515-YF and 926R primers. These primers are also capable of amplifying the eukaryotic 18S rRNA gene and have been found to yield accurate estimates of mock community abundances [4]. Amplification was verified by gel electrophoresis. PCR products were subjected to 0.8x KAPA Pure Bead (Kapa Biosystems, Indianapolis, IN, USA) cleanup according to manufacturer's instruction. Barcoded amplicons were sequenced on an Illumina MiSeq (Illumina Inc., San Diego, CA, USA) at the Duke Center for Genomic and Computational Biology, Rayleigh, North Carolina.

Bioinformatics

Purpose: Here we utilize computer programs to sort through our data. The data we receive is essentially long strings of As, Cs, Ts and Gs (the four base pairs of DNA). One of the goals is to figure out what is a "true" read, and not a mistake; chimeric DNA can form during PCR, which is a chunk of DNA formed from two different organisms, and therefore not representative of a real piece of DNA from the sample. Mistakes like this are considered "noise" in the data. We utilize multiple packages and algorithms to sort the samples, weed out "noise", cross check the DNA reads against a database of known organisms, and then generate visual figures to show us what, and how much of what, is in each sample.

Procedure: The DADA2 [5] open source bioinformatics R script package was utilized to infer Amplicon Sequence Variants (ASVs). DADA2 uses a parametric model to identify true biological sequences from reads. DADA2 was chosen based upon its high sensitivity compared to similar packages [6]. Forward and reverse DNA sequence reads were trimmed according to primer length and quality of the reads; 20 leading base pairs were removed from forward reads, and 40 from reverse. The forward and reverse reads were then truncated according to quality, while still allowing enough room for overlap between forward and reverse reads. The trimmed reads were then used to generate error rates to infer true unique sequences. In essence, the model relies on relative abundance. The higher the abundance, the more likely it is a true read, and less abundant reads are subjected to a probability threshold that infers if a read is "true" or not. The forward and reverse reads were then merged to generate the full denoised sequences. Similar steps were taken for the 18 S rRNA gene for Eucarya, except that the forward and reverse reads were merged by concatenating to compensate for the larger gene. Sequence tables were constructed for merged Bacteria, Archaea and Eucarya. Chimeras were removed and taxonomy was assigned using the SILVA training file (version 128). In addition, eukaryotic symbiont chloroplast and mitochondrial sequences were excised. Figures displaying both Alpha Diversity and Relative Abundance of genera were then generated using

Collection of samples being conducted at Koda Brewing.

the Phyloseq R Package [7]. Species level assignments were assigned by pulling the most abundant sequences and running them through the NCBI BLAST database [8]. Any match greater than 99% was taken as a true match. Species assignment by the available DADA2 function will only execute for exact matches.

Results & Discussion

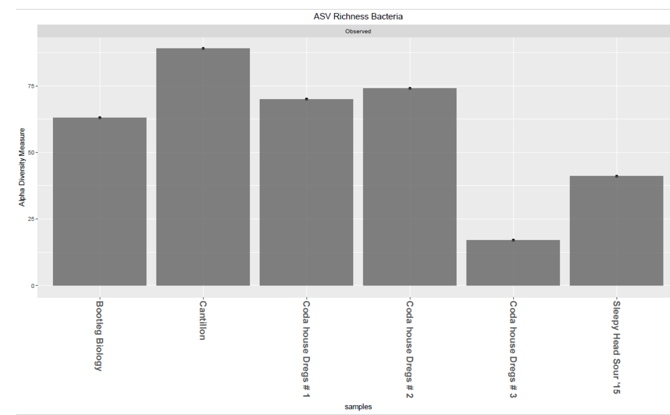

Figure 1: Bacteria Alpha Diversity, This shows how "rich" each culture is in terms of bacteria. In other words, it gives us a rough idea of how many kinds of bacteria are in each sample. This is a measure of ASVs which may belong to the same genus, or even species if the change is small enough.

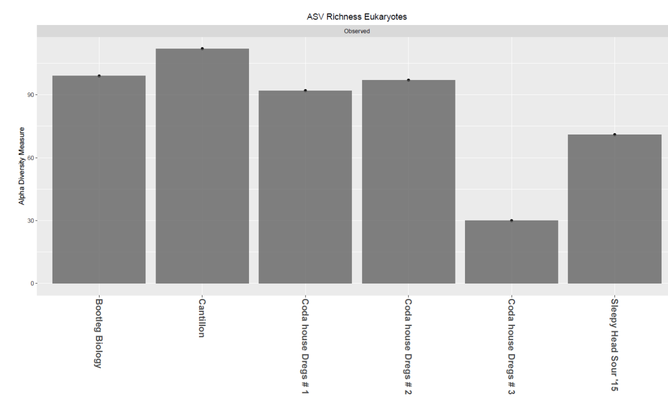

Figure 2: Eukaryotic Alpha Diversity, This shows how "rich" each culture is in terms of Eucarya. In other words, it gives us an idea of how many kinds of Eukaryotes are in each sample.

As seen in Figures 1 and 2, the alpha diversity of both Bacteria and Eucarya display similar trends across the 6 cultures. Screening data revealed that the eukaryotes consisted of 99% *Brettanomyces spp.*, which can produce ethyl caproate and ethyl caprylate during primary fermentation which will add fruity pineapple and floral tropical fruit notes respectively [9]. *Brettanomyces* are also known to produce volatile phenols that may add barnyard "funk" or medicinal aromas [9]. While the latter may not sound ideal, when coupled with other flavors, volatile phenols can add depth and complexity to a brew. A BLAST search of the most abundant sequences revealed that CODA Brewing's Cantillon, House Dregs #1 and #2, and Sleepy Head Sour all contained majority *Brettanomyces custersianus* while Bootleg Biology and House Dregs number #3 all contained majority *Brettanomyces bruxellensis*. *Brettanomyces bruxellensis* is the most encountered 'brett' species in beer and is named after the Senne valley near Brussels, Belgium and is well known for its contribution to the complex Lambic beers of the region [2]. *Brettanomyces custersianus* on the other hand is a newcomer in the beer world, and although it has been found in some wild cultures [1], an exhaustive search only found one commercially available culture listing it by name (East Coast Yeast, Hammonton, NJ, USA). This may be a unique "house" strain of CODA Brewing Company, but further investigation into other small-scale mixed cultures would be needed to verify this. Any genus that was not a *Brettanomyces spp.* was well below 1% relative abundance. The classic brewing yeast, *saccharomyces cerevisiae*, was within the one percent. The absence of *s. cerevisiae*, which was likely present in some of the original cultures before blending, may be attributed to nutrient variabilities during growth that favored the wild 'brett' stains of yeast. For example, it has been demonstrated that the uptake of nitrogen by non-*saccharomyces* species can inhibit the growth of the common brewer's yeast [10]. It may be beneficial in future work to use alternative PCR primers such as the inter transcribed space (ITS; the distance between two genes on a genome) designated primer to target fungal communities to verify the absence of *saccharomyces cerevisiae* [11].

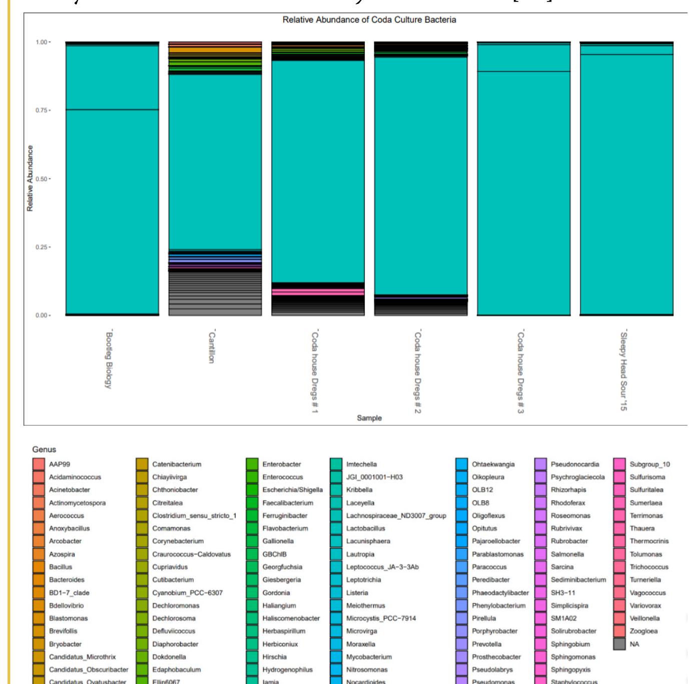

Figure 3: Bacteria Relative Abundance (Each box represents a unique ASV and Gray Represents Taxa not found in the Silva database) Each Relative abundance plot shows these "boxes" representing a unique "read" of DNA. While the DNA is different, it can belong to the same genus, or even the same species if the difference is small enough.

As seen in Figure 3, the Bacteria were dominated by *Lactobacillus spp*. *Lactobacillus* are responsible for the production of lactic acid which is the main source of tartness in sour and mixed culture beers [12]. Of the 15 unique ASVs identified, 14 matched *Lactobacillus acetotolerans*. Literature concerning this specific strain only detail its ability to spoil beer when lactic acid is unwanted [13]. In fact, many of these organisms have historically been defined as contaminates by brewers who sought pure culture beers with limited, and in my opinion boring, yeast characteristics. An extensive search found no commercially available cultures which listed this bacterium by name. *L. acetotolerans* display low pH tolerance and high lactic acid production making it an ideal strain for souring beers. This may be the house strain "power of sour" in CODA's mixed culture beers. Once again, further investigation would be required to identify this strain with high accuracy.

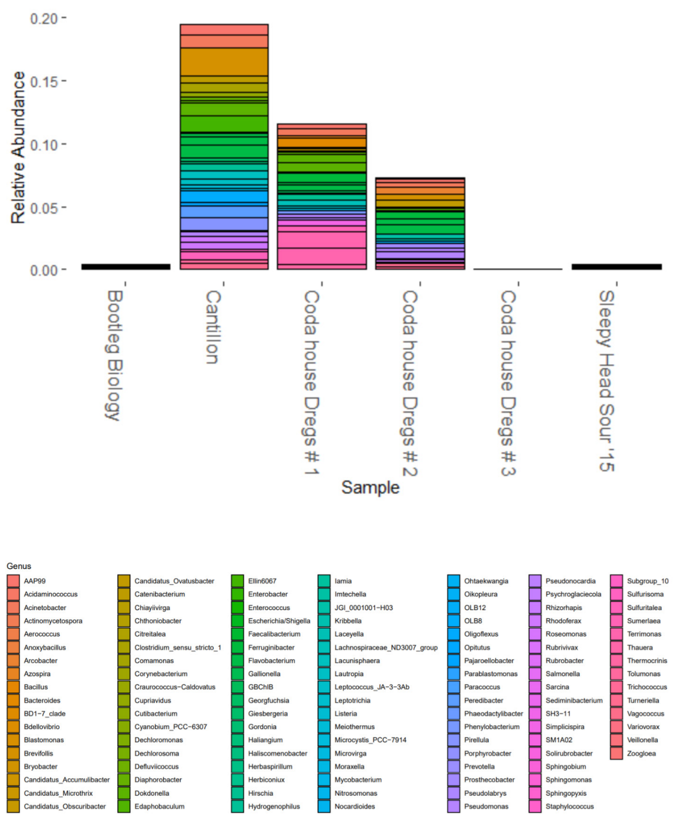

Figure 4: Non-Lacto Genus Abundance. Similar to Figure 3, does not include Lactobacillus, or unmatched genus to highlight the diversity of known organisms.

The role of the one genus of Archaea found in House Dregs # 2, *Methanobrevibacter sp.*, remains a mystery. This small species lives strictly in anaerobic environments and produces methane through the reduction of carbon dioxide via hydrogen [14]. This adds to the conundrum of what exactly is going on in these cultures. What is this specie's function in the community and what is its influence on the other organisms in the environment? For example, are other species in the consortium relying upon the produced methane of this *Methanobrevibacter sp.* as a carbon source or electron donor for their own metabolism to then impart their own flavors to the brew? This question can be expanded to the other organisms that are found in lower abundance. As seen in Figure 4, when Lactobacillus are removed, we are presented with a colorful array of 123 different species of Bacteria. In particular, the 'non-lacto' species that we were able to identify constitute nearly 20% of the relative abundance. Atop this, we can see in Figure 3 that approximately 15% of the ASVs could not be matched to a genus in our database. In other words, approximately 35% of this culture plays some unknown role. It is worth noting that some of these genera may cause alarm because they are known pathogens. Fear not beer drinkers, for these are found in minuscule amounts pre fermentation and are suppressed by the rise in ethanol and drop in pH. In fact, alcoholic fermentation is a widely accepted practice for removing harmful bacteria. These bacteria may still hold a role in early fermentation similar to the Enterobacteriaceae phase of traditional Lambic beers [2], which highlights the dynamic shifts the microbial population sees throughout a fermentation.

The Cantillon culture has the highest diversity, which makes sense given that fact that Cantillon spontaneously ferments their beers. These beers are inoculated by wild microbes that drift in from windows open to the Belgium countryside. Cantillon also blends their beers over years and ages them on mashed fruit, further increasing the possible sources of microorganisms. Much of the microbial world is unknown and uncharacterized, and the wide array of different microorganism sources may account for the lack of genus matching in the Cantillon culture. Only Coda House Dregs #1 and #2 rival the diversity. It is possible that these cultures are also house dregs from spontaneous fermentations. It is likely that Coda House Dregs #3, Sleep Head Sour '15, and Bootleg Biology are so heavily dominated by *Lactobacillus* and *Brettanomyces* that they have outcompeted any rival organisms. The key to the diversity in the other cultures could be housed within symbiotic relationships between the organisms that are still unknown. The starting conditions of each culture is also unknown, but likely influenced symbiosis or competition between organisms. It would be worth exploring how mixed cultures change overtime immediately after blending to draw further insight into competition vs symbiosis.

Conclusion

While many genera of Bacteria were found, approximately 15% of the organisms in the most diverse culture were not identifiable by the employed database. These databases are often incomplete by nature and reveal how incredibly diverse and unknown much of the microbial world is. In addition, so-called 'microbial dark matter' of unknown characterization and function dominate many environments that have been partially characterized. Our study provides a snapshot of the immense diversity of mixed beer cultures from a single brewery. This information could provide a useful steppingstone for further investigation of unique beer cultures and their intricate biochemistry. Continuing these investigations may lead to finer tuned custom cultures, patented cultures, or the discovery of unknown organisms. It's important to recognize that this is just the first stage of a scientific inquiry for the microbial community characterization of beer from one brewery. The next step is to utilize transcriptomics, metabolomics, and qualitative analysis to begin building a full picture of MCF; what are they doing, what are their final or intermediate products, and most importantly how does the end product taste? After all, what's the fun in studying beer microbes if you don't get to enjoy the fine-flavored beverages they produce?

Works Cited

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About the Author

Henry F. Peel is a transfer student from Front Range Community College majoring in Biological and Chemical engineering. He has worked on a multitude of projects in the Geo-Environmental-Microbiology (G.E.M.) Lab here at Mines under Professor John R. Spear and Professor Jonathan O. Sharp.