The Microbial Ecosystem of Beer Spoilage and Souring: Competition and Cooperation in the Age of Bioinformatics

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THE MICROBIAL ECOSYSTEM OF BEER SPOILAGE AND SOURING: COMPETITION AND COOPERATION IN THE AGE OF BIOINFORMATICS

by

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ABSTRACT

The brewing industry generates $350 billion in the US annually, representing 1.9% of the gross domestic product. Spoilage is a persistent problem throughout production and distribution that causes untold economic loss, and is therefore meticulously avoided. Contrarily, artisanal sour beers are necessarily produced by a diverse variety of these spoilage organisms metabolically interacting in symbiosis as a microbial ecosystem. We sought to gain insight into factors driving spoilage and souring by investigating a long-debated Darwinian hypothesis.

Darwin's competition-relatedness hypothesis predicts that closely related species in ecosystems will tend to compete. We isolated a consortium of bacteria and yeast from spoiled and sour beer, then subjected them to co-culture screening in microtiter plates under a variety of controlled abiotic conditions. Competition was measured by comparison of biological output of individuals and co-cultures. Relatedness was quantified from whole genome data using multiple levels of annotation, which allowed for meaningful comparisons to be made between distantly related taxa, such as Bacteria and Eukarya (yeasts).

We found that statistical support for Darwin's hypothesis is dependent upon on both culture conditions and measures of relatedness. Strong positive and negative relationships observed in co-culture screening are the subjects of deeper study, where pathway-level annotations provide insight into potential mechanisms for biotic interactions. A fundamental understanding of these relationships is paramount for both preventing spoilage as well as the controlled production of sour beer. Furthermore, this work sets a precedent for thorough culture-based studies of microbe-microbe interactions in complex communities.

In memory of Dr. William Safranek, my first formal mentor.

Your passion for science and education lives on in the many people you inspired.

We will try to not be so bashful.

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CHAPTER 1: INTRODUCTION

In recent years, astonishing advances have been made in gene sequencing technology. Whole genome sequences have been published for hundreds of thousands of bacteria in just a few decades. Metagenomics have revealed previously unobserved complexity of microbial communities. Microbes which had never been cultured in the laboratory were found to be quite ubiquitous both in nature and in the body [18]. These observations have led renewed interest in understanding the functional role of microbial communities, for example in the human microbiome where they may directly influence health [23].

Microbiologists are now well aware of a great disparity between complex microbial communities and isolated laboratory cultures. Much effort has been directed toword utilizing available metabolomic data from annotated genomes to make predictions about the nature of microbial interactions [17]. Unfortunately, genomic data is being generated at a rate that exceeds the scientific community's ability to validate annotations or predictions with scrutiny, and the majority of annotations lack experimental evidence [46]. Furthermore, computationally-derived predictions of these microbial interactions remain largely unverified [35].

In the present work, the complex problem of verifying predictions of microbial interactions is approached by testing a simple ecological hypothesis in a tractable microbial ecosystem. Following recent publications that seek to predict interactions within the human microbiome, Darwin's competition-relatedness hypothesis is tested. Inspired by Pasteur, who helped revolutionize medicine and microbiology through studies of spoilage, beer seemed an intuitive choice of medium for this investigation. It is hoped that data generated is useful to both the scientific research community as well as brewing industry specialists.

CHAPTER 2: LITERATURE REVIEW

2.1 Historical scientific advancements from beer

Brewing is the first biological engineering process to be utilized by humans and pre-dates written history. The earliest evidence of brewing comes from chemical tests of 7000 year old pottery discovered in what is now Iran. Some of the oldest writings discovered were ancient Sumerian beer receipts. It has been suggested that brewing spawned the agricultural revolution, catalyzing the development human civilization. The process has been invented independently several times and every major civilization has developed unique brewing practices [15].

Louis Pasteur's studies of beer spoilage provided irrefutable evidence for germ theory, which had a revolutionary effects in medicine. This medium, relatively simple with comparisons to the human body, allowed for testing of hypotheses that helped disprove spontaneous generation. As a result aseptic practices became commonplace in medical treatment, vastly reducing mortality from infection [56].

A natural result of advancements aseptic practices was a revolution in food and beverage production. Starter cultures that were cultivated by back-slopping of previous batches began to be produced by growth of pure cultures of singular isolated strains [19]. Such practices in brewing permeated the food industry. Many modern fermented foods and beverages are produced using pure cultures, with some notable exceptions.

The study of beer has not only had far reaching effects in microbiology, but molecular biology as well. The first observation of enzymatic activity was the fermentation of sugar to alcohol using yeast lysate by Eduard Buchner, for which he was awarded the Nobel Prize in Chemistry in 1907 [42].

2.2 Beer industry economic impact

Given the rich humanistic heritage in brewing, it seems unsurprising that brewing is a pervasive practice in modern times. Beer remains the most popular alcoholic beverage in the world by production volume. 189,060 kL (∼$50 million gallons) were consumed in 2014 globally [29]. In the United States the beer industry generates over $350 billion in economic output and represents 1.9% of the gross domestic product. This number includes the economic contribution from breweries, distributors, and retailers [16].

$68 billion of this is from craft beer, defined as breweries that produce less than 6 million barrels per year. While the number of large breweries has remained relatively steady in recent history, the number of craft breweries has been explosive. There were only 124 breweries in 1986 in the United States. By 2006 that number had increased two orders of magnitude. In 2016 there were 5,301 breweries; all but 50 were craft breweries. Craft beer has come to encompass an increasing share of the US beer market and in 2016 craft sales increased by 6.2% [8].

2.3 Beer spoilage

Beer spoilage is persistent threat in modern beer production, and contamination is stringently avoided by most brewers. The process of brewing requires aseptic movement of raw materials between large vessels before being packaged and consumed. With each step in the process there exists risk of contamination. A single fermentation vessel could contain tens or hundreds of thousands of dollars in product, and occupy valuable brewing space for weeks or months.

Spoilage organisms in brewery environments have been extensively studied [5]. In an American craft brewery that produces both modern monoculture beers in addition to sour beers, it was found that substrate and surface contact were key factors in distribution of spoilage microbes. They also found raw materials likely to be contributors of spoilage microbes [6].

Detection of spoilage organisms in modern breweries is often performed by taste or by off-site third parties. Breweries equipped with laboratories may cultivate contaminants on selective and differential media [25]. Newer detection methods include ATP luminescence, which is both cost effective and highly sensitive, but non-specific to spoilage organisms [24]. Other commercially available options for large breweries include the proprietary qPCR-based GeneDisc ® (Pall) that is sensitive and allows for identification of specific microbes. Methods that utilize PCR or next-generation sequencing are of great value to researchers. However, due to price restraints and the necessity for specialized training, these techniques are not often viable options to brewers routine use [20].

Beyond packaging, distribution and handling practices at retailers can be variable. Vinyl beer lines that run from kegs to taps provide an opportunity for contamination that is often beyond control of breweries. Beer lines at proactive retailers are regularly cleaned, but rarely replaced. Other retailers may rarely clean and never replace lines. Beer lines are typically a minimum of one meter in length, but may span great lengths depending on the layout of the establishment. Beer lines can harbor bacteria and yeast that produce off flavors. By serving beer through infected lines, it is possible to harm a brewers reputation. This effect can be especially damaging in the case of fledgling craft breweries in an increasingly competitive market [9].

Spoilage organisms in draft dispense systems are less extensively studied than those of brewing environment. Both draft lines and taps are thought to harbor biofilms that may drastically change beer flavor [43]. The Brewers Association recently issued a $120,000 grant to NSF International Applied Research Center (ARC) and Center for Biofilm Engineering (CBE) to assess factors that lead to biofilm formation and assess efficacy of cleaning methods to combat this process. The Brewers Association describes this research as the first of its kind and the grant to be the largest issued by the trade group to date, reflective of the magnitude of the problem for craft brewers [7].

2.4 Sour beer

Sour beers are a heterogeneous collection of beer styles with one unifying feature, the use of multiple microbes in the fermentation process which produce lactic or acetic acid. The use of microbes that otherwise may spoil beer are utilized in such a way that is considered pleasing to the palate. Examples include the yeast *B. bruxellensis* and bacteria *P. damnosus* that are marketed for commercial and home-brewing use in specialty beers [54]. While little data has been generated regarding the economic impact of specific beer styles, Google Trends indicates increased interest in recent years (Figure 2.1).

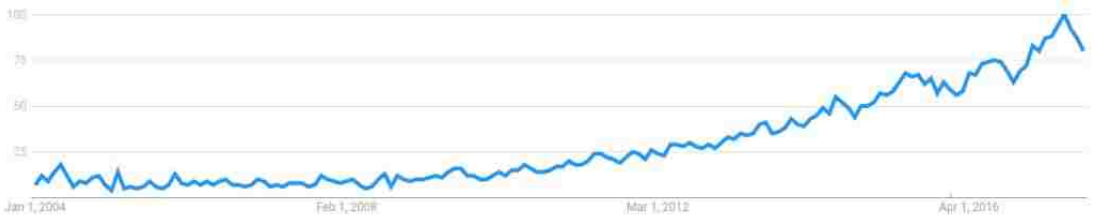

Figure 2.1: **Recent social interest in sour beer.**
Relative frequency of searches worldwide for the term "sour beer" from Google Trends from Jan. 2004 to Oct. 2017. Numbers represent search interest relative to the highest point on the chart for the given region and time.

The fermentation process of historical Belgian styles such as lambic has traditionally been referred to as "spontaneous" although it is well understood by studies of germ theory that this is not the case. These beverages are produced without stringent microbial control processes or starter cultures and are contrasted with modern beers in this way. On the other hand, sour beers like German Berlinerweisse may be produced by a controlled two-step fermentation process with a lactic acid fermentation preceding the primary ethanol fermentation. Brewers of such beers may utilize pure cultures to achieve desired flavor profile. American wild ale is an emerging style with little historical pedigree. Brewing practices are highly variable. Some brewers follow traditional

"spontaneous" fermentation practices while others exhibit tight control using a number of pure cultures [4]. To this end, only a few strains of bacteria and yeast are commercially available and little is known of their effects on each other..

Comprehensive studies of sour beer fermentation date back to at least 1977 [55]. It is well understood that the traditional "spontaneous" fermentation process is the result of a succession of a diverse array of species which contribute the flavor profile of the final product. The fermentation process begins with bacteria of the family *Enterobacteriaceae* which are rapidly displaced, followed by *Lactobacillaceae*, then *Acetobacteraceae*. Yeast populations are initially quite diverse, with little consensus between studies as to which taxa are dominant. Primary fermentation occurs by *Saccharomyces* yeast over the first few months and a secondary fermentation occurs by *Brettanomyces* over several years [50, 51].

Recent studies recapitulate most observations of succession in traditional sour beer in earlier works, with the exception of the initial yeast population. These yeast are short-lived and their contribution to the flavor profile is poorly understood. Early studies that based identification on microscopic observations claimed *Kloeckera apiculata* to be dominant initially in the fermentation process. More recent studies that use genetic techniques to make taxonomic assignments, however found *Pichia spp.* and *Candida spp.* to be abundant in the initial fermentation and found no evidence of *Kloeckera spp*. The cause of the discrepancy is not clear at this time [49].

A common shortcoming of previous studies of sour beer is that their experimental design places little emphasis on the functional role of individuals in the fermentation process, let alone interactions between members of the community. Such observational studies lay a ground work for understanding the traditional fermentation process, but yield little knowledge that is useful to crafting modern sour beers with starter cultures.

2.5 Fermented foods and beverages as microbial ecosystems

Fermented foods and beverages have gained recent attention for the use as model microbial ecosystems. Their value lies in understanding the mechanisms of microbial community formation. There is little consensus about the mechanisms that lead to establishment of persistent communities found in the human microbiome, at least with regard to bacterial species.

A recent review article summarizes numerous studies on fermented foods as microbial ecosystems that have been conducted recently. The article describes that a great challenge in understanding microbial communities lies in determining function, due to vast complexity. The authors suggest a pragmatic approach to simulating the structure of the community is to utilize a subset of representative organisms for deeper study [59].

2.6 Co-cultures

Co-cultures are of great importance in the food industry where cooperative metabolic interactions are regularly employed. A well studied example is yogurt produced by starter cultures consisting of *Streptococcus thermophilus* and *Lactobacillus bulgaricus*. The combined metabolism of these two bacteria are shown to interact positively to produce the desired flavor and aroma compounds of the finished product [2].

While the importance of co-culture cannot be understated, comprehensive study of co-cultures from ecosystems is labor intensive. The number of co-cultures increases exponentially with the number strains investigated. This has led researchers to use robotics in co-culture screening and other labor intensive tasks. Such techniques are known as "culture-omics" and are intended to complement other bioinformatic "-omics" techniques [22].

2.7 Reverse ecology

Making use of whole genome data to explain metagenomic observations would seem a natural goal of systems biology. Numerous so-called reverse-ecology tools have been developed which take different computational approaches [36]. Of particular significance to the present work is a collection of tools that attempt to predict competitive and cooperative interactions in metabolic networks named NetSeed, NetCmpt, and NetCooperate. NetSeed uses Kosaraju's algorithm algorithm to define metabolic "seeds" defined as the minimum reactants required to generate all other products in a network. NetCmpt and NetCooperate compares lists of seeds and non-seeds to generate an asymmetric matrix of competitive and cooperative interactions respectively [34].

2.8 Competition relatedness hypothesis

The group that developed NetCmpt and NetSeed algorithms used these tools and found support for a controversial Darwinian hypothesis known as competition-relatedness or limiting-similarity. This hypothesis makes two logically equivalent predictions about the nature of species interacting in ecosystems; closely related species will compete and more distantly related species will cooperate. While predicted competition and cooperation data are well correlated well with observations of co-occurrence in human microbiome metagenomes, experimental evidence of predicted interactions remains arguably scarce [33]. Despite the intuitive nature of these predictions, recent evaluations produced mixed results [1].

CHAPTER 3: METHODOLOGY

3.1 Sample collection and strain isolation

Sampling sources included contaminated packaged beer from local craft breweries, contaminated tap lines at local bars, sour beer from local crafter brewers and home brewers. Some pure strains of bacteria and yeast for brewing were also purchased from a national supplier. Samples were collected as aseptically as possible using sterile equipment, then kept on ice during transportation and storage.

Samples were plated within 24 hours of collection on modified yeast malt agar (YMA) containing bromophenol blue (BPB) and a selective agent [58, 32]. Either 10 μg/mL cycloheximide or 100 μg/mL chloramphenicol were added to select against the growth of yeast or bacteria respectively [48, 40]. A variety of techniques were employed to effectively isolate microbes from samples with varying concentrations of microbes. These include serial dilution and spot plating, spread plating, and streak isolation. Approximately 100 strains were isolated in total.

3.2 Taxonomic assignment and phylogenetic analysis

Isolated strains were identified on the basis of genetic barcoding. Genomic DNA was extracted from 1 mL liquid cultures using a rapid two-step technique. First, cells from a 1 mL culture (ideally OD 1.0) were pelleted and lysed using zirconia-silica bead beating in 200 μL lysis buffer consisting of 10 mM Tris-Cl pH 8.0, 5 mM EDTA, 1 mM sodium dodecyl sulfate (SDS), and 10 μg/mL RNase A [39]. 100μL of lysate was then purified by silica column binding in 500μL 5 M guianidine solution thiocyanate pH 5.0 with 100 μL isopropanol [11]. Previous reports demonstrate RNase A unfolding to prevail above 1 mM SDS concentration [41]. To our knowledge, this is the first time this technique has been employed, which uses RNase A during lysis with SDS at sub-inhibitory concentrations with resect to enzyme activity.

Hypervaribale regions of ribosomal RNA genes were amplified using polymerase chain reaction (PCR). Bacterial primers (S-D-Bact-0341-b-S-17/S-D-Bact-0785-a-A-21) targeted the V3/V4 region of the 16S ribosomal RNA genes [31]. Fungal primers (ITS1F_KYO1/ITS4_KYO1) targeted the ITS region [53]. Alternative fungal primers (LR0R/LR3) targeting D1/D2 of the large ribosomal subunit RNA genes were also used [45]. Amplicons were purified by gel extraction and then Sanger sequenced commercially. Sequences were used to query Silva using BLAST [44, 12]. Taxonomic assignments were made based on $\geq$98% sequence homology to known specimens.

Bacterial and yeast sequences were aligned separately in MEGA 7 software using the Muscle algorithm [38]. These alignments were used to generate independent phylogenetic reconstructions via the Maximum Likelihood method [52]. Since sequences were trimmed, all gap sites were used, increasing resolution of the reconstruction [57]. Alignments in MEGA were also used to generate relatedness data for our initial analysis with bacteria only. A distance matrix based on the homology of these alignments was exported from MEGA, and ribosomal relatedness was defined as 1 - Distance. Measures of relatedness can be literally interpreted as homology between two ribosomal genetic sequences.

3.3 Bioninformatic analyses using representative whole-genome data

Representative whole genome data were obtained from GenBank type specimens, having been identified by ribosomal taxonomic assignments [10]. FASTA formatted lists of proteins were downloaded for calculations of protein relatedness. GenBank formatted files were downloaded for metabolic competition modeling. Python scripts used in this study are included in Appendix B.

A python script was written to compute the relatedness of organisms on the basis of protein homology. This script uses protein sequences of a given genome as BLAST queries against protein sequences of another genome. Functionally equivalent “positives” are tallied and protein relatedness is reported as the weighted average of positives returned divided by the number of amino

acids queried between two proteomes. Protein relatedness representative of the average functional homology between two proteomes. This approach allowed meaningful comparison to be made between two distantly related genomes, such as between prokaryotes and eukaryotes.

To more accurately predict competition between organisms, higher levels of annotation were required. GenBank formatted sequence files were loaded into Pathway Tools and PathoLogic was used to generate a metabolic network reconstruction [26, 27]. The reconstructed reaction network was then exported into SBML format [28]. The SBML files were loaded into the NetSeed online webtool which generated a list of "seed" compounds for each organism [13]. Seeds are defined as the minimum reactants required to generate all other reactions within a network [33]. A python script was written to compare lists of seeds between organisms. Metabolic seed relatedness is reported as the number of common seeds divided by the total number of non-redundant seeds of two organisms.

3.4 Co-culture experimental setup

All co-cultures were grown in filter sterilized malt extract broth (MEB). Malt extract broth is similar to what brewers refer to as "wort", the precursor to beer. It is derived from the enzymatic conversion of starch from malted barley to sugar and is composed mostly of the disaccharide maltose. Other components include larger sugars such as maltotriose and dextran, and to a lesser extent components such as protein, lipids, and tannins [37]. Malt extract is generated by drying the liquid wort into a powdered product that can be rehydrated at a later time. Brewers typically boil the rehydrated malt extract for one hour, then cool the wort as rapidly as possible before adding pure yeast cultures.

Great effort was taken in the aseptic preparation of growth media to generate a product qualitatively similar to brewers wort that was transparent and thus suitable for optical analyses. Malt extract was added to deionized water at a rate of 10-15% w/v. Once fully dissolved, it was

boiled for 30 minutes allowing steam to escape. The malt extract broth was cooled rapidly by copper coil, then the vessel was transferred to an ice bath overnight to promote the precipitation of haze-forming proteins. The broth was centrifuged twice at 4000 RCF at 0°C to remove insoluble proteins which interfere with filtration. The centrifuged broth was then filtered by 0.2 μm nitrocellulose membrane in a HEPA laminar flow cabinet. Finally, the filtered broth was stored refrigerated in autocalved bottles prior to use.

Cells used as inoculum for co-cultures were washed and "snap" frozen. Individual isolates were grown in MEB for one to five days, depending on growth rate. Cells were centrifuged and washed with 50 mM phosphate buffered saline (PBS), then resuspended in a cell storage buffer consisting of 50 mM PBS with 12.5% glycerol for storage. Cells were homogenized, and subsamples were distributed into multiple microcentrifuge tubes and rapidly frozen in dry ice ethanol slurry. Tubes were stored at -80°C and thawed only once. For each batch of snap frozen cultures, one tube was sacrificed for plate counting. This was thawed serially diluted, then spotted onto agar plates. Colony forming units (CFUs) were enumerated after incubation at 25°C. This was done in an effort to ensure consistent inoculation of co-cultures.

Setup for co-culture screening took place entirely in a HEPA laminar flow hood to prevent contamination. Snap frozen cells were thawed, then normalized in the cell storage buffer. Normalized cells were added to aliquots of MEB resulting in approximately 500 CFU per 100 μL. These 100 μL aliquots were added to microtiter plates pairwise in a quasi-randomized configuration. Plate configurations were generated using a random number generator, then selected for satisfying certain criteria. Plate configurations were chosen where exposure to edge effects was equal and such that like cultures were not adjacent. After addition of inoculated media, microtiter plates were sealed with sterile breathable film to prevent cross contamination, minimize evaporation, and allow for out-gassing of cultures. All co-cultures were incubated in microtiter plates for two weeks at 25°C.

Abiotic conditions varied were media concentration, the addition of hops in media preparation, and atmospheric conditions. Media concentrations was measured by hydrometer and reported as specific gravity (SG), which was varied between SG = 1.040 and 1.060. Concentration of hop compounds in growth media was measured by spectrophotometric absorbance of iso-alpha-acids at 275 nm following solvent extraction in iso-octane [3].

Atmospheric conditions were varied using an in-house high-pressure anaerobic chamber crafted from home-brewing equipment (Figure 3.1). This consists of two soda kegs turned on their side, allowing for the insertion or removal of microtiter plates. The outlet of the soda kegs are attached to a mason jar with an air-lock housing a Resazurin anaerobic indicator strip. The soda kegs are supplied by a CO_2 tank with a pressure regulator. For low pressure anaerobic incubation, one psi of pressure was maintained while allowing outgassing through the air-lock until anaerobic condition was confirmed by indicator strip, then the outlet was sealed and CO_2 supply was turned off. High pressure incubation followed the same initial purging protocol, but pressure was increased to 15 psi for the remainder of the incubation.

Figure 3.1: **High pressure anaerobic incubation chamber.**
The anaerobic chamber, adapted from home-brewing equipment, allows for stationary incubation of microtiter plates in high pressure. Anaerobic condition is indicated by test strips in airlock.

3.5 Co-culture data collection and analysis

Following two weeks of incubation, the two measurements of biotic output were growth, measured by light scattering, and acidification, measured by a colorimetric assay. It should be noted that, while light scattering increases with cell density, it is not consistent across cell types as a reliable metric of biomass. In this sense, reported measurements are merely an estimate of biomass. Alternative evaluations would include CFU counting and direct biomass measurement. Given the large number of samples, these methods were determined to be too laborious for practical application in the present work.

Acidification of media was measured using a colorimetric method developed in-house. Bromophenol blue (BPB) is added to supernatant from cultures, which changes color within a range of pH 3.0 - 6.0. Following light scattering readings of OD 600 nm, plates were centrifuged at 1000 RCF for 30 minutes to pellet cells. 100 μL of supernatant was aspirated from each well and transferred to fresh microtiter plate. 20 μL of 0.5 mg/mL BPB in 10% ethanol was added to each well. Peak optical absobances of BPB are observed at 440 nm and 590 nm. As pH shifts from basic to acidic, absorbance at 440 nm increases 590 nm peak decreases. Using the ratio of absorbance of the two peaks, the pH of the media was reliably determined for a large number of samples relatively rapidly. Spectral scan and standard curve of blank-subtracted absorbances of BPB in growth media are seen in Figure 3.2.

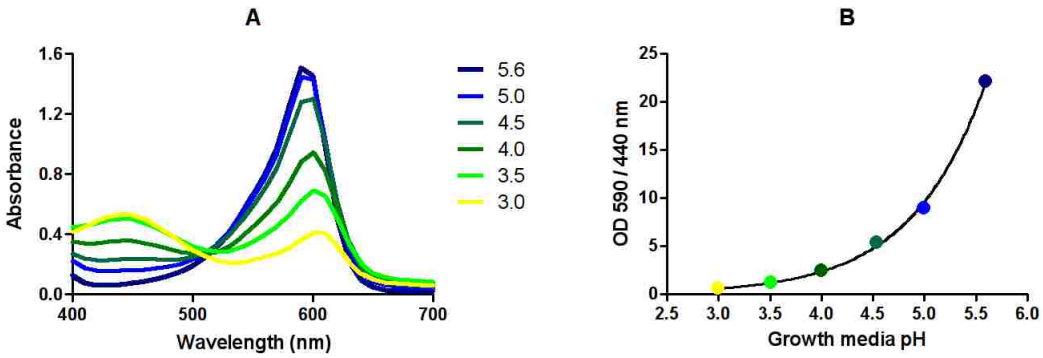

Figure 3.2: **Spectral scan of bromophenol blue in media of variable pH.**
Spectral scan of BPB in growth media shows two local peak absorbances at 440 and 590 nm that vary inversely with pH (A). Semi-log fit of OD 590/440nm ratio is well correlated (R^2 = 0.9984) with pH of growth media (B).

Analysis of data was primarily performed using LibreOffice (OpenOffice.org) spreadsheet software to determine averages and variances, then Prism ® (GraphPad) was used for statistical analyses. First, corrections were made to account for pipetting errors. Next, data were derandomized to a common intuitive configuration. Measurements were blank corrected by subtracting the average measurements of un-inoculated media. For each co-culture and isolated culture, the average growth and coefficient of variation was calculated. Competition and cooperation are calculated as the difference between co-culture growth and average growth of isolates, weighted by the average of the isolates. Co-acidification is measured as an unweighted difference between acidification co-cultures and the average acidification of the isolates.

CHAPTER 4: RESULTS

4.1 DNA extraction, PCR, and sequencing

DNA samples extracted from bacterial and yeast isolates were of sufficient quantity and quality for downstream use in PCR. The DNA extraction technique used here allowed for reliable cell lysis and purification of sufficient quantities of high quality genomic DNA. Genomic DNA extraction using this method was effective for all cell types used in this study including bacteria and yeast. Several samples can be processed in less than one hour and minimal hazardous chemical waste is produced. Ethidium bromide gel electrophoresis indicates that samples are free of RNA that may impede downstream analyses (Figure 4.1).

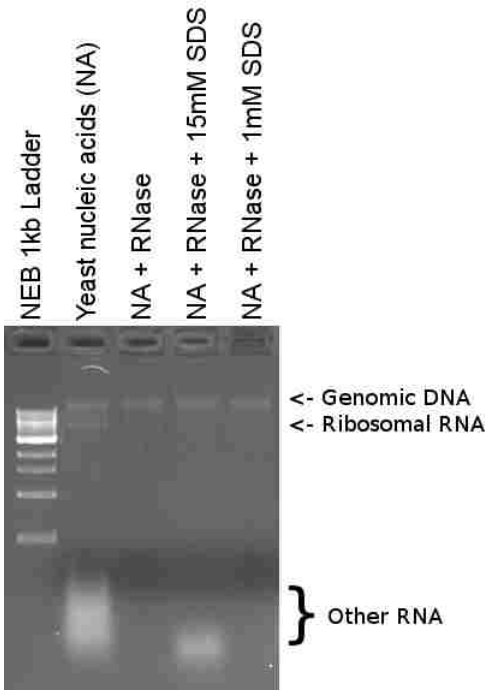

Figure 4.1: **Genomic DNA extracted from beer spoilage and souring isoaltes.**
Agarose gel electrophoresis of nucleic acid samples visualized with ethidium bromide UV transillumination. RNase A treatment is effective in lysis buffer with 1 mM SDS but not 15 mM SDS.

For bacteria, PCR amplification of V3/V4 from 16S RNA encoding genes produced consistently sized ∼500 bp amplicons that generated high-quality Sanger sequence data. For fungi, primers targeting the ITS region generated products of variable size, dependent upon the fungal species (Figure 4.2). A notable problem in the case of brewing yeast *Saccharomyces cerevisae* and *S. pasteurianus* was the production of heterogeneous ITS amplicons, which obfuscated interpretation of Sanger sequencing [60]. Further investigation of WGS data for *S. cerevisiae* strain QA23 revealed two unique species of ribosomal operons, one with 13 base pair deletions interspersed throughout the ∼1kb diagnostic sequences. For *S. pasteurianus* strain W34/70 the WGS data was lacking complete ITS sequences, implying such heterogeneity is indeed problematic for next-generation sequencing as well. Primers targeting the D1/D2 region of the large ribosomal subunit (LSU) were used to generate sequences of acceptable quality for taxonomic analyses of yeast.

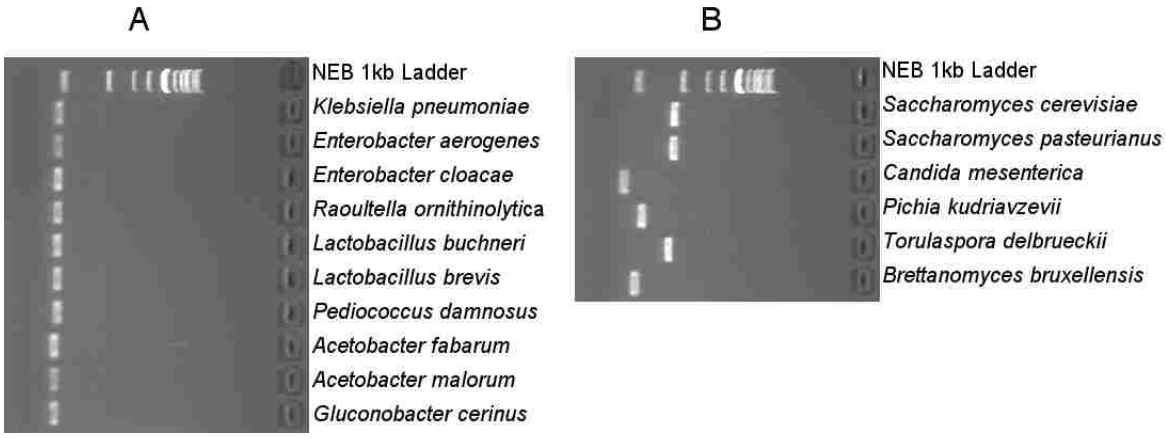

Figure 4.2: **Purified PCR products from beer spoilage and souring isoaltes.**
Agarose gel electrophoresis of purified PCR products visualized with ethidium bromide UV transillumination. Bacterial V3/V4 amplicons (A) are of relatively consistent size (∼500bp), while fungal ITS amplicons (B) are of variable size (500-1000bp).

4.2 Taxonomic assignment and phylogenetic analysis

All isolates collected were identified on the basis of sequence homology to Silva specimens [44]. Taxonomic assignment of yeast by ITS and LSU yielded qualitatively similar results. From approximately 100 isolated strains, 16 were selected for inclusion in co-culture experiments and bioinformatic analyses. Both *Candida sp.* and *Pichia sp.* yeast were identified in beer samples used in this project, and no evidence was found for *Kloeckera spp.* yeast. Numerous isolates of *Brettanomyces bruxellensis* were found in aged sour beer exhibiting an array of colony morphologies. Some strains marketed as *B. anomalus* were indistinguishable from *B. bruxellensis* by Sanger sequencing, therefore a single *B. bruxellensis* isolate was chosen for further study.

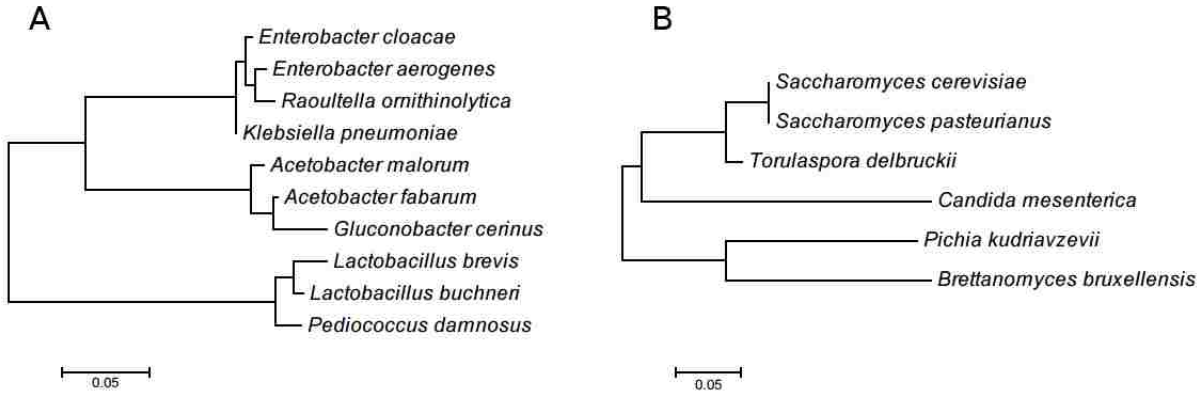

Figure 4.3: **Phylogenetic reconstruction of bacteria and yeast isoaltes.**
Figures are generated in MEGA 7 software using ∼500 bp V3/V4 16S ribosomal sequences of bacteria (A) and D1/D2 26S ribosomal sequences of yeast (B). The tree is drawn to scale, with branch lengths measured as the number of base substitutions per site.

4.3 Validation of bioinformatic tools for relatedness metrics

Assignment of WGS data from type specimens was unproblematic for bacterial strains used in this study. Each strain had a well-annotated counterpart on GenBank that could be readily used for proteomic BLAST-based analysis or metabolomic analysis via PathwayTools. Yeast WGS data however, proved to be much more elusive and certain exceptions needed to be made to continue with bioinformatic analyses. In the case of *C. mesenterica* WGS data has yet been published, so data from closely related *C. dublinensis* was used to serve as a "surrogate". For the yeasts *S. pasteurianus* and *T. delbruckii*, the available WGS data were not functionally annotated, and could be used only for proteomic analyses, but not metabolomic analyses. A summary of the strains used in co-cultures along with the representative WGS strains is provided below in Table 4.1.

Linear regression of ribosomal relatedness derived from ribosomal sequence homology is significantly correlated ($p < 0.0001$) with both protein relatedness and seed competition among bacterial isolates (Figure 4.4). Protein relatedness was found to be significantly correlated to seed competition, and both metrics allow for pairwise analysis of relatedness between bacteria and eukaryotic yeast (Figure 4.5). This validates the use of these tools for further investigation. Metrics of protein relatedness and seed competition have a more uniform distribution than ribosomal relatedness, which may allow for greater resolution of taxonomic and functional differences.

Table 4.1: **Bacteria and yeast isolated from spoiled and sour beer used for co-cultures.**
The species listed here were isolated from sour and spoiled beer, then used for co-culture screening. Bioninformatic analyses were performed using the WGS reference type specimens listed here.
AAB = Acetic acid bacteria, LAB = Lactic acid bacteria.
† = Not functionally annotated , * = *Candida dublinensis* "surrogate" data used.

Group	Species	Isolation Source	Date	WGS Reference
Enteric	*Enterobacter aerogenes*	Sour beer	09-30-2016	KCTC 2190
Enteric	*Enterobacter cloacae*	Sour beer	09-30-2016	ATCC 13047
Enteric	*Klebsiella pneumoniae*	Sour beer	07-24-2016	HS11286
Enteric	*Raoultella ornithinolytica*	Sour beer	09-30-2016	B6
AAB	*Acetobacter fabarum*	Sour beer	09-30-2016	LMG 1590
AAB	*Acetobacter malorum*	Draft line	01-06-2015	LMG 1746
AAB	*Gluconobacter cerinus*	Draft line	01-06-2015	CECT 9110
LAB	*Lactobacillus brevis*	Canned beer	11-03-2014	ATCC 367
LAB	*Lactobacillus buchneri*	Purchased	01-10-2017	CD034
LAB	*Pediococcus damnosus*	Purchased	01-10-2017	TMW 2.1535
Yeast	*Saccharomyces cerevisiae*	Sour beer	07-24-2016	S288c
Yeast	*Saccharomyces pasteurianus*	Purchased	01-10-2017	CBS 1513†
Yeast	*Torulaspora delbrueckii*	Draft line	01-06-2015	CBS 1146†
Yeast	*Candida mesenterica*	Draft line	01-06-2015	CD36*
Yeast	*Pichia kudriavzevii*	Sour beer	07-24-2016	Strain 129
Yeast	*Brettanomyces bruxellensis*	Sour beer	09-30-2016	AWRI1499

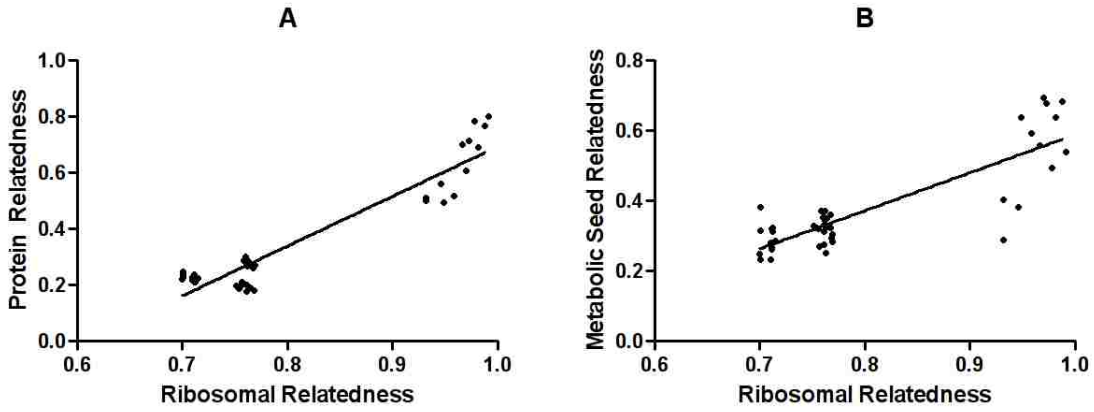

Figure 4.4: **Validation of bioinformatic relatedness metrics.**
For bacteria only, linear regression of ribosomal relatedness determined from alignement of ribosomal sequences compared with proteomic relatedness determined by Python BLAST script (A) and metabolic seed relatedness determined using NetSeed (B).
$R^2 = 0.8935$ and 0.7241 respectively ($p < 0.0001$ for both).

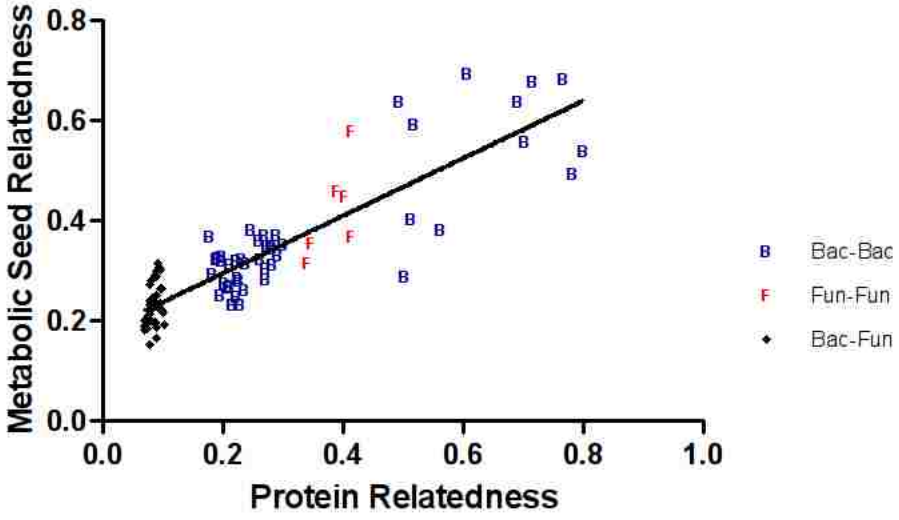

Figure 4.5: **Comparison of bioinformatic relatedness metrics.**
Linear regression of proteomic relatedness and metabolic seed relatedness including bacteria, fungi, and inter-domain bacterial-fungal comparisons. $R^2 = 0.7714$ and $p < 0.0001$.

4.4 Results of co-cultures not reliant on distance metrics

Data from co-cultures are displayed in the form of heat maps found in Appendix A. Both cooperation index and co-acidification data provide useful insight to industrial microbiologists such as brewers and future researchers intending to exploit of study relationships among individual species. There were stark differences between aerobic and anaerobic co-cultures. For example, a strict patterning is seen for co-acidification among low pressure anaerobic cultures in Figure A.5 that is not seen in other physiological conditions.

These analyses indicate a high prevalence of metabolic cooperativity among pairs of microbes shown in Figure 4.6. Co-cultures tend to grow to a significantly greater optical density than the average of their isolates. Co-cultures tend to acidify media to a greater extent than the average of their isolates. By paired t-test $p < 0.0001$ for all data sets (not shown). It is reasoned that relatedness is maximal among the clonal individuals in isolation, and that relatedness for co-cultures is arbitrarily less. These data support the competition-relatedness hypothesis under this assumption.

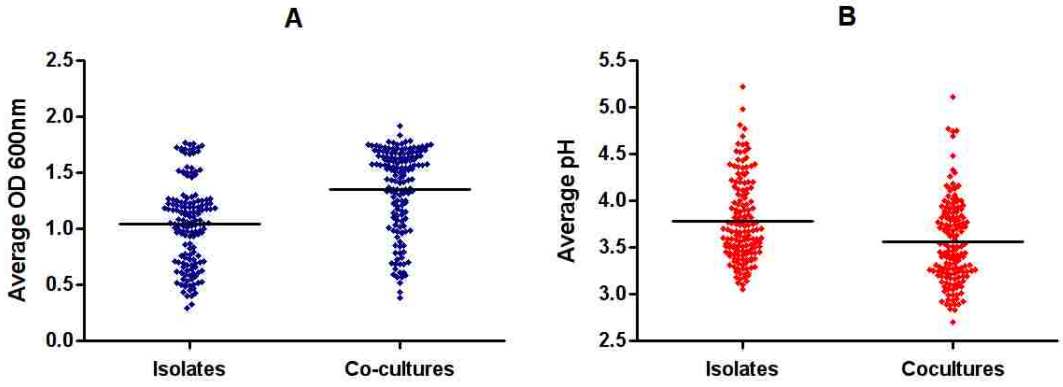

Figure 4.6: **Overall comparisons of co-cultures with average of isolates.**
Dot plot of averaged results from 12 replicates (microaerobic, SG=1.040). Co-cultures tend to grow to a greater optical density than the average of their isolates (A), and acidify media more than isolates (B). In all growth conditions tested $p < 0.0001$ by paired t-test.

4.5 Independent analyses of bacterial and yeast co-cultures using ribosomal data

The following data are included for comparison of competition-relatedness analyses using conventional relatedness metrics with bioinformatic analyses. Using conventional taxonomic relatedness metrics based on ribosomal homology, analyses of the competition-relatedness hypotheses were limited to a single domain (Bacteria or Eukarya). Figure 4.7 shows the analysis of bacterial co-cultures under micro-aerobic conditions. A statistically significant positive trend ($p = 0.0004$) is observed for linear regression of ribosomal relatedness and competition, supporting the central hypothesis. Linear regression of co-acidification with ribosomal relatedness also produces a positive trend line, but with weak statistical support ($p = 0.1293$).

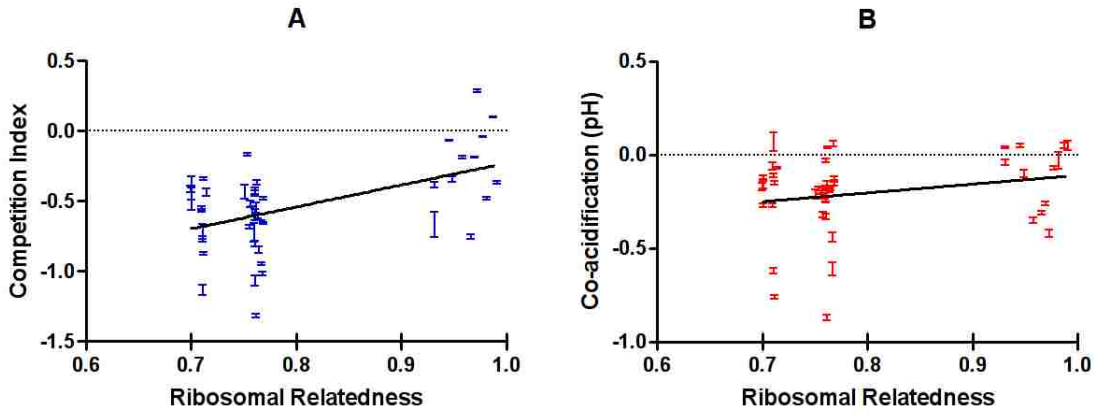

Figure 4.7: **Analysis of bacterial co-cultures by ribosomal relatedness.**
Linear regression of ribosomal relatednesss, determined from alignment of V3/V4 16S sequences, with competition index (A) and co-acidification (B) for microaerobic (SG = 1.040 and n = 12) bacterial isolates and co-cultures only. Error bars represent the standard error of the mean (SEM).

4.6 Analysis of co-cultures using representative whole-genome data

Proteomic data from type specimens allowed for inclusion of all co-cultures in analyses of the competition-relatedness hypothesis. Results of the analyses of the CRH using this proteomic relatedness metric are shown in Figure 4.8 for co-cultures in malt extract broth of SG=1.040 under microaerobic atmospheric conditions. In this case, linear regression of growth based competition generates a positive trend but with weak statistical support ($p = 0.0620$) while statistical support for the co-acidification model is higher ($p = 0.0029$). Similar results are seen for other microaerobic co-cultures when media conditions were varied (see Section 4.7).

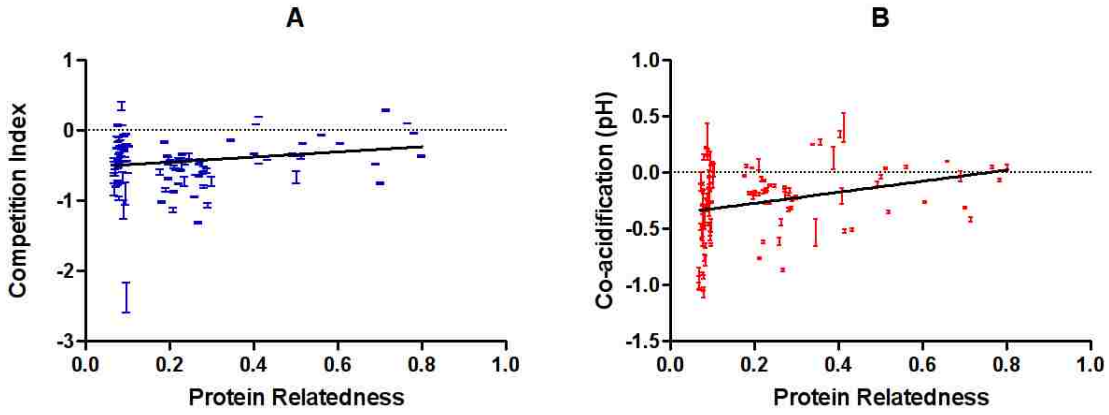

Figure 4.8: **Analysis of co-cultures using proteomic relatedness metric.**
Linear regression of protein relatedness, determined using a BLAST-based Python script, with competition index (A) and co-acidifcation (B). Bacteria and yeast are included in the same analysis. Shown here are the results for microaerobic experiments (SG = 1.040 and n = 12). Error bars represent the SEM.

Metabolic modeling of competition for nutrient seeds (dependencies) also allowed for analysis of bacteria-yeast co-cultures. Results of linear regression (Figure 4.9) are similar to proteomic analyses. Growth based metrics show a positive trend with weak statistical support (p = 0.1596) while the co-acidification model is more strongly supported (p = 0.0103). Similar to the results using proteomic relatedness metrics, these trends are fairly consistent among microaerobic co-cultures. A more even distribution is seen among the metabolic seed relatedness data here than protein relatedness. It should be noted that these results do not include *S. pasteurianus* or *T. delbruckii* due to a lack of annotated WGS data.

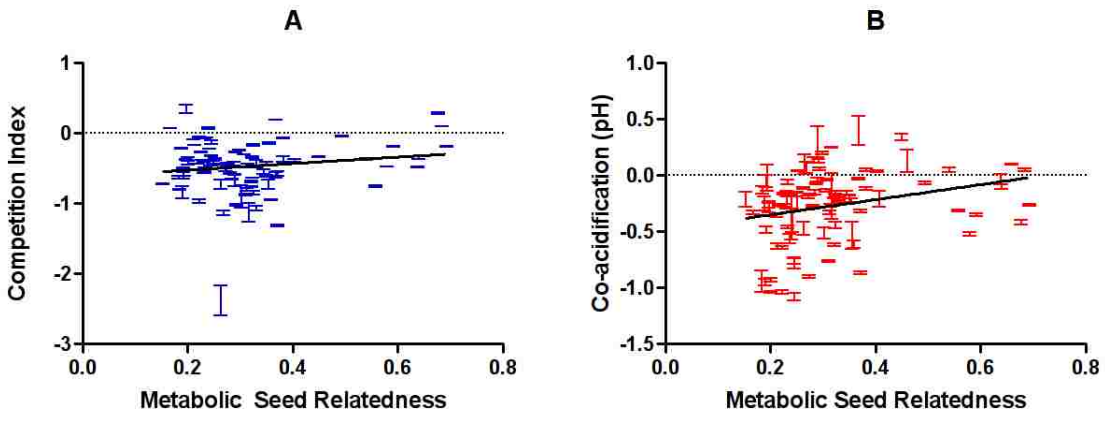

Figure 4.9: **Analysis of co-cultures using metabolic seed relatedness metric.**
Linear regression of metabolic seed-relatedness, determined by NetSeed derived algorithm, with competition index (A) and co-acidication (B). Bacteria and yeast are included in the same analysis (SG = 1.040 and n = 12). Error bars represent the SEM.

Atmospheric conditions drastically changed the growth of individuals and co-cultures. In high-pressure anaerobic experiments linear regression of competition index with proteomic relatedness and metabolic seed relatedness result in a negative trend with weak statistical support (Figure 4.10). Contrarily, the co-acidification model is statistically supported for both protein and metabolic seed relatedness ($p = 0.0003$ and $p = 0.0050$ respectively). In low pressure anaerobic experiments (1 psi), a positive trend is observed for linear regression of competition with weak statistical support. Curiously, a negative trend is observed for the co-acidification model. This is the only growth condition tested where this was the case (Table 4.2).

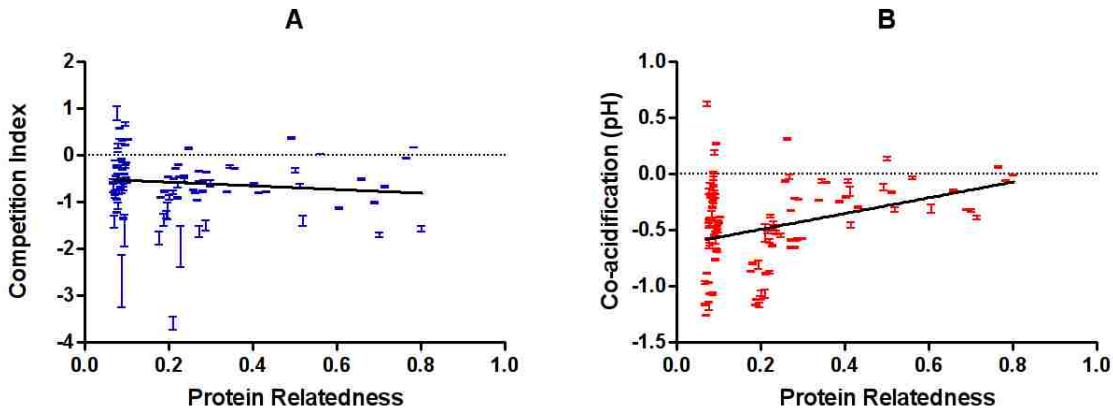

Figure 4.10: **Analysis of 15 psi anaerobic co-cultures by proteomic relatedness.**
Linear regression of protein relatedness with competition (A) and co-acidification (B) for high-pressure anaeobic co-cultures (15 psi, SG = 1.040, n=8). Bacteria and yeast are included in the same analysis. Error bars represent the SEM.

4.7 Meta-analysis of growth conditions and relatedness metrics

The following data were generated to summarize the results of various analyses of the competition relatedness hypothesis. The slope and p-value are provided for both optical density and co-acidification for each culture condition using each metric of relatedness. These data are found in Table 4.2. The results of all aerobic cultures are averaged, weighted by the number of replicates used in the analysis, then summarized in Table 4.3.

Table 4.2: **Summary of collected data.**
Linear regression statistics are summarized for competition and co-acidification analyses of beer microbes from this study. Results of 1 psi and 15 psi anaerobic co-cultures are listed at the bottom.

Growth condition	**Relatedness Metric**	**Competition Index Slope**	**Competition Index p-value**	**Acidification slope**	**Acidification p-value**	**Reps (n)**
SG = 1.04	Protein	0.3638	0.0620	0.4905	0.0029	12
SG = 1.04	Pathway	0.4608	0.0705	0.4479	0.0369	12
SG = 1.04	Seed	0.4620	0.1596	0.6700	0.0103	12
SG = 1.05	Protein	0.2170	0.2648	0.6794	0.0001	4
SG = 1.05	Pathway	0.2597	0.2968	0.6432	0.0022	4
SG = 1.05	Seed	0.2719	0.3845	0.9002	0.0006	4
SG = 1.06	Protein	0.2816	0.1746	0.6485	0.0001	4
SG = 1.06	Pathway	0.4432	0.0940	0.6490	0.0031	4
SG = 1.06	Seed	0.3645	0.2744	0.9035	0.0010	4
40 IBU	Protein	0.6025	0.0690	0.6691	0.0001	4
40 IBU	Pathway	0.9561	0.0242	0.6482	0.0004	4
40 IBU	Seed	1.0110	0.0585	0.9700	0.0001	4
1 psi	Protein	0.2024	0.4422	-0.1198	0.5227	8
1 psi	Pathway	0.1820	0.5774	-0.4882	0.0336	8
1 psi	Seed	0.2910	0.4775	-0.6546	0.0229	8
15 psi	Protein	-0.3909	0.2586	0.7030	0.0003	8
15 psi	Pathway	-0.1942	0.6623	0.8103	0.0013	8
15 psi	Seed	-0.3927	0.4814	0.8929	0.0050	8

Table 4.3: **Comparison of relatedness metrics in micro-aerobic co-cultures.**
Linear regression statistics are summarized for competition and co-acidification analyses of beer microbes from this study. Average slopes and p-values shown are weighted by number of co-culture replicates for each growth condition. Results of 15 psi and 1 psi anaerobic co-cultures are omitted.

Relatedness Metric	**Competition Index Slope**	**Competition Index p-value**	**Acidification slope**	**Acidification p-value**
Protein	0.3654	0.1157	0.5781	0.0015
Pathway	0.5069	0.1044	0.5474	0.0194
Seed	0.5056	0.1994	0.7973	0.0054

4.8 Rare and common pathways

Annotated WGS data provide information with additional utility beyond taxonomic analysis that is pertinent to microbe-microbe interactions. These data can be readily mined to better qualitatively or mechanistically understand the nature of these interactions and the influence of individuals within the community. As an example, the pathway-level annotations generated via PathwayTools were analyzed using the in-house Python script called "Ubique" (Chapter B.4). Analysis of pathway frequency among genomes revealed the abundance of rare pathways among beer isolates (Figure 4.11). Over one hundred uniquely occurring pathways exist among these genomes, demonstrating the substantive influence that individual members may have in a community. Only twenty pathways occur in all genomes and are listed in Table 4.4.

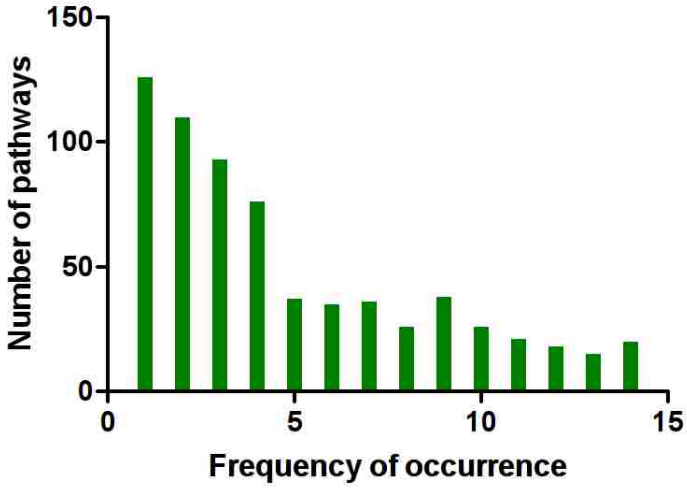

Figure 4.11: **Frequency of pathway occurrence among genomes of beer isolates.**
All pathways identified in bacteria and yeast WGS data by Python script 'Ubique' are binned by the number of genomes in which they occur.

Table 4.4: **Pathways common to all genomes of beer isolates.**
Pathways listed were identified using 'Ubique' from WGS sequences. These pathways were found to be present in all strains used in this analysis, including bacteria and yeast.

Common Pathways
Adenosine deoxyribonucleotides de novo biosynthesis
Adenosine ribonucleotides de novo biosynthesis
CMP phosphorylation
Glutathione-glutaredoxin redox reactions
Glycine biosynthesis I
Guanine and guanosine salvage
Guanosine deoxyribonucleotides de novo biosynthesis I
L-glutamine degradation I
PRPP biosynthesis I
Pyrimidine deoxyribonucleotide phosphorylation
Pyrimidine nucleobases salvage I
S-adenosyl-L-methionine biosynthesis
Tetrahydrofolate biosynthesis
Thioredoxin pathway
tRNA charging
UDP-alpha-D-glucose biosynthesis I
UTP and CTP de novo biosynthesis
Xanthine and xanthosine salvage

4.9 Measurable sources of error in co-culture screening

In the early stages of analysis of co-culture data it became apparent that well-to-well influence was an inevitable confounding factor. Breathable films were used that allow the out-gassing resultant of fermentation. An unintended side-effect is that such films also allow gas exchange into wells. Evidence for this is seen in Figure 4.12 where the pH of un-inoculated media is correlated with the average pH of cultures within plates. These observations affirm the necessity of randomization in co-culture experimental design.

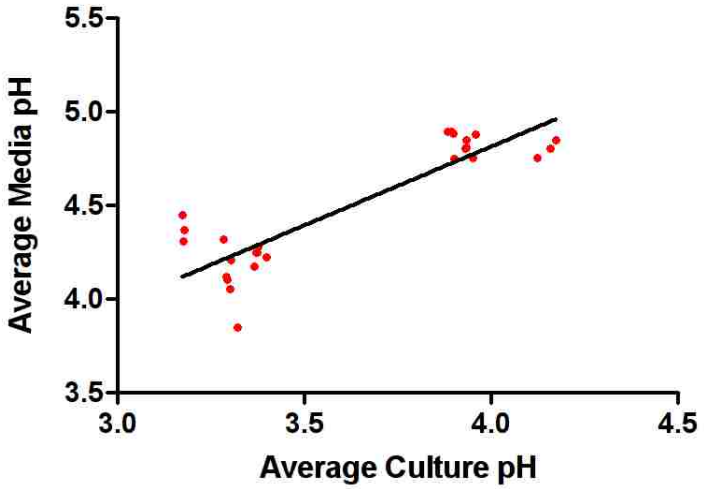

Figure 4.12: **Acidification of un-inoculated growth media.**
Linear regression of the average final pH of cultures after two weeks of incubation with the average pH of un-inoculated growth media from microtiter plate. $R^2 = 0.78$ and $p < 0.0001$.

CHAPTER 5: CONCLUSIONS

5.1 Beer is a robust medium for studies of microbial ecology

This work demonstrates the value of beer as a tractable microbial ecosystem. The majority of microbes found in both sour and spoiled beer by metagenomic studies are readily cultivable. These include a wide variety of both closely and distantly related prokaryotes and eukaryotes. This property was of particular interest in this study by allowing for a somewhat even spread of relatedness among co-culture pairs. Results of these co-culture experiments reflect the importance of inclusion of multiple species within genera in studies of microbial communities.

Physical properties of the growth media made beer an ideal system for co-culture studies. The *in situ* growth medium of brewer's wort can be readily replicated in the lab. Malt extract broth once filtered is transparent and suitable for optical measurements. This was critical for our experiments where spectrophotometry was the primary method of data collection. Experiments were scalable to a 96-well microtiter format and allowed for screening of many unique co-culture combinations. The enumeration of many replicate co-cultures made proper statistical analyses possible. Culture conditions were readily manipulated and shown to have a strong influence on growth of individuals, co-culture interactions, and analysis of the central hypothesis.

5.2 Bioinformatic tools improve measurements of relatedness

Proteomic and metabolomic analyses were developed that allowed for genome-wide comparisons to be made between prokaryotes and eukaryotes. Both bioinformatic metrics of relatedness were well correlated with conventional relatedness based on homology of bacterial ribosomal sequences validating the efficacy of these tools. These comparisons offer better resolution of genome-wide differences between recently diverged species that are not apparent by ribosomal sequences. This can be seen in Figure 4.7 where points tend to cluster into three main groups

with respect to the X-axis (based on ribosomal sequence homology) but not the Y-axis (protein relatedness or seed competition).

These tools provide more functional information about the nature of microbial interactions as well. Data generated by these scripts includes results BLAST hits from each protein-protein comparison as well lists of common ”seed” nutrients between each organism. These data can be mined in deeper investigations of individual relationships or used to better understand the community as a whole, as seen in Figure 4.11 and Table 4.4.

5.3 Key inter-relationships among brewing microbiota are identified

Regardless of the evaluation of competition-relatedness hypothesis, this investigation allowed for the observation of interactions among brewery microbes at large. This information should assist brewers in exhibiting greater control of fermentation processes. For example the growth of the yeast *Brettanomyces bruxellensis* was found to be detrimentally effected by yeast *Pichia kudriavzevii* and the bacterium *Gluconobacter cerinus*. An obscure yeast *Candida mesenterica* exhibited strong interactions among many members of the community and in co-cultures in some cases grew to greater optical density than the sum of isolated strains (see Figure A.1). Such relationships provide leads for development of starter cultures or possibly bio-control agents.

5.4 Support for Darwin’s competition-relatedness hypothesis is circumstantial

A consortium of representatives from the microbial ecosystem of sour beer were vetted for a measurable trend between relatedness and competition. Evidence supporting this hypothesis was dependent primarily on culture conditions and measurements of biological output and relatedness. Large differences in growth rates and co-culture interactions were observed between aerobic and anaerobic experiments. In micro-aerobic conditions, there exists strong statistical support for a relationship between co-acidification and relatedness. A positive trend is a consistent feature of

linear regression comparing co-culture competition evidenced by light scattering (OD 600 nm) and relatedness, but statistical support is not strong enough to accept the hypothesis. Under anaerobic conditions there is little support for the competition relatedness hypothesis (see Figure 4.2).

As microbes used in this study were isolated under aerobic conditions, it could be argued that co-cultures conducted in similar conditions are most appropriate to evaluate the hypothesis. Isolated cultures and co-cultures alike grew to substantially higher OD 600 nm in micro-aerobic conditions than in anaerobiosis indicating this is the preferred growth condition for these organisms. On the other hand, the rationale for experimentation inside anaerobic chambers was to replicate in situ brewing micro-environemnts, which are primarily anaerobic.

These highly contradictory observations make outrightly accepting or rejecting the strict interpretation of the competition-relatedness hypothesis categorically impossible. Relatedness was certainly not a definitive predictor of competitive interactions, yet more observations were recorded in favor of a positive correlation than were contradictory. Darwin postulated that such a trend would "by no means invariably" predict interactions [14]. Under this premise, evidence does seem to corroborate Darwin's statement in the case of the sour beer microbial ecosystem.

5.5 Present limitations are subjects for technological refinement

The simultaneous culture of microbes with diverse physiology proved to be technically challenging. Many microbes used in this study produce generous amounts of carbon dioxide during fermentation and may also produce various volatile organic compounds. We observed pH changes of un-inoculated growth media resultant of cross-talk between wells of microtiter plates. While many innovative co-culture techniques have been developed in recent years, none address the specific need to allow for out-gassing while remaining environmentally isolated [21]. This situation is not unique to beer microbes and future studies, for example of the human microbiome, will be wrought with similar challenges.

Efforts to determine relatedness among highly divergent microbial taxa led to development of some powerful bioinformatic tools. Still, these metrics only crudely estimate interactions between microbes. More elegant approaches such as flux-balance analyses may prove to more accurately predict such interactions [30]. The implementation of these tools was beyond the scope of this project. The refinement of predictive bioinformatic algorithms is an important but relatively new challenge in the growing field of systems biology.

APPENDIX A: COOPERATIVE INDEX AND CO-ACIDIFICATION HEAT MAPS

A.1 SG = 1.040

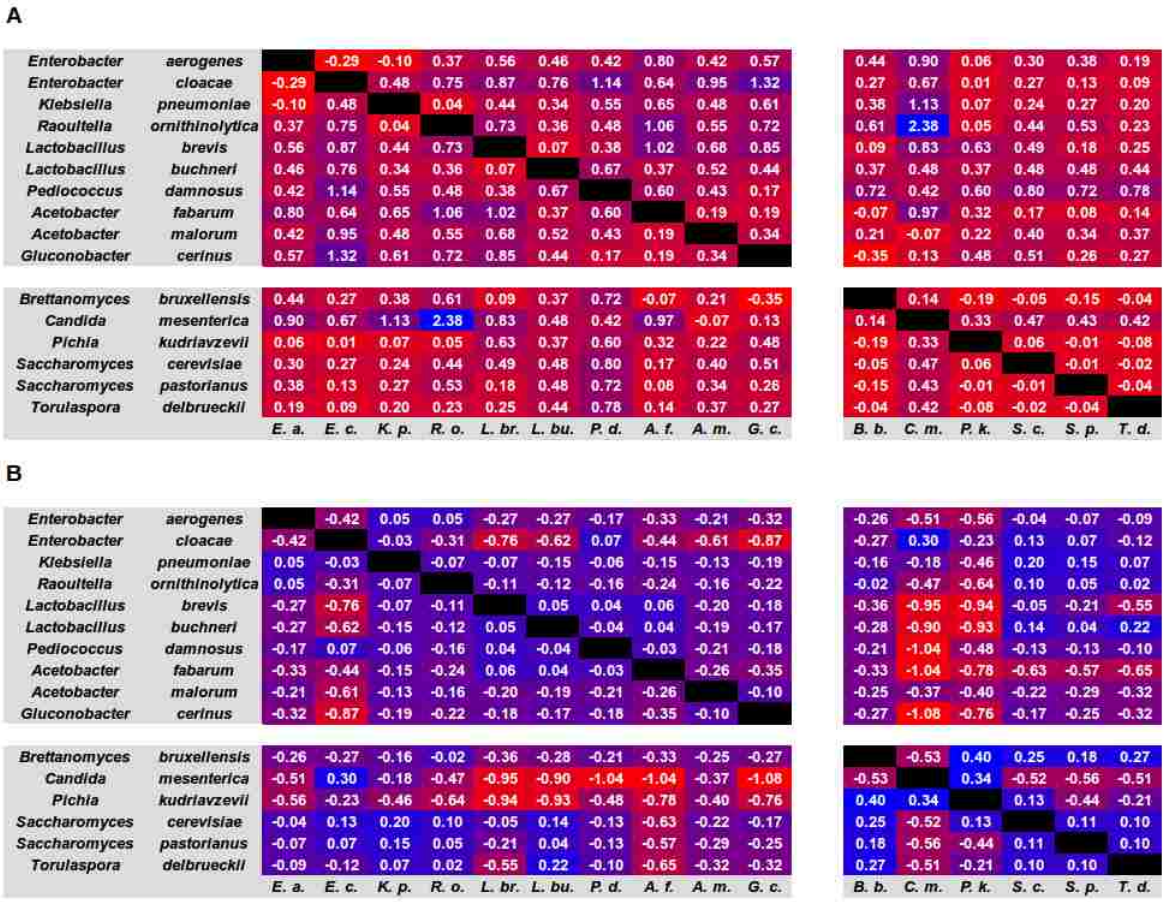

Figure A.1: **Co-culture cooperation and co-acidification: SG = 1.040.**
Data shown are averaged from 12 replicates grown in malt extract broth (MEB) with density of SG = 1.040 with no hop compounds under micro-aerobic conditions. Cooperative index is defined as the difference between the growth of co-cultures and the average growth of respective isolates, weighted by the average growth of the isolates (A). Co-acidification is defined as the difference between the pH of co-cultures and respective isolates (B).

A.2 SG = 1.050

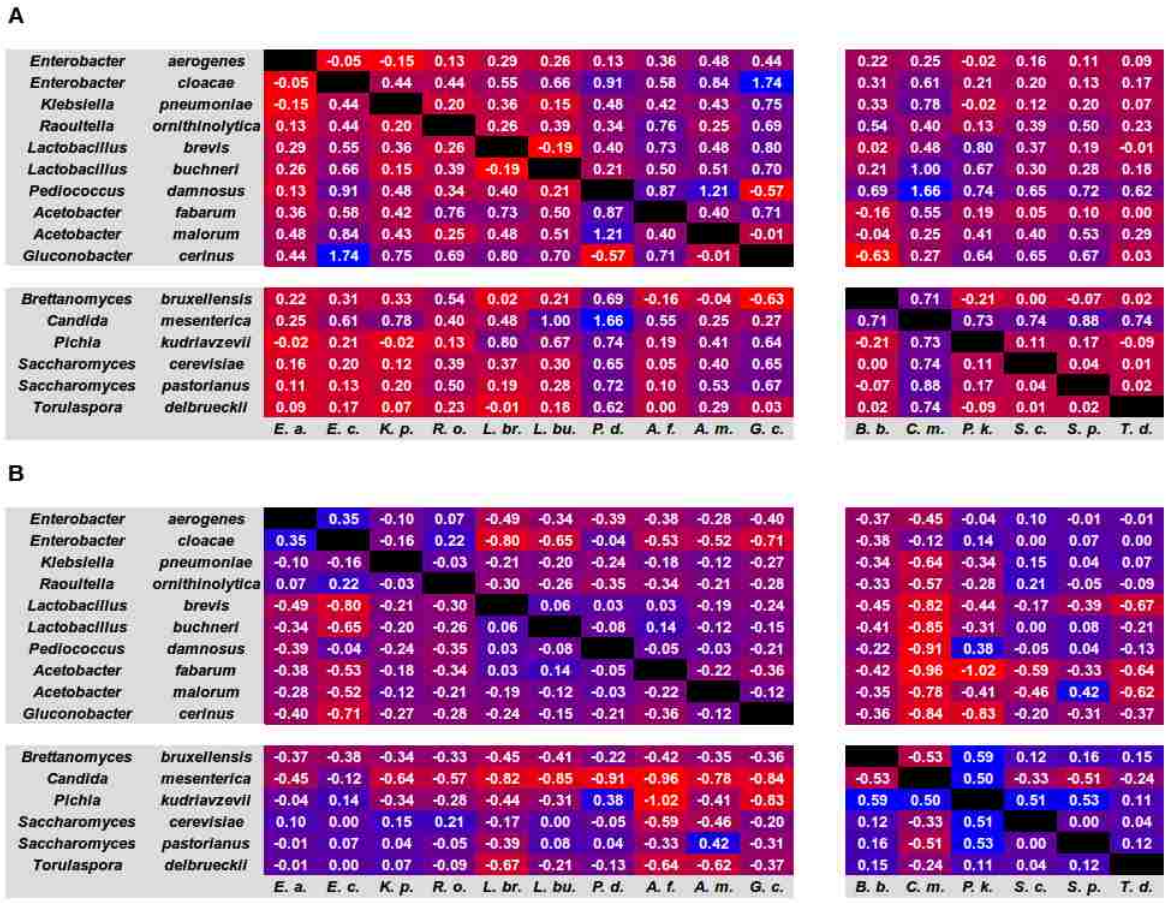

Figure A.2: **Co-culture cooperation and co-acidification: SG = 1.050.**
Data shown are averaged from 4 replicates grown in MEB with density of SG = 1.050 with no hop compounds under micro-aerobic conditions. Cooperative index is defined as the difference between the growth of co-cultures and the average growth of respective isolates, weighted by the average growth of the isolates (A). Co-acidification is defined as the difference between the pH of co-cultures and respective isolates (B).

A.3 SG = 1.060

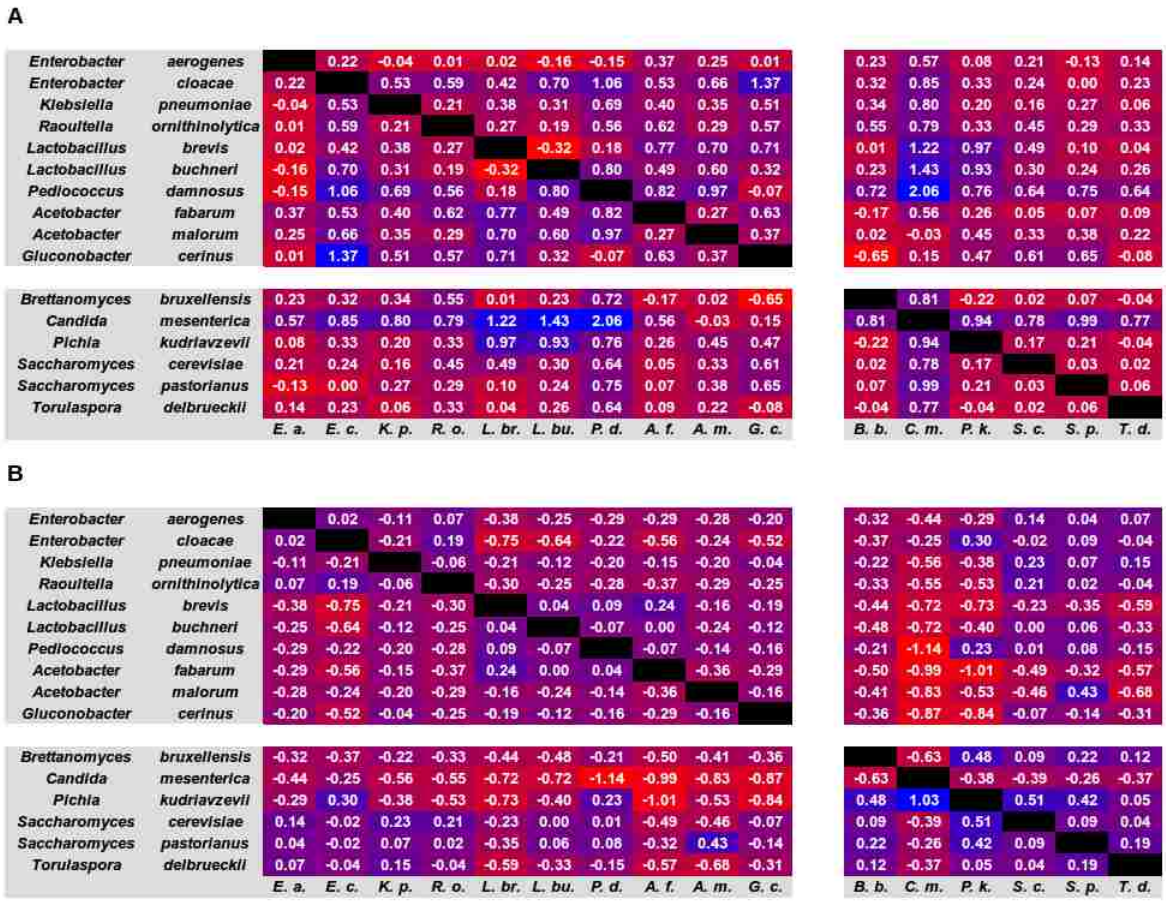

Figure A.3: **Co-culture cooperation and co-acidification: SG = 1.060.**
Data shown are averaged from 4 replicates grown in MEB with density of SG = 1.060 with no hop compounds under micro-aerobic conditions. Cooperative index is defined as the difference between the growth of co-cultures and the average growth of respective isolates, weighted by the average growth of the isolates (A). Co-acidification is defined as the difference between the pH of co-cultures and respective isolates (B).

A.4 40 IBU

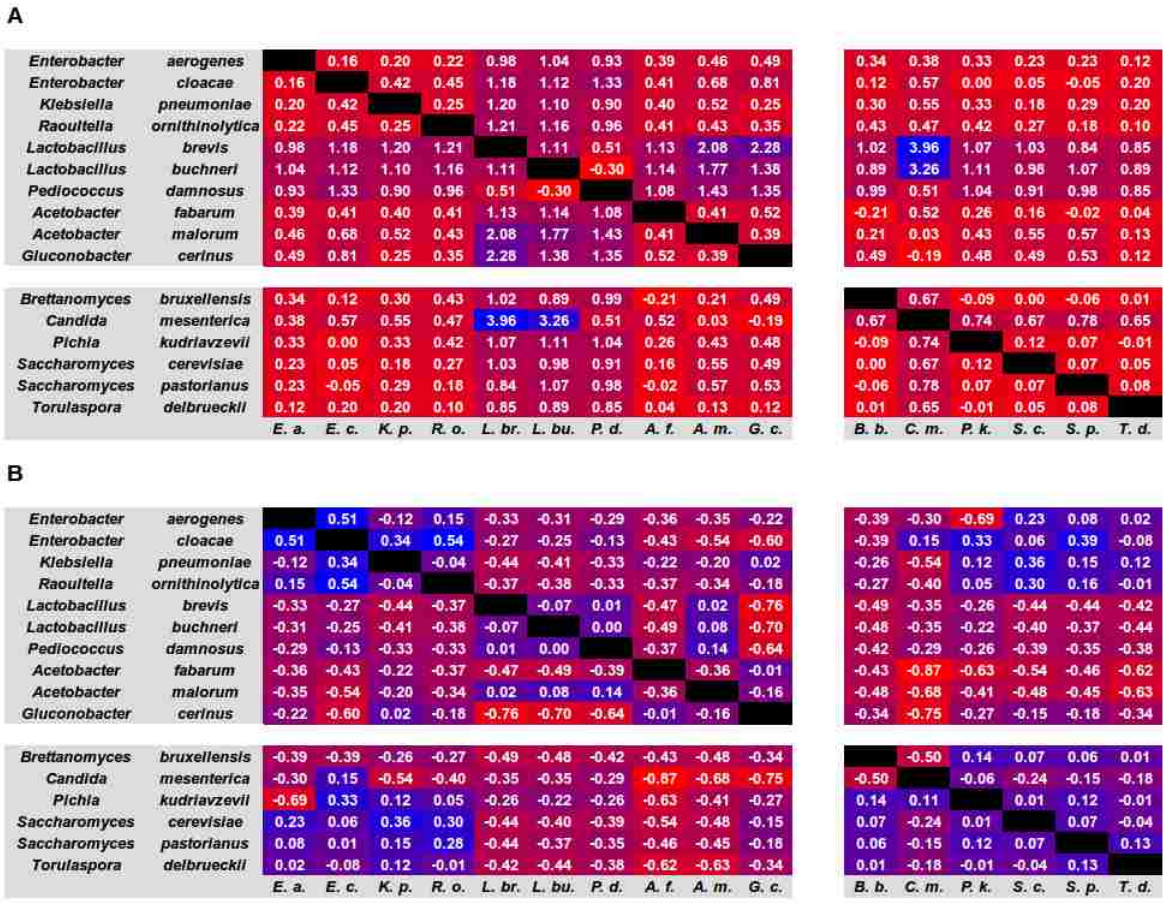

Figure A.4: **Co-culture cooperation and co-acidification: 40 IBU.**
Data shown are averaged from 4 replicates grown under micro-aerobic conditions in MEB with density of SG = 1.050 with hop compounds measured at 40 IBU. Cooperative index is defined as the difference between the growth of co-cultures and the average growth of respective isolates, weighted by the average growth of the isolates (A). Co-acidification is defined as the difference between the pH of co-cultures and respective isolates (B).

A.5 Anaerobic 1 psi

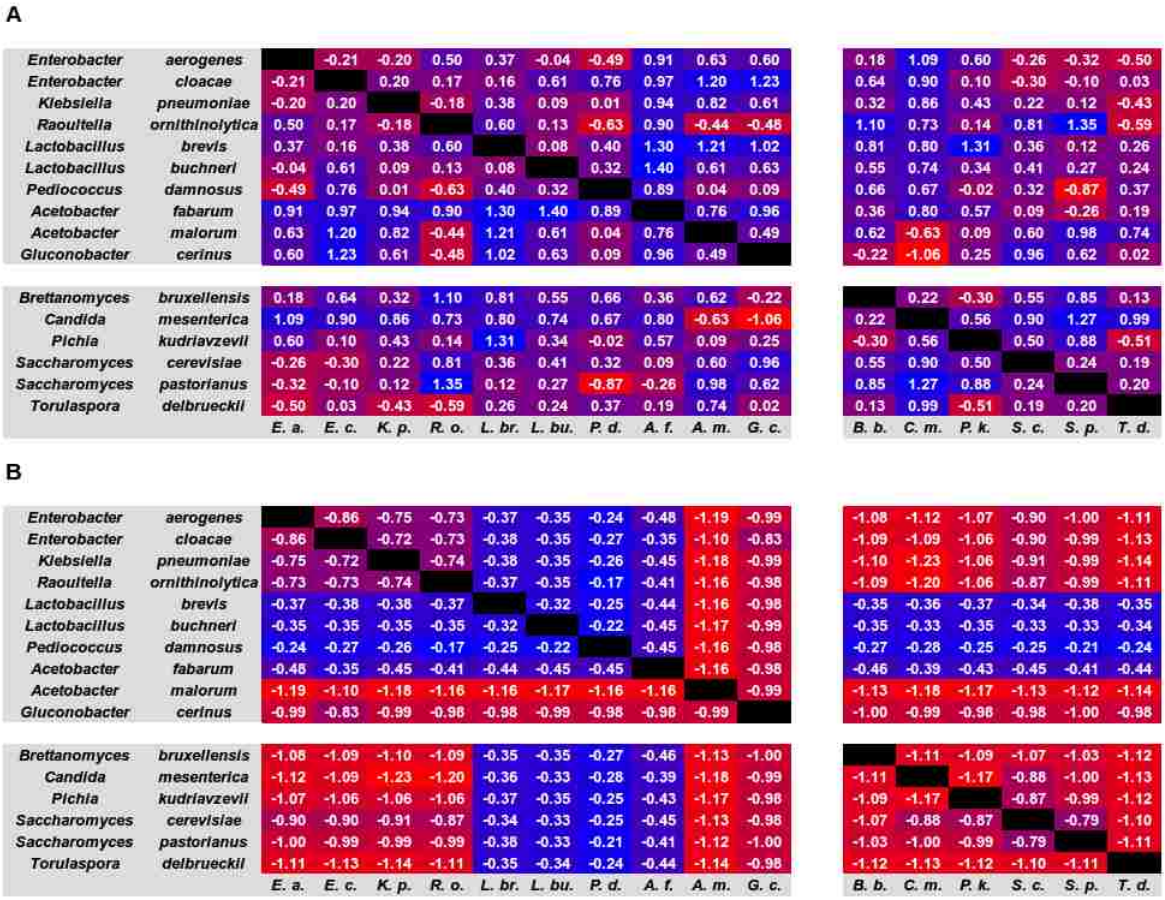

Figure A.5: **Co-culture cooperation and co-acidification: Anaerobic 1 psi.**
Data shown are averaged from 8 replicates grown under anaerobic conditions with atmospheric pressure of 1 psi in MEB with density of SG = 1.040 with no hop compounds. Cooperative index is defined as the difference between the growth of co-cultures and the average growth of respective isolates, weighted by the average growth of the isolates (A). Co-acidification is defined as the difference between the pH of co-cultures and respective isolates (B).

A.6 Anerobic 15 psi

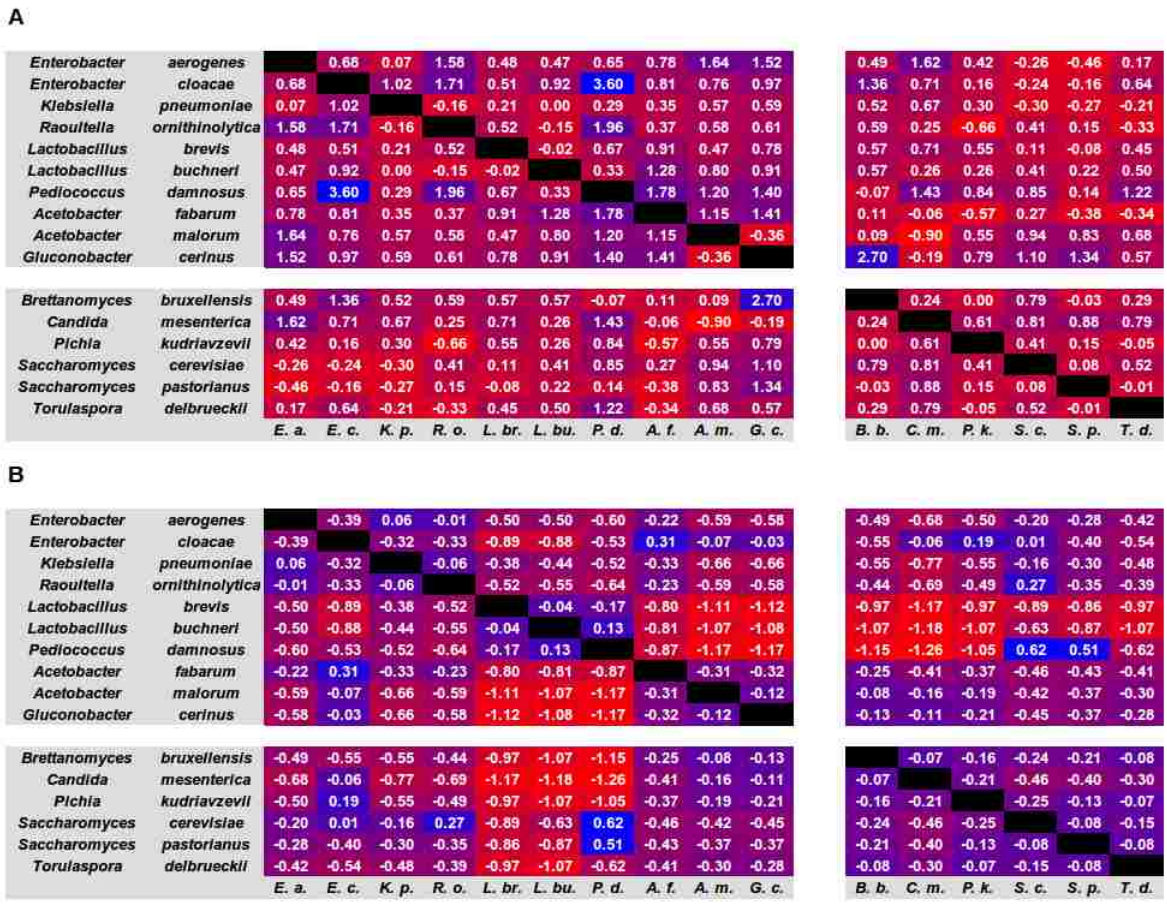

Figure A.6: **Co-culture cooperation and co-acidification: Anaerobic 15 psi.**
Data shown are averaged from 8 replicates grown under anaerobic conditions with atmospheric pressure of 15 psi in MEB with density of SG = 1.040 with no hop compounds. Cooperative index is defined as the difference between the growth of co-cultures and the average growth of respective isolates, weighted by the average growth of the isolates (A). Co-acidification is defined as the difference between the pH of co-cultures and respective isolates (B).

APPENDIX B: PYTHON SCRIPTS FOR BIOINFORMATIC ANALYSES

B.1 Pairwise Protein BLAST

```
#PairwiseProt v 0.1 by Andrew Kettring

#Runs on Python v.2.7.6
#Tested in Linux Mint 17.3

#input is faa fasta protein file
#wgs broser -> bioproject -> protein # (bottom) -> send to file

import os, csv, sys, re, subprocess
import pandas as pd
import numpy as np
from Bio import SeqIO
from Bio.Blast.Applications import NcbiblastpCommandline
from Bio.Blast import NCBIXML
from multiprocessing import Pool
from Bio.Seq import Seq
from Bio.SeqRecord import SeqRecord
import shutil

threadz = 4

#make list of infiles and sort
infiles = []
indir = './infiles/'
for file in os.listdir(indir):
    if file.endswith('.faa'):
        infiles.append(os.path.join( file))
infiles.sort()
#make a list without file extensions
filenames = [k.replace('.faa','') for k in infiles]


#make outifiles directory
outdir = './outfiles/'
if not os.path.exists(outdir):
    os.mkdir(outdir)
#make fasta directory
fasdir = outdir + 'fasta/'
if not os.path.exists(fasdir):
```

```
    os.mkdir(fasdir)
#make blast db directory
dbdir = outdir + 'blastdb/'
if not os.path.exists(dbdir):
    os.mkdir(dbdir)
#make pairwise blast directory
blastdir = outdir + 'blast/'
if not os.path.exists(blastdir):
    os.mkdir(blastdir)


print 'Trimming input files...'
def trim(fasname):
    og = indir + fasname + '.faa'
    ng = fasdir + fasname + '_new.faa'
    tg = fasdir + fasname + '_tmp.faa'
    shutil.copy(og, ng)
    n = 1
    n_tot = 0
    while n > 0:
        with open(ng, "rU") as input_handle, open(tg, "w") as
           output_handle:
            n=0
            for seq_record in SeqIO.parse(input_handle , 'fasta')
               :
                length = len(seq_record.seq)
                last = seq_record.seq[-1:]
                if 'X' in last:
                    new_seq = str(seq_record.seq[:-1])
                    old_id = seq_record.id
                    old_name = seq_record.name
                    old_desc = seq_record.description
                    rec = SeqRecord(Seq(new_seq), id=old_id, name
                       =old_name, description=old_desc)
                    SeqIO.write(rec, output_handle, 'fasta')
                    n += 1
                else:
                    #print seq_record.seq
                    SeqIO.write(seq_record, output_handle, 'fasta
                       ')
            n_tot += n
        shutil.copy(tg, ng)
```

```
    os.remove(tg)
    print 'Trimmed', n_tot, 'terminal Xs from', fasname
for file in filenames:
    trim(file)
print ''


print 'Making BLAST databases...'
cmds = []
for file in filenames:
    bashCommand = 'makeblastdb -in ' + fasdir + file + '_new.faa
       -dbtype prot -out ' + dbdir + file
    cmds.append(bashCommand)
FNULL = open(os.devnull, 'w')
def dater(cmd):
    print cmd
    p = subprocess.Popen(cmd, shell=True, stdout=FNULL, stderr=
       subprocess.STDOUT)
    p.wait()
pool = Pool(threadz)
for cmd in cmds:
    pool.apply_async(dater, [cmd])
pool.close()
pool.join()
print ''


print 'Pairwise BLAST...'
#make csv for pairs
o = outdir + 'pairs.csv'
with open(o, 'w') as f:
    writer = csv.writer(f)
    for x, y in [(x,y) for x in filenames for y in filenames]:
        z = [x,y]
        writer.writerow(z)
#appropriate blast commands
cmds = []
p1 = outdir + 'pairs.csv'
with open(p1, 'r') as f1:
    reader = csv.reader(f1)
    for row in reader:
        i = fasdir + row[0] + '_new.faa'
```

```
        d = dbdir + row[1]
        o = blastdir + row[0] + '_' + row[1] + '.xml'
        blasty = NcbiblastpCommandline(query=i, db=d, out=o,
           outfmt=5, max_hsps_per_subject=1, num_alignments=3)
        cmds.append(str(blasty))
#run them in parallel
def blaster(cmd):
    print cmd
    p = subprocess.Popen(cmd, shell=True)
    p.wait()
pool = Pool(threadz)
for cmd in cmds:
    pool.apply_async(blaster, [cmd])
pool.close()
pool.join()


#make csv for pairs
#o = outdir + 'pairs.csv'
#with open(o, 'w') as f:
#    writer = csv.writer(f)
#    for x, y in [(x,y) for x in filenames for y in filenames]:
#        z = [x,y]
#        writer.writerow(z)

print ''
print 'Analyzing BLAST results...'
q_list = []
p_list = []
with open('./outfiles/pairs.csv', 'r') as f:
    reader = csv.reader(f)
    for row in reader:
        pf = blastdir + row[0] + '_' + row[1] + '.xml'
        print pf
        h = open(pf)
        que_tot = 0
        pos_tot = 0
        for blast_records in NCBIXML.parse(h):
            blast_records.alignments.sort(key = lambda align: max
               (hsp.positives for hsp in align.hsps), reverse=
               True)
            que = blast_records.query_letters
```

```
                que_tot += que
                alignz = iter(blast_records.alignments)
                if blast_records.alignments != []:
                    aligny = next(alignz)
                    hspz = iter(aligny.hsps)
                    hsp = next(hspz)
                    escore = hsp.expect
                    if escore < 0.01:
                        pos = hsp.positives
                        pos_tot += pos
            q_list.append(que_tot)
            p_list.append(pos_tot)
#make a dataframe
r = outdir + 'pairs.csv'
df = pd.read_csv(r, names=['Bug 1', 'Bug 2', 'AAs Queried', '
   Positives'])
#dump data
df['AAs Queried'] = q_list
df['Positives'] = p_list
#write to file
o = outdir + 'pairwise.csv'
df.to_csv(o, index=False)

# Find and add inverse pairs
pw = outdir + 'pairwise.csv'
ps = []
qs = []
ds = []
with open(pw, 'r') as f1:
    reader = csv.reader(f1)
    next(f1)
    for row in reader:
        a1 = row[0]
        a2 = row[1]
        q1 = float(row[2])
        p1 = float(row[3])
        with open(pw, 'r') as f2:
            reader = csv.reader(f2)
            next(f2)
            for row in reader:
                b1 = row[0]
                b2 = row[1]
```

```
                    q2 = float(row[2])
                    p2 = float(row[3])
                    if a1==b2 and b1==a2:
                        qs.append(q2)
                        ps.append(p2)
                        dd = 1- (p1 + p2) / (q1 + q2)
                        ds.append(dd)
#make a dataframe
r = outdir + 'pairwise.csv'
df = pd.read_csv(r)
#dump data
df['AAs_2'] = qs
df['Pos_2'] = ps
df['Distance'] = ds
#rewrite pairwise file
o = outdir + 'pairwise.csv'
df.to_csv(o, index=False)

#pairwise to distance matrix
df = pd.DataFrame(filenames)
num=len(filenames)
distx = ds[:]
for p in filenames:
    disty = distx[:]
#delete up to first 3
    del disty[num:]
    df[p] = disty
#delete fist three
    del distx[:num]
o = outdir + 'matrix.csv'
df.to_csv(o, index=False)

print ''
print 'All_done!'
```

B.2 Pairwise Paths

```
#PairwisePaths v 0.1 by Andrew Kettring

#Runs on Python v.2.7.6
#Tested in Linux Mint 17.3

import os, csv, sys, re, subprocess
import pandas as pd

#make list of files and sort
infiles=[]
for file in os.listdir("./infiles"):
    if file.endswith(".paths"):
        infiles.append(os.path.join( file))
infiles.sort()

#make a list without file extensions
filenames=[k.replace(".paths",'') for k in infiles]

#make directory if needed
directory='./outfiles'
if not os.path.exists(directory):
    os.mkdir(directory)

#extract pathways from files to new file
for p in infiles:
    n = './infiles/' + p
    m = './outfiles/' + p

#split at tab and keep second half
    with open(n) as f:
        with open(m, 'w') as f1:
            for line in f:
                line2 = line.split("\t", 1)[-1]
                line3 = re.sub("\t", '', line2)
                f1.write(line3)
#remove first line
    with open(m, 'r') as fin:
        data = fin.read().splitlines(True)
    with open(m, 'w') as fout:
        fout.writelines(data[1:])
```

```
#sort via bash
    bashCommand = 'sort -u ' + m + ' > ./outfiles/paths.tmp'
    subprocess.call(bashCommand, shell=True)
    bashCommand = 'mv ./outfiles/paths.tmp ' + m
    subprocess.call(bashCommand, shell=True)


# Pairwise Comparisons

#write pairs from filenames to a file
with open("./outfiles/pairs.csv", 'wb') as f:
    w=csv.writer(f)
    for x, y in [(x,y) for x in filenames for y in filenames]:
        z=[x,y]
        w.writerow(z)

#make lists
combined = []
common = []
distances = []

with open('./outfiles/pairs.csv', 'r') as f:
    reader = csv.reader(f)
    for row in reader:

#combine via sort via bash
        bashCommand = 'sort -u ./outfiles/' + row[0] + '.paths ./
            outfiles/' + row[1] + '.paths > ./outfiles/combined_'
             + row[0] + '_' + row[1] + '.paths'
        subprocess.call(bashCommand, shell=True)

#comm via bash
        bashCommand = 'comm ./outfiles/' + row[0] + '.paths ./
            outfiles/' + row[1] + '.paths -1 -2 > ./outfiles/
            common_' + row[0] + '_' + row[1] + '.paths'
        subprocess.call(bashCommand, shell=True)

#count number of lines
        file_comb = './outfiles/combined_' + row[0] + '_' + row
            [1] + '.paths'
        flen_comb = sum(1 for line in open(file_comb))
```

```
        combined.append(flen_comb)

        file_comm = './outfiles/common_' + row[0] + '_' + row[1]
           + '.paths'
        flen_comm = sum(1 for line in open(file_comm))
        common.append(flen_comm)

#do math
        dist = 1 - (flen_comm / float(flen_comb))
        distances.append(dist)

#make a bamf file
df = pd.read_csv('./outfiles/pairs.csv', names=['Bug 1', 'Bug 2',
    'Common', 'Combined', 'Distances'])

#data dump
df['Common'] = common
df['Combined'] = combined
df['Distances'] = distances

#write to file
df.to_csv('./outfiles/pairwise.csv', index=False)
#print df
#print ''


#Pairwise to distance matrix
df = pd.DataFrame(filenames)

num=len(filenames)
distx = distances[:]
for p in filenames:
    disty = distx[:]
#delete up to first 3
    del disty[num:]
    df[p] = disty
#delete fist three
    del distx[:num]

df.to_csv('./outfiles/matrix.csv', index=False)

#print df
```

```
#PairwiseSeeds v 0.1 by Andrew Kettring

#Runs on Python v.2.7.6
#Tested in Linux Mint 17.3

import os, csv, sys, re, subprocess
import pandas as pd

indir = './infiles/'

#make directories if needed
outdir = './outfiles/'
if not os.path.exists(outdir):
    os.mkdir(outdir)
tmpdir = outdir + 'tmp/'
if not os.path.exists(tmpdir):
    os.mkdir(tmpdir)

#make list of bugs from infiles
bugs=[]
for file in os.listdir(indir):
    if file.endswith('_seeds.txt'):
        bug = file.replace('_seeds.txt','')
        if bug not in bugs:
            bugs.append(os.path.join(bug))
    elif file.endswith('_nonseeds.txt'):
        bug = file.replace('_nonseeds.txt','')
        if bug not in bugs:
            bugs.append(os.path.join(bug))
bugs.sort()

#write pairs from filenames to a file
with open(tmpdir + 'pairs.csv', 'wb') as f:
    w=csv.writer(f)
    for x, y in [(x,y) for x in bugs for y in bugs]:
        z=[x,y]
        w.writerow(z)

print 'Preparing infiles...'
for bug in bugs:
```

```
    inseed = indir + bug + '_seeds.txt'
    innonseed = indir + bug + '_nonseeds.txt'
    outseed = tmpdir + bug + '_seeds.txt'
    outnonseed = tmpdir + bug + '_nonseeds.txt'
    tmpseed = tmpdir + bug + '_seeds_tmp'
#verify complementary infiles exist
    if os.path.exists(inseed) and os.path.exists(innonseed):
        pass
    else:
        print 'Missing a complementary infile for' , bug
        sys.exit()
#sort via BASH
    bashCommand = 'sort -u ' + inseed +  ' > ' +  tmpseed
    subprocess.call(bashCommand, shell=True)
    bashCommand = 'sort -u ' + innonseed + ' > ' + outnonseed
    subprocess.call(bashCommand, shell=True)
#trim confidence scores from seed list
    with open(tmpseed) as f:
        with open(outseed, 'w') as f1:
            for line in f:
#split at tab and first half + a return
                line2 = line[:-3] + "\n"
                f1.write(line2)
    os.remove(tmpseed)
    print bug

print ''
print 'Computing competition...'
#make lists
combined = []
common = []
competition = []
#define pariwise files
with open(tmpdir + 'pairs.csv', 'r') as f:
    reader = csv.reader(f)
    for row in reader:
        inseed1 = tmpdir + row[0] + '_seeds.txt'
        inseed2 = tmpdir + row[1] + '_seeds.txt'
        outcomb = tmpdir + 'combined_'  + row[0] + '_' + row[1] +
            '_seeds.txt'
        outcomm = tmpdir + 'common_'  + row[0] + '_' + row[1] + '
           _seeds.txt'
```

```
#combine via sort via bash
        bashCommand = 'sort -u ' + inseed1 + ' ' + inseed2 + ' >
            ' + outcomb
        subprocess.call(bashCommand, shell=True)
#comm via bash
        bashCommand = 'comm -1 -2 ' + inseed1 + ' ' + inseed2 + '
            > ' + outcomm
        subprocess.call(bashCommand, shell=True)
#count number of lines
        flen_comb = sum(1 for line in open(outcomb))
        combined.append(flen_comb)
        flen_comm = sum(1 for line in open(outcomm))
        common.append(flen_comm)
#do math
        comp = (flen_comm / float(flen_comb))
        competition.append(comp)
#make a dataframe
df = pd.read_csv(tmpdir+'pairs.csv', names=['Bug 1', 'Bug 2', '
    Common', 'Combined', 'Competition'])
#data dump
df['Common'] = common
df['Combined'] = combined
df['Competition'] = competition
#write to file
df.to_csv(outdir+'competition_pairs.csv', index=False)
#print df
#Pairwise competition matrix
df = pd.DataFrame(bugs)
num=len(bugs)
compx = competition[:]
for b in bugs:
    compy = compx[:]
    del compy[num:]
    df[b] = compy
    del compx[:num]
df.to_csv(outdir+'competition_matrix.csv', index=False)
print 'Done.'

#compare seeds and non-seeds
print ''
print 'Computing complementarity...'
#make lists
```

```
seedz = []
commonsns = []
complementarity = []
#define pairwise files
with open(tmpdir + 'pairs.csv', 'r') as f:
    reader = csv.reader(f)
    for row in reader:
        inseed = tmpdir + row[0] + '_seeds.txt'
        innonseed = tmpdir + row[1] + '_nonseeds.txt'
        outcomm = tmpdir + 'common_'  + row[0] + '_' + row[1] + '
            _sns.txt'
#comm via bash
        bashCommand = 'comm -1 -2 ' + inseed + ' ' + innonseed +
            ' > ' + outcomm
        subprocess.call(bashCommand, shell=True)
#count number of lines
        flen_seeds = sum(1 for line in open(inseed))
        seedz.append(flen_seeds)
        flen_comm = sum(1 for line in open(outcomm))
        commonsns.append(flen_comm)
#do math
        comp = (flen_comm / float(flen_seeds))
        complementarity.append(comp)
#make a dataframe
df = pd.read_csv(tmpdir+'pairs.csv', names=['Bug 1', 'Bug 2', '
   Seeds', 'Common_SNS', 'Asymmetric'])
#data dump
df['Seeds'] = seedz
df['Common_SNS'] = commonsns
df['Asymmetric'] = complementarity
#write to file
df.to_csv(outdir+'complementarity_pairs.csv', index=False)

#Append complementary complementarity scores
pw = outdir + 'complementarity_pairs.csv'
ss = []
cs = []
sc = []
with open(pw, 'r') as f1:
    reader = csv.reader(f1)
    next(f1)
    for row in reader:
```

```
            a1 = row[0]
            a2 = row[1]
            s1 = float(row[2])
            c1 = float(row[3])
            with open(pw, 'r') as f2:
                reader = csv.reader(f2)
                next(f2)
                for row in reader:
                    b1 = row[0]
                    b2 = row[1]
                    s2 = float(row[2])
                    c2 = float(row[3])
                    if a1==b2 and b1==a2:
                        ss.append(s2)
                        cs.append(c2)
                        symcom =  (c1 + c2) / (s1 + s2)
                        sc.append(symcom)
#make a dataframe
r = outdir + 'complementarity_pairs.csv'
df = pd.read_csv(r)
#dump data
df['Seeds_S2'] = ss
df['SNS_2'] = cs
df['Symmetric'] = sc
#rewrite pairwise file
df.to_csv(r, index=False)

#Pairwise asymmetric matrix
df = pd.DataFrame(bugs)
num=len(bugs)
compx = complementarity[:]
for b in bugs:
    compy = compx[:]
    del compy[num:]
    df[b] = compy
    del compx[:num]
df.to_csv(outdir+'complementary_asym_matrix.csv', index=False)

#Pairwise symmetric matrix
df = pd.DataFrame(bugs)
num=len(bugs)
compx = sc[:]
```

```
for b in bugs:
    compy = compx[:]
    del compy[num:]
    df[b] = compy
    del compx[:num]
df.to_csv(outdir+'complementary_sym_matrix.csv', index=False)

print 'Done.'
```

B.4 Ubique

```
#Ubique v 0.1 by Andrew Kettring

#Runs on Python v.2.7.6
#Tested in Linux Mint 17.3

import os, csv, sys, re, subprocess
import pandas as pd
from pandas.io.parsers import count_empty_vals

indir = './infiles/'

#make directories if needed
outdir = './outfiles/'
if not os.path.exists(outdir):
    os.mkdir(outdir)

tmpdir = outdir + 'tmp/'
if not os.path.exists(tmpdir):
    os.mkdir(tmpdir)



#make list of bugs from infiles
bugs=[]
for file in os.listdir(indir):
    if file.endswith('.paths'):
        bug = file.replace('.paths','')
        if bug not in bugs:
            bugs.append(os.path.join(bug))
bugs.sort()



entries=[]
print 'Preparing infiles...'
for bug in bugs:
    inbug = indir + bug + '.paths'
    tmpbug = tmpdir + bug + '.tmp.paths'
    outbug = outdir + bug + '.sorted.paths'
```

```
#split at tab and keep second half
    with open(inbug) as f:
        with open(tmpbug, 'w') as f1:
            for line in f:
                line2 = line.split("\t", 1)[-1]
                line3 = re.sub("\t", '', line2)
                f1.write(line3)
#remove first line
    with open(tmpbug, 'r') as fin:
        data = fin.read().splitlines(True)
    with open(tmpbug, 'w') as fout:
        fout.writelines(data[1:])

#sort via bash
    bashCommand = 'sort -u ' + tmpbug + ' > ' + outbug
    subprocess.call(bashCommand, shell=True)

for bug in bugs:
    bugy = outdir + bug + '.sorted.paths'

#find unique entries
    with open(bugy, 'r') as b:
        for entry in b:
            entrz = entry[:-1]
            if entrz not in entries:
                entries.append(entrz)
entries.sort
print entries
print ''


print 'Counting...'
returns = []
for entry in entries:
    county=0
    for bug in bugs:
        bugy = outdir + bug + '.sorted.paths'
        with open(bugy, 'r') as b:
            for line in b:
                liney = line[:-1]
                if entry == liney:
                    county += 1
```

```
    returns.append(county)
print returns
print ''

df = pd.DataFrame(columns=['Entry', 'Count'])
df['Entry']=entries
df['Count']=returns

df.to_csv(outdir+'ubique.csv', index=False)
```

LIST OF REFERENCES

[1] Alexandrou M.A., Cardinale B.J., Hall J.D., Delwiche C.F., Fritschie K., Narwani A., Venail P.A., Bentlage B., Pankey M.S., Oakley T.H. (2014) Evolutionary relatedness does not predict competition and co-occurrence in natural or experimental communities of green algae. Proc. R. Soc. B. 282:20141745. doi: 10.1098/rspb.2014.1745.

[2] Angelov M., Kostov G., Simova E., Beshkova D., Koprinkova-Hristova P. (2009) Proto-cooperation factors in yogurt starter cultures. Revue de genie industriel.

[3] ASBC Methods of Analysis, online. (2011) Method 23-A. Manual Isooctane Extraction: Reduced Solvent Technique. American Society of Brewing Chemists, St. Paul, MN, U.S.A. doi: 10.1094/ASBCMOA-Beer-23

[4] B.J.C.P. (2015) Beer Judge Certification Program 2015 Style Guidelines.

[5] Bokulich N.A., Bamforth C.W. (2013) The Microbiology of Malting and Brewing. Microbiology and Molecular Biology Reviews. 77(2):157172. doi: 10.1128/MMBR.00060-12.

[6] Bokulich N.A., Bergsveinson J., Ziola B., Mills D.A. (2015) Mapping microbial ecosystems and spoilage-gene flow in breweries highlights patterns of contamination and resistance. Elife. 2015;4:e04634. doi:10.7554/eLife.04634.

[7] Brewers Association (2016) BA addresses draught beer quality best practices with groundbreaking study. https://www.brewersassociation.org/industry-updates/ba-addresses-draught-beer-quality-best-practices-groundbreaking-study/.

[8] Brewers Association (2017) Brewers Association Economic Impact Study. https://www.brewersassociation.org/statistics/economic-impact-data.

[9] Brewers Association (2017) Draught Beer Quality Manual. Third Edition. http://www.draughtquality.org/wp-content/uploads/DBQM17.pdf.

[10] Benson D.A., Karsch-Mizrachi, I., Lipman D.J., Ostell J., Wheeler D.L. (2005). GenBank. Nucleic Acids Research. 33:D34D38. doi: 10.1093/nar/gki063.

[11] Boom R., Sol C.J., Salimans M.M., Jansen C.L., Wertheim-van Dillen P.M., van der Noordaa J. (1990) Rapid and simple method for purification of nucleic acids. Journal of Clinical Microbiology. 28(3):495-503.

[12] Camacho C., Coulouris G., Avagyan V., Ma N., Papadopoulos J., Bealer K., Madden T.L. (2008) BLAST+: architecture and applications. BMC Bioinformatics. 10:421.

[13] Carr R., Borenstein E. (2012) NetSeed: a network-based reverse-ecology tool for calculating the metabolic interface of an organism with its environment. Bioinformatics. 28(5):734-735. doi:10.1093/bioinformatics/btr721.

[14] Darwin C. (1859) The Origin of Species by Means of Natural Selection. ISBN: 0451529065.

[15] Dietler M., Hayden B. (2010) Feasts: Archaeological and Ethnographic Pespectives on Food, Politics, and Power. University Alabama Press. ISBN-10: 081735641X.

[16] Dunham J. *et al.* (2017) Beer Serves America. The Beer Institute and National Beer Wholesalers Association. http://beerservesamerica.org/wp-content/uploads/2017/05/2017-Beer-Serves-America-Report.pdf

[17] Faust K., Raes J. (2012) Microbial interactions: from networks to models. Nature Reviews Microbiology. 10:538550. doi: 10.1038/nrmicro2832

[18] Fodor A.A., DeSantis T.Z., Wylie K.M., Badger J.H., Ye Y., Hepburn T., Hu P, Sodergren E, Liolios K., Huot-Creasy H., Birren B.W., Earl A.M. (2012) The "Most Wanted" Taxa from the Human Microbiome for Whole Genome Sequencing. PLoS ONE. 7(7):e41294. doi:10.1371/journal.pone.0041294.

[19] Gallone B., Steensels J., Prahl T., Soriaga L., Saels V., Herrera-Malaver B., Merlevede A., Roncoroni M., Voordeckers K., Miraglia L., Teiling C., Steffy B., Taylor M., Schwartz A., Richardson T., White C., Baele G., Maere S., Verstrepen K.J. (2016) Domestication and Divergence of Saccharomyces cerevisiae Beer Yeasts. Cell. 166(6)1397-1410 doi: 10.1016/j.cell.2016.08.020.

[20] Garofalo C., Osimani A., Milanovic V., Taccari M., Aquilanti L., Clementi F. (2015) The Occurrence of Beer Spoilage Lactic AcidBacteria in Craft Beer Production. Journal of Food Science. 80(12): M2845 doi: 10.1111/1750-3841.13112.

[21] Goers L., Freemont P., Polizzi K.M. (2014) Co-culture systems and technologies: taking synthetic biology to the next level. J R Soc Interface. 11(96): 20140065. doi: 10.1098/rsif.2014.0065.

[22] Greub G. (2012) Culturomics: a new approach to study the human microbiome. Clinical Microbiology and Infection. doi: 10.1111/1469-0691.12032.

[23] Guarner F., Malagelada J.R. (2003) Gut flora in health and disease. The Lancet. 360:512-519.

[24] Hammond J., Brennan M., Price A. (1999) The Control of Microbial Spoilage of Beer. Journal of The Institute of Brewing. 105(2):113-120

[25] Jespersen L., Jakobsen M. (1996) Specific spoilage organisms in breweries and laboratory media for their detection. International Journal of Food Microbiology (33):139-155.38

[26] Karp P.D., Paley S., Romero P. (2002) The Pathway Tools software. Bioinformatics. 18:S225S232. doi: 10.1093/bioinformatics/18.suppl_1.S225.

[27] Karp P.D., Latendresse M., Caspi R. (2011) The Pathway Tools Pathway Prediction Algorithm. Standards in Genomic Sciences. 5(3):424-429. doi: 10.4056/sigs.1794338.

[28] Keating S.M., Le Novere N. (2013) Supporting SBML as a Model Exchange Format in Software Applications. In Silico Systems Biology. 1021:201-225.

[29] Kirin Holdings Company, Limited. (2016) Kirin Beer University Report Global Beer Consumption by Country in 2015. http://www.kirinholdings.co.jp/english/news/2016/1221_01.html.

[30] Khandelwal R.A., Olivier B.G., Rolling W., Teusink B., Bruggeman F.J. (2013) Community Flux Balance Analysis for Microbial Consortia at Balanced Growth. PLoS ONE. 8(5):e64567. doi: 10.1371/journal.pone.0064567.

[31] Klindworth A., Pruesse E., Schweer T., Peplies J., Quast C., Horn M., Glockner F.O. (2012) Evaluation of general 16S ribosomal RNA gene PCR primers for classical and next-generation sequencing-based diversity studies. Nucleic Acids Research. 41(1). doi: 10.1093/nar/gks808

[32] Lee H., Lee Y. (2008) A differential medium for lactic acid-producing bacteria in a mixed culture. Letters in Applied Microbiology. 46(6):676-681. doi: 10.1111/j.1472-765x.2008.02371.x.

[33] Levy R., Borenstein E. (2013) Metabolic modeling of species interaction in the human microbiome elucidates community-level assembly rules. PNAS. 110:31. doi: 10.1073/pnas.1300926110.

[34] Levy R., Carr R., Kreimer A., Freilich S., Borenstein E. (2015) NetCooperate: a network-based tool for inferring host-microbe and microbe-microbe cooperation. BMC Bioinformatics. 16:164. doi: 10.1186/s12859-015-0588-y.

[35] Li C., Lim K., Chng K.R., Nagarajan N. (2016) Predicting microbial interactions through computational approaches. Methods. 102:12-19. doi: 10.1016/j.ymeth.2016.02.019.

[36] Li F.Y., Costello J.C., Holloway A.K., Hahn M.W. (2008) Reverse ecology and the power of population genomics. Evolution 62:12 doi: 10.1111/j.1558-5646.2008.00486.x

[37] MacWilliam I.C. (1968) Wort Composition- A Review. Journal of the Insititute of Brewing. 74:38-54.

[38] Kumar S., Stecher G., Tamura K. (2015) MEGA7: Molecular Evolutionary Genetics Analysis version 7.0. Molecular Biology and Evolution.

[39] Micic M., Whyte J.D., Karsten V. (2016) High Performance Bead Beating Based Lysing, Homogenization and Grinding for DNA, RNA and Proteins Extraction with FastPrep® Systems. Springer Protocols Handbooks. Humana Press, New York, NY

[40] Monro R., Vazquez D. (1967) Ribosome-catalysed peptidyl transfer: Effects of some inhibitors of protein synthesis. Journal of Molecular Biology. 28(1):161-165. doi: 10.1016/s0022-2836(67)80085-8

[41] Naidu K.T., Prabhu N.P. (2011) Protein-Surfactant Interaction: Sodium Dodecyl Sulfate-Induced Unfolding of Ribonuclease A. Journal of Physical Chemistry B. (115):1476014767. doi: 10.1021/jp2062496.

[42] Nobel Media AB (2014) The Nobel Prize in Chemistry 1907. Nobelprize.org.

[43] Quain D.E. (2016) Draught beer hygiene: cleaning of dispense tap nozzles. Journal of the Institute of Brewing. (122):388396. doi: 10.1002/jib.335.

[44] Quast C., Pruesse E., Yilmaz P., Gerken J., Schweer T., Peplies P., Glockner F.O. The SILVA ribosomal RNA gene database project: improved data processing and web-based tools. (2013) Nucleic Acids Research. 41(D1):D590D596 doi: 10.1093/nar/gks1219.

[45] Rehner S.A., Samuels G.J. (1995) Molecular systematics of the Hypocreales: a teleomorph gene phylogeny and the status of their anamorphs. Canadian Journal of Botany. 73:S816-S823.

[46] Schnoes A.M., Ream D.C., Thorman A.W., Babbitt P.C., Friedberg I. (2013) Biases in the Experimental Annotations of Protein Function and Their Effect on Our Understanding of Protein Function Space. PLOS Computational Biology. 9(5):e1003063.

[47] Simpson W.J., Fernandez J.L., Hammond J.R.M. (1992) Differentiation of Brewery Yeast Using a Disc-Diffusion Test. Journal of the Institute of Brewing. (98):33-36.

[48] Sisler H.D., Siegel M.R. (1967) Cycloheximide and Other Glutarimide Antibiotics. Mechanism of Action. 283-307. doi: 10.1007/978-3-642-46051-7_21

[49] Spitaels F., Wieme A.D., Janssen M., Aerts M., Daniel H.M., Van Landschoot A., De Vuyst L, Vandamme P. (2014) The Microbial Diversity of Traditional Spontaneously Fermented Lambic Beer. PLoS ONE. 9(4). doi:10.1371/journal.pone.0095384.

[50] Spitaels F., Wieme A.D., Janssens M., Aerts M, Van Landschoot A., De Vuyst L., Vandamme P., (2015) The microbial diversity of an industrially produced lambic beer shares members of a traditionally produced one and reveals a core microbiota for lambic beer fermentation. Food Microbiology. 49:23-32.

[51] Steensels G., Verstrepen K.J. (2014) Taming Wild Yeast: Potential of Conventional and Non-conventional Yeasts in Industrial Fermentations. Annu. Rev. Microbiol. 68:6180.

[52] Tamura K. and Nei M. (1993). Estimation of the number of nucleotide substitutions in the control region of mitochondrial DNA in humans and chimpanzees. Molecular Biology and Evolution. 10:512-526. PMID: 8336541.

[53] Toju H., Tanabe A.S., Yamamoto S., Sato H. (2012) High-Coverage ITS Primers for the DNA-Based Identification of Ascomycetes and Basidiomycetes in Environmental Samples. PLOS ONE. 7(7): e40863. doi: 10.1371/journal.pone.0040863

[54] Tonsmeire M. (2014) American Sour Beers: Innovative Techniques for Mixed Fermentations. Brewers Publications. ISBN-10: 1938469119.

[55] Van Oevelen D., Verachtert H. (1977) Microbiological Aspects of Spontaneous Wort Fermentation in the Production of Lambic Gueuze. Journal of the Institute of Brewing. 83:356-360.

[56] Vander S.H. (2011) Louis Pasteur: Groundbreaking Chemist & Biologist. Minnesota, US: ABDO Publishing Company. pp. 8112. ISBN 978-1-61758-941-6.

[57] Warnow T. (2012) Standard maximum likelihood analyses of alignments with gaps can be statistically inconsistent. PLOS Currents Tree of Life. doi: 10.1371/currents.RRN1308

[58] Wickerham L.J. (1951) Taxonomy of Yeasts. Technical Bulletin No. 1029. U.S. Dept. of Agriculture. p.3.

[59] Wolfe B.E., Dutton R.J. (2015) Fermented Foods as Experimentally Tractable Microbial Ecosystems. Cell. 161(1):49-55. doi: 10.1016/j.cell.2015.02.034.

[60] Zhao Y., Tsang C., Xiao M., Cheng J., Xu Y., Lau A., Woo P. (2015) Intra-Genomic Internal Transcribed Spacer Region Sequence Heterogeneity and Molecular Diagnosis in Clinical Microbiology. International Journal of Molecular Sciences. 16:25067-25079. doi: 10.3390/ijms161025067