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Avram M. Shayevitz for the degree of Master of Science in Food Science and Technology presented on September 17, 2018

Title: Do Oak Barrels Contribute To The Variability of The Microbiome of Barrel-Aged Beers?

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Lambic and other barrel-aged beer styles are gaining popularity in the United States and Europe and are often treated as a premium product that can command a premium price. However, these styles can be prone to spoilage during the barrel-aging process, which represents a significant time and product commitment by a brewery, and thus it is important to understand what exactly is happening within these barrels from a microbiological point of view.

Previous studies have used microbiome analyses to establish the similarity in microbial succession between traditional Belgian Lambic beer and America Coolship Ales, but to date no studies have been performed on a large number of barrels. The focus of this study was on the influence of oak barrels on the microbiome of three distinct beers produced and matured within the state of Oregon, USA and aged in 102 barrels.

It was evident that traditionally fermented beer produced outside of Belgium exhibited a similar microbial profile to traditional Lambic beers during the first 36 weeks of fermentation, with eventual dominance of *Dekkera* (syn. *Brettanomyces*) *bruxellensis* and *Lactobacillus*. During this time, previously unreported instances of *Gluconoacetobacter* were observed, a genera more often associated with vinegar and kombucha production than with beer. Analysis of beer that had aged up to five years in barrels showed that yeast and bacterial communities follow a conserved trend, with the eventual dominance of *Dekkera* (syn. *Brettanomyces*) *bruxellensis* and acetic acid bacteria. At any given point in time, however, there was substantial variation between individual barrels, meaning the beer's rate of progress towards terminal microbiome composition was impacted by the barrel. These temporal aspects played a larger role in microbiome variability than barrel origin, which could not be linked to specific changes in the microbiome.

Do Oak Barrels Contribute To The Variability of The Microbiome of Barrel-Aged Beers?

by

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I understand that my thesis will become part of the permanent collection of Oregon State University libraries. My signature below authorizes release of my thesis to any reader upon request.

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Avram M. Shayevitz, Author

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1 Introduction

Fermentation is one of humanity's oldest technologies, with evidence of its routine use dating back 9000 years. Whether through accidental discovery or purposeful pursuit, our ancestors discovered that a sweet, alcoholic beverage could be produced through the treatment and fermentation of grain-sugar extract. Though its origins remain unknown, the earliest evidence of beer-like drinks dates back 7000 years to Neolithic China. Over time, the practice spread, with beer being produced wherever suitable grains could be farmed. By the height of Sumer Empire 5000 years ago, the production of barley-based beer was an established tradition. For the next 5000 years, continuous and inexorable improvements to the brewing process and lore were established. This, in a sense, encompasses the "traditional fermentation": a spontaneous event once attributed to divine intervention, transforming a broth of extracted grain sugars and nutrients into a gift from the gods. Over the centuries, the combination of tradition, science, and technology have turned beer production into a truly global industry, with annual production reaching 195 billion liters in 2017. While this is predominantly comprised of lager-style beer made using inoculated yeast monocultures, a small but significant portion of that volume is dedicated to the ancient art of spontaneously inoculated "traditionally fermented" beers. These encompass a number of styles, ranging from Umqobothi of southern Africa, to the Berliner Weiss of northern Germany, to perhaps one of the most well-known representatives of traditional fermentation in the Western world: the barrel-aged Lambic beers of Belgium.

Particularly within Europe and the United States, these traditionally fermented beers are often treated as a premium product that can command a premium price. A number of breweries solely focus on these types of beers, forgoing modern styles and techniques in favor of more traditional approaches. With a projected 7000 operating craft breweries in the United States by 2019, the number of breweries engaging in traditional fermentation and barrel maturation is bound to increase

as well. As such, the use barrels in beer production is on the rise. However, the barrel-aging process itself represents a significant time and product commitment by a brewery, and thus it is important to understand what exactly is happening within these barrels from a microbiological point of view.

Spontaneous fermentation of beer is a complex process that is difficult to control and profile. Metabolic processes of a diverse range of yeast and bacterial taxa slowly develop the distinctive aroma and flavor profiles of Lambic beers. Understanding the development of microbial communities within these beers, and how local environment will affect fermentation ecology is an important step in improving production techniques and ultimately reducing loss. The focus of this study is on the reproducibility of Lambic fermentation outside of its traditional region of production and the influence of oak barrels on the microbial communities of three commercially available barrel-aged beers, produced and matured within the state of Oregon, USA.

The collection of all microorganisms within a system, such as spontaneously fermenting beer, is broadly referred to as a microbiome. The fermentation microbiome encompasses the unique ecology that is responsible for the transformation of wort into beer. Therefore, understanding microbiome variability introduced into spontaneously fermenting beer systems by abiotic influences such as barrels will provide insight into the development of improved production techniques and quality control. This study used high-throughput sequencing metabarcoding to profile the yeast and bacterial communities of 102 barrels containing three distinct beers (Beer 1, Beer 2, and Beer 3) sampled over a period of ten months.

Beer 1 was used to profile the microbial successions during spontaneous fermentation of a beer produced in the "*Méthode Traditionnelle"* outside of Belgium. Two barrels were sampled over the course of nine months from July 2017 to April 2018, starting from 24-hours post brewing. Beer 1 was found to closely follow the distinctive microbial succession observed in Lambic beer produced in its traditional location.

Beer 2 allowed exploration of the effects of extended aging on the microbiome of Lambic-like beer. The beer was produced in three batches between May/June 2013 and November 2016, with batches produced approximately 20 months apart. Fifty barrels representing all three batch years were sampled at three separate time points between July/August 2017 and February 2018. Metadata analysis revealed that microbiome variability was linked to the brewing batch and time. Yeast populations were dominated by *Dekkera* (Syn. *Brettanomyces*) *bruxellensis*, and quickly stabilized within the first year of brewing. No divergence of yeast populations were observed after 12 months. Acetobacter, Gluconacetobacter, Acetobacteraeacea, and Lactobacillus dominated bacterial populations. The acetic acid bacteria were observed to primarily dominate the older beer batches while the youngest beer batch exhibited a wide distribution among samples, containing acetic acid bacteria and *Lactobacillus*. Differences in the makeup of acetic acid bacteria populations were evident throughout the 5 years of maturation covered by successive batches of this beer.

Beer 3 was studied to determine the effects of a large number of barrels, and barrel origin, on a single batch of pre-fermented beer. Fifty representative barrels selected from a population of bourbon, port, sherry, cognac, rum, maple syrup, and vanilla extract were sampled at two different time points from July/August 2017 and February 2018. Metadata analysis revealed that barrel type had no effect on microbiome composition, rather differences in yeast composition were observed to be linked with sample time point. Specifically, *S. cerevis*iae and *D. bruxellensis* populations diverged, with some barrels trending towards dominance of one yeast species over the other. In addition, barrels introduced microbiome variability as was noted by an increasing number of barrels yielding microbiome data between sample time points. No changes in bacterial profiles could be linked to specific metadata categories, but were again found to differ across the set of representative barrels.

Based upon the microbiome data collected, there is evidence to support that traditionally fermented beer placed into multiple barrels will diverge in terms of its microbiome, but trend towards a conserved profile over time. The observed fermentation microbiome profile of Beer 1 suggests that Lambic fermentations are not necessarily unique to a single geographic location. Time, more so than any other factor, appears to play the biggest role in microbiome variability, as barrels trend towards a conserved profile but at different rates.

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2 Review of Literature

2.1 A Brief History of Beer

2.1.1 Definition of Beer

Beer and beer-like beverages have existed since antiquity, predating recorded history by thousands of years (Meussdoerffer 2009). While beer's true origins remain unknown, surviving Sumerian cuneiform tablets dating back to 3200 – 3500 BCE provide tantalizing hints, with abundant and detailed records of grain harvests, grain allocations, and sacred grain-based alcoholic beverage recipes (Boulton & Quain 2001; Damerow 2012). The sophistication of these written records, along with archeological evidence suggest that fermented grain-based beverages developed soon after the development of agriculture, with advanced brewing capabilities developing in Neolithic China as early as 5000 BCE (Wang et al. 2016). Indeed, it is a common and important theme among emerging civilizations to quickly discover, and take advantage of, the phenomenon of food fermentation (McGovern et al. 2004; Damerow 2012).

Because of the age and cultural significance, the term "beer" represents diverse products of discrete and complex processes. Therefore, it is important to establish a definition of "beer" in the context of this study. Within the scope of this review, fermentation and microbiome monitoring methods therein will be the primary focus. As such, beer as a whole will be regarded in the most basic modern definiton: the combination of water, malted grain, yeast, and hops. Various traditional definitions may be applied, including the strict *Reinheitsgebot* (purity decree) of Germany, which stipulates beer may only be produced from malted barley, water, hops, and yeast. Alternatively, legal definitions such as the American Code of Federal (27 CFR Part 25 - BEER) defines taxable beer as:

"Beer, ale, porter, stout, and other similar fermented beverages (including sake and similar products) of any name or description containing one-half of one percent or more of alcohol by volume, brewed or produced from malt, wholly or in part, or from any substitute for malt."

In the modern sense, beer is the end goal of the brewing process, which can be broken down into four steps: (1) mashing, (2) lautering, (3) boiling, and (4) fermenting. Mashing involves the milling of malted grains to produce a grist, which is infused with hot water. Milling and infusion serve the purpose of exposing damaged starch granules to endogenous enzymes for efficient conversion into fermentable sugars. After conversion, the mash is drained, leaving behind the spent grist. The result is a nutrient-rich liquid extract called wort, which is then boiled. Boiling serves the purpose of sterilization, concentration, bitterant extraction, and protein denaturation. After the boil, the wort is cooled, inoculated, and then place into a hermetically sealed vessel to undergo fermentation. Though the process may differ slightly between breweries and beer styles, the basic principles remain the same: extract sugar and nutrients from a grain source for conversion into beer via fermentation.

2.1.2 The Traditional Fermentation

For the majority of beer history, and fermented food production, fermentation was accomplished through spontaneous action of autochthonous organisms from the surrounding environment or within the food itself (McGovern et al. 2004; Sieuwerts et al. 2008; Meussdoerffer 2009; Capozzi & Spano 2011). The concept of defined starter cultures used in many industrial food fermentations today are a recent advancement, with first widespread use in beer brewing dating back to 1904, around 50 years after Louis Pasteur and his contemporaries discovered *Saccharomyces cerevisiae's* role in the production of beer and wine. The dairy and meat industry would soon follow, with the development of defined starter cultures for routine use in the 1930's (Claussen 1904; Deibel et al. 1961; O'Toole 2004).

Fermented foods and beverages produced without addition of starter cultures are often referred to as a “traditionally fermented” product. While food fermentation will typically rely on microorganisms present within the raw material to initiate fermentation (Rantsiou et al. 2005), spontaneous beer fermentation is accomplished via direct exposure to air or previously-used fermentation vessels (Sparrow 2005). The result is a complex, mixed fermentation involving both yeast and bacteria across many taxa and genera (Spitaels et al. 2017). The fermentation process may last days or years, depending on the desired product. This process is the antithesis of modern industrialized beer brewing practices, which consider any organism other than the intended fermenter to be a spoilage organism (Bokulich et al. 2012a; Spitaels et al. 2017).

One traditional beer style in particular, Lambic, is of interest in that it undergoes both spontaneous fermentation and extended aging, often spending up to three years in oak barrels. The Lambic-style beer is believed to have originated in the Pajottenland region of Belgium as a cultural remnant of Roman rule over 2000 years ago. Over time, these traditions eventually evolved into the belief the unique environment and microbial ecology of the Pajottenland itself produced the Lambic beer (Van Oevelen et al. 1976; De Keersmaecker 1996; Martens et al. 1997; Capozzi & Spano 2011). Though its true origin is lost to history, Lambic beer production represents one of the oldest brewing methods still in use today (De Keersmaecker 1996). Production is characterized by an extended mashing and boiling process, referred to as “turbid mashing” (Fig. 2.1). The result is a wort rich in a wide range of saccharides, from simple monosaccharides to complex maltodextrins, capable of supporting a host of yeast and bacteria. The boiled wort is allowed to cool overnight in open, shallow vessels called “coolships”, where spontaneous inoculation is believed to occur

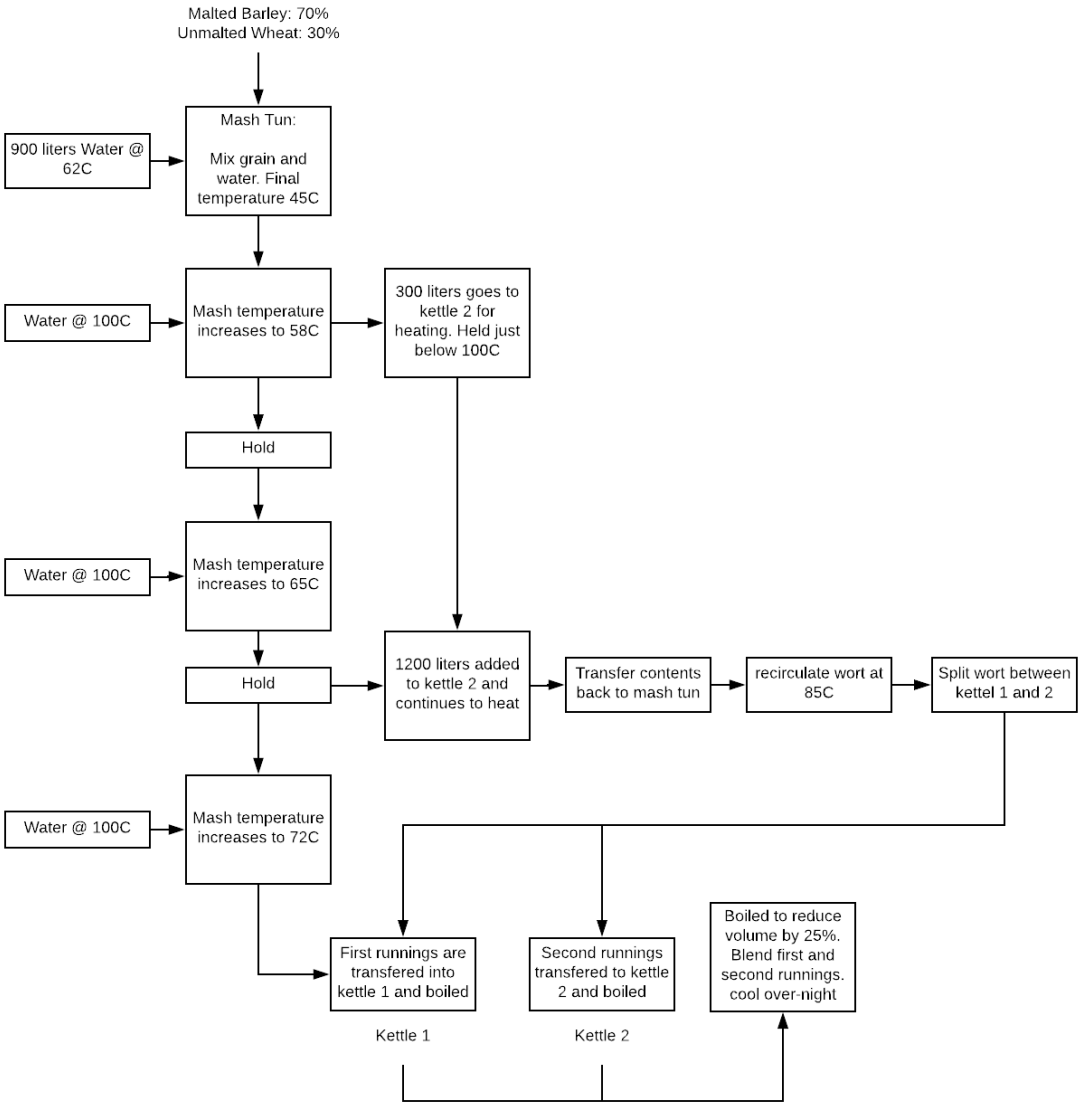

Figure 2.1 Simplified flow diagram of the turbid mashing process. Total elapsed time from start of mashing to end of boil may run 6 to 8 hours. Extended low-temperature mashing allows endogenous plant enzymes within the barley and wheat to break down starch granules and wheat protein. Water is added as needed, making the pre-boil volume variable.

Once cooled to around 20°C, the wort is placed into wooden barrels where a combination of microbial action and stochastic chemical reactions transforms the wort into an acidic, wine-like beverage (Van Oevelen et al. 1977; Verachtert & Iserentant 1995; Spitaels, Van Kerrebroeck, et al. 2015; Snauwaert et al. 2016). Much like *méthode Champenoise* wine, the Lambic style of beer is protected by both Belgian and European Union ordinances, and *appellation controlée* set forth by the European Beer Consumers Union (Sparrow 2005). As such, outside of Belgium, similar traditional fermentations are labeled under different style names. The American Beer Judge Certification Program (BJCP) lists Lambic-type beers brewed in the United States as "American Wild Ales" (Strong 2015). Other common colloquial style terminology includes "mixed fermentation beers" or "American Coolship Ales (ACA)", if produced in the traditional Lambic method (Bokulich et al. 2012b).

2.1.3 Oak Barrel Aged Beers

Wooden vessels play an important role in the development of traditionally fermented beer. Barrels and their larger cousins, foudres, allow for gas exchange (micro-oxidation), a key requirement for the microorganisms associated with Lambic beer maturation (Sparrow 2005). Due to the longevity, ease of manipulation, desirable organoleptic qualities, and general robustness, oak of both American and European varieties are highly desired among brewers, vintners, and distillers (Mosedale & Puech 1998; Sparrow 2005; Sterckx et al. 2012). In wine and spirits, oak barrels play an important role in the maturation and development of stylistic flavors and aroma. Extended contact with the oak surface results in the extraction of highly desirable oak lactones and aldehydes that impart toasty, coconut, vanilla, and nutty characteristics to the wine or spirit (Towey & Waterhouse 1996; Cerdán et al. 2002; Spillman et al. 2004; Sterckx et al. 2012). In modern brewing practices, the use of oak barrels to condition finished beers has been employed to complete the transformation of the final product. Though not a true traditional fermentation in the same capacity as described above, maturation of strong "Imperial" and dark beers is increasingly common among

American craft brewers (Dornbusch 2015). These barrels will have a wide range of origins: from new American oak originally used once for maturation of bourbon, to second (or third, or fourth, etc.) -use wine, whiskey, rum, and sherry barrels. Brewers may desire the character of the previous contents to be imparted on their beer, requiring brief contact time in the order of weeks. Alternatively, a brewer may seek a softening of the harsher aspects of stronger beers through the stochastic chemical reactions and micro-oxidation seen in wine production, requiring extended aging on the order of months, or possibly years.

This phenomenon is well studied among matured wines and spirits, but little work has been performed on beer (Sterckx et al. 2012). Prior to the invention of insulated stainless-steel fermenters and storage tanks, much of beer production, maturation, and transportation took place within wooden vessels. As such, the quality of containers would likely influence the quality of beer (Bokulich & Bamforth 2013). Beer is susceptible to spoilage from microorganisms harbored within the wood of the barrels, including *Brettanomyces spp.* (syn. *Dekkera*) and acetic acid bacteria. In most cases, these organisms impart negative quality characteristics that ruin an otherwise good product. However, depending on beer style, these influences may not be detrimental, and even desired. Traditional styles such as the *Saison* and *Bier De Garde* spend three to six months in previously used barrels, enough time to undergo a brief spontaneous secondary fermentation initiated by autochthonous *Brettanomyces*. During this time spicy, leathery, and earthy character may develop, a hallmark of *Brettanomyces* influence (Markowski 2004).

2.2 Traditional Fermentation: Culture-Dependent Analysis

To fully capture the microbial complexity of traditionally fermented beer, parallel examples of other traditionally fermented food and beverage products will be discussed. Fermentation is an ancient technology used to improve the safety and quality of various foodstuffs. Unlike industrial fermentation products that rely on defined starter cultures, traditional foods such as Lambic beer,

kimchi, komoto sake, sauerkraut, traditional Italian sausage, and various dairy products rely on microorganisms present within the raw ingredients with minor biotic interference from humans. Food produced in this manner develops over a period of several months to years, with advanced age being an indication of high quality. (Nout 1992; Rantsiou et al. 2005; Roh et al. 2010a; Jung et al. 2011; Smid & Lacroix 2013; Bokulich et al. 2014). Prior to the discovery of microorganisms, little was known about fermentation other than what could be empirically derived (Meussdoerffer 2009). A semblance of control could be exerted through process refinement and standardization. Recurring production within each season cycle, re-using fermentations vessels, and consistent maturation conditions would have facilitated batch-to-batch consistency, but the final product is ultimately the result of capricious microbial activity and their interactions (Bokulich et al. 2012a). Understanding population composition of fermentation microbial communities are key to maintaining good functional practices for greater process control, consistency, and quality.

2.2.1 Culture-Dependent Techniques

Early methods of profiling the microbial communities of food systems relied on ecology-derived culture-dependent techniques (Fleet 1999). Culture-dependent techniques have been vital to understanding microbial contributions to an ecosystem, including food systems. When culturing is successful, the physiology and metabolism of representative organisms can be investigated under tightly controlled conditions (Swanson et al. 2016). By understanding the basic physiology of representative microorganisms, a better understanding of fermentation development can be built, which in turn can help to refine the production process and, ultimately, quality.

For over 150 years, general and selective enrichment media have been the primary method of pure culture isolation. From there, qualitative morphological and physiological assays determine the physiology and thus taxonomical identification of isolated organisms (Fry 2004). Often times a complex array of assays are required to make even basic determinations of a single organism's

taxonomic identity. In some instances as many as 73 discreet assays are required to make a definitive identification, thus limiting routine use (Boulton & Quain 2001). Biochemical assays, such as coagulase activity, fermentation capabilities, nitrate reductase activity, pH growth curves, and temporal heterogeneity over the course of community development are used to infer specific metabolic contributions by different microbial species. Various compendia exist detailing these assays, including *Bergey's Manual of Determinative Bacteriology*, *Bergey's Manual of Systematic Bacteriology*, and *The Yeasts: A Taxonomic Study*. In the United States, the Food and Drug Administration (FDA) maintains a Microbiological Methods & Bacteriological Analytical Manual (BAM) specifically designed for monitoring microbiological contamination in food and food-related industries, and is based on the Association of Official Agricultural Chemists (AOAC) International Official Methods of Analysis (FDA 2016). Table 1 outlines examples of culture-dependent methods used in identifying microorganisms in food and food systems.

Table 1. Common culture-dependent methods as outlined by the FDA's Bacteriological Analytical Manual (BAM), 8th edition

Assay	Purpose	Media	Type	Time Requirement	Method
Aerobic plate count (APC)	Enumeration of viable microorganisms present in a food product	Undefined. Yeast-extract peptone dextrose (YPD) agar	Non-selective, non-differential	3 - 7 days	BAM Media M124
Spread-plate method (fungi)	Enumeration of viable molds and yeast present in a food product	Defined, Dichloran rose bengal chloramphenicol (DRBC) agar	Selective, differential	5 days	BAM Media M183
Gram staining	Differentiation between Gram positive and Gram negative bacteria	N/A	N/A	30 minutes, not including culturing time	Various, no set BAM method
Detection of fecal coliforms	Assessing fecal coliform contamination and differentiation among species	Multiple	Selective, differential	3 - 7 days	BAM 4: Enumeration of E. coli and Coliform Bacteria
Coagulase test	Assessing the ability of microorganisms to convert fibrogen into fibrin	Undefined, Brain-Heart Infusion (BHI) broth	Non-selective, differential	18 - 30 hours	BAM Media M24
Visual assessment of Staphylococcus aureus	Isolation of S. aureus from food samples	Undefined, Baird-Parker agar	Selective, differential	45 - 48 hours	BAM Media M17
Thermostable nuclease production	Differentiating between coagulase negative and coagulase positive S. aureus	Defined, Toluidine Blue-DNA (TBD) agar	Selective, differential	4 hours	BAM Media M148
Visual assessment of Salmonella serotypes	Visual identification and isolation of Salmonella Shigella species	Multiple, Undefined, Hektoen Entric (HE) agar, Xylose Lysine Desoxycholate (XLD) agar, Bismuth Sulfite (BS) agar	Selective, differential	24 hours, not including enrichment phase	BAM Media M61, M179, M19
Urease test (conventional), pure cultures	Differentiating between suspected Proteus species and Enterobacteriacea	Defined, Stuart's urea broth	Non-selective, differential	24 hours, not including enrichment and isolation phases	BAM Media M171
Triple sugar iron assay	Assessing the ability of microorganisms to produce hydrogen sulfide	Defined, Triple sugar iron agar (TSI)	Non-selective, differential	24 hours, not including enrichment and isolation phases	BAM Media M149
Carbohydrate fermentation assay	Assessing specific carbohydrate fermentative abilities of isolated microorganisms	Defined, Cystine tryptic agar (CTA)	Non-selective, differential	24 - 48 hours	Various, no set BAM method
Chromogenic differentiation	Differentiation of Saccharomyces cerevisiae strains based on alpha-galactosidase activity	Undefined, YPD+X GAL*	Non-selective, differential	3-5 days	Boulton and Quain (2001)
Direct inhibition	Differentiating between yeast species capable of growing in the presence of a selective inhibitor	Undefined, YPD with cycloheximide	Selective, non-differential	3-5 days	Boulton and Quain (2001)

*bromo-4-chloro-3indoyl-alpha-D-galactoside

2.2.2 Microbial Diversity as Revealed by Culture-Dependent Analysis

A plethora of culture-dependent studies exists exploring microbial population dynamics of complex food ecosystems. As mentioned previously, understanding the contributions of specific organisms to a fermentation system is key to developing model industrial practices. In the context of traditional fermentations, kimchi fermentation represent a well-documented, complex traditional fermentation. Kimchi is a traditional vegetable-based Korean food that undergoes on a month-long spontaneous fermentation (Jung et al. 2011). As a fermentation system, industrial production of kimchi will typically rely upon autochthonous organisms to initiate and complete the transformation of raw vegetable material into the finished product.

To illustrate, previous studies have used culture-dependent techniques to establish the role of lactic acid bacteria (LAB) in the production of kimchi. Methods such as Unweighted Pair Group Method with Arithmetic Mean (UPGMA) hierarchical clustering based on the ability of LAB to oxidize certain carbon sources has revealed the diversity of LAB within spontaneous kimchi fermentations (Lee et al. 1997). Diverse subpopulations of 12 to 15 identifiable *Leuconostoc*, *Lactococcus*, *Pediococcus* and *Weissella* species are believed to play a significant role in the acidification and breakdown of raw material (Lee et al. 1997; Cho et al. 2006; Jung et al. 2011). Microbial diversity and population dynamics are shown to be strongly impacted by abiotic factors, such as temperature. Traditional kimchi is fermented at refrigeration temperatures, thus favoring cryotolerant species such as *W. koreensis*, which are ideal in flavor and texture development of the final product (Cho et al. 2006). Correlation of specific species and fermentation conditions to desired quality characteristics has ultimately improved industrial production protocols, leading to more efficient and consistent process.

2.2.3 Major Limitations of Culture-Dependent Analysis

Despite the prevalence and usefulness of culture-dependent food fermentation studies available, there are known limitations in reproducibility and taxonomic identification of cultured organisms (Jung et al. 2011; Spitaels et al. 2014). Outside of industrial applications, it is rare that microorganisms will exist as isolated species. Due to the inherent capriciousness of living organisms, a complete profile of species distribution and metabolic interactions remains difficult (Fleet 1999; Rantsiou et al. 2005; Carraro et al. 2011). Many organisms rely on the metabolic activity of other organisms to thrive, requiring a complex web of interactions. Thus, one of the significant limitations of culture-dependent techniques is that many microorganisms are simply uncultivable under laboratory conditions. Fastidious metabolic requirements and environmental conditions may be difficult to reproduce, resulting in delayed or inhibited growth. Even if metabolic requirements are met, microorganisms may exist in a viable but noncultureable (VBNC) state due to non-lethal cell damage (Swanson et al. 2016).

Another major limitation of culture-dependent techniques are the introduction of strong bias through enrichment. Culturing conditions will favor organisms suited to those particular conditions, potentially skewing quantitiave analysis preferentially towards those individuals whose metabolic requirements can be artificially met (Muyzer et al. 1993; Giraffa 2004; Spiegelman et al. 2005; Carraro et al. 2011; Swanson et al. 2016). It has proven exceptionally difficult and impractical to artificially mimic ecological niches required for a complete culture-dependent assessment of microbial diversity (Carraro et al. 2011; Swanson et al. 2016)

Comprehensive ecological assessments rely on specific information to assess microbial communities. Food systems are no different. Culture-dependent community profiling requires specific information to be useful: (1) reliable taxonomic diversity data, (2) quantitative growth cycle data, (3) spatial distribution, (4) biochemical process of inoculation, (5) impact of intrinsic

and extrinsic factors, and (6) correlation between growth and quality – including safety (Fleet 1999).

Sample preparation may negatively influence qualitative assessment. Food microbiology relies on four basic principles of analysis: (1) maceration/blending/homogenization (2) homogenate dilution (3) plating on appropriate media (4) isolation and identification (Fleet 1999). Errors may be introduced during homogenization and dilution, while quantitative errors will inevitably accumulate during plating reducing the accuracy of dominant organism identification (Giraffa 2004).

Lastly, living communities are not static constructs. Microbial communities will always exhibit both spatial and temporal heterogeneity. It is typical to find food microbiology studies describing dominant species at singular points through a production process (Giraffa 2004). However, due to complex interactions between the food matrix, other organisms present, and meta-interactions between abiotic and biotic events, a "dominant" isolated species may in reality represent a singular organism selected to thrive in the isolation media rather than a population as a whole (Fry 2004; Giraffa 2004; Jung et al. 2011). Attempts to take into consideration all environmental, metabolic, and nutritional requirements for complex community profiling may result in an impractical amount of cultures.

2.3 Traditional Fermentation: Culture-Independent Analysis

With the deciphering of the generic code in 1960's, the hunt began to find novel methods for rapid and accurate identification of microorganisms in clinical and industrial applications. By the late 1980's it was understood that the limitations of culture-dependent microbiology required the use of more powerful alternatives (Smalla 2004). The advent of DNA-based technology helped revolutionize the field of microbiology. Through chemical manipulation of environmental DNA, a staggering community diversity in previously unexplored ecological niches have been brought to

light (Muyzer et al. 1993; Cocolin et al. 2000; Wan et al. 2006; Martínez et al. 2011; Wolfe et al. 2014; Reuter et al. 2015; De Filippis et al. 2017; Spanoghe et al. 2017). Quantitative real-time polymerase chain reaction (qPCR), denaturing gradient gel electrophoresis (DGGE), flow-cytometry (FC), and DNA sequencing have all made significant contributions to understanding both environmental microbial ecology and food ecosystems. Collectively, these technologies are referred to as culture-independent methods, as they do not rely on the classical culturing methods of mid-20th century microbiology. The primary advantages of culture-independent techniques lay in direct analysis of DNA, thus adding specificity and accuracy to quantitative and qualitative analyses of complex microbial ecologies.

Of note, sequencing technologies opened the door for a wide range of applications outside the realm of medicine, finding specialized uses in the food and beverage industry. The driving force behind this surge in applied food industry applications is attributed to the dramatic drop in cost. To illustrate, the first human genome sequence, comprising of over 3 billion base pairs and encoding 19,000 to 20,000 protein-encoding genes, was estimated to have cost nearly $1 billion in 2001. By 2015, this would drop to under $2000, and costs continue to fall (Reuter et al. 2015).

Despite the wide range of culture-independent techniques available, their ecological application falls within two applications. Within literature, these two categories are split into (1) targeted analysis and (2) community profiling. Targeted analysis is used to identify taxa, specific species, individual strains, or the presence of specific genes depending on the requirements of the analysis. A targeted analysis is typically much narrower and requires some prior knowledge of organisms present. Community profiling is a technique used to simultaneously detect and differentiate all taxonomical groups present via universal marker gene polymorphism or entire genomes. Community profiling is less dependent on accessory knowledge of what may or may not be present. (Hugenholtz & Tyson 2008; Schoch et al. 2012; Franzosa et al. 2015; Yang et al. 2016).

2.3.1 Culture-independent analytical techniques (non-HTS based)

2.3.1.1 Real-Time Quantitative PCR (qPCR)

qPCR is a common targeted analysis that is capable of quantitatively measuring target populations within a sample. It is related to the standard PCR methods, but differs in that a fluorogenic probe is introduced into the system (e.g. TaqMan Assay), allowing for direct, real-time quantification based on fluorescent intensity (Heid et al. 1996; Muniesa et al. 2014)

qPCR is extremely sensitive, capable of detecting targeted organisms in concentrations as low as 0.2 CFU/g (Cheng et al. 2009). However, several disadvantages complicate its use in the context of profiling active fermentation systems. It is important to define quantitative and qualitative biases associated with PCR-based techniques. DNA extraction efficiency, preferential amplification and variable copies of target gene can negatively influence quantification (Keisam et al. 2016). Additionally, genomic DNA from dead microorganisms will persist in most food environments, further confounding quantitative analysis. A common method to reduce those biases is to make use of reverse transcribed-RNA as the template. RNA will degrade quickly upon cell death, making it a better indicator of living microorganisms (Carraro et al. 2011). Another disadvantage of qPCR is that community profiling is generally prohibitively time-consuming and at the time of this writing cannot be used to characterize complex mixed communities of unknown composition beyond that of coarse taxonomic identification (Fierer et al. 2005; Bokulich et al. 2012a).

Despite the limitations of complete community profiling, qPCR does have application as a rapid and specific detection technique for spoilage organisms. (Wan et al. 2006; Martínez et al. 2011; Spanoghe et al. 2017). Carraro et al (2011) describes targeted qPCR analysis applications in monitoring temporal heterogeneity of specific lactic acid bacteria found in ripening cheese. Additionally, using clone library sequencing, they show that at least a general profile of microorganisms present throughout a fermentation is required to take full advantage of qPCR

monitoring, including monitoring previously uncultivated lactic acid bacteria. Indeed, because of this study, Carraro et al (2011) speculate that instances and as-yet-to-be determined impact of the fish pathogen *Lactococcus piscium* are highly underestimated in dairy fermentations.

2.3.1.2 Denaturing gradient gel electrophoresis (DGGE)

DGGE is a common and effective qualitative community profiling method used to study microbial diversity in complex systems (Muyzer et al. 1993; Cocolin et al. 2000; Randazzo et al. 2002; Rantsiou et al. 2005; Bokulich et al. 2012a). DNA is extracted from a community sample and PCR is used to amplify a highly conserved universal marker gene. Amplified DNA fragments are separated based on the electrophoretic mobility of partially melted DNA molecules, which is inversely related to melting point (Muyzer et al. 1993). The result is a visual assessment of DNA banding through a specialized acrylamide gel, sensitive to single-point nucleic polymorphisms. Unique banding patterns establish community fingerprints and community changes over time, revealing the degree of diversity among sample sets. Bands of interest can be physically retrieved and sequenced for added qualitative depth. Sequence polymorphism can then be used to taxonomically profile complex communities (Muyzer et al. 1993; Kisand & Wikner 2003; Cébron et al. 2004; Bokulich et al. 2012a).

DGGE has provided significant insight into food systems, and numerous studies have been published that incorporate DGGE in fermentation community profiling. Examples include direct profiling of dynamic yeast populations in wine fermentation (Cocolin et al. 2000), profiling bacterial diversity of artisanal Sicilian cheese production (Randazzo et al. 2002), understanding the evolution of solid-state fermentation pits in the production of traditional Chinese spirits (Liu et al. 2017), and profiling microbial communities present during the fermentation of traditional Italian sausage (Rantsiou et al. 2005). Combined with DNA sequencing technology, novel and previously

unknown microbial actors have been characterized as part of those particular fermentation processes.

Rantsiou et al (2005) showed, to generally good effect, that additional PCR-DGGE investigation of fermenting sausages demonstrates the limitations of culture-dependent methods. Highly diverse microbial ecosystems not previously observed using standard culturing methods were revealed. A key aspect of which demonstrated that temporal heterogeneity may play an important role sausage quality. Application of culture-independent techniques also reveal potentially unknown microbial actors. For instance, within that same study, the identification of an aquatic angiosperm, *Mayaca fluviatilis*, is not necessarily cause for alarm. Rather, the authors note that the detection of *M. fluviatilis* coincides with the universal 26S ribosomal RNA gene commonly used in fungal community profiling. The sequence itself was reported to have a poor match with known libraries (86.5% identical). Thus, it is therefore more likely to represent a previously undocumented fungus rather than a subtropical flowering plant (Rantsiou et al. 2005). Implementation of targeted approaches can then determine the prevalence of these unknown taxa, which may lead to a novel understanding of fermentation profiles. Yet, despite its versatility and established reliability, DGGE has distinct disadvantages in its technical difficulty, time commitment, relative expense, and complications of PCR bias, making it unsuitable for industry applications outside of a research setting (Bokulich et al. 2012a).

2.3.1.3 Flow Cytometry (FC)

Originally designed for cell quantification in biomedical applications, FC is a highly versatile technique that is capable of both quantitatively and qualitatively analyzing microbial populations based on specific parameters (Swanson et al. 2016). FC coupled with cell sorting is capable of physically sorting individual microorganisms present in a phenotypically heterogeneous community. VBNC cells along with dormant, damaged, and dead cells can be distinguished from

each other, representing a huge advantage over culture-dependent techniques (Diaper et al. 1992; Spiegelman et al. 2005; Comas-Riu & Rius 2009).

FC works on the principle of light scattering and cell fluorescence, and is capable of measuring thousands of cell per second. Sorting can be done using physical and physiological parameters, including size, membrane permeability, internal refractive index, intercellular pH, and DNA density. FC is highly effective when paired with fluorogenic dyes, nucleic acid-binding fluorogenic probes or antibodies (see below). FC has seen significant use in studying industrial fermentations and the decline in yeast and bacteria population viability over time. Other food industry applications are directly related to rapid detection of specific spoilage or pathogenic organisms. It holds applications in the dairy industry for monitoring the quality of defined starter cultures and detection of bacteriophages that can disrupt yogurt fermentation (Comas-Riu & Rius 2009).

Bearing this in mind, FC is a still a targeted approach, requiring DNA or RNA-flurogenic probes for phylogenic profiling, similar to primers used in qPCR. Primary applications in industry are linked to studies limited to controlled monoculture-yeast and malolactic fermentation cultures found in the production of ciders and wine (Herrero et al. 2006). By itself, FC is limited in its ability to characterize complex, undefined communities (Spiegelman et al. 2005).

2.3.1.4 Fluorescence *in situ* Hybridization (FISH)

Using the same concepts applied to qPCR and targeted FC analysis, specific fluorogenic primers can be designed to hybridize with complementary DNA or RNA inside living organisms. Universal rDNA primers are especially useful for targeted identification of yeast, bacteria, and archaeal species. Though useful by itself, FISH can offer significant advantages in community characterization and fermentation monitoring when paired with other culture-independent techniques.

FISH PCR-DGGE analysis has yielded insight into the methanogen archaeal communities found in Luzhou mud pits used in the production of the Chinese liquor Baijiu. The development of essential flavor and aroma-active compounds are contingent on complex interactions between hydrogen-producing bacteria and hydrogen-consuming archaea. Understanding microbial communities of these mud pits can lead to process improvements for greater consistency and product quality (Tao et al. 2014; Wu et al. 2015)

Coupled with culture-dependent techniques, FISH-qPCR has been shown to be able to overcome the limitations of standard plating methodologies for assessing targeted microbial populations. FISH-qPCR has seen significant use in monitoring population density of *Saccharomyces cerevisiae* and *Hanseniaspora guillermondii* during wine fermentation. (Andorra et al. 2011). Additionally, FISH-qPCR has been shown to be more accurate for quantifying acetic-acid bacteria than culture-dependent methods (Sengun 2016).

2.3.2 Culture-independent analytical techniques (HTS-based)

Sequencing-based approaches have revolutionized the fields of biology and microbial ecology. Advances in massively parallel high-throughput sequencing (HTS) technology and the growing availability of affordable computational power have changed the way complex microbial populations are explored and profiled. A sequencing-based approach can provide high-resolution insight into the functional potential of almost any microbial population, providing faster and more comprehensive results than classical culture-dependent techniques alone (Hugenholtz & Tyson 2008; Park et al. 2011; Cao et al. 2017). The power behind HTS techniques lies in the ability to produce enormous amounts of data with minimal starting material, mapping out entire taxa regardless of organism cultivability based upon all the DNA present in the sample. This is, essentially, the definition of the microbiome – all of the DNA representing all of the microbes present in a given ecosystem. While older sequencing technologies, such as Sanger sequencing,

offer high confidence results, their quantitative community profiling capabilities are severely lacking due to decreased throughput (Churko et al. 2013), providing at best a partial view of the microbiome.

2.3.2.1 Technology

At its core, HTS techniques rely on three basic principles: sample collection, DNA extraction, and DNA sequencing. How the DNA is handled post-extraction depends on the downstream application. Downstream HTS microbiome profiling is accomplished in one of two ways: metabarcoding and metagenomics, which will be discussed in detail below. While relatively new, both approaches have already played a significant role in profiling of microbial communities relevant to food quality, safety, and production (Abdelfattah et al. 2016; De Filippis et al. 2017; Cao et al. 2017). Within the domain of these two approaches, several technology platforms exist that are capable of providing inexpensive, reliable data. Roche 454 Life Sciences Pyrosequencing and Illumina sequencing by synthesis represent two platforms through which major strides in understanding food-microbiome community structures have been made (De Filippis et al. 2017; Cao et al. 2017). It should be noted, however, that as of 2016 the Roche 454 technology platform is no longer supported.

2.3.2.2 Metabarcode Analysis

At the time of writing, metabarcoding is the most common and cost-effective HTS approach to the study of microbial communities. Barcoding in taxonomy works on the principle of using short regions of conserved, cross-taxa genomic DNA. This region, or regions, are referred to as a "barcode" – a unique identifier that can be easily and quickly amplified and sequenced. High-confidence taxonomic identification of species or genera can be made using these unique barcodes (Hebert et al. 2003). Metabarcoding refers to the simultaneous detection of multiple taxonomic barcodes in a single sample. By virtue of the highly-parallel nature of HTS, the process allows

sequencing of multiple PCR amplicon libraries simultaneously, meaning multiple individual samples. Each library is built using a series of unique tags during PCR amplification that identify each individual sample and allow downstream analysis to separate multiple libraries (samples) from a single pooled sequence run It can be considered the next logical development of DGGE methodologies, in that PCR products are directly sequenced rather than first visualized in a gel, and rather than having to run a separate gel lane per sample, all were combined into a single lane. As with DGGE, metabarcoding analysis targets highly conserved microbial housekeeping-genes or ribosomal RNA (rRNA) genes. Community diversity is determined based on genetic polymorphisms within these target genes.

In the context of food and traditional fermentations, bacterial communities are profiled using 16S rRNA gene amplicons. Because of the highly conserved nature and ubiquity of the 16S rRNA gene among prokaryotes, sequencing the 16S rRNA gene (hereafter referred to as 16S) is, at present, the best option for rapid phylogenetic studies of bacterial communities (Weisburg et al. 1991). The gene, which contains nine hypervariable regions (designated V1 trough V9), can be correlated to genus and, in some instances, species. Yang et al (2016) suggest that regions V4-V6 offer the best phylogenic resolution for bacterial communities.

Fungal community profiling makes use of the highly conserved ribosomal internal transcribed spacer (ITS) region. The ITS region (hereafter referred to as ITS) fulfills two major components for ideal short-sequence barcoding identification: high PCR amplification performance and superior species discrimination across a large range of fungal families, making it ideal as a universal fungal marker (Schoch et al. 2012). Additionally, both 16S and ITS PCR can make use of respective universal primers, adding to the efficiency and efficacy of metabarcoding approaches.

The capacity for HTS approaches to reveal previously unreported genera in food systems is immense. As discussed previously, techniques such as DGGE, and PCR clone libraries have

revealed novel microbial actors that remained unnoticed when only culture-dependent techniques were used. Metabarcoding goes a step further, offering insight into the taxonomic diversity and the mechanisms that underlie assembly of microbial communities (Wolfe et al. 2014; De Filippis et al. 2017). In a deep, large-scale analysis of the microbial communities of cheese rinds, Wolfe et al (2014) was able to demonstrate the prevalence of *Yaniella* and *Nocardiopsis*, previously unreported genera of bacteria in food fermentation systems. Additionally, halotolerant marine bacteria such as *Vibrio*, *Halomonas*, and *Pseudoaltermonas* are found to be widespread among traditional cheeses regardless of geographic origin.

Metabarcoding is fast, economical, and capable of a good phylogenic resolution. However, similar disadvantages seen in DGGE and qPCR also apply here: environmental contamination, PCR bias – which include preferential amplification, amplification of non-living DNA, DNA extraction efficiency and biases, and target gene redundancy are an area of concern (Glassing et al. 2016; Keisam et al. 2016).

2.3.2.3 Metagenomics

Unlike metabarcoding, metagenomics is a fully untargeted approach that takes into account the *total DNA present* in a representative environmental sample. The result is the true "metagenome" of an environment: biodiversity represented by the overall genome contributed by each species/strains' respective genome. The advantage of a metagenomics approach over other sequencing approaches is that it does not rely on PCR. Thus, a complete community profile can be constructed in addition to the abundance of microbial genes without the PCR biases associated with metabarcoding (Hugenholtz & Tyson 2008; De Filippis et al. 2017). Additionally, it is possible to directly asses the overall functional metabolic potential within a food or beverage matrix. In the context of food systems, the same cheese study mentioned above (Wolfe et al. 2014) highlights the ability to assess the functional potential of complex food microbiomes. Their metagenomic analysis

was able to establish potential function of halotolerant marine bacteria in cheese production. Metabolic pathways associated with flavor development were enriched in cheeses that exhibited the highest population of these bacteria, particularly *Pseudoaltermonas*. The prevalence of cold-adapted lipase genes could be associated with lipolysis and related flavor development of cheese.

However, like metabarcoding, metagenomic analyses cannot directly provide information on the metabolic activity of active communities. Furthermore, metagenomics is also associated with significantly higher costs and computational processing than metabarcoding and is often used as a secondary analysis on a subset of samples (Hugenholtz & Tyson 2008; Chen et al. 2017; Cao et al. 2017)**.**

2.3.2.4 Microbial Diversity as Revealed by Culture-Independent Analysis

Culture-independent techniques, particularly Illumina based HTS metabarcoding analysis, are now playing an important role in the study of food systems, both in industrial and research capacities. Improvements ranging from safety, quality control to fermentation management and tracking are gaining traction (Cocolin et al. 2000; Bokulich et al. 2012a; Wolfe & Dutton 2015; Leonard et al. 2015; De Filippis et al. 2017; Spanoghe et al. 2017; Chen et al. 2017). As a product of complex fermentation, Lambic and other barrel-aged beers provide rich grounds in which to take advantage of these powerful techniques, particularly within fermentation management.

The key to understanding and reproducing large-scale traditional fermentations in a controlled and tractable manner is a fundamental understanding of fermentation ecologies. As Roh et al (2010b) and Bokulich et al (2012a) discuss, deep sequencing technologies can be used to develop innovative new ways to study fermentation systems, potentially answering questions that have yet to be asked. Pyrosequencing has been used in archaeal and bacterial diversity studies in Korean seafood fermentations, and have shed light on the previously invisible role of viral communities on kimchi, sauerkraut, and fish fermentation (Roh et al. 2010b; Park et al. 2011). Illumina sequencing has been

used to understand community structures of traditional fermentations, revealing previously undescribed bacterial involvement in foods such as traditional Italian sausages, beer, and sake (Rantsiou et al. 2005; Bokulich et al. 2012b; Bokulich et al. 2014). Recently, Leonard et al (2015), demonstrated the potential for metagenomic analysis to reveal high resolution typing of Shiga-toxin producing *Escherichia coli* in bagged leafy greens, without the extended enrichment typically required for *E. coli* characterization. However, given the limitations of both culture-dependent and culture-independent methods, a combination of both types of methods are often required to obtain a complete profile of a microbial ecosystem.

2.4 Microbial Ecology of Traditionally Fermented Beer

The microbial community of fermentation is one of the primary influences on the final product. Extensive modern studies on microbial community profiling in food systems have been published and reviewed, but of those, only a handful pertain to beer and its raw materials (Bokulich et al. 2012a; Bokulich et al. 2016; Spitaels et al. 2017; De Filippis et al. 2017)**.** The majority of modern beer production is performed under monoculture conditions. Despite this practice, beer production is influenced by a diverse range of microorganisms, both upstream and downstream of the brewing process (Van Oevelen et al. 1976; Vanderhaegen et al. 2003; Bokulich & Bamforth 2013; Pires et al. 2014). As such, raw materials, geographic location, environment, and preparation methods may have profound influence on the outcome of a finished beer. Traditionally fermented beer represents an interplay of all these factors, under a degree of freedom or stochasticity not seen in modern beer production. Thus, the ability to qualitatively and quantitatively analyze microbial communities of traditional fermentations is important to understand predictive measures of quality in the final product.

The earliest comprehensive efforts to profile the microbial communities of traditionally fermented beer were made using the tools available at the time: culture-dependent isolation biochemical

assays for taxonomic classification. These methods, though limited, made important progress that guides later studies. Van Oevelen et al (1977) established that fermenting Lambic beer undergoes complex temporal microbiome shifts, representing sequential depletion of increasingly complex carbon sources and compositional changes of the beer itself (Fig 2.1**)**. In this defining work that kick-started the quest to demystify the microbiology of Lambic beer, Van Oevelen et al put fort the following questions:

> From this study it seems apparent that the main microbial groups active in Lambic fermentation are wort Enterobacteriaceae, yeasts such as Kloeckera, Saccharomyces and Brettanomyces and bacteria such as Pediococcus. Many problems remain to be solved such as: (1) Where do the different organisms find their origin? (2) Can the development of acetic acid bacteria be controlled? (3) Which organisms cause ropiness when it occurs and can it be controlled? (4) What is the nature of the mousy smell produced by Brettanomyces? (5) Are the events occurring in other Lambic breweries similar? (6) Can Lambic be made with pure cultures? (7) Are there other unknown fermentation products in Lambic?

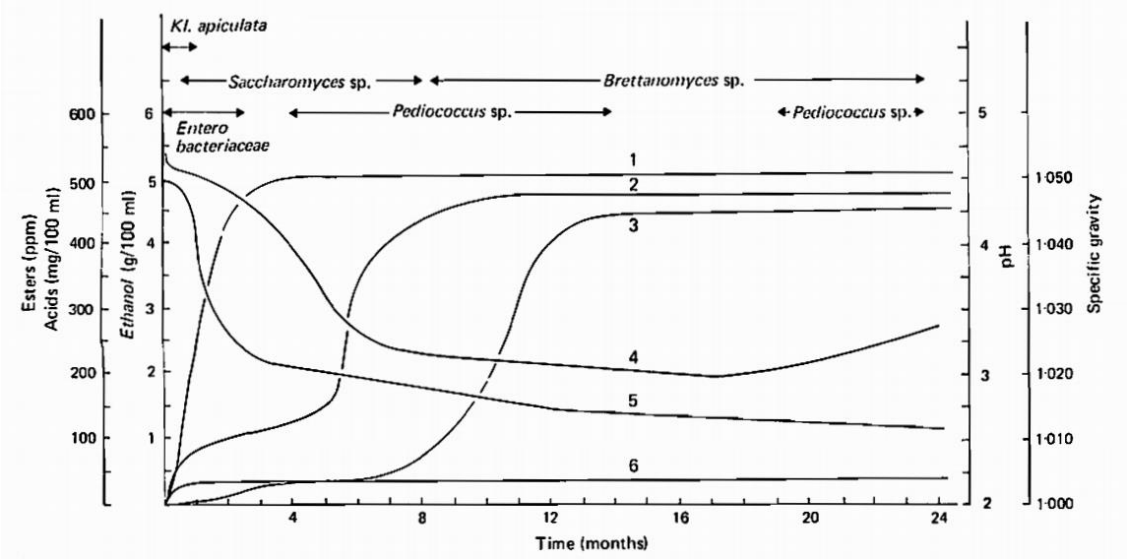

Figure 2.2 The microbial successions of spontaneously fermented beer as profiled using classical culture-dependent techniques. The appearance of microorganisms correspond to compositional changes of the beer. Isolated organisms (top of chart) are shown relative to the time (bottom X-axis) of their appearance. Inner-left Y-axis scales ethanol production (1). Outer-left Y-axis scales lactic acid (2), acetic acid (6) and ethyl lactate (3). Inner-right Y-axis scales pH (4) and outter-right Y-axis scales real extract (5). Reproduced from Van Oevelen et al (1977).

Between 1977 and 1997, much of the information gleaned was limited to what could be cultured and identified based upon now-outdated taxonomical information (Bokulich et al. 2016; Spitaels et al. 2017). As stated previously, one of the significant limitations of culture-dependent techniques is that many microorganisms are simply uncultivable under laboratory conditions. Organisms of interest may exist in a VBNC state, are too fastidious for current culturing techniques, or require syntrophic communities to grow. Thus what is cultured may not be fully representative of the microbial population (Herrero et al. 2006; Wu et al. 2015; Swanson et al. 2016). Additionally, when dealing with casks, barrels, or other large vessels, it is unclear whether a representative microbial sample is obtainable. This problem is illustrated by the fact modern sampling techniques are all

derived from the original Van Oevelen et al (1977) methods with the working assumption that viable organisms remain in suspension and thus sampling from this point is representative of the important (viable) part of the microbial population (Van Oevelen et al. 1977; Snauwaert et al. 2016).

2.4.1 Microbial Phases of Traditionally Fermented Lambic Beer

Some of the earliest comprehensive work done on the ecology of Lambic beer had begun in the 1970's, with preliminary culture work starting as early as the 1930's in Belgium. Using culture dependent techniques on a 24-month Lambic fermentation, Van Oevelen et al (1977) was able to build a temporal model of the progression of *Enterobacteriaceae* genera, *Kloeckera apiculate* (now known as *Hanseniaspora uvarum*), *Pediococcus spp*., *Saccharomyces cerevisiae*, and *Brettanomyces spp*. (Fig. 2.1). This was later expanded upon to separate successive blooms into five overlapping phases (Table 2). By this point, correlation between microbial population trends to aroma and flavor development of Lambic beer had begun to be established (Priest et al. 1974; Van Oevelen et al. 1976; Van Oevelen et al. 1977; Verachtert & Iserentant 1995).

Table 2 Five microbial phases of Lambic beer as outlined by Veracher and Iserntant (1995)

Enterobacterial phase	Occurs immediately after wort cooling. Characterized by a strong presence of *Enterobacter*, *Klebsiella* and *Citrobacter*. Consumes glucose.
Saccharomyces phase	Begins approximately one month after the start of Enterobacterial phase. Enterobacteria decline significantly. *Sacharomycese* spp. increase. Marks the start of primary ethanol fermentation. Consumes remaining glucose, maltose, maltotriose. pH begins to rapidly drop.
Acidification phase	Begins approximately four months after the start of Enterobacterial phase. Characterized by *Pediococcus* and *Lactobacillus*. Marks the decline of *Saccharomyces* spp.
Ripening phase	Begins approximately ten months after the start of Enterobacterial phase. Primarily dominated by *Brettanomyces* (Syn. *Dekkera*) spp. and *Pediococcus* spp. Acetic acid bacteria begin to increase in numbers.
Bottle re-fermentation phase	Depending on the style, mature Lambic beer will be packaged with younger beer to initiate a bottle conditioning phase with the intention of carbonating beer in bottle. Microbial diversity may rise due to the introduction of younger beer.

Recent work has revealed the limitations of culture-dependent techniques in understanding the microbiome of Lambic beer. A combination of culture-dependent and culture-independent methods explicate a more complex sequential process in the development of Lambic and Lambic-like beers. The current model for Lambic beer temporal heterogeneity views the process as a gradual gradient of phases, with periodic bursts of activity. The *Enterobacteriaceae* phase is reported to start three to seven days after transfer to coolship has occurred and typically lasts 30 to 40 days. During this time, *Enterobacter cloacae*, *Klebsiella aerogenes*, *Enterobacter aerogenes*, *Citrobacter freundii*, *Escherichia coli*, and *Hafnia pralvei* can be detected. Other known organisms include *Enterobacter hormaechei*, *Enterobacter kobei*, *Klebsiella oxytoca*, *Citrobacter gillenii*, and *Raoultella terrigena*. *Hanseniaspora uvarum*, *Naumovia dairensis*, and *Saccharomyces uvarum* have been reported to be the main yeasts present during the *Enterobactericeae* phase. Variation in genera and species distribution occur depending on the sources cited. *Enteribacteriaceae* begin to decline rapidly,

disappearing almost entirely within 30 to 40 days, attributed to depletion of glucose and the drop in wort pH to below 4.2 (Van Oevelen et al. 1977; Bokulich et al. 2012b; Spitaels et al. 2014; Spitaels et al. 2017). Most of these organisms represent spoilage organisms in unfermented wort and pitching yeast, but may represent important metabolic activity instrumental to the sensory qualities of finished Lambic beer (Bokulich & Bamforth 2013; Spitaels, Wieme, et al. 2015)

Debaryomyces hansenii and *Saccharomyces cerevisiae* can be detected immediately after transfer into wooden casks, but will significantly lag behind Enterobacteriaceae-phase organisms. *Saccharomyces pastorianus* and *Naumovia castelii* are detectable within a week afterwards. Primary, or alcoholic, fermentation will pick up after 30 to 40 days, coinciding with the decline of *Enteribacteriaceae*. This will last three to four weeks, with a steady decline in *Saccharomyces cerevisiae* in favor of *Saccharomyces pastorianus*.

After the primary fermentation phase, *Cryptococcus*, *Candida*, *Pichia*, and *Torulopsis spp*. will form a pellicle at wort-air interface. Around this time, *Pediococcus damnosus* will be detected. Additionally, acetic acid bacteria begin to make more of an appearance, with *Acetobacter lambici* and *Gluconobacter cerevisiae* appearing sporadically throughout fermentation (Spitaels et al. 2014; Spitaels et al. 2017).

Two to three months after transfer, *Pediococcus damnosus* will be consistently present. This will also coincide with depletion of carbon sources usable by *Saccharomyces spp*. and a significant drop in pH (acidification phase). The acidification phase is rapid, occurring somewhere between three to six months, at which point almost all Saccharomyces will be out-competed by *Brettanomyces spp.* (Van Oevelen et al. 1977; Spitaels et al. 2017).

Four to eight months after transfer, both lactic acid bacteria and *Brettanomyces spp*. become increasingly prevalent. It is during this phase that Lambic beer reaches high attenuation.

Brettanomyces produces alpha-glucosidase, allowing for breakdown and use of complex maltodextrins that could not be used by *Saccharomyces spp*. during primary fermentation.

Ten months after transfer, most bacteria populations decrease, marking the start of the *Brettanomyces*-dominated phase of fermentation (Van Oevelen et al. 1977; Verachtert & Iserentant 1995). Modern analysis reveals that two to three-year old Lambic beer is dominated by *Acetobacter lambici, Pseudomonas membranifaciens, Brettanomyces bruxellensis, Brettanomyces anomalus, Candida patagonica,* and *Wikerhamomyces anomalus* (Spitaels, Van Kerrebroeck, et al. 2015).

2.5 Aims and Approach

The primary aim of this study is to evaluate the repeatability of traditional Lambic fermentations outside of Belgium and the impact of oak barrels on the microbial populations of barrel-aged beer. Though it is generally assumed that barrels will introduce variation, the degree to which this occurs is uncertain. Previous use, treatments, and care could potentially affect the microbial population distribution between barrels. To study this, three different commercially available beers using different production methods will be examined. Microbial community profiling will primarily make use of metabarcode analysis to build a profile based upon ITS and 16S sequencing. Culture-dependent analysis will also be performed. Because of the limitations of culture-dependent techniques, and the scope of the sampling involved, culture-dependent analysis will be limited to the set parameters based on preliminary media and incubation conditions. Multiple, in-depth culture-dependent studies have been performed on the isolation and enumeration of dominant microorganisms of Lambic and other spontaneously fermented beer (Martens et al. 1992; Verachtert & Iserentant 1995; Martens et al. 1997; Bokulich et al. 2012b; Spitaels et al. 2014; Spitaels, Van Kerrebroeck, et al. 2015; Spitaels, Wieme, et al. 2015).

The similarity between American Coolship Ales (ACAs) and traditional Lambic beer provide an excellent opportunity to explore the microbiome of traditionally fermented beer across multiple

geographic ranges. Bokulich et al. (2012) have established that resident brewery microbiomes were responsible for the inoculation of ACAs in a similar fashion to that of traditional Lambic style beer, and that ACAs exhibit microbial diversity and subsequent population succession previously thought to be unique to beer production in the Pajottenland region of Belgium, as established by Van Oevelen et al (1977), Verachtert & Iserentant (1995), and Martens et al (1997).

The similarity between traditional Belgian Lambic beers and ACAs suggests that enrichment of certain microorganism particularly suited to a beer environment occur through the movement raw materials (grain, hops, etc.), people, and regular brewery activity (Bokulich et al. 2012b; Bokulich et al. 2015). Other recent studies have made significant contributions to understanding microbial diversity of traditional fermentations through the use of culture-independent techniques (Spitaels et al. 2014; De Roos et al. 2018).

Differences in brewing cultures and age have led to a divergence among traditional European brewers and the American craft beer industry. Many breweries in the United States simply have not had enough time to develop an ideal brewery microbiome to serve as a primary inoculant for a spontaneously fermented beer. By virtue of the fact, American craft breweries are producing many different beers alongside other more “wild” offerings. Rigorous cleaning and sanitation processes are a standard requirement to prevent cross contamination, potentially eliminating an enriched environment suitable for producing Lambic-like beers. Thus, relying on barrel microbiomes is believed to be a suitable alternative, reasoning that the barrel biomes are rich in similar microorganisms thought to be required to produce a satisfactory Lambic-style beer. At present, there is very little microbiological management or control in place for traditionally fermented beer, and breweries within the United States have no standardized methods in place, relying on trial and error for individual circumstance. The trend within American craft beer, and the rising popularity of ACAs, has led to the increased demand for oak barrels of both known and unknown histories.

With the rising use of barrels, it is becoming increasingly important to understand the effect of diverse microbiomes on the quality of a finished product. With much of the quality control relying on subjective sensory experiences rather than quantifiable microbial and metabolic data, understanding the effect of individual barrel microbiomes on the final product is necessary to maintaining a high quality, consistent product.

Two Lambic-inspired beers are examined at different stages of production. The first beer is designated Beer 1, a small-batch beer brewed and matured in Oregon, USA. Beer 1 was sampled the day after brewing and followed over the course of eight months. The second beer, designated Beer 2, was also produced and matured in Oregon, USA. Beer 2 represents a cross section of maturation already in progress when the study was initiated, and follows a staggered aging process, with samples being examined at three time points from a population of near-identical batches brewed and aged approximately 20 months apart. These samples represent a five, three, and two-year old beers prior to blending. Ageing took place in French Pinot Noir barrels from four different vineyards, and white American oak barrels used for Repasado and Anejo Tequila.

The third beer, designated as Beer 3, does not represent a traditional fermentation. Rather this beer has been aged for ten months with the intent of taking on specific organoleptic properties of the barrel's previous contents for later blending. Aging took place in bourbon, cognac, maple syrup, port, rum, sherry and vanilla-extract barrels. Barrel maturation in this case is done primarily as a stylistic choice, with any secondary fermentation considered unintentional. However, extended aging in barrels provides a unique opportunity to compare different beer styles and insight into microbiome variability introduced by a large inventory of barrels.

3 Materials and methods

3.1 Sample collection and processing

Samples were collected from two commercial breweries located in Oregon, USA, predominantly taken from beer held in traditional oak barrels undergoing fermentation and/or maturation. All barrels were of standard cooperage construction, at a capacity of 235 liters and fitted with two access points: a bunghole located on the side of the barrel and a nail port located on the head. The primary bunghole was inaccessible for sampling due to stacking. As such, samples were collected as aseptically as possible from sample ports located in the head of the barrel, approximately 7.5 – 10 cm above the rim of the barrel. 100 ml of beer was allowed to flow from sample port before 100 ml were collected into two sterile 50-ml centrifuge tube. The tubes were designated "A" and "B", where tube B acted as a backup for later HPLC analysis should insufficient volume remain after processing tube A. Samples were immediately stored in a cooler at 4°C and brought back to the lab for processing within 24 hours.

Samples were handled aseptically at all times. The centrifuge tube labeled "A" was vortexed on high for five seconds to ensure sample was fully homogenized. A 10 ml sterile serological pipette was used to aseptically distribute two 10 ml aliquots into two separate 15 ml centrifuge tubes designated "Extraction" and "Cryo". The tubes were then centrifuged (Eppendorf 5810 R) at 3900 xG for 15 minutes. After which the supernatant was carefully removed, leaving behind the resulting pellet.

Samples for backup cryogenic storage were re-suspended in 1 ml sterile yeast-extract peptone dextrose (YPD) broth. The re-suspended material was then transferred to a 2 ml cryogenic storage tube. 1 ml of 30% v/v sterile glycerol solution was then added to each tube and mixed. The tubes were then labeled and stored under -80°C conditions for long-term storage.

3.1.1 Beer

Three beers were sampled from two breweries. Beers were designated as Beer 1, Beer 2, and Beer 3. Beer 1 was brewed and held by Brewery 1, an independent craft brewery located in the Willamette Valley region of Oregon. Beer 1 was an Oud Lambic-style beer, brewed in the traditional Lambic method (Fig. 2.1). The purpose of this beer was to follow microbial succession and compare observed trends to those outlined in literature for both Belgian Lambic beers and American Coolship Ales, and to act as a frame of reference for the other two beers. The beer was placed into two replicate stainless steel fermenters after spending the night in a coolship. Sampling began 24 hours after brewing and coolship inoculation. The beer was then transferred into two designated barrels (barrel ID 114 and 115) five days after brewing to undergo primary alcoholic fermentation and maturation. Sampling took place at regular intervals for eight months, totaling ten sample points for both barrels. As part of the standard brewery protocol, regular top-offs and mixing between previous batches of the same beer occurred during the fermentation process to minimize headspace.

Beer 2 and Beer 3 were brewed by Brewery 2 in Eastern Oregon. Two separate styles of barrel-aged beers were sampled from Brewery 2. Beer 2 was a Lambic-inspired beer using a modified malt bill and mashing process. Three batches of Beer 2 were prepared between May 2013 and November 2016, and placed into 235-liter oak barrels previously used for other aging projects (Appendix A1). The barrels were allowed to undergo spontaneous fermentation. In instances where primary alcoholic fermentation took longer than a week to begin, the barrels were inoculated with a house blend of yeast and bacteria species commonly found in Lambic and American Brett beers. Fifty barrels were randomly selected from a population of 175 barrels across the entire age range (2013 – 2016), resulting in 23 representative barrels from 2013 (Batch 1), 15 representative barrels from 2015 (Batch 2) and 12 representative barrels from 2016 (Batch 3). None of the barrels were topped off during aging.

Beer 3 was a strong Imperial porter and brewed and fermented in three batches during the month of June 2017 with the intention of aging for a single year. The beer had undergone standard primary alcoholic fermentation in stainless steel fermentation vessels prior to being placed into 235-liter oak barrels. As with Beer 2, the oak barrels used for ageing had been previously used for other projects. Fifty barrels were randomly selected from a population of 440 barrels. The brewers were neither expecting nor desiring microbial growth to occur within these barrels. Barrels that had been topped off were noted (Appendix A2).

Sampling was done at three different intervals for Beer 2 and twice for Beer 3. Sampling began on July 13, 2017 and extended through to February 28, 2018. During that time, Beer 2 was sampled at four and two-month intervals from the initial sampling in July 2017, representing a collective sample point from July/August, December, and February. Beer 3 was sampled in July/August 2017 just after initial placement into oak barrels for aging. Beer 2 was sampled again in February 2018, marking the midpoint of its aging cycle.

3.2 Culture-Dependent Analysis:

3.2.1 Media Selection

Microbiological media was selected out of 13 possible media combinations, derived from common laboratory media types outlined by the American Society of Brewing Chemists (ASBC) methods for microbial enumeration and differentiation (ASBC Methods: Microbiological Control 4; Microbiological Control 5; http://methods.asbcnet.org/toc.aspx#microbiology) (Appendix A3). Further refinement of the remaining media was performed using pure cultures of control yeast and bacteria species. Yeast species were assessed using Lee's Multi-differential Agar (LMDA; Weber Scientific), Lysine agar (Sigma-Aldrich), De Man, Rogosa and Sharpe (MRS; Sigma-Aldrich) agar, Universal Beer Agar (UBA; Sigma-Aldrich), Wallerstein (WL; Webber Scientific) agar, and yeast

extract peptone dextrose (YPD; Sigma-Aldrich) agar. Bacteria species were assessed on LMDA, Lysine, MRS, and WL agar.

The media was tested with two types of bottled barrel-aged beer supplied by Deschutes Brewery and a maturing, microbial-active Flanders Red ale (FRA) supplied by Jeff Clawson of Oregon State University. For the bottled beers, volumes of 10 ml per sample were filtered through membrane filtrations was used to concentrate viable organisms onto a MicroCheck® II Beverage Monitor 0.45 um sterile filters (Pall Corporation). The process was repeated with serial dilutions of each beer, this time including the FRA. Three spread plates of each media were prepared using 100 ul of serially diluted beer, with dilution factors of 0, 10, and 100. All media were incubated aerobically at 30°C for five days, and gross colony morphologies were observed and imaged. Colony types were confirmed through the use of wet-mount microscopy to ensure the presence both bacteria and yeast.

Further testing was performed using the Miles, Misra and Irwin method of microbial quantification (Miles et al. 1938). The purpose of this test was to ensure that representative species expected to occur naturally in spontaneously fermented beer (Spitaels et al. 2017) would grow as expected on available media, and to determine expected colony morphology for gross observation. Control yeast and bacteria species were cultured in 5.0 ml liquid media. Yeast was cultured in yeast-extract peptone dextrose (YPD; Sigma-Aldrich) broth, lactic acid bacteria were cultured in De Man, Rogosa and Sharpe (MRS; Sigma-Aldrich) broth, acetic acid bacteria were cultured in yeast-extract mannitol peptone (M0013; Sigma-Aldrich) broth. Control yeast were centrifuged and washed with HyPure™ molecular biology-grade water prior to creation of serial dilutions to remove excess growth medium. Bacteria were not washed. Serial dilutions of 100, 1000, 10000 and 100000 dilution factors were prepared for each control species. A 10 µl aliquot of each dilution was then

placed in designated rows on the test media plate. All media were incubated aerobically at 30°C for five days, and gross colony morphologies were observed and imaged.

Selection of final experimental media was based on the observation of overlapping growth patterns between two media types. As discussed previously, behavior of known species guided the final media selection. All samples were serially diluted twice in sterile DI water to obtain 10-fold and 100-fold dilutions. Direct spread plates were prepare using 100 µl of the undiluted sample and each subsequent dilution. Dilutions were used to ensure at least some quantifiable data could be collected. Each dilution was spread in singlet onto general media yeast-extract peptone dextrose (YPD; Sigma-Aldrich) with 10 mg/L chloramphenicol (Sigma-Aldrich) for yeast enumeration and identification. Lee's Mult-Differential Agar (LMDA; Webber Scientific) with 10 mg/L cycloheximide (Sigma-Aldrich) for bacterial and cycloheximide-resistant yeast enumeration and identification. MicroCheck® II Beverage Monitor 0.45 um sterile filter were used in addition to spread plates in the event that viable and culturable organisms were too diffuse. MicroCheck® II Beverage Monitor membranes were incubated on same media used for spread plating. Isolation media was selected based on preliminary testing performed on a mid-process barrel-aged wild Flanders Rad ale supplied by Oregon State University pilot brewery. Plates were allowed to incubate for 7 days under aerobic conditions at 30°C. Plates were imaged at 5 days and 7 days and the colonies were counted.

3.2.2 Yeast and Bacteria Enumeration

Microbial growth was quantified where colonies were in statistically significant range of ≥25 colonies to ≤ 250 colonies. Separate tallies were determined for each media type and sample time points. Quantification were treated as single replicates per sample and was therefore treated as an estimated measurement of Log_{10} CFU/ml per sample. Average Log_{10} CFU/ml were calculated across all sample time points for each media type. JMP® Pro 13 (v. 13.0.0) was used to perform

one-sided analysis of variance (ANOVA) to determine the difference in average Log_{10} CFU/ml across batch years. Post hoc analysis was performed using Tukey-Kramer Honestly Significant Difference to determine group differences. Paired t-tests were performed to determine differences in average Log_{10} CFU/ml between sample time points for each media type within each batch year ($\alpha = 0.05$).

3.2.3 Isolate Selection

Plates were split into quadrants depending on the amount of growth exhibited post incubation. Six to eight randomized colonies were selected for identification from single quadrants, ensuring that all gross morphology types on each plate were accounted for. The process was repeated for both media types. Colonies were selected based on least crowded dilution sets, from plates with 50 – 250 colonies where possible. For sample sets where < 10 colonies were present, all colonies were selected. Wet-mount light microscopy was used to differentiate between yeast and bacteria colonies. Selected colonies were plated onto UBA agar in a 6 x 6 grid using the patch-plate method and incubated for five days. Colony isolates were then transferred into 250 µl YPD broth in a 1000 µl 96 deep-well plate and incubated for 5 days. 250 µl 30% v/v sterile glycerol was then added to each well and aseptically sealed. The deep-well plates (referred to hereafter as cryoblocks) were placed in -80°C storage until sub-culturing and Sanger sequencing could be performed. Ten cryoblocks, containing 827 individual isolates were prepared (784 yeast, 43 bacteria).

3.2.4 Sanger Sequencing Identification

Colony isolates were identified using Sanger sequencing of the internal transcribed spacer (ITS) region 2 PCR amplicon for yeast and 16S amplicon for bacteria. Two yeast isolates were randomly selected from each cryoblock, and all bacterial isolates were selected for DNA extraction and PCR. Streak plates were prepared directly from each cryoblock by aseptically streaking onto YPD for yeast isolates and LMDA plates for bacterial isolates. Plates were incubated aerobically at 30°C for

five days. A 96-well lysis plate was then prepared from single colony picks of each plate. Small amounts of biomass (estimated 1 – 20 ng) were placed into 200 µl of 5% w/v Chelex® 100 (Bio-Rad) in HyPure™ molecular biology-grade water and heated with agitation at 90°C and 1000 RPM for 20 minutes using a ThermoMixer C (Eppendorf) to lyse cells and chelate potential PCR-inhibitory substances. The lysis plate was centrifuged at 3900 xG for 2 minutes and a 1:10 dilution plate of extracted material was prepared. Table 3 and 4 list reagents and primers used for Sanger sequencing.

Table 3 PCR mastermix recipe for Sanger sequencing preparation

Reagent:	**1 Reaction – 50 ul**	**n-Reaction Master Mix**
EconoTaq® DNA Polymerase (SigmaAldrich):	25.0 ul	(25.0 ul)n
Forward primer:	0.4 ul	(0.4 ul)n
Reverse Primer:	0.4 ul	(0.4 ul)n
HyClone™ HyPure H2O:	19.2 ul	(19.2 ul)n
Template DNA:	5.0 ul	--
Total:	**50.0 ul**	**(30.8 ul)n**

Table 4 Primer sequences for ITS and 16S Sanger sequencing PCR

ITS Primers - 10 mM working stock (Invitrogen, Thermo Fisher Scientific):	
ITS1(F):	TCCGTAGGTGAACCTGCGG
ITS4(R):	TCCTCCGCTTATTGATATGC
16S Primers - 10 mM working stock (Invitrogen, Thermo Fisher Scientific):	
27F:	AGAGTTTGATCMTGGCTCAG
1492R:	TACGGYTACCTTGTTACGACTT

The following thermocycler parameters were used for both ITS and 16S PCR: initial denaturation phase at 95°C for five minutes (x1) and x35 cycles of denaturation at 95°C for one minute, annealing at 55°C for two minutes, and extension at 72°C for two minutes, with a final extension at 72°C for ten minutes.

PCR products were visualized on a 1.8% w/v agarose gel to determine success of individual reactions. The final PCR product was cleaned using EZ-10 Spin Column BS364 Kit (BIO Basic

Inc.) and quantified using SpectraMax® Quant™ AccuClear™ Nano dsDNA Assay Kit (Molecular Devices, Danaher Life Sciences Platform). DNA was normalized to 50 ng DNA for ITS product and 250 ng DNA for 16S product. DNA was submitted to the Oregon State University Center for Genomics Research and Biocomputing for sequencing (https://cgrb.oregonstate.edu/core/sanger-sequencing). Sequencing was performed using BigDye® Terminator v. 3.1 Cycle Sequencing Kit. Equipment used was ABI Prism® 3730 Genetic Analyzer using ABI Prims® Data Collection v 3.0 and DNA Sequencing Analysis Software v. 5.2. Species identification was performed using nucleotide Basic Local Alignment Search Tool (BLAST) through NCBI (https://blast.ncbi.nlm.nih.gov/Blast.cgi)

3.3 Culture-Independent Analysis:

3.3.1 DNA Extraction

Samples were re-suspended in 1 ml sterile, HyPure™ molecular biology-grade water and place into 2 ml PowerFood® DNA extraction collection tube (QIAGEN, DNeasy PowerFood® Microbial Kit 21000-100). The samples were then centrifuged at 15,000 xG for 5 minutes and supernatant was removed as completely as possible with extra care not disturb the pellet. The prepared samples were stored -20°C until extractions were ready to be performed.

DNA extraction was performed on 223 beer samples as per DNeasy PowerFood® Microbial Kit 21000-100 extraction protocol with minor adjustments. Extractions were performed in batches of 24 samples when possible. Extraction efficiency was evaluated using 75 µl ZymoBIOMICS® Microbial Community Standard (Zymo Research; Table 5) control included as a discrete sample with each extraction batch. Omni Bead Ruptor 24 (Omni International, INC.) replaced traditional bead-vortexing, using 15 s pulses at 8.00 M/s for with a 55 s pause between each pulse for 10 cycles, based on previous testing to determine extraction efficiency (Appendix A4). Extracted DNA

was quantified using SpectraMax® Quant™ AccuClear™ Nano dsDNA Assay Kit (Molecular Devices, Danaher Life Sciences Platform) at 468/507 nm.

Table 5 Microbial Community Standard composition

Family:	**Species:**	**Theoretical Composition:**
Gram(+) Bacteria:		12%
Listeriaceae	*Listeria monocytogenes*	12%
Bacillaceae	*Bacillus subtilis*	12%
Lactobacillaceae	*Lactobacillus fermentum*	12%
Enterococcaceae	*Enterococcus faecalis*	12%
Staphylococcaceae	*Staphylococcus aureus*	12%
Gram(-) Bacteria:		
Pseudomonadaceae	*Pseudomonas aeruginosa*	12%
Enterobacteriaceae	*Escherichia coli*	12%
Enterobacteriaceae	*Salmonella enterica*	12%
Yeast:		
Saccharomycetaceae	*Saccharomyces cerevisiae*	2%
Tremellaceae	*Cryptococcus neoformans*	2%

3.3.2 Sample Indexing

Metabarcoding libraries were prepared using the extracted DNA. Separate libraries were prepared for yeast and bacterial community analysis. All PCR libraries were constructed as outlined by Comeau et al. 2017 (https://github.com/LangilleLab/microbiome_helper/wiki) with minor adjustments made as necessary. For yeast community profiling the internal transcription spacer (ITS) region 2 was amplified using BITS-F and B58S3-R universal primers. For bacterial community profiling the V4-V5 domain of the bacterial 16S rRNA gene was amplified using F515 and R926 universal primers. Two PCR were performed for each yeast and bacteria communities to build duplicate libraries with identical barcode designations. The first PCR was performed using 2.5 ul of undiluted template DNA. The second PCR was performed using 2.5 ul of a 1:10 dilution of the template DNA. A total of 968 individual PCRs were performed in six 96-well PCR plates (Eppendorf twin.tec; Fig. 3.1).

INDEX ID →		N701	N702	N703	N704	N705	N706	N707	N710	N711	N712	N714	N715
↓		1	2	3	4	5	6	7	8	9	10	11	12
S502	A												
S503	B												
S505	C												
S506	D												
S507	E												
S508	F												
S510	G												
S511	H												

Figure 3.1 Demonstration of ITS 96-well PCR plate indexing set up. Forward set 1 primers are loaded column-wise and reverse set 1 primers are loaded row-wise. Ninety-six unique index combinations are created. e.g., the PCR amplicons in well A1 will be tagged with the index ID S502/N701, enabling downstream sample demultiplexing and differentiation.

Differentiation between samples and libraries was accomplished through the use of sample indexing. Specialized primers were used in sequential combination to index individual PCRs with a unique identification tag (Fig 3.2). This allows for the use of single-round PCR, which reduces overall PCR bias. Table 6 and 7 list reagents and primers used for HTS library preparation.

S502	S503	S505	S506	S507	S508	S510	S511	←	Forward Set 1		
S513	S515	S516	S517	S518	S520	S521	S522	←	Forward Set 2		
N701	N702	N703	N704	N705	N706	N707	N710	N711	N712	N714	N715
								↖	Reverse Set 1		
N716	N718	N719	N720	N721	N722	N723	N724	N726	N727	N728	N729
								↖	Reverse Set 2		

Figure 3.2 Original primer indexing table. Primers are laid out column-wise (Forward Set 1 and 2) and row-wise (Reverse Set 1 and 2). Sequential combination of primer sets (e.g. Forward Set 1 x Reverse Set 1, etc.) allows for the 384 unique index combinations. Figure reproduced from Comeau et al. 2017.

Table 6 PCR mastermix recipe for HTS sequencing preparation

Reagent:	**1 Reaction – 25 ul**	**n-Reaction Master Mix**
Platinum™ Hot Start PCR Master Mix, 2x (ThermoFisher Scientific):	12.5 ul	(12.5 ul)n
Forward primer:	5.0 ul	(5.0 ul)n
Reverse Primer:	5.0 ul	(5.0 ul)n
HyClone™ HyPure H2O:	9.0 ul	(9.5 ul)n
Template:	2.5 ul	(2.0 ul)n
Total:	**25.0 ul**	**(25.0 ul)n**

Table 7 Primer sequences for ITS and 16S HTS sequencing PCR

ITS Primers - 10 Mm working stock (Nextera Ultramer II, Integrated DNA Technologies):	
BITS (F):	ACCTGCGGAR GGATCA
B58S3 (R):	GAGATCCRTTGYTRAAAGTT

16S Primers - 10 Mm working stock (Nextera Ultramer II, Integrated DNA Technologies):	
515F:	GTGYCAGCMGCCGCGGTAA
926R:	CCGYCAATTYMTTTRAGTTT

3.3.3 HTS PCR Parameters

The following thermocycler parameters were used for both ITS and 16S PCR: initial denaturation phase at 94°C for three minutes and 30 cycles of denaturation at 94°C for 45 seconds, annealing at 55°C for 45 seconds, and extension at 72°C for one minute thirty seconds with a final extension at 72°C for 10 minutes and hold at 4°C.

Each PCR plate was accompanied by a positive control (ZymoBIOMICS® Microbial Community Standard Isolated DNA; Table 5) and negative control containing no template. The PCR products were then combined and visualized on a 1.8% w/v agarose gel to determine the success of individual reactions After which the combined PCR products for each sample were normalized using a 96-well SequalPrep™ Normalization Plate Kit (Applied Biosystems™), following manufacturer's instructions. Each normalized sample was then pooled into a single library, in a 1.5 ml Eppendorf tube. Invitrogen Qubit 4 HS dsDNA assay was used to determine the concentration of DNA in each pooled library. Library concentrations were adjusted as needed using a Savant® DNA 120 Speed Vac (Thermo Fisher Scientific). 3 µl aliquots at a concentration of 50 ng/ul DNA were submitted to the Oregon State University CGRB to determine fragment size distribution using Agilent Bioanalyzer 2100 High Sensitivity DNA assay.

3.3.4 HTS library preparation

Based upon the concentration obtained by the Qubit 4 assay, and the fragment size distribution obtained from the Bioanalyzer analysis, molar concentrations of each library was calculated:

$$\left(\frac{500\ bp}{average\ fragment\ size, Library - n}\right) \times \left(concentration\ \frac{ng}{ul}\right) \times (3.29\ nM)$$

For ITS a single pooled sample was prepared at equimolar concentrations of 10 mM, spiked with 5% PhiX to overcome low sequence diversity. The process was repeated for 16S pooled library

preparation. Final pooled libraries were submitted and run by Oregon State University CGRB on MiSeq chemistry v3 2x300 bp.

3.3.5 Data Analysis and Raw read processing

Raw Illumina FASTQ files were analyzed using QIIME1 (v. 1.9.1; Caporaso et al. 2010) in Microbiome Helper v. 2.0.0 virtual box (Comeau et al. 2017). Bacterial profiling was performed using a 16S QIIME1 pipeline. Operational taxonomic units (OTUs) were picked using pick_otu_indexdb_rna/97_otus reference database and QIIME1 implemented sortmerna_sumaclust method comparison with 80% coverage and 97% pair-wise identity. Low confidence OTUs were then removed. Samples were then rarified with a cutoff of 1000 reads per sample to produce a high-confidence OTU table. The same process was repeated for yeast profiling using sh_refs_qiime_ver7_dynamic_01.12.2017_dev.fasta database reference database with 50% coverage and 97% pair-wise identity.

3.3.5.1 Paired-read stitching

Illumina sequencing generates sequencing data using both the forward and reverse sense of DNA fragments to increase data robustness. Raw Illumina files were received as FASTQ files and processed using Paired-End Read Merger (PEAR v. 0.9.8; Zhang et al. 2014) to stitch the forward and reverse read together into one cohesive file set. The same process was repeated for both ITS and 16S data files.

3.3.5.2 Filtering

FastQC (Andrews 2016) was used to check the quality of the stitched reads prior to filtering with read_filter.pl. A quality score of 30 over 90% of the bases used as the parameters for filtering. A fragment cut-off size (L) was based on the FastQC quality metrics to ensure all relevant detected fragments were included in downstream analysis. Fragments length of L > 100 bp for ITS data and L > 379 bp for 16S data were used.

3.3.5.3 Chimera detection

FASTQ files were converted to FASTA format using fastq_to_fasta.pl, which also removes fully ambiguous reads. Chimeric reads were then detected and removed using the QIIME-wrapped VSEARCH chimera_filter.pl command (Rognes et al. 2016). Chimera search removal for ITS data sets were compared to uchime_reference_dataset_ITS1_28.06.2017.fasta and 16S data sets were compared to Bacteria_RDP_trainset15_092015.fasta.

3.3.5.4 OTU picking

Operational taxonomic unit (OTU) define a taxonomic group based on sequence similarity of all organisms present in a sample (Comeau et al. 2017). OTUs for ITS and 16S microbiome data were picked using open-reference methods. ITS region 2 was sequenced for this experiment. Since this specific region has a large degree of variability, reference alignment is not possible. Therefore, alignment and tree building were suppressed. ITS OTU picking was done sortmerna and sumaclust using pick_open_reference_otus.py against sh_refs_qiime_ver7_dynamic_01.12.2017_dev.fasta database. 16S data was processed the same way, and OTU picking was performed using pick_otu_indexdb_rna/97_otus reference database. Alignment and tree building were not suppressed for 16S OTU picking. Low confidence OTUs were removed using remove_low_confidence_otus.py, based on the accepted criteria of OTUs identified by fewer than 0.1% of total reads (Comeau et al. 2017).

3.3.5.5 Rarefaction

Rarefaction was performed using single_rarefaction.py to subsample all samples to the same read depth. Both ITS and 16S were rarefied to $d_{rarefaction} = 1000$. Meaning, 1000 random reads were taken from every sample that provided >1000 reads. Additional rarefaction was done to find an optimum balance between highest rarefaction value possible while retaining the greatest number of

meaningful samples. Iterations of $d_{rarefaction}$ = 1000, 2000, 5000 and 10000 were performed, with $d_{rarefaction}$ = 1000 offering greatest number of reads while retaining the highest number of samples.

3.3.5.6 Metadata statistical analysis

Beta diversity was assessed using QIIME1-wrapped python script compare_categories.py to determine statistical significance of metadata grouping based upon PERMANOVA analysis of distance matrices ($\alpha = 0.05$). Default permutations (n) of 999 were used.

The processed microbiome data was also analyzed using Statistical Analysis of Metagenomic Profile (STAMP) software (v. 2.1.0; Parks & Beiko 2010), which was used to generate Unweighted Pair Group with Arithmetic Mean clustered heatmaps. Metadata-derived groups (beer_type, barrel_type, sample_time_points, beer_average_age, and batch year) were used to perform one-way analysis of variance (ANOVA) to find statistically significant OTUs.

Other data such as colony count data, was analyzed using JMP® Pro v. 13.0.0.

3.3.5.7 Manual post-processing

Completion of rarefaction generated high-confidence OTU tables, in which contained all OTUs that made it past filtration criteria. High-confidence OTU tables were further refined manually. Read depth ($d_{rarefaction}$) selection of high-confidence OTUs resulted in some taxa being represented at < 0.1% relative abundance in some samples and were pruned. OTUs where relative abundance was > 0.1% but in < 5% of samples was used to as additional criteria to remove potentially spurious taxa from further analysis for this study.

4 Results

This study focused upon samples of beer undergoing spontaneous barrel fermentation and maturation. In the context of spontaneous fermentation community profiling, the microbiology of beer has remained poorly understood. While the last eight years have seen ubiquitous application of culture-independent high-throughput sequencing microbiome approaches being applied to food systems, to date only two studies of beer and its raw materials specifically using HTS techniques have been published (Bokulich et al. 2012b; Justé et al. 2014). Furthermore, these studies drew upon relatively small sample sizes, which may give an incomplete view of the microbiome given the inherent variability likely due to maturation in non-sterile, difficult to clean, oak barrels. In this study, sampling was performed across a large barrel set (n_{total} = 102) in which three distinct beer types were undergoing fermentation and/or maturation. Beer 1 was brewed using the traditional turbid mashing process used for Lambic production and allowed to cool overnight in an indoor coolship prior to being placed into two replicate fermenters for five days, and was then transferred to two replicate barrels. Beer 2 was a Lambic-inspired beer brewed using a brewery-dependent method and standard operating procedures put forth by the brewery of origin and relied primarily on barrel microbiomes to inoculate the wort. Beer 3 was brewed using high-gravity brewing practices to produce a high-ethanol, monoculture beer specifically intended to age in multiple barrel types over the course of a year. These sample sets were gathered from two craft breweries in the state of Oregon. Within the constraints of production requirements, best endeavors were made to minimize uncontrolled variation. The main methodology used in this study was microbiome sequencing, which allowed for simultaneous, massively paralleled analysis of hundreds of samples, vastly improving the efficacy of microbial community profiling. Culture-based methodology was used for some sample sets to provide support for the microbiome data but was not performed routinely due to the large sample size.

4.1 Choice of media for culture-based analysis of beer microbiota

Prior to initiating sampling from large barrel sets for Beer 2 and Beer 3, preliminary work was performed to select appropriate culture media.

YPD spiked with 10 mg/L chloramphenicol supported the greatest degree of growth across all of the control yeast species with little to no inhibition. Lysine was second, displaying moderate inhibition of *S. cerevisiae* while the remaining species were unaffected. It should be noted that Lysine media would ideally completely inhibit *S. cerevisiae* due to the inability of *S. cerevisiae* to metabolize lysine. The presence of residual nitrogen compounds from culturing will allow weak growth to occur. Sequential washing of control yeast prior to plating would likely result a more complete inhibition. Additionally, beer samples will contain nitrogen compounds in sufficient concentrations that could hamper the Lysine media's ability to fully inhibit non-lysine metabolizing yeast (Gorinstein et al. 1999; Fumi et al. 2009). LMDA spiked with 10 mg/L cycloheximide completely inhibited *S. cerevisiae* and *Z. bailii*. LMDA supported the greatest degree of growth across all the known bacteria species, with low but visible growth of *Pediococcus spp.* and *A. pasteurianus* under aerobic conditions. LMDA also supported *Lactobacillus plantarum.* and *Lactobacillus brevis* growth. MRS completely inhibited the growth of both *Pediococcus spp.* and *A. pasteurianus*, while displaying moderate *Lactobacillus plantarum.* and *Lactobacillus brevis* growth. YPD spiked with 10 mg/L chloramphenicol completely inhibited control bacterial species with the exception of *A. pasteurianus* (data not shown). Based on observed growth trends a final selection of two media types was made. YPD spiked with 10 mg/L chloramphenicol was selected for enumeration of total yeast. *A. pasteurianus* was capable of growing in the presence of chloramphenicol and exhibited a distinct morphology that was readily distinguishable from yeast colonies. LMDA spiked with 10 mg/L cycloheximide was selected for enumeration of bacteria and cycloheximide-resistant yeast. Lysine media was used for some isolation purposes at sample time point 1 and are noted in section 4.5.3.

4.2 Description of datasets

Data from Beer 1 displays the progression of yeast and bacterial communities over the course of 36 weeks through the lens of microbiome analysis. Yeast and bacterial OTU changes over time were observed and noted. It should be noted specifically that the OTU reference database documented in section 3.3.5.4 classifies *Brettanomyces* species under the synonym genus *Dekkera* across all Beers and will be referred to as such throughout the remaining sections. Results are shown in section 4.4.

Data from Beer 2 was used to explore the effect of extended barrel aging on the microbiome of the same beer brewed in separate batches over the span of 2 to 5 years. Beer 2 is split into three batch years: 2013, 2015, and 2016. While the beer recipe and brewing procedures were consistent across the three batches, there was some variation in the amount and type of hops used in the 2016 batch year, with higher alpha-acid content going into the 2016 batch compared to 2013 and 2015. Statistical analyses were performed to compare microbiome composition according to batch years and by sample time points. Culture-dependent techniques were used to quantify the yeast and bacterial populations and obtain pure isolates from selected sample points to enable verification of microbiome data. Results are shown in section 4.5.

Data from Beer 3 was used to explore the effect of multiple barrel types (origins) on the microbiome of one batch of an imperial porter-style beer. Statistical analyses were performed to compare microbiome composition according to barrel type and sample time points. Culture-dependent techniques were used for the purpose of observing the frequency of viable microbe detection on a per-barrel basis. Results are shown in section 4.6.

4.3 Summary of microbiome sequencing data

A total of 506 samples were sequenced and comprised of 253 ITS sequencing samples and 253 16S sequencing samples. Sequencing was performed in two batches. The first batch, comprised of 11

replicate Beer 1 samples obtained in the months of August through December, was sequenced in December 2017. The remaining 484 samples were processed and sequenced in May of 2018. Table 8 outlines raw sequence read data spread across all beer samples. Variation in median sequence reads generated per sample for each dataset reflects uneven pooling of samples and libraries, and the likelihood that some sample sets were generated from beer that contains low numbers of microbes.

Table 8 Summary of stitched sequence reads generated for samples from each beer

	Beer 1 (ITS)	**Beer 1 (16S)**	**Beer 2 (ITS)**	**Beer 2 (16S)**	**Beer 3 (ITS)**	**Beer 3 (16S)**
Min:	86.0	0.0	178.0	53.0	1401.0	1472.0
Max:	293663.0	248398.0	294134.0	441526.0	292121.0	327700.0
Median:	5637.0	1499.5	997.0	629.0	27852.0	86966.0
Mean:	33320.160	33377.83	22414.320	6468.092	73914.714	106622.667
St. dev.:	54769.0	58878.601	63143.073	47286.304	93000.953	95935.301

4.3.1 Microbiome sequencing data QC

In order to directly compare the microbiome of each sample, it is necessary to simulate equivalent ‘sequencing effort’ per sample. This is achieved by randomly rarefying each dataset to an equivalent number of sequence reads. Rarefaction of each set of sequencing data was performed at four depths (d) independently for each dataset. By comparing the number OTUs detected at each level of rarefaction (d = 1000, 2000, 5000, 10000), a read depth of 1000 was selected as a compromise to maintain the greatest number of samples while still providing meaningful OTU distribution. Figure 4.1 provides an example of the lack of impact of rarefaction on relative OTU abundance, in this case for Beer 3. Relative proportions of detected OTUs remain consistent.

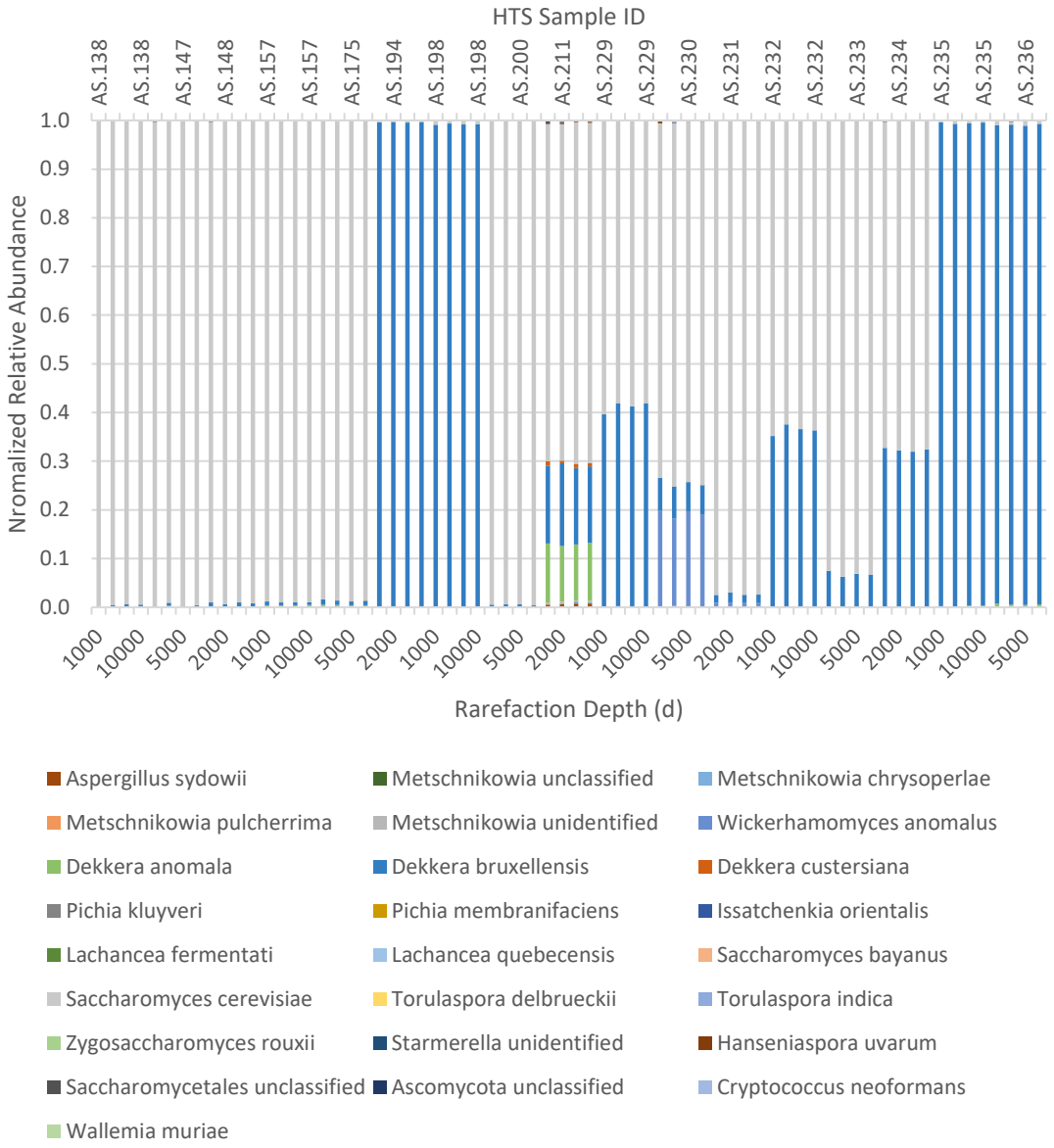

Figure 4.1 Stacked bar chart displaying relative proportions of selected fungal OTUs detected in beer three at four rarefaction levels. Bottom X-axis represents rarefaction depth (d). The top X-axis represents samples with sufficient sequencing reads generated to remain across all four rarefaction depths. Y-axis represents relative abundance. Bars are stacked in ascending order from d = 1000 to 10000 and sorted by HTS sample ID.

A higher d-value attributed to rarefaction resulted in a loss of samples retained in the dataset, and an overall loss in detected OTUs. For this example, 47 samples are represented at d = 1000 and 17 at d = 10000. At d = 10000, 35% of detected OTUs were lost due to removal of samples from the dataset. Where d = 1000, the relative abundance of those OTUs made up 0.0 to 30.4% of total reads within their respective samples (Table 9). OTUs absent where d = 10000 made up 4.5% ± 7.2% relative abundance in each sample at d = 1000.

Table 9 OTUs detected at d = 1000 not present in dataset at d =10000

OTU	**Max Value**
Alternaria unclassified	4.0%
*Sclerotinia sclerotiorum**	0.1%
Lachancea fermentati	9.2%
Torulaspora indica	1.0%
Zygosaccharomyces rouxii	1.4%
*Armillaria mellea**	0.5%
Agaricales unidentified*	3.7%
Atheliaceae unidentified*	1.8%
*Trametes cubensis**	1.8%
*Ceriporia lacerate**	1.9%
Cabalodontia unidentified*	2.5%
*Stereum hirsutum**	2.0%
Malassezia restricta	0.8%
Sporobolomyces roseus	5.0%
*Filobasidium wieringae**	2.2%
Vishniacozyma victoriae	0.3%
Cryptococcus neoformans	30.3%
Wallemia muriae	5.0%

(*) indicates multicellular fungi not normally associated with beer production

As discussed in section 2.3.2.2, DNA extraction efficiency and biases are an area of concern for microbiome analyses. For that reason, a quantitative and qualitative community standard was used throughout sample processing. Each sample extraction batch was accompanied by a community standard that was extracted and sequenced alongside the beer microbiome samples. The use of DNA extraction community standards serves to assess extraction efficiency of specific organisms

based upon physiology and to assess potential biases in absolute quantitation of detected OTUs. Additionally, a manufacturer pre-extracted DNA community standard was included in each PCR batch. Both the extraction and PCR controls were prepared from a cocktail of microbes at the proportions outlined in Table 5. Figures 4.2 and 4.3 display the relative distribution of community standards sequenced with beer microbiome samples.

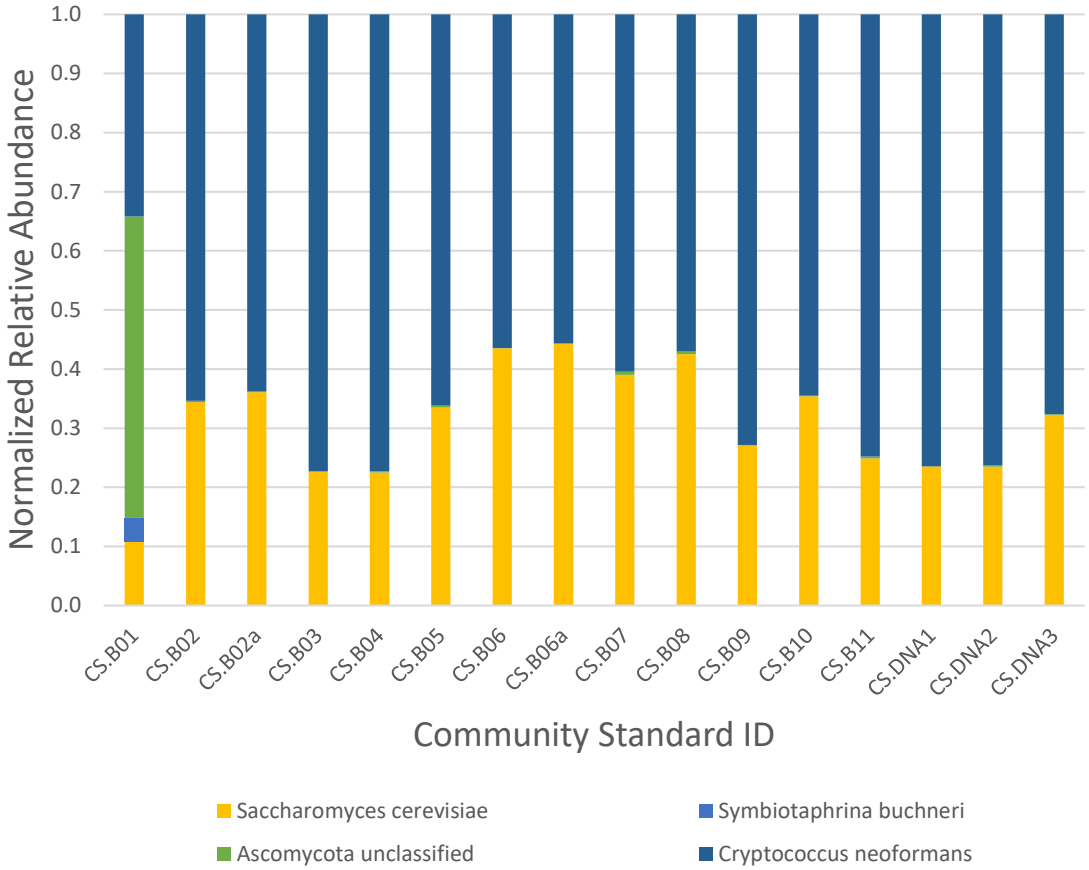

Figure 4.2 Stacked bar chart displaying relative proportions of yeast OTUs detected in each extraction and PCR community standards at d = 1000. Bottom X-axis represents community standard ID and representative extraction batch. Y-axis represents percent relative abundance. Community standard CS.B02a and CS.B06a represent extractions performed on a 37.5 µl aliquot

community standard in addition to the standard 75 µl outlined in §3.3.1. CS.DNA 1, CS.DNA2, and CS.DNA3 represent microbial DNA community standard for each PCR batch.

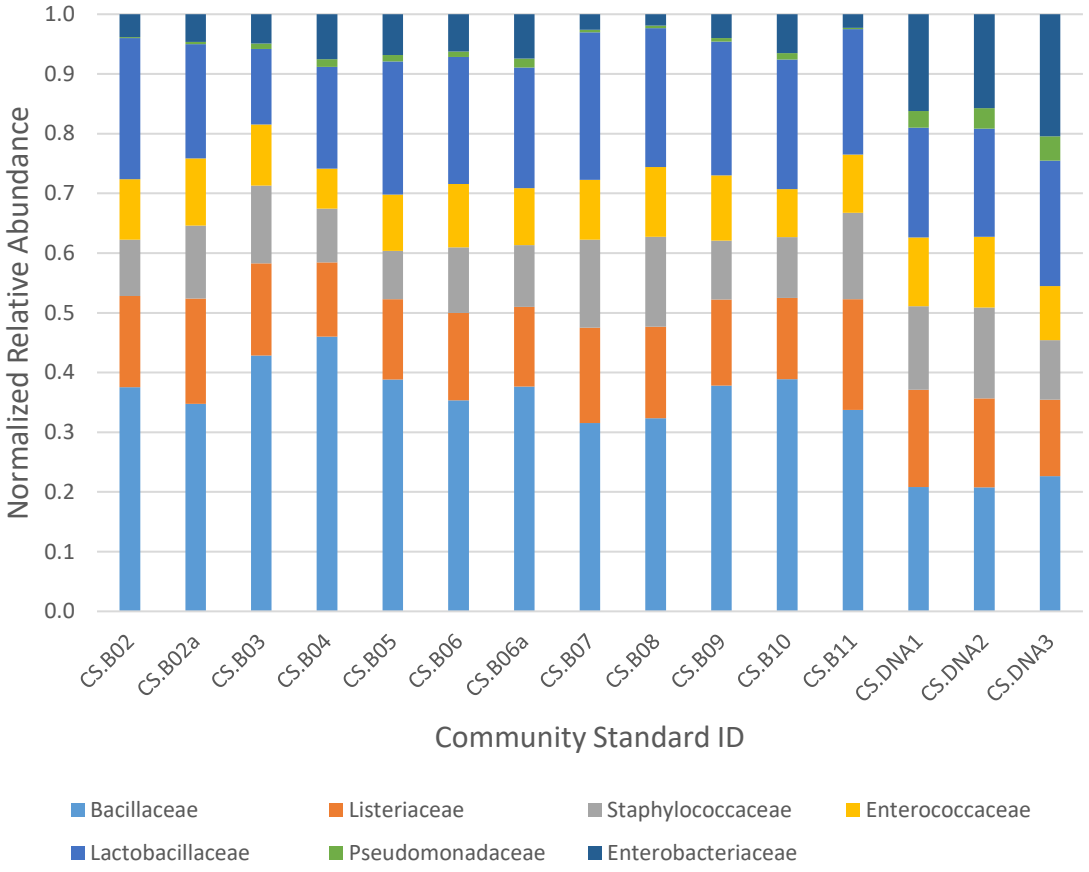

Figure 4.3 Stacked bar chart displaying relative proportions of bacterial OTUs detected in each extraction and PCR community standards at d = 1000. Bottom X-axis represents community standard ID and representative extraction batch. Y-axis represents percent relative abundance. Community standard CS.B02a and CS.B06a represent extractions performed on a 37.5 µl aliquot community standard in addition to the standard 75 µl outlined in §3.3.1. CS.DNA 1, CS.DNA2, and CS.DNA3 represent community standard DNA for each PCR batch. CS.B01 did not yield 16S data.

Comparing the differences of the average relative abundance for each taxa across all extraction standards to the average relative abundance of each microbial DNA standard displayed no statistically significant difference between extracted *S. cerevisiae* ($p = 0.3671$) and *C. neoformans* ($p = 0.1736$) DNA and the microbial DNA standards. Bacillaceae, Enterobacteriaceae, Pseudomonadaceae ($p < 0.0001$ respectively) displayed statistically significant differences between relative abundance of extracted DNA standards when compared to microbial DNA standards. Listeriaceae ($p = 0.7976$), Staphylococcaceae (0.3183), Enterococcaceae (0.3135), Lactobacillaceae (0.4482) displayed no statistically significant difference between standards. Extraction batch control CS.B01 did not display the expected profile of yeast genera and did not yield 16S data. Close inspection of data for samples extracted in the same batch and comparison alongside equivalent samples (same beer, same time-point) did not reveal notable aberrations, therefore we did not exclude these data from subsequent analyses.

ITS PCR no-template negative (NT) control sequence data of replicate PCR plate 1 through 3 fell well below the rarefaction value of $d = 1000$. 16S PCR NT control sequence data of replicate PCR plate 1 and 2 also fell below rarefaction value of $d = 1000$. The exception was NT control of PCR plate 2 (NT.002), displayed 1220 sequence reads, representing 12 bacterial OTUs (Fig. 4.4).

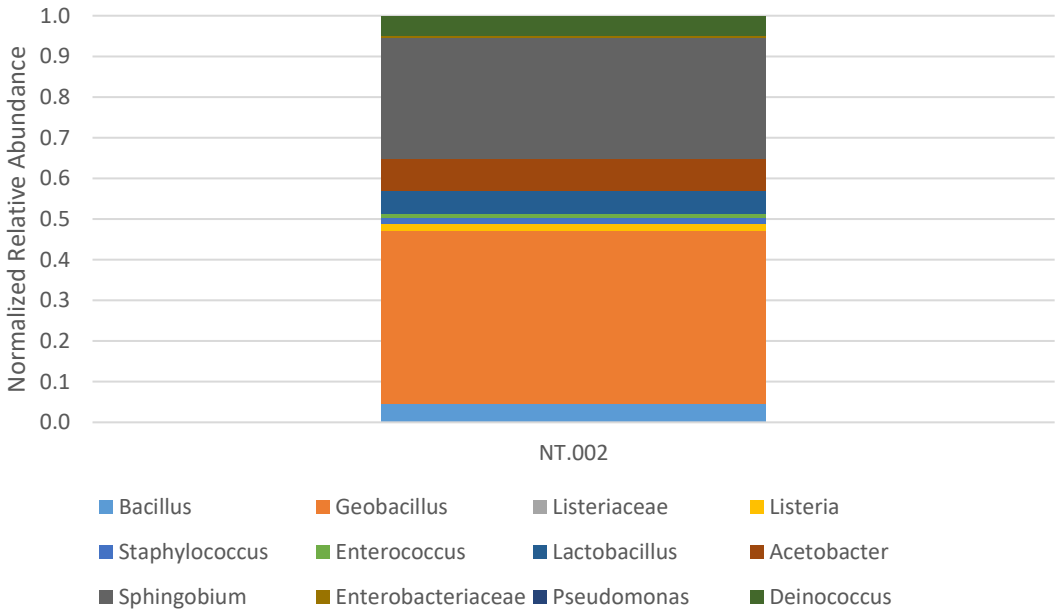

Figure 4.4 Stacked bar chart displaying relative proportions of bacterial OTUs detected in each the no template negative control of combined replicate PCR plate 2 at d = 1000. X-axis represents no template negative control ID. Y-axis represents percent relative abundance.

4.4 Beer 1

4.4.1 Beer 1 fungal community composition as revealed by ITS microbiome data

A total 21 fungal operational taxonomic unites (OTUs) were detected across the Beer 1 sample set, of which 11 were retained for community analyses based upon relative abundance cutoff criteria. Relative abundance of these OTUs is shown in Figure 4..5, where it can be seen that initial samples taken 24 hours after brewing displayed a significant presence of *S. cerevisiae* (58.2% abundance sample A, 72.7% abundance sample B) and *D. bruxellensis* (41.7% abundance sample A, 6.4% abundance sample B). *Wickerhamomyces anomalus* was also detected in one of the initial samples (19.7% abundance fermentor sample B). Other species detected were *Hanseniaspora uvarum*,

Metschikowia pulcherrima, *Cryptococcus neoformans* (not shown), and the mold *Cladosporium ramotenellum*.

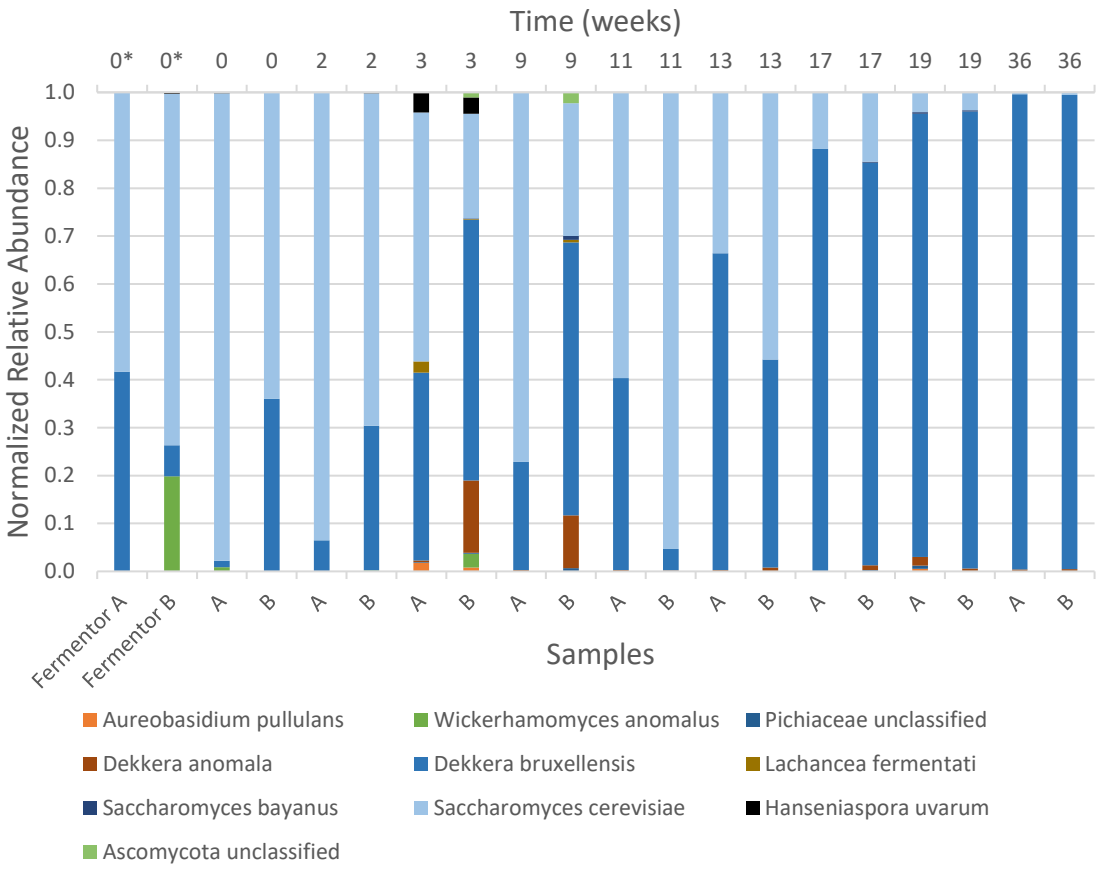

Figure 4.5 Stacked bar of fungal OTUs at greater than 0.1% abundance in Beer 1 measured using HTS sequence data. Species and family-level identification, and normalized relative abundance from 0 to 100%. Y-axis indicates relative OTU abundance, bottom X-axis represents replicate sample from Fermentor A and B, and then from barrel A and barrel B. Top axis represents sample time point in weeks relative to brew day. (*) on top X-axis indicates samples taken 24 hours post brewing.

The relative abundance of detected fungal OTUs changed over time, with *S. cerevisiae* and *D. bruxellensis* making up the bulk of the sequenced population throughout fermentation. The number of detectable fungal OTUs peaked by week 3, with 15 OTUs present above the cut off criteria. These OTUs were assigned to yeast species including *H. uvarum, S. cerevisiae, S. bayanus, Lachancea fermentati, D. bruxellensis, D. anomala, W. anomalus, Aureobasidium pullulans*, along with various unclassified *Ascomycota*, and *C. ramorenellum*.

The number of detectable fungal OTUs decreased by week 11, with *S. cerevisiae, D. bruxellensis, D. anomala, W. anomalus, and H. uvarum* representing the most abundant yeast species. *S. cerevisiae* had significantly declined by week 17, with *D. bruxellensis* becoming the dominant fermentative species. *D. anomala, A. pullulans, W. anomalus* and unclassified *Pichiaceae* were also detectable at this time but in relatively low abundance (0.1 – 1.0%). By week 36, *D. bruxellensis* (98.6-99.2%) had become completely dominant, with *D. anomala* (0.4-0.5%) and *S. cerevisae* (0.4-0.5%) present at relatively low abundance.

A degree of variation between replicate samples was observed. Slight variations in which fungal OTUs were detected accounted for some of this variation, with *D. anomola* detected in greater relative abundance in barrel B at both 3 (13.2% relative abundance) and 9 weeks (8.3% relative abundance), before ultimately leveling off at 36 weeks (0.4-0.5% relative abundance). Relative abundance of *S. cerevisiae* and *D. bruxellensis* appeared to be in constant flux for the first half of fermentation, only stabilizing between 13 and 17 weeks. *Lachancea fermentati* made a brief appearance in barrel A at 3 weeks (2.2% relative abundance), coinciding with peak number of detectable OTUs for this beer. By this time a total of 12 unique OTUs were identified in barrel A and 15 unique OTUs were identified in barrel B.

Fungal taxa such as *Cladosporium ramotenellum, Penicillium roqueforti*, and *Malassezia restricta*, which are not normally associated with beer fermentation were detected throughout. Data for these

OTUs are not displayed in Figure 4.5. A complete list of all detectable fungal OTUs and their relative abundances are provided in Appendix A5.

4.4.2 Beer 1 bacteria community composition as revealed by 16S microbiome data

Compared to ITS microbiome data, fewer OTUs were detected in 16S microbiome data. Eighteen bacterial OTUs were identified after data processing and rarefaction. Of these , seven were retained based upon previously established cutoff criteria, with their relative abundance shown in Figure 4.6.

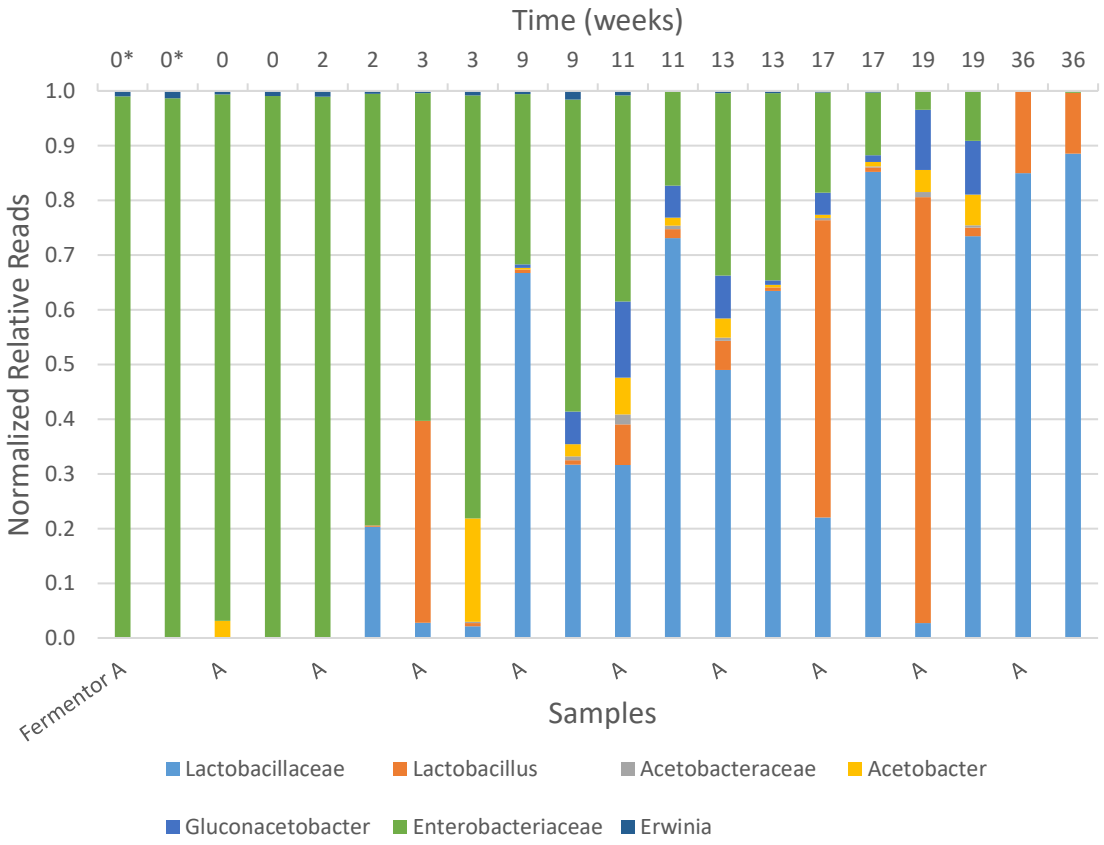

Figure 4.6 Stacked bar of bacterial OTUs at greater than 0.1% abundance in Beer 1 measured using HTS sequence data. Taxon identification and normalized relative abundance from 0 to 100%. Y-axis indicates relative OTU abundance, bottom X-axis represents replicate sample from

Fermentor A and B, and then from barrel A and barrel B. Top axis represents sample time point in weeks relative to brew day. (*) on top X-axis indicates samples taken 24 hours post brewing.

The first two sample time points displayed complete dominance by bacterial family *Enterobacteriaceae* (98.6-98.1%). Genus *Erwinia* (1.0-1.4%) belongs to family *Enterobacteriaceae* but was maintained as a separate OTU in the dataset. The total number of detectable bacterial OTUs increased from two to seven by week 9, with *Acetobacter*, *Gluconoacetobacter*, *Lactobacillus*, *Acetobacteraceae*, and *Lactobacillaceae* present at varying relative abundance. By week 11, *Lactobacillaceae* had begun to dominate the bacterial community, marking the decline of *Enterobacteriaceae*. *Lactobacillaceae*, along with *Lactobacillus* (maintained as separate OUT), continued to dominate the bacterial community, reaching > 98% relative abundance by 36 weeks. *Acetobacter*, *Gluconacetobacter*, and *Acetobacteraceae* were detected from week 3 through week 19, prior to *Lactobacillaceae* dominating the bacterial community. As seen with the detected fungal OTUs, there was a degree of variation between sample replicates. Barrel A exhibits a higher relative abundance of *Lactobacillus* than barrel B that carried through most of the fermentation. Barrel A also tended to exhibit a higher relative abundance of *Gluconacetobacter* than barrel B.

Bacterial taxa such as *Geobacillus*, *Sphingobium*, *Staphylococcus*, and *Listeriaceae*, which are not normally associated with beer fermentation, were detected throughout the sampling period even after rigorous OTU. A complete list of all detectable bacterial OTUs and their relative abundances are provided in Appendix A6.

4.5 Beer 2

4.5.1 Beer 2 fungal community composition as revealed by ITS HTS data:

Through regular brewery operations, eight barrels were removed from the sample pool over the course of sampling. By the end of the sampling period, a total of 135 individual samples for Beer 2 were collected and sequenced, represented by 48 from sample time point 1, 45 from sample time point 2, and 42 from sample time point 3. Of those, 106 remained in the dataset after rarefaction (d = 1000). Amongst the rarefied samples, 86 fungal OTUs were identified of which 19 were retained according to established relative abundance cutoff criteria. The proportion of samples for each fill year and sample time point that yielded microbiome data is listed below in Table 10.

Table 10 Proportion of barrels from each batch year that yielded ITS microbiome data at each time point

	Sample Time Point 1			**Sample Time Point 2**			**Sample Time Point 3**		
Batch Year:	2013	2015	2016	2013	2015	2016	2013	2015	2016
Proportion:	86%	87%	92%	71%	80%	92%	33%	80%	58%

Metadata analysis of Beer 2 ITS microbiome data revealed a statistically significant difference by batch year (p = 0.035). However, clustering of samples according to overall ITS profile similarity (Fig 4.7) suggests a the lack of association between these clusters and batch year.

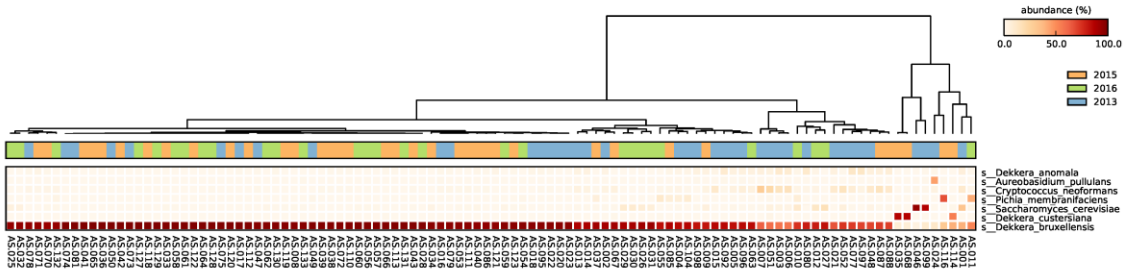

Figure 4.7 Truncated heatmap of fungal OTUs at greater than 0.1% abundance in Beer 2 clustered according to overall microbiome similarity. Unweighted Pair Group Method with Arithmetic Mean (UPGMA) clustering of samples based upon microbiome profile derived from relative abundance of OTUs represented on a scale of 0 to 100%. Batch year is indicated by color above that sample profile, with orange representing batch year 2016, green batch year 2015, and blue batch year 2013. HTS sample ID are indicated along the bottom of the heatmap. OTU assignments are indicated on the right hand side of the heatmap, where suffix characters "g" indicates genus, "f" indicates family, and "o" indicates order. See Appendix A7 for full heatmap with all detected taxa.

Analysis of abundance for each OTU individually (as opposed to overall sample microbiome similarity) revealed a statistically significant difference between sample time points within 2016 batch, but not the 2013 or 2015 batches. Figure 4.8 shows changes in relative abundance of *S. cerevisiae* ($p = 5.66e-6$) and *D. bruxellensis* (0.011) between sample time points for the 2016 batch. The average relative abundance of *S. cerevisiae* across all samples dropped from 8% to approximately 1.5% between 35 and 52 weeks. *D. bruxellensis* displayed the opposite trend, with a shift to complete dominance across samples over the same period. At 35 weeks, there was substantial variation between samples from individual barrels in their relative proportion of these two species, indicated by wider distribution of boxplot quartiles, whereas for the later time point samples, tighter boxplot quartiles indicated less variation among barrels within the 2016 batch year.

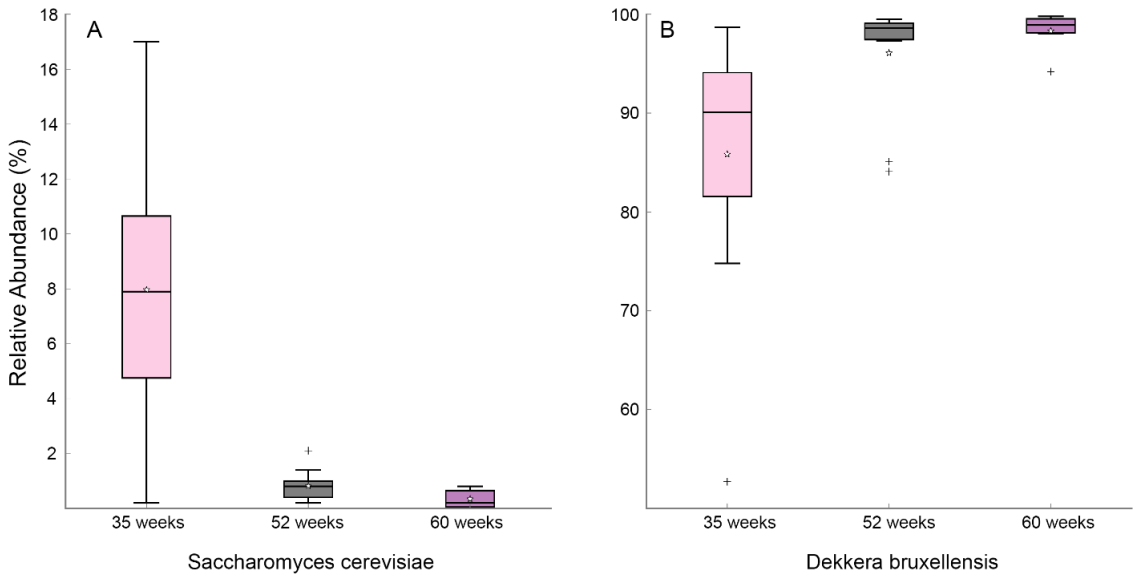

Figure 4.8 Box and whisker plot of relative abundance of *S. cerevisiae* (A) and *D. bruxellensis* (B) between sample time points of Beer 2 2016 batch year. Change in relative proportions of *S. cerevisiae* (p = 5.66e-6) and *D. bruxellensis* (0.011) between sample time points of the 2016 batch

Fungal taxa such as *Penicillium corylophilum*, *Phlebia radiata*, *Filobasidium chernovii*, and *Mucor circinelloides*, which are not normally associated with beer fermentation, were detected even after rigorous fungal OTU rarefication. A complete list of all detected OTUs present are provided in Appendix A8.

4.5.2 Culture-dependent yeast enumeration of Beer 2 samples

Colonies on LMDA and YPD displaying yeast-like morphology were enumerated (Fig. 4.9). Average counts across all sampled barrels differed significantly between batch years 2013 and 2016 on LMDA at sample time point 1 (p = 0.0137), and at sample time point 3 (p = 0.0267), and on

YPD at sample time point 1 ($p = 0.0023$). No statistically significant difference was found for YPD at sample time point 2 ($p = 0.4230$).

Because the LMDA medium contained cylohexamide to inhibit *Saccharomyces* yeasts, counts on the two media were also compared at each time point for each batch year to determine the relative abundance of *Saccharomyces* and non-*Saccharomyces* yeasts. In each case there were no significant differences ($p > 0.05$), suggesting that the culturable yeast population was predominantly composed of cycloheximide tolerant species.

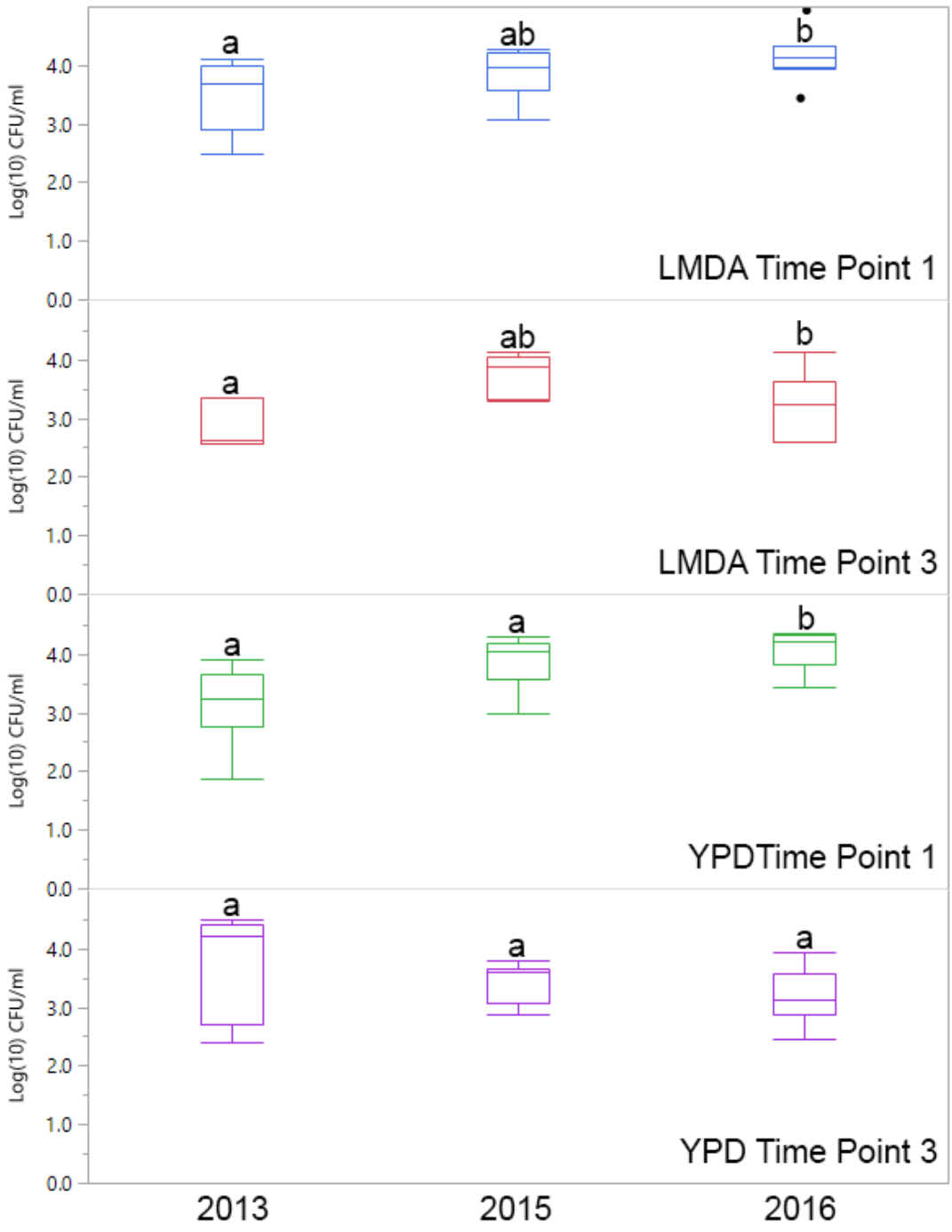

Figure 4.9 Box and whisker plots of yeast population size as evaluated on each media type in Log_{10} CFU/ml across three batch years. Significant differences of means within each panel are indicated by letters above box plot. Two outliers (●) are accounted for in LMDA Time Point 1 for the 2016 batch year.

It should be noted that the lower limit of quantitation by spread-plating was 250 CFU/ml, and samples below this threshold were excluded from statistical analysis. A clear trend was observed that more barrel samples from the older beer batches were in this category (Table 11), supporting the observation that yeast population size was higher in the youngest beer batch.

Table 11 Summary of relative proportion of agar plates below lower quantitation limit (LQL) within each batch year.

	LMDA + Cycloheximide		**YPD + Chloramphenicol**	
Fill Year	**Sample Time Point 1**	**Sample Time Point 3**	**Sample Time Point 1**	**Sample Time Point 3**
2013	52%	62%	62%	76%
2015	20%	47%	33%	40%
2016	25%	50%	33%	33%

4.5.3 Sanger sequencing identification of random Beer 2 yeast isolates

A total of 784 representative yeast colonies were randomly picked from available plates. From this representative set a random number generator was used to select 44 isolates for molecular identification. A laboratory strain of *S. cervisiae* (S288c) was used as a control for PCR and sequencing quality control purposes. The randomly-selected isolates consisted of 12 from the 2013 batch year, 16 from 2015 batch, and 15 from the 2016 batch. Isolates from samples 170810-07, 170810-17, 170810-18, 170810-23, 170810-28, 170810-29 were obtained through plating on Lysine media. Lysine media was not used for any other sample time point or for quantification purposes. Forty-two of the sequenced isolates were identified as *D. bruxellensis*. Isolate No. 31 returned as a 92% match with *Pichia membranifaciens*. Isolate No. 44 was unable to be identified. Complete Sanger identification are listed in Appendix A9.

4.5.4 Beer 2 bacterial community composition as revealed by 16S microbiome data:

16S microbiome data was processed according to a similar rarefaction process to that applied for ITS microbiome data, where d = 1000. Of the original 135 collected samples, 70 remained after processing and rarefaction. From these remaining samples, 75 bacterial OTUs were identified. Of those OTUs, 39 were retained according to established relative abundance cutoff criteria. The proportion of samples for each fill year and sample time point that yielded microbiome data is listed below in Table 12.

Table 12 Proportion of barrels from each batch year that yielded 16S microbiome data at each time point

	Sample Time Point 1			**Sample Time Point 2**			**Sample Time Point 3**		
Batch Year:	2013	2015	2016	2013	2015	2016	2013	2015	2016
Proportion:	0%	67%	92%	33%	27%	75%	5%	60%	75%

Metadata analysis of the Beer 2 16S microbiome data revealed a statistically significant difference according to batch year ($p = 0.001$) and by sample time point within each batch year ($p = 0.001$). Figure 4.10 shows clustering of the microbiome profiles according to overall similarity, which revealed four distinct clusters. Cluster A is comprised of samples from the 2015 (orange) and 2016 (green) batch years. Cluster B is comprised of samples solely from the 2016 batch year. Cluster C is made up of samples from the 2016 and 2013 (blue) batches. Cluster D is comprised of samples from all three batch years, though most were from 2013.

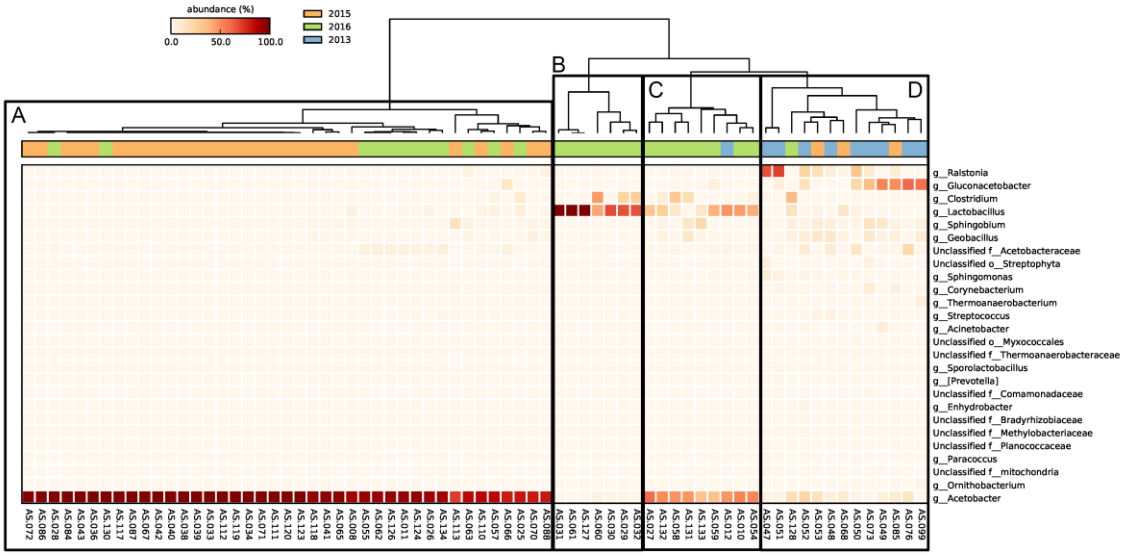

Figure 4.10 Truncated Heatmap of bacterial OTUs in Beer 2 clustered according to overall microbiome similarity. UPGMA clustering of samples based upon microbiome profile derived from relative abundance of 26 bacterial OTUs represented on a scale of 0 to 100%. Batch year is indicated by color code above that sample profile, with orange representing batch year 2016, green batch year 2015, and blue batch year 2013. OTU assignments are indicated on the right hand side of the heatmap, where suffix characters “g” indicates genus, “f” indicates family, and “o” indicates order.

The observed clusters were differentiated primarily by relative abundance of OTUs corresponding to acetic and lactic acid bacteria, which make up the bulk of the overall bacterial population across all samples. Figure 4.11 shows the relative abundance of the key acetic acid and lactic acid bacteria OTUs that differ significantly according to batch year.

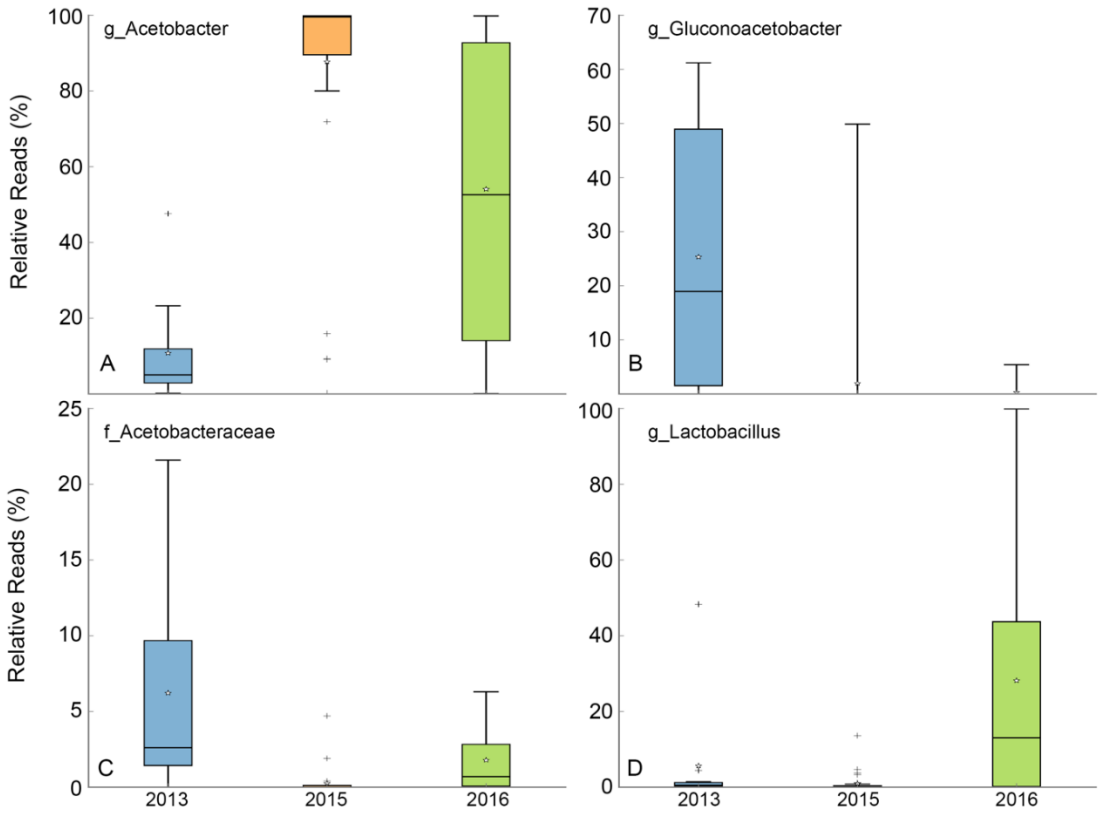

Figure 4.11 Box and whisker plot displaying the average distribution of *Acetobacter* (A), *Gluconoacetobacter* (B), *Acetobacteraceae* (C), and *Lactobacillus* (D) that drive differences in clustering in each batch year of Beer 2. Differences in clustering are driven by these four primary taxa, within the 2016 batch year displaying the greatest relative abundance of *Acetobacter* and *Lactobacillus*.

Acetobacter (p = 3.64e-9) was detected across all three batch years, with relative abundance ranging between 0 and 100% in the 2016 batch year, 80 to 100% relative abundance in the 2015 batch year, 0 to approximately 23% abundance in the 2013 batch year.

Gluconoacetobacter (p = 1.09e-7) was also detected across all three batch years, with relative abundance ranging between 0.5 to 5.4% in the 2016 batch year, 0 to 58% relative abundance in the 2015 batch year, and 0 to 61% relative abundance in 2013 batch year.

Unclassified *Acetobacteraceae* (p = 3.07e-6) appeared in all three batch years, with relative abundance ranging between 0 to 5.4% in the 2016 batch year, 0 to 0.3% in the 2015 batch year, and 0 to 21.6% in the 2016 batch year. Given the low proportion of samples from the 2013 batch year (n = 9), unclassified *Acetobacteraceae* represents the lowest relative abundance of these four taxa, and it was almost entirely absent from the 2015 batch year.

Lactobacillus (p = 4.16 e-5) appeared in all three batch years, ranging from 0 to 100% in the 2016 batch year, 0 to 13.6% relative abundance in the 2015 batch year and 0 to 4.7% relative abundance in the 2013 batch year.

Bacterial taxa such as *Agrococcus*, *Sphingobium*, *Sphingomonas*, and *Ralstonia*, which are not normally associated with beer fermentation were detected even after rigorous fungal OTU rarefication. A complete list of all detectable OTUs present are provided in Appendix A10.

4.5.5 Culture-dependent bacterial enumeration of Beer 2 samples

Few Beer 2 samples when plated (~3%) exhibited bacterial colony morphology, and of those only a small number were able to be enumerated, the results of which are summarized in Table 13. Enumeration was problematic due to the dominance of yeast colonies on most plates, combined with relative size of colonies vs. the particulate background of LMDA media. There were insufficient enumerated samples within valid counting ranges to allow for statistical comparison of bacterial population size according to batch year, time point, or media type. It is worth noting that only samples from the 2015 and 2016 batch years contained culturable bacteria above the limit of detection (10 CFU/ml).

Table 13 Average Time Point (ATP) colony counts of samples yielding both detectable and quantifiable bacterial growth

		LMDA + Cycloheximide		YPD + Chloramphenicol	
Barrel ID	**Fill Year**	**ATP 1 (Log10 cfu/ml)**	**ATP 2 (Log10 cfu/ml)**	**ATP 1 (Log10 cfu/ml)**	**ATP 2 (Log10 cfu/ml)**
142820	2015	--*	--*	--*	4.0
142826	2015	--*	--*	--*	--*
155125	2016	4.9	>5.4	3.8	--*
155134	2016	>5.4	--*	>5.4	>5.4
155185	2016	4.3	--*	>5.4	--*
142816	2015	3.3	--*	--*	3.5
155124	2016	--*	4.1	--*	4.2
155130	2016	--*	--*	--*	4.5
155179	2016	--*	3.5	--*	3.4

(*) bacterial colony morphology evident
Upper limit of quantitation (LOQ) 5.4 log10 cfu/ml

4.5.6 Sanger Sequencing of isolates

A total of 43 bacterial isolates were collected and stored, however only 33 were able to be re-cultured from cryopreserved stocks at the time of analysis. *Acetobacter aceti* DNA (BCCM LMG 00005) was used as a PCR reference and for sequencing quality control. Of the 33 isolates, 24 were identified as belonging to the *Acetobacter* genus, with *A. fabarum*, *A. malorum*, *A. pomorum* and *A. pasterurianus* being the four represented species. Four colonies were identified as *Staphylococcus* spp. and three were *Bacillus* spp. The reference species was positively identified as *A. aceti* BCCM LMG00005 (Appendix A11).

4.6 Beer 3

4.6.1 Beer 3 fungal community structure as revealed by ITS HTS data:

Random selection of Beer 3 barrels provided a cross-section analysis of the entire barrel inventory. Table 14 shows the relative proportion of barrel types within Beer 3 sample sets. Beer 3 was collected at two sample points spaced 27 weeks apart. As seen with Beer 2, regular brewery operations removed three barrels from sample pool over the course of collection, resulting in 46 samples in sample set 1 and 47 samples in sample set 2. A total of 93 samples were collected and sequenced. Of those samples, 37 remained in the dataset after rarefaction (d = 1000). Sample point 1 at 9 weeks represented 13 barrel microbiomes. Sample point 2 at 36 weeks represented 24 barrel microbiomes, with six barrels intersecting between both sample time points (HTS sample ID AS.145, 147, 148, 143, 168, 175, 221, 223, 194, 212, 216, 220). From those remaining samples, 47 fungal OTUs were identified of which 12 fungal OTUs were kept according to established relative abundance cutoff criteria.

Table 14 Barrel types and their relative proportions with Beer 3 sample sets.

Barrel type (origin)	No. of barrels	% population
Bourbon	26	52%
Cognac	2	4%
Maple syrup	8	16%
Port	1	2%
Rum	8	16%
Sherry	3	6%
Vanilla Extract	2	4%

Metadata analysis of Beer 3 ITS microbiome data found there was no statistically significant difference between barrel types ($p = 0.152$). Figure 4.12 illustrates this as samples clustered according to microbiome profile similarity do not group according to barrel type.

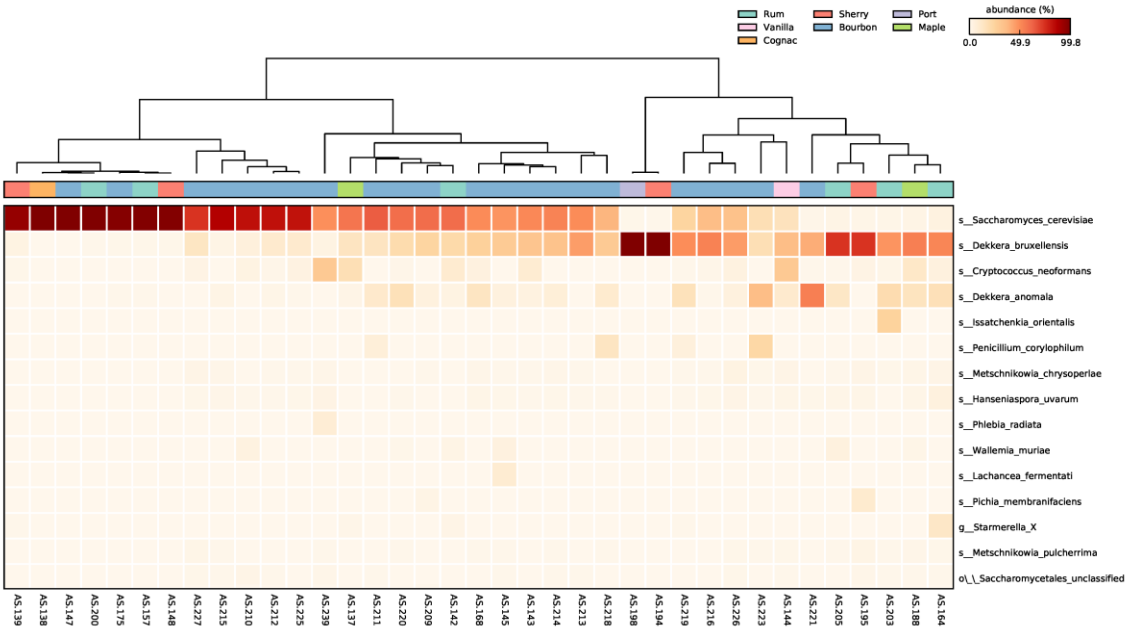

Figure 4.12 Truncated Heatmap of fungal OTUs at greater than 0.1% abundance in Beer 3 clustered according to overall microbiome similarity and barrel type. UPGMA clustering of samples based upon microbiome profiles derived from relative abundance of each OTU represented on a scale from 0 to 100%. Samples were taken from different barrel types, indicated by color, with blue representing bourbon, green maple syrup, pink vanilla extract, blue rum, orange cognac, purple port, red sherry. OTU assignments are indicated on the right hand side of the heatmap, where suffix characters "g" indicates genus, "f" indicates family, and "o" indicates order. See Appendix A12 for full heatmap with all detected taxa.

Further metadata analysis of Beer 3 ITS microbiome data found a statically significant difference between sample time points (p = 0.0439). Clustering based upon similarity of microbiome composition by sample time point, as shown in Figure. 4.13, shows a trend in statistically significant composition of *S. cerevisiae* (p = 0.033) and *D. bruxellensis* (0.034) changes over time, along with unclassified *Saccharomycetales* (p = 0.033) and *Metschnikowia* (0.013), both present below 6.5% and 0.6% relative abundance respectively in all samples. Variations in the relative

abundance of *S. cerevisiae* and *D. bruxellensis* display a temporal trend in the gradual dominance of *D. bruxellensis* over time, (Fig. 4.6.2 D and C), with samples beginning to display a presence of *S. cerevisiae* at sample time point 2 (Fig. 4.6.2 B)

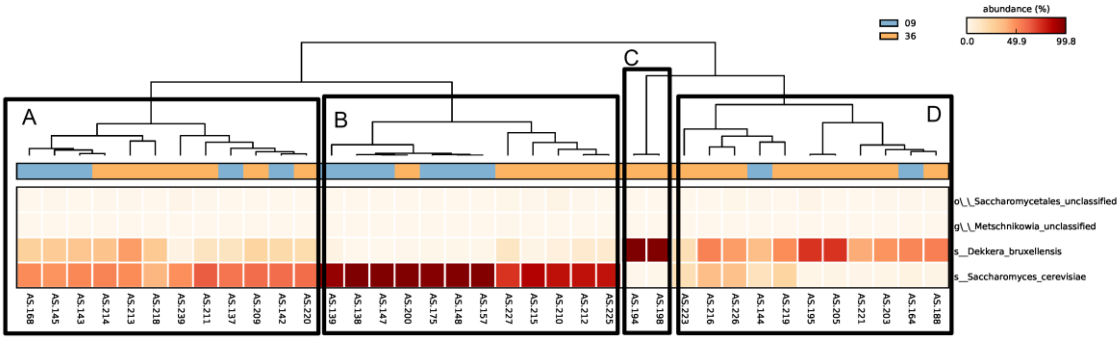

Figure 4.13 Heatmap of statistically significant fungal OTUs at greater than 0.1% abundance in Beer 3 clustered according to overall microbiome similarity. UPGMA clustering of samples based upon microbiome profiles derived from relative abundance of each OTU on a scale from 0 to 99.8%. Sample are grouped according similar microbiome composition of four statistically significant taxa. Temporal variations are outlined by cluster, with OTU assignments are indicated on the right hand side of the heatmap, where suffix characters "g" indicates genus, "f" indicates family, and "o" indicates order.

Figure 4.14 shows the distribution of the relative abundance of *S. cerevi*siae and *D. bruxellensis* at sample time point 1 (9 weeks) and sample time point 2 (36 weeks). The *S. cerevisiae* proportion of the population declined slightly as the beer aged while the *D. bruxellensis* proportion increased slightly with beer age, as shown by percent relative abundance per sample time point.

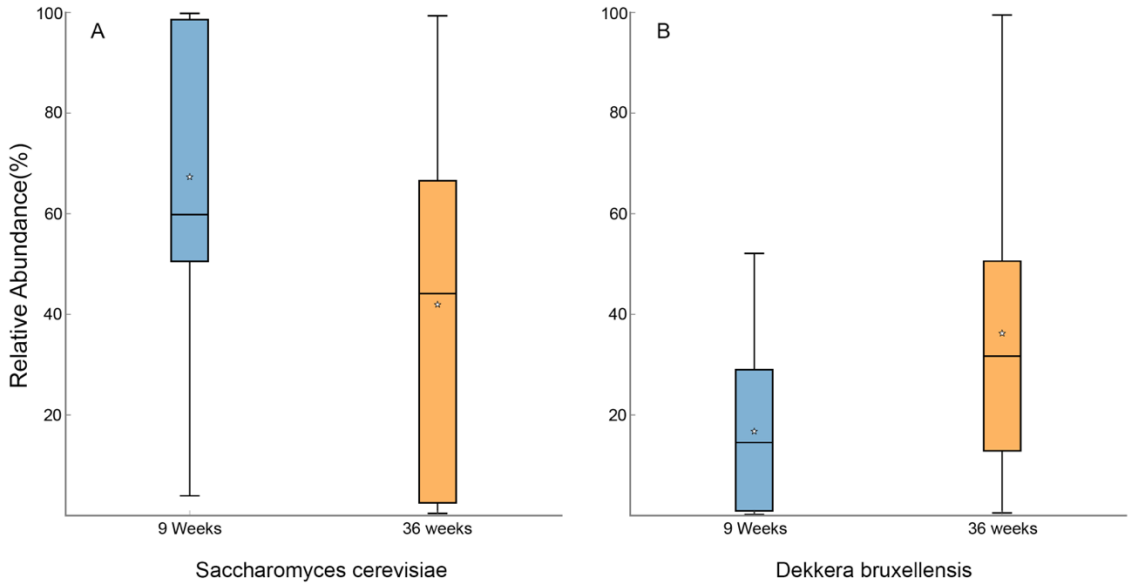

Figure 4.14. Box and whisker plot of relative abundance of S. cerevisiae (A) and D. bruxellensis (B) between sample time points of Beer 3. One-way ANOVA was performed to determine the relationship individual OTUs and sample time points, with a measurement value of relative OTU abundance and a nominal value represented by sample time point. *S. cerevisiae* (p = 0.033) and *D. bruxellenis* (0.034) were detected in all barrels that yielded microbiome data. All samples at each time point were treated as replicates. Sample time points represent the average age of Beer 3.

4.6.2 Culture-dependent microbiome analysis

Beer 3 was initially treated the same way as Beer 2, with 276 plates across three dilutions at sample point 1, including single replicates of 10 ml filter plates on each media type for a total of 92 filter plates. No growth occurred on any of the spread plates. Beer 3 sample time point 2 was performed using filter plates only. A total of 96 filter plates were prepared using 25 ml of each Beer 3 sample, representing single replicates on each media type. Filter plates yielded some microbial growth as

outlined by Table 15. No isolates were obtained from Beer 3 cultures and therefore no Sanger sequencing was performed at this time.

Table 15 Beer 3 barrels yielding microbial growth.

Barrel	HTS ID	Volume (ml)	Counts LMDA	Estimated CFU/mL	Counts YPD	Estimated Log CFU/mL
175303	AS.141	10.0	TNTC	>25	<LOQ	<25
175258	AS.144	10.0	TNTC	>25	<LOQ	<25
175554	AS.147	10.0	8	<25	<LOQ	<25
175258	AS.185	25.0	3	<10	<LOQ	<10
175303	AS.191	25.0	TNTC	>10	TNTC	>10
175340	AS.195	25.0	TNTC	>10	TNTC	>10
175352	AS.196	25.0	1	<10	TNTC	>10
175361	AS.198	25.0	TNTC	>10	TNTC	>10
175386	AS.200	25.0	<LOQ	<10	9	<10
175391	AS.201	25.0	<LOQ	<10	TNTC	>10
175424	AS.206	25.0	3	<10	<LOQ	<10

TNTC = filter completely overgrown
LQL = lower quantitation limit

4.6.3 Beer 3 bacterial community structure as revealed by ITS HTS data:

Beer 3 16S microbiome data was processed and analyzed using the same pipeline as Beer 2 16S microbiome analysis (d = 1000). Eighteen samples remained out of the original 93 samples collected following rarefaction. In total, 84 bacterial OTUs were identified, which was reduced to 34 bacterial OTUs according to established relative abundance cutoff criteria

Metadata analysis of overall Beer 3 16S microbiome data did not show a statistically significant difference between barrel types ($p = 0.094$) and no differences was found when sample time points were compared ($p = 0.615$). Figure 4.15 shows clustering of 16S microbiome data compared to barrel type, exemplifying this lack of association. Four samples displayed relatively high abundance of *Acetobacter*, *Lactobacillus* and *Sphigobium* (AS.164, AS.205, AS.191, AS.190), none of which were associated with barrel type or sample time point.


abundance (%)
0.0
50.0
100.0
Rum
Vanilla
Cognac
Sherry
Bourbon
Port
Maple
g__Acetobacter
g__Sphingobium
g__Lactobacillus
g__Meiothermus
g__Bacillus
g__Geobacillus
g__Streptococcus
Unclassified f__Oxalobacteraceae
g__Paracoccus
Unclassified o__Streptophyta
g__Gluconacetobacter
Unclassified f__Enterobacteriaceae
g__Staphylococcus
Unclassified f__Listeriaceae
g__Acinetobacter
g__Cloacibacterium
g__Micrococcus
g__Sphingomonas
g__Thermus
Unclassified f__Planococcaceae
g__Lactococcus
g__Deinococcus
g__Dermacoccus
Unclassified f__Comamonadaceae
g__Enterococcus
g__Corynebacterium
g__Pseudomonas
g__Dechloromonas
g__Paenibacillus
Unclassified f__Caulobacteraceae
g__Janthinobacterium
Unclassified f__mitochondria
Unclassified f__Bradyrhizobiaceae
Unclassified o__Myxococcales
g__Brevundimonas
g__Ethanoligenens
Unclassified f__Microbacteriaceae
g__Hymenobacter
g__Sphingopyxis
g__Facklamia
g__Propionibacterium
g__[Prevotella]
g__Rubellimicrobium
g__Novosphingobium
g__Candidatus Portiera
Unclassified o__Lactobacillales
Unclassified f__Micrococcaceae
g__Enhydrobacter
Unclassified o__Rhizobiales
Unclassified f__Methylobacteriaceae
Unclassified f__Ellin6075
g__Salinibacterium
g__Caldicellulosiruptor
g__Dorea
g__Conchiformibius
g__Anaerococcus
g__Thermoanaerobacterium
Unclassified f__Acetobacteraceae
g__Veillonella
Unclassified f__Streptococcaceae
Unclassified o__Bacillales
g__Neisseria
g__Gemella
g__Salinicoccus
g__Exiguobacterium
g__Clostridium
g__Methyloversatilis
g__Megasphaera
Unclassified f__Bacillaceae
Unclassified f__Pseudomonadaceae
g__Providencia
g__Listeria
g__Haemophilus
Unclassified f__Coriobacteriaceae
g__Tepidimonas
Unclassified f__Enterococcaceae
g__Comamonas
g__Methylobacterium
g__N09
AS.164
AS.205
AS.191
AS.190
AS.156
AS.226
AS.141
AS.206
AS.146
AS.143
AS.142
AS.144
AS.145
AS.192
AS.181
AS.216
AS.140
AS.139

Figure 4.15 Heatmap of bacterial OTUs at greater than 0.1% abundance in Beer 3 clustered according to overall microbiome similarity. UPGMA clustering of samples based upon microbiome profiles derived from relative abundance of each OUT represented on a scale from 0 to 100%. Samples were obtained from different barrel types: indicated by color, bourbon (blue), maple syrup (green,) vanilla extract (pink), rum (blue), cognac (orange,) port (purple), and sherry (red). OTU assignments are indicated on the right hand side of the heatmap, where suffix characters "g" indicates genus, "f" indicates family, and "o" indicates order.

Analysis of abundance for each individual OTU revealed that *Lactococcus* (Fig 4.16) differed significantly ($p = 0.049$) between sample time point 1 and sample time point 2.

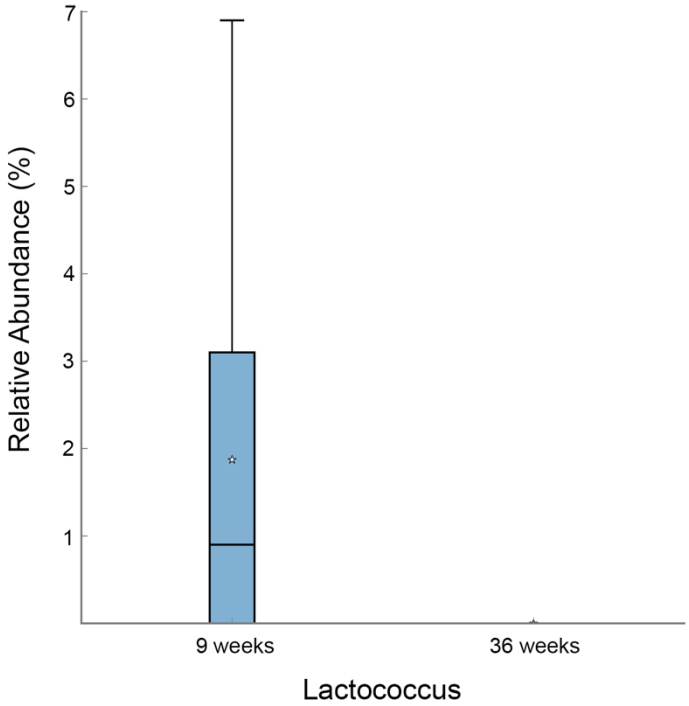

Figure 4.16 Box and whisker plot of relative abundance of Lactococcus between sample time points of Beer 3.. One-way ANOVA was performed to determine the relationship individual OTUs and sample time points, with a measurement value of relative OTU abundance and a nominal value represented by sample time point. *Lactococcus* ($p = 0.049$) was only detected at 9 weeks in five barrels at an abundance greater than 0.1%, and was not represented in the microbiome of any barrels at 36 weeks.

5 Discussion

As technology becomes more affordable and automated, the applications of culture-independent techniques are becoming more practical within the food and beverage industry. Though mainstream use of metabarcoding HTS technology (hereafter referred to as HTS in the context of this discussion) as it applies to complex microbiome profiling is gaining traction, its application within the beer industry is still lagging. Sixty-six published studies from 2010 to 2016 offer in-depth analysis of food systems through HTS technology, and of those studies only two are directly related to traditional beer fermentation and its raw materials (Bokulich et al. 2012b; Justé et al. 2014; De Filippis et al. 2017). Primary application has been to reveal complex bacterial communities of common fermentation systems, using 16S HTS to explore changes in bacterial populations overs time, often focusing on single-family dominated microbiomes (Justé et al. 2014; De Roos et al. 2018). Despite a narrow focus, those studies combined with works making use of other culture-independent techniques, such as DGGE, have begun to reveal the complex microbial consortia of spontaneously fermented beer.

Culture-independent studies, including those outlined in section 2.3 and 2.4.1, of traditionally fermented beer have been accompanied by culture-dependent techniques to help reinforce derived microbiome data. While those studies were limited in sample sizes to around 20 barrels, routine and in-depth use of culture-dependent techniques are an important validation method for culture-independent techniques in previously unexplored food systems. Taking this into consideration, the increasingly common use of barrels in the American craft beer industry, and with some breweries working with upwards of 4000 individual barrels, the impact of large-scale barrel fermentation and maturation has yet to be explored from a microbiome point of view. Application of HTS technology is potentially a boon to barrel-aging programs and large-scale traditional fermentations, allowing for simultaneous processing and monitoring of hundreds of barrel samples at once. The primary goal of this study was to use HTS techniques to investigate the impact of a large number of oak

barrels on the microbiome of a traditionally fermented beer and barrel aged beer. Additionally, we sought to investigate the microbiome progression of a traditional Lambic-style beer brewed in a geographically distinct location from those previously described in the literature.

5.1 Culture-independent data QC

An important aspect of HTS technology is the ability to quantitative and qualitatively assess microbial profiles. Maximum recovery of DNA is critical to fully exploring any microbial ecological niche and as such, extraction methods will play an important role in how a given microbiome is assessed and described (Keisam et al. 2016). This holds particularly true for systems where expected biomass recovery is low. Understanding the impact of commercially available DNA extraction kits and methods on DNA recovery are still being explored (Glassing et al. 2016; Keisam et al. 2016; Hermans et al. 2018). The application of a single standardized extraction procedure is generally unfeasible when applied to food systems. The diversity of both mechanical and chemical properties of fermented foods often makes it difficult to apply a standardized process to any one food system (Quigley et al. 2012; Thomas et al. 2013; Vojkovska et al. 2015; Keisam et al. 2016). Additionally, different organisms will display varying degrees of 'extractability'. Fungi are protected by a tough cell wall made of chitin and Gram-positive bacteria contain a thick peptidoglycan layer, which may further complicate DNA extraction.

The validation and curation of microbiome data is one of the most important aspects of microbiome profiling. *In situ* extractions from a liquid matrix does offer some advantages, in as much that inherently lower concentrations of tough structural biopolymers compared to solid food are expected to have interference with the extraction process. To be able to properly assess extraction efficiencies and PCR amplicon generation, manufactured microbial standards comprised of representative taxa are used to great effect, though by no means are they universally applied. By

processing community standards with unknown samples, estimations of overall extraction efficiencies and validity of relative quantitation can be made.

Community extraction and PCR standard results are dependent on the DNA content, and may not necessarily reflect the theoretical composition in terms of 1:1 proportions. Rather, microbiome data will reflect the percent composition of targeted genes. In the extraction standards used for this study, yeast genome copies were expected to be 0.57% *C. neoformans* and 0.37% *S. cerevisiae*, based upon approximately equal proportions of total genomic DNA. As a further complication, the *C. neoformans* genome encodes approximately 60 copies of the ribosomal RNA gene cluster, while *S. cerevisiae* has approximately 109 copies. As seen in Figure 4.2, *C. neoformans* made up roughly 66 to 75% relative abundance of the extracted community standards, illustrating one the challenges in relating microbiome data to the true microbial community composition. Recent work using these extraction standards have yielded similar results, displaying a preferential amplification of *C. neoformans* DNA within the same ITS region 2 (Hermans et al. 2018). Ultimately, there are a number of reasons as to why this may occur. Assuming the community standards contained the correct proportions of DNA, the three main possibilities come down to PCR bias, extraction efficiency, or 'tag-jumping'/'index-hopping'. *C. neoformans* was preferentially amplified, either through higher ITS primer affinity, or by virtue of there being more *C. neoformans* DNA physically available. Misidentification could occur through a process known as 'tag jumping' or 'index hopping', in which metabarcode tags will get swapped through recombination events during sequencing or library construction, leading to the appearance of sample contamination (Schnell et al. 2015). However, due to the data processing pipelines in place, it is expected that the effect of tag-jumping should be mitigated to 0.1 – 2.0% (Minimizing Index Hopping). The relative consistency of *C. neoformans* versus *S. cerevisiae* distribution, as noted by the lack of statistical difference between extracted DNA and PCR standards, would support the notion of preferential PCR amplification. More work would have to be done to determine extraction efficiency using the

protocols outlined in section 3.3.1. Relative quantitation of *S. cerevisiae* based upon our microbiome data is therefore expected to be skewed slightly, and may be more abundant than reported. Likewise, presence of *C. neoformans* in the dataset may be over-represented due to a combination of tag-jumping and preferential amplification.

Bacterial community standards behaved in the manner expected by the Zymo Research Corp microbial community standard protocol. Extraction replicates of bacterial community standards remained roughly proportional to each other, with *Enterobacteriaceae*, *Pseudomonadaceae*, and *Bacillaceae* showing the widest variability. It should be noted that *Enterobacteriaceae* comprises both *Salmonella* and *Escherichia* from the community standard. Expected composition, based upon theoretical 16S composition (10.4% and 10.1% respectively) was approximately 18 to 20% of relative abundance, as seen the PCR community standards and outlined in the microbial community standard protocol. (ZymoBIOMICS™ Microbial Community Standard Instruction Manual). Preferential amplification of *Bacillaceae* was observed, while *Lacotobacillaceae*, *Staphylococcaeae*, and *Listeriaceae* were more in line with expected recovery. At the time of writing, one of the major limitations of HTS technology is the difficulty of resolving bacterial genera and species using 16S microbiome data alone. The universal primers used in this study, while useful in allowing the detection of many taxa, are not ideal for genera resolution in highly diverse bacterial families. Therefore, it is impossible to assess the recovery and extraction efficiency of both *S. enterica* and *E. coli* from the extraction standards alone, as their overall extraction and amplification was much lower than expected. Despite this, all family-level OTUs are accounted for, and present in relative abundances above the established cut-off criteria. As such, bacterial DNA recovery from samples was expected to be representative of barrel microbiomes. Extraction data overall highlights the necessity of further extraction protocol optimization. In terms of potential impact upon the beer microbiomes generated in this study, the desired outcome was the ability to ensure that representative DNA would be extracted from barrel samples. Absolute

abundance, while important, is not the primary goal. To determine microbiome variability introduced by specific metadata categories, repeatable extraction events are required. Thus, while high extraction efficiency is ideal, consistent quantifiable results are more important at this stage. As noted earlier, the extraction concern over low extraction efficiency of Gram-positive bacteria was a concern coming into this study, but ultimately was shown to be a non-issue

The low number of reads for NT controls for each PCR plate indicated negligible contamination. The detectable OTUs found in NT.002 indicates that there may have been minor bacterial contamination during processing. PCR plate 2 was comprised of samples from Beer 2 at sample time point 3 and Beer 3 at sample time point 1. The implications of this potential contamination event are discussed in sections 5.3 and 5.4.

5.2 Beer 1

Beer 1 provided the opportunity to profile the microbiome of a traditional Lambic beer produced outside of Belgium. Only one published work (Bokulich et al. 2012) has previously described the microbiome of traditionally fermented beer produced in the United States, or indeed anywhere outside of Belgium. In that study, Bokulich et al. found strong evidence to support that American Coolship Ales undergo a similar microbial succession seen in the traditional Belgian Lambic. What had previously been established as important taxa required for Lambic beer production (Van Oevelen et al. 1977; Martens et al. 1997; Spitaels et al. 2014), were all accounted for with evidence supporting a much richer and more dynamic microbiome than previously thought. Bearing this in mind, what was once thought to be a geographically unique process could potentially be quite reproducible and tractable elsewhere.

The fermentation of Beer 1 over the course 36 weeks displayed strong resemblance to Lambic beer microbiomes previously profiled using non-HTS culture independent techniques, with some

notable differences. Both yeast and bacterial communities were profiled in parallel using HTS technology, allowing for a high-resolution analysis of both communities at the same time.

Yeast community profiling was able to achieve species-level identification of detectable OTUs, with the exception of five taxa that could only be matched to their respective families, and in one instance, phylum. These represent a minority of detected OTUs, making up an average 0.094±0.052% relative abundance. Yeast community profiling revealed a core population of *S. cerevisiae* and *D. bruxellensis* within 24 hours post-brewing, approximately three weeks ahead of previously described isolation points (Van Oevelen et al. 1977; Spitaels et al. 2017). Early detection may be due to a yeast-enriched environment in the facility where Beer 1 was brewed and fermented relative to other studies.

Three key factors play into this assumption. The brewery environment in which this Lambic-style beer was brewed is an isolated area of the brewery that serves as a quarantine for beers that contain or will contain *Dekkera* species (*Brettanomyces*), and is not subject to the same level of hygiene as the rest of the brewery. Brewing is performed in an open manner, allowing for the accumulation of steam and water vapor from the process, providing an ideal environment for the proliferation of many fungal and bacterial species within the brewery. The presence of exposed wooden beams may also play a role in environmental enrichment, as they are believed to sequester a wide range of microorganisms (Bokulich et al. 2012b; Spitaels et al. 2014; Bokulich et al. 2015). Lastly, the barrels used for fermentation, A and B, were ozone-treated French oak wine barrels used in the production of Pinot Nior and were obtained from a vineyard within the same geographic region as the brewery itself. While ozone treatment is effective at reducing microbial populations, it will not eliminate everything (Guzzon et al. 2017). Resident yeast within those barrels may act to inoculate the wort, however sampling began from two sterile fermenters prior to the beer being placed into barrels, suggesting that the presences of both *S. cerevisiae* and *D. bruxellensis* originated from the

local environment rather than the barrels. To determine the origins of these yeasts, environmental sampling of both pre-filled barrels and brewery environments would need to be performed.

Other fungal species not normally associated with beer fermentation were detected and identified over the course of sampling. In the case of Beer 1, these species are represented only sparsely, making up an average 5.8±8.5% observed relative abundance. For instance, *Cladosporium ramotenellum* is a common environmental fungi found in soil, dust, and is associated with woody plants and food (Schubert et al. 2007). This detected 3 weeks into fermentation but not detected afterwards. *Wickerhamomyces anomalus* has been associated with malting and grain ecosystems relevant to beer production (Laitila et al. 2011) and was detected sporadically during the during the first half of the fermentation. Of note, *C. neoformans* appears in 12 out of 20 samples, ranging from 0.1 – 21.4%, albeit at low media (0.3%) abundance. *C. neoformans* has not been described to have any role in beer fermentation, and it is likely that for most samples its detection at low abundance is the result of Illumina sequencing bleed over (tag-hopping), given the presence of *C. neoformans* in the microbial community standards.

By comparing higher-level taxonomic composition, Beer 1 closely followed previously established temporal community changes of Lambic beer fermentations (Van Oevelen et al. 1977; Spitaels et al. 2014). Bacterial community profiling was able to achieve genus-level taxonomic identification for nine of the 17 detected bacterial OTUs. The remaining detected OTUs could be identified at the family level, with one unassigned bacterial OTU not associated with listed taxa. Other bacterial OTUs not normally associated with beer microbiology were detected as well, at varying relative abundances. Taxa such as *Staphylococcus*, *Lysteriaceae*, *Sphingobium*, and *Geobacillus* were detected among Beer 1 samples. Of those, both *Staphylococcus* and *Lysteriaceae* were part of the extraction community standard and again, their relatively low abundance and frequency of occurrence within sample sets is consistent with potential tag-hopping as discussed previously.

However, genera such as *Geobacillus* and *Sphingobium* are more likely indicative of low-level environmental contamination than any meaningful contribution to the 16S microbiome of traditionally fermented beer. Both *Geobacillus* and *Sphigobium* are known environmental bacteria genera, associated with soil, fresh water, and human habitats (McMullan et al. 2004; Kelley et al. 2004; Glaeser & Kämpfer 2014). Their occurrence was limited to two samples, AS.237 and AS.238, with *Sphingobium* only appearing in AS.238. Given their relatively high abundance (5.4 – 31% relative abundance), and the close spacing of early sample points for Beer 1, both genera would likely be detectable at earlier and later dates if they were an important contributor within the beer, due to persistence of DNA within the environment and the overall extraction efficiency seen in the community standards. Negative PCR controls back this up, as no bacterial OTUs were associated with Beer 1 PCR (data not shown). A more rigorous brewery environmental profiling would be ideal to assess origin of potential environmental contaminants in the beer samples.

5.3 Beer 2

Beer 2 provided the opportunity to observe microbiome changes across a larger set of replicated beer samples, across different maturation phases. Primary fermentation had already been completed for all barrels at the time this study was initiated. At the start of sampling, the age of Beer 2 ranged from 8 to 50 months and thus were in what would be considered the final maturation phase of a Lambic beer (Spitaels et al. 2014). During this period, *D. bruxellensis* has been previously shown to slowly attenuate the remaining complex sugars left behind by early yeast and bacteria species. This also marks the slow decline of lactic acid bacteria populations and the gradual increase in abundance of acetic acid bacteria.

By eight months into maturation, the 2016 batch started with a small, but significant population of *S. cerevisiae*. One of the key features of the 2016 batch year was the substantial variation among barrels at the start of sampling. As shown in Figure 4.8, there was a rapid decline of the remaining

S. cerevisiae population between the first and second sample time points in favor of *D. bruxellensis*. Variation amongst barrels quickly stabilized in first year of maturation. Behavior observed in Beer 1, where *S. cerevisiae* stabilized to similar relative abundances in the same period (8 to 9 months), appears to reinforce this trend. This behavior could potentially explain the small, but statistically significant difference found when Beer 2 ITS metadata was analyzed according to batch year. Indeed, intra-batch ITS microbiome variation was conserved in both the 2015 and 2013 batches, favoring a homogenous yeast microbiome among barrels within those batch years. The trend that the 2016 batch displayed, combined with observed yeast communities in the 2015 and 2013 batches provides strong evidence that yeast populations will quickly reach a conserved profile. This observation was backed up by culture-dependent analysis where it was evident that the bulk of the yeast population was cycloheximide tolerant, and 95% of identified isolates belonged to *D. bruxellensis*.

Beer 2 bacterial community structures were more dynamic than the observed yeast communities, but were represented by fewer barrels. This fits with the relative scarcity of barrel samples where culturable bacteria could be observed. 16S microbiome metadata analysis showed a distinct clustering by batch year that was primarily driven by four main bacterial taxa. Clustering shown in figure 4.10 displays a distinct trend within each batch year. Cluster C clearly shows barrels in 2016 batch shows containing distinct populations of both Acetobacter and Lactobacillus while Cluster B is primarily dominated by Lactobacillus. Cluster A contains a portion of the 2016 batch which are almost completely dominated by Acetobacter. Barrels from the 2015 batch are observed in Cluster A and D, and dominated by Acetobacter and contain almost no Lactobacillus. Lastly, the 2013 batch, which represents the lowest relative number of barrels, is found in Cluster C and D, and dominated by all three acetic acid taxa while hosting some Lactobacillus. This is further illustrated in Figure 4.11, where these four taxa displayed distinct trends within each batch year. Though these batches cannot be meaningfully compared as a continuum, their individual trends appear to show a

strong temporal element associated with bacterial communities. That some individual barrel samples from the 2016 batch year clustered with barrel samples from the 2013 batch year, and vice versa, suggests that while the bacterial populations trend towards a conserved profile, the rate at which this occurs will vary.

While it may seem surprising that none of the bacterial isolates collected from the culture-dependent portion of this study corresponde to lactic acid bacteria, this makes sense based upon their relatively low average abundance according to microbiome data. All isolated bacteria belonged to the genus *Acetobacter*, with *A. fabarum, A. malorum, A. pomorum* and *A. pasterurianus* being the four represented species. No Gluconacetobacter was isolated from any sample, again consistent with its relatively low abundance. Given that this genera has yet to be described as part of traditional beer fermentations, it would prove interesting to further explore its origins and effect on the sensory aspects compared to other acetic acid bacteria, once it's presence as an active member of the microbial consortia is established.

The other sequenced isolates returned as *Staphylococcus* and *Bacillus* species (Appendix A11). While not ruling out their presence in beer samples, there is a high likelihood that these represent contamination during subsequent re-culturing rather than presence during isolation from beer samples. Noted detected bacterial OTUs such as *Staphylococcus* and *Propionibacterium* are unable to grow at pH levels associated with Lambic beers (< 4.5) and are strongly associated human skin (Korting et al. 1992). They also appear in low relative abundances in Beer 2 data, ranging from 0 0 to 6% relative abundance (median xx%). The exception would be Bacillus, and in particular, Bacillus subtilis. This species is a ubiquitous environmental bacteria, associated with humans and capable of persisting in harsh environments as spores (Setlow 2006; Earl et al. 2008). Though used in some food fermentations, these tend to be alkaline and therefore Bacillus is not likely to grow during traditional beer fermentation (Tamang et al. 2016). Therefore, persistent environmental

Bacillus spores may represent a strong environmental contaminant that needs to be accounted for through environmental sampling.

Careful consideration was made when determining what were considered important bacterial taxa. Potentially spurious taxa were evaluated based upon their primary ecological niche, rate of occurrence within sample sets, and overall abundance. Under conditions where the number of returned sequences from a given sample set were considered low, in this case those samples with read values at close the rarefaction value (d = 1000), PCR stochasticity will compound any environmental contamination, which is a known problem for low-template PCR (Alberdi et al. 2018). As seen with Beer 1, a number of taxa not normally associated with beer microbiomes were detected across all three batch years. Because negative PCR controls did not return any significant sequence data (d < 1000) (data not shown), it was assumed that many of the taxa present are the result of environmental contamination. Additionally, unlike Beer 1 samples, which returned a consistently higher number of sequence reads per sample, the concentration of microorganisms present in Beer 2 was significantly reduced. This is due to the fact that older beer is a rarefied environment as shown by the culture-dependent plating data in Tables 10 and 11. Thus, in such a rarefied system, minor levels of environmental contamination, cross contamination, and index jumping will be compounded, significantly increasing the level of genetic background noise. This is particularly true for some bacterial taxa, such as *Ralstonia*, which is a known and fairly ubiquitous contaminant of DNA extraction kits (Salter et al. 2014; Glassing et al. 2016).

5.4 Beer 3

By collecting and analyzing microbiome data on Beer 3, the impact of different barrel types, or origins, could be explored. ITS HTS microbiome data revealed a near complete dominating presence of *S. cerevisiae* and *D. bruxellensis*. Additionally, the number of barrels yielding microbiome data between sample time points doubled. Maximum relative abundance of each

species were observed at 99.1% and 99.9% respectively. Though no correlation between barrel types and ITS microbiome composition could be determined, the presence of detectable fungal OTUs and the increase in microbiome yielding barrels between sample sets indicates that barrels contribute to the variability of barrel-aged beers. As with Beer 2, the temporal aspect revealed a small, but significant difference between sample sets. As Figure 4.13 displays, the ITS microbiome composition displays a temporal trend in barrel clustering by sample time point. Divergence is highlighted by the fact that some barrels exhibited an initial *S. cerevisiae* phase and then became *D. bruxellensis* dominant (e.g. HTS sample ID AS.148 and AS.194), while other barrels appear to become *S. cerevisiae* dominant within the same period (e.g. HTS sample ID AS.168 and AS.212). Figure 4.14 illustrates this divergence between *S. cerevisiae* and *D. bruxellensis*, noting that the average relative abundance of *S. cerevisiae* decreased between sample time points, yet the number of barrels yielding some *S. cerevisiae* sequence data increased. In that same vein, there was an average increase in relative abundance *D. bruxellensis*, along with a slight increase in the number barrels yielding *D. bruxellensis* sequence data within that same period.

The 16S microbiome data revealed a large number of bacterial OTUs, many of which fell below the established cutoff criteria. As with ITS HTS microbiome data, barrel type did not significantly influence the overall bacterial population composition. The only taxa that differed significantly in abundance between sample time points was *Lactococcus*, as shown in Figure 4.16. However, it was only detected in the first sample time point and at low relative abundances. Interestingly, samples AS.141 and AS.191 represent the same barrel at 9 weeks and 36 weeks respectively. This barrel was observed to follow the same trend seen in Beer 2, where *Lactobacillus* (3.1% abundance at 9 weeks, and 0% abundance at 36 weeks) declined over time while *Acetobacter* (10.3% at 9 weeks, and 94.7% at 36 weeks) increased over time.

Representative Beer 3 OTUs for both ITS microbiome data and 16S microbiome data were picked after careful consideration, based upon how Beer 2 data was treated. Sample processing saw extremely low DNA recovery from Beer 3 and as discussed in Beer 2, this could lead to over-representation of environmental contaminants. Low recovery rates are illustrated in the culture-dependent analysis. Standard spread plates using 100 µl of undiluted beer yielded no growth at either sample time points. Microbial growth was not observed until 10 to 25 ml of sample was filtered through 0.45 µm sterile filter, therefore viable microbial density was on average ~25 CFU/ml. And even then, not all barrels yielded microbial growth. Despite best possible practices, truly aseptic collection was impossible due to the practicalities of sampling from barrels. Thus, for many of the detected OTUs, a combination of low overall sequencing reads and low rarefaction depth allows for the overrepresentation of common environmental contaminates, such as *Bacillus*, *Sphigobium*, and *Geobacillus*. It is likely that the 16S microbiome data for Beer 3 represents genetic background noise and artifacts of sequencing. Instances of relatively high abundance of known spoilage organisms (e.g *Acetobacter* observed in sample AS.191) may represent opportunistic spoilage, but more work is required to fully understand the implications of low DNA recovery on the microbiome of rarefied beer.

6 Conclusions

Previous studies have compared and explored the microbial successions of Lambic beer fermentation. The distinct phases that occur during a spontaneous beer fermentation were once believed to be unique to the Belgian Lambic beers, giving them their characteristic aromas and flavors. Beer 1 provided evidence that similar microbial successions will happen regardless of geography, likely owing more to the selective nature of the unfermented wort itself, and a microbially enriched brewery environment, than to microbes occurring in surrounding geographical niches. A beer brewed and fermented in Oregon, USA with ingredients and vessels originating from local sources still displayed the same microbial successions observed in beer produced on an entirely different continent. A caveat to this is the potential importance of strain-level variation. While at a macro level, all relevant taxa are accounted for in both yeast and bacteria communities, differences may arise in the strains of important microorganisms. Strain-level variations may influence the overall metabolic profile of the beer, leading to unexpected development of different organoleptic properties.

Further exploration into the aging process revealed that the microbiomes of barrel-aged beers were strongly influenced by temporal aspects. In the case of Beer 2, the same beer brewed and aged over the course of 5 years displayed a trend towards a conserved yeast and bacterial profile, with *D. bruxellensis* and acetic acid bacteria becoming the dominant organisms over long-term maturation. A clear trend was observed that though barrels began to look similar in terms of microbiome composition, the rate at which this occurred varied. Some barrels will reach what could be thought of as an “end phase” profile in two years, while others may take much longer. This variation may be introduced by barrels in terms of microorganisms present in the barrels themselves, a combination of beer composition and abiotic stressors such as temperature may favor enrichment of one taxa over another, or handling by brewers during filling.

This temporal aspect was further reinforced by observation of the divergence of barrel microbiomes observed in Beer 3, which appeared to occur independent of barrel type. The same beer batch placed into 50 different barrels began to experience microbiome shifts, despite being a finished beer.

To further explore this notion of conserved microbial profiles, the drivers of flavor and aroma are important to consider. It has been long understood that microorganism have a profound impact on the flavor and quality of beer. Introduction of unwanted microbial species within a beer system could result in over-attenuation or the production of off-flavors and aromas, ruining a perfectly good product. However, in the context of the traditional fermentation, previous work has established the importance of microbial influences on flavor and aroma. In Lambic beer, microbiome shifts are directly associated with the production of the specific aroma and flavor compounds sought after in traditionally fermented beers (Van Oevelen et al. 1976; Van Oevelen et al. 1977; Verachtert & Iserentant 1995; Martens et al. 1997; Vanderhaegen et al. 2003; Pires et al. 2014; Snauwaert et al. 2016). Unfortunately, because of inherent capriciousness of living systems (including fermentation, raw materials, human influences), perfect batch to batch consistency for traditionally fermented beer is likely impossible. Should the popularity of these beers continue to grow, however, brewers will need to meet demand. Building a deeper understanding of complex mixed fermentations in the context of beer production, is the first step to creating standardized processes and approaches to maximize production while minimizing waste.

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APPENDICES

Appendix A1: Barrel ID number, Origins, volume and fill date of Beer 2 barrels allocated for sampling

Number	Origin	Character	Vol (liter)	Fill Date
131520	Canoe Ridge	French Pinot 5-yr	227	5/30/13
131488	Canoe Ridge	French Pinot 5-yr	227	5/30/13
131483	Canoe Ridge	French Pinot 5-yr	227	5/30/13
131507	Canoe Ridge	French Pinot 5-yr	227	5/30/13
131523	Canoe Ridge	French Pinot 5-yr	227	5/30/13
131453	Canoe Ridge	French Pinot 5-yr	227	5/30/13
131461	Canoe Ridge	French Pinot 5-yr	227	5/30/13
131468	Canoe Ridge	French Pinot 5-yr	227	5/30/13
131472	Canoe Ridge	French Pinot 5-yr	227	5/30/13
131480	Canoe Ridge	French Pinot 5-yr	227	5/30/13
131486	Canoe Ridge	French Pinot 5-yr	227	5/30/13
131576	Canoe Ridge	French Pinot 5-yr	227	6/12/13
131579	Canoe Ridge	French Pinot 5-yr	227	6/12/13
131529	Canoe Ridge	French Pinot 5-yr	227	6/12/13
131574	Canoe Ridge	French Pinot 5-yr	227	6/12/13
131585	Canoe Ridge	French Pinot 5-yr	227	6/12/13
131586	Canoe Ridge	French Pinot 5-yr	227	6/12/13
131588	Canoe Ridge	French Pinot 5-yr	227	6/12/13
131597	Canoe Ridge	French Pinot 5-yr	227	6/12/13
131599	Canoe Ridge	French Pinot 5-yr	227	6/12/13
131650	Canoe Ridge	French Pinot 5-yr	227	6/12/13
131664	Canoe Ridge	French Pinot 5-yr	227	6/12/13
131665	Canoe Ridge	French Pinot 5-yr	227	6/12/13
142774	Domain Serene	French Pinot 5-yr	227	4/29/15
142836	Adelsheim	French Pinot 5-yr	227	4/29/15
142844	Adelsheim	French Pinot 5-yr	227	4/29/15
142847	Adelsheim	French Pinot 5-yr	227	4/29/15
142770	Domain Serene	French Pinot 5-yr	227	4/29/15
142787	Domain Serene	French Pinot 5-yr	227	4/29/15
142790	Domain Serene	French Pinot 5-yr	227	4/29/15
142792	Domain Serene	French Pinot 5-yr	227	4/29/15
142794	Domain Serene	French Pinot 5-yr	227	4/29/15
142798	Domain Serene	French Pinot 5-yr	227	4/29/15
142816	Adelsheim	French Pinot 5-yr	227	4/29/15
142817	Adelsheim	French Pinot 5-yr	227	4/29/15
142820	Adelsheim	French Pinot 5-yr	227	4/29/15
142826	Adelsheim	French Pinot 5-yr	227	4/29/15

Appendix A1. (continued) Barrel ID number, Origins, volume and fill date of Beer 2 barrels allocated for sampling

Number	Origin	Character	Vol (liter)	Fill Date
142850	Adelsheim	French Pinot 5-yr	227	4/29/15
155134	RMBC	Tequila	227	11/1/16
155184	RMBC	Tequila	227	11/1/16
155185	RMBC	Tequila	227	11/1/16
155188	RMBC	Tequila	227	11/1/16
155124	RMBC	Tequila	227	11/1/16
155125	RMBC	Tequila	227	11/1/16
155130	RMBC	Tequila	227	11/1/16
155135	RMBC	Tequila	227	11/1/16
155149	RMBC	Tequila	227	11/1/16
155157	RMBC	Tequila	227	11/1/16
155179	RMBC	Tequila	227	11/1/16
155183	RMBC	Tequila	227	11/1/16

Appendix A2: Barrel ID number, Origins, volume and fill date of Beer 2 barrels allocated for sampling

Number	Origin	Character	Vol (liter)	Fill Date
175277	River Drive Cooperage	Maple Syrup	199	6/8/17
175330	River Drive Cooperage	Maple Syrup	199	6/8/17
175303	River Drive Cooperage	Vanilla Extract	199	6/8/17
175391	River Drive Cooperage	Rum	199	6/8/17
175258	River Drive Cooperage	Vanilla Extract	199	6/8/17
175262	River Drive Cooperage	Maple Syrup	199	6/8/17
175263	River Drive Cooperage	Maple Syrup	199	6/8/17
175271	River Drive Cooperage	Maple Syrup	199	6/8/17
175289	River Drive Cooperage	Maple Syrup	199	6/8/17
175323	River Drive Cooperage	Maple Syrup	199	6/8/17
175332	River Drive Cooperage	Maple Syrup	199	6/8/17
175380	River Drive Cooperage	Rum	199	6/8/17
175401	River Drive Cooperage	Rum	199	6/8/17
175340	River Drive Cooperage	Sherry	223	6/12/17
175361	River Drive Cooperage	Port	223	6/12/17
175339	River Drive Cooperage	Sherry	223	6/12/17
175352	River Drive Cooperage	Sherry	223	6/12/17
175357	River Drive Cooperage	Cognac	223	6/12/17
175386	River Drive Cooperage	Rum	199	6/12/17
175396	River Drive Cooperage	Rum	199	6/12/17
175397	River Drive Cooperage	Rum	199	6/12/17

Appenix A2 (continued) Barrel ID number, Origins, volume and fill date of Beer 2 barrels allocated for sampling

Origin	Origin	Origin	Origin	Origin
175411	River Drive Cooperage	Rum	199	6/12/17
175424	River Drive Cooperage	Rum	199	6/12/17
175437	Great Lakes Bourbon	Bourbon Whisky	199	6/13/17
175460	Great Lakes Bourbon	Bourbon Whisky	199	6/13/17
175473	Great Lakes Bourbon	Bourbon Whisky	199	6/13/17
175477	Great Lakes Bourbon	Bourbon Whisky	199	6/13/17
176192	Great Lakes Bourbon	Bourbon Whisky	199	6/13/17
175430	Santa Ynez BR	Cognac	223	6/14/17
175710	Bendistillery	Black Butte Whisky	199	6/14/17
175467	Great Lakes Bourbon	Bourbon Whisky	199	6/14/17
175442	Great Lakes Bourbon	Bourbon Whisky	199	6/14/17
175454	Great Lakes Bourbon	Bourbon Whisky	199	6/14/17
175469	Great Lakes Bourbon	Bourbon Whisky	199	6/14/17
175628	Great Lakes Bourbon	Bourbon Whisky	199	6/14/17
175637	Great Lakes Bourbon	Bourbon Whisky	199	6/14/17
175677	Great Lakes Bourbon	Bourbon Whisky	199	6/14/17
176198	Great Lakes Bourbon	Bourbon Whisky	199	6/14/17
175538	Great Lakes Bourbon	Bourbon Whisky	199	6/15/17
175496	Great Lakes Bourbon	Bourbon Whisky	199	6/28/17
175547	Great Lakes Bourbon	Bourbon Whisky	199	6/28/17
175550	Great Lakes Bourbon	Bourbon Whisky	199	6/28/17
175554	Great Lakes Bourbon	Bourbon Whisky	199	6/28/17
175492	Great Lakes Bourbon	Bourbon Whisky	199	6/28/17
175514	Great Lakes Bourbon	Bourbon Whisky	199	6/28/17
175521	Great Lakes Bourbon	Bourbon Whisky	199	6/28/17
175526	Great Lakes Bourbon	Bourbon Whisky	199	6/28/17
175555	Great Lakes Bourbon	Bourbon Whisky	199	6/28/17
175574	Great Lakes Bourbon	Bourbon Whisky	199	6/28/17
175578	Great Lakes Bourbon	Bourbon Whisky	199	6/28/17

Appendix A3. Total colony forming unit (CFU) data in singlet.

		LMDA + Cycloheximide		YPD + Chloramphenicol	
Barrel	**Fill Year**	**Avg Time Point 1 (Log cfu/ml)**	**Avg Time Point 3 (Log cfu/ml)**	**Avg Time Point 1 (Log cfu/ml)**	**Avg Time Point 3 (Log cfu/ml)**
131453	2013	3.8	< LQL*	< LQL	< LQL
131461	2013	< LQL	< LQL	< LQL	< LQL
131468	2013	< LQL	--	< LQL	--
131480	2013	4.1	2.7	< LQL	2.4

Appendix A3. (continued). Total colony forming unit (CFU) data in singlet

		LMDA + Cycloheximide		YPD + Chloramphenicol	
Barrel	**Fill Year**	**Avg Time Point 1 (Log cfu/ml)**	**Avg Time Point 3 (Log cfu/ml)**	**Avg Time Point 1 (Log cfu/ml)**	**Avg Time Point 3 (Log cfu/ml)**
131483	2013	4.0	2.6	3.6	< LQL
131486	2013	2.6	< LQL	< LQL	< LQL
131488	2013	3.6	< LQL	3.7	< LQL
131507	2013	< LQL	< LQL	< LQL	< LQL
131520	2013	< LQL	< LQL	3.2	< LQL
131523	2013	< LQL	< LQL	< LQL	< LQL
131529	2013	4.1	2.7	3.3	2.6
131574	2013	< LQL	2.6	< LQL	2.4
131576	2013	2.5	< LQL	< LQL	< LQL
131579	2013	3.7	< LQL	3.9	< LQL
131586	2013	< LQL	< LQL	< LQL	< LQL
131588	2013	< LQL	< LQL	< LQL	< LQL
131597	2013	< LQL	--*	< LQL	--
131599	2013	< LQL	--*	< LQL	--
131650	2013	< LQL	< LQL	1.9	< LQL
131664	2013	3.0	3.3	2.9	3.1
131665	2013	3.0	< LQL	2.7	3.3
142770	2015	3.7	< LQL	< LQL	4.2
142774	2015	< LQL	--*	< LQL	--*
142787	2015	4.3	< LQL	4.0	4.3
142790	2015	< LQL	3.4	< LQL	3.0
142792	2015	3.1	< LQL	3.1	< LQL
142794	2015	< LQL	2.4	< LQL	2.4
142798	2015	3.6	4.1	< LQL	< LQL
142816	2015	4.2	3.3	4.3	2.9
142817	2015	4.3	< LQL	4.3	< LQL
142820	2015	3.6	4.0	3.7	< LQL
142826	2015	3.8	< LQL	3.9	3.1
142836	2015	4.2	< LQL	4.1	3.6
142844	2015	4.1	--	4.1	--*
142847	2015	3.1	< LQL	3.0	4.2
142850	2015	4.2	3.8	4.1	3.6
155124	2016	< LQL	< LQL	3.4	< LQL
155125	2016	4.9	< LQL	< LQL	< LQL
155130	2016	4.0	< LQL	3.8	4.8
155134	2016	4.3	--*	< LQL	--
155135	2016	4.3	< LQL	4.3	3.6
155149	2016	4.2	4.1	4.2	3.8
155157	2016	< LQL	4.9	< LQL	4.8
155179	2016	< LQL	3.4	4.4	3.2
155183	2016	4.1	< LQL	4.0	2.5
155184	2016	3.9	3.6	4.2	3.5
155185	2016	4.0	< LQL	4.3	< LQL

Appendix A3. (continued). Total colony forming unit (CFU) data in singlet

		LMDA + Cycloheximide		YPD + Chloramphenicol	
Barrel	**Fill Year**	**Avg Time Point 1 (Log cfu/ml)**	**Avg Time Point 3 (Log cfu/ml)**	**Avg Time Point 1 (Log cfu/ml)**	**Avg Time Point 3 (Log cfu/ml)**
155188	2016	3.4	3.3	< LQL	3.0

(--) barrels missing at this sample time point. (*) Lower Quantitation Limit, Log(10) 2.4 CFU/ml

Appendix A4. Triplicate extraction runs performed on 24-hour single-strain liquid cultures of **Saccharomyces cerevisiae** (Y 1- 3) and Pediococcus spp. (B1-3). DNA extraction concentrations of BeadRuptor 24 method vs. Vortex method. SpectraMax® Quant™ AccuClear™ Nano dsDNA Assay Kit used for DNA quantification.

	BeadRuptor24		Vortex	
Replicate:	**Response**	**ng/µl**	**Response**	**ng/µl**
Y1	2788.54	51.63	551.779	9.55
Y2	3065.842	56.85	324.284	5.27
Y3	3979.978	74.04	355.05	5.85
		60.84 ±9.58		**6.89±1.90**
B1	2365.418	43.67	918.143	16.44
B2	2703.475	50.03	1383.078	25.19
B3	2609.405	48.26	860.723	15.36
		47.32±2.68		**19.00±4.40**

Appendix A5. Complete list of fungal OTUs detected in Beer 1, their maximum relative abundance, mean relative abundance and rate of occurrence (No.) at greater than 0.1% out of 21 samples.

OTU ID	**Maximum**	**Median**	**Mean**	**No. > 0.1%**
Cladosporium ramotenellum	0.017	0.000	0.001333	3
Aureobasidium pullulans	0.017	0.000	0.001429	4
Penicillium roqueforti	0.005	0.000	0.000238	1
Metschnikowia chrysoperlae	0.026	0.000	0.001571	2
Metschnikowia pulcherrima	0.017	0.000	0.001619	4
Wickerhamomyces anomalus	0.197	0.000	0.011143	6
Pichiaceae unclassified	0.007	0.000	0.000905	7
Dekkera anomala	0.132	0.003	0.013381	15
Dekkera bruxellensis	0.992	0.430	0.51319	21
Lachancea fermentati	0.022	0.000	0.001286	3
Saccharomyces bayanus	0.006	0.000	0.000714	5
Saccharomyces cerevisiae	0.975	0.485	0.429	21
Zygosaccharomyces parabailii	0.001	0.000	4.76e-05	1

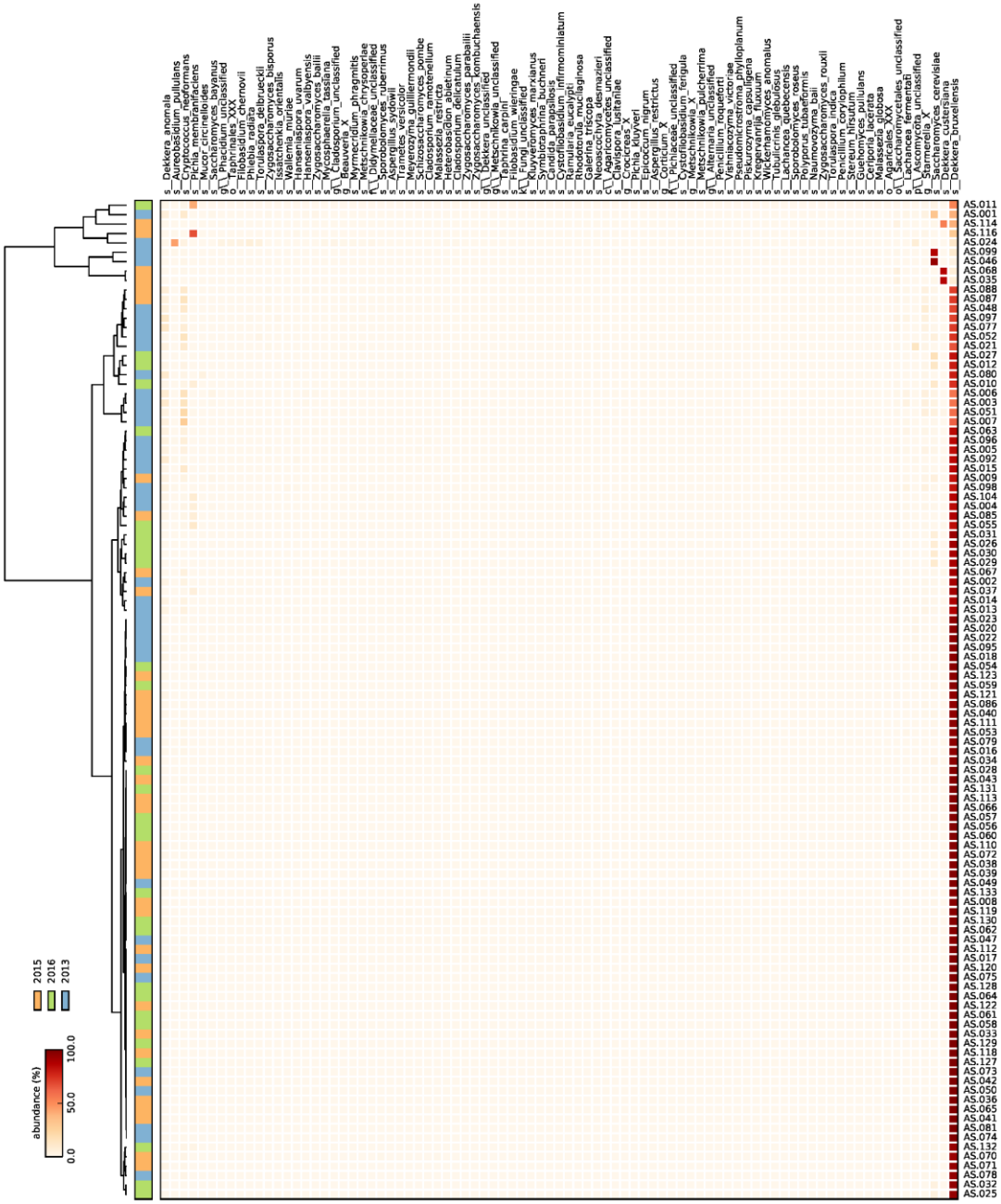
Appendix A5. (continued) Complete list of fungal OTUs detected in Beer 1, their maximum relative abundance, mean relative abundance and rate of occurrence (No.) at greater than 0.1% out of 21 samples.

OTU ID	Maximum	Median	Mean	No. > 0.1%
Starmerella;s unidentified	0.029	0.000	0.001619	5
Hanseniaspora uvarum	0.039	0.000	0.003667	7
Saccharomycetales unclassified	0.002	0.000	0.000143	2
Saccharomycetales unclassified	0.008	0.000	0.000619	4
Ascomycota unclassified	0.017	0.000	0.001333	4
Malassezia restricta	0.006	0.000	0.000333	2
Sporobolomyces roseus	0.056	0.000	0.002667	1
Cryptococcus neoformans	0.214	0.001	0.013762	12

Appendix A6. Complete list of bacterial OTUs detected in Beer 1, their maximum relative abundance, mean relative abundance and rate of occurrence (No.) at greater than 0.1% out of 21 samples.

OTU ID	Maximum	Mean	Mean	No. > 0.1%
Unassigned	0.032	0.0016	0.002	2
Streptophyta	0.091	0.0044	0.004	1
Bacillus	0.006	0.0013	0.001	7
Geobacillus	0.310	0.0232	0.023	2
Listeriaceae;Other	0.004	0.0004	0.000	3
Staphylococcus	0.073	0.0040	0.004	4
Enterococcaceae	0.001	0.0002	0.000	2
Lactobacillaceae	0.885	0.3306	0.331	16
Lactobacillus	0.777	0.1304	0.130	17
Acetobacteraceae	0.018	0.0033	0.003	10
Acetobacter	0.114	0.0235	0.024	13
Gluconacetobacter	0.139	0.0353	0.035	11
Sphingobium	0.054	0.0026	0.003	1
Enterobacteriaceae (Other)	0.003	0.0007	0.001	6
Enterobacteriaceae	0.986	0.4324	0.432	20
Enterobacter	0.004	0.0008	0.001	7
Erwinia	0.016	0.0052	0.005	16

Appendix A7: Full heatmap version of Figure 4.7



Appendix A8. Complete list of Fungal OTUs detected in Beer 2, their maximum relative abundance, mean relative abundance and rate of occurrence (No.) at greater than 0.1% out of 168 samples.

OTU ID	Maximum	Median	Mean	No. > 0.1%
Fungi unclassified	0.033	0.000	2.976E-04	6
Cladosporium unclassified	0.020	0.000	1.786E-04	7
Cladosporium delicatulum	0.015	0.000	3.988E-04	29
Cladosporium ramotenellum	0.012	0.000	4.464E-04	27
Mycosphaerella tassiana	0.041	0.000	4.702E-04	13
Ramularia eucalypti	0.002	0.000	1.190E-05	1
Aureobasidium pullulans	0.449	0.000	3.881E-03	46
Didymellaceae unclassified	0.013	0.000	8.333E-05	2
Epicoccum nigrum	0.006	0.000	5.952E-05	5
Neoascochyta desmazieri	0.002	0.000	1.190E-05	1
Alternaria unclassified	0.039	0.000	2.500E-04	4
Aspergillus restrictus	0.001	0.000	5.952E-06	1
Aspergillus sydowii	0.019	0.000	5.060E-04	15
Penicillium corylophilum	0.234	0.000	2.935E-03	4
Penicillium roqueforti	0.008	0.000	8.929E-05	4
Crocicreas	0.003	0.000	1.786E-05	1
Phacidium unclassified	0.033	0.000	1.964E-04	1
Meyerozyma guilliermondii	0.007	0.000	4.762E-05	2
Clavispora lusitaniae	0.002	0.000	2.381E-05	3
Metschnikowia unclassified	0.008	0.000	5.595E-04	37
Metschnikowia chrysoperlae	0.031	0.000	2.226E-03	64
Metschnikowia pulcherrima	0.019	0.000	8.571E-04	27
Metschnikowia;s unidentified	0.014	0.000	4.464E-04	23
Wickerhamomyces anomalus	0.192	0.000	1.357E-03	9
Pichiaceae unclassified	0.001	0.000	5.952E-06	1
Dekkera unclassified	0.002	0.000	6.548E-05	9
Dekkera anomala	0.569	0.002	2.610E-02	103
Dekkera bruxellensis	1.000	0.834	6.256E-01	165
Dekkera custersiana	0.870	0.000	1.402E-02	26
Kregervanrija fluxuum	0.001	0.000	1.190E-05	2
Pichia kluyveri	0.003	0.000	9.524E-05	10
Pichia membranifaciens	0.674	0.000	1.057E-02	37
Issatchenkia orientalis	0.265	0.000	1.738E-03	8
Kluyveromyces marxianus	0.002	0.000	3.571E-05	4
Lachancea fermentati	0.103	0.000	2.280E-03	33
Lachancea quebecensis	0.008	0.000	1.548E-04	11

Appendix A8. (continued) Complete list of Fungal OTUs detected in Beer 2, their maximum relative abundance, mean relative abundance and rate of occurrence (No.) at greater than 0.1% out of 168 samples.

OTU ID	Maximum	Median	Mean	No. > 0.1%
Naumovozyma baii	0.001	0.000	5.952E-06	1
Saccharomyces bayanus	0.025	0.000	5.238E-04	25
Saccharomyces cerevisiae	0.994	0.010	1.908E-01	145
Torulaspora delbrueckii	0.048	0.000	5.952E-04	17
Torulaspora indica	0.012	0.000	7.143E-05	1
Zygosaccharomyces bailii	0.013	0.000	4.345E-04	22
Zygosaccharomyces bisporus	0.008	0.000	3.452E-04	18
Zygosaccharomyces kombuchaensis	0.002	0.000	5.357E-05	7
Zygosaccharomyces parabailii	0.007	0.000	1.131E-04	7
Zygosaccharomyces rouxii	0.015	0.000	8.929E-05	1
Candida parapsilosis	0.002	0.000	1.190E-05	1
Starmerella;s unidentified	0.084	0.000	5.054E-03	53
Hanseniaspora uvarum	0.041	0.000	2.363E-03	47
Hanseniaspora valbyensis	0.015	0.000	2.917E-04	14
Saccharomycetales unclassified	0.067	0.001	4.351E-03	91
Schizosaccharomyces pombe	0.004	0.000	1.131E-04	9
Beauveria unidentified	0.010	0.000	5.952E-05	1
Myrmecridium phragmitis	0.008	0.000	4.762E-05	1
Taphrina carpini	0.003	0.000	1.786E-05	1
Taphrinomycetes	0.032	0.000	1.905E-04	1
Symbiotaphrina buchneri	0.045	0.000	2.917E-04	3
Ascomycota unclassified	0.485	0.001	6.107E-03	87
Agaricomycetes unclassified	0.002	0.000	1.190E-05	1
Galerina triscopa	0.002	0.000	1.190E-05	1
Agaricales	0.027	0.000	1.786E-04	2
Corticium unidentified	0.001	0.000	5.952E-06	1
Tubulicrinis glebulosus	0.001	0.000	5.952E-06	1
Trametes versicolor	0.010	0.000	1.190E-04	5
Ceriporia lacerata	0.026	0.000	1.548E-04	1
Phlebia radiata	0.084	0.000	9.048E-04	3
Polyporus tubaeformis	0.020	0.000	1.190E-04	1
Heterobasidion abietinum	0.005	0.000	2.976E-05	1
Stereum hirsutum	0.023	0.000	1.369E-04	1
Pseudomicrostroma phylloplanum	0.002	0.000	5.952E-05	8
Malassezia globosa	0.031	0.000	1.845E-04	1
Malassezia restricta	0.008	0.000	2.976E-04	18
Rhodotorula mucilaginosa	0.005	0.000	5.952E-05	5
Sporobolomyces roseus	0.058	0.000	3.452E-04	1
Sporobolomyces ruberrimus	0.010	0.000	9.524E-05	4

Appendix A8. (continued) Complete list of Fungal OTUs detected in Beer 2, their maximum relative abundance, mean relative abundance and rate of occurrence (No.) at greater than 0.1% out of 168 samples.

OTU ID	Maximum	Median	Mean	Occurrence at > 0.1%
Cystofilobasidium ferigula	0.001	0.000	5.952E-06	1
Cystofilobasidium infirmominiatum	0.002	0.000	1.190E-05	1
Guehomyces pullulans	0.021	0.000	1.250E-04	1
Filobasidium chernovii	0.037	0.000	2.619E-04	5
Filobasidium wieringae	0.019	0.000	1.429E-04	4
Piskurozyma capsuligena	0.001	0.000	1.190E-05	2
Vishniacozyma victoriae	0.003	0.000	9.524E-05	11
Cryptococcus saitoi	0.001	0.000	5.952E-06	1
Cryptococcus neoformans	0.790	0.006	8.719E-02	130
Wallemia muriae	0.049	0.000	1.149E-03	8
Mucor circinelloides	0.041	0.000	2.560E-04	3

Appendix A9. Sanger sequencing identification of randomized Beer 2 yeast isolates.

Sample ID	Sample ID	Fill Year	Media	Species	Accession	Query Cover	%Identity
01	170810-01	2013	LMDA	D. bruxellensis	KY103320.1	99%	99%
02	170810-06	2013	YPD	D. bruxellensis	KY103319.1	98%	100%
03	170810-07	2013	Lysine	D. bruxellensis	KY103320.1	99%	99%
04	170810-10	2013	YPD	D. bruxellensis	KY103320.1	99%	99%
05	170810-13	2013	LMDA	D. bruxellensis	KY103319.1	97%	100%
06	180228-01	2013	LMDA	D. bruxellensis	KY103319.1	100%	100%
07	180228-01	2013	LMDA	D. bruxellensis	KY103319.1	99%	100%
08	180228-16	2013	LMDA	D. bruxellensis	KY103319.1	99%	100%
09	180228-17	2013	LMDA	D. bruxellensis	KY103319.1	98%	100%
10	180228-17	2013	LMDA	D. bruxellensis	KY103319.1	99%	100%
11	180228-18	2013	LMDA	D. bruxellensis	KY103319.1	99%	100%
12	180228-18	2013	LMDA	D. bruxellensis	KY103319.1	97%	100%
13	170810-23	2015	Lysine	D. bruxellensis	KY103319.1	97%	100%
14	170810-23	2015	YPD	D. bruxellensis	KY103319.1	97%	100%
15	170810-28	2015	LMDA	D. bruxellensis	KY103319.1	99%	99%
16	170810-28	2015	Lysine	D. bruxellensis	KY103319.1	100%	100%
17	170810-29	2015	Lysine	D. bruxellensis	KY103321.1	98%	99%
18	170810-31	2015	LMDA	D. bruxellensis	KY103319.1	99%	100%
19	170810-31	2015	YPD	D. bruxellensis	KY103319.1	97%	100%
20	180228-27	2015	LMDA	D. bruxellensis	KY103319.1	98%	100%
21	180228-27	2015	LMDA	D. bruxellensis	KY103319.1	97%	100%
22	180228-27	2015	LMDA	D. bruxellensis	KY103319.1	99%	99%

Appendix A9. (continued)Sanger sequencing identification of randomized Beer 2 yeast isolates.

Sample ID	Sample ID	Fill Year	Media	Species	Accession	Query Cover	%Identity
23	180228-27	2015	LMDA	D. bruxellensis	KY103319.1	99%	99%
24	180228-27	2015	LMDA	D. bruxellensis	KY103320.1	98%	99%
25	180228-28	2015	LMDA	D. bruxellensis	KY103319.1	99%	100%
26	180228-29	2015	LMDA	D. bruxellensis	KY103319.1	98%	99%
27	180228-30	2015	LMDA	D. bruxellensis	KY103319.1	98%	99%
28	180228-30	2015	LMDA	D. bruxellensis	KY103319.1	100%	100%
29	170810-16	2016	LMDA	D. bruxellensis	KY103319.1	100%	100%
30	170810-17	2016	LMDA	D. bruxellensis	KY103319.1	99%	100%
31	170810-17	2016	Lysine	P. membranifaciens	KY104622.1	100%	92%
32	170810-17	2016	YPD	D. bruxellensis	KY103319.1	98%	100%
33	170810-17	2016	YPD	D. bruxellensis	KY103320.1	99%	99%
34	170810-19	2016	YPD	D. bruxellensis	KY103319.1	97%	100%
35	170810-20	2016	YPD	D. bruxellensis	KY103320.1	99%	99%
36	170810-20	2016	YPD	D. bruxellensis	JQ327829.1	97%	99%
37	170810-21	2016	LMDA	D. bruxellensis	KY103319.1	99%	100%
38	180228-32	2016	LMDA	D. bruxellensis	KY103319.1	97%	99%
39	180228-32	2016	LMDA	D. bruxellensis	KY103319.1	97%	99%
40	180228-33	2016	LMDA	D. bruxellensis	KY103319.1	100%	100%
41	180228-33	2016	LMDA	D. bruxellensis	KY103319.1	98%	100%
42	180228-42	2016	LMDA	D. bruxellensis	KY103319.1	97%	100%
43	180228-42	2016	LMDA	D. bruxellensis	KY103319.1	97%	99%
44	170810-18	2016	Lysine	Too short, no significant similarity found			
45	PCR Control			S. cerevisiae	MH380196.1	99%	99%

Appendix A10. Complete list of bacterial OTUs detected in Beer 2, their maximum relative abundance, mean relative abundance and rate of occurrence (No.) at greater than 0.1% out of 70 samples.

OTU ID	Maximum	Median	Mean	No. > 0.1%
Acidobacteria	0.006	0.000	8.571E-05	1
Corynebacterium	0.084	0.000	3.043E-03	16
Dermacoccus	0.003	0.000	4.286E-05	1
Geodermatophilaceae	0.010	0.000	1.429E-04	1
Agrococcus	0.006	0.000	8.571E-05	1
Micrococcaceae	0.006	0.000	8.571E-05	1
Arthrobacter	0.001	0.000	1.429E-05	1
Propionibacterium	0.032	0.000	1.629E-03	14
Parabacteroides	0.011	0.000	1.571E-04	1

Prevotella	0.007	0.000	1.000E-04	1

Appendix A10. (continued) Complete list of bacterial OTUs detected in Beer 2, their maximum relative abundance, mean relative abundance and rate of occurrence (No.) at greater than 0.1% out of 70 samples.

OTU ID	Maximum	Median	Mean	No. > 0.1%
Hymenobacter	0.001	0.000	1.429E-05	1
Ornithobacterium	0.029	0.000	4.143E-04	1
Chloroflexi, Ellin6529	0.015	0.000	2.143E-04	1
Cyanobacteria	0.005	0.000	1.143E-04	2
Streptophyta	0.067	0.000	1.800E-03	5
Anoxybacillus	0.001	0.000	1.429E-05	1
Bacillus	0.223	0.000	8.443E-03	26
Geobacillus	0.131	0.001	1.679E-02	38
Virgibacillus	0.024	0.000	3.429E-04	1
Listeriaceae	0.159	0.000	3.429E-03	20
Planococcaceae	0.012	0.000	2.286E-04	4
Sporolactobacillus	0.010	0.000	1.429E-04	1
Staphylococcus	0.067	0.000	4.471E-03	26
Lactobacillales	0.001	0.000	1.429E-05	1
Facklamia	0.001	0.000	1.429E-05	1
Enterococcus	0.102	0.000	3.571E-03	23
Lactobacillus	0.999	0.003	1.249E-01	49
Lactococcus	0.286	0.000	1.309E-02	17
Streptococcus	0.047	0.000	2.300E-03	15
Clostridium	0.461	0.000	3.010E-02	15
Thermoanaerobacterium	0.067	0.000	9.857E-04	2
Caldicellulosiruptor	0.032	0.000	7.429E-04	5
Thermoanaerobacteraceae	0.008	0.000	2.857E-04	5
Thermoanaerobacter	0.026	0.000	4.143E-04	2
Caulobacter	0.026	0.000	3.714E-04	1
Mycoplana	0.002	0.000	7.143E-05	3
Bradyrhizobiaceae	0.017	0.000	3.571E-04	4
Methylobacteriaceae	0.014	0.000	4.143E-04	3
Paracoccus	0.017	0.000	4.429E-04	3
Acetobacteraceae	0.216	0.000	1.040E-02	33
Acetobacteraceae	0.054	0.000	6.800E-03	31
Acetobacter	1.000	0.857	6.328E-01	70
Gluconacetobacter	0.612	0.000	4.630E-02	16
Gluconobacter	0.004	0.000	1.000E-04	2
Rhodospirillaceae	0.048	0.000	6.857E-04	1
mitochondria	0.028	0.000	4.429E-04	2
Novosphingobium	0.065	0.000	1.200E-03	4

Appendix A10. (continued) Complete list of bacterial OTUs detected in Beer 2, their maximum relative abundance, mean relative abundance and rate of occurrence (No.) at greater than 0.1% out of 70 samples..

OTU ID	Maximum	Median	Mean	No. > 0.1%
Sphingobium	0.210	0.001	2.064E-02	36
Sphingomonas	0.071	0.000	3.043E-03	14
Sphingopyxis	0.058	0.000	1.143E-03	2
Burkholderia	0.005	0.000	1.286E-04	3
Comamonadaceae	0.001	0.000	1.429E-05	1
Comamonadaceae	0.017	0.000	4.286E-04	4
Delftia	0.002	0.000	2.857E-05	1
Tepidimonas	0.004	0.000	5.714E-05	1
Oxalobacteraceae	0.009	0.000	1.714E-04	4
Oxalobacteraceae	0.033	0.000	8.143E-04	6
Ralstonia	0.708	0.000	3.603E-02	19
Neisseriaceae	0.039	0.000	1.029E-03	3
Neisseria	0.004	0.000	5.714E-05	1
Vogesella	0.006	0.000	1.714E-04	2
Dechloromonas	0.006	0.000	1.714E-04	3
Myxococcales	0.011	0.000	1.571E-04	1
Myxococcales	0.021	0.000	3.571E-04	2
Enterobacteriaceae	0.020	0.000	4.857E-04	7
Enterobacteriaceae	0.066	0.000	2.000E-03	19
Haemophilus	0.003	0.000	1.143E-04	4
Acinetobacter	0.074	0.000	1.686E-03	9
Enhydrobacter	0.022	0.000	3.286E-04	2
Moraxella	0.008	0.000	2.714E-04	4
Pseudomonas	0.041	0.000	1.343E-03	5
Opitutus	0.006	0.000	8.571E-05	1
Deinococcus	0.085	0.000	3.529E-03	16
Meiothermus	0.031	0.000	1.343E-03	13
Thermus	0.142	0.000	5.800E-03	26

Appendix A11. Sanger sequencing identification of all Beer 2 bacterial isolates.

Sanger ID	Sample ID	Media	Fill Year	Species	Accession	Query Cover	%Ident
01	170810-01	LMDA	2013	Staphylococcus capitis	FJ380955.1	100%	100%
02	170810-01	LMDA	2013	Staphylococcus epidermidis	KU922146.1	100%	99%
03	170810-23	LMDA	2015	Staphylococcus epidermidis	MH732996.1	100%	100%
04	170810-23	Lysine	2015	Bacillus velezensis	MH373544.1	100%	99%
05	170810-23	YPD	2015	Bacillus subtilis	KT945024.1	100%	99%
06	170810-32	LMDA	2015	Staphylococcus epidermidis	KU922146.1	100%	99%
07	170810-32	Lysine	2015	Bacillus subtilis	KY515422.1	100%	99%
08	180228-31	LMDA	2015	Acetobacter pasteurianus	HM357879.1	100%	99%
09	180228-32	LMDA	2015	Acetobacter fabarum	MH633719.1	100%	99%
10	180228-32	LMDA	2015	Acetobacter fabarum	KX150620.1	100%	100%
11	180228-32	LMDA	2015	Acetobacter fabarum	MH549126.1	100%	99%
12	180228-32	YPD	2015	Acetobacter fabarum	MH633719.1	100%	99%
13	180228-32	YPD	2015	Acetobacter fabarum	MH549126.1	100%	99%
14	180228-34	LMDA	2015	Acetobacter malorum	MH424758.1	100%	99%
15	180228-34	LMDA	2015	Acetobacter malorum	MH424758.1	100%	99%
16	180228-34	LMDA	2015	Acetobacter malorum	FJ157243.1	100%	100%
17	180228-34	LMDA	2015	Acetobacter malorum	KX131150.1	99%	99%
18	180228-34	YPD	2015	Acetobacter pomorum	CP023189.1	100%	99%
19	170810-16	YPD	2016	Acetobacter pomorum	CP023189.1	100%	99%
20	170810-17	Lysine	2016	Acetobacter pomorum	CP023189.1	100%	99%
21	170810-18	LMDA	2016	Acetobacter pasteurianus	MF179549.1	100%	99%
22	170810-18	LMDA	2016	Acetobacter pasteurianus	HM357879.1	100%	99%
23	170810-18	LMDA	2016	Acetobacter pomorum	CP023189.1	100%	100%
24	170810-18	Lysine	2016	Staphylococcus epidermidis	KU922146.1	99%	99%
25	170810-18	Lysine	2016	Acetobacter pomorum	CP023189.1	100%	99%
26	170810-18	Lysine	2016	Acetobacter pomorum	CP023189.1	100%	100%
27	170810-18	YPD	2016	Acetobacter pomorum	CP023189.1	100%	99%
28	170810-18	YPD	2016	Acetobacter pomorum	CP023189.1	100%	99%
29	170810-18	YPD	2016	Acetobacter pasteurianus	MF179549.1	100%	99%
30	170810-18	YPD	2016	Acetobacter pomorum	CP023189.1	100%	100%
31	170810-18	YPD	2016	Acetobacter pasteurianus	MF179549.1	100%	100%
32	170810-19	YPD	2016	Acetobacter pomorum	CP023189.1	100%	100%
33	170810-20	Lysine	2016	Staphylococcus capitis	KT719458.1	100%	99%
34	PCR Control			Acetobacter aceti	CP014692.1	100%	100%

Appendix A12. Full heatmap of fungal OTUs at greater than 0.1% abundance in Beer 3 clustered according to overall microbiome similarity and barrel type.