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Phylogenetic Diversity of the Bacterial Communities in Craft Beer

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Phylogenetic Diversity of the Bacterial
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Abstract

The craft beer industry is increasing in popularity in the United States. The craft brewing process typically does not use a pasteurization step, therefore the boiling process is the primary critical control step. Any microorganisms introduced after boiling, or those that are not killed during boiling, are likely to participate in fermentation and persist in the final product. Previous culture-based studies have isolated bacteria and yeast from craft beers at specific time points, but little research has been done on the process as a whole. The objectives of this research are to (1) track bacteria development throughout the brewing process and (2) compare these results to environmental samples. Two craft breweries in Arkansas were used. Five beer styles were sampled, each for two batches. Swab samples were taken of the mash tun, boil kettle, and the fermentation tank. Samples of the raw material include the grain, hops, and any additional ingredient added during the process. Beer samples were taken at each stage of the brewing process, starting at the mash tun and ending with the final product. High throughput sequencing using the Illumina MiSeq was used to identify bacterial DNA. Results show that there were few differences between the breweries. Equipment swab microbiota was similar in bacterial composition to the beer microbiota associated with that process. Most of the bacteria found in the malt is typically isolated from soil and the environment. The boiling step reduced some bacteria abundance, but some bacteria were introduced after this step. Filtering had no impact on reducing microbial abundance. This research provides the first extensive microbiota research of craft beers in Northwest Arkansas, allows craft brewers to have a better understanding of the microbiology of their product, and will initiate further research about the role that microorganisms play on the quality of the beer.

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1. Overview of craft brewing specificities and potentially associated microbiota

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1. Introduction

Beer is the third most popular drink worldwide after tea and coffee, and is the most preferred alcoholic beverage (Swot, 2016). In contrast with wine or other spirits, beer (including non-alcoholic types in countries that forbid alcohol consumption and sales) is produced and easily available commercially in most countries (Jernigan, 2000). China produced the largest volume of beer in 2014 at 44,933,300 kiloliters with the United States ranked at number two, producing 22,547,400 kiloliters (Anonymous, 2015a). Beer consumption per capita ranges from less than 50 liters to more than 150 liters in Ireland and Czech Republic. The United States consumes around 75 liters per capita. (Alcázar et al., 2002). Overall, the brewing industry is a global business dominated by a few multinational companies and thousands of smaller producers, producing tens of billions of liters and generating several hundred billion dollars in global revenues (Anonymous, 2015b; Jernigan, 2009).

Most beer consumed in the United States is produced by large, industrial breweries which rely on very stringent practices to limit spoilage risk and variation in the final product (Vrellas and Tsiotras, 2015). While the economic benefits of these industrial processes are numerous, many consumers have been drawn to craft beer due to their novel organoleptic properties. Increased demand for original beer products has resulted in a drastic increase in the home-brewing and microbrewery markets (Aquilani et al., 2015).

As a fermented beverage, beer inherently relies on microbial metabolism for production. Traditionally, the yeast *Saccharomyces cerevisiae* is almost always the primary fermentation microorganisms involved in ethanol and carbon dioxide production (Lodolo et al., 2008). It is also known that *S. cerevisiae* imparts sensory characteristics through a variety of other metabolic pathways (Cocolin et al., 2011). Industrial beer production processes, especially pasteurization of

the final product, are purported to reduce the presence of other microbes to less than detectable levels (Jeon et al., 2015). On the other hand, craft brewing processes are known to only limit the development of such microbes. Common beer spoilage microbes are relatively well described, but very little is known about the arguably more diverse and variable microbiota associated with craft beer.

The objective of this chapter is to review current literature about the craft brewing industry from a microbiology perspective. The craft brewing specificities will be delineated in relation with the potential for uncontrolled microbes' establishment. Potential sources of contamination and strategies to reduce microbial load will be presented. Furthermore, the types of microorganisms and their detection methods will be discussed. This review will emphasize the limited knowledge on craft beer microbiology and the need for further research.

2. Evolution of beer production and craft brewing emergence

2.1 Historical perspective

Modern beer is an alcoholic beverage made from four main ingredients: malted grain, water, hops, and yeast; which has been perfected through time (Meussdoerffer, 2009). The origin of fermented beverages is unclear, and it is argued that they may have been consumed by nomadic Neolithic populations. Between the years 2000 and 4000 B.C., the Egyptians and Sumerians developed the process for brewing beverages that more closely resemble modern beer, though a variety of fermented beverages based from different food were independently developed by other civilizations (Correa-Ascencio et al., 2014; McGovern et al., 2004; Paul Ross et al., 2002). Beer brewing remained largely artisanal until the industrial revolution, with a few European countries (Germany, Belgium, and England) taking the lead in mastering brewing processes and developing

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specific styles. With the discovery of America, German immigrants brought with them lager beer recipes (Meussdoerffer, 2009). Lager was the preferred beer style because of its light color and flavor (Olson et al., 2014). Many people also distrusted the quality of the water, therefore beer was the preferred beverage (Beuchat, 1978).

Although the discovery of yeast as the fermenter didn't occur until 1860, fermentation was used as early as 700 BC in China to preserve foods and beverages (McGovern et al., 2004; Sicard and Legras, 2011). Early fermented beverages are assumed to have utilized airborne or plant yeast (Meussdoerffer, 2009). The yeast *Saccharomyces cerevisiae* was and currently is used to ferment beer, but also wine and bread. The history of the domestication of yeast is not completely known. This is mainly because very few yeast strains have been isolated from nature (Sicard and Legras, 2011). This leads to the common belief that *Saccharomyces cerevisiae* has been domesticated as a result of mankind's use of the yeast in fermented alcoholic beverages and bread (Fay and Benavides, 2005).

2.2 Craft brewing emergence

As mentioned, lagers were originally the beer of choice in the United States dating back to its discovery. However, today there has been an increase in popularity of beers with rich flavors and aromas that utilize new ingredients (Aquilani et al., 2015; Canonico et al., 2014). The market share of craft beers has been gaining on that of international and national breweries, with most attention on microbreweries and brew pubs (Murray and O'Neill, 2012). Craft beer does not have a specific definition or clear boundaries, but the Brewer's Association describes a craft brewery as small, independent, and traditional (Anonymous, 2016). A craft brewery has an annual production of no more than 6 million barrels of beer. No more than 25% of the brewery can be owned by an

alcohol industry member that is not a craft brewer. Finally, a craft brewery is traditional in that most of the beverage alcohol by volume comes from beer brewed with traditional or innovative ingredients and is fermented by yeast (Anonymous, 2016). The addition of fruits, herbs, and spices can transform ordinary beer into specialty beer, along with other flavorings and fermentable substrates (Aquilani et al., 2015). Craft breweries are focused on the production of traditional ales, lagers, and even beer styles that do not fit in any of the two main styles; and compete on the market on the criteria of high quality and diversity (Marongiu et al., 2015).

The craft brewing industry has become increasingly popular in the United States just in the last several years. Craft breweries in the United States are seeing large growth in production, sales, brewing capacity, and employment (Marongiu et al., 2015; Anonymous, 2016). There was a 16.2% increase in the number of craft breweries nationally from 2015 to 2016, with a total of 5,234 in 2016. Craft breweries account for 98.7% of the total number of breweries in the United States, as of 2016 (Anonymous, 2016).

In Arkansas, as of 2015, there are twenty-six craft breweries with 1.2 breweries per 100,000 21 and older adults. This number increased drastically from six breweries in 2011. About 24,623 barrels of craft beer are produced in the state per year. This trend is similar among the rest of the states in the country, and it appears to be continually increasing as craft beer becomes more popular (Anonymous, 2016). A beer is considered craft when it is produced in small breweries and follows traditional recipes without pasteurization. Pasteurization is usually a practice only found in commercial production breweries (Jeon et al., 2015). This makes craft beer more vulnerable to microbial contamination than industrial beers (Giovenzana et al., 2014).

3. **Craft brewing process specificities**

In this section, the emphasis will be on the differences between craft brewing process compared to industrial-scale processes which are well described and reviewed elsewhere (Beuchat, 1978; Priest and Campbell, 2003).

3.1 Raw ingredients and mashing

Beer has commonly been produced from barley and less often from wheat. However, novel consumer trends have led to the evaluation of different grain types for beer production. To develop gluten-free beers (Hager et al., 2014), sorghum (Agu and Palmer, 1998; Owuama, 1997) and rice (Teramoto et al., 2002) are now used by several craft breweries. Other grains or seeds used by craft brewers include rye, millet, spelt, and buckwheat (De Meo et al., 2011; Phiarais et al., 2010). While rhizosphere microbiota (Lindow and Brandl, 2003; Bulgarelli et al., 2015) and plant pathogens (Beattie and Lindow, 1995; Goodwin et al., 2011) have been studied extensively, there is only limited indirect knowledge on the commensal microbiota associated with cereals and grain crops (Sultan et al., 2016; Duniere et al., 2017; Granzow et al., 2017). It is suspected that grain associated microbes may end up to a certain extent in final beer products, but this has not been demonstrated.

The grains used contain large amounts of starches and sugars which will later serve as nutrients for brewing yeast and sometimes bacteria (Mascia et al., 2014). Starches are converted into fermentable sugars and polysaccharides through germination enzymes released through grain germination, the main step of malting. Malting consists of steeping (increasing humidity), germination, and kilning (heat treatment to dry malted grains). While industrial beer relies on standard malting processes, a staggering diversity of malts is now produced and made available to home and craft brewers (Anonymous, 2015c). The most important variation in malts' processing

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are the intensity of kilning, which is sometimes described as roasting when very high heat is used (Hämäläinen and Reinikainen, 2007). Steeping and kilning can greatly influence grain-associated microbiota dynamics in composition.

It is also known that barley varieties will strongly influence fermentation and final product properties (Hager et al., 2014; Kihara et al., 1998) and possibly indirectly microbiota. Malts are milled and/or crushed by the malting company or on site by brewers, and diluted in hot water to become the mash. Milling and crushing may influence grain-associated microbiota, though they should be relatively resilient to coarse mechanical treatments (Manthey et al., 2004).

3.2 Sparging and boiling

After the sugars are made available, the sweet liquid, also known as wort, is separated from the spent grains. During this process, wort is pumped through to the boil kettle as the spent grains are sparged, or sprayed with hot water, to extract any other dissolved substances (Beuchat, 1978). The wort is then boiled at a temperature between 103 and 110°C for approximately one hour (Ormrod, 1986). Hops are added during the boil, at different times depending on the desired use of the hops. Boiling isomerizes the hops, causes proteins to coagulate for easy removal, concentrates the liquid, causes Maillard reactions to enhance the color and flavor of the wort, and drives off sulfur componds which could lead to a cooked corn or cabbage aroma in beer if not removed (Beuchat, 1978; Vriesekoop et al., 2012). Boiling can also drastically reduce the microbial load in wort to undetectable levels. After boiling, the wort is cooled and microorganisms can increase in abundance due to its high sugar content and lower temperature (Kim et al., 2015).

The cooled wort is transferred to a fermentation tank in which yeast is added and left to ferment for several days up to one week. Yeast is often re-pitched in a craft brewery, meaning the yeast from one batch of beer is used to ferment a future batch. Re-pitching yeast is generally limited to less than ten times to avoid yeast quality degradation (Jenkins et al., 2003). Yeast in better condition will produce less fusel alcohols and more sulfite than old or contaminated yeast (Guido et al., 2004). The practice of re-pitching yeast can cause deterioration by cross contamination with other cultures or wild microorganisms, causing genetic changes to the original culture or causing physiological changes due to stress (Lodolo et al., 2008). While genetic drift and eventual speciation of novel strains/species could be expected, it has been reported that *Saccharomyces* strains used for brewing are genetically stable (Powell and Diacetis, 2007). Pitching rate of yeast also affects final quality of beer. Higher pitching rates allow for an increased rate of fermentation, but it creates large quantities of yeast biomass. Excessive pitching rates can degrade the health of the yeast culture (Kucharczyk and Tuszyński, 2015).

Some beers are filtered for clarity before packaging, depending on the brewer's preferences and style. Filtering can be done using cellulose fibers or particles of diatomite as a medium (Gan et al., 2001; Niemsch and Heinrich, 2000). Isinglass can also be used as a fining agent to clarify beer (Walker et al., 2007). Simple filtering removes flocculant yeast but has no effect on reducing bacterial load (Sensidoni et al., 2011). However, more elaborate alternative methods, such as high hydrostatic pressure, have shown potential to reduce microbial load in beer as efficiently as pasteurization (Buzrul et al., 2005). Industrial breweries may use pasteurization to sterilize beer, and fill the beer into sterilized containers (Dilay et al., 2006). In a craft brewery there is usually not a pasteurization process, though. Unpasteurized beer has a more appealing and fresh taste to

modern consumers (Asano et al., 2007), but this makes craft beer more prone to bacterial spoilage. For example craft brewers have reported loss of canned beers due to gas production of unidentified microbes (personal communication).

4. Beer parameters and impact on microbial load

4.1 Beer styles defined

Beer is classified in numerous styles based on their properties including alcohol content, color, bitterness, clarity, flavor, and ingredients. Alcohol content is measured in alcohol by volume (ABV). ABV is calculated using the original and final gravity of the beer. Beer ABV typically ranges from 3 to 14% when normal fermentation is used, but the most commonly consumed styles don't exceed 6%. Alcohol content has traditionally been considered an inherent antimicrobial, however it has become known that several microbes are able to tolerate low to medium alcohol content (Ingram, 1990). Alcohol tolerance in *Saccharomyces* is a trait that has been considered beneficial and sought after, especially in winemaking (Fujita et al., 2006).

Bitterness is measured in International Bitterness Units, or IBUs. IBU is calculated using the percentage of alpha acids, the utilization of iso-α-acids based on the strength of the wort (original gravity), the boil time, and the volume of the recipe (Anonymous, 2012). A higher alpha acid hop will result in a more bitter beer and a longer boil will also increase IBUs. Hops provide antimicrobial properties, to be described in detail in section 4.2.

The color of a beer can be measured by the Standard Reference Method, or the SRM scale. The colors correspond to a number ranging from 1 to 40. The rating is based on the absorbance of turbidity free beer in a ½ inch cell at a wavelength of 430 nm (Anonymous, 1958). A light beer

such as a lager will have an SRM of 2 to 4. A dark imperial stout beer has an SRM of 40 (Strong and England, 2015).

Gruit (a mixture of herbs and spices) previously was the distinguishing factor of ales from other fermented beverages, whereas today beers are categorized as ales and lagers by the yeast used for fermentation. Ales are brewed with top-fermenting yeast, typically *Saccharomyces cerevisiae* strains, with fermentation conducted at 20°C (Beuchat, 1978). Common styles in craft breweries include: American Pale Ale, Wheat beers, India Pale Ale (often abbreviated as IPA), American Brown Ale and Belgian Golden Ale. (Strong and England, 2015).

Saccharomyces pastorianus (or *Saccharomyces carlsbergensis*) is generally accepted as the fermentation yeast used for lagers and fermentation is carried out at 13°C. Lager yeasts congregate at the bottom of fermentation tanks and result in a lighter, cleaner flavor than ales (Beuchat, 1978). Lager and ale yeasts have specific fermentation temperature ranges and an increase in temperature could deteriorate the yeast, reduce foam stability, decrease pH, and reduce bittering compounds (Solgajová et al., 2013).

Ales and lagers are the most common beer styles today, however, there are several other different variations of the beverage. For example, lambic beers are those that use spontaneous fermentation, rather than inoculation with a yeast strain. These beers are fermented and aged anywhere from one to three years in oak barrels and are native to Belgium. The unique flavors of this style are fruity and sometimes sour (De Keersmaecker, 1996). During the first couple months, *Enterobacteriaceae* are the most prominent bacteria, but disappear later in fermentation. The first yeast to appear, *Kloeckera*, occurs within the first couple of weeks after wort boiling. This yeast is quickly taken over by *Saccharomyces,* which perform the main fermentation over the next several months (Van Oevelen et al., 1977). Finally, *Brettanomyces* takes over as the last main yeast

to impart characteristic flavors and aromas (Van Oevelen et al., 1976). Although lambic beers have a diverse microbiota at the beginning stages of fermentation, the diversity and quantity of microorganisms stabilizes by 18 months (Spitaels et al., 2014).

In the United States, a similar lambic-style beer is being brewed called the American coolship ale. This style is an attempted replica of a lambic beer utilizing spontaneous fermentation and using the same production practices as the brewers in Belgium of lambic beers. The successions found in American coolship ales closely mimic those of the lambic beers, with *Enterobacteriaceae* being the starting bacteria and *Lactobacillaceae* taking over. *Saccharomyces* is the starting yeast with the disappearance of it coinciding with the growth of *Brettanomyces* (Bokulich et al., 2012a).

Barley and wheat are the most common grains used in brewing, but several other fermented beverages are made using different starch sources. Although these beverages are described elsewhere (Blandino et al., 2003), there are notable characteristics of the microbiota of some. For example, 'cauim' is a fermented beverage produced in South America made from cassava root. This beverage starts as a porridge and ferments for a couple of days. Typical microbiota of the 'caium' beverage is predominately lactic acid bacteria and species belonging to *Enterobacter*, *Serratia*, *Pseudomonas*, and *Streptococcus* genera. Yeast begin playing a role in this product's fermentation after the first day (Almeida et al., 2007). Chicha beer is another traditional South American beer produced from corn, cassava, or palm. The bacterial community of chicha beer consists mainly of *Lactobacillus fermentum*, *Lactococcus lactis*, *Leuconostoc mesenteroides*, and *Streptococcus salivarius*, with other bacteria species being less abundant (Freire et al., 2016). A similar microbiota has been shown for a rice-based Brazilian beer, with *Bacillus*, *Enteroccocus*, *Leuconostoc*, and *Lactobacillus* being in highest abundance (Puerari et al., 2015).

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4.2 Antimicrobial properties

There are several factors that contribute to the preservation of beer which have been studied extensively. These characteristics include intrinsic factors such as pH and ethanol concentration, the use of hops, and sanitation in the brewery.

The two most important intrinsic antibacterial properties of beer are pH and ethanol. Most pathogenic microorganisms prefer a more neutral environment and beer ranges in pH between 3.8 and 4.7 (Jespersen and Jakobsen, 1996). Lower pH values allow for acidification of cells, destroys enzyme systems, and reduces nutrient uptake (Vriesekoop et al., 2012). Alcohol is usually found in a concentration of 0-8% alcohol by volume (ABV) (Jespersen and Jakobsen, 1996). Most microorganisms do not tolerate high ethanol concentrations because it can inhibit cell growth and metabolism (Fujita et al., 2006).

Carbon dioxide that is produced by the yeast and added by the brewers can be an antimicrobial hurdle. Carbon dioxide is typically found in a concentration of 0.5% weight by volume (Jespersen and Jakobsen, 1996). Carbon dioxide helps to provide an anaerobic environment, decreases pH, and has a direct inhibitory effect on cell growth (Vriesekoop et al., 2012). A reduction in CO_2 concentration in beer can ultimately reduce shelf life (Brocklehurst and Lund, 1990).

Fermentation yeast are often competitive with other microorganisms, thus eliminating the contaminants from the final product. There are only trace amounts of substances for yeast nutrition, so the yeast will consume the sugars before any other bacteria or yeast can (Sakamoto and Konings, 2003; Vriesekoop et al., 2012).

Hops were originally used in beer because of their bitterness. However, it was eventually discovered that hops were a big factor in controlling spoilage. Hops contain alpha acids which isomerize into iso-α-acids during boiling, in concentrations of 17-55 mg, of which impart bitterness and antimicrobial properties (Jespersen and Jakobsen, 1996). Hops dissipate the transmembrane pH gradient to prevent spoilage organism growth in beer, acting as protonophores (Simpson, 1993). However, hops have a bactericidal effect on Gram positive bacteria only (Shimwell, 1937). Some lactic acid bacteria have developed resistance to hops and can grow in beer (Richards and Macrae, 1964; Sakamoto and Konings, 2003).

4.4 Heat treatment and sanitation

The overall brewing process affects the microbiological status of beer. Because mashing is a temperature intense process, most microorganisms present in the raw materials are unlikely to be transferred in large numbers to the final product (Couto et al., 2005; Kim et al., 2015). However, aerobic bacteria, lactic acid bacteria, coliforms, _Pseudomonas_, and yeast can still be present in low numbers after the mashing process (O'Sullivan et al., 1999). The boiling process also uses high heat, so pathogens that could be present before boil are not likely to remain post-boil. In one study where _Salmonella_ Typhimurium, _Staphylococcus aureus_, _Listeria monocytogenes_, and _Bacillus cereus_ were inoculated in wort before boiling showed that all pathogens were reduced to undetectable levels by culture dependent methods (Kim et al., 2015). Many craft breweries often utilize additional ingredients such as fruit juices and flavoring ingredients in innovative beers. These extra ingredients are often heat treated before being added. Many of the fermentation tanks in small breweries require multiple batches to fill. Because of

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this, the yeast is pitched with the first batch of wort, to protect the beer from bacterial growth (Priest and Campbell, 2003).

The microbiological safety of beer also depends on proper cleaning and sanitation practices in the brewery. Cleaning uses a detergent and removes soil from the substrate, whereas disinfection refers to the destruction of microorganisms to reduce the microbial load to a level that is not harmful to health or quality. Equipment in the brewery is made of stainless steel and the equipment is closed off to the environment (Priest and Campbell, 2003). The equipment is also designed for easy cleaning. For example, the fermentation tanks have a cone shape at the bottom, which is mainly used for harvesting yeast after fermentation, but is also helpful in removing sanitizer and cleaning agents straight out of the bottom (de Oliva Neto et al., 2004). Breweries utilize cleaning-in-place (CIP), cleaning loops, and tank recirculation systems (Bremer et al., 2006; Chen et al., 2012). Cleaning is usually done immediately after use, while sanitization occurs immediately before use to be the most effective. Disinfectants that are used should be compatible with plant materials, tolerant of hard water, non-foaming, nonirritating, economical, and have a low environmental impact. Hot caustic soda is the most common cleaning agent, used in a cycle of pre-rinse, cleaning with caustic, and a post- rinse (Manzano et al., 2011). Little research has been done on the effectiveness of current brewery cleaning practices on reducing/eliminating microbial contamination.

Even with these control measures to prevent spoilage in beer, some bacteria and yeast proliferate in the beverage imparting off-flavors and aromas. This can be desirable or undesirable depending on the style. As mentioned previously, lambic beers thrive on the diverse microbiota and depend on it to provide unique flavors and aromas (Van Oevelen et al., 1977). However, in

typical ales and lagers, any microorganisms present in the final product are considered contaminants.

5. Sources of beer microbiota

It is generally accepted that beer is safe of pathogens, however, it is not uncommon for beer to be colonized with undesirable microorganisms. Sources of contamination can be from the raw materials, the process, and from the brewery environment.

5.1 Raw materials

The raw materials used in craft brewing include water, hops, malted grain, and yeast. Due to an increased market for special beers (Yeo and Liu, 2014), some additional ingredients can be used to add unique flavors and aromas to the beer such as fruit additives, spices, and flavoring ingredients. The microbiota of the ingredients is likely to influence the microbiota of the final product.

Barley is the most commonly used grain for brewing beer. In fact, 10% of the world barley crop is used for the production of beer (Kaur et al., 2015). The barley grain is covered in a husk that is normally inhabited by *Eubacteria, Actinomycetes*, filamentous fungi, and yeasts (Priest and Campbell, 2003). The grain is malted, milled, and mashed for the starch to convert to sugar to be used for fermentation. Lactic acid bacteria are naturally present on barley, so they can be found throughout the brewing process (Giusto et al., 2006). Cereal grains and fruits used in beer production can be contaminated in the field, during storage, or malting by mycotoxin-producing-fungi (Kaaya and Kyamuhangire, 2006). Spores can be found in the air when the conditions for temperature, moisture, and oxygen are favorable. The spores then grow and produce mycotoxins.

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Mycotoxins are generally thermostable and can remain in crops when all signs of the fungi itself have been removed (Inoue et al., 2013).

Craft breweries use pre-malted grain as the starting point for brewing. This grain can be stored for months before use, which can affect the microbiota of the beer. At high water activities (0.8-0.9), visible mold can develop on the malted grain after just one month of storage. At slightly lower water activities (0.693), visible mold will appear after three months of storage. Malted grain can last up to 12 months of storage at low water activities below 0.529 (Hoff et al., 2014).

Many toxins have been known to metabolize into less toxic compounds or decrease in concentration due to adsorption of the spent grain during brewing. Zearalenone and patulin are two of the mycotoxins that were metabolized during the beer fermentation process, posing little risk to contamination in the final product. Aflatoxins B1 and B2, along with Fusarium1 and Orchatoxin A decreased in residual concentrations to less than 20% during the mashing process when inoculated artificially into the raw materials. This led to the disappearance of the toxins throughout the rest of the brewing process, showing that they are only of small health risk in beer (Inoue et al., 2013). Although these particular mycotoxins were not a threat to the final product in this study, other toxins can be of concern.

During the brewing process, Aflatoxin B1 and Fumonisin B1 present on barley can contaminate beer. Although fermentation has antimicrobial effects, it does not decrease the amount of Fumonisin B1 when the toxin is found in the barley. Clarification processes fail to reduce amount of the toxin as well. Fumonisin has a high solubility in water and is relatively stable to heat treatments, therefore it can be found in finished beer products when it is present on the raw material (Pietri et al., 2010).

Grain is not the only source of unwanted microorganisms in the raw ingredients. Fruits are sometimes used in brewing because they can be a source of natural yeasts that ancient brewers utilized for fermentation (McGovern et al., 2004; McGovern, 2009). However, in the controlled craft brewing atmosphere today, these natural yeasts can be unwanted contaminants. Yeasts such as *Geotrichum candidus*, *Hanseniaspora guilliermondii*, *Hanseniaspora uvarum*, *Metschnikowia pulcherrima*, *Pichia kluyveri*, *Pichia kudriavzevii*, and *Saccharomyces cerevisiae* are commonly isolated from fruits (Vadkertiova et al., 2012).

Spices can also contain microorganisms that could persist in the brewing environment. Spices can be contaminated from the environment by unsanitary conditions or when hygienic handling is not carried out. High microbial levels in spices are not acceptable to use in ready-to-eat foods, and they can be a source of intoxication when added to foods in which pathogen growth is favorable (Sospedra et al., 2010).

Although the brewing process includes many control steps to eliminate microorganism growth (mashing, boiling, and fermentation), some flavor additives are often added at the final step of craft brewing in the bright tank. Fruit juices, honey, and other flavoring ingredients can be added to craft beer to provide a unique flavor profile, but also their own foodborne microbes (Janisiewicz et al., 2014; Abdelfattah et al., 2016). Some of these may be heat treated before adding, however, the increased amount of sugar could increase the overall susceptibility of the beer to spoilage. After the beer is finished aging in the bright tank, it usually is immediately packaged, thus if there are any microorganisms present in the ingredients added here, it will persist in the finished product.

5.2 Brewery environment

The last major source of contamination in a brewery is the brewery environment. Air, pipework, and equipment can all be potential sources. The microbiota of a brewery can be quite diverse and most equipment shows distinct microbial clustering based on function. For example, fermenter samples cluster around *Bacillaceae* whereas wort prep is associated with *Enterobacteriaceae*, *Leconostocaceae*, *Candida*, *Pichia*, and *Rhodotorula* found in one study (Bokulich et al., 2015). Sources of surfaces' microbiota were predicted using the Bayesian technique source tracker (Knights et al., 2011). Grains contributed mostly to the mash, pre- boil, and post- boil stage microbiota whereas hops contributed to fermentation areas and equipment. Outdoor air, soil, human skin, saliva, and feces were all shown to play a very minor contribution to the microbiota of the brewery environment (Bokulich et al., 2015).

Seasonality plays a role in determining microorganism presence in the brewery as well. *Saccharomyces cerevisiae* is primarily found during fermentation and packaging areas in the fall, however, in the spring and summer the yeast is found throughout the entire brewery. *Candida santamariae* was found clustered around the mash and boil steps in fall, but in the cellar during the spring and summer months (Bokulich et al., 2015).

Contaminants within the brewery will play a role on the microbiota of the product during the process. Thermotolerant bacteria and yeast that are present during mashing and boiling could attach and survive on these vessels in a biofilm (Fielding et al., 2007) and thus contaminate other batches. During the mash process, airborne contaminants can drift from the mill to fermentation vessels, bright tanks, and packaging equipment. Microorganisms can also blow in from outside depending on the set up of the brewery (Priest and Campbell, 2003). Contaminated wort could

further infect the pipes that carry the wort throughout the brewery. Leaking or contaminated heat exchangers could cause an unsanitary work environment in the brewery (Bokulich et al., 2015).

6. Types of microbes associated with beer

The craft brewing industry faces spoilage contamination problems similar to those of early brewers in the nineteenth century (Priest and Campbell, 2003). Many different microorganisms can be introduced during the brewing process and cause spoilage. Spoilage in a brewery is defined as any organism not introduced intentionally (Bokulich et al., 2012b). Some microorganisms present may not influence the flavor or taste of the final product, but they can retard the progress of fermentation (Takahashi et al., 2015).

The types of microorganisms found depend on the beer style and process. The microbial community of fermenting beer is often diverse and bacteria could survive in it. In a study designed to trace microbial diversity in a pilot scale brewing process using next generation sequencing and quantitative polymerase chain reaction detected that the bacterial population decreased during boiling, increased at early fermentation, slightly increased at late stage fermentation, and slightly increased again by filtration (Takahashi et al., 2015). These spoilage organisms can be divided into bacteria and fungi, and bacteria further dived by phylum.

6.1 Firmicutes

Firmicutes are a phylum of Gram positive bacteria. Gram positive bacteria are classified by their thick single layer of peptidoglycan, which stains purple by performing a Gram stain. The two classes of Firmicutes commonly reported in beer are Bacilli and Negativicutes.

Lactic acid bacteria belong to the Bacilli class and can cause spoilage characterized by silky, turbid aspect and/or a buttery flavor caused by diacetyl production. *Lactobacillus brevis* is the most common beer spoiler. It is generally hop tolerant and grows at 30°C and between pH 4 and 5 (Sakamoto and Konings, 2003). *Lactobacillus lindneri* has been isolated from lagers and grows at 19°C (Priest and Campbell, 2003). Other spoilage strains include *L. maloefermentans, L. paracbuchnerie* (Farrow et al., 1988) *L. collinoides*, and *L. paracasei* subsp. *paracasei* (Hollerova and Kubizniakova, 2001). *Streptococcus lactis* can produce slime and gas in final products (Banwart, 1979). Spoilage is also characterized by an acidic off-flavor (Storåards et al., 1998).

Pediococci are another genus among Bacilli. In beer, *P. damnousus* and *P. inopinatus* are spoilers that produce diacetyl (Dobson et al., 2002). Other *Pediococci* that have been found in breweries include *P. acidilactici*, *P.dextrinicus*, and *P. halophilus* (Collins et al., 1990). These bacteria can adapt to the brewery environment. *Pediococcus damnousus* is also very resistant to the iso-α-acids in hops. Acid formation and the buttery aroma of diacetyl formation is associated with contaminant strains of *Pediococcuss* in beer. Ropiness is also an unfavorable characteristic caused by *Pediococcus* (Priest and Campbell, 2003). Although *Pediococcus* is responsible for beer spoilage, the incidence of this has decreased recently due to improved sanitation conditions (Sakamoto and Konings, 2003).

The third most common Gram positive bacteria that causes spoilage in beer is *Leuconostoc*. This is a heterofermentative cocci or oval, short rod. They are found in pairs or short chains. The natural reservoir for *Leuconostoc* is vegetables and fruits, but they can occur rarely in breweries (Priest and Campbell, 2003). In beer, they are also diacetyl producers (Speckman and Collins, 1968).

Pectinatus and *Megasphaera* are two beer spoilers among the Negativicutes. *Pectinatus frisingensis* has been isolated from pitching yeast. *Pectinatus* can grow in beer with ethanol concentrations lower than 5% ABV and in pH above 4.3 (Jespersen and Jakobsen, 1996; Lee et al., 1980). *Megasphaera* causes cloudiness and unpleasant odors. Both genera also form butyric acid, but are sensitive to alcohol production and low pH. Because modern brewery practices include reduction of oxygen to as low as possible, these aerobic bacteria are not as prominently found in beer today (Jespersen and Jakobsen, 1996).

6.2 Proteobacteria

A major phylum of Gram negative bacteria is the Proteobacteria. Gram negative bacteria, rather than a thick layer of peptidoglycan, have a multilayered envelope that contains a thin layer of peptidoglycan and a hydrophobic outer membrane (Priest and Campbell, 2003). Acetic acid bacteria are a large group of Gram negative bacteria that are rod shaped and can convert ethanol into acetic acid. They can grow in and spoil beer, but only under aerobic conditions (Sakamoto and Konings, 2003). Acetic acid bacteria are used in the food industry to make vinegar, soft drinks, and alcoholic beverages (Camu et al., 2007; Wu et al., 2012). In general, acetic acid bacteria spoil beer by producing acid, off-flavors, turbidity, and ropiness. They are resistant to hops, low pH, and ethanol. This group of bacteria is further divided into *Acetobacter* and *Gluconobacter*. *Acetobacter* can oxidize ethanol into acetate, CO_2, and water. *Gluconobacter* is similar to *Acetobacter* but reduces ethanol to acetic acid (Priest and Campbell, 2003) and is responsible for ropy texture in beer (Banwart, 1979).

Another class of Gram negative spoilers is Enterobacteriaceae, which are facultative anaerobic rods. They are indirect beer spoilers because they are not normally found in the finished

product but can cause negative characteristics if present throughout the process. Characteristics of spoilage by Enterobacteriaceae include fermentation retardation or acceleration and off-flavor and aroma production (Prest et al., 1994).

Enterobacteriaceae include *Citrobacter*, *Enterobacter*, *Hafnia*, *Klebsielia*, *Obesumbacterium*, *Proteus*, *Rahnella*, and *Serratia* that have all been isolated from breweries. *Obesumbacterium proteus* is a bacterium that has only been isolated from brewery environments and is often found in pitching yeast (Koivula et al., 2006). This Enterobacteriaceae can result in a beer with a high final specific gravity and pH and can give fruity odors or flavors (Keevil et al., 1979). *Rahnella aquatilis* can grow well in hopped or unhopped wort. It also survives the brewing process when the wort has normal gravity (Hamze et al., 1991). *Rahnella aquatilis* can increase levels of acetaldehyde and methyl acetate and can give a fruity, milky, or sulfur taste and aroma (Priest and Campbell, 2003). *Hafnia protea* is found strictly in breweries (Priest et al., 1974).

Other characteristics of anaerobic Gram negative spoilage include acetic acid and propionic acid production. (Priest and Campbell, 2003). Anaerobic bacteria incidence has increased due to the practice of non-pasteurized beer and improved technology to reduce oxygen in the brewery (Jespersen and Jakobsen, 1996).

6.3 Other bacterial phyla

Although Firmicutes and Proteobacteria are the most common of the brewery phyla, some others have been detected. *Micrococcus*, belonging to the Actinobacteria phylum, have been reported in breweries (Sakamoto and Konings, 2003). Using next generation sequencing to detect microorganisms in beer, other phyla besides Firmicutes and Proteobacteria have been identified in

beer. Acidobacteria, Actinobacteria, Bacteroidetes, Chlamydiae, and Planctomycetes can all be present in low numbers throughout the brewing process (Takahashi et al., 2015).

6.4 Fungi

The third category of spoilage microorganisms found in beer is contamination fungi, which include yeast and mold. Before the boiling process, yeasts are of little concern to the brewery because yeast are not thermotolerant and cannot survive even the briefest of boiling procedures. There are four separate groups that wild yeasts can fall into: fermentative contaminants, killer yeasts, wrong type of culture yeast, or nonfermentative yeasts (Priest and Campbell, 2003).

Contamination yeast sometimes grow slightly faster than the pitching yeast and will take-over the pitching yeast culture through successive fermentations. In a study that aimed at detecting wild yeast in lager breweries, wild yeasts were detected in 41 out of 101 brewery yeast samples (van der Aa Kühle and Jespersen, 1998). Killer yeast attack sensitive yeast cultures and become the dominant yeast in fermentation. It is unlikely that these yeast will be detected in a brewery until the killer yeast has completely taken over the pitching yeast. Contamination yeast cultures can affect the rate of fermentation, final attenuation, and the production of flavor by-products.

Typically, contamination yeasts are divided into *Saccharomyces* and non-*Saccharomyces* wild yeasts. Non-*Saccharomyces* wild yeasts include a variety of species. *Brettanomyces* produces acetic acid (Coton et al., 2006; Gray et al., 2011) and has a high level of resistance to carbonation (Ison and Gutteridge, 1987). *Pichia* and *Williposis* can produce esters in beer. The most common characteristics of a spoiled beer by yeast is off-flavor, turbidity due to the nonflocculent properties of wild yeast, production of surface film, and granular deposits (Priest and Campbell, 2003). *Candida* is another contamination yeast that can produce fruity off-flavors and turbidity (Banwart,

1979). The fermentation yeast can also be considered a contaminant after the beer is filtered (Manzano et al., 2011).

Mold and toxins can sometimes be found on raw materials and negatively impact the barley. Usually, fungi causes deterioration of grain which results in discoloration, decreased germination, formation of mycotoxins, and mustiness (Banwart, 1979). Mycotoxins are produced by Fusarium and are fairly heat stable. They are common contaminants of corn, wheat, sorghum, and fruits (Shale et al., 2012). More information on the types of mold and toxins found on the raw material can be found in the sources of contamination section.

6.5 Beer as a pre-probiotic food

There are a few health benefits from beer drinking, which may contribute to the increase in popularity. For example, beer can reduce the risk of cardiovascular disease (Grønbæk et al., 1995), reduce blood cholesterol levels, and reduce the risk for dementia (Ruitenberg et al., 2002). Beer can also be a source of minerals, vitamins, fiber, and polyphenols which are good for human health (Yeo and Liu, 2014). Future research could involve adding functional ingredients in beer such as chitosan, which would inhibit the growth of lactic acid bacterial strains without affecting the viability of yeast (Gil et al., 2004). Using the beverage as a vehicle for delivering probiotics would be another example of furthering the functionality of beer (Yeo and Liu, 2014).

7. Microbiota detection techniques

In the quality control department of breweries, if they have one, the main tasks are to confirm sterility, determine that the microbiological count does not exceed the limit to cause spoilage, and examine for presence of specific organisms (Priest and Campbell, 2003). Analysis

in the brewery is predominately retrospective, meaning that the brewers typically expect a quality product and the objective is to confirm this.

7.1 Culture dependent methods

Culturing is a method of using specific media to grow and enumerate bacteria. It is the preferred method used by craft breweries to detect spoilage organisms, however it does not provide specificity and sensitivity (Jespersen and Jakobsen, 1996; Manzano et al., 2011). Species-specific media has been developed to detect beer microorganisms (Manzano et al., 2011), but there is not one single media that can be used to detect all beer spoilage specific microorganisms (Jespersen and Jakobsen, 1996). MRS (de Man, Rogosa and Sharpe) agar can be used to detect *Lactobacillus* and *Pediococcus* bacteria and is often supplemented with cycloheximide to prevent yeast and mold growth. The detection of *Pectinatus* and *Megasphaera* can be accomplished with a beer enrichment step and using one or more types of media such as Universal Beer Agar (UBA), Nachweismedium für bierschädliche Bakteriën agar (NBB), and Raka-Ray media (Sakamoto and Konings, 2003). UBA has been used to isolate *Enterobacter agglomerans* from lager beer (van Vuuren et al., 1978). Some media, along with detecting the desired microorganisms, can also detect non-spoilage species. Although there are compounds that can be added for selectivity, this could require longer incubation times (Sakamoto and Konings, 2003).

It is understood that less than 1% of microbiota in high diversity environments can be cultured using these traditional methods (Amann et al., 1995; Torsvik et al., 1990). Cultivating the microorganisms can also be a long and tedious process (Manzano et al., 2011). It can take a week or more for bacteria to form visible colonies on agar plates or to develop turbidity in broths (Sakamoto and Konings, 2003). Detection is also difficult because microorganisms present in beer

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are found in low numbers (Jespersen and Jakobsen, 1996). Product that is found to have been contaminated with a spoilage organism has likely already been released for sale, which can lead to recalls and economic damages to a brewery (Sakamoto and Konings, 2003). Therefore, there is a need for the development of faster methods to detect microorganisms in beer. Other reasons for a need of improved methods of detection include an increased awareness of the consumer in the area of product quality, tightened government regulations, increased competition among brewers (in particular, craft breweries of the same region), growing market volumes for non-pasteurized beer in cans and bottles, more low or non-alcoholic beers, increasing numbers of flavored sweetened type beverages, and technological advancements (Priest and Campbell, 2003).

7.2 Culture Independent Methods

Several molecular methods have been identified for the use of detecting spoilage organisms in beer. Molecular methods involve analysis of deoxyribonucleic acid (DNA), ribonucleic acid (RNA), proteins, or lipids. Nucleic acids are informational macromolecules that have defined sequences which serve as blueprints for the cells (Priest and Campbell, 2003). Polymerase chain reaction (PCR) methods have been developed for the use of fast detection and can be used to detect *Megasphaera* and *Pectinatus* in beer (Satokari et al., 1998). Real time PCR can be used for early detection and quantification of contaminant yeast species, such as *Dekkera*, during fermentation and testing in final beer and beverage products (Gray et al., 2011). Anaerobic beer spoilage *Clostridia* bacteria have been targeted and detected in beer using real time PCR methods (Juvone et al., 2008). Random Amplification of Polymorphic DNA polymerase chain reaction (RAPD PCR) has been used to develop primers and genetic markers

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to distinguish between beer spoilage and non-spoilage strains of *Lactobacillus* (Fujii et al., 2005).

Temporal Temperature Gradient Electrophoresis (TGGE) and Denaturing Gradient Gel Electrophoresis (DGGE) are different fingerprinting methods that can be performed after PCR (Manzano et al., 2011). The principle behind gel electrophoresis is that DNA will migrate through a gel under the influence of an electric field (Priest and Campbell, 2003). DGGE and TGGE separates sequences of DNA according to different melting conditions and forms a gradient on a polyacrylamide gel (Fischer and Lerman, 1983; Muyzer and Smalla, 1998). TGGE and DGGE have been used to compare microbiota of beer before and after a cleaning process, and also to distinguish different strains of *Saccharomyces* (Manzano et al., 2011). Terminal restriction fragment length polymorphism (TRFLP) is also a fingerprinting method that is used for rapid profiling of complex microbial populations. This method has been used to compare barley microbiota from different geographical regions (Kaur et al., 2015). Microarrays can be used as a fast, sensitive, and specific method to identify different bacterial species in a sample. For example, one study used this method to detect viable spoilage bacteria in beer (Weber et al., 2008).

Whole Genome Sequencing (WGS) gives the most complete understanding of the genetic information of a single microorganism, such as the beer fermentation yeast *Saccharomyces cerevisiae*, and can provide the most in-depth comparisons between related species (Chen et al., 2016). High throughput sequencing (HTS) has been proven to profile highly complex and diverse communities from a wide variety of sources, such as those of fermentation products (Reuter et al., 2015). Sequencing techniques have the accuracy of a using digital system. HTS uses publicly available databases which are continually enhanced (Priest and Campbell, 2003). Sequencing

methods have been used to determine potential hop resistance genes in order to develop new methods of detecting beer spoilage *Lactobacilli* (Sami et al., 1997).

In a study that evaluated the microbial diversity in a brewing process by culture dependent and independent methods, culture dependent methods detected 88 genera from the most diverse sample of beer. Almost all bacteria that were recovered belonged to Proteobacteria or Firmicutes. However, more than 190 different genera belonging to several phyla were detected using culture independent methods. The most predominate genera belonged to the Firmicutes and Proteobacteria phyla (Takahashi et al., 2015). The specificity, sensitivity, and time reduction of molecular methods is preferred over the cost efficient and ease of culturing techniques.

8. Conclusions

Beer is a microbiological product, but a diversity and abundance of microbes is typically considered a defect. In contrast with industrial brewing, craft brewing is characterized by less stringent processes to limit microbial load. A few limited studies have confirmed that craft or micro brewed beer harbor relatively diverse and abundant fungal and bacterial microbiota. These observations challenge the common belief that the combination of antimicrobial properties such as alcohol content, acidity and the use of hops should significantly limit microbial load, especially in the final product.

There is a need for further research studies to better understand the normal and detrimental impacts of microbes in the craft brewing industry. The microbiota of malted grain and hops that are ready for use by breweries is virtually unknown. Although some research has been done on the flora of raw barley, brewers do not know the microbiological status of pre-malted grain and the

potential differences in microbiota imparted by different varieties, grain types, or malting processes. Hops are known to be inhibitive of Gram positive bacteria, but it is theoretically possible that they are harboring their own distinct microbiota. There is also little information on the potential for seeding of brewery-resident microbes to brewing products at different production stages. Finally, the impact of the different brewing steps on microbiota dynamics is largely unknown with the exception of the intuitive microbial load reduction incurred by wort boiling.

For economic reasons, small-scale brewers are limited to culture-dependent tests to confirm the safety of beer for consumption and potentially track back the origin of recurrent spoilage. However, such methods are not sufficient to study the full microbiota profiles and dynamics along the brewing process, which may play a role in the organoleptic characteristics and shelf life of the beer. There is a clear need for more culture-independent studies, especially using HTS to explore the role of this microbiota. The few studies conducted on specific beer styles have demonstrated that very diverse bacterial and fungal communities are present along the brewing process. However, the sources of microbes and the parameters driving microbial dynamics in typical craft brewing are unknown.

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2. Bacteria development throughout the brewing process and bacteria persistence on brewing equipment

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Abstract

The craft brewing industry has recently seen a surge in popularity. Beer is a microbiological product relying on stringent cleaning practices, intrinsic antimicrobial hurdles (pH, ethanol, and hops), and intense heat processing (mash and boil). The potential beer microorganisms have been studied, but not at a craft brewery or during the brewing process. The objectives of this research are to track bacteria development throughout the brewing process and observe the brewery environment for sources of microorganisms. Samples were collected at two local breweries of five beer styles, each of two distinct batches. DNA was extracted from the raw material, wort and finished beer product, and environmental swab samples. The bacteria (16S) v4 region of the DNA was amplified and high throughput sequencing was used to analyze the DNA found in the samples. Sequences were analyzed with Mothur. Data were analyzed using Past 3.15 for NMDS with Bray-Curtis index. Kruskal Wallis and Mann-Whitney tests with $p<0.05$ were used to determine statistical significance. Results showed an abundance of environmental bacteria found in the malt and mash samples. A small percentage of hop samples contained bacterial DNA and were diverse in genera. Mashing and boiling had some effect on the bacteria present in the samples, but filtering had no significant effect on reducing microbial abundance. Spoilage bacteria were found in different stages throughout the brewing process. Overall, these were in low abundance. Final samples had a composite microbiota of four main phyla: Actinobacteria, Firmicutes, Bacteroidetes, and Proteobacteria. Different genera in the final product were a combination of bacteria originating in the raw materials, bacteria introduced through the process, and bacteria potentially originating from human contact.

Keywords: bacteria, DNA sequence, brewing process, high throughput sequencing

Introduction

Bacteria are ubiquitous in food produced for human consumption (Forsythe, 2000). Furthermore, bacteria are primary players in most fermented foods with only beer, bread, and wine to a lesser extent produced through yeast fermentation (Bourdichon et al., 2012). In beer products, bacteria are generally considered to be a flaw and many processes are employed to prevent bacterial proliferation, typically by pasteurization in mass-produced beer (Manzano et al., 2011). However, the emerging trends of home and craft brewing rely on less stringent bacterial control and even in a few cases, attempts to favor controlled growth of lactic acid bacteria for different organoleptic properties (Canonico et al., 2014; Aquilani et al., 2015). While bacterial spoilage of beer is well known and reviewed elsewhere (Jespersen and Jakobsen, 1996; Sakamoto and Konings, 2003), there is limited information on the commensal bacterial microbiota that is likely to be associated with craft beer.

Craft brewers are faced with similar microbiological issues as brewers in the 19th and early 20th centuries (Priest and Campbell, 2003), with better access to efficient sanitation procedures. Therefore, the most common spoilage bacteria reported by craft brewers are lactic acid bacteria, acetic acid bacteria, (Sakamoto and Konings, 2003), and Enterobacteriaceae (Koivula et al., 2006). Bacterial spoilage can occur at different brewing steps and viable bacteria can be found in the final product leading to the production of undesired metabolites and organoleptic properties (Fielding et al., 2007). Cereals and grains used for brewing are known to harbor a fungal microbiota (Priest and Campbell, 2003; Kaaya and Kyamuhangire, 2006) but little is known on the bacterial composition. Water and pitching yeast should harbor a limited bacterial load (Furukawa et al., 2011) and hops are known for their antibacterial properties (Simpson, 1993). It has been shown that brewery resident bacteria can be transferred to the wort at different stages, a desired trait in

the brewing of coolship ale and lambics (Van Oevelen et al., 1976; Bokulich et al., 2012). Since bacteria are resilient to environmental stresses, it is known that they can persist and form biofilms in vessels used for brewing (Fielding et al., 2007). Therefore, bacteria can be seeded from different sources, and the wort and beer bacterial microbiota may contain plant-associated and environmental taxa.

In the brewing industry, especially at the small-scale level of craft breweries, microbiological quality testing relies on culture dependent methods. However, it is well known that such methods do not provide the sensitivity and specificity necessary to detect and identify every bacterium present in beer (Jespersen and Jakobsen, 1996; Manzano et al., 2011). Indeed, in one study comparing culture dependent and independent methods, 454 pyrosequencing revealed a large number of genera not detected by culture methods that were never reported in beer before (Takahashi et al., 2015). While bacteria are unlikely to inhibit yeast fermentation, there are numerous examples where bacteria impart different organoleptic properties: malolactic fermentation (Davis et al., 1985) or the sour, fruity flavors produced by bacteria in lambic and gueuze beers (Van Oevelen et al., 1977; De Keersmaecker, 1996). In addition, there is growing evidence that food fermentations are characterized by bacterial successions (Nie et al., 2015; Piao et al., 2015; Van Oevelen et al., 1977) due to the changes in physiochemical properties; but whether bacterial successions occur in the brewing process is unknown.

The objectives of this research are to analyze the bacterial microbiota throughout the brewing process and determine the contributions of raw materials and vessel-associated microbiota to the bacterial content of the final product. Samples from different styles and different batches were collected from two local breweries.

Materials and Methods

Experimental design

For this experiment, five beer styles from two craft breweries in the Fayetteville-Springdale-Rogers Metropolitan Statistical Area (Arkansas, United States) were studied. Samples of an India Pale Ale (IPA) and a Belgian Golden Ale (BGA) were collected at Brewery 1; samples of a Pale Ale (PA), Brown Ale (BA), and Hoppy Wheat (HW, unfiltered) were collected at Brewery 2. Samples were collected along the brewing process for two distinct batches of each style. Samples of the raw materials, malts and other grains, and hops used for each beer styles were collected. During the brewing process a sample was collected during the mash, pre- boil, post-boil, after cooling, fermentation day 0, mid fermentation, pre- filter, post- filter steps, and from the final package. Environmental swabs samples were collected with two repetitions on each vessel from the mash tun, the boil kettle, and the fermentation tanks. The alcohol by volume (ABV), International Bitterness Units (IBU), and Standard Reference Method (SRM) for color were reported by the breweries and the final pH was measured using pH strips for each style (Table 2.1).

Sample collection

Raw material samples, processed hop pellets and pre- malted grain, were aseptically collected in sterile 50 milliliter centrifuge tubes and stored at room temperature until analysis. Wort and beer samples were collected in sterile 50 milliliter centrifuge tubes and were stored at 4°C if analysis was to be conducted within 24 hours, or -20 °C if analyzed later. The samples taken of the raw materials, from the mash process, pre- boil, post- boil, after cooling, and fermentation day 0 were taken on the initial day of sample collection. Samples were placed on ice for the duration of sample collection. Fermentation day 2/3, pre-filter, post-filter and final

product samples were collected when those steps were conducted at the breweries. Final product samples were collected in the containers chosen by the brewery, whether it was cans, bottles or kegs. Keg samples were pumped directly from the tap into the centrifuge tubes. Final products from cans were cleaned with an ethanol wipe, opened, and poured aseptically into the 50 ml centrifuge tube. The pH of liquid samples was measured before microbial DNA extraction.

Swab samples were collected using the Environmental Sampling Kit (Puritan Diagnostics, United States) polyester tipped swabs with a neutralizing buffer solution directly before the beer came into contact with the vessel. A square 25cm x 25cm swab stencil template was made (Ronnqvist et al., 2013). Before swabbing the surface, scissors and the template were cleaned with ethanol. The swab was removed from the buffer and the template was placed in the vessel. The vessel was swabbed diagonally in the template on one side of the cotton swab. The cotton swab was rotated and swabbed diagonally in the opposite direction on the inside of the template. The tip of the cotton swab was cut into a tube containing 0.1 g of 0.1 mm diameter and 0.1 g of 0.5 mm diameter zirconia/silica beads. InhibitX buffer from the QIAamp Fast DNA Stool Mini Kit (Qiagen, Germany) was then added to the swab/bead mixture. The samples were stored on ice until further analysis.

Microbial DNA extraction

All DNA from raw material samples (hops, malted grain, and coriander) was extracted using the PowerPlant Pro DNA Isolation Kit (Mo Bio Laboratories, United States) following the manufacturer's protocol with few modifications. For step 1, the maximum 50 mg of the sample was weighed into the provided bead tubes. The samples were homogenized using the vortex method. For the elution step, 50 µL of Solution PD7 was used instead of 200 µL.

Centrifuge tubes containing ~ 50 ml of wort or beer were centrifuged at 4,000 xg for 15 minutes at 4°C (Bokulich et al., 2012). The supernatant was discarded and pellets from samples collected on or after fermentation day 2 were subject to a decanting step. The decanting step consisted of resting the tubes containing the DNA pellets in a fume hood with the air blowing and the cap off for thirty minutes to evaporate any residual ethanol. A sterile bead mixture consisting of 0.1 g of 0.1 mm diameter and 0.1 g of 0.5 mm diameter zirconia/silica beads was added to the pellet. One milliliter of InhibitX Buffer (Qiagen, Germany) was added to the bead and pellet tube. The mixture was bead-beated with a FastPrep-24^{TM} (MPBiomedicals, United States) three times, each for twenty seconds. The DNA was extracted using the QIAamp Fast DNA Stool Mini Kit (Qiagen, Germany) per the manufacturers protocol. However, instead of eluting with 200 µL of ATE buffer, 50 µL was used.

Environmental swabs were extracted using the QIAamp Fast DNA Stool Mini Kit (Qiagen, Germany). The swab, buffer, and bead mixture was bead-beated as described previously. The swab was then removed from the buffer and DNA was extracted following the manufacturers protocol. Elution was also performed using 50 µL of ATE buffer.

The concentration of all DNA samples were measured using the Nanodrop (Thermo Scientific, United States). Samples with a concentration over 30 ng/ µL were diluted to ~30ng/ µL.

Universal polymerase chain reaction

A univirsal PCR targeting 16S genes were used to amplify the DNA and confirm the presence or absence of bacteria (16S). A mastermix was made using 12.5 µL of GoTaq (Promega, United States), 7.5 µL of sterile, nuclease free water, 1 µL of the forward primer 8F, and 1 µL of the reverse primer 1541R (Carbonero et al., 2014). Three microliters of DNA were added to 22 µL

of the mix for a total PCR reaction of 25 µL. The PCR reaction consisted of 30 cycles of denaturation at 98°C for 30 seconds, annealing at 55°C for 30 seconds, and elongation at 72°C for 1 minute using the Eppendorf thermocycler.

The DNA was separated by molecular size using gel electrophoresis. An agarose gel consisting of 2% agarose (Fisher BioReagents, United States) mixture in TAE 1X buffer (50X from Amresco, United States) and 1 µL of SYBR Safe DNA stain (EDVOTEK, United States) was used. The gel was placed in the chamber surrounded by TAE 1X buffer. Five µL of the PCR reaction were loaded on a gel electrophoresis with conditions of 120 Volts and 300mAmps for 45 minutes. Target amplicon length was 1,500 base pairs.

Index polymerase chain reaction

Index PCR was performed according to the approach developed by Kozich et al. (2013) using primers covering the V4 region: 515F (5′-GTGCCAGCMGCCGCGGTAA-3′) and 806R (5′-GGACTACHVGGGTWTCTAAT-3′) (Caporaso et al., 2011); with addition of pad, link and index leading to 16 forward primers and 24 reverse primers allowing up to 384 samples to be assigned a specific index combination.. The mastermix consisted of 22 µL of Accuprime (Invitrogen, United States), 2 µL of each primer (each reaction having a different combination of primers), and 3 µL of DNA for a total reaction of 27 µL. Denaturation was done at 95°C for 30 seconds, annealing at 55°C for 30 seconds, and elongation at 72°C for one minute using the Eppendorf thermocycler. Random reactions were chosen from the PCR plate to load on an agarose gel to verify that the amplification was successful. Gel electrophoresis was performed as described in the Universal PCR protocol. Target amplicon size was 250 base pairs.

Amplicon libraries preparation and quality control

The SequalPrep Normalization Plate (96) Kit (Invitrogen, United States) was used to purify and normalize the PCR product reactions from the index PCR. The protocol was followed per the manufacturer's instructions with minor modifications. During the elution step, instead of incubating for 5 minutes the plate was left to incubate overnight. The purified DNA was pooled the following morning.

The two bacterial pools were analyzed for length of the amplicon fragments on a TapeStation (Agilent, United States). The concentrations of the pools were determined using quantitative PCR and the PerfeCta NGS Library Quantification Kit for Illumina (Quanta Biosciences, United States) according to the manufacturers protocol. This qPCR reaction involved making a master mix and using five standards to create a standard curve. Efficiency of the standards was 92.71%. The qPCR reaction was 35 cycles of denaturation at 95°C for 15 seconds, annealing at 60°C for 20 seconds, and extension at 72°C for 45 seconds. A final melting curve was added at the end of the reaction.

Sequencing

Both amplicon pools were diluted to 0.083 nM with 0.2 N fresh NaOH and HT1 buffer according to the MiSeq System Guide. Denatured DNA was combined with 20% PhiX control v3. Final concentration of the reagent and library was 20 pm. Index primer, Read 1, and Read 2 sequencing primers along with the samples were loaded into a v3 Illumina MiSeq reagent cartridge (Kozich et al., 2013).

Sequence and statistical analysis

The sequencing reads were downloaded from the Illumina Basespace server in Fastq files format. The sequences were demultiplexed in Read 1 and Read 2 with approximately 250 base pairs in length. The sequencing analyses were carried out using SILVA database as reference for assignation of operational taxonomic units (OTUs) with 97% of identity. Further analysis was done using Mothur 1.39.5 pipeline (Schloss et al., 2009). Non-metric multidimensional scaling (NMDS) plots and analysis of similarities (ANOSIM), both based on the Bray-Curtis index, were obtained using PAST 3.15. In addition Kruskal Wallis and Mann-Whitney tests were performed to detect significant differences in bacterial taxa between samples and time points (by convention, differences were considered significant when $p<0.05$).

Results and Discussion

The purpose of this research was to provide a survey of the bacterial populations that could be present in craft breweries using HTS. Table 2.2 shows the total number and percentage of samples that were positive for bacterial DNA based on 16S PCR. Overall, 56% of the samples were positive. Only 15% of environmental swabs contained bacterial DNA, which could be a result of effective cleaning practices in the brewery or extraneous methodology issues. The majority of malted grain samples were positive, supporting the hypothesis that plant-associated microbiota may play an important role in seeding communities in subsequent stages. Five out of 28 hop samples also contained bacterial DNA, which is in line with a previous report (Bokulich et al., 2015). Bacteria were found all along the brewing process with some intriguing findings: more positive samples were obtained from post- boil samples than pre- boil ones. A low number of samples taken during active fermentation (Fermentation day 2) were positive, which may be

due to an over-representation of *Saccharomyces* DNA in the DNA extract inhibiting successful 16S rRNA gene sequence amplification. The vast majority of post fermentation samples, including final products, were positive, suggesting the presence of viable bacteria in craft beer, since it is unlikely that extracellular DNA left from bacterial cell death are likely to be degraded by yeasts' DNAses or denatured by high temperature (Nielsen et al., 2007).

Amplicon sequencing yielded high quality sequences for 125 samples, for a total of 6,681,355 reads (52,198±80,973). Notably, malts and other grain samples yielded very significant higher numbers of reads.

1. Microbiota profiles by sample type

1.1. Raw materials

The raw materials in brewing consist of water, malted grain, hops, and yeast. For the purpose of this study, the malted grain and hops were analyzed for their microbiota. The microbiota of the hops and malts are separate and distinct (Figure 2.2).

1.1.1. Malts

Looking at clusters of data can allow an overview of the differences. Though the malts divided by Brewery 1 and Brewery 2 show no significant differences, there does appear to be a core microbiota shared between the two (Figure 2.3). The NMDS plot for the four types of malt show a very distinct microbiota for the wheat malt and smaller, somewhat linked groups for the barley malts (Figure 2.4).

Overall, the malts bacterial microbiota were dominated by Proteobacteria (50%), followed by Actinobacteria, Firmicutes and Bacteroidetes (Figure 2.5), a profile resembling

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reported plant associated bacterial microbiota (Bulgarelli et al., 2013). Specifically, several

genera were found to be more prominent in the malted grain samples. *Arthrobacter* was the

prominent Actinobacteria (Figure 2.6), and is found primarily in soil (Conn and Dimmick, 1947;

Busse, 2016), so the abundance of this bacteria in malt is not surprising. *Brachybacterium* is

another bacterium found in the environment (Singh et al., 2016; Liu et al., 2014b) that was

present predominately in malt samples. This bacterium has also been isolated from corn steep

liquor (Takeuchi et al., 1995). Other malt-borne Actinobacteria included *Corynebacterium*,

unclassified Microbacteriaceae, *Brachybacterium*, and *Sanguibacter*. *Corynebacterium* include

species involved in diphtheria disease (Bolt et al., 2010), however plant-associated species are

typically only plant pathogens (Vidaver, 1982). *Sanguibacter* has been reported as a non-

pathogenic blood resident (Pascual et al., 1996) as well as to be present in marine samples, but

not from plants (Huang et al., 2005). Of note, a significantly greater abundance of

Brachybacterium was found in malt samples from Brewery 2 compared to Brewery 1 (Figure

2.7).

Other bacteria that were found in high abundance in the malt samples were *Bacteroides*,

Sphingobacterium, and *Prevotella* (Figure 2.8). *Prevotella* (Ueki et al., 2007) and

Sphingobacterium (Choi and Lee, 2012) are found in the environment and in the malt samples.

Bacteroides is known as being part of a healthy gut microbiome (Eckburg et al., 2005) but is

found in malt samples, more predominant in Brewery 2, although not significant.

DNA from *Pseudomonas*, *Stenotrophomas*, *Xanthomas*, and *Methylobacterium* were

recovered from malt samples as well (Figure 2.9). These bacteria are also found in high

abundance in the environment (Jun et al., 2016; Dow et al., 2009; Jones et al., 2011; Madhaiyan

and Poonguzhali, 2014; Tani and Sahin, 2013). Between the two breweries, Brewery 1 had a

significantly greater abundance of *Pseudomonas* and *Stenotrophomas*; Brewery 2 had a

significantly greater abundance of *Methylobacterium*.

Four different types of specific malts were used in the beer styles: pale ale base malt,

pilsner malt, wheat malt, and acidulated malt. Some of the malts had significantly different

microbiota from others. *Arthrobacter* was higher in abundance in the pilsner malt than the other

three malts (Figure 2.10). However, *Weissella* was in lower abundance in the pilsner malt than

the other three (Figure 2.11). *Weissella* is found in many different food fermentations such as

chocolate (Snauwaert et al., 2013) and cheese (Settanni et al., 2012). *Wautersiella*, a rare human

pathogen (Velden et al., 2012; Giordano et al., 2016), was found to be significantly lower in

abundance in the acidulated malt, which coincides with its optimum pH for growth of 7.0-7.5

and the fact that an acidulated malt will have a lower pH (Zhang et al., 2014) *Pedobacter,*

another environmental bacterium (Yoon et al., 2007; Zhou et al., 2012), was found in lower

abundance in the white wheat malt than in the barley malts (Figure 2.12).

1.1.2. Hops

Hops are the bittering ingredient added in beer, but they are also used as an antimicrobial

(Simpson, 1993) to target Gram positive bacteria (Shimwell, 1937). The microbiota of hop

samples in this study consisted of several different phyla with Actinobacteria, Bacteroidetes,

Firmicutes, and Proteobacteria being the most dominant (Figure 2.13). Other phyla consisting of

Acidobacteria, Chloroflexi, and Fusobacteria made up only a very small percentage of the

bacteria found in the samples. Actinobacteria were very diverse in the hop samples, with

Leucobacter being the predominant genus (Figure 2.14). *Leucobacter* is an environmental

bacteteria that has been isolated from soil and cow manure (Weon et al., 2012; Her and Lee,

2015). There appears to be no clustering of the microbiota of the hop samples, presumably because of important PCR biases due to low bacterial counts in the samples (Figure 2.15). It is well known that using high templates of DNA reduces risk of observing biased microbiota profiles because of unbalanced amplification of DNA strands (Polz and Cavanaugh, 1998). Nested PCR has commonly been used to remedy this issue, however it has also been shown to introduce significant biases (Fan et al., 2009).

CTZ hops were used in the three beers from Brewery 2 and were responsible for two out of the five positive hop samples. *Soonwooa* and *Larkinella* were two bacteria that were significantly more abundant in the CTZ hops than the other hop samples averaged. (Figure 2.16). *Soonwooa*, which belongs to the Flavobacteriaceae family, has been isolated from seawater (Joung et al., 2010) and *Larkinella* has been isolated from bovine products (Anandham et al., 2011). Both of these bacteria are Gram negative.

Bokulich, et al. found hop pellet samples to contain lactic acid bacteria populations, which is not surprising considering lactic acid bacteria's ability to develop hop resistance (Bokulich et al., 2015; Richards and Macrae, 1964; Sakamoto and Konings, 2003). Hops were also cited as a contributor of microorganisms to the cellar fermentation areas and fermentation equipment in breweries and could be possible vectors of spoilage organisms (Bokulich et al., 2015).

1.2. Mash tun

The first step of the brewing process is mashing. The malted grain gets steeped in hot water to release the starches and convert them to sugars. The mash process is completed between 64.5 and 70°C, so the mash tun is not cleaned as frequently as other brewing equipment. One out

of 20 mash tun swabs were positive for bacteria DNA. Although significant differences cannot be shown for one swab sample, the microbiota of the swab closely resembles that of the mash samples from the IPA beer (Figure 2.17).

There were only four bacteria that showed a significant difference in the samples of the mash: *Legionella*, *Carnobacterium*, *Novosphingobium*, and *Xanthomonadaceae* unclassified (Figure 2.18). Brewery 1 had a greater abundance of *Carnobacterium*, the other three were more prominent in Brewery 2. However, the four bacteria were present at less than 0.1% abundance in the samples, so the likelihood of any of the four bacteria playing a major role in the microbiota of craft beer is limited.

Few significant differences were found between the mash tun samples according to style. *Arthrobacter* was found to be in significantly higher abundance in the Belgian Golden Ale, the Pale Ale, and the Hoppy Wheat mash tun samples (Figure 2.19). *Corynebacterium* was found in lowest abundance in the Pale Ale mash tun sample. This bacterium has been isolated from cows and sheep ill with mastitis (Fernandez-Garayzabal et al., 1997; Hommez et al., 1999) mastitis. *Sanguibacter* and *Rhodococcus* are both environmental bacteria, isolated from water sources (Hong et al., 2008; Kämpfer et al., 2014). *Chryseobacterium* and *Pedobacter* are two other environmental organisms (Kämpfer et al., 2014; Yoon et al., 2007; Zhou et al., 2012) found in significantly different abundances (Figure 2.20).

1.3. Boil kettle

The boiling process of brewing consists of the wort being boiled and hops being added at different time periods according to their use. Environmental swabs were taken of the boil kettle. Wort samples were taken from the kettle pre- boil, post- boil, and after flowing through the wort

chiller. The post- boil and the environmental swabs had a distinct microbiota from the wort samples (Figure 2.21).

Several bacteria were impacted by the boiling process. For example, combining all of the beer samples together, *Bacteroides*, *Lachnospiraceae*, *Ruminococcaceae*, and *Porphyromonadaceae* were more abundant in post- boil and after cooling samples than the pre- boil samples (Figure 2.22). *Bacteroides* (Eckburg et al., 2005), *Ruminococcaceae* (Ze et al., 2012), and *Lachnospiraceae* are commonly known as good gut microorganisms (Kittelmann et al., 2013; Gosalbes et al., 2011), so it is more relevant that they are found after the pre- boil step and throughout the brewing process, possibly from human origin, rather than in the raw materials.

Enterobacteriaceae, *Acinetobacter*, *Pseudomonas*, *Pantoea*, and *Stenotrophomonas* had the opposite effect of the previous four bacteria, decreasing in abundance from pre- boil to post- boil (Figure 2.22). *Acinetobacter* and *Pantoea* are environmental bacteria (Nemec et al., 2016; Brady et al., 2009) that likely originated in the raw materials.

Beer spoilage bacteria was found in low abundance during the boil stage (Figure 2.23). *Leuconostoc* is a bacteria commonly isolated from fruits and vegetables (Priest and Campbell, 2003), but can produce diacetyl in beer (Speckman and Collins, 1968). This bacterium was negatively affected by the boil step, decreasing in abundance in the post- boil sample. *Lactobacillus* is the most common beer spoiler, providing silky and turbid characteristics, and is growing in hop resistance (Sakamoto and Konings, 2003). *Lactobacillus* increased in relative abundance from pre- boil samples to post- boil samples. This is most likely caused by a reduction in the abundance of other bacteria present in these samples.

The IPA, Pale Ale, Brown Ale, and Hoppy Wheat showed very few significant differences during the boiling process, but the Belgian Golden Ale displayed significant differences in bacteria abundance between the pre- boil, post- boil, and after cooling stage. The Belgian Golden Ale showed a significant increase in abundance of *Bacteroides*, *Blautia*, *Paenibacillus*, *Tumebacillus*, and *Clostridium XI* after the boil (Figure 2.24). *Blautia* is known as a good gut microorganism (Park et al., 2012). Both *Paenibacillus* and *Tumebacillus* are isolated from the environment, specifically plant root and soil respectively (Kim et al., 2015; Her et al., 2015). *Tumebacillus* has also been isolated from wastewater (Wang et al., 2013) and river water (Prasad et al., 2015), therefore the source of the bacteria may potentially be from the water used by the brewery or the wastewater that is produced by the brewery. *Xanthomonas* significantly decreased to very low abundance during the boil.

The pre- boil stage showed few significant differences between the two breweries in relation to abundance of bacteria (Figure 2.25). *Arthrobacter*, *Weissella*, and *Ochrobactrum* were significantly higher in abundance in the pre- boil samples of Brewery 2. *Ochrobactrum* is another environmental bacterium that has been isolated form soil and wheat root (Lebuhn et al., 2000). Brewery 1 had a higher abundance of *Lachnospiraceae* and *Ruminococaceae* at the pre- boil stage.

Three significant differences were found between the two breweries at the post- boil step (Figure 2.26). *Clostridiales* and *Blautia* were significantly greater in abundance in Brewery 1 than Brewery 2; and *Bacilliales* was more abundant in Brewery 2.

Environmental bacteria *Acinetobacter*, *Pseudomonas*, and *Stenotrophomonas* were found in higher abundance after cooling in Brewery 2 (Figure 2.27). *Prevotellaceae* and *Clostridium IV*

were significantly more abundant after cooling in Brewery 1. Overall, the microbiota of samples post- boil and after cooling were similar.

1.4. Fermenter

Fermentation samples were taken on the brewing day (Day 0) and mid fermentation for four styles: IPA, Belgian Golden Ale, Pale Ale, and Brown Ale. Day 0 and end of fermentation samples were taken for the Hoppy Wheat, since there was no filter step with this style. Four bacteria were shown to be significantly different in abundance between Fermentation Day 0 and Mid Fermentation (Figure 2.28). *Arthrobacter*, *Paenibacillus*, and *Bacillales* all decreased in relative abundance during the fermentation process, and *Clostridiales* increased in abundance. This is likely due to the fermentation yeast competing for nutrients, with some bacteria more so than others.

The Hoppy Wheat beer showed significant differences for bacteria between Fermentation Day 0 and End of Fermentation samples (Figure 2.29). *Lactococcus*, *Chryseobacterium*, and *Betaproteobacteria* all significantly increased in abundance during fermentation, and *Streptococcus* was eliminated during fermentation. Again, this is likely due to the competition of specific bacteria with the fermentation yeast decreasing in relative abundance.

Between the styles, there were some differences in abundance of bacteria (Figure 2.30). The Hoppy Wheat beer had a significantly higher abundance of *Corynebacterium* than the Belgian Golden Ale. *Chryseobacterium* was in significantly higher abundance in the Brown Ale than in the Pale Ale and Belgian Golden Ale.

1.5. Filtering

Filtering is mainly a clarifying process in brewing but is sometimes considered a critical control step to reduce microbial load, typically targeting the brewing yeast (Manzano et al., 2011). Filtering in the craft breweries from this research consists of using a plate and frame filter. Beer is pumped from the fermentation tank through filter plates and travels to the bright tank. Overall there is no significant effect of filtering on the microbial abundance in the beer styles sampled (Figure 2.31). With all beer styles combined, there were only three significant differences in bacteria abundance pre- and post-filter (Figure 2.32). *Blautia*, *Streptococcus*, and *Clostridium IV* all significantly increased in abundance after filtering, which could be a result of contamination in the brewery or the decreasing relative abundance of other bacteria. However, these bacteria were in abundance at less than 2.5% in the beer, so the possibility that they play a major role in the final microbiota is small.

The Belgian Golden Ale also showed very minor significant changes during the filter stage (Figure 2.33). Both *Ruminococcus* and Proteobacteria unclassified decreased significantly by filtering. Again, both of these bacteria were found in very low abundance, so it is unlikely that they participate strongly in the final product microbiota.

1.6. Final products

The four phyla that predominate the final product samples were Actinobacteria, Bacteroidetes, Firmicutes, and Proteobacteria (Figue 2.34). Actinobacteria made up a small abundance of the final product, and most of the phylum was composed of *Arthrobacter,* and a smaller percentage of *Corynebacterium* and *Microbateriaceae* (Figure 2.35). Bacteroidetes in the final product were fairly diverse, with most consisting of *Porphyromonadaceae*, *Prevotellacea*,

and *Chryseobacterium. Microbacteriaceae* are environmental microorganisms, having been isolated from salty soil (Kook et al., 2014) and salt marshes (Fidalgo et al., 2016) (Figure 2.36). There were many highly prominent Firmicutes present in the final samples, some being important beer spoilers like *Leuconostoc*, *Lactococcus*, and *Lactobacillus* (Figure 2.37). Of the Proteobacteria, *Enterobacteriaceae*, *Acinetobacter*, and *Pseudomonas* were found in the highest abundance in the final products (Figure 2.38).

The microbiota of the final products differed between styles (Figure 2.39). For example, the Belgian Golden Ale had a significantly higher abundance of *Porphyromonadaceae*, *Prevotellaceae,* and *Lactobacillus*. The Brown Ale had a greater abundance of *Chryseobacterium, Leuconostoc,* and *Lactococcus*.

Between the two breweries, there were significant differences as well (Figure 2.40) *Microbacteriaceae*, *Chryseobacterium*, *Lactococcus*, *Enterobacteriaceae*, and *Sphingomonas* were all in significantly greater abundance in Brewery 2.

2. Bacterial dynamics and style/brewery specificities

2.1. General dynamics

The dynamics of the 5 beer styles combined shows that the most of the beer microbiota throughout the process was encompassed by four phyla: Actinobacteria, Bacteroidetes, Firmicutes, and Proteobacteria (Figure 2.41). The raw ingredients and samples taken before the boil were dominated by Proteobacteria, but after boiling showed an overtake of the Firmicutes phylum. The final product microbiota appears to be more evenly distributed between the three phyla: Firmicutes, Bacteroidetes, and Proteobacteria; with Actinobacteria being abundant in a relatively small amount.

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Although most of the bacterial DNA present in the samples belonged to four phyla, there were several other phyla represented in low abundance (Figure 2.42). None of these phyla encompassed more than 10% of the total microbiota, however they could prove to be of importance throughout the brewing process. Verrucomicrobia were relatively abundant in the boil kettle swab, after cooling, in the fermenter, and the final product. This phylum is isolated almost exclusively from soil (Janssen et al., 2002; Zhang and Xu, 2008).

Tenericutes were found in samples after boiling and in the swab of the fermentation tank. Mollicutes are the only class belonging to the phylum (sometimes included in the Firmicutes phylum) and have been isolated from a diverse range of plants and animals. Most predominantly they have been isolated from humans, cows (*Anaeroplasma*), pigs, goats, and insects and are parasitic to their hosts (Weisburg et al., 1989). Only very trace amounts were shown to be present in the final product. Plantomycetes make up over 6% of the microbiota of the fermentation tank swab. This phylum is largely associated with aquatic environments, isolated from the Arctic Mid Ocean Ridge and the Southern Mariana Trough (Kato et al., 2010; Storesund and Øvreås, 2013).

2.2. Dynamics by breweries and styles

2.2.1. Breweries

The two breweries showed very similar microbial profiles, with bacteria that were predominant in the malt decreasing throughout the process, and bacteria that were more abundant during the process being more likely to end up in the final product. Some bacteria were in significantly greater abundance in one brewery than the other (Figure 2.43). Two subgroups of Acidobacteria, Gp 17 and Gp 10, were found exclusively in Brewery 1, and *Thermosporothrix*

was found exclusively in Brewery 2. Other significant differences included the increased abundance of *Arthrobacter*, *Chryseobacterium*, *Leuconostoc*, and *Curtobacterium* in Brewery 2. Brewery 1 had a greater abundance of *Lactobacillus*, *Ralstonia*, *Thermus*, and *Alistipes*. *Ralstonia* is an environmental bacterium (Yabuuchi et al., 1995) and can create biofilms (Liu et al., 2014a) which may explain its significantly greater presence in one brewery. *Alistipes* is a natural part of the human microbiota (Rautio et al., 2003; Song et al., 2006), so the origin of this bacterium in Brewery 1 could be from operators.

Brewery 1 consisted of the IPA and the Belgian Golden Ale beers. *Arthrobacter* and *Acinetobacter*, *Leuconostoc*, and *Pseudomonas* were all found in high abundance in the malt samples (Figure 2.44). These four bacteria slowly decreased through the process, and increased in abundance in the final product, presumably a result of decreasing abundances of other bacteria. *Lactobacillus* and *Akkermansia* were not found abundant in the malts, but throughout the process. *Akkermansia* is known as a good gut microorganism (Derrien et al., 2004; Hatayama et al., 2014), so it is possible the bacteria originated from human contamination.

Brewery 2 showed similarities in abundant bacteria and trends throughout the process (Figure 2.45). Again, *Arthrobacter*, *Acinetobacter*, and *Pseudomonas* were abundant most in the malts and decreased through the process. *Brevibacillus* became highly abundant after boiling and persisted through fermentation, with an increase after filtering. This bacterium is predominately found in the environment (Hatayama et al., 2014). *Paenibacillus* steadily increased through the brewing process as well, but was present in very low abundance in the final product. Just as in Brewery 1, *Akkermansia* also increased steadily through the brewing process.

2.2.2. Style

The dynamics on the phylum level for each individual style based on two repetitions shows similarity to the combined data (Figure 2.46). Firmicutes and Proteobacteria were the dominating phyla with Bacteroidetes and Actinobacteria being the third and fourth most abundant. Other phyla make up a small portion of total microbiota. Actinobacteria seem to be more prominent in malts, decreasing in number throughout the process, and again increasing in abundance in the final product samples. Verrumicrobia is primarily limited to hop samples and samples taken after boiling.

The IPA beer from Brewery 1 has some distinct characteristics found during the brewing process in reference to the bacteria present (Figure 2.47). This style had a significant amount of *Gluconobacter* found during the filter step but eliminated in the final product, which could possibly be a contamination in the process. *Gluconobacter* is a beer spoiler that reduces ethanol to acetate and creates a ropy texture (Banwart, 1979). *Staphylococcus* had a high abundance (26%) in the hops of the IPA, but was not found throughout the rest of the process. *Acinetobacter* and *Arthrobacter* were in higher abundance in the malt, but slowly decreased throughout the process with little remaining in the final product.

The Belgian Golden Ale from Brewery 1 had similar dynamics of *Arthrobacter* as was seen in the IPA (Figure 2.48). *Pseudomonas* was another bacterium in high abundance in the malt, but slowly decreased in abundance through the process. However, *Prevotellaceae*, *Lachnospiraceae*, and *Akkermansia* were all absent in the raw materials for this beer, but introduced later in the process and participated in the final microbiota.

The beer styles from Brewery 2 showed similar profiles of the bacteria present in malt, but some other distinct bacteria were found in variety throughout the brewing process. The Pale

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Ale had a high abundance of *Chryseobacterium* and *Leuconostoc* in the malts, but by post- filter were almost eliminated (Figure 2.49). *Streptococcus* was found in greater abundance during fermentation, but by the final product was in very low abundance. During the boil stage, *Flavobacterium* was found in greatest abundance, but was eliminated by the fermentation. This microorganism is environmental, having been isolated from soil (Dong et al., 2013). *Lactobacillus* showed the most dramatic change through the Pale Ale brewing process, not being present in the malt or mash, but increasing in abundance during the boil stage and again at fermentation. The abundance of *Lactobacillus*, a beer spoiler, was about 7.5% in the final product. *Clostridium XI* also increased in abundance steadily throughout the process.

The Brown Ale showed a dramatic decrease in abundance of several malt – dominating bacteria during the boiling process (Figure 2.50). Again, *Arthrobacter* was highest in abundance in the malt samples, with a near zero abundance after boiling. *Pantoea* was also prominent in the malt and mash and eliminated during the boil step. *Lactococcus, Sphingobacterium* and *Leuconostoc* were found in the malt samples, decreased during boiling, and increased in abundance again during fermentation. *Stenotrophomonas* was found more abundant in the mash samples, but again, decreased during the boil process.

The last style from Brewery 2 was the Hoppy Wheat beer. The dynamics of this style were very sporadic (Figure 2.51). *Chryseobacterium* and *Acinetobacter* were present in the malts, and increased and decreased in abundance throughout the process. *Stenotrophomonas* and *Pantoea* also had sporadic increases and decreases in abundance. *Lactococcus* steadily slightly decreased in abundance during brewing. *Akkermansia* was not present in the samples until the post- boil stage, and it persisted through fermentation.

Conclusions

The microbiota of the brewing process was diverse at the genera level between the five styles and two breweries. Overall, there were no major differences between the two breweries, and bacteria that were present in one brewery and not the other were in very low abundance. Between the styles there were also minor differences in bacteria present. One difference was that the IPA had a greater abundance of *Gluconobacter*, which was likely to be a contamination at the one step of the process during that one batch of beer.

The raw material microbiota contributed greatly to the bacteria in the mash tun and the mash tun swab. Most of the bacteria that were in high abundance in the raw material are typically isolated from the environment, and were reduced in abundance during the boiling process. The presence of identical bacteria in the raw material and final product samples could lead one to believe that the microbiota of the raw material is important for the final product, even with as intense brewing processes as mashing and boiling, and intrinsic hurdles including low pH (3.8-4.7) and ethanol concentration (0-8% ABV) (Jespersen and Jakobsen, 1996).

Bacteria not present (or in low relative abundance) in the raw material that were potentially introduced during the process were more likely to persist in the final product. The presence of bacterial spoilers such as *Lactobacillus* and *Leuconostoc* were in low abundance throughout the process, but had some persistence in the final product. Some bacteria that were present on the swabs of the brewing equipment are biofilm formers, which explains their persistence on the brewing equipment. *Ralstonia* was found on swabs of the boil kettle and the fermenter, and its biofilm forming abilities (Liu et al., 2014a) explain its low abundance in malt samples but high abundance throughout the process. *Actinomyces* was also found on the swab of the boil kettle and is able to form biofilms on teeth (Li et al., 2004).

Mashing and boiling were fairly effective at reducing microbial abundance from the raw material, however filtering had no significant impact. Filtering is predominately used as a clarifying practice in craft breweries, and sometimes to remove flocculant yeast. However, in this research, it was shown to be ineffective at reducing bacterial abundance.

Final product microbiota consisted mainly of four phyla: Actinobacteria, Bacteroidetes, Firmicutes, and Proteobacteria. The genera of each were diverse, and were a composite of bacteria found in the raw material that were reduced during boiling and increased in abundance during fermentation, and bacteria that were more abundant during the brewing process. The data obtained confirms that the bacterial microbiota are associated with the raw materials, the brewing environment, and the craft beer final product. It further shows relatively consistent bacterial successions along the brewing process, including the critical control steps of mashing, boiling, and filtering.

Throughout this study, some limitations were considered and should be discussed. One of the greatest obstacles was working with brewers and having limited access to the brewery environment and the brewing schedule. Some samples were not collected because of this limitation. The sample size for this study was two batches per brewery style. For future studies, increasing the number of batches could improve repeatability and statistical significance. Finally, since this research was the one of the first to use HTS to track bacteria development throughout the process, method optimization was not necessarily achieved prior to the study due to time constraints.

Even with these limitations, this research provides the first extensive microbiota research of craft beer throughout the process. It will initiate other research on the potential organoleptic

properties that these diverse bacteria are may provide to the craft beer and the role that the

microbiota plays on the quality of craft beer. This research also allows craft brewers to have a

better understanding of their product.

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Figure 2.1: The brewing process.

Mash Tun	Malted grain is steeped in hot water	Sweet wort is pumped to boil kettle	Spent grain is sprayed with water
Boil Kettle	Wort is boiled	Hops are added	
Cooling	Wort is pumped though wort chiller		
Fermen-tation	Wort is pumped to fermentation tank	Yeast is added	Fermentation occurs for between one to two weeks
Bright Tank	Beer is further aged after fermentation	Other ingredients and flavors can be added	
Filtering	Beer may be filtered for clarity		
Final Product	Beer is packaged into cans, bottles, or kegs		

Table 2.1: Specificities of the beer styles.

Beer	Brewery	ABV	IBU	SRM	Final pH
Belgian Golden Ale	1	6.3%	36	3	4.0
Brown Ale	2	6.0%	18	20-25	4.0
Hoppy Wheat	2	5.5%	50	5-8	4.25
IPA	1	5.5%	56	10	4.25
Pale Ale	2	5.5%	35	10-15	4.0

ABV – alcohol by volume; IBU – International Bitterness Units; SRM – Standard Reference Method (beer color)

Table 2.2: Positive bacteria samples determined by Universal PCR.

Sample	#	%
Malted Grain	43/51	84.3%
Hops	5/28	17.9%
Environmental Swabs	9/60	15%
From Mash Tun	10/10	100%
Pre-Boil	6/10	60%
Post-Boil	9/10	90%
After Cooling	10/10	100%
Fermentation Day 0	8/10	80%
Fermentation Day 2	2/10	20%
End of Fermentation	2/2	100%
Pre-Filter	4/5	80%
Post-Filter	7/7	100%
Final Product	9/10	90%
Total	124/223	55.6%

Figure 2.2: Raw material NMDS plot with Bray-Curtis index. Blue represents the malts, red represents hops.
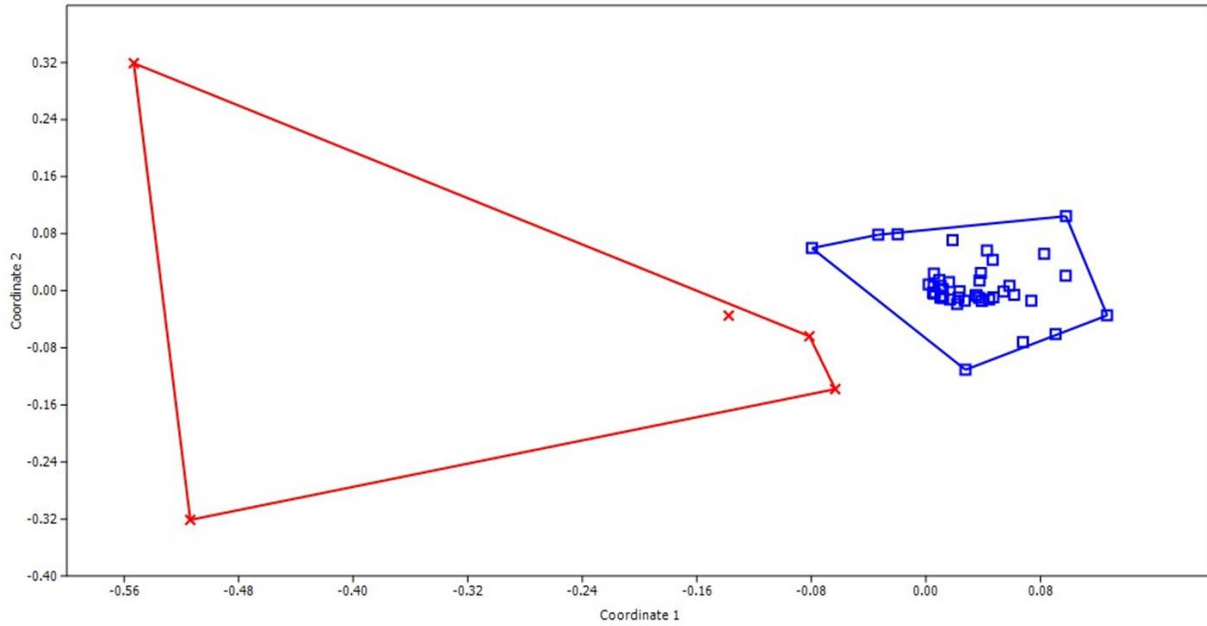
Figure 2.3: NMDS plot with Bray-Curtis index for the malts of the two breweries. Open squares represent Brewery 1, filled squares represent Brewery 2.
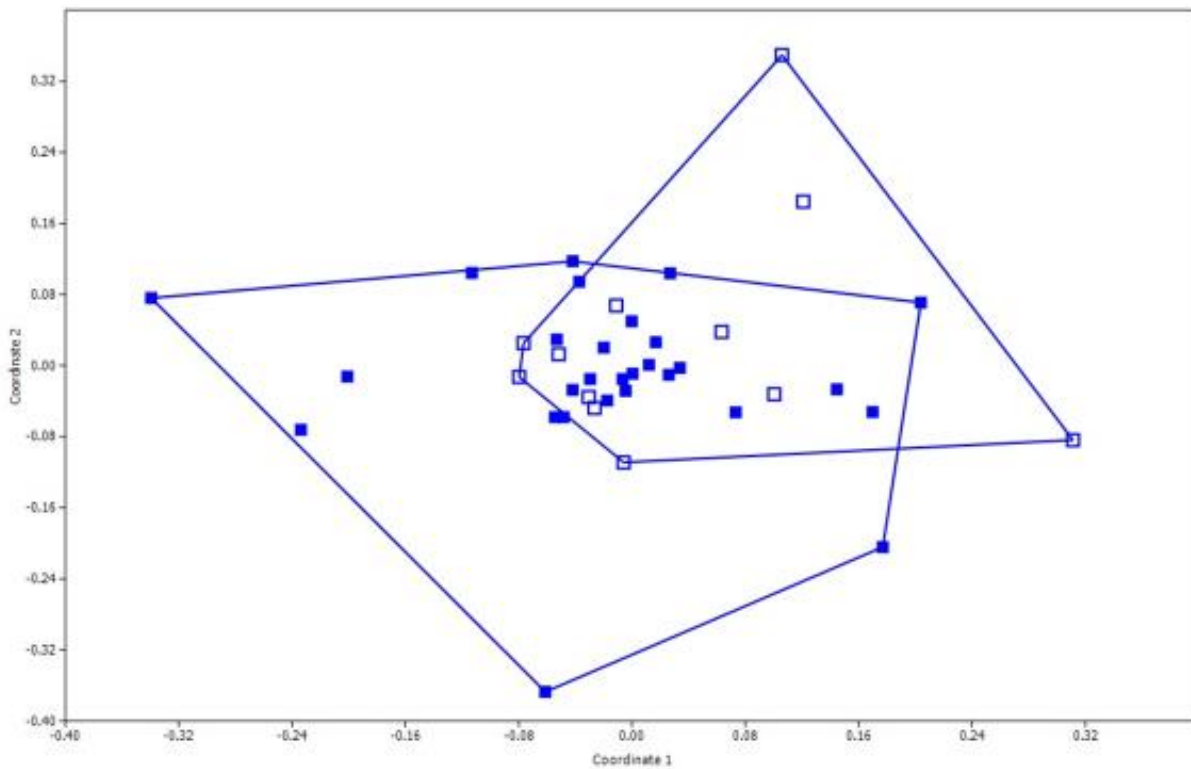
Figure 2.4: NMDS plot with Bray-Curtis index for the different types of malts. Green represents the wheat malt and the blue clusters are the pilsner, pale ale, and acidulated malt.
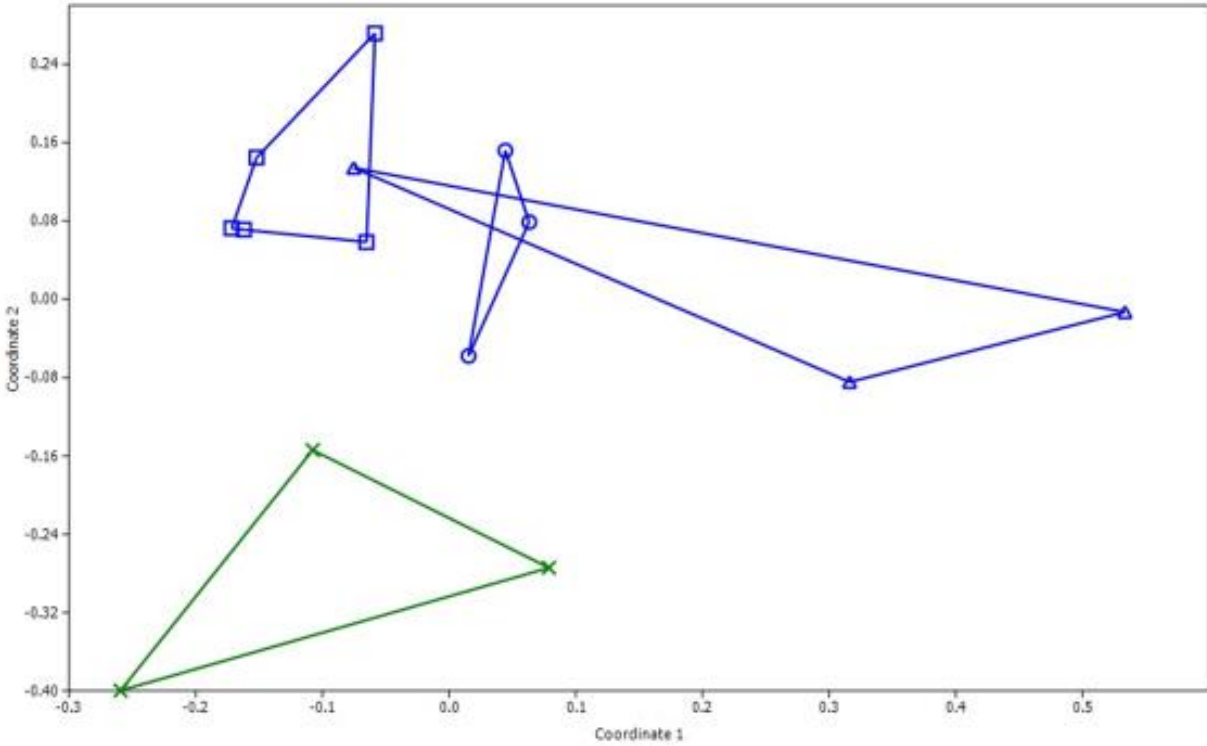
Figure 2.5: Abundance of phyla in malt samples for all styles.

Malts

- Actinobacteria
- Bacteroidetes
- Firmicutes
- Proteobacteria

80
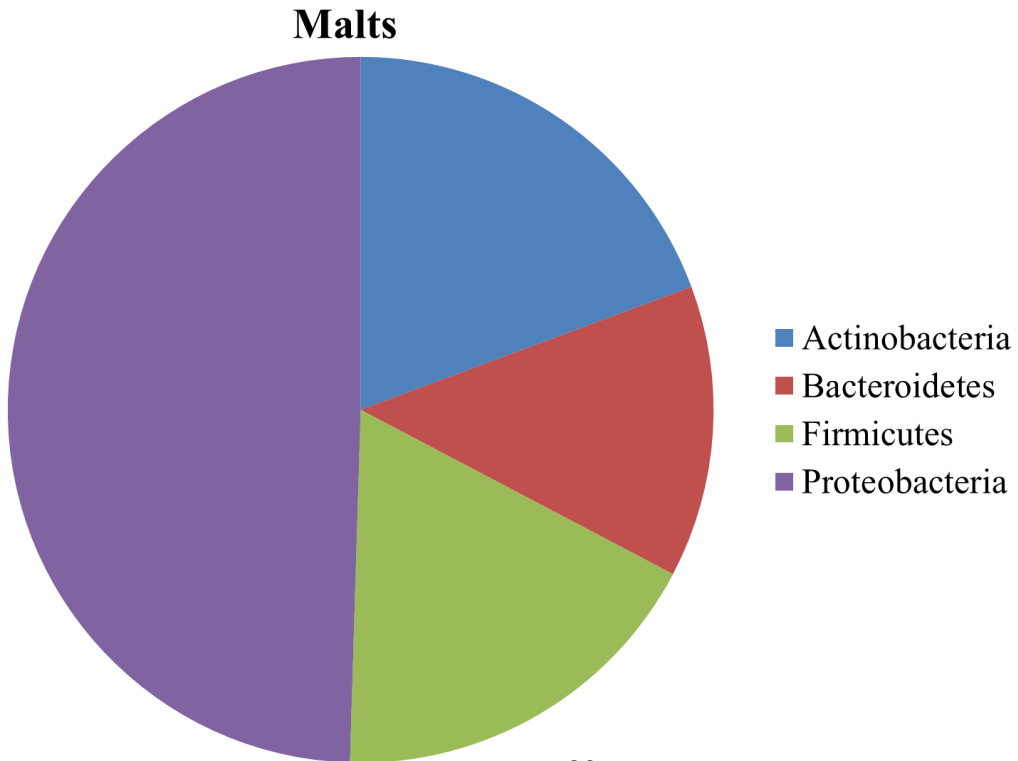
Figure 2.6: Abundance of specific genera in all malt samples (A-D)

A)

Legend:
- Leuconostoc
- Lactococcus
- Lactobacillus
- Streptococcus
- Paenibacillus
- Weissella
- Exiguobacterium
- Saccharibacillus
- Planococcaceae_unclassified
- Paenibacillaceae_1_unclassified
- Lactobacillales_unclassified
- Bacillales_unclassified
- Staphylococcus
- Other

Firmicutes

B)

Legend:
- Enterobacteriaceae_unclassified
- Acinetobacter
- Pseudomonas
- Pantoea
- Stenotrophomonas
- Comamonas
- Sphingomonas
- Janthinobacterium
- Xanthomonas
- Rhizobium
- Oxalobacteraceae_unclassified
- Comamonadaceae_unclassified
- Massilia
- Methylobacterium
- Paracoccus
- Duganella
- Gluconobacter
- Other
- Aeromonas

Proteobacteria

Bacteroidetes

C)

Legend:
- Bacteria_unclassified
- Alistipes
- Bacteroides
- Sphingobacterium
- Chryseobacterium
- Wautersiella
- Flavobacteriaceae_unclassified
- Pedobacter
- Flavobacterium
- Other

81

D)

Legend:
- Arthrobacter
- Corynebacterium
- Microbacteriaceae_unclassified
- Brachybacterium
- Sanguibacter
- Leucobacter
- Rhodococcus
- Microbacterium
- Other
- Curtobacterium

Actinobacteria

Figure 2.7: Differences in *Arthrobacter*, *Brachybacterium*, and *Microbacterium* in the malts of the two breweries. Letters denote significant differences within genera at $p<0.05$.
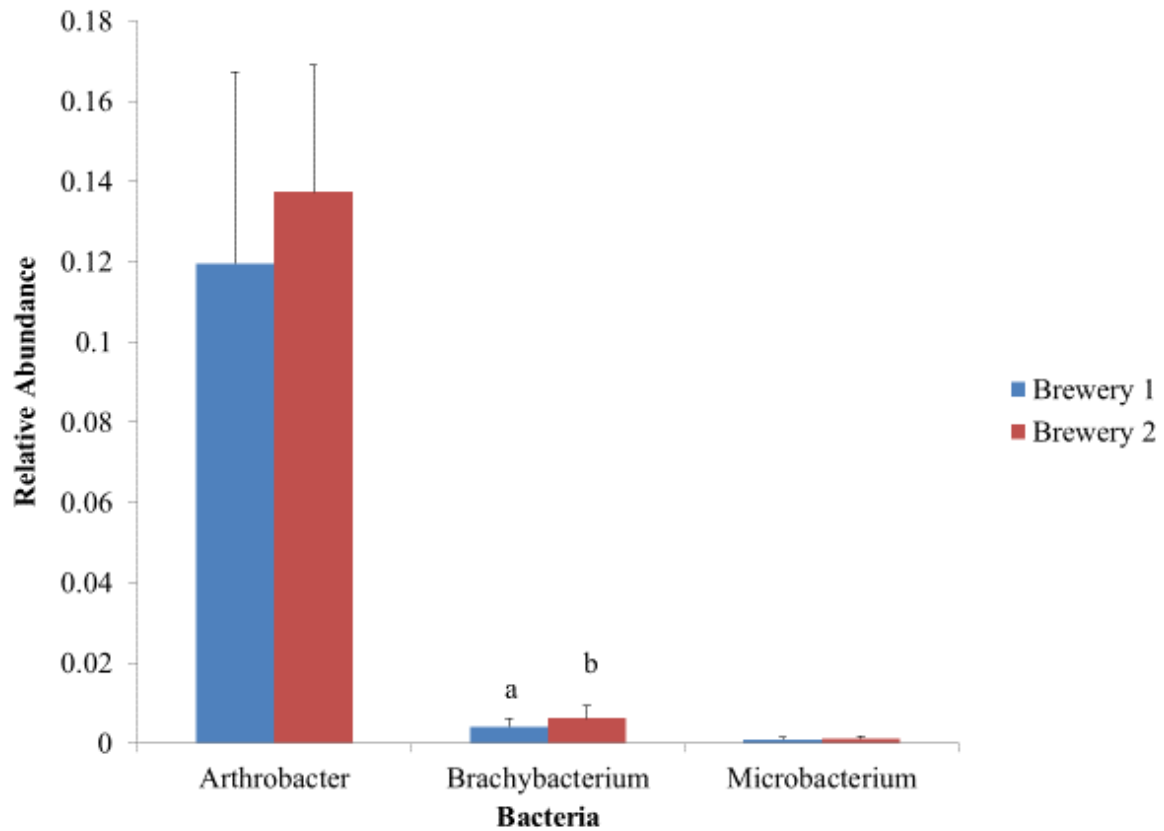
Figure 2.8: Differences in *Bacteroides*, *Sphingobacterium*, and *Prevotella* in the malts of the two breweries.
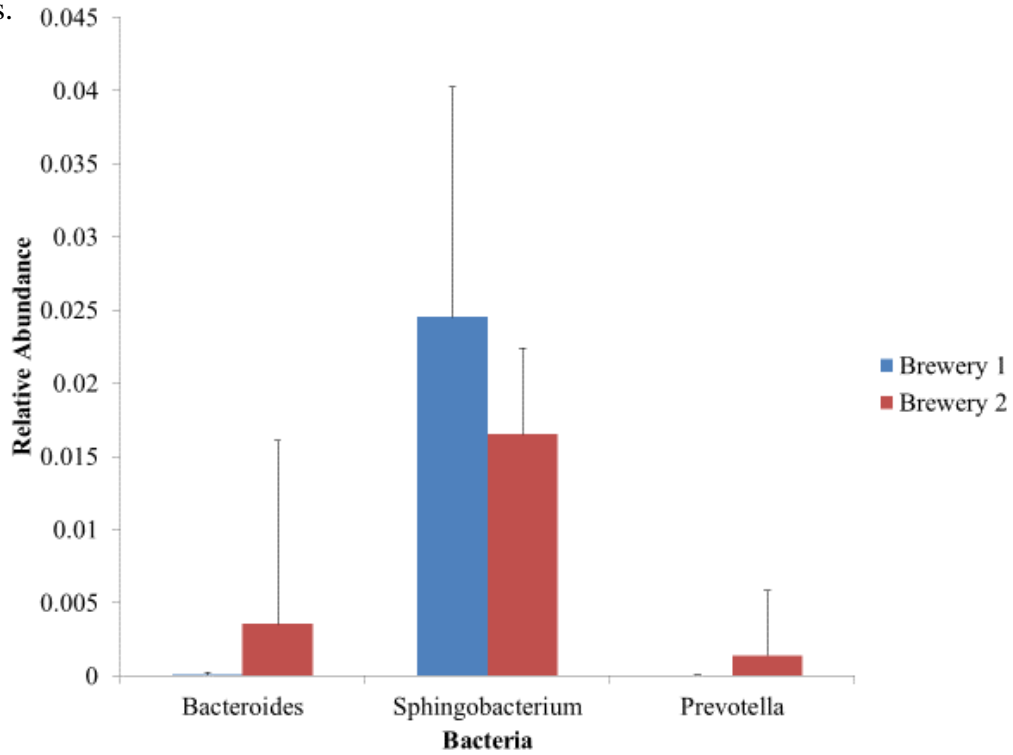
Figure 2.9: Differences in *Pseudomonas*, *Stenotrophomas*, *Xanthomonas,* and *Methylobacterium* in the malts of the two breweries. Letters denote significant differences within genera at $p<0.05$.
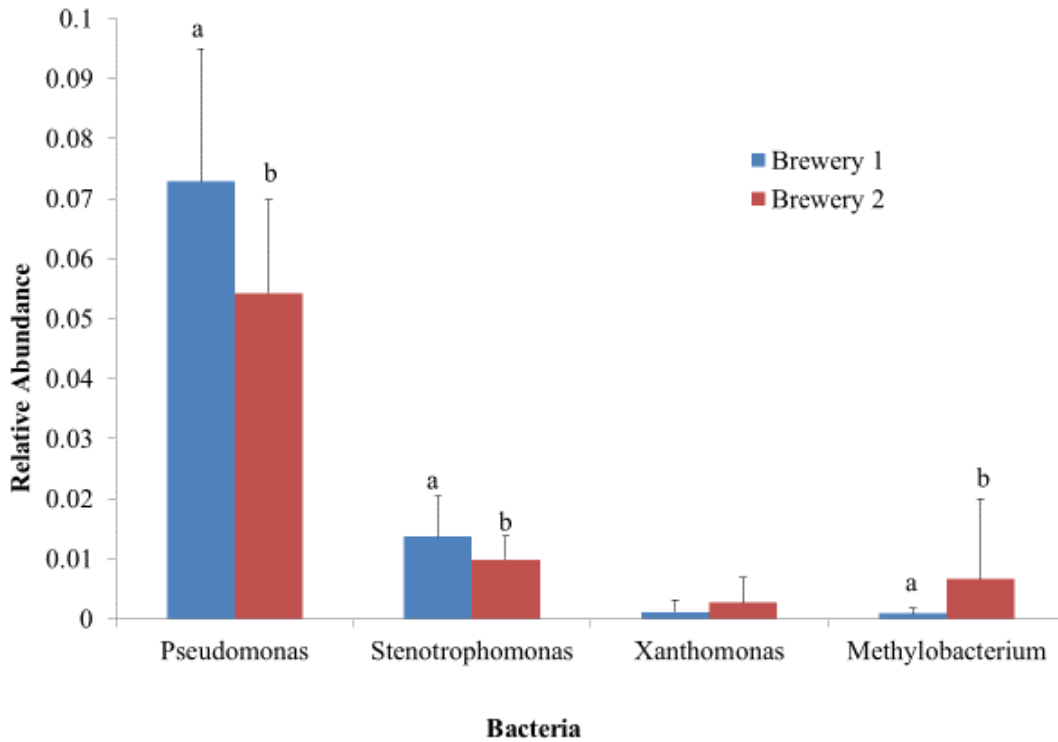
Figure 2.10: *Arthrobacter, Corneybacterium*, and *Brachybacterium* abundance for four malt types. Letters denote significant differences within genera at p<0.05.
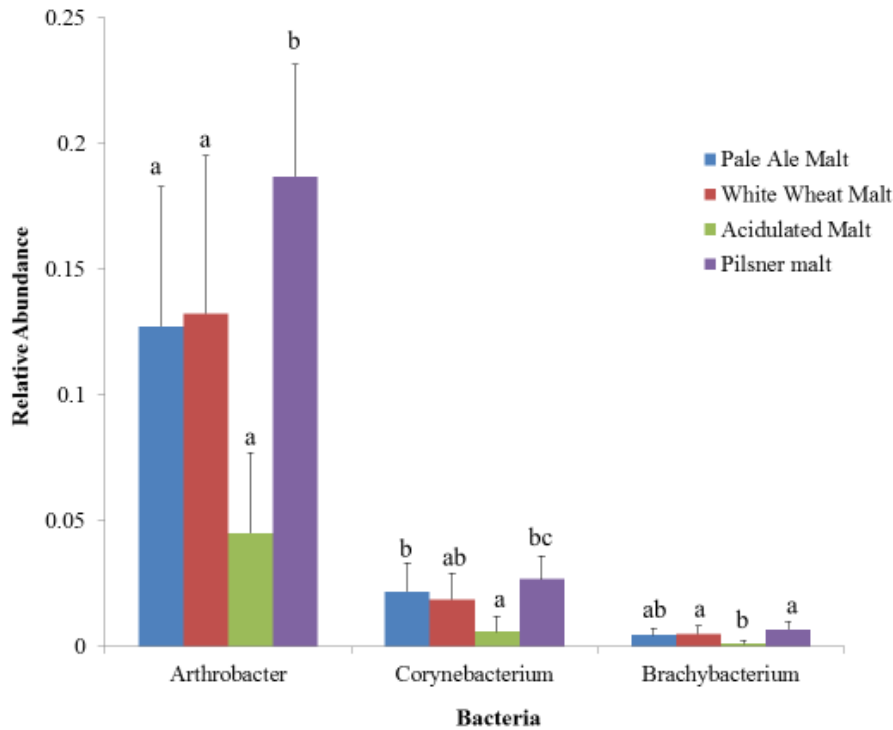
Figure 2.11: *Lactoccus, Weissella, Saccharibacillus* and *Enterococcus* abundance for four malt types. Letters denote significant differences within genera at p<0.05
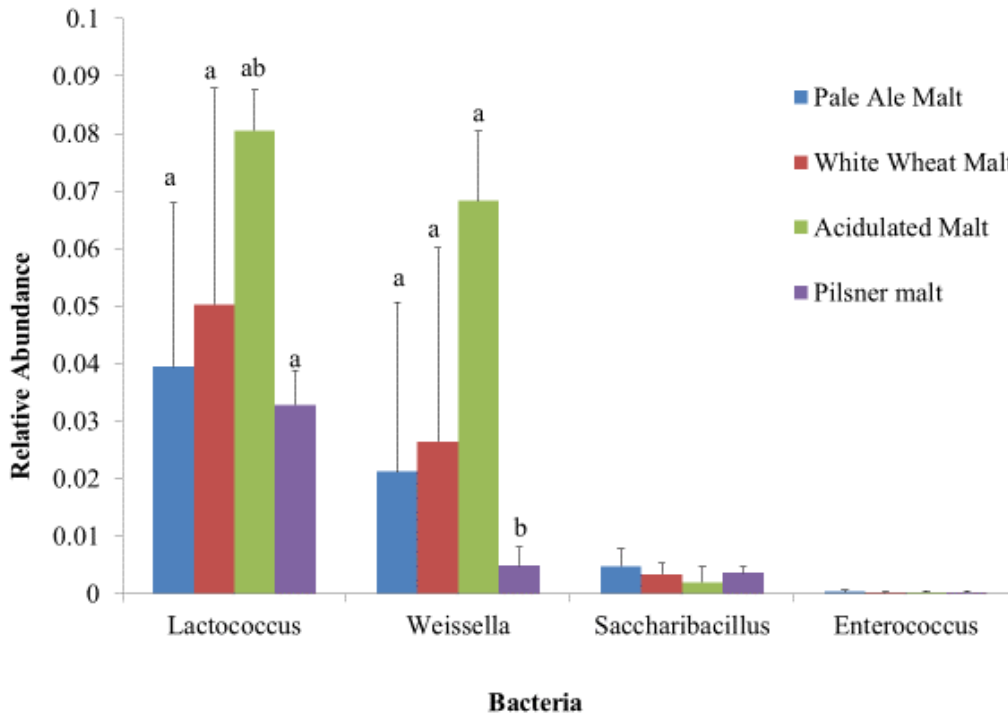
Figure 2.12: *Sphingobacterium, Chryseobacterium, Wautersiella, Pedobacter,* and *Flavobacterium* abundance for four malt types. Letters denote significant differences within genera at $p<0.05$.
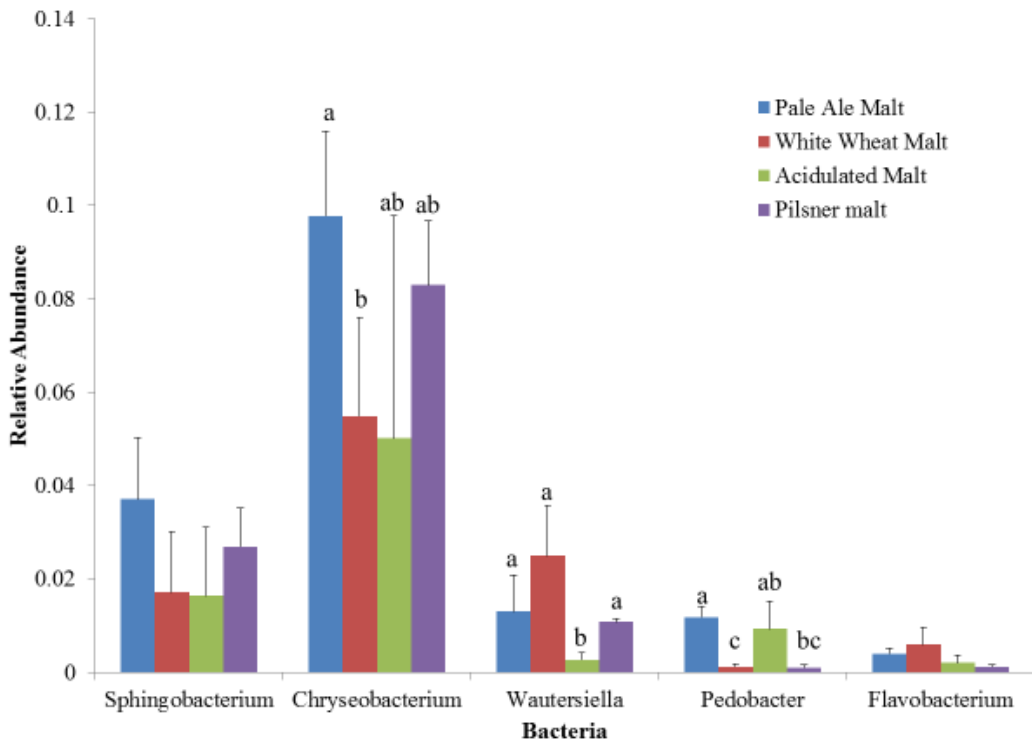
Figure 2.13: Abundance of different phyla in all hop samples.
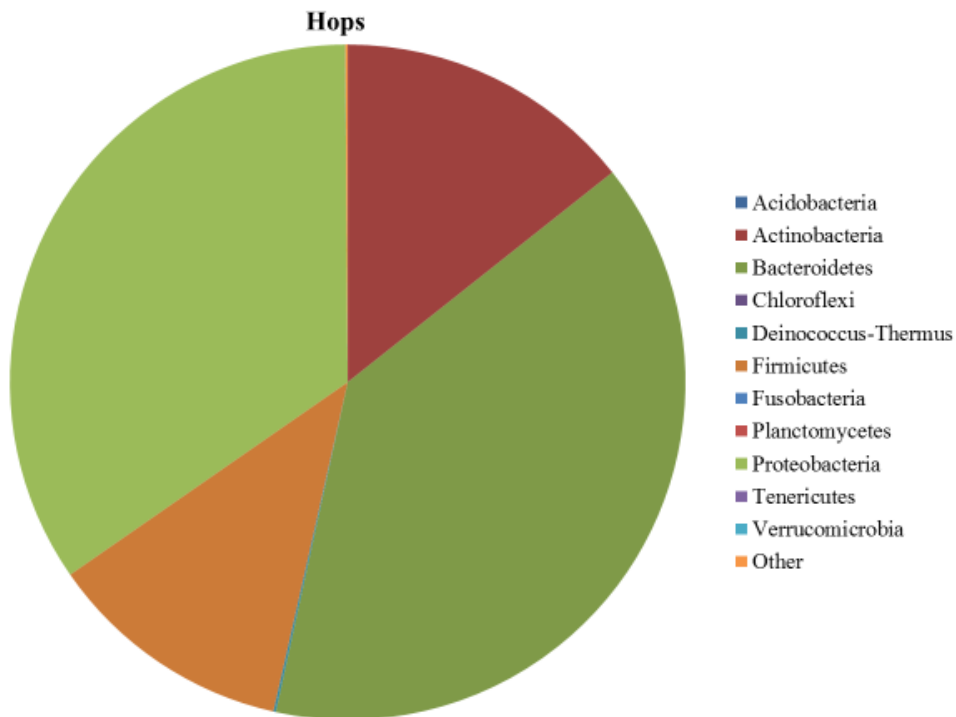
Figure 2.14: Abundance of specific genera of Actinobacteria in hop samples.

Legend:
- Arthrobacter
- Bifidobacterium
- Corynebacterium
- Microbacteriaceae_unclassified
- Leucobacter
- Actinomyces
- Atopobium
- Actinomycetaceae_unclassified
- Dermacoccus
- Kocuria
- Rathayibacter
- Cellulomonas
- Kineococcus
- Mycobacterium
- Gordonia
- Collinsella
- Curtobacterium
- Demequina
- Tessaracoccus
- Isoptericola
- Piscicoccus
- Renibacterium
- Other

Actinobacteria

Figure 2.15: NMDS with Bray-Curtis index plot of the five hop samples.

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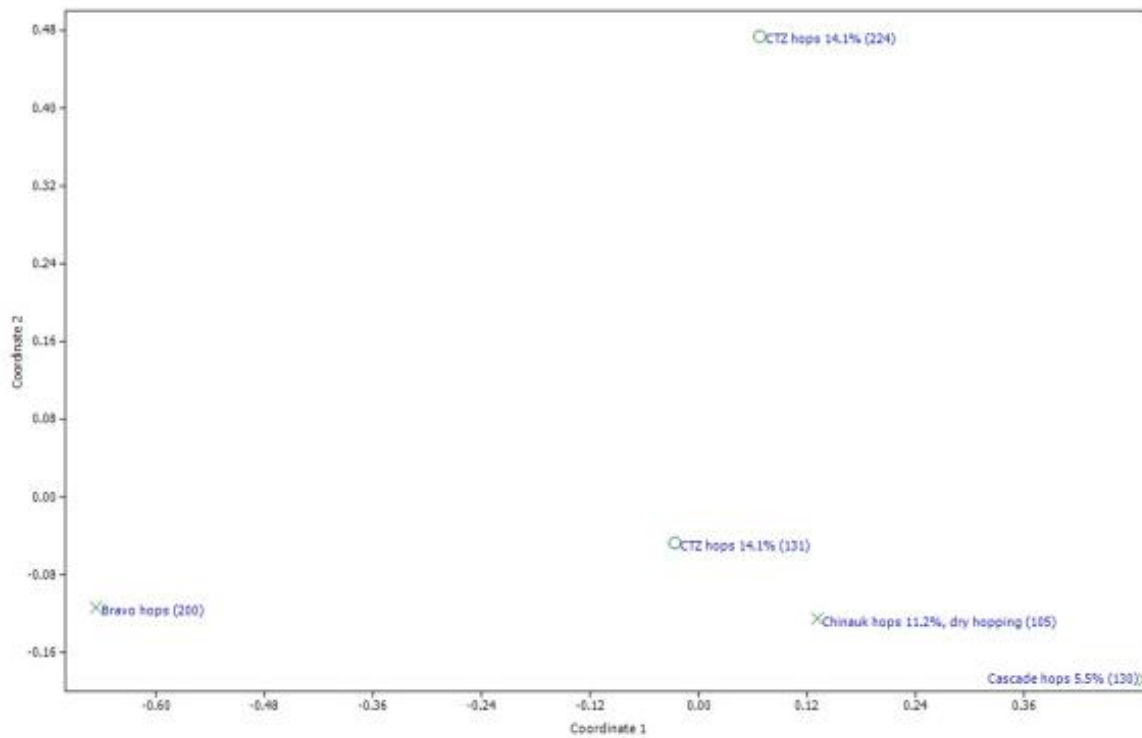
Figure 2.16: Bacteria abundance in CTZ hops and other hop samples. Letters denote significant differences within genera at p<0.05.
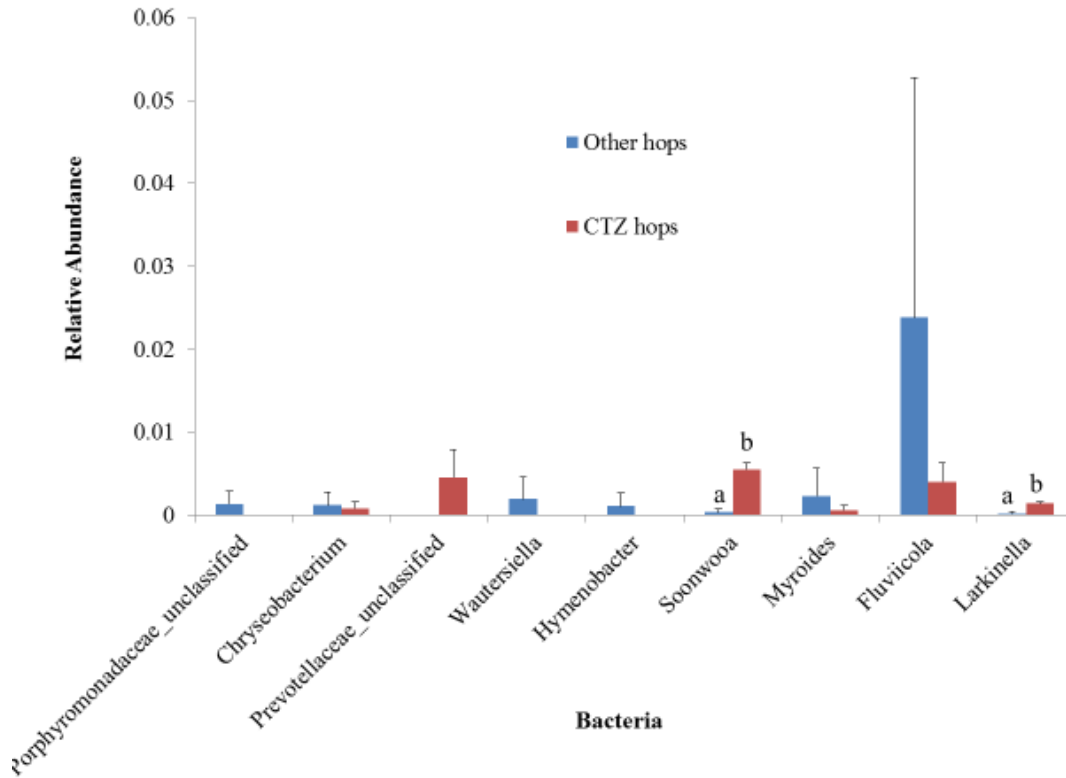
Figure 2.17: IPA mash tun swab sample microbiota compared to the IPA beer during the mash
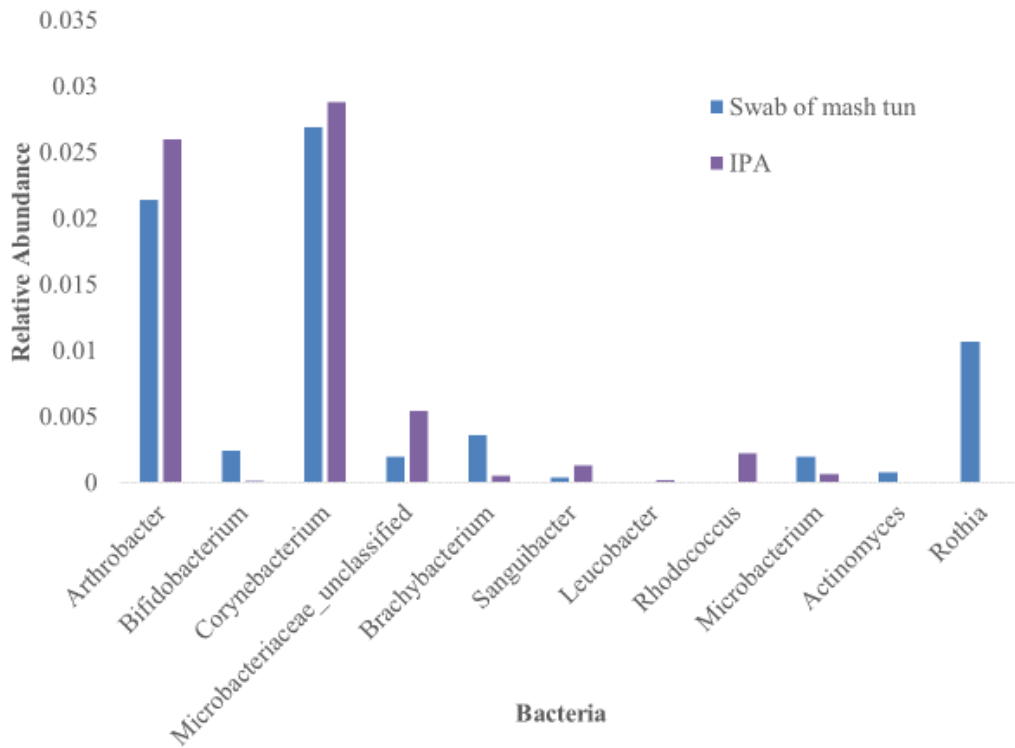
Figure 2.18: *Legionella*, *Carnobacterium*, *Novosphingobium*, and *Xanthomonadaceae* unclassified abundances shown for samples of the mash tun of the two breweries. Letters denote significant differences within genera at p<0.05.
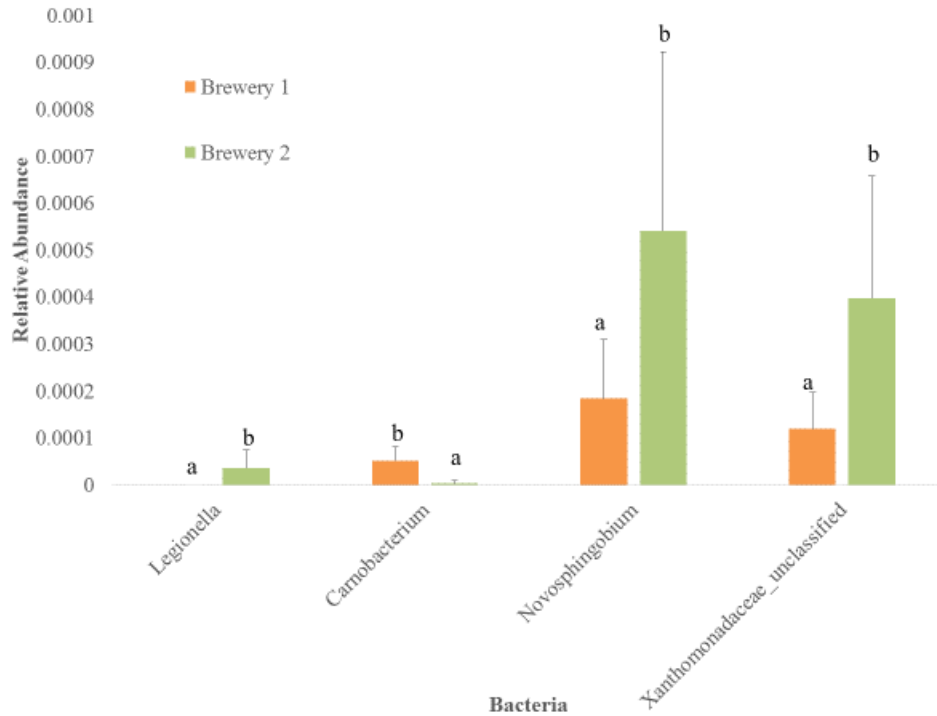
Figure 2.19: *Arthrobacter*, *Croynebacterium*, *Sanguibacter*, and *Rhodococcus* abundance in mash samples of the five styles. Letters denote significant differences within genera at p<0.05.
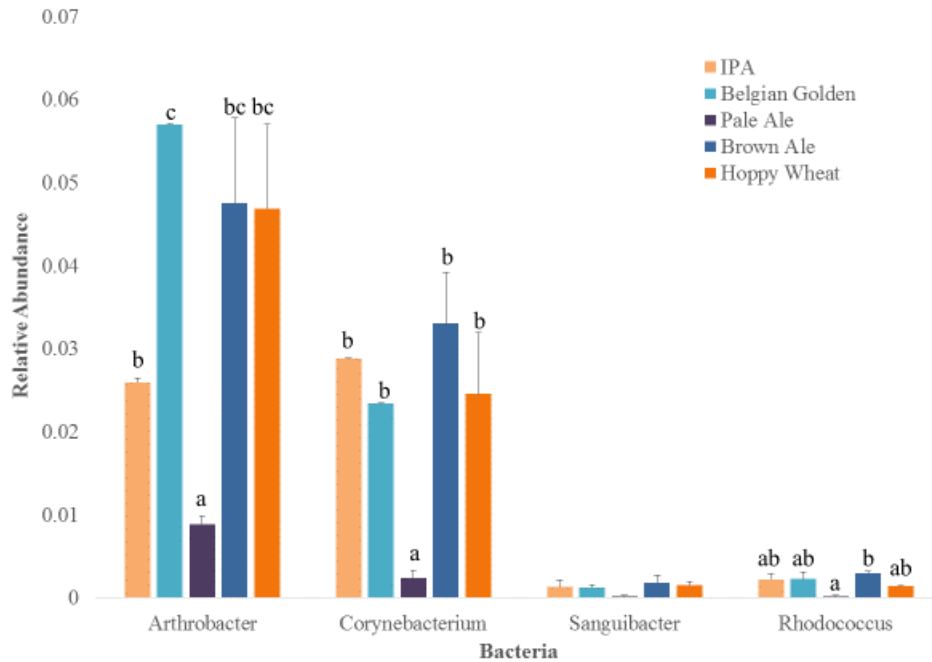
Figure 2.20: *Chryseobacterium*, *Wautersiella*, *Flavobacteriaceae* unclassified, and *Pedobacter* abundance in mash samples of the five styles. Letters denote significant differences within genera at p<0.05.
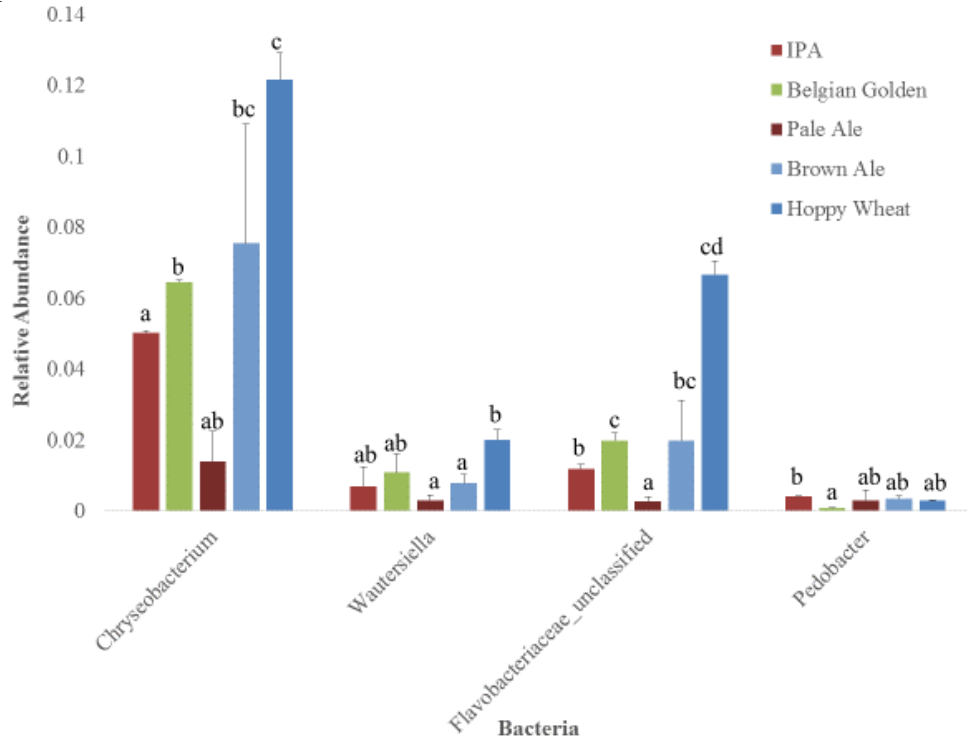
Figure 2.21: NMDS plot with Bray-Curtis index of the boil kettle swabs (red crosses) pre- boil (green), post- boil (red open square), and after cooling (red filled square) samples.
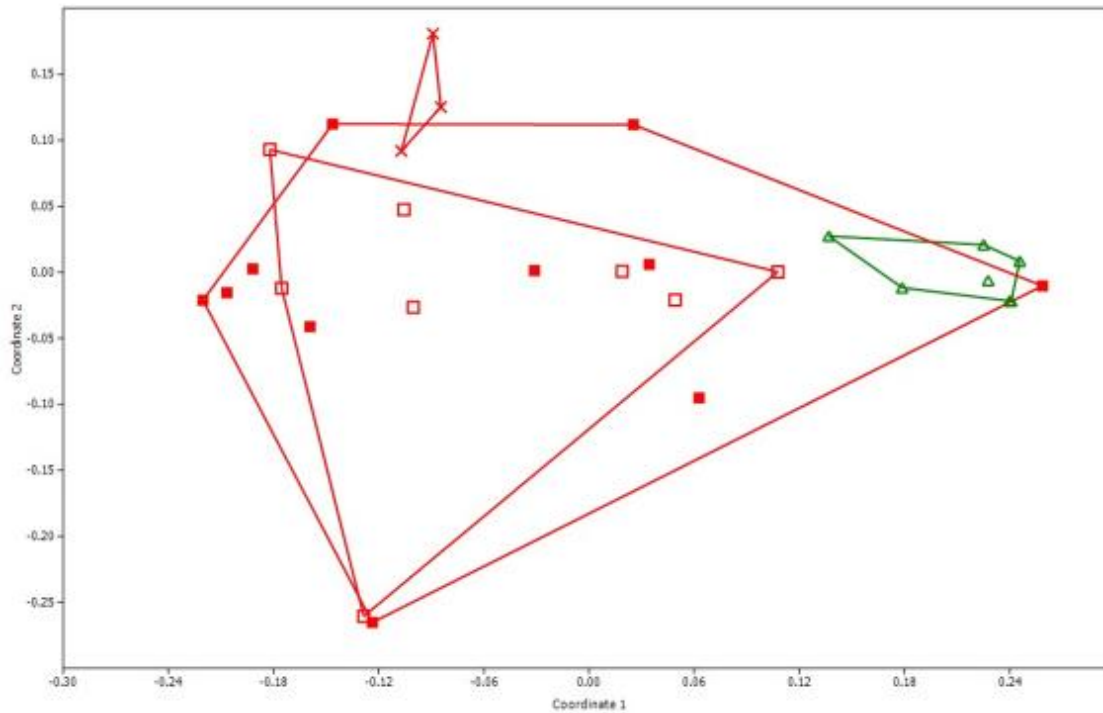
Figure 2.22: Abundance of bacteria combined for all beer styles during the boil stage. Letters denote significant differences within genera at p<0.05.
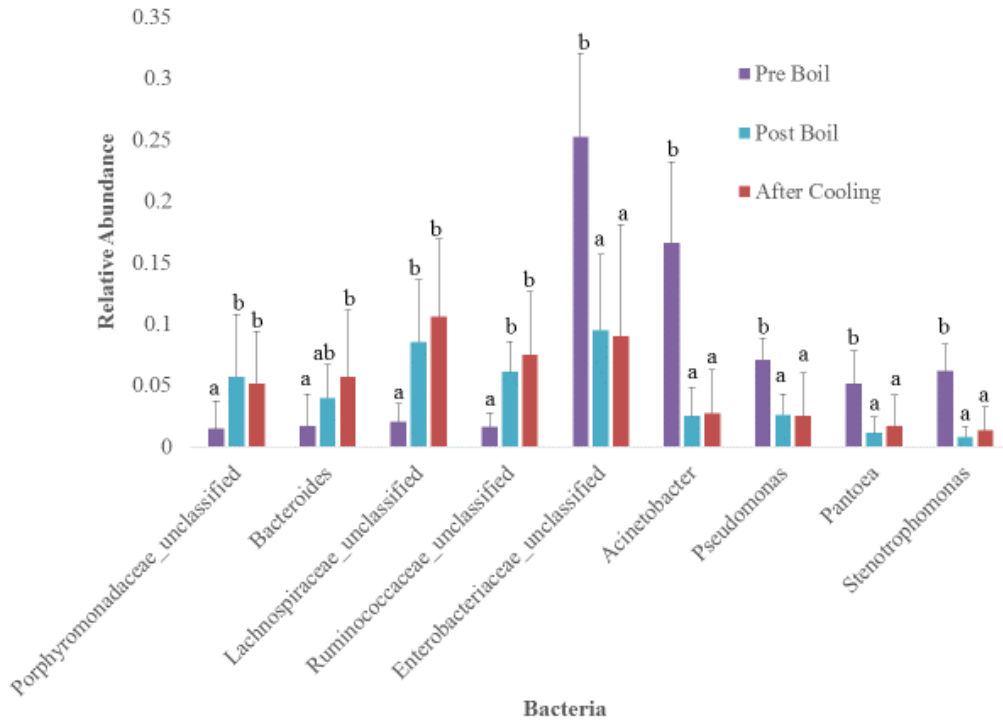
Figure 2.23: Abundance of spoilage bacteria for all beer styles during the boil stage. Letters denote significant differences within genera at p<0.05.
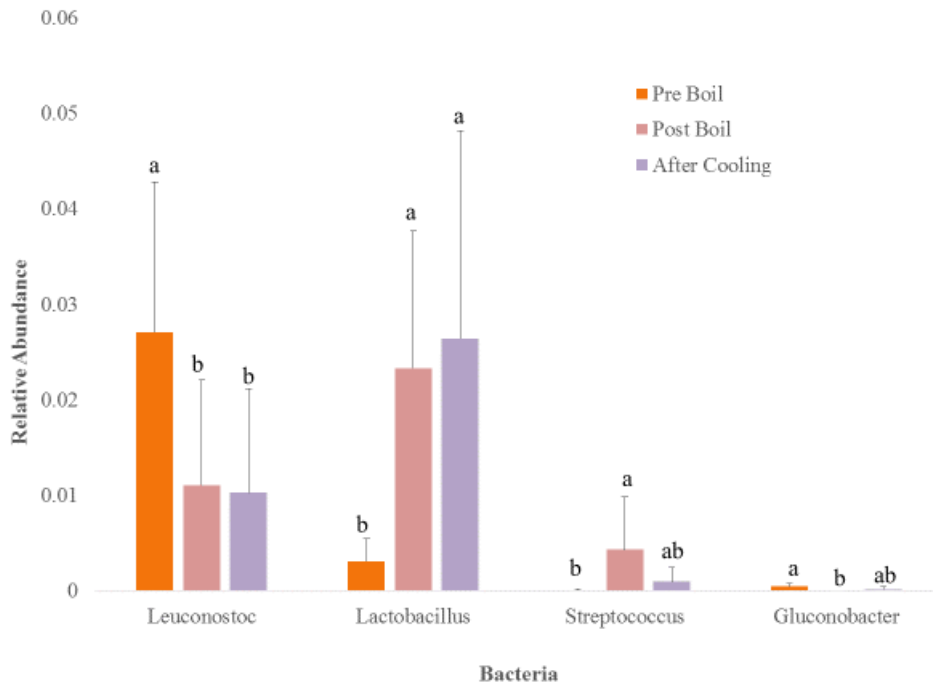
Figure 2.24: Abundance of bacteria during the boiling process of the Belgian Golden Ale. Letters denote significant differences within genera at p<0.05.
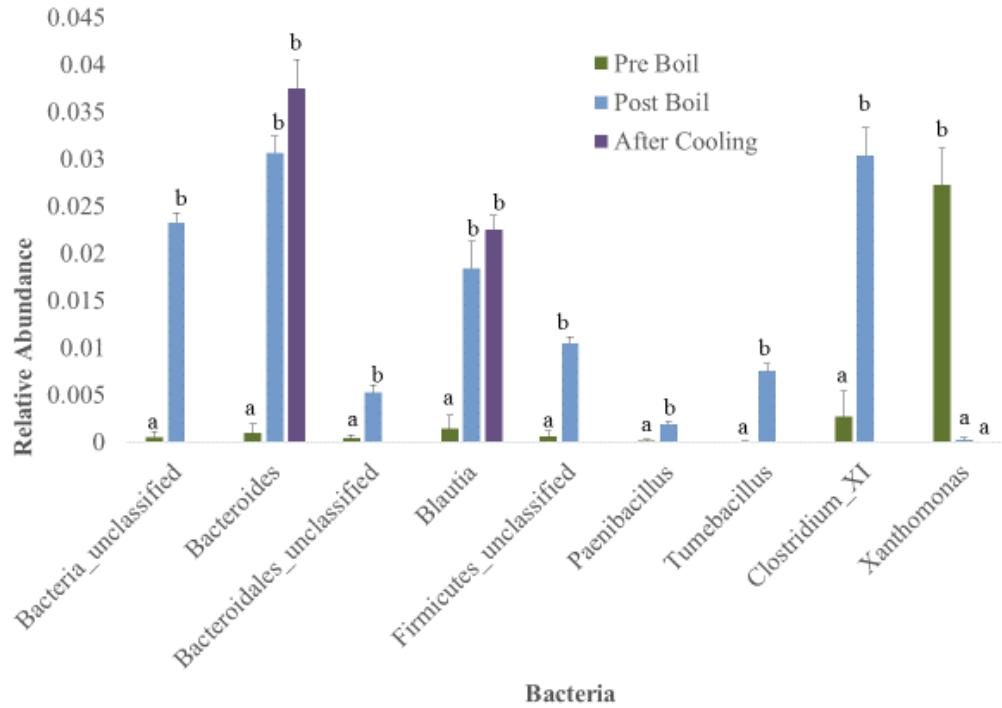
Figure 2.25: *Arthrobacter*, *Lachnospiraceae* unclassified, *Ruminococaceae* unclassified, *Weissella*, and *Ochrobactrum* differences during pre- boil stage. Letters denote significant differences within genera at p<0.05.
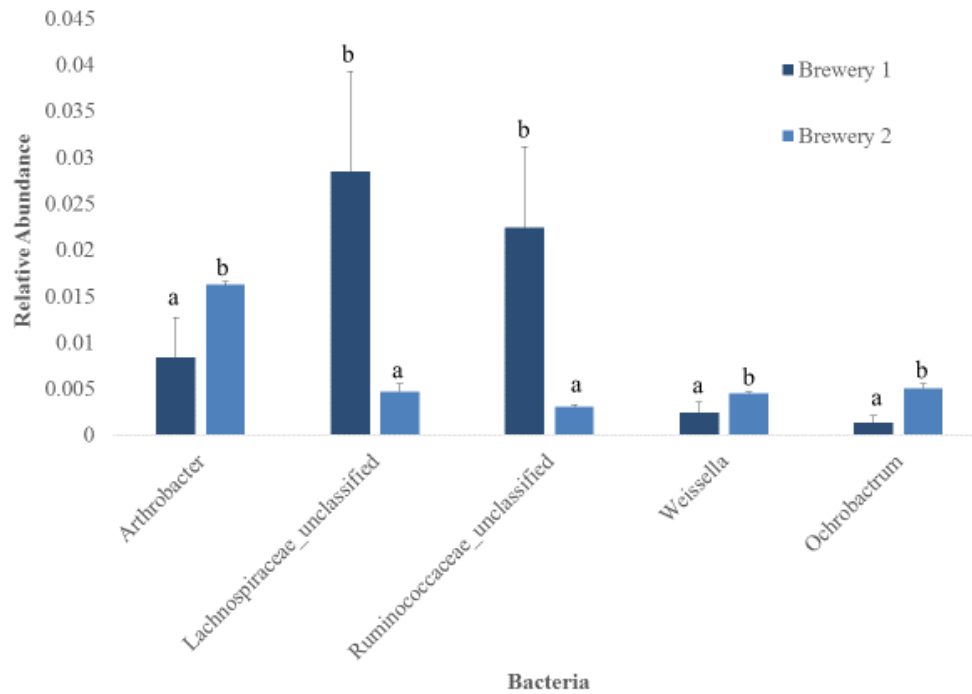
Figure 2.26: *Clostridiales* unclassified, *Blautia*, and *Bacilliales* differences during post- boil stage. Letters denote significant differences within genera at p<0.05.
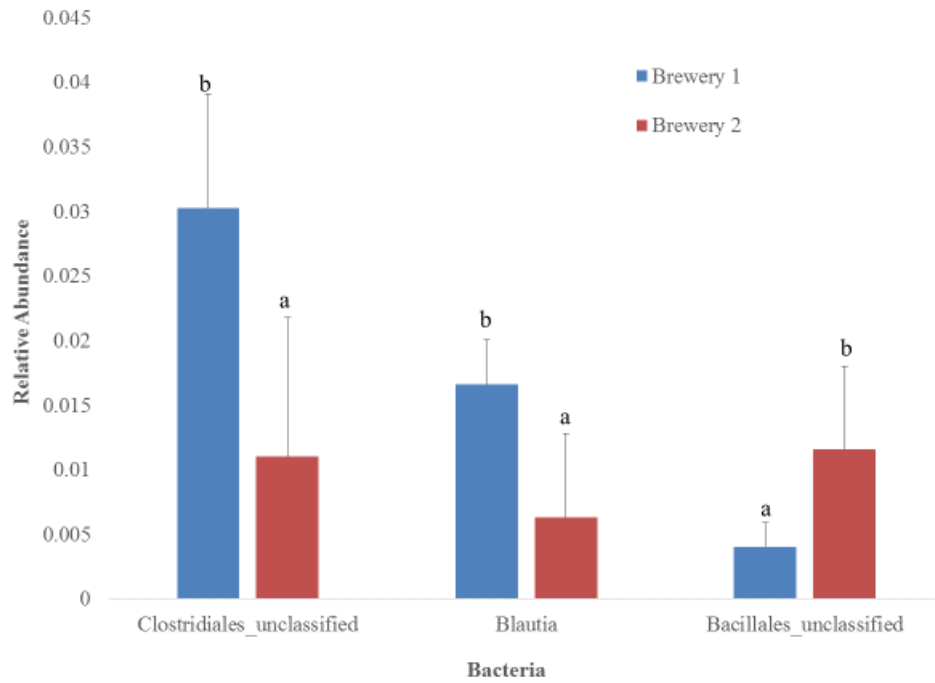
Figure 2.27: Bacteria abundance differences after cooling. Letters denote significant differences within genera at p<0.05.
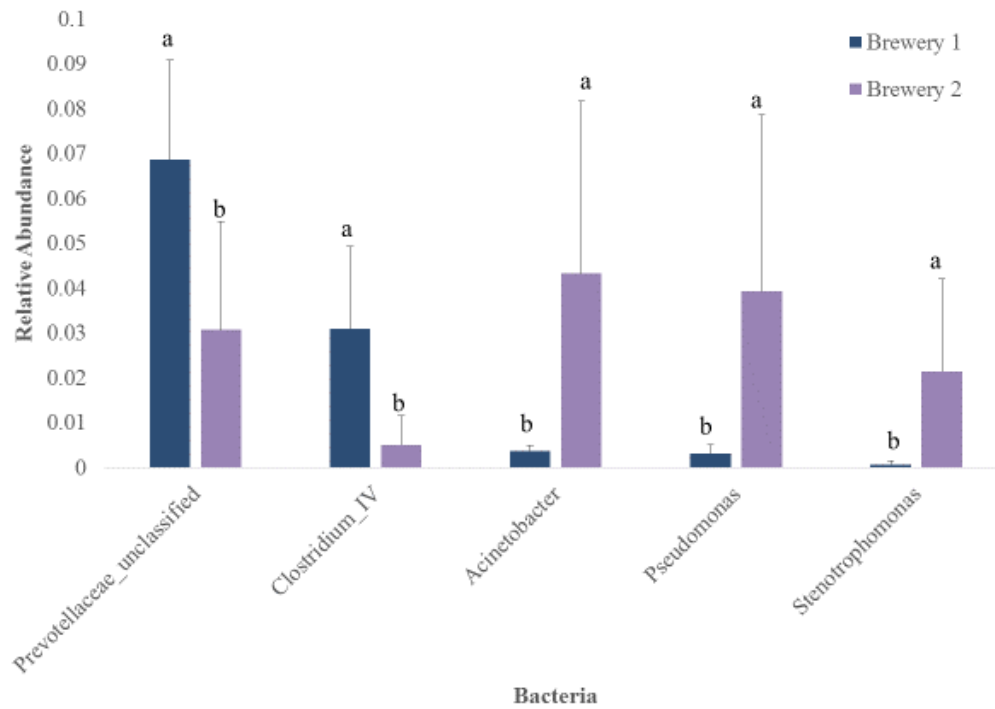
Figure 2.28: Bacteria abundance during fermentation of IPA, Belgian Golden Ale, Brown Ale, and Pale Ale combined. Letters denote significant differences within genera at p<0.05.
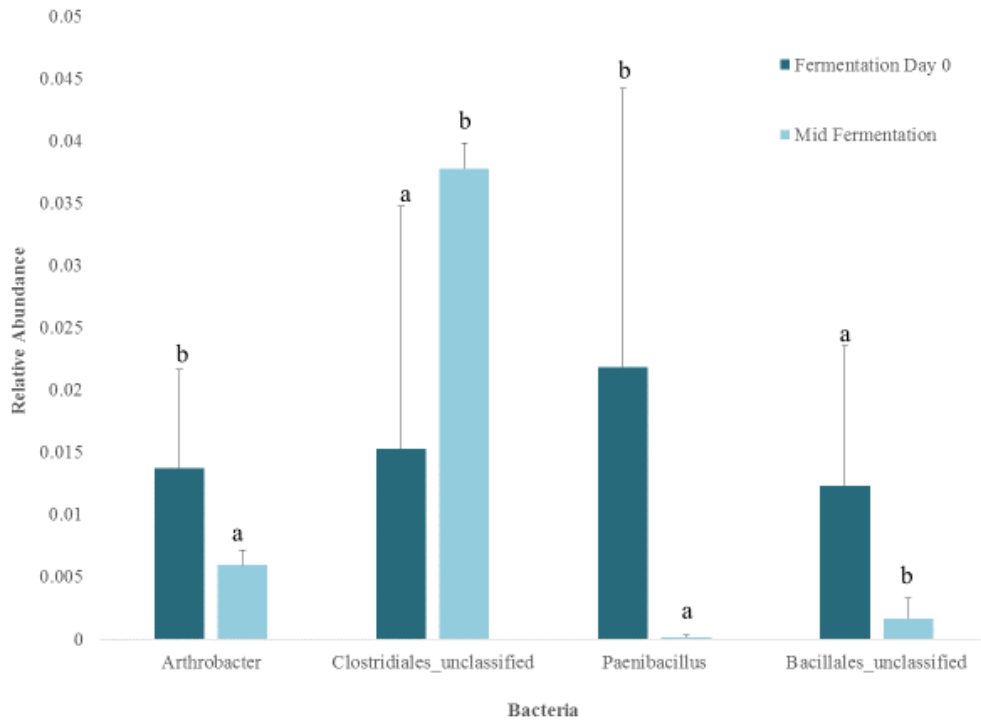
Figure 2.29: Hoppy Wheat bacteria during fermentation. Letters denote significant differences within genera at p<0.05.
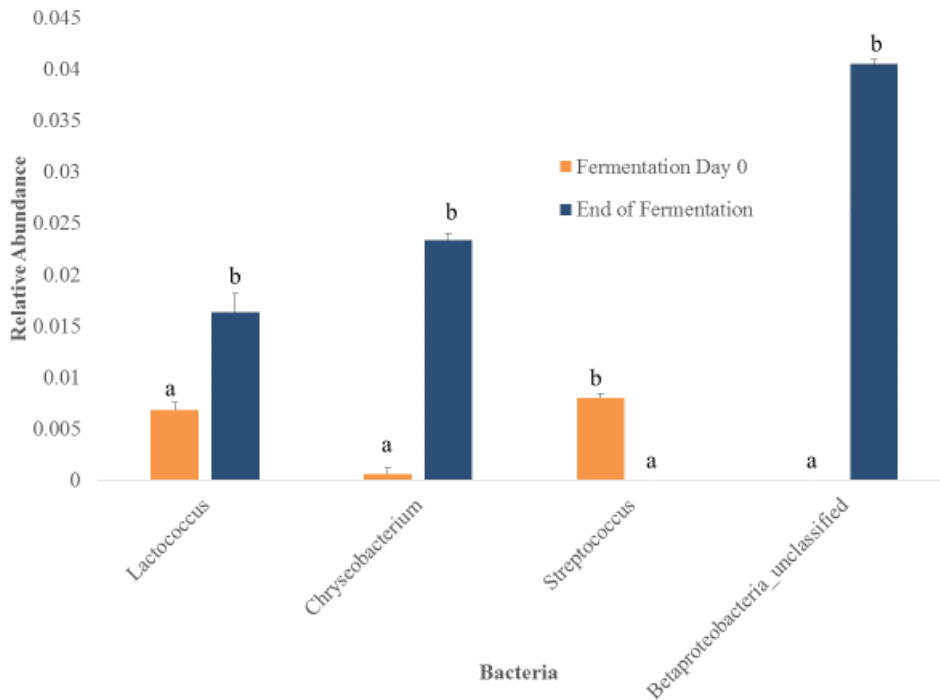
Figure 2.30: Abundance of bacteria during fermentation in four styles. Letters denote significant differences at p<0.05.
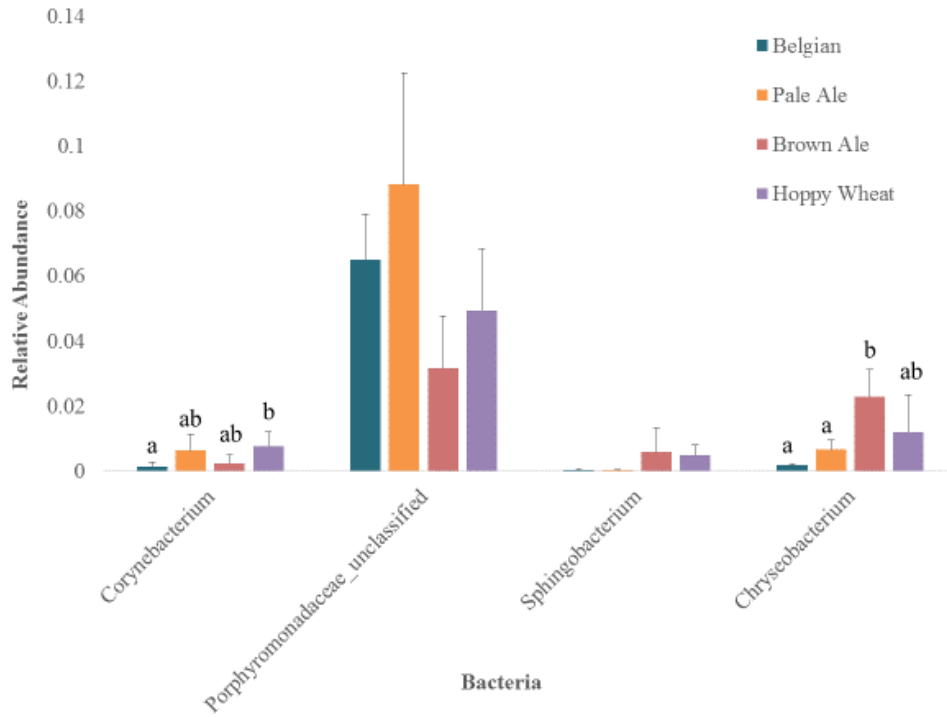
Figure 2.31: NMDS plot with Bray-Curtis index of filtering. Pre- filter is represented by crosses, post- filter is represented by open circles.
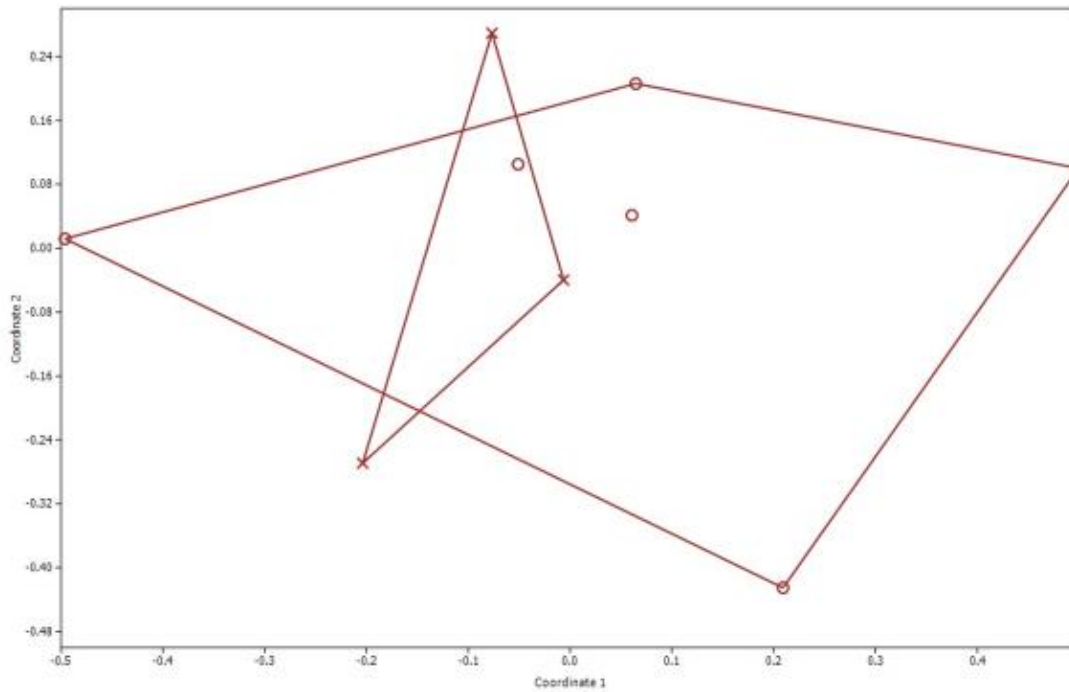
Figure 2.32: Abundance of *Blautia*, *Streptococcus*, and *Clostridium IV* in pre- and post- filter samples for all beer styles. Letters denote significant differences within genera at p<0.05.
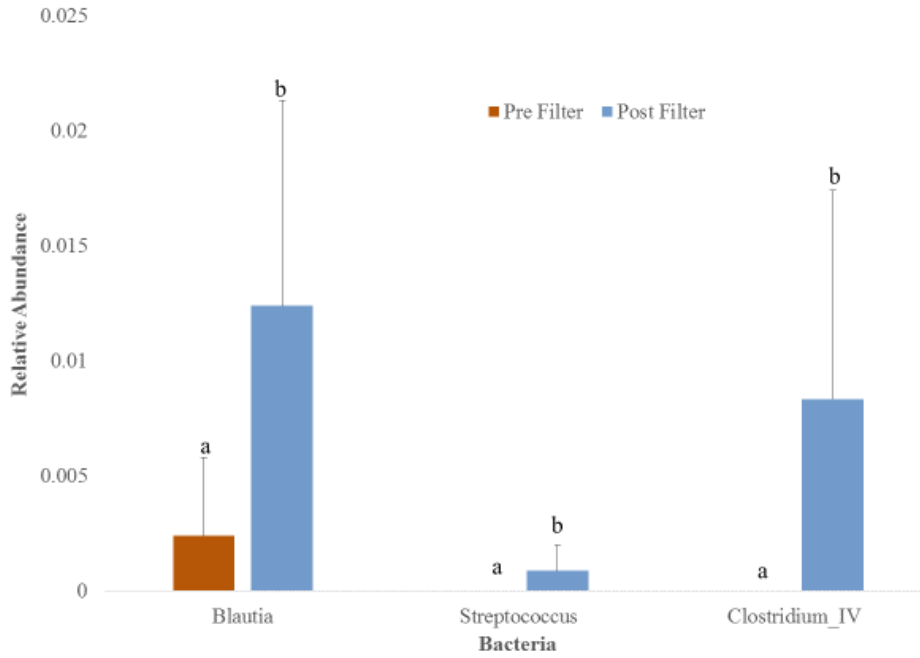
Figure 2.33: Ruminococcus and Proteobacteria unclassified abundance in the Belgian Golden Ale during filtering. Letters denote significant differences within genera at p<0.05.
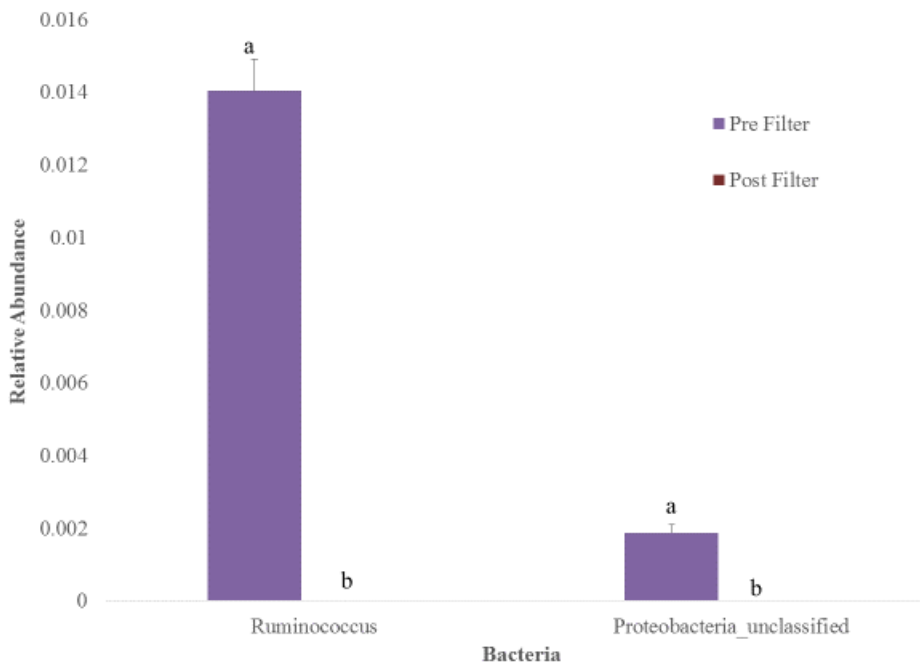
Figure 2.34 Final product phyla for all styles combined.
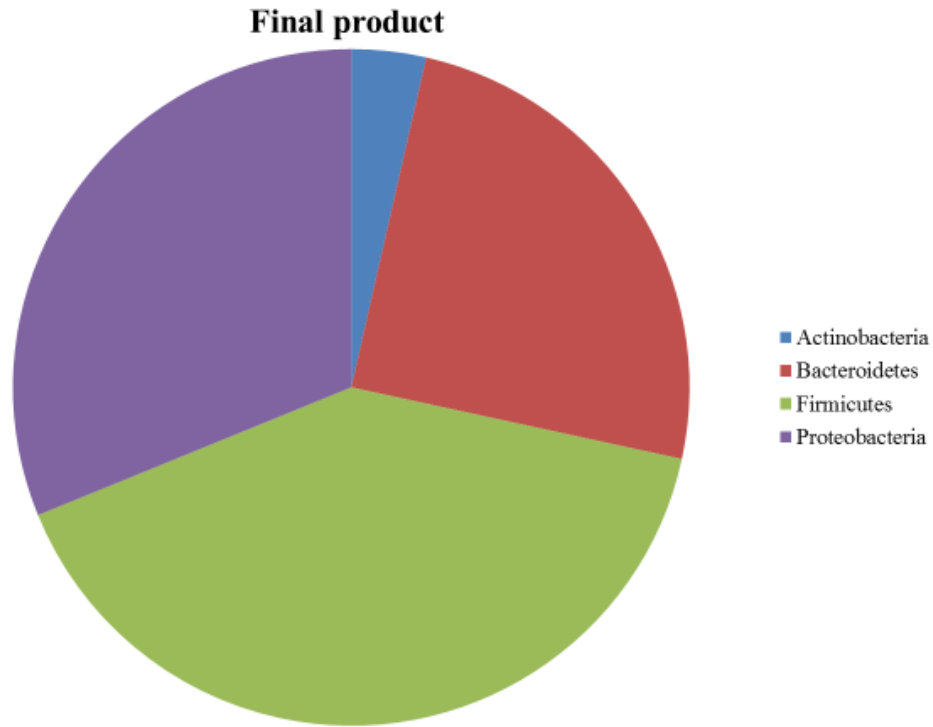
Figure 2.35 Actinobacteria genera in final samples for all styles combined.
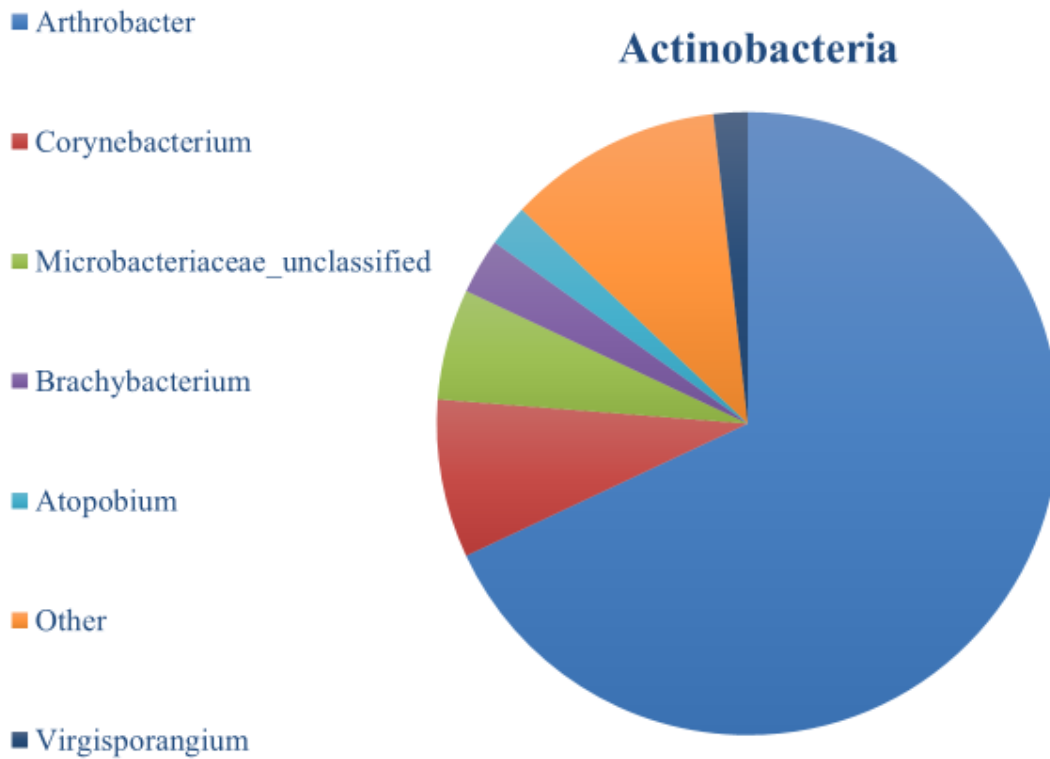
Figure 2.36: Bacteroidetes genera for final product samples combined.

Legend:
- Porphyromonadaceae_unclassified
- Alistipes
- Bacteroides
- Sphingobacterium
- Chryseobacterium
- Prevotellaceae_unclassified
- Flavobacteriaceae_unclassified
- Other

Bacteroidetes
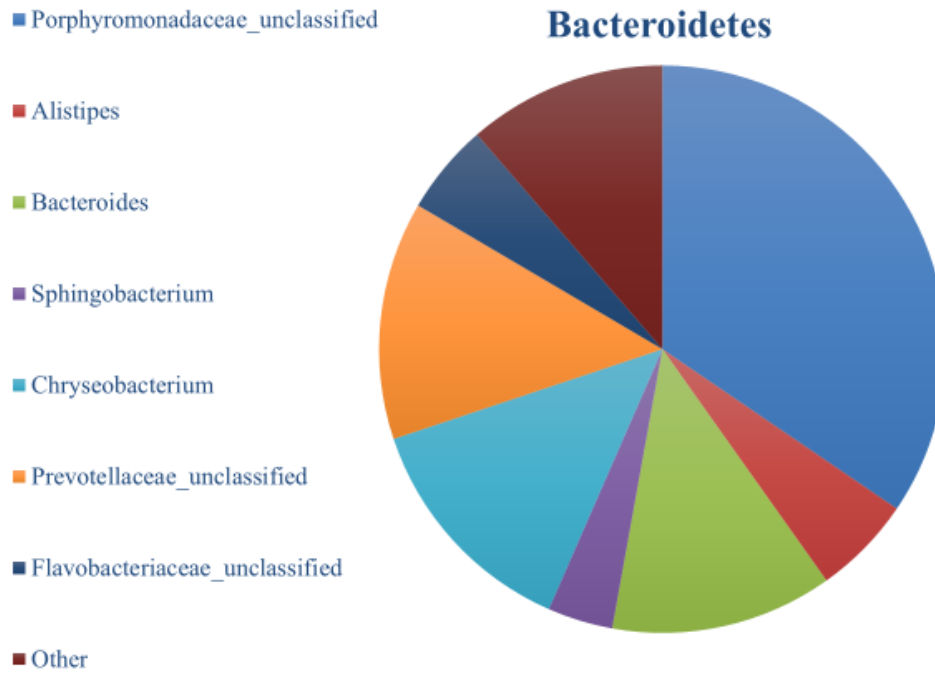
Figure 2.37: Firmicutes genera for final product samples combined.

Legend:
- Lachnospiraceae_unclassified
- Ruminococcaceae_unclassified
- Clostridiales_unclassified
- Brevibacillus
- Leuconostoc
- Lactococcus
- Lactobacillus
- Blautia
- Weissella
- Clostridium_XI
- Turicibacter
- Clostridium_IV
- Other
- Butyricicoccus

Firmicutes
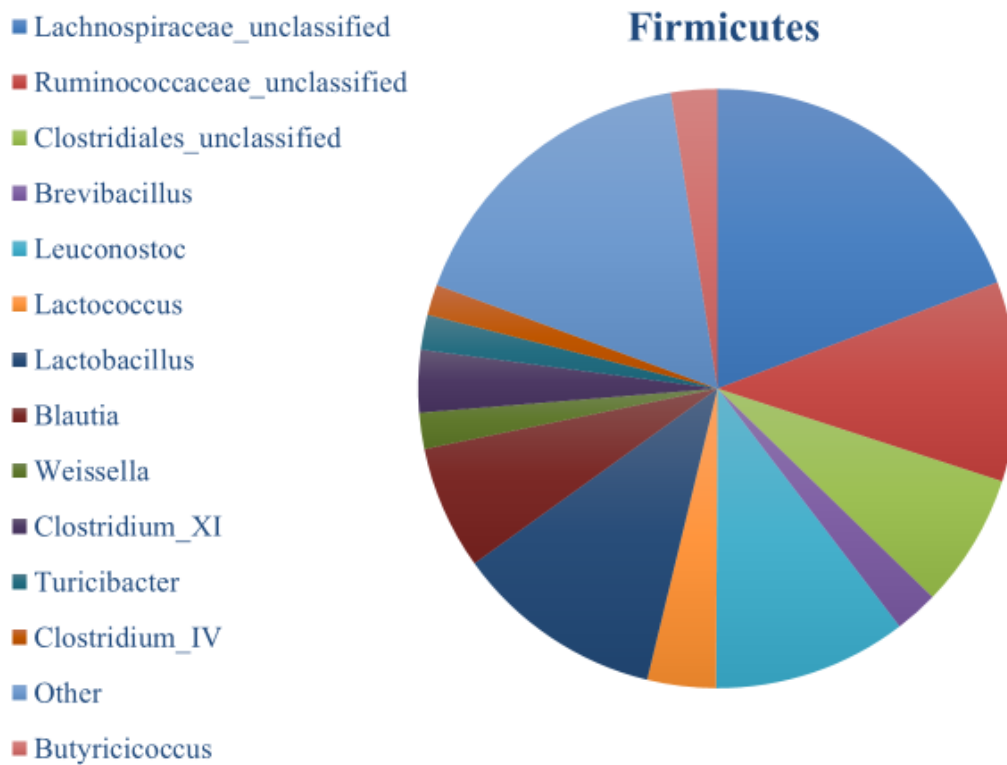
Figure 2.38: Proteobacteria genera for final product samples combined.
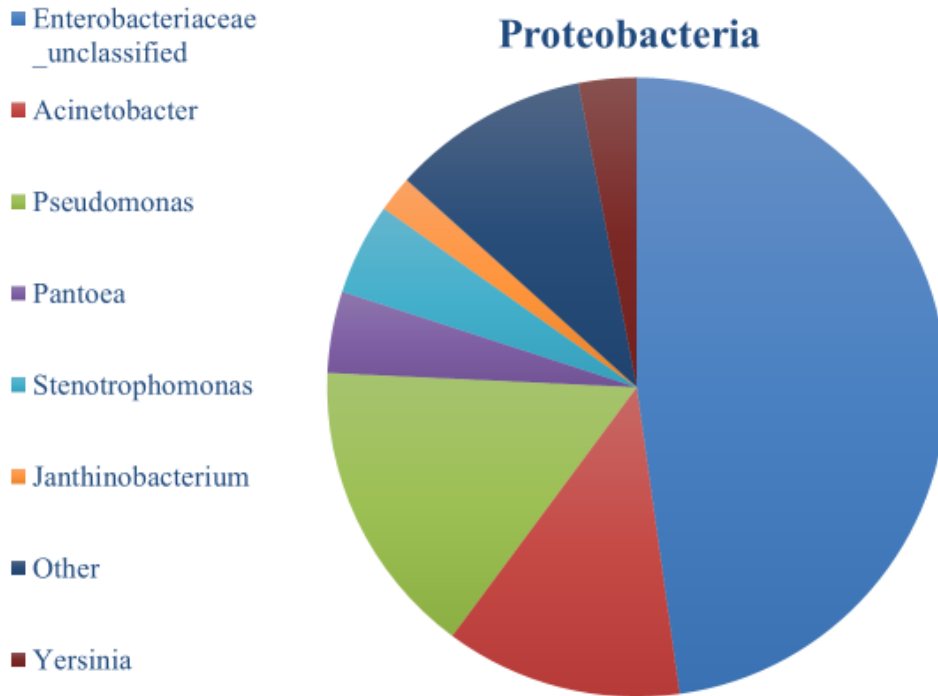
Figure 2.39: Final product differences in abundance of the five styles. Letters denote significant differences within genera at $p<0.05$. There was only one positive final product of the Hoppy Wheat, therefore there is no standard error bar or letters of significance.
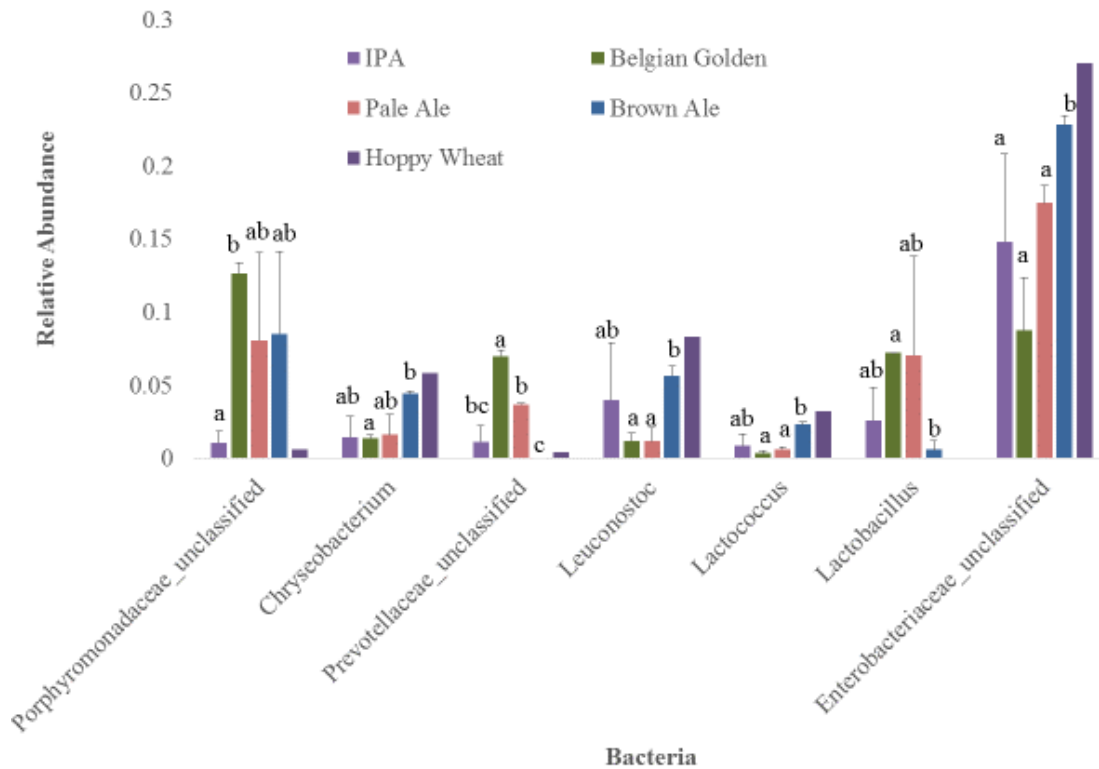
Figure 2.40: Bacteria abundance in the final product of the two breweries. Letters denote significant differences within genera at p<0.05.
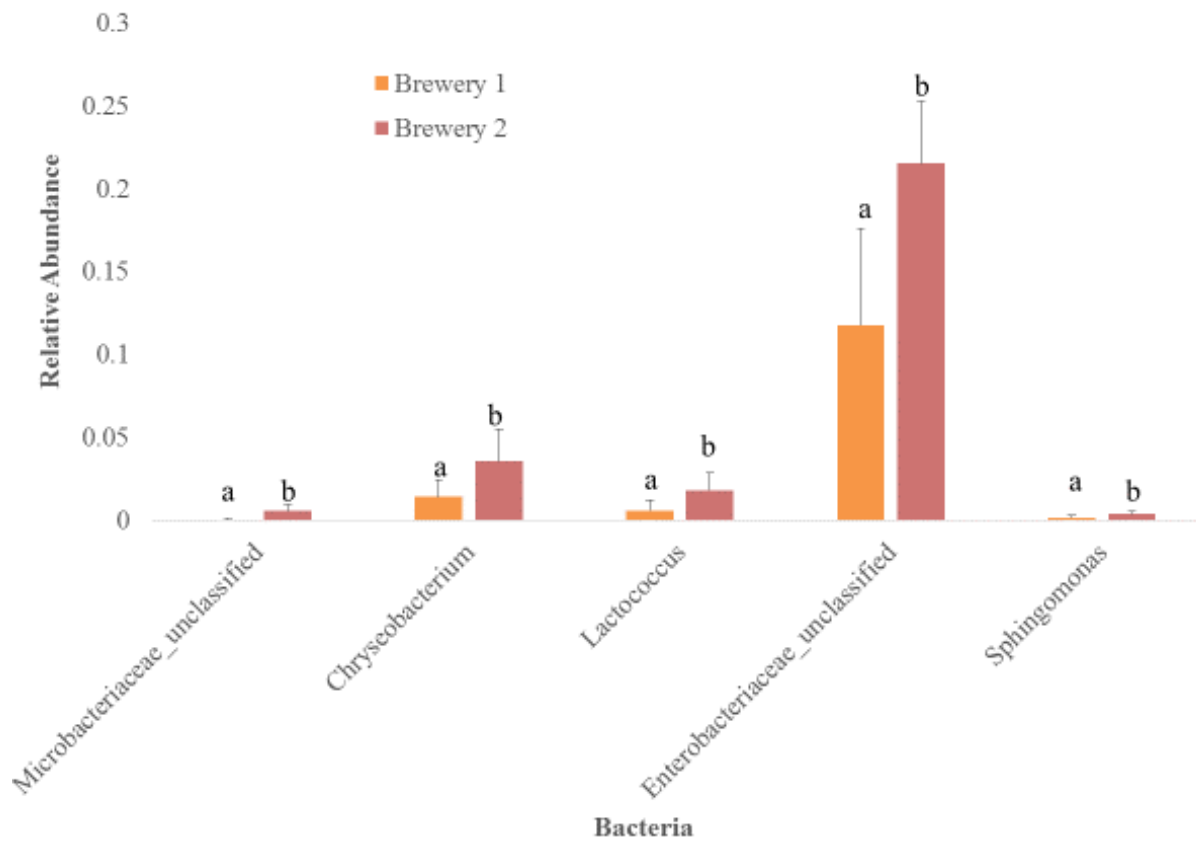
Figure 2.41: Overall beer dynamics by phylum for styles combined.
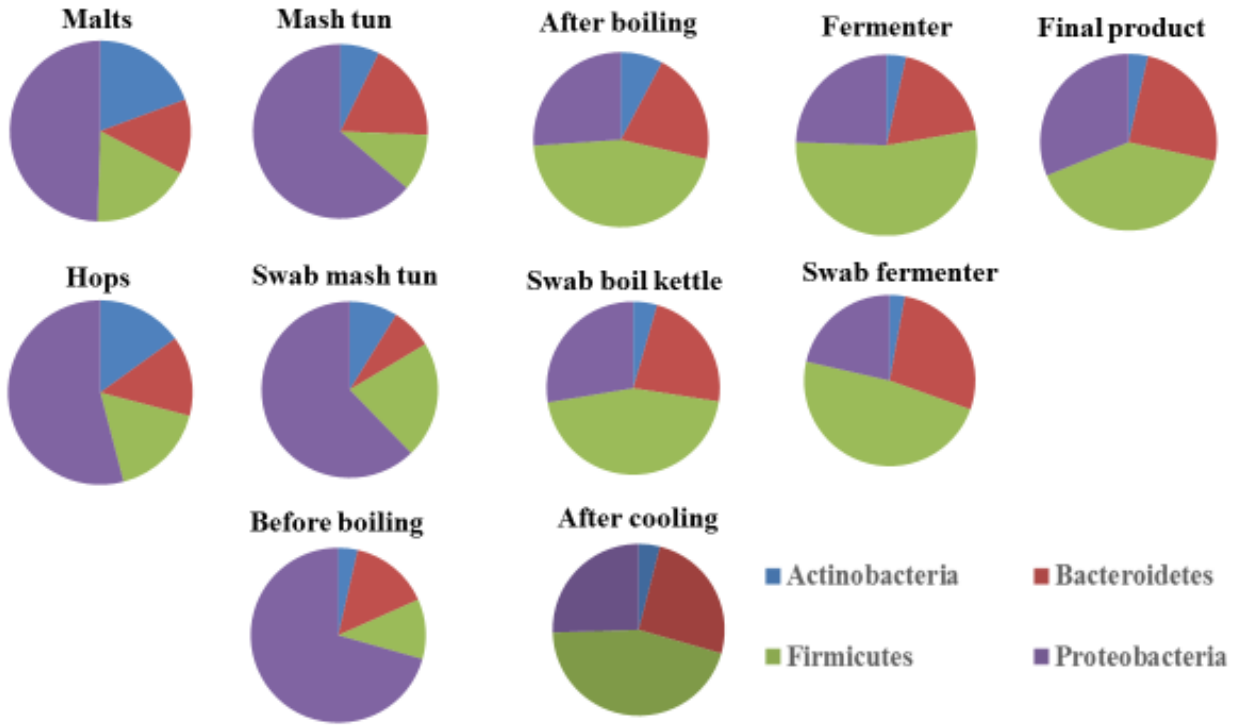
Figure 2.42: Rare phyla found throughout the brewing process.
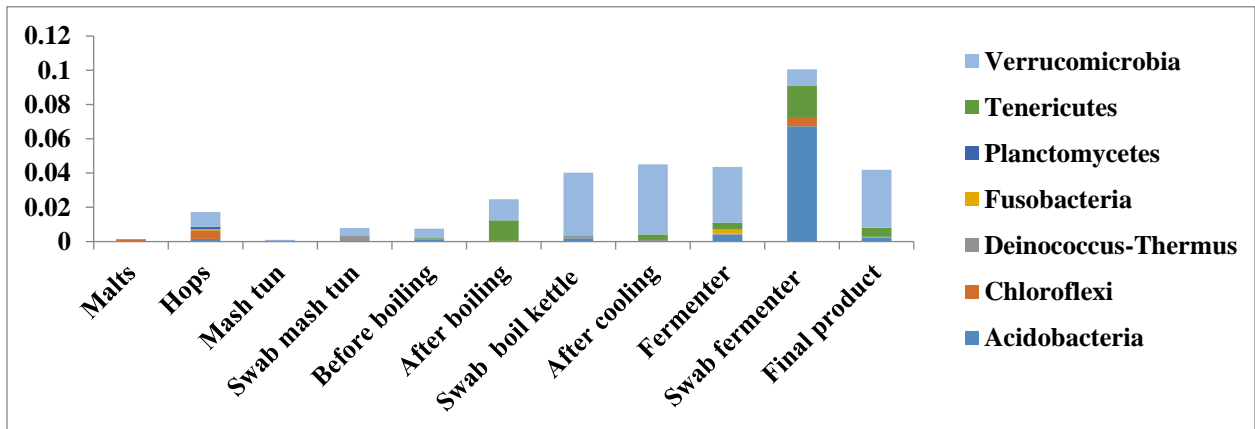
Figure 2.43: Significant differences in bacteria abundance between the two breweries.
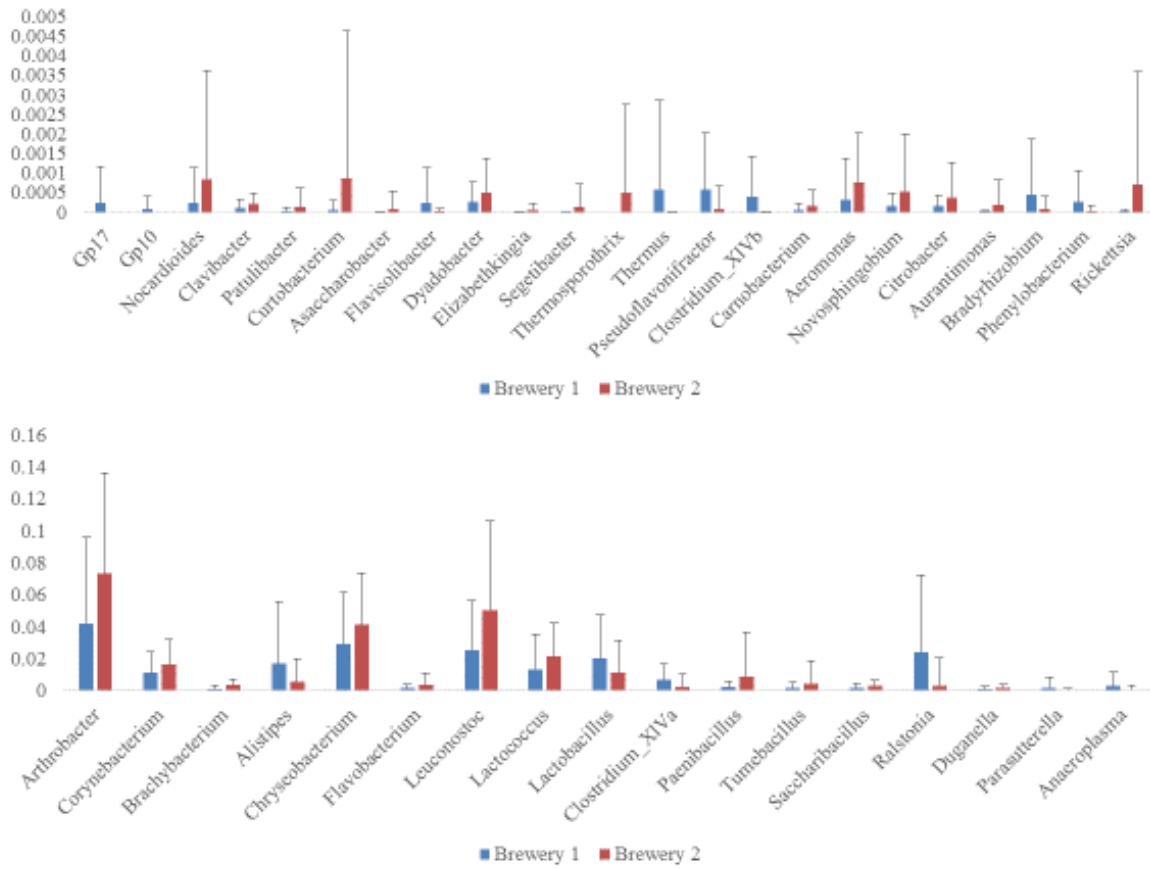
Figure 2.44: Bacteria dynamics of Brewery 1 through the process.
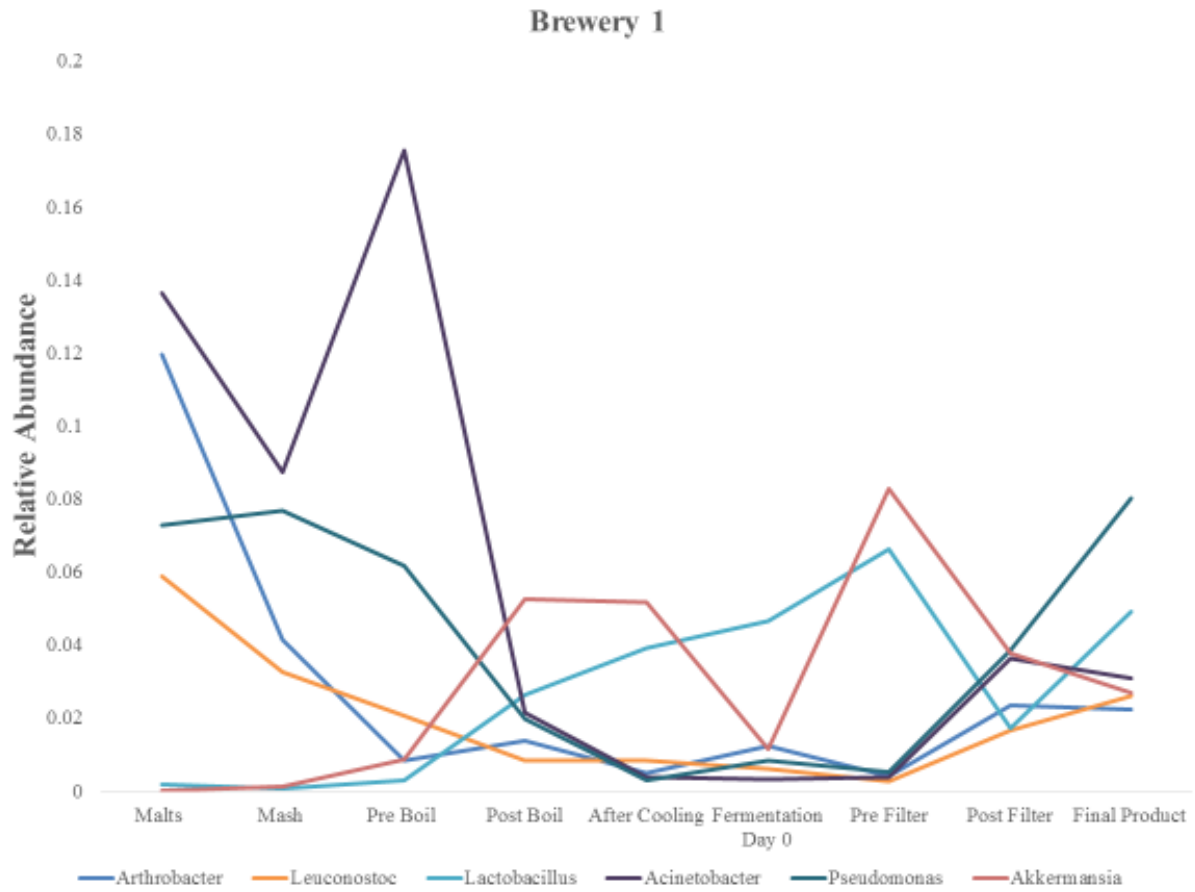
Brewery 1

Figure 2.45: Bacteria dynamics of Brewery 2 through the process.
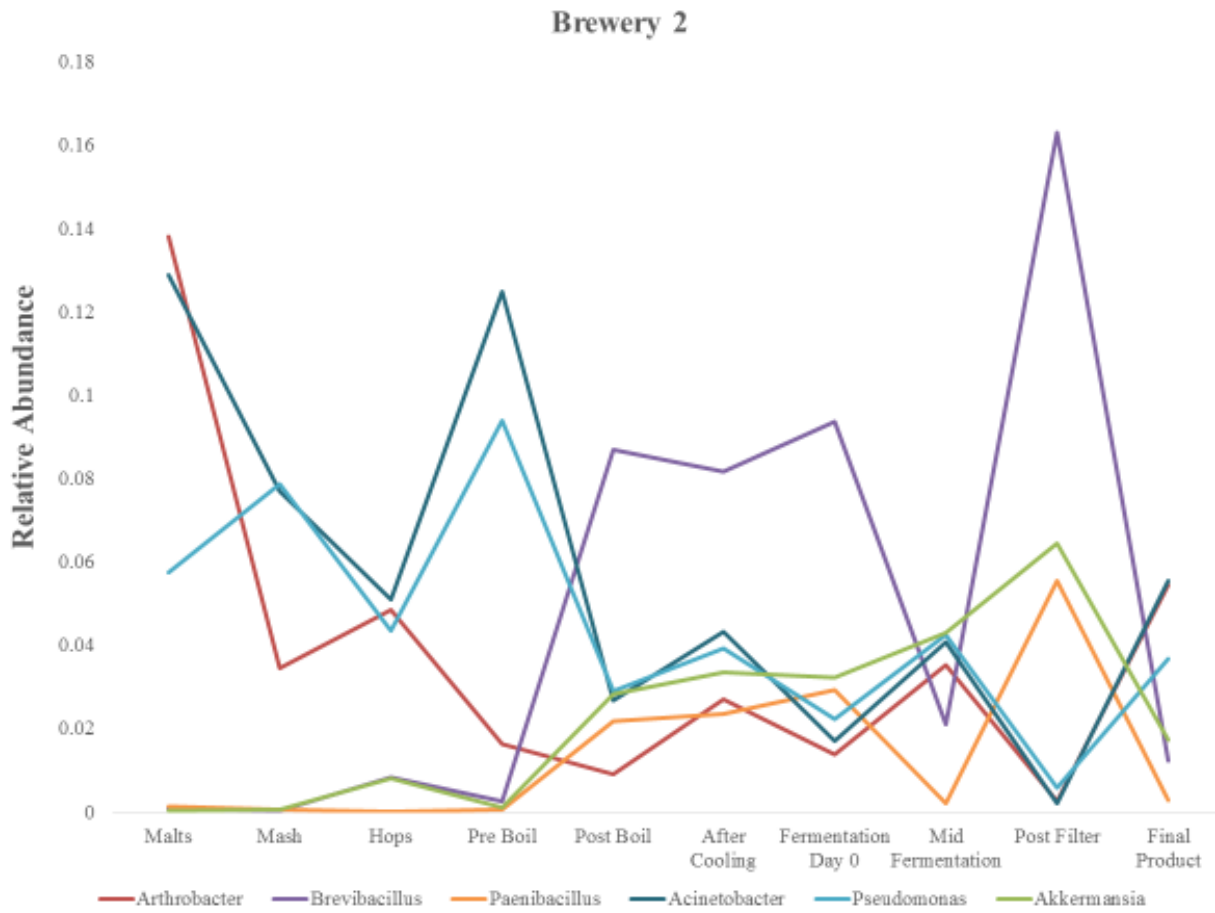
Figure 2.46: Beer dynamics by phyla for each style.
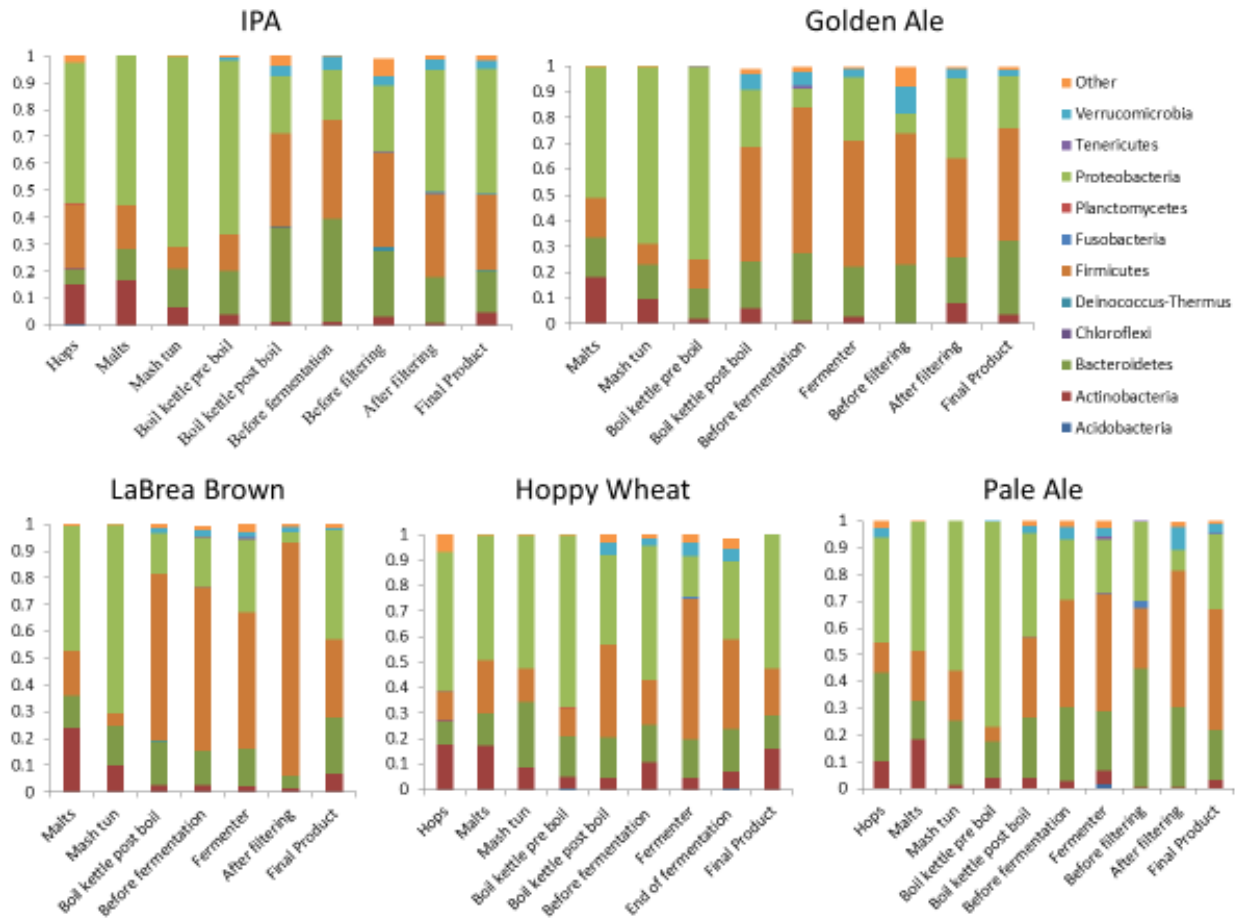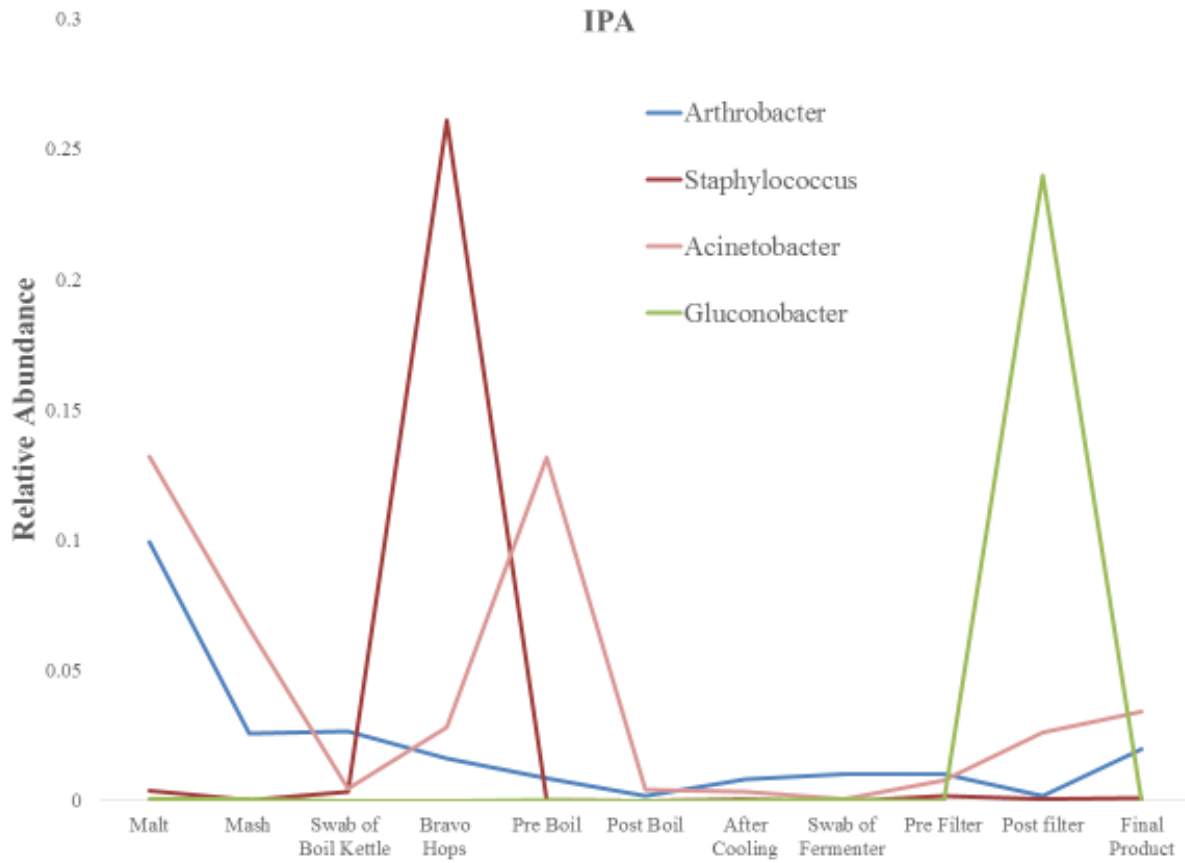
Figure 2.47: IPA dynamics through the process.

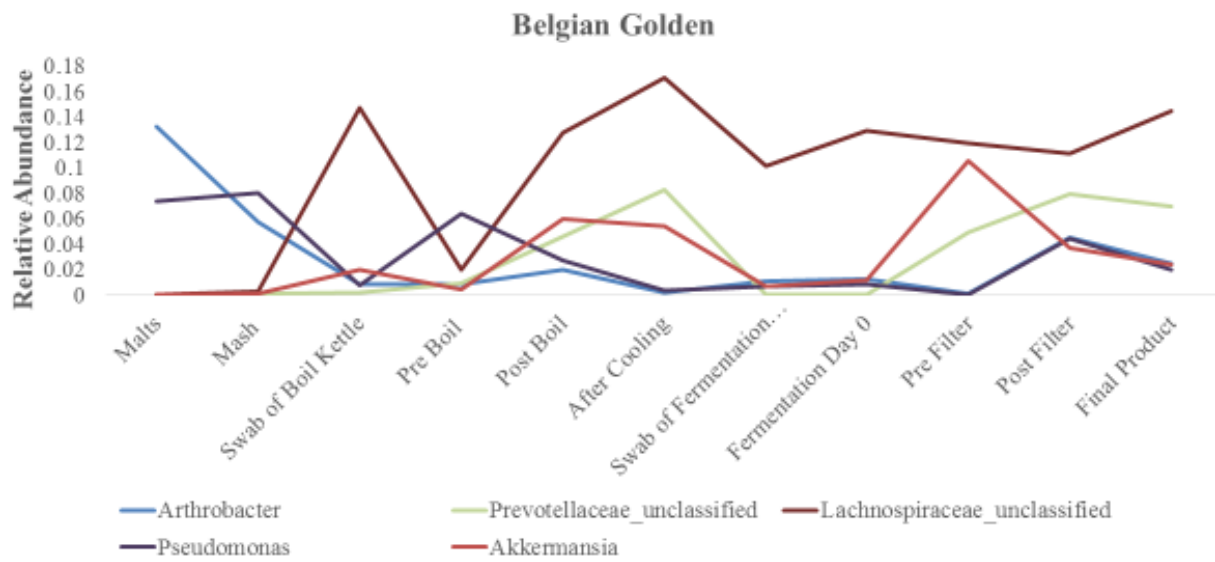**Figure 2.48:** Belgian Golden Ale dynamics through the process.

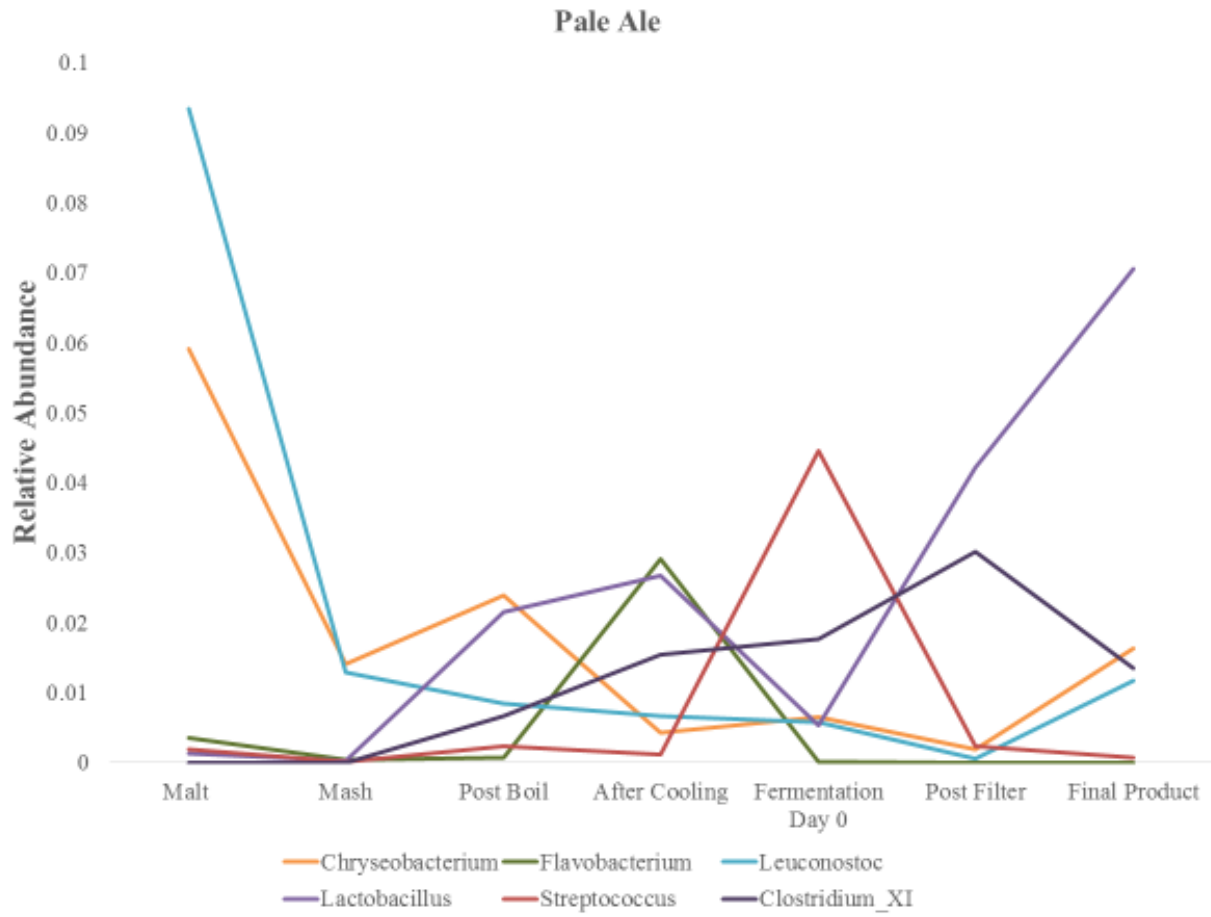**Figure 2.49:** Pale Ale dynamics through the process.
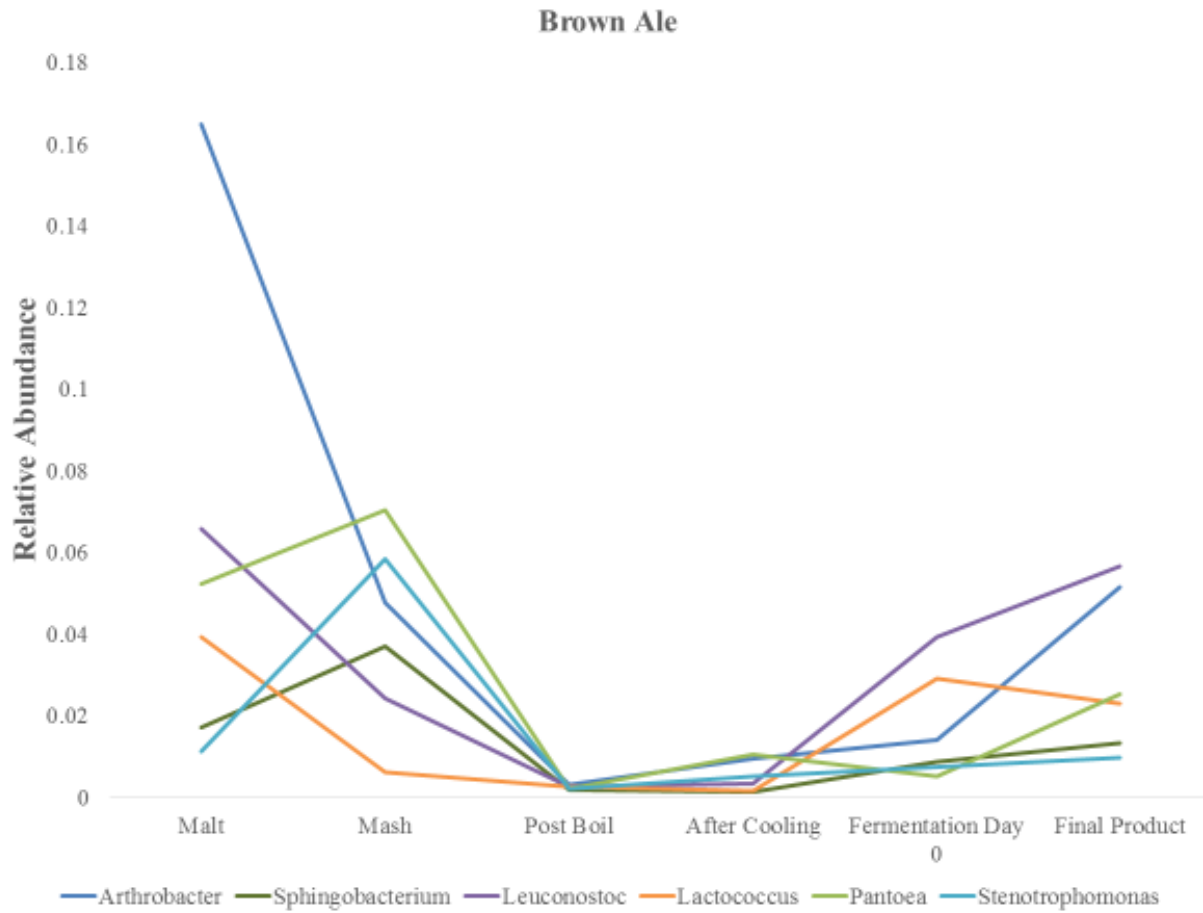

Pale Ale

Figure 2.50: Brown Ale dynamics through the process.

Figure 2.51: Hoppy Wheat dynamics through the process.

Hoppy Wheat