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Identification of Critical Points for Bacterial Contamination in the Microbrewery Environment

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IDENTIFICATION OF CRITICAL POINTS FOR BACTERIAL CONTAMINATION IN THE MICROBREWERY ENVIRONMENTS

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ABSTRACT

There are 8,884 craft breweries producing over 23 million barrels of beer in the United States as of 2020. These 23 million barrels of craft beer account for 12.3% of the United States beer consumption in 2020. The American craft beer industry is substantial and needs to protect its product from bacterial contamination.

Overall, beer is a microbially stable product. Beers pH, ethanol levels, CO_2 concentrations, the presence of hop-derived antimicrobial compounds, and low levels of O_2 make beer a highly unfavorable environment for most bacterial species. Furthermore, the brewing process, which involves heat treatments and chemical sanitizers further protect beer from bacterial contamination. However, beer sometimes does become contaminated by unwanted bacteria. Primarily these bacteria are members of the Genera *Lactobacillus*, *Pediococcus*, *Pectinatus*, and *Megasphaera*. These bacteria contaminate beer due to a variety of factors that allow them to evade beer's antibacterial properties or the process hurdles of the brewing process. Protecting beer from these spoilage organisms is crucial to maintain quality and shelf stability. Microbreweries and brewpub are especially vulnerable to bacterial contamination due to the unique challenges they face. These challenges include the use of modular chlorobutyl hose systems and mobile pumps, the overall "open" nature of smaller brewhouses and cellars exposed to the outside environment (such as opening tanks to pitch yeast, dry hop, or introduction of additions), and the proximity of the production and packaging areas to the public.

Research was conducted to determine steps in the brewing process that bacterial species were most likely to contaminate the microbrewery/brewpub environment. Samples were collected from three breweries of similar size and scale in Upstate South Carolina, from eleven

common locations throughout the brewing process and analyzed via HybriScan *D* Beer rapid molecular testing kits for the cell counts at each location. The racking arm valve had the highest levels of bacterial contamination.

DEDICATION

I would like to first dedicate this work to my wife Katie. Her support, love, ideas, and diligent proofreading has been valuable throughout this paper and my entire career. I would also like to dedicate this to my parents Alan and Vickie Thompson and my brother Jeffery Thompson who have always been my biggest supporters, motivators, and occasional hecklers. Last, I would also like to dedicate this work to Kimberly Ann Kolak and Victor and Mary Crews. This work would also not be possible without their support.

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CHAPTER I: Literature Review

Part 1: A Brief Bit on Beer and Brewing

Alcoholic beverages are an integral part of human civilization. Most historians agree alcoholic fermentation was serendipitously discovered by early hunter-gatherer humans. Gathered sources of fermentable sugar were stored in containers open to circulating microorganisms. These circulating microbes, somewhat "hunter-gatherers" themselves, colonized the sugar sources and began to grow. The growth of these microbes, who use sugars as an energy source, produces ethanol as a byproduct. One organism's trash is another's $1,587 billion industry. This figure was presented in the Alcoholic Beverages Market: Global Industry Trends, Share, Size, Growth, Opportunity and Forecast 2021-2026, published by the IMARC Group in May 2021 (40). Beer accounted for $552.4 billion of this total. Beer is the third most popular beverage in the world behind water and tea (42).

Beer is an ancient beverage. Historical evidence shows that Mesopotamians brewed beer. The Egyptians were accomplished brewers and large consumers of beer. All Egyptians from commoners to the pharaohs drank beer. Egyptian beer was most likely very low in alcohol and was brewed daily and consumed immediately (41). This beer would have been wildly different from the beer of the 21st century.

Modern beer has its roots in Europe. The cereal grains used to brew beer grew widely across Europe from the British Isles to the steppes of western Russia. These grains were gathered and cultivated by the various ancient tribes that inhabited Europe at the time and was brewed into beer. Roman society viewed beer and the culture surrounding its consumption as something foreign and "un-Roman", declaring beer "a barbarian's beverage" (42). However, the Roman

conflict with the various Gallic and Germanic tribes, beginning with Vercingetorix and ending with Odoacer's sacking of Rome and the forced abdication of Emperor Romulus Augustus, brought European beer drinking culture back into favor.

Charlemagne, the first Holy Roman Emperor, considered beer an integral part of daily life and is believed to be an accomplished brewer himself (41,44). Pepin, the father of Charlemagne, gifted a hop garden to a friend in 768A.D. (45). Monasteries controlled closely guarded recipes of a spice and herb blend known as "gruit" that was used to flavor beers (46). The famous Benedictine polymath and abbess Hildegarde of Bingen wrote a treatise on hop growing in 1150 A.D (45). Beer production, once a cottage industry, became a commercialized in 13th century Bohemia and the trend spread to Holland, Flanders, and Belgium in the 14th century and to England by the 15th century (44). William IV, Duke of Bavaria, passed the "Reinheitsgebot" or purity order, which codified the allowable ingredients in beer in 1516. The Reinheitsgebot is known as one of the oldest written food regulations in human history. The British Empire sent its beer and beer drinking culture across the globe, from their New World Colonies to garrisons of the East India Trading Company. In fact, the wildly popular beer style of India Pale Ale (colloquially known as IPA) was developed in England to be sent to India. Barrels of traditional English pale ale were packed full of hops (a known preservative) to assist its survival in the arduous sea journey around the Cape of Good Hope to the British East Indies. These extra hops gave IPA its distinctive bitterness and aroma. Beer has also been a source for scientific advancement and discovery.

Louis Pasteur wrote his book *Studies on Fermentation: The Diseases of Beer, Their Causes, and the Means of Preventing Them* out of anger at the result of the Franco-Prussian War, hoping to give France a competitive edge against Germany and their largest export, beer (47).

Johan Kjeldhal developed the Kjeldahl Method to determine nitrogen content of a sample while analyzing grain protein content at Carlsberg Laboratory at the Carlsberg Brewery in Copenhagen, Denmark in 1883. William Sealy Gosset, the head brewer of Guiness in Dublin, Ireland developed Student's (his pen name) t-distribution in 1907 and became a pioneer of modern statistics (50).

Beer's popularity in America originated with its Founding Fathers. George Washington, Thomas Jefferson, and Samuel Adams all brewed beer. American brewers produced some of the most recognizable beer brands in the world. David Gottlieb Yuengling founded the Eagle Brewery (now D.G. Yuengling and Son) in Pottsville, Pennsylvania in 1829, Frederick Miller started the Miller Brewing Company in Milwaukee, Wisconsin in 1853, Eberhard Anheuser and Adolphus Busch partnered 1869 to form Anheuser-Busch (now known as AB InBev), the producer of Budweiser, and Adolphus Coors and Joseph Schuler started the Coors Brewing Company in Golden, CO in 1873.

Since then, new American brewers have made names for themselves as pioneers of the craft beer movement. Ken Grossman and Paul Camusi found the Sierra Nevada Brewing Company in Chico, California in 1979. Jim Koch founded the Boston Beer Company (the producer of Samuel Adams) in Boston, Massachusetts in 1984. Kim Jordan and Jeff Lebesch founded New Belgium Brewing Company in Fort Collins, CO in 1991, and Sam Calagione founded Dogfish Head Brewery in Milton, DE in 1994. Since then, craft beer in America has exploded.

According to the Brewers Association, there were 8,884 craft breweries in the United States, producing over 23 million barrels of beer, accounting for 12.3% of the total U.S. Beer

market in 2020 (20). It is estimated that most Americans live within 10 miles of a craft brewery (49). Beer and especially craft beer is here to stay.

Part 2: Microbial Contamination and Persistence in the Microbrewery/Brewpub Environment

Beer is typically considered a microbiologically stable product. Beer can still become contaminated with specific bacterial species that are capable of evading beer's intrinsic antibacterial properties and the processing hurdles of brewing that lower bacterial contamination risk. Research thus far is lacking is protecting the growing community of microbreweries and brewpubs. These producers face a unique set of challenges in protecting their product that larger breweries do not.

Definitions, Market Share, and Economic Impact

The Brewers Association, a 501(c)(6) not-for-profit trade association, defines a craft beer producer as a small and independent brewer. "Small" is defined as an annual production of six million barrels of beer or less. "Independent" is defined as less than twenty five percent of the producer is owned by a beverage alcohol producer that itself is not a craft brewer. Microbreweries are a subcategory of craft beer producers defined by the Brewers Association. Microbreweries have been defined by an annual production of fifteen thousand barrels of beer or less and must sell 25% or more of their annual beer production on site. Per the Brewers Association 2020 Industry Statistics Report, there are 1,854 microbreweries in the US. Brewpubs are identical in definition to microbreweries with the exception that 100% of their annual beer production is sold on site. According to the same report, there are 3,219 brewpubs in the United States (20).

Craft beer sales make up 12.3% of annual beer sales for a total of 22,815,258 barrels in 2020. Beer produced by microbreweries and brewpubs make up 32.8% of the craft beer market for a total of 7,483,404 barrels in 2020. The craft beer industry contributed $62.1 billion dollars and 400,000 jobs to the US economy in 2020. 140,000 of these jobs were directly tied to microbreweries and brewpubs.

Need to Protect Product

Craft beer has a substantial footprint in the US beverage market with. Microbreweries and brewpubs being a major component of that footprint. Most microbreweries and brewpubs are small family-owned operations that operate on very tight margins (which have only become tighter due to the COVID-19 pandemic). The loss of product for any reason can have a major financial impact on the owners of a microbrewery or brewpub. The primary reason for product loss is microbial contamination. Microbial contamination can occur at different stages along the production stream and can lead to substantial negative impacts on beer aroma, flavor, appearance, and overall quality to the point that the product is unsaleable. Furthermore, contamination that is not identified prior to packaging can lead to the rupturing and bursting of packaged products. This can lead to product loss, cross contamination of nearby products, and even injury to those around the package at the time of failure. Product contamination being discovered by consumers outside the brewery can have a substantial impact of the producer's reputation and dramatically impact sales.

Beer as a Microbial Product

Beer is generally recognized as a microbially stable product. This stability is due to various hurdles to microbial contamination. Hurdle technology is defined by Leistnar as a "combination of food preservation methods" (1,21). Leistnar further elaborates on the concept of

hurdle technology by describing the "hurdles" as both the intrinsic properties of the food including pH, a_w, Eh, and competitive microbes (1,21) as well as the "influence of food preservation methods on the physiology and behavior of microorganisms in food" (1,21).

In beer these hurdles include pH (typically 3.9-4.4), the presence of ethanol (ranging from 3.2% to 14% by volume), the presence of hop derived compounds (iso-α acids typically in the range of 17-55ppm), increased CO_2 (typically 0.5% w/w), and low oxygen levels (<0.1ppm) (2). Furthermore, the process of brewing also presents hurdles to the microbial contamination of beer.

Heat is first introduced in the brewing process during the mashing stage. Mashing is the process in which hot water (ranging from 60-70℃, 140 – 158℉) is mixed with crushed cereal malt (typically malted barley) and is held at the previously stated temperature range for 30 to 90 minutes. The primary purpose of mashing is to convert the complex starches contained in the malted barley to fermentable sugars. Mashing also acts as a form of Low-Temperature Long-Time (LTLT) pasteurization of the cereal malts and the wort (sugar rich liquid produced during the mash). Malted barley typically contains multiple lactic acid bacteria (LAB) species that can potentially spoil beer further along the processing stream (3).

Next, the wort is lautered (lautering is the separation of the wort from the grain bed in the mash tun) into a boil kettle. Once the desired volume is collected, the wort is typically held at a rolling boil for 30 to 90 minutes. This boiling is a form of heat treatment that brings the beer to a point of commercial sterility (4). During the boil, hops are typically added as well. Hops are added at various times throughout the boiling to achieve a balance of the production of bittering compounds and the retention of volatile aromatics. Hops (*Humulus lupulus)* contain a variety of chemical compounds known colloquially as α-acids. These α-acids are isomerized when they are

added to boiling wort and generate the production of both *cis*- and *trans*- stereoisomers (5,37). The rate of α-acid isomerization has a positive linear relationship with the length of boiling in wort (5). The presence *trans*-Iso α-acids act as ionophores which stimulate the dissociation of H+ protons from the other isomerized α-acids. The dissociated H+ protons act as a proton pump inhibitor that interact with the cell membrane of Gram-positive bacterial species (35). This interaction causes a shift in the pH gradient across the cellular membrane, which disrupts the functions of the Gram-positive cellular membrane and results in cell death (6,22,28,29,37,39).

The boiled and hopped wort is then cooled and transferred into a fermentation vessel. Wort is typically cooled via plate heat exchangers. Plate heat exchangers use multiple sets of plates with separate flow channels for hot and cold liquids to pass through. Heat from the hot liquid rapidly transfers to the cold liquid via conduction (7). Once the wort reaches temperatures below 60℃ (140℉) it has reached what is known as the "Temperature Danger Zone". The temperature danger zone is defined by food safety institutions such as the United States Department of Agriculture (USDA) as the area between 4.4 and 60℃ (40 and 140℉) (8). This temperature range is considered dangerous because most bacteria grow rapidly at these temperatures and any cells present in a food product at that range can double in cell count in as quickly as 20 minutes (8). Beer then remains within this danger zone throughout the fermentation process where temperatures typically range from 13 to 20.5℃ (55 to 69℉). Beer is then cooled to around 0-1℃(33℉) for packaging into either kegs, cans or bottles. All surfaces that beer contact such as stainless-steel pipes, valves, fermentation vessels, chlorobutyl lined transfer hoses and polytetrafluroethylene tubing at packaging are typically sanitized prior to contact with product with peroxyacetic acid (PAA). Beer produced at larger facilities is typically

pasteurized in some way prior to packaging (25). Pasteurization is highly uncommon in microbreweries and brewpubs.

PAA is a commercially available disinfecting agent typically available in a quaternary equilibrium mixture containing acetic acid, hydrogen peroxide, and water (9). PAA combines the oxidative properties of peroxide with acetic acid molecules to provide strong bactericidal, virucidal, fungicidal, and sporicidal properties (9,10). Furthermore, dilute PAA solutions used in brewing environments rapidly degrade into acetic acid, oxygen, and water. This allows PAA to be used as a no-rinse sanitizer allowing minimal surface exposure to the outside environment between surface sanitation and product contact with the surface (9).

StarSan, a commercial sanitizer produced by Five Star Chemicals & Supply, Inc., is occasionally used by microbreweries to quickly spot sanitize during sample collections from fermentation vessels and during packaging. StarSan is an acid-based sanitizer consisting of 50-60% phosphoric acid and 10-20% benzenesulfonic acid and 4-C10-13-sec-alkyl derivatives (11).

Common Bacterial Contaminates of Beer

As previously stated, beer is typically considered a microbially stable product due to the protective factors of its intrinsic properties. Furthermore, several processing hurdles work in unison to decrease the likelihood of beer becoming contaminated with undesirable microbes. Despite these factors, beer still does occasionally become contaminated with undesirable microbes. While beer is theoretically susceptible to contamination from multitudes of microorganisms that can use beer as a substrate, there are a handful of common microbes that most often spoil beer (2,12,14,24,26,29). These microbes include *Lactobacillus spp.*, *Pediococcus spp., Pectinatus spp.,* and *Megasphaera cerevisiae* (12,14,26,29,33).

Lactobacillus spp. and *Pediococcus spp.* fall into a category of microorganisms known as lactic acid bacteria (LAB). LAB's are classified as Gram-positive, non-spore forming, anaerobic, rod or cocci- shaped bacteria that metabolize sugars into lactic acid as an end-product (13,32). The genus *Lactobacillus* contains several different species that commonly contaminate beer. The first of these species is *Lactobacillus brevis*. In a study conducted between 1980 and 2002 *L. brevis* was the most identified organism in beer, ranging from 39 to 51% of reported spoilage cases (12). *L. brevis* is also a highly hop resistant strain using *hor*A genetic modifications to remove H+ ions (22,31). Beers contaminated with *L. brevis* may become hazy, acidified, or show increased sedimentation when packaged (12). Furthermore, packaged beers contaminated with LAB such as *L. brevis* can cause the swelling or rupture of single-serve packaging such as bottles or cans.

Lactobacillus lindneri is another common strain of LAB found in spoiled beers. *L. lindneri* differs from *L. brevis* in several ways. First, it is considered a hard to detect bacteria, meaning it is hard to grow on common growth media used in brewing industry QC protocols. This difficulty leads to *L. lindneri* contaminations often going undetected. Second, *L. lindneri* has a smaller individual cell size than *L. brevis* which allows it to pass through common microfilters used to cold pasteurize finished beer (12). Lastly, *L. lindneri* displays minimal hop resistance compared to *L. brevis.* Despite the increased difficulty in dealing with *L. lindneri* versus *L. brevis*, *L. lindneri* contaminations do cause minimal damage to finished products. The main characteristic of *L. lindneri* contaminations in finished beer is slight haze and increased sedimentation (12).

L. plantarum, L. casei/paracasei, and *L. coryniformis* are other common *Lactobacillus* strains that can spoil beer. These strains occur in beer at lower rates than *L. brevis* and *L. lindneri*

due to extremely weak hop resistance (13). Common spoilage characteristics of these strains include haziness, increased sedimentation, decreased pH, and production of diacetyl which is characterized by a butter-like off flavor in finished beer (15). *L. backi* is a recently identified strain found to contaminate beer. *L. backi* is morphologically identical to *L. plantarum, L. casei/paracasei,* and *L. coryniformis.* It is mainly differentiated from these strains due to its inability to ferment maltose and sucrose, as well as its lack of diacetyl production (14).

The genera *Pediococcus* is also a significant group of spoilage organisms in beer. Two prevalent species of *Pediococcus* found in spoiled beer are *P. damnosus* and *P. claussenii*. *P. damnosus* is commonly identified as the source of spoilage in reported contaminations (12,30). *P. damnosus* is extremely well-adapted to the biological environment of beer. It has a well-developed folate synthesis system as well as *hor*A genes that is easily transferred to other species such as *P. claussenii* should both species be involved in contamination (14,30,34). The *hor*A gene contributes to a high level of hop resistance with the *Pediococcus* genus. Beers contaminated with *Pediococcus* strains exhibit high levels of diacetyl production and lowered pH. Certain strains of *P. damnosus* and most strains of *P. claussenii* have the potential to produce exopolysaccharides. The primary exopolysaccharide produced by *P. damnosus* is β-glucan (14,30). When present in beer, the excess β-glucan can cause a viscous and thick texture, often describes as "ropy" (14,30). Exopolysaccharide producing strains of *P. damnosus* when present in beer are harder to control due to higher-than-average ethanol and pH tolerance (14,30).

The genus *Pectinatus* and *Megasphaera* are relatively recently identfied Gram-negative microorganisms, being discovered in 1978 and 1979 respectively, capable of spoiling beer (12). These microbial genera contaminate beers at lower rates than Gram-positive beer spoiling organisms, typically at rates lower than 10% of total occurrences of contamination (12,16).

These bacteria are immune to the antimicrobial effects of hops due to their Gram-negative membrane structures. Due to their strict anaerobic nature, contamination by these organisms typically occurs in packaging equipment where CIP methods are more difficult due to the mechanical complexities of the machinery. Furthermore, contaminations by the organisms are more likely to be discovered by consumers as they occur during packaging, which is a highly unfavorable situation for producers. Beer spoiled by *Pectinatus* exhibits hazy appearances, heavy sedimentation, small clots forming throughout the beer, and strong odors and flavors from the production of hydrogen sulfide (which smells of rotten egg). *Megasphaera* contaminations produce next to no haze, sediment, or clotting but produce a variety of unpleasant odors and flavors. These include butyric acid (bile, rancid), caproic acid (pungent, cheesy), and hydrogen sulfide (12).

Sources and Solutions

Research thus far has identified several methods in which beer spoilage bacteria overcome beers intrinsic protective properties and the processing hurdles that decrease microbial risk. These methods include genetic mechanisms of hop resistance and tolerance to the intrinsic properties of beer (ethanol, pH, O_2 and CO_2 levels) (2,12,23,26). More recent research has begun to look at the possibility of beer spoiling bacteria forming biofilms alongside *Saccharomyces cerevisiae* that allow the bacteria to survive processing hurdles (17,18,19,36). Research conducted by Mazano et al looked to identify common bacterial species in microbreweries through PCR-TTGE/DGGE and traditional microbiology. This research focused on the product itself more than surfaces and steps in the brewing process (38). Little research has been done to identify where during production beer spoilage bacteria are most likely to evade processing hurdles. Research is also lacking in looking into how problems unique to microbreweries and

brewpubs increase the likelihood of bacterial contamination. These problems include the use of modular chlorobutyl hose systems and mobile pumps, the overall "open" nature of smaller brewhouses and cellars to the outside environment (such as opening tanks to pitch yeast, dry hop, or introduction of additions), and the proximity of the production and packaging areas to the public. Also, many microbreweries and brewpubs intentionally produce beer with LAB's (sour ales) in the same facilities (2). Future research should look to identify where in the microbrewery/brewpub process bacterial contamination is most likely occur. This knowledge can help develop methods and protocols that will help microbreweries and brewpubs better protect their product quality, reputation, and financial investments from beer spoilage bacteria.

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CHAPTER II: "Identification of Critical Points for Bacterial Contamination in the Microbrewery Environment"

Abstract

Research was conducted to identify the critical points for bacterial contamination in the microbrewery environment. Three breweries of similar size and scale in Upstate South Carolina were sampled. Eleven locations within each brewery were sampled and analyzed using HybriScan *D* Beer rapid molecular testing for bacterial cell counts. The locations of the racking arm valve and the blow off valve were the most highly contaminated by bacteria.

Introduction

Beer is generally recognized as a microbially stable product. This stability is due to various hurdles to microbial contamination. Hurdle technology is defined by Leistnar as a "combination of food preservation methods" (1,2). Leistnar further elaborates on the concept of hurdle technology by describing the "hurdles" as both the intrinsic properties of the food including pH, a_w , Eh, and competitive microbes (1,2) as well as the "influence of food preservation methods on the physiology and behavior of microorganisms in food" (1,2).

In beer these hurdles include pH (typically 3.9-4.4), the presence of ethanol (ranging from 3.2% to 14% by volume), the presence of hop derived compounds (iso-α acids typically in the range of 17-55ppm), increased CO_2 (typically 0.5% w/w), and low oxygen levels (<0.1ppm) (3). Furthermore, the process of brewing also presents hurdles to the microbial contamination of beer. The introduction of heat during the mashing, lautering, and boiling process works to protect the beer from bacterial contamination. The use of chemical sanitizers such as peracetic acid also

protect the beer as in passes through a plate heat exchanger and into the fermentation vessel. Hops, a key ingredient in modern beer, also play a role in protecting beer from bacterial contamination. Hops achieve this by releasing H+ ions into boiled beer. The dissociated H+ protons act as a proton pump inhibitor that interact with the cell membrane of Gram-positive bacterial species (8). This interaction causes a shift in the pH gradient across the cellular membrane, which disrupts the functions of the Gram-positive cellular membrane and results in cell death (4,7,10,12,13,14). However, some bacterial species that commonly contaminate beer have developed resistance to this mechanism of actions via a mutation *hor*A gene that allows the bacteria to remove the H+ and protect its cellular membrane (12,18).

Despite these hurdles, beer still can become contaminated with unwanted bacterial species. These most common genus that contaminate beer are *Lactobacillus, Pediococcus, Pectinatus,* and *Megasphaera,* within these genera there are multiple species that are likely to contaminate beer and causes a variety of product defects (5,6,9,15).

Microbreweries have more difficulty protecting their beer from these organisms due to unique challenges. These challenges include the use of modular chlorobutyl hose systems and mobile pumps, the overall "open" nature of smaller brewhouses and cellars to the outside environment (such as opening tanks to pitch yeast, dry hop, or introduction of additions), and the proximity of the production and packaging areas to the public. Also, many microbreweries and brewpubs intentionally produce beer with LAB's (sour ales) in the same facilities (3).

Research Objective

The goal of the research was to identify possible bacterial contamination points in the microbrewery process.

Materials and Methods

Materials

Hybriscan™ *D* Beer kits were obtained from Millipore Sigma (St. Louis, MO). Bacto Peptone was obtained from Beckton, Dickinson and Company (Sparks, MD). Sterile cotton tipped applicators were obtained from Puritan Medical Products Company LLC (Guilford, ME). Peracetic acids samples were obtained from Envirotech (Modesto, CA) and Birko (Henderson, CO). Beer samples were collected from three breweries located in Upstate South Carolina.

Brewery Selection

The three breweries were chosen due to their similar brewing system size, annual production level, floor plan, and location.

Brewery A – Located in Upstate South Carolina. Two vessel (Mash Tun and Kettle) 7 brewery barrel (BBL, 1 BBL = 177.35 L) brewhouse with five 7 BBL fermentation tanks. Estimated annual output of 450 BBL (52,807.5L).

Brewery B – Located in Upstate South Carolina. Two vessel (Mash Tun and Kettle) 5 BBL brewhouse with two 10 BBL, three 5 BBL, and one 2 BBL fermentation tanks. One 5 BBL tank was sampled for this study. Estimated annual output of 500 BBL (58,675 L).

Brewery C – Located in Upstate South Carolina. Two vessel (Mash Tun and Kettle) 10 BBL brewhouse with four 10 BBL fermentation tanks and 2 10 BBL horizontal lagering tanks. Estimated annual output of 600 BBL (70,410 L).

Sample Location Selection

Sampling locations were chosen by identifying commonalities between the production processes of the participating breweries. The participating breweries were surveyed, and a flow chart of the facility production process was generated. The purpose of the flow charts and floor plans was to eliminate the variability in design and layout of each brewery. Floor plans of the breweries were also generated and were divided into hygienic zones. Eleven target locations were chosen for sampling. These eleven zones were chosen due to their commonality between all three breweries and their high risk for bacterial contamination. The eleven-target locations were the spray ball valve, sample tap, racking arm valve, blow-off valve, bottom valve, the carb stone valve, yeast pitch, the cooled wort leaving the heat exchanger, and early, mid, and late packaging runs. All samples across four repetitions were collected from the same fermentation tank to control the variation between tanks.

Figure 3.1 Brewery Floor Plans

Hygienic Zones

Zone 1 – Areas of direct contact with product such as brewhouse, hot and cold liquor tanks, grain mill, heat exchanger, and fermentation tanks. High risk of product contamination in these areas.

Zone 2 – Ingredient storage or other areas that do not directly contact product but are directly adjacent to production areas with no physical barrier. Medium risk of product contamination in these areas.

Zone 3 – Areas completely removed from all production areas. Low risk of product contamination in these areas.

Color Codes

Red (Hygienic Zone 1) – Production Areas

Yellow (Hygienic Zone 2)– Ingredient Storage, Employee Walking Areas, Customer Seating, Tap/Bar Areas, or Lab Areas Adjacent to Production Area with No Physical Barriers

Green (Hygienic Zone 3) – Offices, Retail Space, Finish Product Storage, Taps and Bar, Customer Seating not Adjacent to Production Area

Figure 3.1A - Brewery A

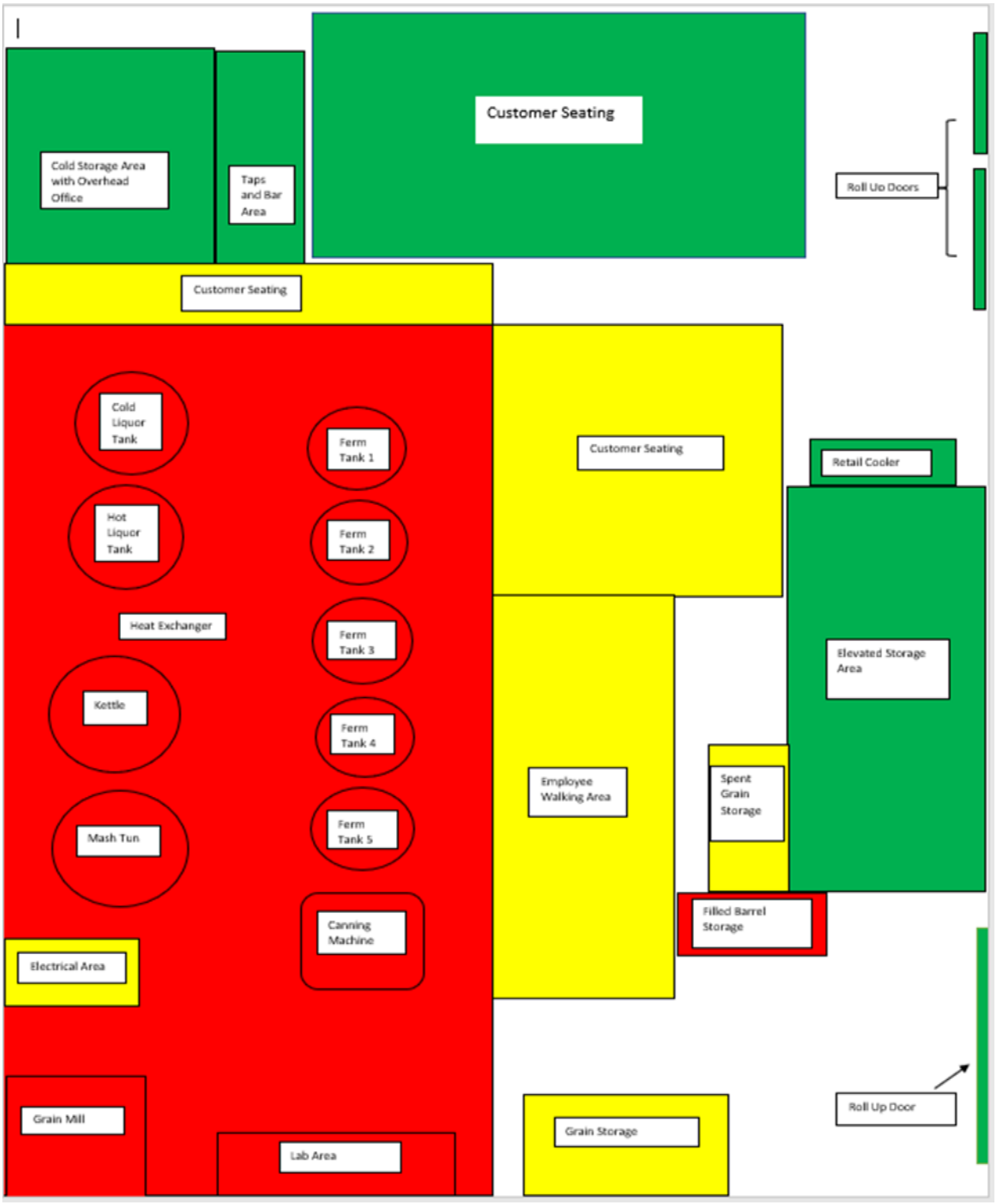


Figure 3.1B - Brewery B

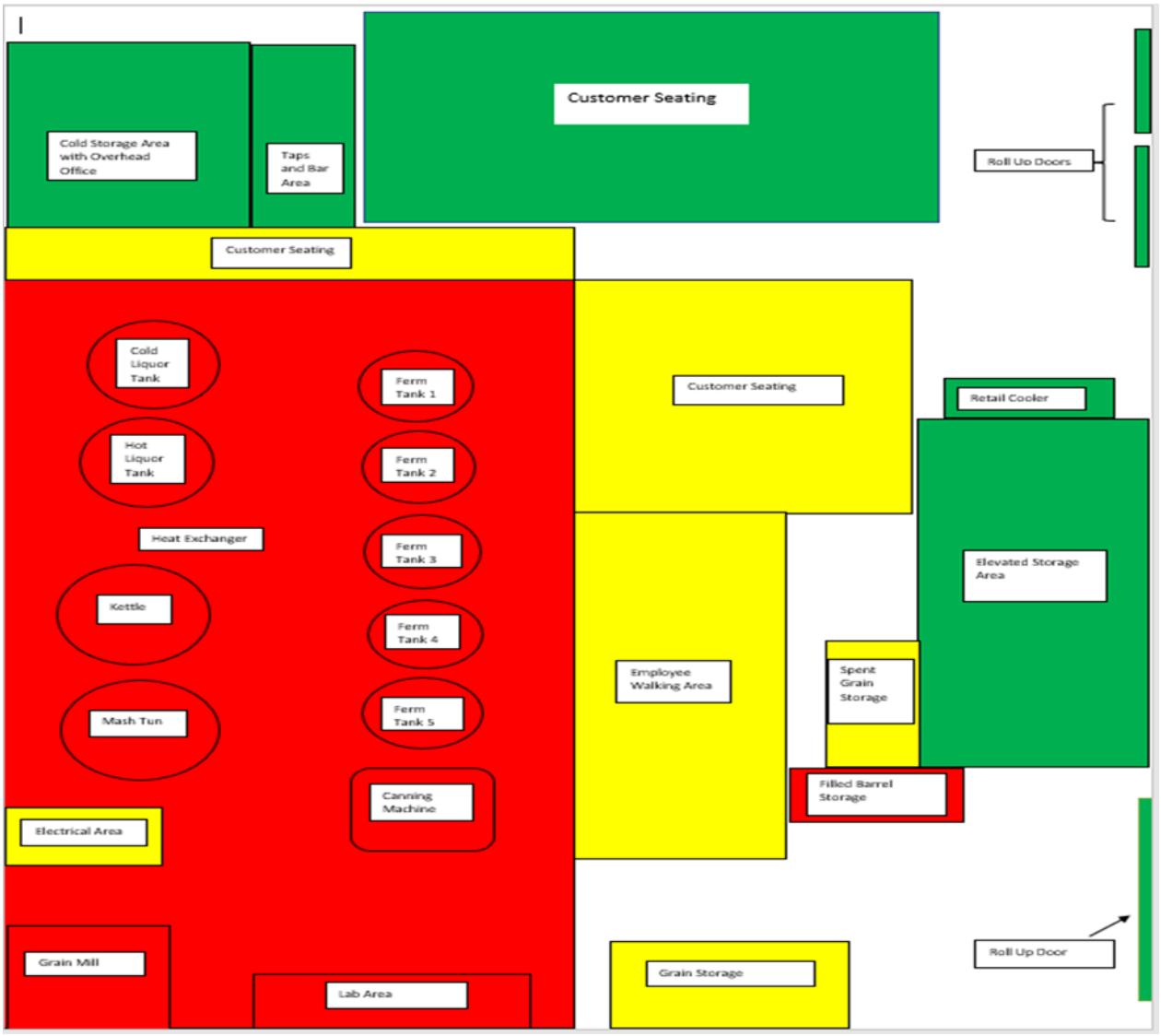
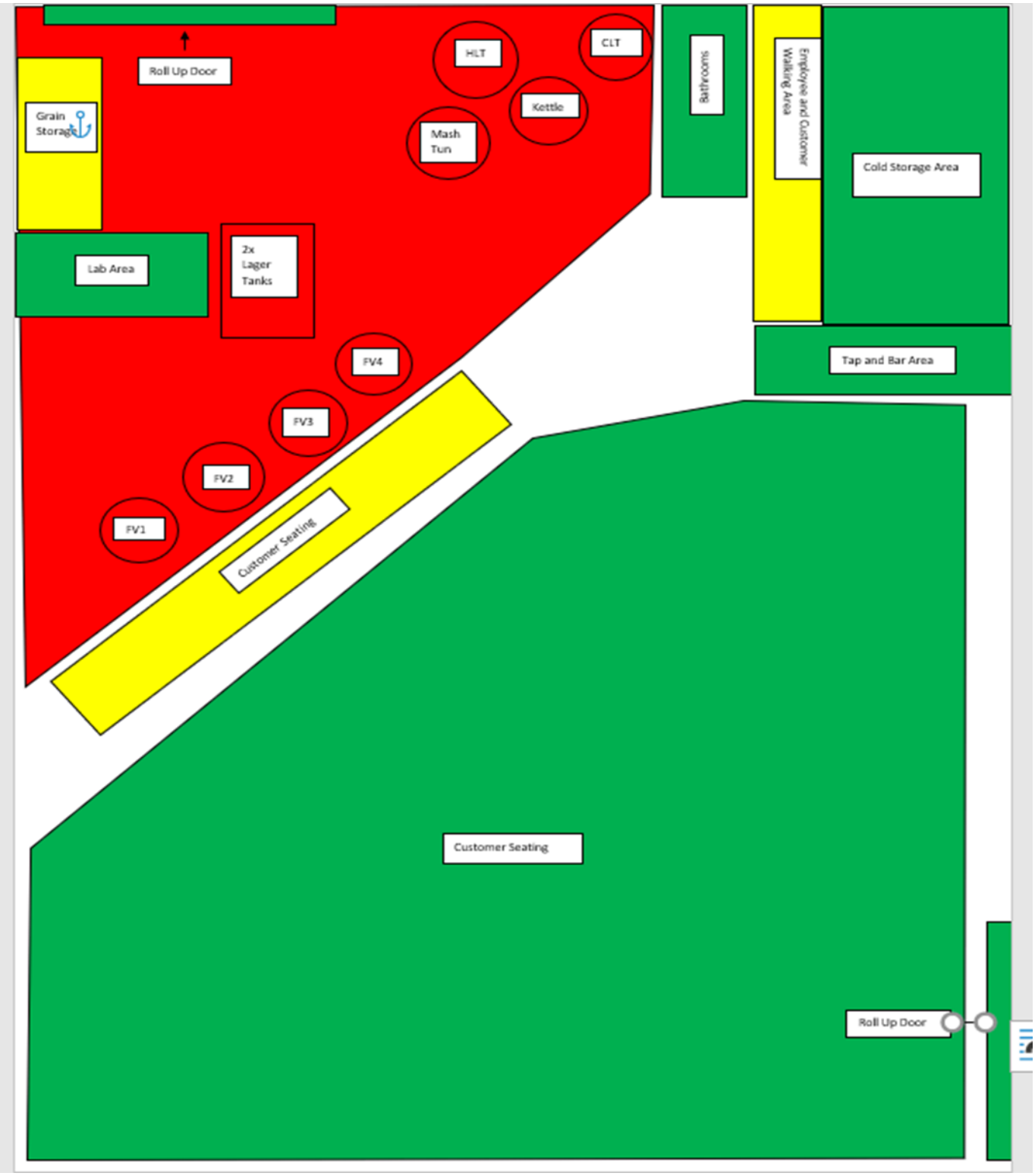


Figure 3.1C – Brewery C



Descriptions of Surface Swab Sampling Locations (Figure 3.2)

Spray Ball Valve – The butterfly valve that closes the pipe running from the CIP spray ball located at the top of the fermentation tank. Typically, 1" to 1.5" in diameter and made of 304 sanitary stainless steel and attached to the tank by a tri-clamp and gasket.

Sample Tap – The horizontal valve attached the fermentation tanks just above the cone of the tank used to collect samples of the beer during and after fermentation. Typically, 1" to 1.5" in diameter and made of 304 sanitary stainless steel and attached to the tank by a tri-clamp and gasket.

Racking Arm Valve – The butterfly valve that closes off the racking arm attached to the fermentation tank at the top of the cone. Typically, 1.5" to 2" in diameter and made of 304 sanitary stainless steel and attached to the tank by a tri-clamp and gasket. The racking arm is a curved piece of 304 sanitary stainless steel used to transfer beer from the fermentation tank without disturbing the sediment collected in the cone of the tank.

Blow Off Valve – The butterfly valve that closes off the pipe running from the top of the fermentation tank that allows carbon dioxide released during fermentation to escape. Typically, 1" to 1.5" in diameter and made of 304 sanitary stainless steel and attached to the tank by a tri-clamp and gasket. This valve is left open during fermentation and is closed when fermentation is complete.

Bottom Valve – The butterfly valve that closes the opening at the bottom of the fermentation tank. This is where cooled wort is pumped into the tank after leaving the heat exchanger. Typically, 1.5" to 2" in diameter and made of 304 sanitary stainless steel and attached to the tank by a tri-clamp and gasket.

Carb Stone Valve – The ball valve the closes the opening of the carb stone assembly. The carb stone assembly is an apparatus attached to the fermentation that aids in forcing carbon dioxide into solution during the carbonation process. Typically, 1.5” to 2” in diameter and made of 304 sanitary stainless steel and attached to the tank by a tri-clamp and gasket

Yeast Pitch – Yeast added to the fermentation tank once the cooled wort has been transferred into the tank. Yeast pitches are either obtained from yeast suppliers, often called a “fresh pitch” or collected from the cone of a fully fermented beer, a “harvested pitch”.

Description of Liquid Sampling Locations

Packaging Run Early – Samples collected at initial flow from keg filler head before first kegs are filled.

Packaging Run Mid – Samples collected from keg filler head after approximately half the total volume of product had been packaged.

Packaging Run Late – Samples collected from keg filler head before the final keg of the packaging run was filled.

Cooled wort leaving the heat exchanger - Cooled wort coming off the heat exchanger and into the fermentation tank. Cooled wort bacterial counts represent the overall bacterial counts of the transfer lines between the heat exchanger and the fermentation tank as well as the heat exchanger itself.

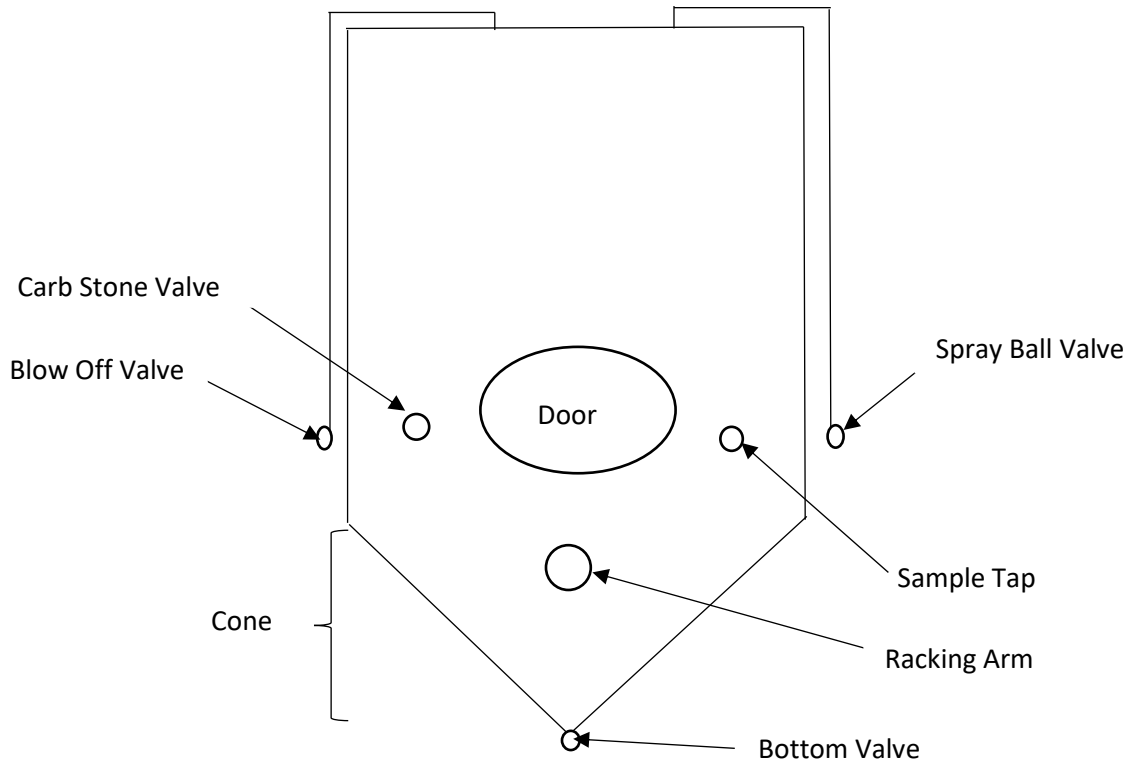


Figure 3.2 – General Fermentation Tank Diagram

Open Air Sampling Results

During preliminary data collection, open air samples were collected from Brewery A. Trypticase soy agar plates were produced with sufficient thickness to not dry out during exposure. These plates were placed on the left and right side of the brew deck, above and below the fermentation vessel being sampled, in the grain storage area, in the barrel storage area, and on the heat exchanger. The plates were exposed to the open air at the brewery for 24 hours. During this 24 hour both productions operations occurred, and customers visited the tap room. The plates were then collected and incubated at 98.6° F (37° C) for 24 hours. Next, colonies were removed from the media using aseptic techniques and washed into 2mL of peptone. The 2 mL of sample was then subjected to the Hybriscan assay. All samples were positive for the spoilage

bacteria that Hybriscan detects. However, due to the pre-enrichment of the samples via incubation of the TSA, quantification of the cell count was not possible.

Sample Collection Method

Surface Swabs - Sterile 15mL plastic test tubes were loaded with 3mL 0.1% peptone water then used to collect samples at the 11 sampling sites (Figure 3.3). Sterile cotton tipped applicators were used to swab the target surfaces in a crosshatch pattern (Figure 3.4) then swabs were placed into the test tubes. Yeast pitch samples were collected by swabbing yeast residue from inside fresh pitch packaging or inside of the brink from harvested pitches. The area swab was an 8.04 cm^2 area. All samples were immediately stored on ice post collection and analyzed within 24 hours. All samples were collected in triplicate.

Liquid Samples – Liquid Samples were collected directly into sterile 15mL test tubes. All samples were immediately stored on ice post collection and analyzed within 24 hours. All samples were collected in triplicate.

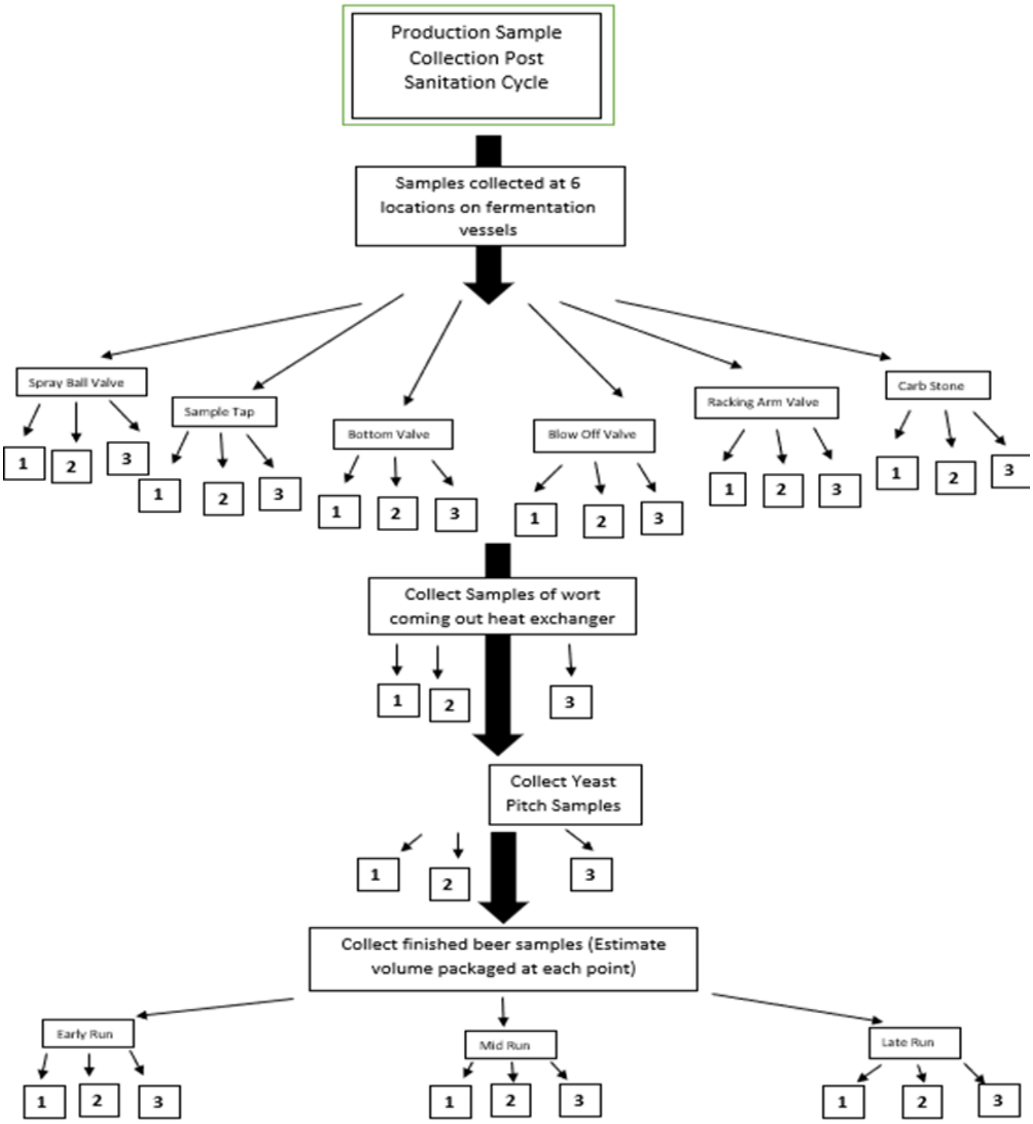


Figure 3.3 – Sample Collection Diagram

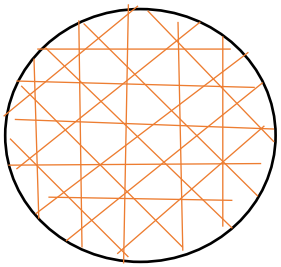


Figure 3.4 – Sample Surface Swabbing Pattern

Sample Analysis Method

A 2mL aliquot was removed from the 15mL test tubes and transferred into a 2mL microreaction tube. A spatula-tip of glass beads was added each microreaction tube. The tubes were centrifuged for 2 minutes at 13,000 rpm. The supernatant was then removed and discarded. 40 µL of Lysis Buffer B* and 10 µL of Lysis Buffer A* were added to the cell pellet in each microreaction tube. The samples were then incubated for 15 minutes at 98.6° F (37° C) at 1,400 rpm in a thermoshaker. 50 µL of Lysis Buffer C* was added to each microreaction tube. The samples were then incubated for 15 minutes at 98.6° F (37° C) at 1,400 rpm in a thermoshaker. The samples were then centrifuged for 10 minutes at 13,000 rpm. During the centrifugation of the samples, 45 µL of Test Solution D was loaded into each well of a microplate and incubated for 5 minutes at 122° F (50° C). 10 µL of the supernatant of each sample was transferred into the wells of the microplate containing Test Solution D. The microplate was covered with a lid and incubated at 122° F (50° C) at 500 rpm for 10 minutes. 50 µL of the reaction mixes were transferred to a streptavidin coated microplate and incubated 122° F (50° C) at 500 rpm for 10 minutes. During this incubation cycle an appropriate amount of Enzyme solution F* was diluted with Washing Solution E* at a ratio of 1:100. After the incubation cycle, the liquid in the wells of the microplate was discarded and 200 µL of Washing Solution E* was added the each well at incubated at room temperature for 2 minutes. 100 µL of the 1:100 Enzyme solution F:Washing Soltuion E mixture was added to each well of the microplate. The plate was then covered with a lid and incubated at 77° F (25° C) for 10 minutes. The liquid in each well was then discarded and the wells were filled with 200 µL of Washing Solution E* and incubated at room temperature for 1 minutes and discarded. The step was repeated once. 100 µL of Substrate Solution G* was added to each well of the microplate. The microplate was covered with a lid and incubated at 77°

F (25°C) for 15 minutes. 50 µL of Stop Solution H* was added to each well of the microplate. Absorbance was then measured at 450nm.

**** All reagents used are included in the Millipore Sigma Hybriscan™ D Beer kits.***

Bacterial Species Sensitivity of Millipore Sigma *Hybriscan™ D Beer kits*

Genus *Lactobacillus*

Lactobacillus acidophilus

Lactobacillus brevis

Lactobacillus brevisimilis

Lactobacillus buchneri

Lactobacillus casei

Lactobacillus collinoides

Lactobacillus coryniformis

Lactobacillus curvatus

Lactobacillus fermentum

Lactobacillus frucivorans

Lactobacillus linderi

Lactobacillus malefermentans

Lactobacillus parabuchneri (frigidus)

Lactobacillus paracasei

Lactobacillus paraplantarum

Lactobacillus plantarum

Lactobacillus rhamnosus

Genus *Pediococcus*

Pediococcus acidilactici

Pediococcus claussenii

Pediococcus damnosus

Pediococcus inopinatus

Pediococcus parvulus

Pediococcus pentosaceus

Genus *Pectinatus*

Pectinatus cerevisiiphilus

Pectinatus frisigensis

Genus *Megasphaera*

Megasphaera cerevisiae

Data Analysis Method

Absorbance values were converted into cell counts (cfu/10µL) using the standard curve (Appendix A) provided in the QC sheet of each Millipore Sigma Hybriscan™ *D* Beer kits. The

value was then converted to cfu/ml. Surface swab cfu/ml counts were divided by 5.36 (brewery valves with diameter of 1.25" have a surface area of 8.04 cm^2 and 2ml out of 3 ml peptone collected was sampled) to give the units cfu/cm^2. The absorbance, cfu/mL, and cfu/cm^2 data were entered into SAS Studio. The data was analyzed using a general linear model (GLM) and standard deviation for absorbance and cfu/ml were generated for sampling locations and breweries. Main effects for breweries and locations were significant ($p \leq 0.05$) and means were separated at the $p \leq 0.05$ level using the pdiff command.

Results and Discussion

The racking arm valve had statistically higher bacterial contamination compared to other sampling locations (Table 3.1).

The racking arms of most 5-10BBL fermentation tanks are attached to their respective tanks by threaded couplers with an appropriate gasket sealing the connection. These racking arm assemblies may not always completely disassemble during the CIP protocols at some breweries. Furthermore, racking arms themselves stay vertically set during fermentation but are slowly lowered just above the trub line (trub is a brewing term for the sediment that collects in the cone of the fermentation tank after fermentation is complete). This means racking arms tend to collect residues from the trub at higher rates that other points in the tank. These trub deposits could become reservoirs of bacterial contamination and support the growth of biofilms (11,16). Furthermore, research conducted by Ismail et al, found that growth of both aerobic and anaerobic bacteria and their subsequent growth in micropits (small pitting in stainless steel caused by abrasion, this would be common on a frequently rotated apparatus like the racking arm) can decrease the passive film of 304 stainless steel and caused increased rates of corrosion over time.

This phenomenon could cause bacterial contaminations and biofilms centered around the racking arm to have a snowball like effect, causing greater risk of contamination overtime.

Issues relating to the potential for residue-based contamination and steel degradation in the racking arm stress the importance of proper fermentation vessel cleaning and sanitation. During peak fermentation (12-36 hours after the introduction of yeast to the wort) krausen is formed. Krausen is a brewing term originating from the German word kräus which means “curly or fizzy”. Krausen refers to the thick, brown/off-white foam formed by the active yeast during fermentation. Research conducted by Wang et al identified the metabolites contained within krausen (19). These metabolites within the krausen residues could adhere to the surfaces of the fermentation tank as well as the blow off piping, valve, and tubes. These residue deposits could again be reservoirs for bacterial contamination and cause damage to the structural integrity of 304 stainless steel (11,16,17). Ensuring all residues are removed from these areas using hot water, caustic solutions, brushes, and visual inspections allow for proper sanitizing agent surface contact. A proper cleaning and sanitation protocol that accounts for the unique attributes of the racking arm and blow off apparats will protect the beer from bacterial contamination based in these areas.

While the racking arm and blowoff apparatus had statistically overall bacterial counts than other surface swab locations, this does not mean that the racking arm and blow off apparat are the sole source of potential bacterial contamination in a microbrewery. All other sampling locations showed levels of bacteria that could cause beer to become contaminated. No surfaces swabbed were below the detection threshold of the assay used. Therefore, it is important for brewers at microbreweries to understand that spoilage bacteria are always present during the process. It is important for brewers to closely follow their facilities cleaning and sanitation

protocols, cap tank valves when possible, monitor production surfaces for signs of wear, and always follow good manufacturing processes for microbreweries.

Table 3.1 – Mean colony forming units (cfu)/cm^2 for each surface swab sampling location, across all three breweries

Sampling Location	cfu/cm^2	Standard Deviation	Log Values	Standard Deviation
Blow Off Valve (BOV)	57307^{a,b}	64622	4.76^{a,b}	0.51
Bottom Valve (BV)	49482^{a,b}	36238	4.69^{a,b}	0.38
Carb Stone Valve (CSV)	39543^{b}	35700	4.60^{b}	0.45
Racking Arm Valve (RAV)	76845^{a}	77574	4.89^{a}	0.57
Sample Tap (ST)	30028^{b}	22019	4.48^{b}	0.46
Spray Ball Valve (SBV)	39049^{b}	40515	4.59^{b}	0.53
Yeast Pitch (YP)	49559^{a,b}	57429	4.70^{a,b}	0.46

^{a,b} means with different superscripts are significantly different ($p \leq 0.05$) n = 36

There was no statistically significant difference in the four liquid samples between the three breweries (Table 3.2)

Table 3.2 - Mean colony forming units (cfu)/ml for each liquid sampling location, across all three breweries

Sampling Location	cfu/ml	Standard Deviation	Log Values	Standard Deviation
Packaging Early Run (PER)	228033	200583	5.30	0.42
Packaging Mid Run (PMR)	259734	250661	5.40	0.43
Packaging Late Run (PLR)	229751	248731	5.40	0.45
Wort Out Heat Exchanger (WO)	183660	130780	5.26	0.40

Breweries A and C had higher overall bacterial level compared to Brewery B (Table 3.3) across surface swabbing locations. This may be due to a variety of differences between the breweries sampled. Despite the commonalities in size, scale, geographical locations the three

breweries are different in many ways. These include floor plan, equipment layout, recipes, ingredient suppliers and cleaning/sanitation protocols.

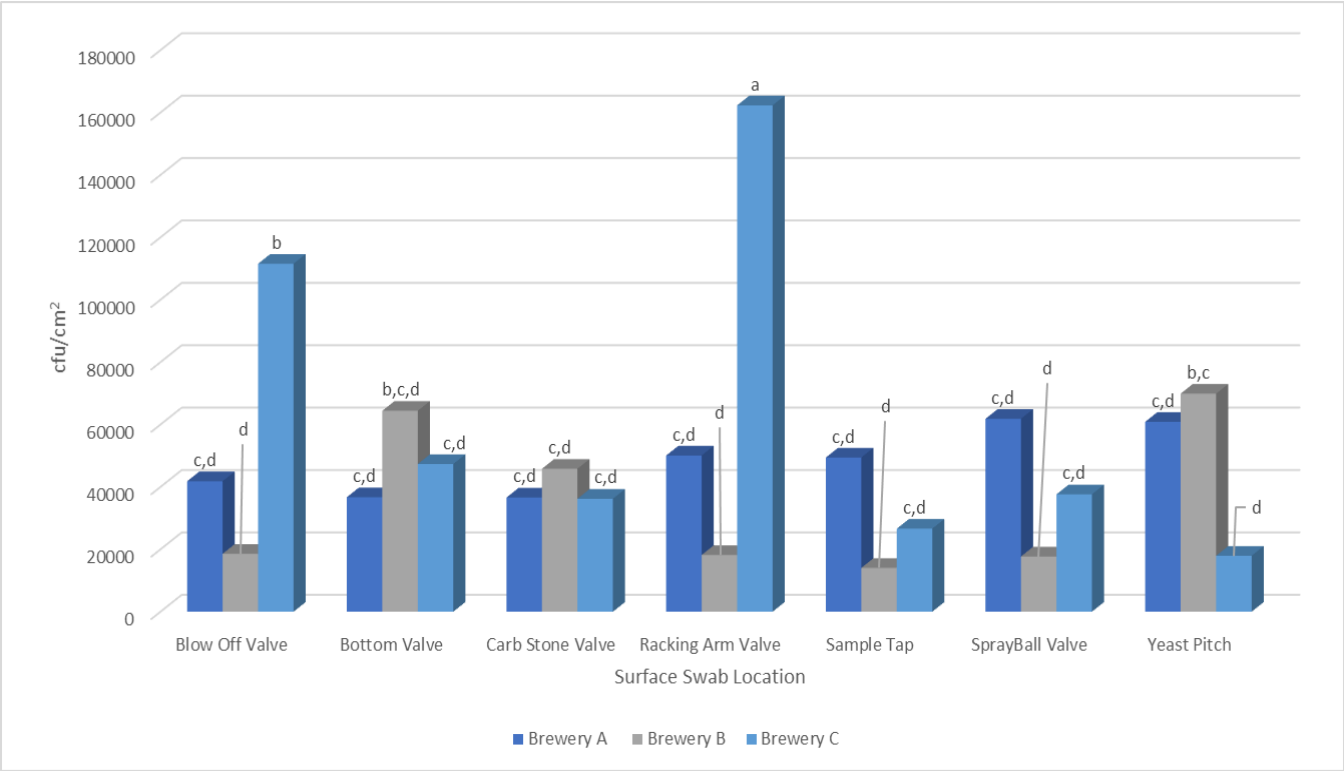
Table 3.3 – Mean colony forming units (cfu)/cm^2 for each brewery, across all surface swab sampling locations

Brewery	cfu/cm^2	Standard Deviation	Log Values	Standard Deviation
A	48184^{a}	50700	4.68^{a}	0.09
B	35511^{a,b}	26708	4.55^{a,b}	0.28
C	62796^{a}	65779	4.80^{a}	0.31

^{a,b} means with different superscripts are significantly different ($p<0.05$)

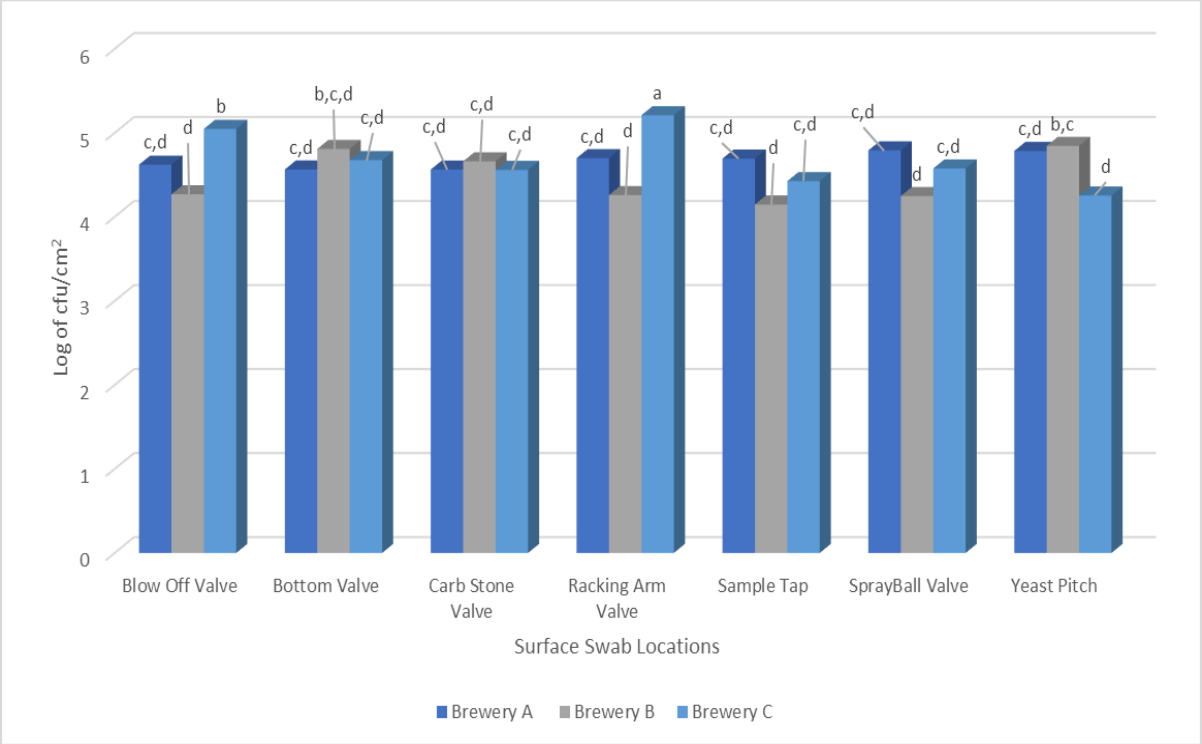
The racking arm valve and blow off valve at Brewery C had higher levels of bacterial contamination that the other surface swab locations (Figure 3.5). This may show an issue with micro-pitting and steel degradation in these areas causing increased chance of bacterial contamination (11,16,17,19). This may cause the typical cleaning and sanitation protocols to not be effective enough to remove bacterial contamination in these areas.

Figure 3.5 – Mean colony forming units (cfu)/cm^2 at each surface swab sampling location across all breweries.



Standard Error:3960 n=12

Figure 3.6 – Log of Mean colony forming units (cfu)/cm^2 at each surface swab sampling location across all breweries.



Standard Error: 0.036 n=12

Liquid samples collected from Breweries A, B, C were all statistically different from one another (Table 3.4). This may be due to differences in the types of kegging rigs used and the variety in cleaning and sanitation protocols used at each brewery during packaging.

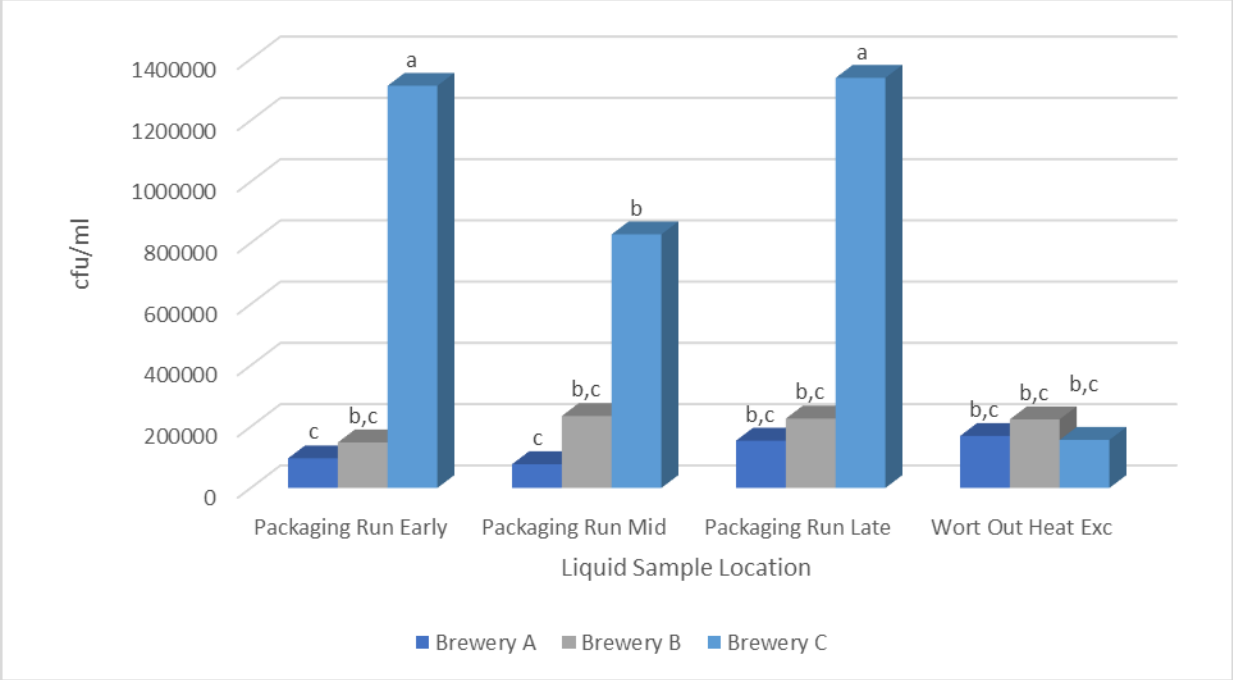
Table 3.4 – Mean colony forming units (cfu)/ml for each brewery across all liquid samples

Brewery	cfu/ml	Standard Deviation	Log Values	Standard Deviation
A	124700^{c}	115160	5.06	0.14
B	208680^{b}	118340	5.07	0.08
C	796748^{a}	1021013	6.01	0.38

^{a,b} means with different superscripts are significantly different ($p<0.05$)

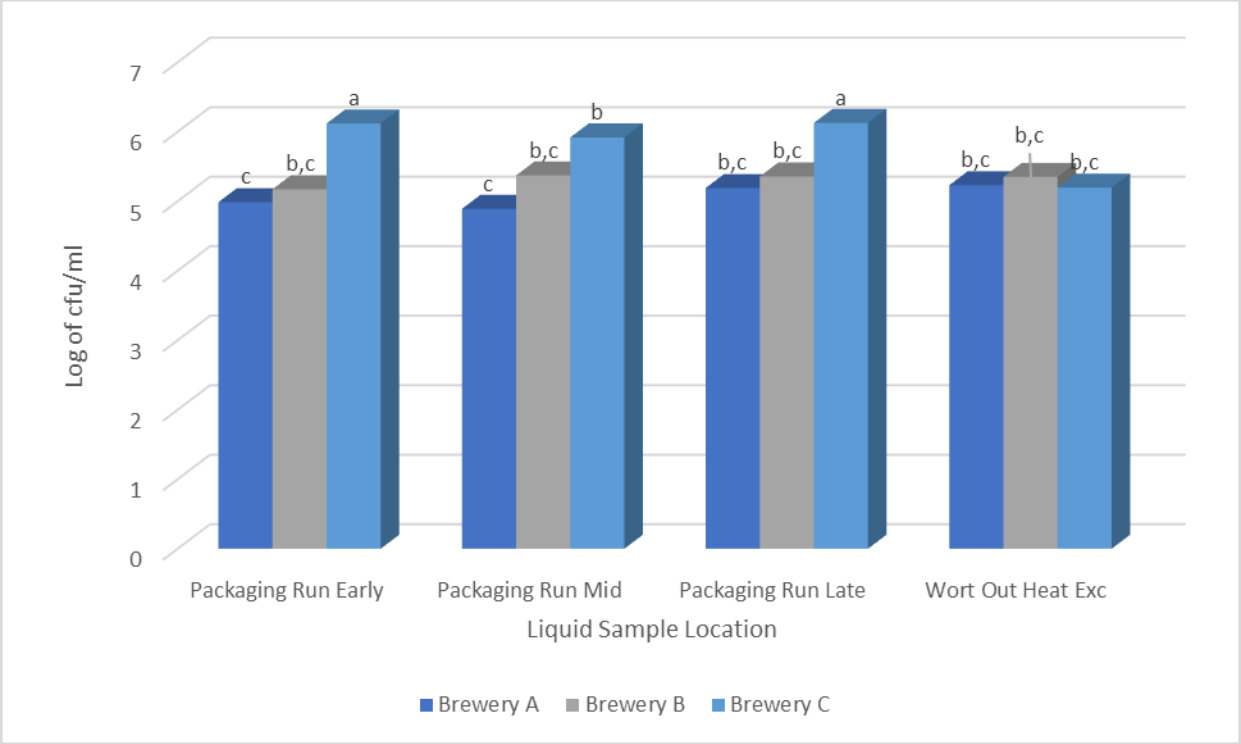
The Packaging Run Early, Mid and Late at Brewery C was statistically different from the other liquid sample locations across the three breweries (Figure 3.6). This difference is most likely due to packaging equipment becoming contaminated during the packaging run. This equipment is most likely contaminated by the kegging rig touching the floor or some other unclean surface during packaging and transfer bacteria into the finished beer.

Figure 3.7 – Mean colony forming units (cfu)/ml at each liquid sampling location across all breweries.



Standard Error: 22241 n=12

Figure 3.8 – Log of Mean colony forming units (cfu)/ml at each liquid sampling location across all breweries.



Standard Error: 0.081 n=12

Conclusion

Overall, this study showed some vulnerabilities in the standard cleaning protocols used by most microbreweries and brewpubs. Future research could look to identify enhanced cleaning protocols that could decrease the overall likelihood of bacterial contamination of beer. However, this research also found very low levels of bacterial counts in finished beer despite higher counts on various surfaces during the production of the beer. The fact that high bacterial counts not always causing contaminated final beer emphasizes the microbiological stability of beer (3).

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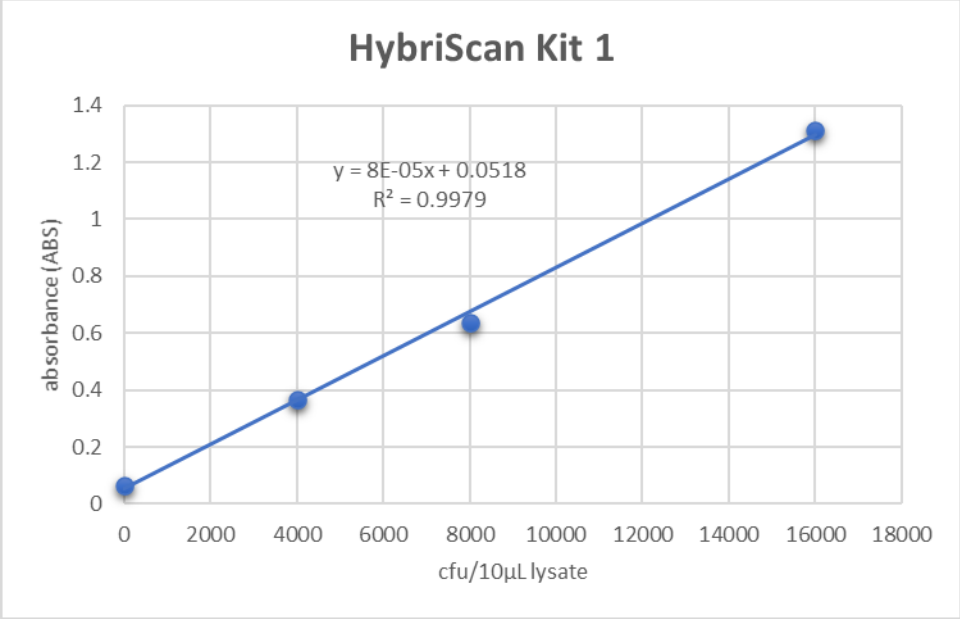
Appendices

Appendix A – HybriScan QC DATA from Millipore Sigma Used for Data Analysis

HybriScan Kit 1

Mean value ABS 450nm

Cfu/µL Lysate	ABS (450nm)
0	0.067
4000	0.366
8000	0.639
16000	1.313

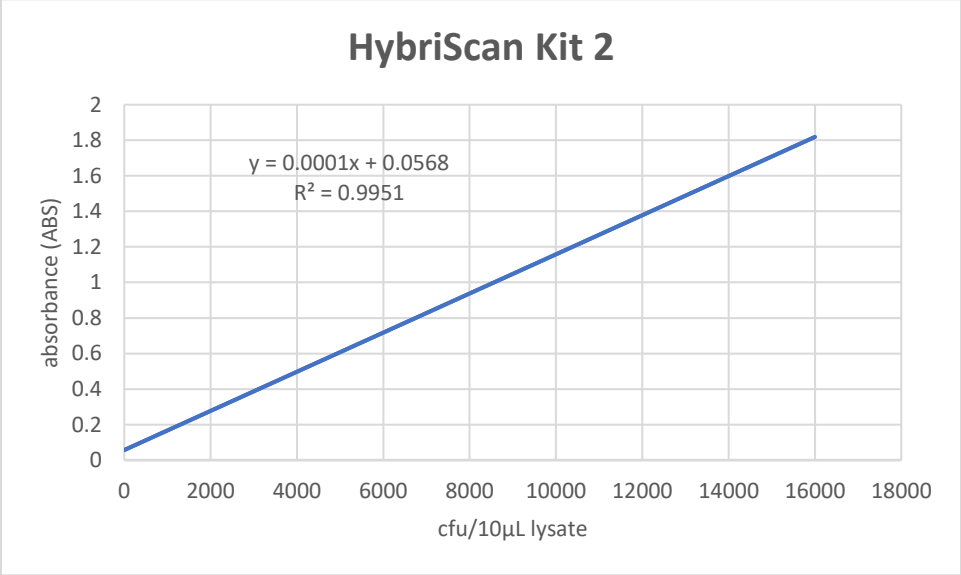


Appendix A - Continued

HybriScan Kit 2

Mean value ABS 450nm

Cfu/µL Lysate	**ABS (450nm)**
0	0.068
4000	0.438
8000	1.004
16000	1.800

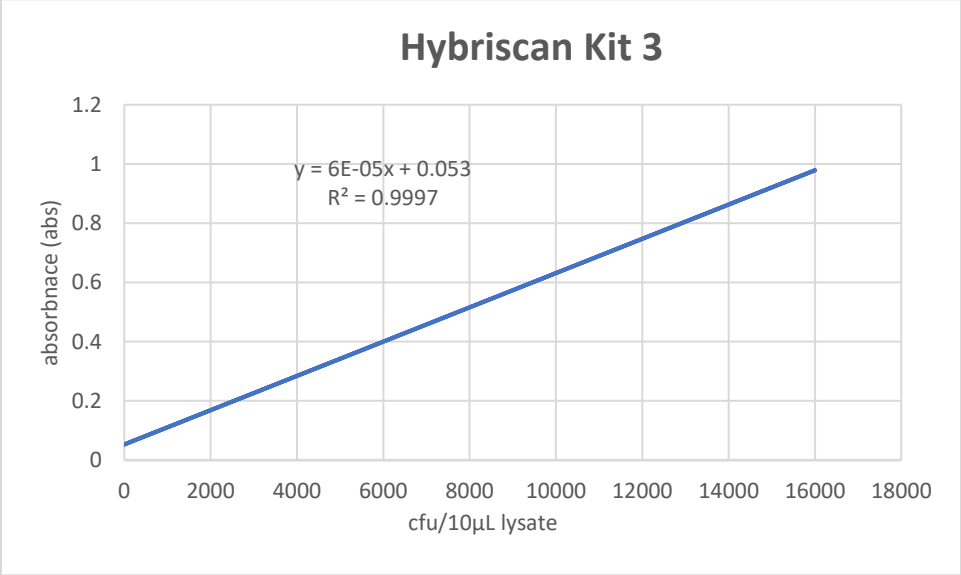


Appendix A - Continued

HybriScan Kit 3

Mean value ABS 450nm

Cfu/µL Lysate	ABS (450nm)
0	0.055
4000	0.289
8000	0.505
16000	0.983

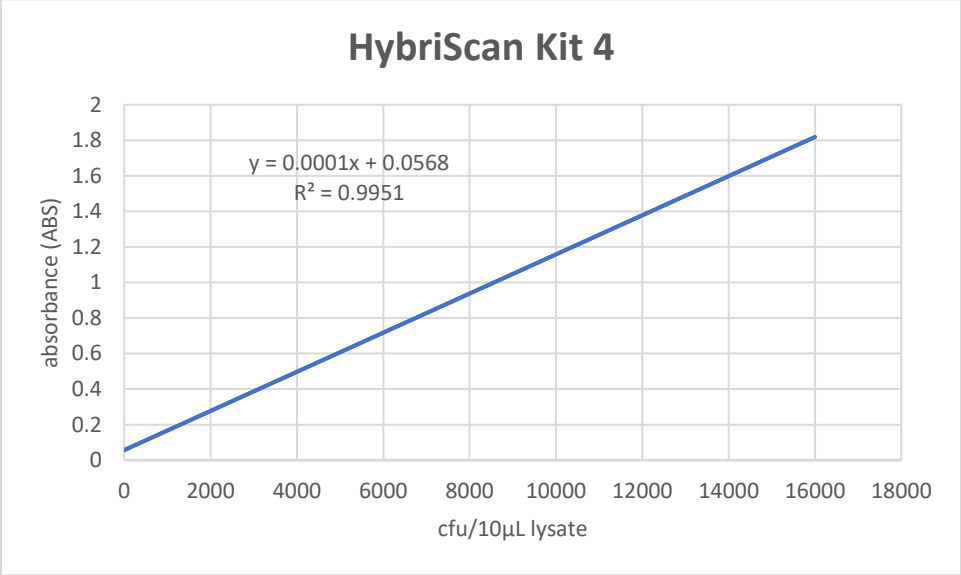


Appendix A - Continued

HybriScan Kit 4

Mean value ABS 450nm

Cfu/µL Lysate	**ABS (450nm)**
0	0.068
4000	0.438
8000	1.004
16000	1.800

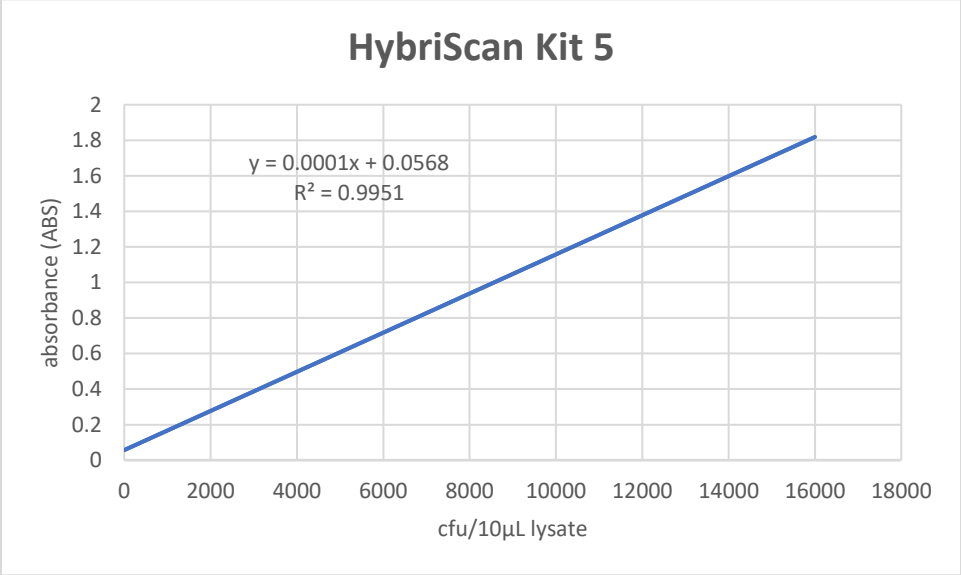


Appendix A - Continued

HybriScan Kit 5

Mean value ABS 450nm

Cfu/µL Lysate	**ABS (450nm)**
0	0.068
4000	0.438
8000	1.004
16000	1.800



Appendix B – SAS Studio Code

```
data brewery;
infile datalines dlm='09'x;
input rep brew $ loc $ obs abs cfu;
cards;
```

rep	brewery	location	obs	abs	cfu
1	fire	sprball 1	0.089	46500	
1	fire	sprball 2	0.087	44000	
1	fire	sprball 3	0.126	92800	
1	keowee	sprball 1	0.197	18160	
1	keowee	sprball 2	0.23	222760	
1	keowee	sprball 3	0.216	205260	
1	kite	sprball 1	0.076	19200	
1	kite	sprball 2	0.07	13200	
1	kite	sprball 3	0.086	29200	
1	fire	samtap 1	0.092	5060	
1	fire	samtap 2	0.155	129000	
1	fire	samtap 3	0.116	80260	
1	keowee	samtap 1	0.113	76500	
1	keowee	samtap 2	0.096	75260	
1	keowee	samtap 3	0.093	45260	
1	kite	samtap 1	0.071	14200	

1	kite	samtap	2	0.09	33200
1	kite	samtap	3	0.119	62200
1	fire	rackarm	1	0.158	132760
1	fire	rackarm	2	0.138	107760
1	fire	rackarm	3	0.105	66500
1	keowee	rackarm	1	0.096	55260
1	keowee	rackarm	2	0.178	157740
1	keowee	rackarm	3	0.118	82760
1	kite	rackarm	1	0.086	29200
1	kite	rackarm	2	0.076	19200
1	kite	rackarm	3	0.071	14200
1	fire	blowoff	1	0.095	54000
1	fire	blowoff	2	0.102	62760
1	fire	blowoff	3	0.085	41500
1	keowee	blowoff	1	0.134	102760
1	keowee	blowoff	2	0.125	91500
1	keowee	blowoff	3	0.201	186500
1	kite	blowoff	1	0.087	30200
1	kite	blowoff	2	0.117	60200
1	kite	blowoff	3	0.075	18500
1	fire	bottomvalve	1	0.108	70260
1	fire	bottomvalve	2	0.099	59000
1	fire	bottomvalve	3	0.083	39000

1	keowee	bottomvalve	1	0.375	404000
1	keowee	bottomvalve	2	0.202	187760
1	keowee	bottomvalve	3	0.806	942760
1	kite	bottomvalve	1	0.355	298200
1	kite	bottomvalve	2	0.248	191200
1	kite	bottomvalve	3	0.315	258200
1	fire	carbstone	1	0.148	120260
1	fire	carbstone	2	0.131	99000
1	fire	carbstone	3	0.147	119000
1	keowee	carbstone	1	0.138	107760
1	keowee	carbstone	2	0.129	92760
1	keowee	carbstone	3	0.124	90260
1	kite	carbstone	1	0.331	274200
1	kite	carbstone	2	0.299	242200
1	kite	carbstone	3	0.268	211200
1	fire	yeast	1	0.127	94000
1	fire	yeast	2	0.095	54000
1	fire	yeast	3	0.09	47760
1	keowee	yeast	1	0.172	150260
1	keowee	yeast	2	0.159	134000
1	keowee	yeast	3	0.321	336500
1	kite	yeast	1	0.115	58200
1	kite	yeast	2	0.125	68200

1	kite	yeast	3	0.108	51200
1	fire	wort	1	0.156	130260
1	fire	wort	2	0.097	56500
1	fire	wort	3	0.166	142760
1	keowee	wort	1	0.109	71500
1	keowee	wort	2	0.123	89000
1	keowee	wort	3	0.126	92760
1	kite	wort	1	0.091	34200
1	kite	wort	2	0.067	10200
1	kite	wort	3	0.13	73200
1	fire	packearly	1	0.132	100260
1	fire	packearly	2	0.098	57760
1	fire	packearly	3	0.134	102760
1	keowee	packearly	1	0.102	45200
1	keowee	packearly	2	0.083	26200
1	keowee	packearly	3	0.08	23200
1	kite	packearly	1	0.127	70200
1	kite	packearly	2	0.15	93200
1	kite	packearly	3	0.13	73200
1	fire	packmid	1	0.101	61500
1	fire	packmid	2	0.133	101500
1	fire	packmid	3	0.142	112760
1	keowee	packmid	1	0.675	618200

1	keowee	packmid	2	0.161	104200
1	keowee	packmid	3	0.243	186200
1	kite	packmid	1	0.164	107200
1	kite	packmid	2	0.156	99200
1	kite	packmid	3	0.249	189200
1	fire	packlate	1	0.128	95260
1	fire	packlate	2	0.141	111500
1	fire	packlate	3	0.136	112460
1	keowee	packlate	1	0.096	39200
1	keowee	packlate	2	0.224	167200
1	keowee	packlate	3	0.141	84200
1	kite	packlate	1	0.094	37200
1	kite	packlate	2	0.1	43200
1	kite	packlate	3	0.112	55200
2	fire	sprball	1	0.176	155260
2	fire	sprball	2	0.681	786500
2	fire	sprball	3	0.858	1007760
2	keowee	sprball	1	0.095	38200
2	keowee	sprball	2	0.19	133200
2	keowee	sprball	3	0.089	32200
2	kite	sprball	1	0.327	455680
2	kite	sprball	2	0.335	470000
2	kite	sprball	3	0.257	340000

2	fire	samtap	1	0.418	361200
2	fire	samtap	2	0.355	298200
2	fire	samtap	3	0.279	222200
2	keowee	samtap	1	0.101	44200
2	keowee	samtap	2	0.092	35200
2	keowee	samtap	3	0.097	40200
2	kite	samtap	1	0.109	93334
2	kite	samtap	2	0.142	148334
2	kite	samtap	3	0.22	278334
2	fire	rackarm	1	0.247	190200
2	fire	rackarm	2	0.183	126200
2	fire	rackarm	3	0.232	175200
2	keowee	rackarm	1	0.123	66200
2	keowee	rackarm	2	0.115	58200
2	keowee	rackarm	3	0.115	58200
2	kite	rackarm	1	0.748	1158334
2	kite	rackarm	2	1.378	2208334
2	kite	rackarm	3	1.088	1725000
2	fire	blowoff	1	0.246	189200
2	fire	blowoff	2	0.441	384200
2	fire	blowoff	3	0.362	305200
2	keowee	blowoff	1	0.09	33200
2	keowee	blowoff	2	0.09	33200

2	keowee	blowoff	3	0.073	16200
2	kite	blowoff	1	1.095	1736667
2	kite	blowoff	2	1.449	2276667
2	kite	blowoff	3	1.212	1931667
2	fire	bottomvalve	1	0.185	128500
2	fire	bottomvalve	2	0.309	252200
2	fire	bottomvalve	3	0.261	204200
2	keowee	bottomvalve	1	0.283	226200
2	keowee	bottomvalve	2	0.221	164200
2	keowee	bottomvalve	3	0.257	200200
2	kite	bottomvalve	1	0.312	431667
2	kite	bottomvalve	2	0.326	455000
2	kite	bottomvalve	3	0.315	436667
2	fire	carbstone	1	0.217	160200
2	fire	carbstone	2	0.15	93200
2	fire	carbstone	3	0.148	91200
2	keowee	carbstone	1	0.31	253200
2	keowee	carbstone	2	0.27	213200
2	keowee	carbstone	3	0.3	243200
2	kite	carbstone	1	0.164	185000
2	kite	carbstone	2	0.184	395000
2	kite	carbstone	3	0.189	395000
2	fire	yeast	1	0.293	236200

2	fire	yeast	2	1.903	1846200
2	fire	yeast	3	0.29	233200
2	keowee	yeast	1	0.314	257200
2	keowee	yeast	2	0.311	254200
2	keowee	yeast	3	0.344	287200
2	kite	yeast	1	0.099	76667
2	kite	yeast	2	0.122	115000
2	kite	yeast	3	0.077	40000
2	fire	wort	1	0.323	266200
2	fire	wort	2	0.193	136200
2	fire	wort	3	0.307	250200
2	keowee	wort	1	0.236	179200
2	keowee	wort	2	0.187	130200
2	keowee	wort	3	0.256	199200
2	kite	wort	1	0.233	300000
2	kite	wort	2	0.116	105000
2	kite	wort	3	0.21	261667
2	fire	packearly	1	0.205	148200
2	fire	packearly	2	0.281	224200
2	fire	packearly	3	0.171	114200
2	keowee	packearly	1	0.25	193200
2	keowee	packearly	2	0.198	141200
2	keowee	packearly	3	0.23	173200

2	kite	packearly	1	0.156	99200
2	kite	packearly	2	0.155	98200
2	kite	packearly	3	0.136	79200
2	fire	packmid	1	0.16	127200
2	fire	packmid	2	0.184	127200
2	fire	packmid	3	0.133	76500
2	keowee	packmid	1	0.242	185200
2	keowee	packmid	2	0.212	155200
2	keowee	packmid	3	0.109	49500
2	kite	packmid	1	0.226	169200
2	kite	packmid	2	0.18	123200
2	kite	packmid	3	0.3	243200
2	fire	packlate	1	0.109	52200
2	fire	packlate	2	0.1	43200
2	fire	packlate	3	0.096	39200
2	keowee	packlate	1	0.38	323200
2	keowee	packlate	2	0.202	145200
2	keowee	packlate	3	0.25	193200
2	kite	packlate	1	0.216	159200
2	kite	packlate	2	0.246	189200
2	kite	packlate	3	0.134	77200
3	fire	sprball	1	0.136	79200
3	fire	sprball	2	0.188	131200

3	fire	sprball	3	0.212	155200
3	keowee	sprball	1	0.072	31660
3	keowee	sprball	2	0.071	30000
3	keowee	sprball	3	0.076	38340
3	kite	sprball	1	0.244	187200
3	kite	sprball	2	0.281	224200
3	kite	sprball	3	0.655	598200
3	fire	samtap	1	0.2	245000
3	fire	samtap	2	0.231	296700
3	fire	samtap	3	0.333	466680
3	keowee	samtap	1	0.108	91660
3	keowee	samtap	2	0.086	73340
3	keowee	samtap	3	0.097	73340
3	kite	samtap	1	0.563	506200
3	kite	samtap	2	0.379	322200
3	kite	samtap	3	0.194	137200
3	fire	rackarm	1	0.285	386680
3	fire	rackarm	2	0.233	300000
3	fire	rackarm	3	0.187	223340
3	keowee	rackarm	1	0.086	55000
3	keowee	rackarm	2	0.097	73340
3	keowee	rackarm	3	0.116	105000
3	kite	rackarm	1	0.748	1158334

3	kite	rackarm	2	1.378	2208334
3	kite	rackarm	3	1.088	1725000
3	fire	blowoff	1	0.27	361660
3	fire	blowoff	2	0.244	318340
3	fire	blowoff	3	0.281	380000
3	keowee	blowoff	1	0.108	91660
3	keowee	blowoff	2	0.085	53340
3	keowee	blowoff	3	0.097	73340
3	kite	blowoff	1	0.386	329200
3	kite	blowoff	2	0.3	243200
3	kite	blowoff	3	0.351	294200
3	fire	bottomvalve	1	0.068	25000
3	fire	bottomvalve	2	0.092	65000
3	fire	bottomvalve	3	0.081	46680
3	keowee	bottomvalve	1	0.126	121660
3	keowee	bottomvalve	2	0.19	228340
3	keowee	bottomvalve	3	0.183	216660
3	kite	bottomvalve	1	0.265	208200
3	kite	bottomvalve	2	0.3	243200
3	kite	bottomvalve	3	0.085	28200
3	fire	carbstone	1	0.087	56680
3	fire	carbstone	2	0.087	56680
3	fire	carbstone	3	0.085	53340

3	keowee	carbstone	1	0.169	193340	
3	keowee	carbstone	2	0.18	211660	
3	keowee	carbstone	3	0.229	293340	
3	kite	carbstone	1	0.066	9200	
3	kite	carbstone	2	0.094	37200	
3	kite	carbstone	3	0.063	6200	
3	fire	yeast	1	0.064	18340	
3	fire	yeast	2	0.068	25000	
3	fire	yeast	3	0.099	76680	
3	keowee	yeast	1	0.179	157500	
3	keowee	yeast	2	0.888	1391660	
3	keowee	yeast	3	0.192	238340	
3	kite	yeast	1	0.087	30200	
3	kite	yeast	2	0.103	46200	
3	kite	yeast	3	0.112	55200	
3	fire	wort	1	0.066	21660	
3	fire	wort	2	0.074	35000	
3	fire	wort	3	0.095	70000	
3	keowee	wort	1	0.186	221700	
3	keowee	wort	2	0.171	196660	
3	keowee	wort	3	0.186	236100	
3	kite	wort	1	0.139	82200	
3	kite	wort	2	0.091	34200	

3	kite	wort	3	0.07	13200
3	fire	packearly	1	0.088	58340
3	fire	packearly	2	0.116	105000
3	fire	packearly	3	0.105	86660
3	keowee	packearly	1	0.214	268340
3	keowee	packearly	2	0.207	256660
3	keowee	packearly	3	0.194	235000
3	kite	packearly	1	0.481	424200
3	kite	packearly	2	0.37	313200
3	kite	packearly	3	0.626	569200
3	fire	packmid	1	0.099	76660
3	fire	packmid	2	0.102	81660
3	fire	packmid	3	0.62	945000
3	keowee	packmid	1	0.185	220000
3	keowee	packmid	2	0.181	213340
3	keowee	packmid	3	0.218	275000
3	kite	packmid	1	0.579	522200
3	kite	packmid	2	0.612	555200
3	kite	packmid	3	0.522	465200
3	fire	packlate	1	0.161	180000
3	fire	packlate	2	0.093	66660
3	fire	packlate	3	0.113	100000
3	keowee	packlate	1	0.231	296660

3	keowee	packlate	2	0.256	338340
3	keowee	packlate	3	0.244	318340
3	kite	packlate	1	0.956	899200
3	kite	packlate	2	1.046	989200
3	kite	packlate	3	0.7	643200
4	fire	sprball	1	0.591	534200
4	fire	sprball	2	0.122	65200
4	fire	sprball	3	0.936	879200
4	keowee	sprball	1	0.189	132200
4	keowee	sprball	2	0.171	114200
4	keowee	sprball	3	0.199	142200
4	kite	sprball	1	0.084	27200
4	kite	sprball	2	0.077	20200
4	kite	sprball	3	0.092	35200
4	fire	samtap	1	0.686	629200
4	fire	samtap	2	0.19	133200
4	fire	samtap	3	0.36	312200
4	keowee	samtap	1	0.162	108200
4	keowee	samtap	2	0.176	119200
4	keowee	samtap	3	0.177	120200
4	kite	samtap	1	0.075	18200
4	kite	samtap	2	0.114	57200
4	kite	samtap	3	0.099	42200

4	fire	rackarm	1	0.254	197200
4	fire	rackarm	2	0.251	194200
4	fire	rackarm	3	1.176	1119200
4	keowee	rackarm	1	0.24	183200
4	keowee	rackarm	2	0.191	134200
4	keowee	rackarm	3	0.197	140200
4	kite	rackarm	1	0.105	48200
4	kite	rackarm	2	0.11	53200
4	kite	rackarm	3	0.149	92200
4	fire	blowoff	1	0.306	249200
4	fire	blowoff	2	0.263	206200
4	fire	blowoff	3	0.194	138200
4	keowee	blowoff	1	0.233	176200
4	keowee	blowoff	2	0.24	183200
4	keowee	blowoff	3	0.209	152200
4	kite	blowoff	1	0.123	66200
4	kite	blowoff	2	0.132	75200
4	kite	blowoff	3	0.169	112200
4	fire	bottomvalve	1	0.324	267200
4	fire	bottomvalve	2	0.546	489200
4	fire	bottomvalve	3	0.771	714200
4	keowee	bottomvalve	1	0.697	640200
4	keowee	bottomvalve	2	0.419	362200

4	keowee	bottomvalve	3	0.505	448200
4	kite	bottomvalve	1	0.236	179200
4	kite	bottomvalve	2	0.216	159200
4	kite	bottomvalve	3	0.213	156200
4	fire	carbstone	1	0.26	203200
4	fire	carbstone	2	0.901	844200
4	fire	carbstone	3	0.514	457200
4	keowee	carbstone	1	0.411	354200
4	keowee	carbstone	2	0.501	44200
4	keowee	carbstone	3	0.563	850000
4	kite	carbstone	1	0.202	145200
4	kite	carbstone	2	0.294	237200
4	kite	carbstone	3	0.248	191200
4	fire	yeast	1	0.676	619200
4	fire	yeast	2	0.443	386200
4	fire	yeast	3	0.334	277200
4	keowee	yeast	1	0.609	552200
4	keowee	yeast	2	0.454	397200
4	keowee	yeast	3	0.396	339200
4	kite	yeast	1	0.272	215200
4	kite	yeast	2	0.209	152200
4	kite	yeast	3	0.302	245200
4	fire	wort	1	0.327	270200

4	fire	wort	2	0.426	369200
4	fire	wort	3	0.347	290200
4	keowee	wort	1	0.457	400200
4	keowee	wort	2	0.456	399200
4	keowee	wort	3	0.531	474200
4	kite	wort	1	0.308	251200
4	kite	wort	2	0.325	268200
4	kite	wort	3	0.507	450200
4	fire	packearly	1	0.121	64200
4	fire	packearly	2	0.108	51200
4	fire	packearly	3	0.105	48200
4	keowee	packearly	1	0.109	52200
4	keowee	packearly	2	0.231	174200
4	keowee	packearly	3	0.252	195200
4	kite	packearly	1	1.393	1336200
4	kite	packearly	2	1.176	1119200
4	kite	packearly	3	1.046	989200
4	fire	packmid	1	0.141	84200
4	fire	packmid	2	0.099	42200
4	fire	packmid	3	0.079	19200
4	keowee	packmid	1	0.234	177200
4	keowee	packmid	2	0.281	224200
4	keowee	packmid	3	0.37	313200

```
4       kite    packmid     1       0.817   760200
4       kite    packmid     2       0.773   716200
4       kite    packmid     3       0.88    823200
4       fire    packlate    1       0.084   27200
4       fire    packlate    2       0.098   41200
4       fire    packlate    3       0.119   62200
4       keowee      packlate    1       0.286   229200
4       keowee      packlate    2       0.416   359200
4       keowee      packlate    3       0.384   327200
4       kite    packlate    1       0.807   750200
4       kite    packlate    2       0.645   588200
4       kite    packlate    3       0.982   925200
proc print;
run;
proc sort;
by loc brew;
proc means mean stddev stderr;
var abs cfu;
by loc brew;
run;
proc glm;
class rep loc brew;
model abs cfu = rep loc brew loc*brew;
```

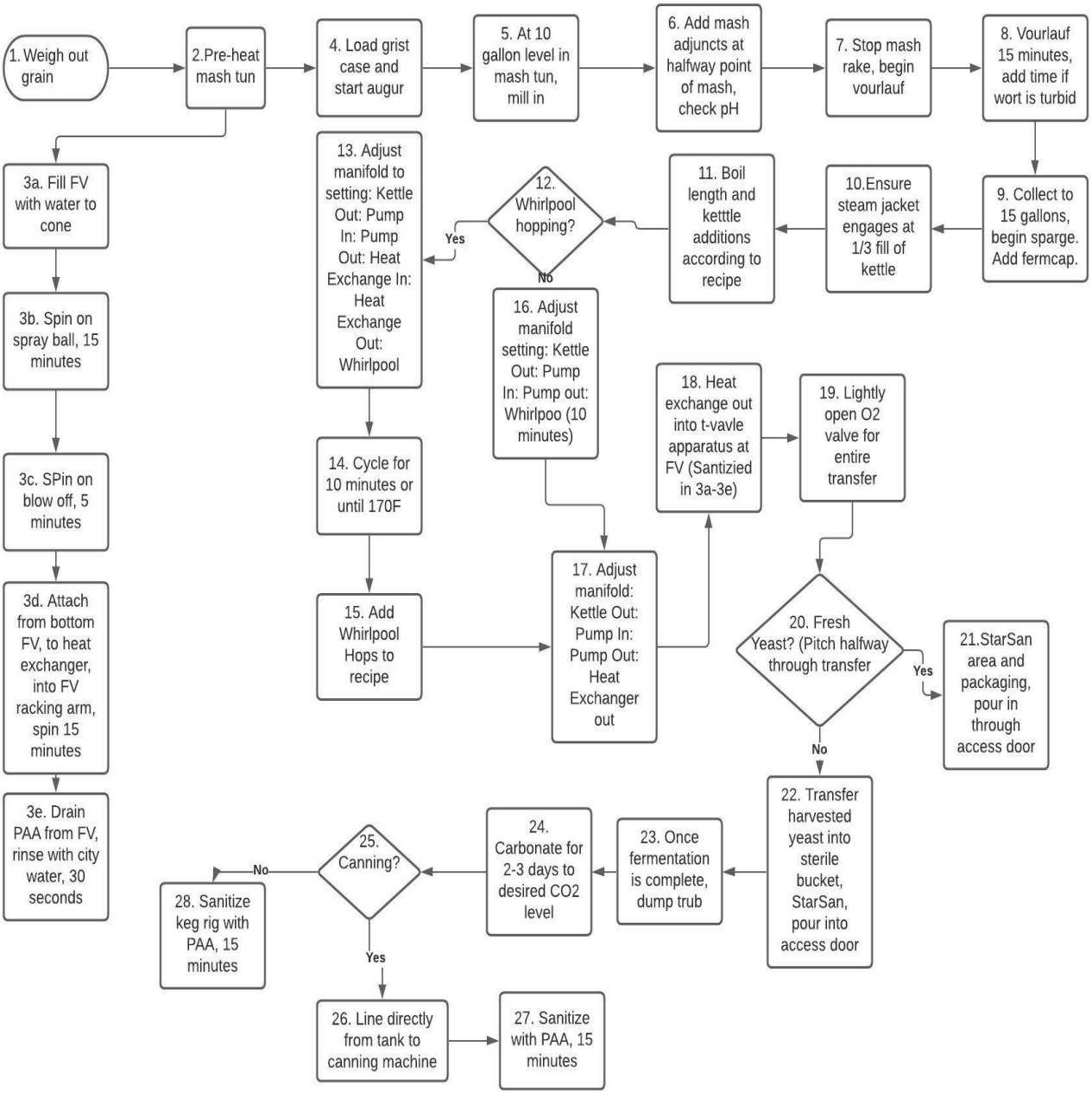
```
lsmeans loc brew loc*brew/pdiff;

run;

quit;
```

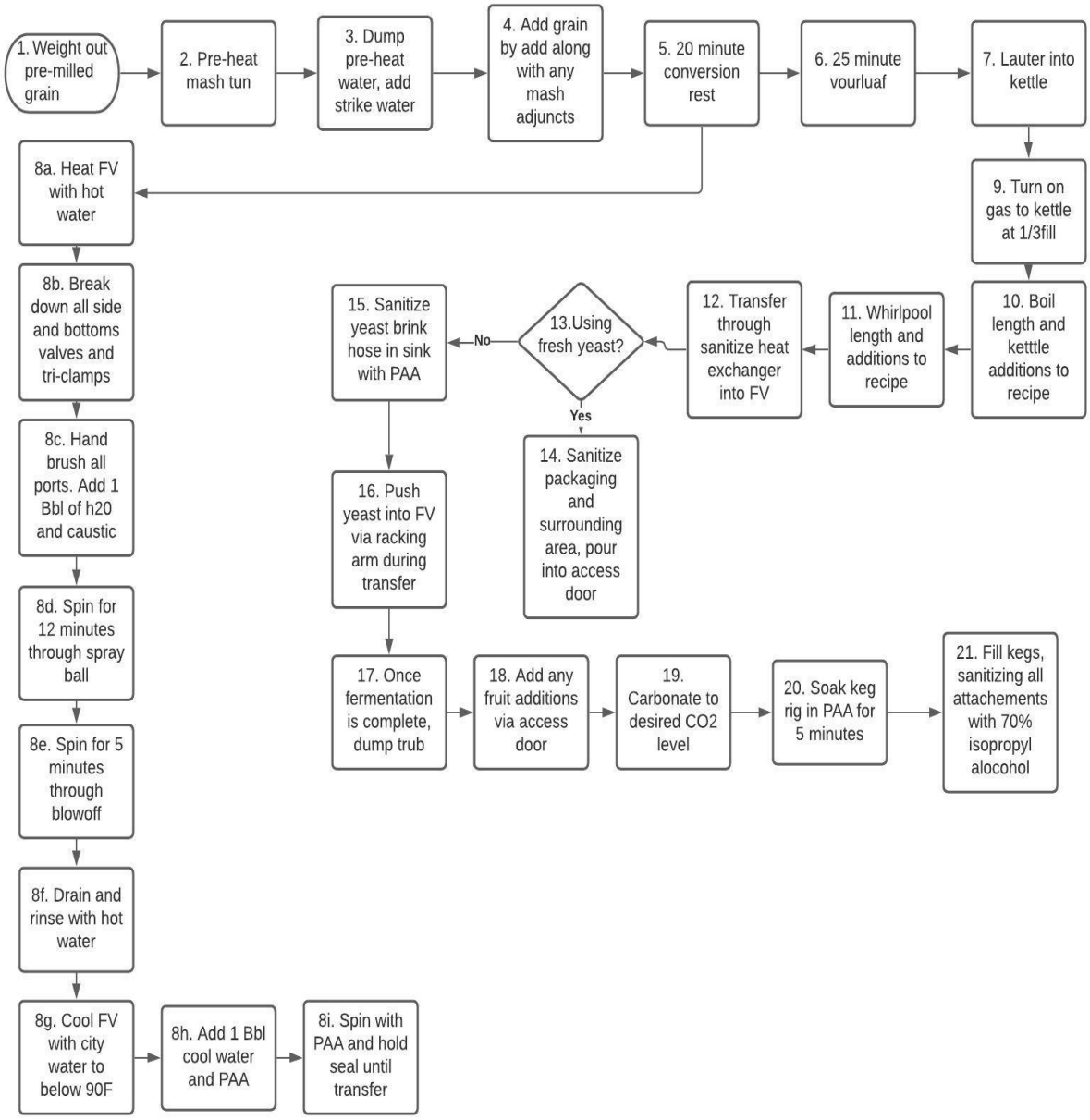
Appendix C – Brewery Process Flow Charts

Brewery A



Appendix C – Continued

Brewery B



Appendix C – Continued

Brewery C