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The Rapid Enumeration of a Mixed Culture of S. cerevisiae & L. plantarum in Beer Using Image-based Cytometry

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THE RAPID ENUMERATION OF A MIXED CULTURE OF *S. CEREVISIAE & L. PLANTARUM* IN BEER USING IMAGE-BASED CYTOMETRY

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Abstract

Mixed cultures are prevalent in the food industry. From sour beer to sourdough bread, mix culture popularity is growing. A number of microbiological mixtures are used in these unique fermenting processes to create distinctive flavor profiles of consumers' favorite foods. Although mixed cultures seem ubiquitous, they are often not well enumerated. The aim of this thesis was to create a novel rapid image-based cytometry method to enumerate mixed cultures in beer. Imaged-based cytometry can be used to rapidly enumerate mixed microbial cultures, as opposed to traditional plate counting methods that can take days to grow and count. A novel method was developed using the Nexcelom Cellometer X2 (X2) using fluorescent dyes and size exclusion automated counting on a mixed culture containing the microorganisms, lactic acid bacteria and yeast (*L. plantarum* and *S. cerevisiae)*. Traditional spread plating using MRS and ADPA selective plates were used to validate this new methodology. The enumeration results were not found to be significantly different ($P > 0.05$) when analyzed by ANOVA comparing X2 to traditional plating for log(CFU/mL). The difference between the log(CFU/mL) of the X2 and

traditional plating was also within a ±0.5 log(CFU/mL). The difference was noted as having a negligible impact on commercial brewing applications. The X2 counts were most precise when the CFU/mL for yeast were between approximately 5 to 7 log(CFU/mL) and 6 to 8 log(CFU/mL) for lactic acid bacteria cells. These ranges are important as they encompass sour beer fermentation pitch rates and can be actively used by brewers today. Overall, the novel image-based cytometry method accomplished the goal of precisely and consistently counting a mixed culture (*L. plantarum* and *S. cerevisiae*) in beer media within the identified ranges and correlated well with the standard process of traditional plating methods. This technique enables brewers to make proactive decisions during fermentation, saves brewers time, and can be a cost effective alternative to other enumeration methods.

DEDICATION

To my loving and beautiful wife, Hope Kennedy, and parents, Norman and Angela Kennedy, with whose support has made this all possible.

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1.1.1   Introduction

Mixed cultures have long been a staple in food preparation, with use documented as early as 10,000 BC (Prajapati & Nair, 2003). For tea, brewing, or food products, mixed cultures are noted for their ability to create products with unique taste profiles, health benefits, and a capacity to preserve food (Smid & Lacroix, 2013). Foods that utilize mixed cultures undergo fermentation, a process occurring when a food product is produced by microbial growth converting one product, usually carbohydrates, to another via enzyme activity (Dimidi et al., 2019). Fermentations can use one microorganism, such as yeast for traditional beer, or lactic acid bacteria for sauerkraut. Mixed cultures fermentation utilize two or more different microorganisms and can include microorganism from the same genus (e.g. bacteria) or different kingdoms (e.g. fungi and bacteria). Cultures containing *Lactiplantibacillus plantarum* and *Levilactobacillus brevis* are considered a mixed culture. (Hesseltine, 1992). Mixed culture fermentations are popular for diverse food products such as sour beers, kombucha, and sourdough breads. In recent years, culture-fermented food products have gained in popularity (Staticia, 2022). The importance of understanding and enumerating these cultures has grown with their increased use. Brewers and manufacturers strive to create products that uses consistent populations of mixed cultures. Understanding and enumerating these cultures can lead to more consistent products with higher quality.

Mixed cultures used by manufacturers are traditionally enumerated by conventional plating methods such as pour plating and spread plating. These methods render mixed cultures difficult to enumerate quickly and analysis can take as long as a week to produce acceptable

results. Further, cultures can be inconsistent in distribution of microorganisms, leading to variation in concentration and number of cells used in beverage products resulting in inconsistent batches (Harrison, 2021). The research described in this manuscript explores the creation of a novel image-based method for the enumeration of yeast and lactic acid bacteria in beer using image-based cytometry. Image-based cytometry has previously been used to evaluate monocultures of yeast and lactic bacteria strains (Saldi et al., 2014; Hodgkin, 2019). This novel technique can provide a faster, simpler approach for brewers and manufacturers to enumerate a mixed culture consistently and precisely.

1.1.2   Fermentation History

The advent of fermentation was largely accidental. The earliest known cases of fermentation have been documented to around 10,000 BC, far before any knowledge of microbial organisms and their capabilities. Still, a variety of foods have historically been fermented including meat, fish, legumes and fruit. Many variations of fermented products and examples survive to this day (Dimidi et al., 2019). It is believed that fermented cheese was first made approximately 8000 years ago in a region between the Tigris and Euphrates rivers in what is now known as Iraq. (Fox, 1993). Alcoholic fermented beverages were first produced between 2000 and 4000 BC by the Sumerians, Mesopotamians, and Egyptians. Sumerians were the first to brew beer followed by the Mesopotamians and Egyptians. In addition, sourdough and leaven breads are attributed to the Egyptians during this period (Ross, 2002). Fermentation has long been utilized, but detailed understanding of the process was evasive for centuries, until a string of scientific discoveries. Microorganisms, specifically fungi, were first recognized by Robert Hooke in 1665 (Gest, 2004). Two centuries later, Louis Pasteur developed his namesake process, pasteurization, in 1863. Pasteurization was significant, as it changed the way producers

viewed food and its relationships to microorganisms. Pasteurization demonstrated how microorganisms can affect food spoilage, safety, and quality (Smith, 2012). During this time period a German scientist, Oskar Korschelt, recorded the first breakthrough in the understanding of fermentation. In 1878, he documented the role of fungus, *Aspergillus oryzae,* in the production of koji. His contribution help spur the study of *Rhizopus oligosporus,* expanding interest in fermentation (Ojha, 2016). These late nineteenth century events coincide with the beginning of the industrial revolution, the social upheaval also credited with starting the paradigm shift of food production from small scale and personal production to large populations and mass markets. Beer was able to be brewed with improved vessels and furnaces that could maintain constant temperatures which led to more consistent batches (Oliver, 2011).

Alcoholic beverages have long been spontaneously produced with yeast and wild lactic acid bacteria has historically been utilized for dairy products, vegetables, and meats (Ross, 2004). Wild fermentation, also known as spontaneous fermentation, relies on microorganisms indigenous to the environment or product (Dimidi et al., 2019). Often found on the peels of fruit or in the air, wild microorganisms can often colonize a suitable food matrices. Over time, manufactures began using starter cultures of known genera and species to induce more controlled fermentation. Starter cultures create more consistent products with known shelf life and reliable product characteristics. Today, however, there is a movement to re-explore using spontaneous and mixed culture products, as mixed cultures can often create unique products with novel taste and texture profiles. This trend has drawbacks, as resulting products have a higher potential to vary when organisms are improperly enumerated or balanced. Presently, work is being conducted to better understand the microorganisms that are used in mixed culture fermentation.

This thesis explores the methods used for the determination of concentration and enumeration of mixed cultures containing lactic acid bacteria and yeast in beer.

1.1.3   Lactic Acid Bacteria

Lactic acid bacteria (LAB) are a group of gram-positive, anaerobic bacteria that produce lactic acid. There are 269 species of LAB with varying benefits and status for human use. Recent genome sequencing has shown that the catch-all genus *Lactobacillus* was too broad. Many organisms in this genera are more heterogeneous than previously thought (Pot et al., 2019). For this reason, the International Journal of Systematic and Evolutionary Microbiology updated names in 2020. *Lactobacillus plantarum*, evaluated in this paper, is now known as *lactiplanticacillus plantarum*.

LAB are commonly utilized for their probiotic properties and unique flavors in foods such as fermented fish, dairy, and vegetables (Mathur et al., 2020). Products that feature LAB have an emblematic sour taste due to the production of lactic acid. In homolactic fermentation, two molecules of lactic acid are produced during fermentation. In contrast, heterolactic fermentation yield one molecule of lactic acid, in addition to alcohol, and carbon dioxide (Wang et al, 2021). Different LAB species are capable of homolactic or heterolactic fermentation. Sometimes both fermentations are possible, depending on the species and environment (Bintisis, 2018). This distinguishing metabolism can be exploited to create different food products. It is important to note that these bacteria can produce different metabolites, depending on environmental conditions or species (Ganzle, 2015).

During fermentation LAB primarily metabolize sugar. Carbohydrates often offer the most direct route to the metabolic pathway, producing lactic acid, alcohol, and CO_2. However, LAB can also breakdown proteins, amino acids, other non-nutritive substances (Wang et al., 2021). The degradation of proteins and amino acids come with many benefits. For example, in dairy products, the protein casein is considered an allergen for a significant segment of the human population. During fermentation LAB are able to hydrolyze casein protein in milk, which reduces the overall allergen-inducing properties of the product (Iwamoto et al., 2019). In another study, LAB had proteolytic activity which could hydrolyze bread proteins including albumins, globulins and gliadins and observed a reduced IgE protein response, indicating a reduced allergenicity of bread (Stefanska et al., 2016). Such advances in fermentation can make food palatable to a wider range of consumers. These benefits may be constrained to specific strains/species of LAB, however, producers of fermented beverage may take the liberty to market and educate consumers to imply LAB is synonymous with health regardless of strain of bacteria.

When LAB metabolize amino acids, removal of unwanted flavor substances can often be accomplished. LAB can also metabolize amino acids and prevent formation of biogenic amines (BAs) in food products. Most BAs, result from decarboxylation of amino acids and some, including histamine, are considered a public health concern due to their toxicological effects. At elevated concentrations BAs can cause food poisoning or histamine poisoning. The toxicity threshold in humans is approximately 10 – 100 mg p.o. (Diel et al., 2009). The reduction of BA-producing amino acids result in safer food products (Ruiz-Capillas & Herrero, 2019). Again, these benefits may be limited to certain strains/species of LAB and producers of fermented beverages should be knowledgeable of the strain added and its documented benefits.

In a mixed culture context, different lactic acid bacteria will grow at different points during fermentation, aiding in culture viabilities. The expression of these LAB can be caused by different tolerances to alcohol, pH, and presence of metabolites. A study following LAB probiotic in coffee brews found that mixed cultures enabled strains of bacteria to maintain longer viability than their monoculture counterparts (Chan, 2021). This extension of viability is considered an important goal of co-cultured lab products.

LAB are also promoted for health benefits, namely for probiotic potential. Probiotics are products containing live microorganisms that can positively affect well-being, especially gut health. They do this using a number of highly variable mechanisms, which include but are not limited to adhering to mucosal and epithelial surfaces, such as the small and large intestine, aiding in immune support by competitively excluding adhesion and propagation of pathogens. Additionally, some probiotics show antimicrobial activity and the ability to hydrolyze bile salt (Kechagia et al., 2013). To be effective, a probiotic must be able to tolerate a digestive system environment including low pH, bile salts, and competition from antagonist microorganisms. Many LAB work well as probiotics as they have high tolerance to all these challenges, in addition to their ability to co-aggregate with of other bacteria (Cikkadi, 2007). Some probiotic strains of *Lactiplantibacillus plantarum* were shown to adhere to gastrointestinal cells while being able to ferment silage and produce antimicrobial substances, such as plantarcins, that help reduce pathogens (Soundharrajan et al., 2019). The ability of LAB to adhere to gastrointestinal cells allows colonization of the gut and enables more growth over time. This colonization has been reported to provide a barrier preventing the colonization of harmful pathogens (Collado et al., 2007).

L. plantarum's ability to metabolize non-digestable substances also aids in digestion and nutrient absorption by the host organism. *L. plantarum* can produce large amounts of β-galactosidase which promote lactose digestion. Lactose, a major component of milk, is a disaccharide composed glucose and galactose. Lactose malabsorption, also known as lactose intolerance, can cause abdominal pain, distension, diarrhea, and other ill effects. Ability to digest lactose decrease with age as lactase activity decreases (Deng, 2015). *L. plantarum* may help supplement a decreased lactase expression and aid in reducing digestive conditions. Further, LAB can ferment fructooligosaccharides, indigestible sugars that can cause dehydration (Cebeci & Gurakan, 2003). Fructooligosaccharides can contribute to bowel irritability, dehydration, and diarrhea. *L. plantarum's* ability to metabolize these largely indigestible molecules helps facilitate the digestion process and provide overall health benefits.

1.1.4 Yeast

Yeast are members of the fungi kingdom and are eukaryotic, single celled microorganisms. They are found naturally in the environment and are common to fruit skins, plant surfaces, and attached to some insects. Yeast are well known for their ability to participate in alcoholic fermentation or anaerobic respiration. Alcohol fermentation follows the glycolysis pathway then NADH regeneration. During glycolysis two pyruvate molecules are generated. During NAD+ regeneration 2 pyruvate molecules are converted to CO_2 and 2 acetaldehyde molecules. NDDH is converted to NAD+ which results in the generation of 2 ethanol molecules (Ciani et al, 2013). Yeast fermentation yields ethanol and a myriad of other desirable and undesirable metabolic byproducts. Examples of yeast-fermented products cover a broad range and include alcoholic beverages, kombucha, bread, and bio-fuels.

Although producers of fermented foods often use pure cultures to create their products, the use of mixed cultures is increasing. Many times, yeast is employed in conjunction with bacterium to create different versions of products. Sourdough bread, kombucha, and sour beer are all examples of products using yeast/bacterial mixed cultures. A recent study found increased health benefits for sour beers that used mixed cultures. *S. cerevisiae* (S.B strain) fermented with mixed starters, had a positive potential health effect, increasing antioxidant activity and polyphenol content (Capece, 2018). Free radicals are best described as unstable atoms that can cause oxidative stress on the body (Lobo, 2010). Phenolic compounds, produced during beer fermentation, work as antioxidants by reacting with free radicals. Phenolic compounds accomplish this by transferring hydrogen atoms, having stabilizing resonance structures, and/or acting as chelators (Zeb, 2020). Although alcohol drinks are not considered to be healthy, American consumers always seek healthier alternatives 43% of the time, while 52% of Americans sometimes looked for healthy alternatives sometimes (Buchholz, 2019). As a marketing tool, brewers and fermented beverage producers could conceivably educate consumers on the potential health benefits provided by some alcohol- containing fermented beverages.

1.2 Fermented Beverage Industry

1.2.1 Industry Growth

A new generation of beverage products featuring yeast/bacteria co-culture are on the forefront of innovation for beverage companies. The ever-growing beverage industry predicts non-alcoholic beverages could to produce a revenue of $537 million by 2026 and boasted 0.5% growth in 2020 despite a global pandemic (Statista Non-alcoholic, 2021). In addition, carbonated beverages lead the non-alcoholic beverage category. This trend bodes well for yeast/bacteria co-cultured products such as kombucha, a natural effervescent beverage that embodies these

growing segments. This research could be used to create an expanded mixed culture method to include gram negative bacteria that could be applied to fermented drinks such as kombucha.

Likewise, alcohol beverage industry is expected to see beer sales increase to $145 million by 2025 (Statista Alcoholic, 2020). This growth is highlighted by beer, ale, and cider's 38% increase in sales from 2019-2020 and carbonated beverages sales growth of 26.5% (Statista, Alcoholic, 2020). These beverages often use yeast and lactic acid bacteria that can be enumerated by the novel mixed culture method validated by this research.

To maintain commercial growth, beverage manufactures must rely on innovation to satiate ever-shifting consumer appetites. As health-conscious consumers turn toward more natural products, beverage manufactures could employ mixed cultured products to fill the market gap. To do this, marketing and education of the potential health benefits to consumers must be taken. Increasingly, "unfiltered" or "hazy" is featured on product labels containing mixed cultures. Consumers want the probiotic and perceived natural benefits that these products may provide and beverages.

The craft brewing industry continues to expand in North America. According to the Brewers Association craft brewing was a 22.2 billion dollar market in 2020 and has taken America by storm with almost 9000 operating craft breweries, with a 373 breweries opened between 2019 and 2020 (CBA, 2021). Along with these new breweries comes demand for sour beers. From 2018 to 2019 sour beers enjoyed a 40 percent increase in sales, totaling 14.8 million in total sales (Hurt, 2020). These brews employ mixed cultures to create sourer and sweeter beverages. They often utilize different mixtures of yeast or bacteria to achieve a novel taste. Kettle sours, for example, are well known for their sour taste created by lactic acid bacteria.

1.2.2   Mixed Culture Fermented Beverage Products of Interest

1.2.2.1 Kombucha

Kombucha, a mixed culture product, is one of the fastest growing grocery segment in the past

decade (Market Watch, 2020) (Figure 1.2.1). Kombucha is a fermented tea beverage made from

green or black tea, sugar and a symbiotic culture of bacteria and yeast (SCOBY). SCOBYs

generally contains mainly yeast, lactic acid bacteria and acetic acid bacteria (Antolak, 2021).
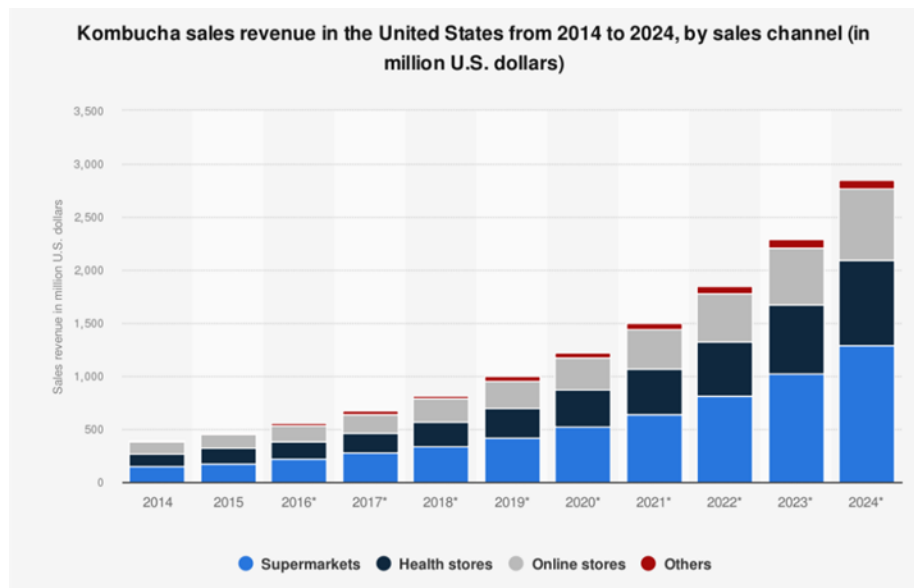
Figure 1.2.1*: Kombucha Revenue in the United States from 2014 to 2024, by sales channel
(Credit: Statista Kombucha, 2022)

Touted for its health benefits, kombucha is known for probiotic characteristics and anti-

oxidants capabilities. These attributes are promoted to help boost consumer's immune system

and help reduce cancer-causing free radicals (Massound et al, 2022). In addition, kombucha

contains nutrients that other sugary carbonated drinks may lack including thiamine, riboflavin,

niacin, folate, B6, B12, and vitamin C. The B vitamins are best known for their role as co-

enzymes, and aid in many cellular functions. Vitamin C participates in fortifying the immune system and has antioxidant activity (Jakubczyk et al, 2020).

The kombucha mixed culture is not generally well enumerated and can be unique for both commercial brewers and at-home enthusiasts. This is because SCOBY composition is highly differentiated, making sampling inconsistent between batches (Harrison & Curtin, 2021). Each SCOBY can be novel if allowed to grow. Environment, stressful events, or time can affect the ratio of bacteria to fungi changes over time (Mas et al, 2022). This variables, in addition to brewing method, can cause a fluctuation in the flavor profile. Difficulty enumerating microbial populations causes quality changes that can make consistency challenging for any brewer and can be especially daunting for industrial-scale producers.

Image-based cytometry should help to reduce many of these consistency issues, allowing brewers to take counts of yeast and lactic acid bacteria simultaneously and better enumerate products over the course of fermentation (Hodgkin, 2019). The use of image based cytometry on complex multi-organism cultures should be the next step after the creation of an image-based method for LAB and yeast. The expansion to kombucha or a fermented beverage with more organism will make adopting the image cytometer more enticing to brewers as the additional methods can highlight the image cytometers expanded capabilities.

1.2.2.2 Sour Beers

Although less popular than modern light beers (pilsners) and a variety of craft beers, sour beers make up approximately 10.3 percent the beer market in 2019 (Staticia Alcoholic, 2020). Today sour beers can be created through many methods including, kettle souring, short/long term mixed fermentation, and maturation in wooden barrels. Sour beers have risen in popularity since

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the mid-1990s, when only a few craft brewers produced this style. In 2002, The Great American Beer Festival introduced a "Sour Beer" category that had only 15 entries. By 2013, this category boasted 238 entries (Tonsmeire, 2014). As illustrated in Figure 1.2.2, sour beers now comprise about 11.0% of beer sales and enjoyed a 73% increase in sales growth in 2016 in the U.S. (Kunst, 2017; Statista, 2016).

Sour beers have unique taste that are separated in multiple categories. Within these categories there are many variations of sour beers. The most popular categories include Lambic, Flanders Red Ale, Oud Bruin, Gose, Berliner Weisse, and American style sour ales. These categories are loosely defined and interpreted by brewers, leading to an amalgamation of styles and unique flavors.

Lambic beers are created through wild/ spontaneous fermentation. These beers are fermented by organisms found naturally in the native environment or ingredients. Because these fermentations rely heavily on environmental organisms, lambic fermentations can be difficult to replicate. They are a versatile style of beer and often contain un-malted wheat and barley, or a fruit base. Additionally, they can be barrel aged or mixed to create unique flavors and hops are often used as an antimicrobial agent to impart alpha and beta acids to wort, preventing unwanted microbial growth (Wilmes, 2016).

Flanders Red Ale or Flemish red-brown is a sour ale with Belgium origins. It is often fermented with *Saccharomyces cerevisiae* and *Lactobacillus* spp. This sour has long fermentation times that can last more than a year. The beer is characteristically made with red malt and aged in oak barrels resulting in an intense fruit flavor (Bouckaert, 2012).

Oud Bruin are another Belgium sour like the Flanders Red, but is a brown ale. Like Flanders Red it can be oak cask-aged for up to two years, but commercial brewers usually use stainless steel (Wilmes, 2016). This style is malt forward and is often blended with younger beers to allow for more acidity during maturation (Jackson, 2003).

Gose is a traditional German sour beer. Usually a wheat beer, Gose is known for being an unfiltered sour. Gose beers are commonly brewed with half-malted wheat, malted barley, coriander and salt. Although, traditionally spontaneously fermented, American brewers have begun to kettle-sour beer this style (Wilmes, 2016).

Berliner Wiesse is a German style sour beer. The research described in this thesis used a Berliner Wiesse recipe to evaluate the novel image-based mixed culture method. Berliner wiesse has enjoyed a resurgence in American breweries. This beer is often fermented with *S. cerevisiae* and *Lactobacillus* spp. This sour beer is known for a fruity taste and low alcohol content (Wilmes, 2016).

The American-style sour ales are wide ranging and often imitate on other sour beer styles. Brewing techniques and traditional recipes of other styles to create this unique category of beer. American-style sour ales are known for having low acidity, but are tart, with acidic flavor and widely palatable taste (Wilmes, 2016).

Sales growth

| | -50% | 0% | 50% | 100% | 150% | 200% | 250% | 300% | 350% | 400% |

Herb/spice — 375%

Sour/American wild ale — 73%

Blonde/golden ale — 54%

Shandy/Radler — 42%

Saison/farmhouse — 38%

Fruit/vegetable/pumpkin — 33%

Hefeweizen — -4%
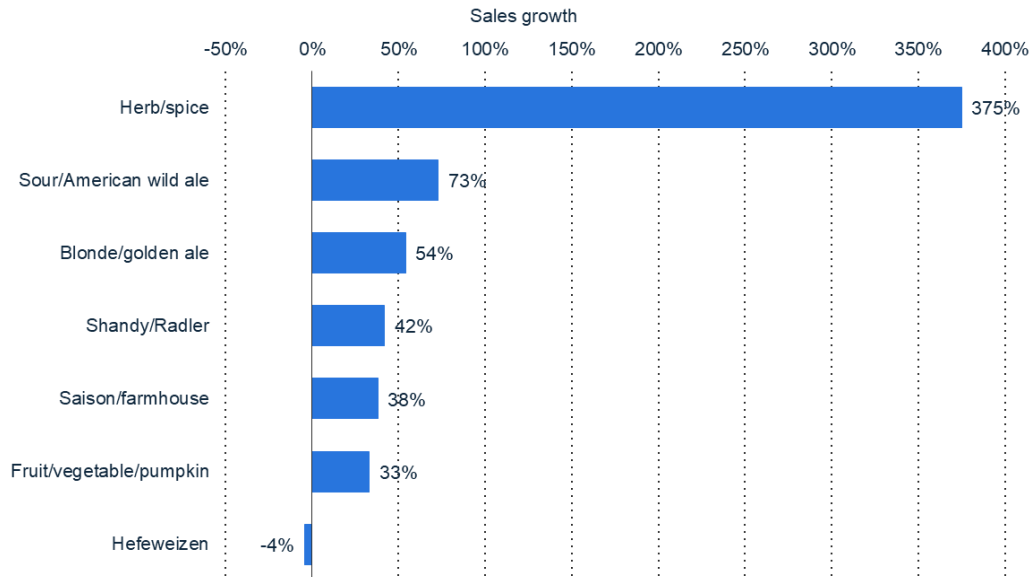
Figure 1.2.2: *Craft beer sales growth in the United States in 2016, by style (Credit: Statistic, Alcoholic Beverages, 2016)*

The process of creating sour beers has changed over time. Brewers rely less on spontaneous or wild fermentation and often opt for established enumerated microbial pitches. Traditional beers such as lambic and gueze that use spontaneous fermentation can take years to mature and achieve the correct flavor (Ciosek et al., 2019). These beers may be prone to spoilage and variation when not properly maintained and can be time consuming endeavors. Because of these difficulties many brewers used controlled, established enumerated pitches to acidify wort. In a kettle sour, wort is acidified with *Lactobacillus* spp, then boiled to kill or inactivate the lactic acid bacteria before yeast is pitched. In this process microorganisms are added stepwise, enabling fermentations at optimal temperatures. This results in beverages created in days instead of months unlike their spontaneous counterparts. When microorganisms are pitched simultaneously, brewers often elect for specialty, well-enumerated pitches that have been optimized for a generic type of beer. Both of these modern methods are much faster than spontaneous fermentations and usually yield more consistent results.

Different combinations of yeast and bacteria species are often used in sour beer production. *Brettanomyces* spp. and *S. cerevisiae* are commonly used yeast while *Lactobacillus, Pediococcus,* and *Acetobacter* are representative genus of the bacteria employed in these co-culture fermentations (Dysvik et al., 2020). The trend away from spontaneous fermentation is largely due to a lack of consistency of the final product. Although the scientific literature is lacking in this area, a recent study examining the enumeration of a mixed culture beer, revealed bottle to bottle metabolite (flavor component) inconsistency overtime (Spitaels et al., 2015). This represents a major problem for brewers as a consistent, high quality products are coveted by consumers. Brewers must know that their pitch rate and growth of yeast and bacteria is consistent. As the starter concentration can change within hours of growth, traditional plating methods may only provide a delayed (errant) snapshot of the fermentation. The speed and ease of image-based cytometry can help alleviate some of the concerns brewers have when using a mixed culture.

1.2 Counting Methods

1.3.1 Traditional Plate Counting

Beverages produced with mixed cultures can contain multiple species or genera of microorganisms found ubiquitously in the environment. As discussed previously, mixed cultures are utilized in kombucha, sour beer, and many other fermented food products. These mixed cultures often contain a variety of yeast, molds, acetic acid bacteria, lactic acid bacteria. The biodiversity in mixed cultures can make microorganisms difficult to enumerate, due to inconsistency in sampling and inconsistency of the cultures themselves. Common methods for enumerating mixed cultures include pour plating and use of selective media.

The pour plating technique is often used to count microorganism in a mixed culture. Molten agar is inoculated with culture and then left to solidify. This method facilitates the even distribution of microorganisms throughout agar, from which viable plate counts can be taken (Sanders 2012). This method works well for microorganisms of the same species or with genera that use common media.

Selective media is used in conjunction with various plating. Selective media uses additives such as salt, acids, or metabolites to inhibit unwanted microorganism growth while promoting selected microorganism growth (Prinze & Rohde, 2020). Spread plating dilutes cultures and spreads them evenly on the surface of the agar plates. Viable cell counts can then be taken.

Pour plating and selective media are both reliable and acceptable methods for accessing the concentration of mixed culture samples to be used for beer wort or kombucha products. Incubation of plates may take from one day to a week depending on targeted microorganisms (BD Difco, 2009). In this study, acidified potato dextrose agar and De Man, Rogosa and Sharpe agar were used as selective media for our mixed culture samples.

1.3.2   Microscopy & Hemocytometer

The hemocytometer was originally designed for counting blood cells, however, its function has expanded to other analytical work, including enumeration of yeast and bacteria. A hemocytometer is a grid etched in a surface of a slide and each square of the grid has a defined area. The dilution applied to the sample before slide loading and the number of cells in the area are used to determine concentration of target organism (Absher, 1973). A hemocytometer utilizes dyes such has trypan blue to stain cells indiscriminately. These instruments have low startup

costs and speed of evaluation give a clear advantages over traditional cell counting methods. Some drawbacks, however, make them less reliable in laboratory settings. A hemocytometer can be prone to bias when manual counting, increasing the variability of results (Cadena-Herrera et al, 2015).

1.3.3   Flow Cytometry

Flow cytometry is used in the fields of biochemistry, immunology, cell biology, and many more scientific areas as the technique can quickly quantify microorganism concentrations. Flow cytometers convey fluorescent cells through a light source, usually a laser, and to send a signal to a computer that can analyze cell populations (McKinnnon, 2018). Parameters such as cell density, purity, and viability can be analyzed. Other applications include protein expression, cell cycle status, and organism identification (Adan et al., 2016).

Flow cytometry allows a large number of cells to be quantified and has the capability to collect more information other than traditional methods. This technique can be used to measure cellular metabolic activity, perform cell sorting and concentration determinations, and conduct immune-phenotyping (SCH, 2022). This technology is also useful for analysis of heterogeneous populations.

Unfortunately, use of flow cytometry for food microbiology work remains low. Capital cost of equipment and extensive training make flow cytometry prohibitive for many breweries or food laboratories. Furthermore, in a heterogeneous population, such as a mixed culture, flow cytometry is a slow process when compared to image-based cytometry. Breweries deal with mixed culture and media with varying debris and opacity, which may interfere with enumeration. The high-speed sorter used on flow cytometers can sort about 10^3 to 10^4 cells per second

(McKinnon, 2018). Flow cytometry manufactures report sample analysis time taking anywhere from hours to a few days. It would take approximately one to two hours to sort a cell population of 2.0×10^7 cells (UV, 2021).

1.3.4   Imaged-Based Cytometry

Imaged-based cytometry is a proven viable alternative to traditional counting methods and flow cytometry. With both research and industry applications, automated counting allows cheaper, more efficient counting. Recent studies have validated its use for enumerating the concentration of yeast and lactic acid bacteria monocultures (Saldi et al., 2014; Hodgkin, 2019). Image-based cytometry equipment often requires fewer resources and maintenance when compared to flow cytometry, while providing results in little as a few a minutes. Notwithstanding, the capitol cost of image -based cytometry can be five times less expensive than flow cytometry, with image-based cytometry costs starting at $20,000 and flow cytometry ranging between $25,000 and $500,000.

Unlike flow cytometry, imaged-based cytometry analyzes pictures to determine cell density. This has been most effective for cell counting and assessing viability of single cell culture suspensions. Image-based cytometry uses single stains to determine concentration or dual stained dyes to determine viability and concentration. Cell suspensions are first diluted then dyed with nucleic stains, allowing for the exclusion of debris. Then a fluorescent microscope takes a digital image and counts the cells. Concentration and viability can be determined from the image (Nexcelom, 2022).

For the work presented in this thesis, an image cytometer, Cellometer X2 (Figure 1.3.1), donated by Nexcelom Biosciences LLC (Lawrence, MA), was used to evaluate the concentration

of monocultures and mixed cultures in beer. This instrument can conduct automated cell analysis including counting cells, measuring cell sizes, and detecting fluorescence properties of cells (Nexcelom, 2022).

Figure 1.3.1: Nexcelom Cellometer X2 and Cellometer X2 Program (Credit: Nexcelom.com)

The assay created to enumerate mixed cultures relies on size exclusion and fluorescent analysis. The Cellometer X2 has two fields to measure fluorescent fields, F1 and F2. Each field can measure at both 535 and 660 nm. Fluorescent microscopy relies on fluorophores to illuminate cells. Fluorophores are chemical compounds that stain fixed or live cells that can then fluoresce upon light excitation (Stockert & Blazquez-Castro, 2019).

Fluorophores excite when a photon of energy provided by the Cellometer X2 light source is absorbed. This creates an electronic singlet state or high energy state. This singlet state causes conformational changes in the fluorophore. When the fluorophore relaxes emission of light begins which then can be imaged and returns the fluorophore to a ground state. The emission or ground state is at lower energy which has a longer wavelength (Johnson, 2010). For this research

fluorescent channel VC-535-402 was used, excited at 470 nm and emitted at 535 nm. The VC-660-502 channel excited at 540 nm and emitted at 660 nm.

The Cellometer X2 uses fluorescent microscopy in conjunction with automated counting. The automated program allows precise, unbiased counting as compared to manual enumeration. Yeast cells are stained with AO/PI and lactic acid bacteria stained with Syto BC. These fluorescent dyes bind nucleic acids and fluoresce at 535 nm. The mixed culture validation experiment and brewing application used a 2:1 ratio of Syto BC to AO. These dyes stain both living and dead cells, allowing only the total enumeration of mixed cultures. Viability and the inclusion of fluorescent dead cells requires evaluation with another confirming technique. The Cellometer X2 is limited to two fluorescent channels, which excludes evaluation of additional parameters.

Once the cells are properly stained, size exclusion is used to discriminate and count cells. The Nexcelom program includes parameters that can help distinguish yeast from lactic acid bacteria cells. These parameters, shown in Table 1.3.1, include Cell Diameter Minimum/Maximum, Roundness, Contrast Enhancement, Decluster Edge Factor, Decluster Th Factor, Background Adjustment, and Very Dim Enhancement.

Table 1.3.1: Cellometer Key Terms and Definitions (Credit: Nexcelom Cellometer X2 Manual)

Term	Definition
Cell Diameter Minimum/Maximum	Minimum and Maximum diameter of the cell included in the count
Roundness	Value indicates shape of cells to be included in the count
Contrast Enhancement	Value indicates what imaging enhancement is needed for cells to be identified
Decluster Edge Factor	Value indicates the degree in which the software needs to enhance the cell edge in order for Declustering Declustering: Distinguishing single cells from aggregated or clumped cells
Decluster Th Factor	Value indicates the threshold ratio between the cell signal and the background
Background Adjustment	Value indicates the threshold adjustment needed to pick up strong or weak cells
Very Dim Enhancement	A special function to count cells with weak contrast

Parameters that pertain to size are important, as yeast and lactic acid bacteria cannot be differentiated solely based on fluorescence. Both microorganism dyes fluoresce at 535 nm, so their size difference is used to distinguish them. One fluorescent field is used for yeast, while the

second is used for lactic acid bacteria. Yeast are generally larger and are ≥ 5 μm with literature

citing mean size approximately 8 μm (Zakhartsev & Reuss, 2018). Lactic acid bacteria are

generally ≤ 5 μm with literature citing size between 3-8 μm (Landete et al, 2010). Both

microorganisms can appear larger or smaller depending on their orientation in slide chamber, but

cell count can be evaluated manually, by post imaging, to ensure proper counting. Additionally,

cell orientation can be mitigated by slide type. The Cellometer X2 employs multiple slide types.

The two most common slides are SD100 and SD025. SD025 provides a smaller chamber that

properly orients bacteria and yeast on the same viewing plane. SD100 are more suited for larger

cells such as yeast, but not bacteria, as small cells can move in and out of the viewing plane,

causing inconsistent focus.

1.3.5   Method Summary

Inexpensive and effective methods for evaluating mixed cultures include traditional

plating methods and image-based cytometry. Other methods prove costly or highly variable. To

support a growing brewing industry, it is important to consider that the most efficient microbial

evaluation method may be the most useful to brewers who value time. Image-based cytometry

gives brewers the flexibility to evaluate samples in under 15 minutes when compared to

traditional plating methods. Further exploration of image-based cytometry is needed to provide

confidence in this emerging technology.

RESEARCH OBJECTIVES

- **Verify the Cellometer X2's ability to precisely count yeast and bacteria individually using established Cellometer X2 methods**

- **Demonstrate the Cellometer X2's ability to count a mixed culture containing yeast and lactic acid bacteria**

  o **Validate the Cellometer X2's ability to determine yeast and bacteria concentrations while keeping one consistent and varying the other**

  o **Validate the Cellometer X2 's ability to reliably evaluate different ratios of yeast vs. bacteria concentration in a mixed culture**

- **Demonstrate that the newly developed image-based mixed culture method can be successfully applied to a sour beer fermentation**

CHAPTER 2 MONOCULTURE VERIFICATION

2.1   Introduction

Monoculture verification was completed with yeast and lactic acid bacteria using the

Cellometer X2. *S. cerevisiae* and *L. plantarum* monocultures were chosen as representative

microorganism for this experiment. *S. cerevisiae*, is the fermentation yeast of choice for beer

brewing and is also employed with various mixed brewing cultures. It should be noted that there

are many strains of *S. cerevisiae* used for brewing. *L. plantarum* monoculture methods were

previously validated with the Cellometer X2 monoculture and is commonly used in sour beers to

acidify wort. This organism is easily sourced and cultures can be grown in 24 hours.

The use of image-based cytometry can result in faster sample analysis, leading to a more

proactive fermentation by brewers. Instead of potentially waiting days to get results, brewers can

sample and have results from multiple time points in as little as 15 minutes. This information

gives brewers the flexibility to be proactive instead of reactive in their brewing regimes. Brewers

can monitor real time changes of how adjusting the pitch affects their final product. This can

prevent over-pitching and under-pitching which can affect the quality of the final product. If a

fermentation has too many cells/mL or too concentrated brewers can dilute to prevent over-

pitching. Conversely if a fermentation has too few cells/mL or too dilute brewers can re-pitch for

a more concentrated fermentation.

Previous research has validated each monoculture using the Cellometer X2 for yeast and

lactic acid bacteria. The goal of this section was to verify previous research monoculture

methods on the Cellometer X2, confirming that the instrument's results were not significantly

different from the respective traditional plating method for each microorganism. Only the total

enumeration was analyzed, as viability cannot be assessed in the mixed culture method due to

instrument constraints. This use of image-based cytometry can result in faster sample analysis, leading to more proactive fermentation regimes.

2.2   Method

2.2.1   Yeast Culture Preparation

Yeast, *Saccharomyces cerevisiae* (Safale S-04) was purchased from Fermentis (Peri Marq-en-Baroeul, France) in dry yeast packets. Dried yeast packets were rehydrated and grown overnight at 30°C in 50 mL of potato dextrose broth (BD Difco). In triplicate plates, yeast culture was isolated using streak plating on acidified potato dextrose agar. Agar was acidified by adding 10% tartaric acid (TA) to the media to create APDA with 0.01% TA. Isolated yeast colonies were aseptically transferred to 10 mL of acidified potato dextrose broth and incubated for 24 hours at 30°C. The 24-hour yeast culture were streaked on acidified potato dextrose agar.

For each experiment, in triplicate, an isolated yeast colony was aseptically transferred to 50 mL of potato dextrose broth. Samples were incubated for 24-hours in a water-bath shaker at 30°C. Samples were taken in triplicate 10 mL aliquots. Aliquots were centrifuged for 5 min at 1968 x g. The pellet was saved and the supernatant was decanted. The pellet was re-suspended in 2 mL of 1X PBS buffer (pH 7.4) yielding an approximate yeast concentration of 10^7 cells/mL. Samples were vortex before use to help decluster or prevent aggregation of yeast cells in the suspension.

2.2.2   Lactic Acid Culture Preparation

Lactiplantibacillus plantarum (ATCC ® 8014[TM] , Manassas, VA) was provided by Dr. Jennifer Perry's microbiology lab at the University of Maine and the strain was stored in 1 mL frozen aliquots at -80°C. In triplicate, each aliquot was transferred to 9 mLs of Demann, Rogosa

and Sharpe (MRS)(BD, Difico) broth and incubated overnight at 30°C. The 24-hour growth culture was then streaked onto MRS plates in triplicate and incubated at 30°C for 2 days.

For each trial, a single isolated colony from an MRS streak plate was aseptically transferred to a tube containing 9 mL sterilized MRS broth. Tubes were prepared in triplicate and were incubated for 24 hours at 30° C.

2.2.3 Yeast Cell Stain

Acridine orange (AO) and Propidium iodide (PI) are nucleic dyes used in the staining of *S. cerevisiae*. AO/PI relies on dual fluorescence viability and AO is permeable to both living and dead cells and fluoresce green at 535 nm. PI can only enter dead cells with perforated membranes and fluoresces red at 635 nm. These complementary stains allow the discrimination of living vs. dead cells. Cells stained with both AO/PI will fluoresce red due to resonance energy transfer, while live cells stained only with AO fluoresce green. AO/PI dye stain was provided by Nexcelom Bioscience (Lawrence, MA). Dye solutions were kept at ambient temperature for the duration of the experiment.

2.2.4 Lactic Acid Bacteria Cell Stain

Syto BC Green Fluorescents is a nucleic acid stain and was purchased by Nexcelom Bioscience (Lawrence, MA) from Thermo Fischer Scientific (Waltham, MA). Syto BC fluoresces green at 535 nm and does not discriminate between living or dead cells rather it is used for the total enumeration of cell cultures and it can stain both gram-negative and gram-positive cultures if nucleic acids are present. Syto BC working solution was prepared by diluting the stock Syto BC 1:100 with deionized water. Dye stock solution was stored at ambient

temperature for the duration of the experiment. Fresh working dye was prepared before each trial.

2.2.5   Cellometer X2

The Cellometer X2 utilizes a bright field (BF) and two fluorescence fields. (F1 & F2). Both fluorescence fields use VC-535-402 and VC-660-502 to evaluate different fluorescent dyes which can then be used to measure viability, cell count, and concentration, the Cellometer X2 implements a 10X magnification to better identify the cell outlines showing about ~0.5 μm^2 /pixel resolution

For yeast evaluations, both cellometer fluorescence fields were used. VC-535-402 was used to detect the presence of cells stained with acridine orange, with exposure times between 500-1000 ms. Cells stained with propidium iodide were evaluated under VC-660-502 channel with exposure times between 2200-2700 ms. Yeast cell suspensions were diluted 1:1 with yeast dilution buffer for 30 seconds then diluted again 1:1 with AO/PI dye for 30 seconds. Five μL of dyed yeast sample was pipetted into the counting chamber (CHT4-SD025). Once inserted into the Cellometer X2, the slide was checked under the bright field for correct morphology and contamination. After the slide was reviewed and focused, The Cellometer X2 took four different images of four different areas in the counting chamber using the three channels BF, F1, and F2. Cell counts, viability, and concentrations were extrapolated from the images. Four replicates were measured for each dyed sample. The Cellometer X2 yeast criteria for brightfield was: Cell Diameter (4-microns), Roundness (0.1), Contrast(0.1), Decluster Edge Factor (0.9), Decluster Th factor (1.0), Background Adjustment (1.0), Fluorescent Criteria: Cell diameter (4.0-20.0 micron), Roundness (0.1) , Fluorescent Threshold (10.0), Decluster Th Factor (0.25). Criteria was taken

from previously validated yeast monoculture methods to verify that the method could be used on a monoculture.

Lactic acid bacteria fields were analyzed under the VC-535-402 channel with exposure times between 300-1500 ms. Lactic acid bacteria were stained 1:1 with Syto BC for 30 seconds. After staining, five μL of lactic acid bacteria stained sample was pipetted into a Nexcelom counting chamber (CHT4-SD025). The ports were taped with extra clear tape (Scotch) to prevent evaporation and to slow cell movement in the chamber. Cells in the taped chamber were allowed to settle for 30 seconds to further stop cell movement. After insertion into the Cellometer X2, the slide was checked under the bright field for correct morphology and contamination. Once the slide was reviewed and focused, the Cellometer took four different images of four different areas in the counting chamber using the two channels BF and F1. Four replicates were measured from each dyed sample. The Cellometer's criteria for evaluation for bright field was: Cell Diameter (0.1-3.9 microns), Roundness (0.1), Contrast (0.2), Decluster Edge Factor (0.5), Decluster Th factor (1.0), Background Adjustment (0.2), Fluorescent Criteria: Cell diameter (0.1-3.9 micron), Roundness (0.1), Fluorescent Threshold (5.0), Decluster Th Factor (0.25). Criteria was taken from previous validated Cellometer X2 methods to verify that it could be used *lactobacillus* culture.

2.2.6   Yeast Verification

Cell concentration log(CFU/mL) of 24-hour incubated yeast culture were compared between the X2 and traditional manual plate counting. Linear cell counts and concentrations were compared between the methods. *S. cerevisiae* cell suspension was decimally diluted with peptone water (1.5%, Sigma-Aldrich).

For Cellometer counting, triplicate diluted yeast samples were transferred to a microcentrifuge tube. Samples were diluted 1:1 with yeast dilution buffer then 1:1 with AO: PI dye solution. Slides were analyzed using the Yeast AO: PI method outlined in Section 2.2.4 and Figure 2.2.1 with the Cellometer X2.

For plate counting controls, serial dilutions were prepared in triplicate. Appropriate dilutions were spread onto acidified potato dextrose plates in duplicate. Inoculated plates were incubated for 2-3 days at 30°C.

2.2.7 Lactic Acid Verification

Cell concentration log(CFU/mL) of 24-hour lactic acid bacteria cultures were compared between the Cellometer X2 and traditional plating methods. Stock cultures for *L. plantarum* were diluted and analyzed for cell count and concentration.

In triplicate, serial dilutions were performed between 10^{-1} and 10^{-5} for *L. plantarum*. Samples were dyed following the staining procedure outlined in Section 2.2.4. Dilutions were stained in triplicate 1:1 with Syto BC for lactic acid bacteria.

Serial Dilutions were prepared triplicate for plating. Appropriate dilutions were spread onto De Man, Rogosa and Sharpe agar plates in duplicate. Inoculated plates were incubated for 1-2 days at 30°C.
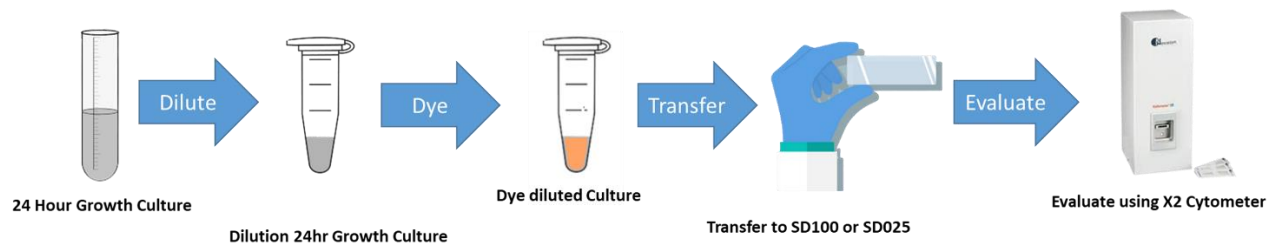
Figure 2.2.1: Cell Staining and Evaluation Workflow

2.2.8   Statistical Analysis

Cell suspension concentrations were transformed to starting log(CFU/mL) for Cellometer

and plating methods. Each dilution factor of cell suspensions were compared using ANOVA. If a

p-value of less than 0.05 was indicated then a tukey hsd was conducted. Cell suspensions with p-

values greater than 0.05 were averaged to give an experimental method average and others were

excluded. The experimental method average log(CFU/mL) was compared between the

Cellometer X2 and respective traditional plating method using ANOVA in JMP Pro 15.2.0

(466311). A p-value of less than 0.5 was considered statistically significant.

2.3   Results

Before validating the novel mixed culture method, monoculture counting methods for

yeast and lactic acid bacteria were first verified. Concentrations for each microorganism were

compared between the Cellometer X2 and respective traditional plating method. Concentrations

were evaluated at different dilutions to confirm linearity of Cellometer counting results for the

Lactiplantibacillus plantarum and *Saccharomyces cerevisiae* with different cell densities present

in the slide viewing field.

S. cerevisiae samples were evaluated using cell concentrations ≈ 4-7 log(CFU/mL). Cell

count consistently increased in the images and is shown in Figure 2.3.1. Some clustering was

viewed during the yeast monoculture trials. Samples were vortexed after staining to facilitate declustering.

Figure 2.3.1: Decimal Dilutions of *S. cerevisiae* viewed with Nexcelom Cellometer X2 between 4 – 7 log(CFU/mL)

Yeast dilution concentrations evaluated were highly linear with an average R^2 value of 0.9976 for samples taken between 5 – 7 log(CFU/mL) across three trials, confirming ability of the Cellometer X2 to precisely establish cell concentrations in this range. Cell counts prepared at concentrations of 4 log(CFU/mL) and lower resulted in an increase in R^2 and standard deviation. This indicated less confidence in the method at concentration 4 log(CFU/mL) and lower. Cells were either not present at 4 log(CFU/mL) or when present caused inflated concentrations.
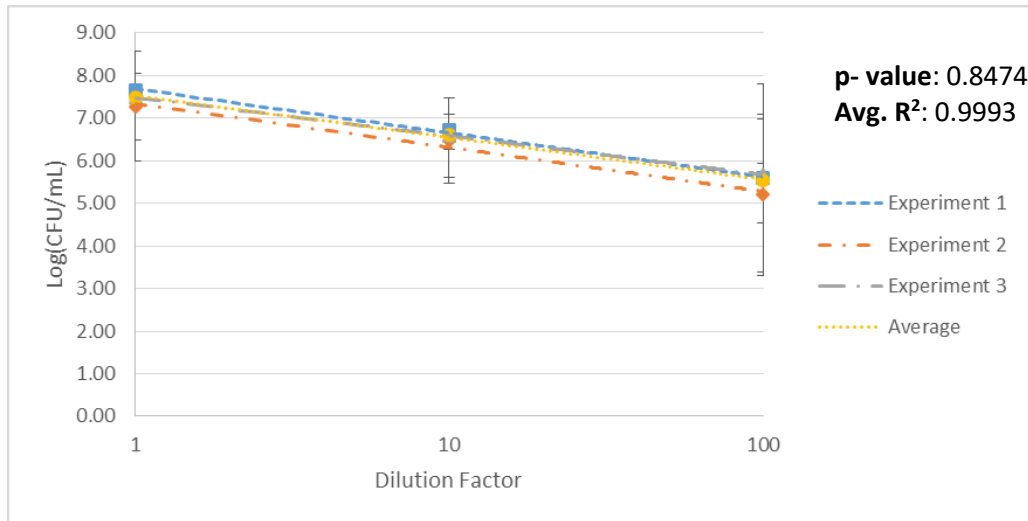
Figure 2.3.2: *S.* c*erevisiae* log(CFU/mL) Monoculture at Various Decimal Dilutions Evaluated with the Nexcelom Cellometer X2

Figure Legend: Each trend line corresponds to a monoculture experiment. The average is the mean of all three trials at each data point. There was no significant difference (p>0.05) between the APDA plating and Cellometer X2 method.

Before ANOVA and statistical analysis, a boxplot was created to filter any outlying data points by IRQ assessments. If an outlier was present, the outlier was excluded. No outlier was present between the concentration 5 log(CFU/mL) and 7 log(CFU/mL).

Next the concentration was converted to log(CFU/mL). The log(CFU/mL) for APDA plated and Cellometer X2 was compared by ANOVA to evaluate the difference between methods. The average log(CFU/mL) for Cellometer X2 and traditional plating was 7.48 ± 0.12 and 7.51 ± 0.08 Log(CFU/mL) respectively. This resulted in an average difference of -0.03 log(CFU/mL). A p-value of 0.8474 confirmed no significant difference between the methods.

Table 2.3.1: ANOVA Comparing Methods, Cellometer X2 & Acidified Potato Dextrose Agar

Plated, for *S. cerevisiae* in a monoculture

Organism	*S. Cerevisiae*	Experiment	Monoculture Verification	
Trial	Plating Log(CFU/mL)	Cellometer X2 Log(CFU/mL)	Difference	P-Value
1	7.66 ± 0.07	7.54 ± 0.17	0.12	
2	7.32 ± 0.12	7.31 ± 0.08	0.01	0.8474
3	7.53 ± 0.08	7.58 ± 0.08	-0.05	

L. Plantarum samples were evaluated using serial dilutions as well. *L. Plantarum* samples were evaluated between ≈4 – 9 log(CFU/mL). At 9 log(CFU/mL) too many cells where in the image, causing overlap and inconsistency during automated counting. At 4 log(CFU/mL) too few cells were in the frame, causing inflated concentration and high standard deviation. These concentrations were omitted from the ANOVA and regression analysis and represent the quantitative limit for the application of the Cellometer method.

10^5 10^6 10^7 10^8 10^9
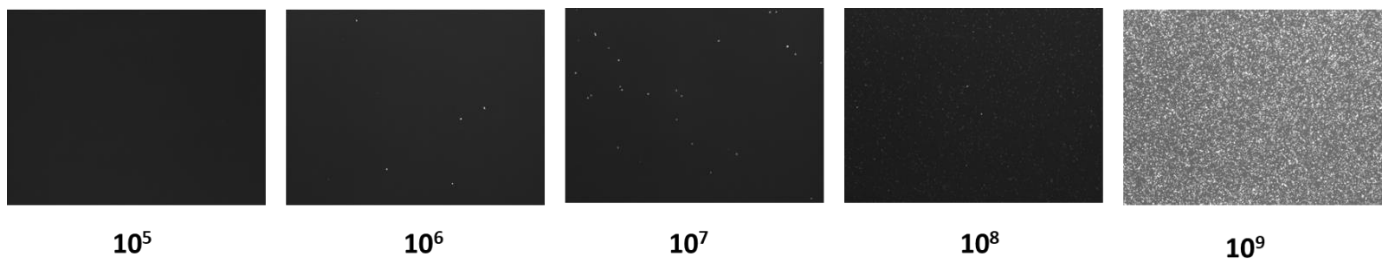
Figure 2.3.3: Decimal Dilutions of *L. plantarum* Viewed with Nexcelom Cellometer X2

between 5 – 9 log(CFU/mL)

Lactobacillus samples were shown to be highly linear with a R^2 of 0.9993 between concentrations 5 – 8 log(CFU/mL) indicating Cellometer X2 ability to precisely establish cell concentrations between this range which was then confirmed by traditional plating methods.
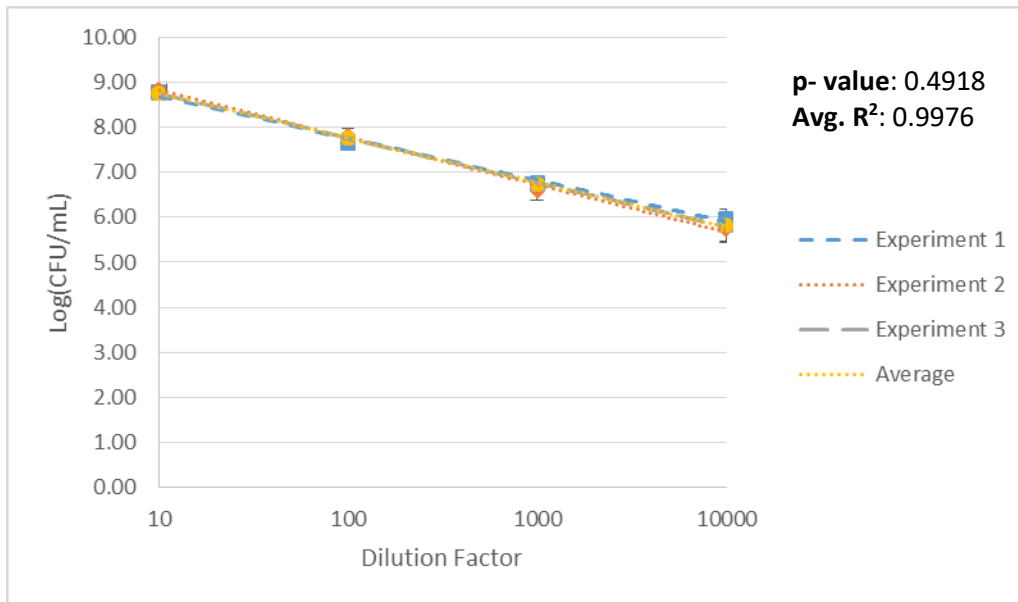
Figure 2.3.4: *L. plantarum* log(CFU/mL) Monoculture at Various Decimal Dilutions Evaluated

with Nexcelom Cellometer X2

Figure legend: Each trend line corresponds to a monoculture experiment. The average is the

mean of all three experiments at each data point. There was no significant difference (p>0.05)

when an ANOVA was conducting comparing MRS plated and Cellometer X2.

Before ANOVA and statistical analysis, a boxplot was created to filter any outlying data

points by IRQ assessments. Concentrations between 5 – 8 log(CFU/mL) were evaluated.

Samples diluted further than 5 log(CFU/mL) were not viable, as they resulted in a large standard

deviation. Inprecise counts were documented at those dilutions. Cell suspensions of 9

log(CFU/mL) were too concentrated to evaluate. Overlapping cells and movement prevented

precise counts. Slides showed fluorescent washout, which made counting inconsistent using

system parameters.

The cell concentrations were converted to log(CFU/mL and the average Cellometer X2 concentration was 9.70 ± 0.05 log(CFU/mL) and plated was 9.74 ± 0.02 log(CFU/mL). The mean difference between the methods was -0.04 log(CFU/mL). An ANOVA was used compare APDA plated and Cellometer X2 resulting in a non-statistically significant p-value of 0.4918.

Table 2.3.2: ANOVA Comparing Methods, Cellometer X2 & De Man, Rogosa and Sharpe Plated, for *L. plantarum* Monoculture

Organism	*L. Plantarum*	Experiment	Monoculture Verification	
Trial	Plating Log(CFU/mL)	Cellometer X2 Log(CFU/mL)	Difference	P-Value
1	9.65 ± 0.04	9.71 ± 0.25	-0.06	
2	9.65 ± 0.06	9.74 ± 0.26	-0.09	0.4918
3	9.80 ± 0.04	9.77 ± 0.19	0.03	

2.4   Discussion

To begin, monoculture verification was conducted, with the goal of confirming that the Cellometer X2 could evaluate monoculture concentration precisely and to ensure that the Cellometer X2 method was not significantly different than the accepted traditional plating method. The first test was a yeast enumeration method, which evaluated *S. cerevisiae* and the second test was a lactic acid bacteria method that evaluated *L. plantarum*. As seen in Table 2.2.1 & 2.2.2 the distribution between the Cellometer and plating were similar. The average difference between plating and Cellometer X2 was -0.04 for *L. plantarum* and -0.03 for *S. cerevisiae*. This difference may be of negligible for commercial brewing that relies on cell concentration of 6 log(CFU/mL) and larger for pitching rates.

Regression analysis was also performed for yeast and lactic acid bacteria for each trial. Yeast had a strong R^2 value of 0.9976 between 5 and 7 log(CFU/mL) concentrations as seen in

Figure 2.3.3. The strong R^2 value indicates the Cellometer X2's ability to consistently enumerate yeast monocultures in an acceptable concentration range. Concentrations that brewers use to pitch are well within the ranges demonstrated by the Cellometer X2 monoculture methods. Brewers would not need to count samples further diluted than 5 log(CFU/mL) making the Cellometer X2's ability to count at those ranges irrelevant.

As the sample became more dilute, less than 5 log(CFU/mL), noise poisoning reduced the R^2 value. Noise poisoning is a description of instrument detection limits and occurs when the sample is too dilute and the instrument is no longer able to evaluate the sample with precision. This phenomena is partially attributed to image cytometry's small sample volume size of 5 μL on a slide. If a sample is not probably vortexed or distributed then the sample may not be representative of the original mixture. Noise poisoning is similar to having too few colonies on a plate for a traditional plating method such as pour or spread plating. Too few colonies on a plate is not representative of a sample and indicates low confidence in the counts. For *L. plantarum* samples regression was analyzed between 5 and 8 log(CFU/mL) and the R^2 value was 0.9993. For monoculture samples, the Cellometer X2 showed strong R^2 values indicating capability to determine concentration for lactic acid bacteria within this range.

An ANOVA comparing the log(CFU/mL) of traditional plating methods and Cellometer X2 resulted in a p-value of 0.8474 and 0.4918 for yeast and LAB respectively. A p-value of below 0.05 was considered significantly different. The p-value for both methods confirmed that there was no significant difference between the traditional plating method and cellometer method for either the yeast or LAB method. This suggest that the imaged-based method is useful for most brewing applications with the expectation of non-statistically different results from plating, assuming that the cell concentrations fall between the validated ranges.

Previous research creating image-based method for monocultures of *S. cerevisiae* and *L. plantarum* were verified in through this research, A p-value of > 0.05 was confirmed for each monoculture method when comparing Cellometer X2 with traditional plating method for both yeast and *L. plantarum* as was reported in previous research (Saldi et al., 2014 & Hodgkins, 2019). Furthermore the difference between the plating method and Cellometer X2 was less than 0.5 log(CFU/ml) for each microorganism. This was confirmed throughout the each experiment and was reported in previous research for both microorganisms.

The Cellometer X2 method required no more than 15 minutes for sample collection, preparation, and evaluation. Traditional plating methods can take up to 48 hours for lactic acid bacteria and 72 hours for yeast to incubate and does not include sample preparation time for plating methods. The time difference in evaluation can result in a major time and financial impact on brewers or other fermented beverage producers. The Cellometer X2 allows brewers to be proactive in addressing a number of quality issues during primary beer fermentation, when using monocultures. Brewers can now adjust for over and under pitching and spend less time enumerating their starter culture before pitching. Both cellometer monoculture methods, show strong correlation with traditional plating methods for enumeration and will potentially reduce time, effort, and expense for commercial brewers.

2.5   Conclusion

The goal of verifying the monoculture method for yeast and lactic acid bacteria was achieved. Each method showed similar results to previous image-based Cellometer X2 papers for each microorganism. Both image-based methods (yeast and LAB) showed a strong regression suggesting precise enumeration of monocultures. In addition, there was statistical difference between the log(CFU/mL) between the Cellometer X2 and traditional plating methods when an

ANOVA was conducted. The validation of this method supports previous studies confirming the Cellometer X2 ability to precisely count *Lactobacillus* and yeast monocultures. The Cellometer X2 can be used by brewers to proactively adjust pitch rates and monitor fermentations. Further studies are encouraged to expand Cellometer X2 enumeration use for microorganisms such as acetic acid bacteria.

CHAPTER 3 MIXED CULTURE VALIDATION

3.1   Introduction

Mixed culture method validation was completed by evaluating a mixed culture containing yeast and lactic acid bacteria. *S. cerevisiae* was chosen for yeast and *L. plantarum* was chosen for lactic acid bacteria. Both microorganisms are commonly included in mixed culture fermentations for sour beer. The validation was divided into three parts. The first was range validation which evaluated constant concentration of yeast while varying lactic acid bacteria concentration. The second range validation evaluated constant concentration of lactic acid bacteria while varying yeast concentration. The final validation was to test the ratio by varying both yeast and lactic acid bacteria concentration. The goal of the mixed culture method validation was to access the capability of the Cellometer X2 to precisely determine yeast and lactic acid bacteria concentration simultaneously before testing the enumeration limits for mixed cultures in a complex food matrix, such as beer.

3.2   Method

3.2.1   Yeast Culture Preparation

S. cerevisiae (Safale-04) was purchased from Fermentis. *S. cerevisiae* (Safale-04) was prepared according to <u>Section 2.2.1</u>.

3.2.2   Lactic Acid Bacteria Culture Preparation

L. Plantarum 8014 (ATCC, Manassas, VA) was provided by Dr. Perry's Lab from the University of Maine. *L. plantarum* was prepared according to <u>Section 2.2.2</u>.

3.2.3 Mixed Culture Stain Preparation

A mixed culture dye was used to stain lactic acid bacteria and yeast simultaneously. Syto BC and Acridine Orange were combined 2:1 (Syto BC: Acridine Orange). Syto BC was prepared following Section 2.2.4. Acridine orange was provided by Nexcelom Biosciences. Syto BC working solution was prepared by diluting stock Syto BC 1:100 in DI water. Syto BC is then combine with Acridine orange in a microcentrifuge tube in a 2:1 ratio and vortexed. A stock solution of mixed culture dye solution was kept at ambient temperature for the duration of the experimental trial. Mixed culture dye was freshly prepared for each experimental trial

3.2.4   Cellometer X2

The Cellometer X2 utilizes one bright field and two fluorescent channels (F1 & F2) for evaluating mixed culture samples. F1 and F2 fields both use the VC-535-402 Channel. F1 is utilized to evaluate yeast while F2 is utilized for lactic acid bacteria. The mixed culture method uses size exclusion to discriminate between yeast and bacteria.

 The criteria for F1 channels was: Cell Diameter (6-50.0 microns), Roundness (0.1), Contrast (0.40), Decluster Edge Factor (0.5), Decluster Th factor (1.0), Background Adjustment (1.0); Fluorescent Criteria: Cell diameter (6.0-50.0 micron), Roundness (0.1), Fluorescent Threshold (15.0), Decluster Th Factor (0.9). Yeast are larger than lactobacillus spp. and are generally larger than 5 microns. LAB in the previous experiment were approximately 3 microns during the monoculture experiments. Yeast criteria was set to 6 microns as anything below 6 microns was considered LAB regardless of its orientation in the slide. A large decluster factor was used to help discriminate clumped yeast cells. In addition yeast had a larger fluorescent threshold as they usually appeared to emit more fluorescence than LAB. Finally roundness did

not appear to affect the automated cell count capabilities of the Cellometer X2 so the parameter was kept the same as with monoculture evaluations.

The Criteria for F2 channel was: Cell Diameter (0.5- 4.9 microns), Roundness (0.1), Contrast (0.2), Decluster Edge Factor (1.0), Decluster Th factor (1.0), Background Adjustment (0.2); Fluorescent Criteria: Cell diameter (1-4.9 micron), Roundness (0.1), Fluorescent Threshold (10.0), Decluster Th Factor (0.9). LAB have a smaller cell diameter than yeast and they were approximately 3 microns in the monoculture experiments. Additionally their fluorescent threshold was lowered as they appeared dimmer than their yeast counterparts. A large Decluster value was used as well to help separate any LAB from yeast colonies. Roundness did not appear to have an appreciable effect on counting so the value remained the same with the monoculture evaluations.

Yeast and lactic acid bacteria were combined in appropriate ratios and vortexed. The mixed cultures were then dyed 1:1 with the mixed culture stain. Dyed mixed cultures were prepared in triplicate. Once inserted into the Cellometer X2, the slide was evaluated under the bright field for correct morphologies and contamination. Once the slide was reviewed and focused, the cellometer took images of four different areas in the counting chamber using BF, F1, and F2. Four replicates were measured from each dyed sample.

3.2.5   Statistical Analysis

For mixed cultures, colony forming units per mL (CFU/mL) and cell counts were taken for both methods, plating and Cellometer X2. CFU/mL was converted to a log scale (base 10) for Cellometer X2 and plating calculations. The log values were compared to their counterpart using

ANOVA in JMP Pro 15.2.0 (466311). A p-value of less than 0.5 was considered not statistically significant.

3.3 Result

3.3.1   Range Validation 1 (Constant Yeast, Varying Lactic Acid Concentration)

Range verification 1 consisted of keeping *S. cerevisiae* concentration constant while varying *L. plantarum* concentrations. *S. cerevisiae* counts were kept constant at a 7 log(CFU/mL). As expected no visible change in concentration for yeast was observed while a reduction in LAB as dilution increased was noted (Figure 3.3.1).

$10^7{:}10^5$ $10^7{:}10^6$ $10^7{:}10^7$ $10^7{:}10^8$ $10^7{:}10^8$
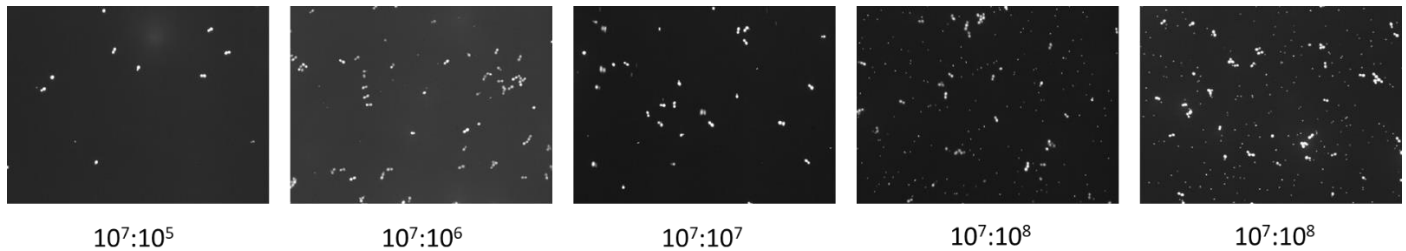
Figure 3.3.1: Decimal Dilutions of varying *L. plantarum* and constant *S. cerevisiae* Viewed with

Nexcelom Cellometer X2

Figure Legend: Each concentration (CFU/mL) is below the image. The format for concentration is Yeast: LAB. From left to right the dilution was 0.001, 0.01, 0.1, and 1:5, 1 for *L. Plantarum*

. Regression was evaluated and the resulting R^2 value confirmed consistent concentrations. The average R^2 value was 0.5927 which as expected, indicted little change in concentration of yeast.
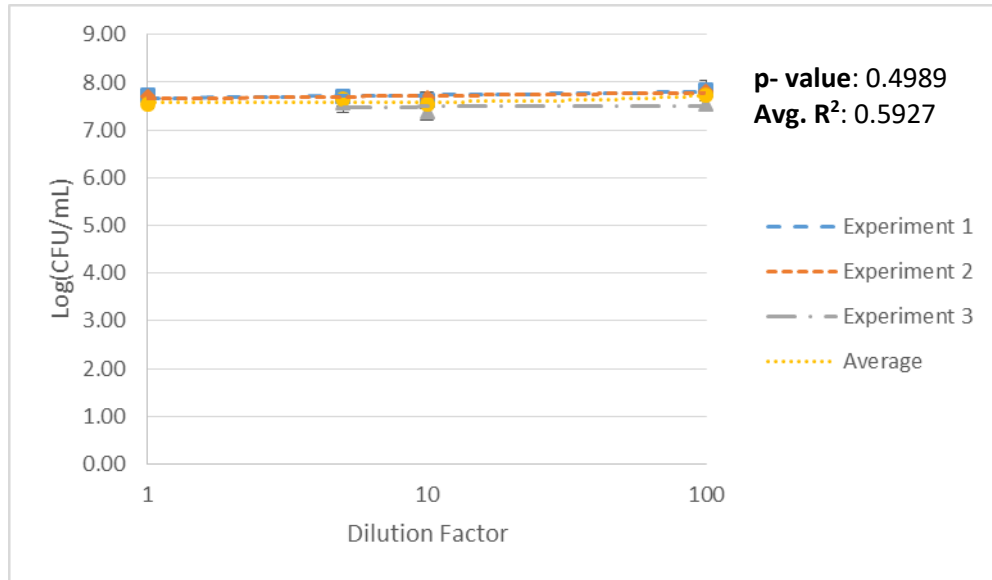
Figure 3.3.2: *S. cerevisiae* log(CFU/mL) at Various Decimal Dilution in a Mixed Culture Evaluated with Nexcelom Cellometer X2 (Constant Yeast Concentration and Varying Lactic Acid Bacteria Concentrations)

Figure Legend: Constant yeast (approximately 7 log(CFU/mL) concentrations were used while diluted LAB sample were prepared at 1, 1:5, 0.1, 0.01 dilutions. When an ANOVA was conducted between APDA plated and Cellometer X2, no statistical significance (p>0.05) was found.

Before an ANOVA was run, a boxplot was generated to exclude outliers. No outliers were noted for samples between 1:1 and 1:(0.01) (Yeast:Lab) for either method.

The average for traditional plating (APDA) of *S. cerevisiae* was 7.58 ± 0.07 log(CFU/mL) and for the Cellometer X2 was 7.61 ± 0.09 log(CFU/mL). The difference between the method concentrations was -0.03 log(CFU/mL. An ANOVA was used to compare the two methods, resulting in a p-value of 0.4989. The p-value suggests there is no significant difference

between the methods. Table 3.3.1 displays the comparison and difference for each of the experiments.

Table 3.3.1: ANOVA Comparing log(CFU/mL) of methods, APDA plated & Cellometer X2, for *S. cerevisiae* in a Mixed Culture (Constant Yeast Concentration, Varying LAB Concentration)

Organism	*S. Cerevisiae*	Experiment	Range Validation 1	
Trial	Plating Log(CFU/mL)	Cellometer X2 Log(CFU/mL)	Difference	P-Value
1	7.66 ± 0.07	7.73 ± 0.08	-0.07	
2	7.57 ± 0.13	7.71 ± 0.07	-0.14	0.4989
3	7.50 ± 0.05	7.49 ± 0.13	0.07	

L. plantarum was evaluated by varying concentrations between 5 to 8 log(CFU/mL). Representative images of concentrations are shown in Figure 3.3.1. Minimal cells can be seen at the 5 log(CFU/mL). Some washout of fluoresce was observed throughout the samples, contributing to diminished sensitivity of the instrument. This phenomenon was observed in most yeast samples, particularly when yeast exhibited clustering.

When evaluating the regression between concentrations 6 to 8 log(CFU/mL), the R^2 value was 0.9927, as shown in Figure 3.3.3. This suggests precise cellometer counting when keeping yeast concentration constant and varying LAB concentration between these dilutions (1X – 0.01X dilutions or 6-8 log(CFU/mL).
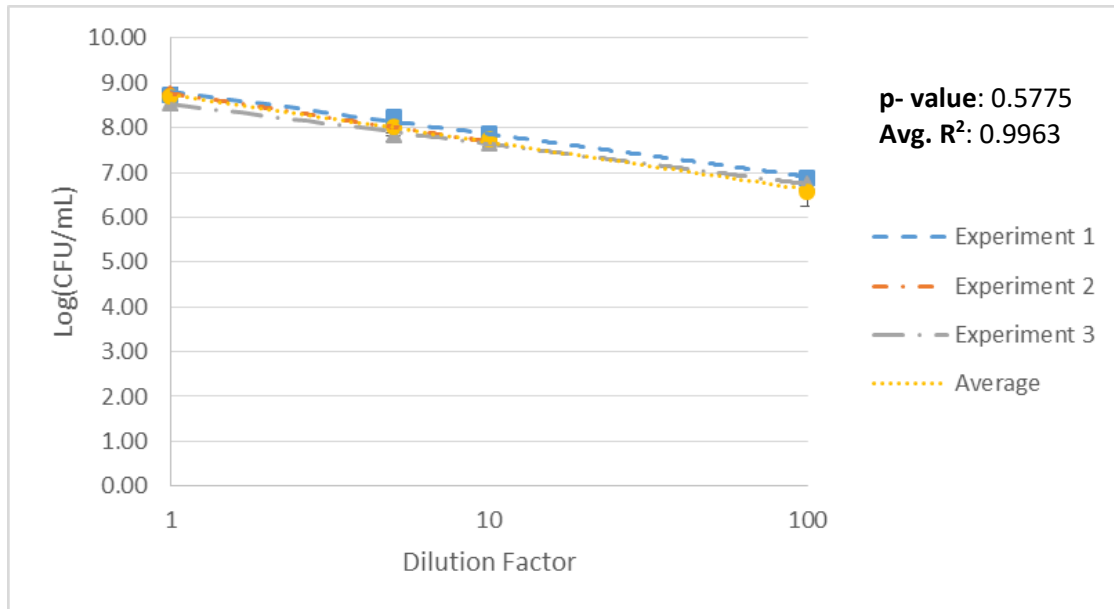
Figure 3.3.3*: L. plantarum* log(CFU/mL) at Various Decimal Dilution in a Mixed Culture

Evaluated with Nexcelom Cellometer X2 (Constant Yeast Concentrations and Varying

Lactic Acid Bacteria Concentrations)

Figure legend: Concentrations between 6 and 8 log(CFU/mL) were evaluated for LAB while

keeping yeast concentrations constant. When an ANOVA was conducted no statistical

significance (p>0.05) was observed.

Before an ANOVA was run, a boxplot was generated. Any outliers discovered were excluded.

Concentrations 6 to 8 log(CFU/mL) were evaluated. Other concentrations were excluded.

An ANOVA was used to compare the starting log(CFU/mL) between the Cellometer X2

and the traditional plating method. Table 3.3.2 shows the average log(CFU/mL) and difference

for both methods. The average concentration for the plating method was 9.79 ± 0.08

log(CFU/mL) and for Cellometer X2 was 9.73 ± 0.09 log(CFU/mL). The difference between the

methods was 0.06 log(CFU/mL).

Table 3.3.2: ANOVA comparing log(CFU/mL) of methods, MRS plated and Cellometer X2, for

L. plantarum in a Mixed Culture (Constant Yeast Concentration, Varying LAB Concentration)

Organism	*L. Plantarum*	Experiment	Range Validaiton 1	
Trial	Plating Log(CFU/mL)	Cellometer X2 Log(CFU/mL)	Difference	P-Value
1	9.68 ± 0.04	9.85 ± 0.07	-0.17	
2	9.89 ± 0.07	9.72 ± 0.04	0.32	0.5775
3	9.79 ± 0.07	9.63 ± 0.08	0.16	

3.3.2   Range Validation 2 (Varying Yeast, Constant Lactic Acid Concentration)

For Range Validation 2, varying lactic acid and constant yeast concentrations were evaluated. *S. cerevisiae* was chosen for yeast samples and *L. plantarum* for the lactic acid samples. *L. plantarum* was held approximately at 8 log(CFU/mL) for this trial. *S. Cerevisiae* varied from 4 to 7 log(CFU/mL).

S. cerevisiae was evaluated between 4 and 7 log(CFU/mL). When 4 log(CFU/mL) was evaluated no cells were visible, or when cells were visible an inflated concentration was noted. For those reasons, 4 log(CFU/mL) was excluded from statistical analysis. Images shown in Figure 3.3.4 display a constant LAB concentration and varying yeast concentration. Some fluorescent washout was observed, but individual LAB colonies could be discriminated during automated counting.

$10^8:10^4$ $10^8:10^5$ $10^8:10^6$ $10^8:10^7$ $10^8:10^7$
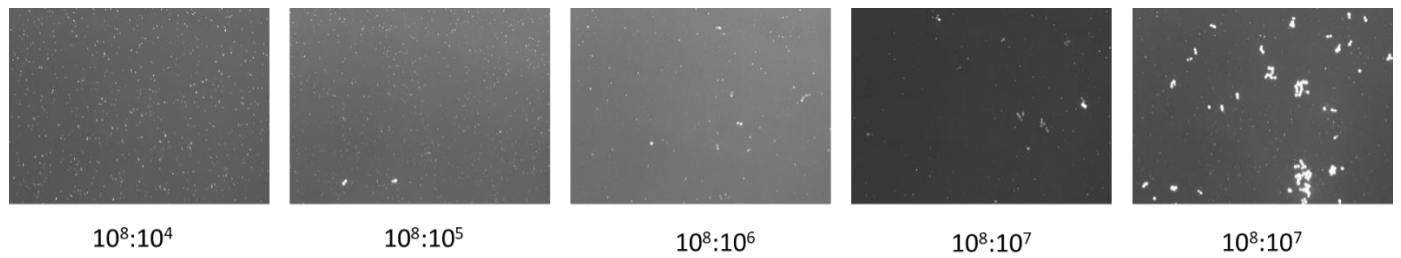
Figure 3.3.4: Decimal Dilutions of constant *L. plantarum* and varying *S. cerevisiae* Viewed with

Nexcelom Cellometer X2

Figure legend: The format for concentration (CFU/mL) of the samples is LAB: Yeast. From left

to right the dilution was 0.001, 0.01, 0.1, 1:5, and 1.

When the regression for *S. cerevisiae* was evaluated between 5 to 7 log(CFU/mL), an R^2

value of 0.9969 (Figure 3.3.5). This strong R^2 value indicated a strong ability to count yeast and

determine concentration between these accepted ranges.

Figure 3.3.5: *S. cerevisiae* log(CFU/mL) at Various Decimal Dilutions Evaluated with

Nexcelom Cellometer X2 (Varying Yeast Concentration, Constant LAB Concentration)

Figure legend: Concentrations between 5 and 7 log(CFU/mL) were evaluated for yeast while

keeping LAB concentration constant. When an ANOVA was conducted no statistical

significance ($p>0.05$) was observed.

Before an ANOVA was run, a boxplot was generated to exclude outliers. Concentrations

4 log(CFU/mL) and lower and pure cultures were excluded. No outliers were noted in these

trials.

For *S. cerevisiae* the average concentration for the Cellometer X2 was 7.67 ± 0.14

log(CFU/mL) and for plated was 7.58 ± 0.08 log(CFU/mL) as listed Table 3.3.3. The difference

between the log(CFU/mL) was -0.09 log(CFU/mL). An ANOVA was used to compare the

plating and Cellometer for each experimental trial and the resulting p-value was 0.4625,

indicating no significant difference between the techniques.

Table 3.3.3: ANOVA Comparing log(CFU/mL) of the methods, APDA Plated &

Cellometer X2, for *S. cerevisiae* in a Mixed Culture (Constant LAB Concentration, Varying Yeast

Concentration)

Organism	*S. Cerevisiae*	Experiment	Range Validation 2	
Trial	Plating Log(CFU/mL)	Cellometer X2 Log(CFU/mL)	Difference	P-Value
1	7.66 ± 0.07	7.87 ± 0.05	-0.11	
2	7.62 ± 0.11	7.62 ± 0.14	0.00	0.4625
3	7.47 ± 0.05	7.54 ± 0.06	-0.10	

For Range Validation 2, concentrations of *L. Plantarum* was kept constant at

approximately of 8 log(CFU/mL). As seen in Figure 3.3.5 the visible number of LAB remained

consistent while the yeast number decline as dilutions increase. Some fluorescent washout was

observed but LAB concentrations remained countable throughout the trials.

Regression analysis was completed for each replicate of consistent *L. plantarum* as

shown in Figure 3.3.6. The average R^2 value was 0.5621. The low R^2 indicated negligible

differences in the concentration when varying *S. cerevisiae* levels.
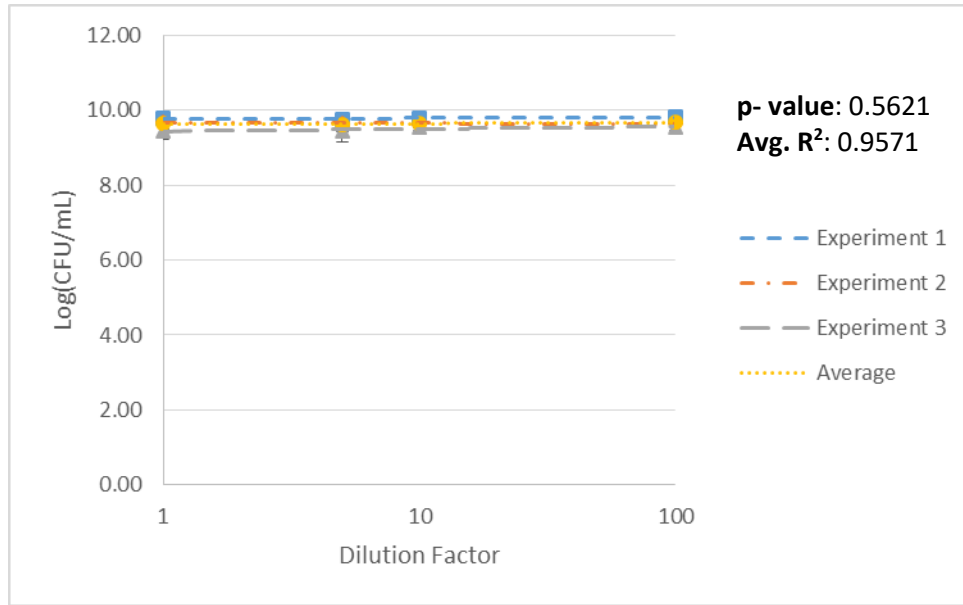
Figure 3.3.6: *L. plantarum* log(CFU/mL) at Various Decimal Dilutions in a Mixed Culture

(Varying Yeast Concentration, Constant LAB Concentration)

Figure legend: Approximately 8 log(CFU/mL) were evaluated for LAB while varying yeast

concentrations and keeping LAB concentration constant. When an ANOVA was conducted no

statistical significance ($p > 0.05$) was observed.

Before an ANOVA was run a boxplot was generated to identify for and exclude outliers.

No outliers were found.

The average MRS plated concentration was 9.65 ± 0.12 log(CFU/mL) and for Cellometer

X2 was 9.64 ± 0.02 log(CFU/mL). The difference between the methods was 0.01 log(CFU/mL).

Differences and p-value are noted in Table 3.3.4.

Table 3.3.4: ANOVA comparing log(CFU/mL of Methods, MRS Plated & Cellometer X2,

for *L. plantarum* in a Mixed Culture (Constant LAB Concentration, Varying Yeast

Concentration)

Organism	*L. Plantarum*	Experiment	Range Validation 2	
Trial	Plating Log(CFU/ml)	Cellometer X2 Log(CFU/ml)	Difference	P-Value
1	9.69 ± 0.03	9.78 ± 0.03	-0.09	
2	9.49 ± 0.11	9.65 ± 0.03	-0.16	0.9571
3	9.76 ± 0.07	9.49 ± 0.05	0.26	

3.3.3   Ratio Validation (Varying Yeast, Varying Lactic Acid Concentration)

Ratio validation experiments used varying concentrations of yeast and lactic acid

bacteria. Various percentages of yeast were added to complementary percentages of lactic acid

bacteria. Visually, both microorganisms fit the expected trends in Figure 3.3.7. Some fluorescent

washout was experienced, but automated counts were not negatively impacted during the

experimental trials.
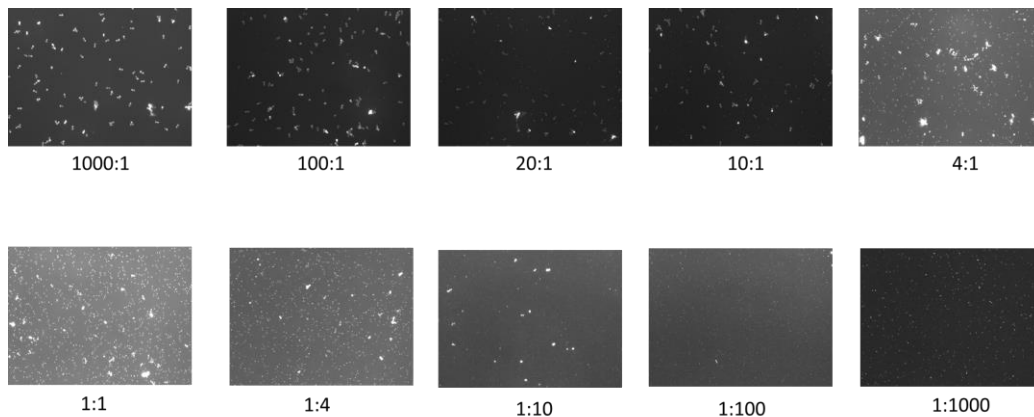
Figure 3.3.7: Decimal Dilutions of *S. cerevisiae* and *L. plantarum* Viewed with the

Nexcelom Cellometer X2 (Varying Yeast & Varying LAB Concentrations)

A regression analysis was conducted and the mean R^2 value was 0.9997 for *S. cerevisiae* and 0.9989 for *L. plantarum*. R^2 is shown in Figure 3.3.8 and 3.3.9 for yeast and lactic acid bacteria, respectively.
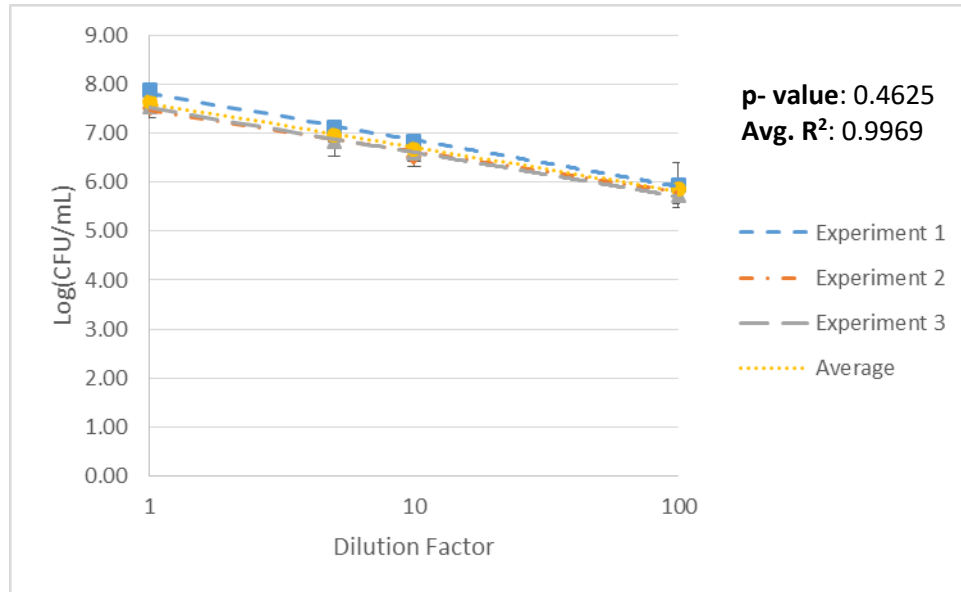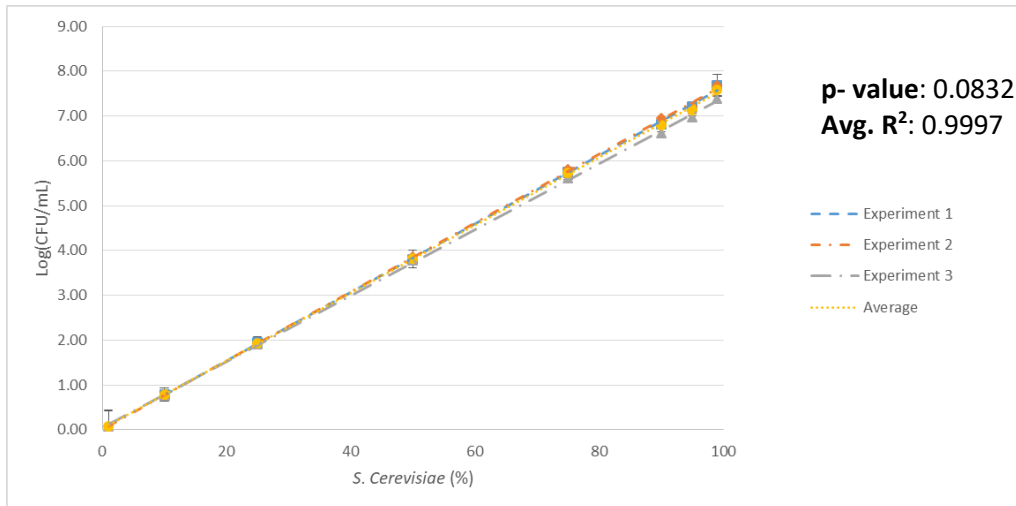

Figure 3.3.8: *S. cerevisiae* log(CFU/mL) at Various Decimal Dilutions Evaluated with Nexcelom Cellometer X2 (Varied Yeast and LAB Concentrations)
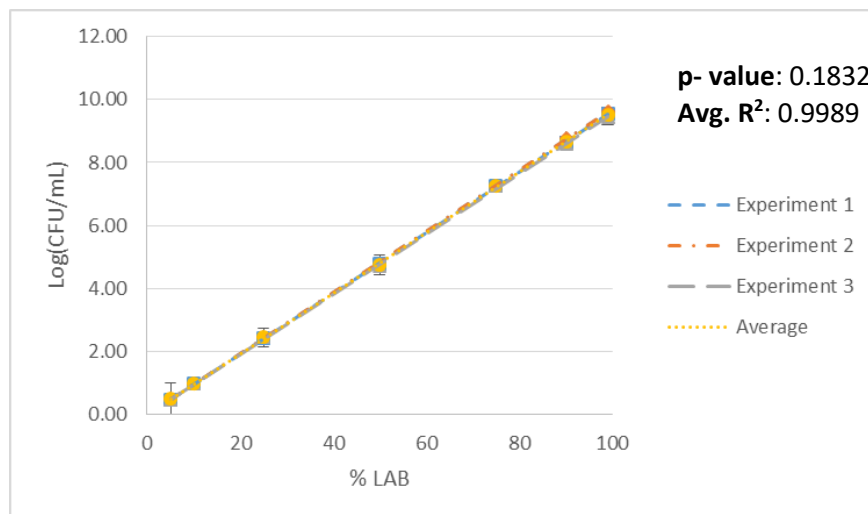
Figure 3.3.9: *L. plantarum* log(CFU/mL) at Various Decimal Dilutions Evaluated with Nexcelom Cellometer X2 (Varied Yeast and LAB Concentrations)

Before an ANOVA was performed, a boxplot for each microorganism was generated in order to exclude outliers. In addition to outliers, pure cultures and 1000:1, 1:1000 (Yeast: LAB) were excluded in the statistical analysis.

An ANOVA was conducted comparing the traditional plating method to the cellometer. *S. cerevisiae* had a p-value of 0.0832 and *L. plantarum* had a p-value of 0.1832. Both p-values were greater than 0.05 indicating no significant difference between the methods for either microorganism.

Table 3.3.5: ANOVA Comparing Methods, APDA plated & Cellometer X2, for *S. cerevisiae* in a Mixed Culture (Varying Yeast and LAB Concentrations)

Organism	*S. Cerevisiae*	Experiment	Ratio Validation	
Trial	Plating Log(CFU/mL)	Cellometer X2 Log(CFU/mL)	Difference	P-Value
1	7.17 ± 0.06	7.67 ± 0.10	-0.50	
2	7.33 ± 0.12	7.74 ± 0.08	-0.41	0.0832
3	7.54 ± 0.15	7.51 ± 0.11	0.03	

Table 3.3.6: ANOVA Comparing Methods, APDA plated & Cellometer X2, for *L. plantarum* in a Mixed Culture (Varying Yeast and LAB Concentrations)

Organism	*L. Plantarum*	Experiment	Ratio Validation	
Trial	Plating Log(CFU/mL)	Cellometer X2 Log(CFU/mL)	Difference	P-Value
1	9.32 ± 0.06	9.68 ± 0.07	-0.36	
2	9.60 ± 0.12	9.76 ± 0.14	-0.16	0.1832
3	9.68 ± 0.08	9.65 ± 0.14	0.03	

The average log(CFU/mL) for *S. cerevisiae* was 7.35 ± 0.15 and for the Cellometer X2 was 7.51 ± 0.01. This led to a difference of -0.16 between the methods. *For L. plantarum* the average log(CFU/mL) for plated was 9.54 ± 0.15 and the Cellometer X2 was 9.65 ± 0.05 resulting in a difference -0.09 between the methods.

3.4 Discussion

The goal of this study was to confirm that cell enumeration of yeast and LAB mixed cultures in a liquid medium can be precisely determined using the Nexcelom Cellometer X2. Three experimental trials were conducted. The first and second objectives were designed to show reliable enumeration could be maintained, keeping yeast or bacteria concentration constant, while varying one or the other in a mixed culture. The third objective was to show reliable enumeration using different ratios of yeast vs. bacteria.

The creation of a novel mixed culture method is important as it provides a new alternative to time consuming, traditional plating methods. The use of image-based cytometers enables the rapid enumeration in mixed cultures in minutes, rather than 1-3 days depending on the microorganisms. In addition, image-based cytometry is potentially less expensive alternative to plating when materials and costs are considered.

For each of the experimental trials regression was analyzed to determine cell count accuracy. Regression was strong for each of the Range Validation experimental trials. When varying yeast between the concentrations of 5 log(CFU/mL) and 7 log(CFU/mL) the mean R^2 value was 0.9969. While varying lactic acid bacteria between 6 log(CFU/mL) and 8 log(CFU/mL) the R^2 value was 0.9927. Each microorganisms was precisely counted using the mixed culture method, while keeping the other microorganism levels constant. Both yeast and LAB resulted in lower R^2 values of below 0.6000 indicating a constant concentration. For the Ratio experimental trials the R^2 value was 0.9997 for *S. cerevisiae* and 0.9999 for *L. plantarum*. The yeast countable in a mixed culture containing 1% yeast while LAB concentration was able to be determined in a mixture containing 5% LAB. The ratio of yeast to bacteria are far below the ratios brewers' would use to pitch a sour beer. Recipes may vary but some general pitching ratios for bacteria:yeast cells range from 10:1 to 2:1 in a kettle sour. Spontaneous fermentations can have concentrations of much less bacteria and yeast. One study enumerated only 4 log(CFU/mL) bacteria and between 3 to 4 log(CFU/ml) in a spontaneous fermentation giving closer to a 1:1 ratio of bacteria to yeast (Spitaels et al., 2014) The Cellometer X2 was capable of enumeration at a 1:1 ratio for each of the range and ratio validation. If a brewers starter cultures are not concentrated enough for the image-based mixed culture method then the starter culture can be centrifuged and suspended until an appropriate concentration is reached. More likely, a common problem among brewers is over pitching or having a starter that is too concentrated. If the starter is too concentrated then the sample can be diluted until an appropriate concentration is reached.

An ANOVA was conducted between the mean log(CFU/mL) for each experiment and the mean log(CFU/mL) for the plating method. Concentrations were converted to starting

concentrations then to log(CFU/mL) for the plating confirmation method. A p-value of ≤ 0.05 was considered significant. For each experimental trial, the p-value was > 0.05, indicating that there is no significant difference between the Cellometer X2 and plating methods for the mixed culture.

The Cellometer X2 counting method was validated for mixed cultures containing *S. cerevisiae* concentrations between 5 to 7 log(CFU/mL) and *L. plantarum* concentrations between 6 to 8 log(CFU/mL). Concentrations below each of these threshold were affected by noise poisoning and could not be precisely counted. Conversely, concentrations above each listed thresholds resulted in the wash out phenomenon, caused by an overabundance of fluorescence, yielding depressed cell counts. To deal with these constraints, dilutions outside these ranges were excluded. Between each accepted ranges the Cellometer X2 precisely determined concentration for yeast and lactic acid bacteria. Finally, although pure cultures were evaluated, they were excluded from ANOVA and regression analysis. The Cellometer X2's program cannot account for dilution factors of zero causing inflated monoculture concentration. Furthermore, a concentration was recorded for observed pure culture of both microorganism despite the bright field verifying purity. This could be due to cell diameter threshold being too strict, as smaller or larger cells close to the threshold could be misidentified. As monoculture methods exist and are validated for the Cellometer X2, they would be better utilized for more precise counts then this novel mixed culture.

In the brewing industry, it is common to pitch lactic acid bacteria at concentrations between 6 to 8 log(CFU/mL) when making sour beer (wyeast, 2022). Yeast pitch rates can vary, but yeast is commonly pitched at about 6 to 7 log(CFU/mL) (wyeast, 2022). As each beer fermentation is unique, pitch rates may vary with product. The mixed culture method validated in

this study provided precise cell counts of the mixed culture in a range useful for the brewing industry.

3.5   Conclusion

The novel mixed culture method created was not significantly different from traditional plating methods. The Range Validation 1 + 2 and Ratio experiments had a p-value greater than 0.05 for yeast concentrations ranging between 5 to 7 log(CFU/mL) and for lactic acid bacteria was 6 to 8 log(CFU/mL) . Range and ratio experimental trials resulted in strong regression (>0.99), suggesting the Cellometer X2 can determine concentrations quickly and precisely.

CHAPTER 4 MIXED CULTURE BREWING APPLICATION

4.1   Introduction

Chapter 3 describes the development and validation of a novel mixed culture method using the Nexcelom image-based cytometer, Cellometer X2, for analysis of microbial mixed cultures. This chapter details the validation of the technique using a beer matrix. A mixed culture of lactic acid bacteria and yeast is typically used for niche brewing applications. The described method was developed for use with fermented beverages including sour beer as the mixed cultures in these products are not generally well enumerated over the course of fermentation

. Knowledge of microbial concentration is especially important, as during the pitching process, a low pitch rate can result in a "stalled fermentation". A stalled fermentation occurs when the yeast become dormant state before fermentation is completed causing quality issue such as oxidation, off flavors, and spoilage. Conversely, over-pitching can result in low oxygen and improper metabolism, leading to a buildup of esters, biogenic amines, and other quality affecting metabolites (Found, 2016). Peer-reviewed data for evaluating pitch rates and sour beer quality is largely unavailable, as most of the brewing industry considers this information proprietary. In lieu of peer-reviewed data, niche brewing enthusiasts or smaller craft brewers must often speculate or estimated pitch rates of yeast or lactic acid starter concentrations. There are general rules for pitching yeast and lactic acid bacteria. Concentrations for yeast are commonly range from 5-8 log(CFU/mL) depending on the beer style. Lactic acid bacteria pitch rates can range from 6-8 log(CFU/mL) depending on the style (Wyeast, 2022). For newer breweries, a simple image-based method for mixed culture evaluation could prove indispensable, as the method would aid in the production of more consistent beverage products.

For this study, beer wort media was inoculated with yeast and LAB culture to test the novel mixed culture method described in Chapter 3. Wort is the sugary liquid extracted from the mashing process and is one of the first products of the brewing process. Wort contains a number of carbohydrates including sucrose, fructose, glucose, maltose and maltotriose (Dong, 2014). These simple sugars are more readily fermentable. Comprised largely of maltose, the ratios and concentrations of these sugars make wort a less consist fermentable product than the media used in the monoculture or mixed culture validation studies described in previous chapters. Wort also contains non-fermentable sugars. Depending on how effective or complete the mashing process is at converting sugars, wort may include long chain non fermentable sugars, such as maltdextrins, that may interfere with enumeration (Paulino et al., 2006).

The goal of this work was to validate the use of newly developed mixed culture method for mixed culture (yeast/LAB) in a beer (wort) matrix, mimicking use in the beer brewing process. Such a method will add value to commercial breweries, enabling rapid and percise evaluations of yeast and LAB throughout the brewing process.

The brewing process contains multiple steps to create sour beer that are often modified or even omitted by brewers to create new variations of beverages. The steps used in this research include milling, mashing, lautering, boiling, cooling, and fermenting.

This research brewing process began by receiving German wheat malt and German grain malt. The wheat and grain were purchased pre-milled malts as would be standard for most small brewing applications.

Next, the grain was added to heated water between 140-159° F. This began the mashing process. Mashing converts carbohydrates and starches into sugars that can be fermented.

Naturally occurring enzymes(amylase) in the malt convert long chain carbohydrates into simple sugars in a process called saccharifiaction (Palmer, 2017). Mashing creates the liquid known as wort and for this research, was completed in 60 minutes, as complete conversion of starch to fermentable sugars is accomplished. At the end of mashing the wort was recirculated. This ensures that wort can be properly pumped for lautering and helps to free some of the sugars. The straining of the wort from the used malt, also known as spent grain, is called lautering and is important for transfer of all fermentable sugars to the kettle. The brewing system used kettles with raised filters to remove the spent grain when siphoning the desired wort.

As lautering was completed, the spent grain was rinsed with additional water (175 ° F) to extract any of the residual sugars left in process called sparging.

To begin the kettling step, sweet wort was moved from the mashtun to a kettle to be boiled. Boiling wort functions to sterilize and end enzymatic reactions. Additionally, boiling can volatize off flavors such as di methyl sulfide precursors which are known to impart notes of garlic and tomato in the final product (Oliver, 2011). At this stage hops are usually added to impart bitterness to the wort. Hops were omitted for this research as they produce anti-bacterial metabolites. The kittling step lasted for 60 minutes during the sour beer brewing process for this study.

Once sterilized wort cooling was conducted via a plate chiller. Wort was rapidly cooled to temperatures between 68-79°F. Cooling the wort prepares it for immediate fermentation temperatures. After cooling dissolved oxygen is added back to the wort to aid yeast reproduction and fermentation.

The cooled wort was transferred to gallon fermentation vessels for these trials. In a commercial brewing facility, wood barrels, stainless steel vessels, bottles can be used for fermentation depending on a beer batch size. Brewers may have primary fermentation vessels, secondary, and long-term fermentation vessels. Sour beers such as the Flanders Red may use wooden barrels due to the microorganism natively present in the wood. This experiment did not rely on native microorganisms as for research purposes the fermentation needed to be controlled.

The workflow of the research presented in this chapter is displayed in Figure 4.1.
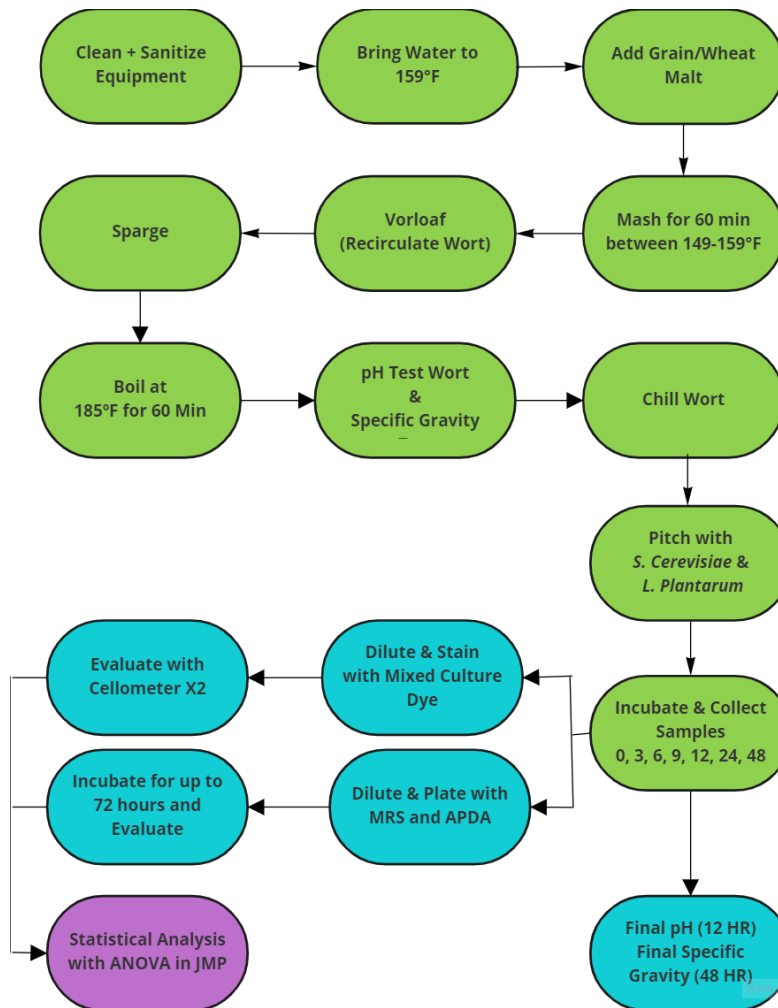
Figure 4.1: Brewing Workflow for Mixed Culture Validation in Beer

4.2 Method

4.2.1 Bacteria Culture Propagation

An isolated colony of *L. plantarum* was transferred to 100 mLs of MRS broth and incubated for 24 hours at 30°C until a concentration of approximately 9 log(CFU/mL) was reached.

4.2.2 Yeast Culture Propagation

A yeast slurry was prepared for each trial. An isolated yeast colony was aseptically transferred to 50 mL of potato dextrose broth and incubated for 24 hours at 30°C. The 24-hour yeast culture was added to 1.040 specific gravity malt dextrose solution. The solution was mixed using a stir bar at room temperature for up to 48 hours or until a concentration of at least 8 log(CFU/mL) was reached.

4.2.3 Cleaning and Sanitation

Kettles and the Sabco Brew-Magic™ (Toledo, OH) were cleaned and sanitized before each trial. Five Star (Arvada, CO) PBW was used for cleaning of debris form the Sabco Brew Magic™ system and equipment. Following cleaning, Five Star Saniclean Low Foam was used according to manufacturer instructions to sanitize the brewing system and equipment.

4.2.4 Wort Preparation

Sabco Brew-Magic™ system (Figure 4.2.1) was used to prepare wort. This system was comprised of three 58.7 L heavy-duty stainless kettles connected by tri-clamp connectors. The system was monitored with thermometers and was temperature controlled with propane gas.

To begin, 6 gallons of wort was prepared to then be divided into three fermentation vessels. Wort was prepared by bringing 3 gallons of water to 140°F. 4.33 lbs. of milled German

pilsner malt, 4.33 lbs. of milled German wheat malt, and 0.66 lbs. rice hulls were weighed and

slowly mashed into three gallons of boiling water. The mash was stirred every twenty minutes

for a total of 60 minutes of the mashing step. At the end of 60 minutes, mash was allowed to

recirculate (Vorloauf) for 10 minutes or until the liquid was clear. For the last 10 minutes during

vorloauf of the mashing step the mixture was brought to 175°F for lautering. The wheat and

grain were then rinsed (sparged) with 4 gallons of water heated to 185°F. The wort was transfer

to a kettle and boiled (kettled) for 60 minutes. Wort is boiled to sanitize the wort and denature

proteins. After boiling, a sample was collected to test the pH and specific gravity. The initial pH

averaged 5.66 and the average initial specific gravity was 1.0336. Wort was chilled using a

Sabco (Toledo, OH) Chill Wizard plate chiller/heat exchanger. 0.75 gallons of chilled wort was

transferred to each of three one-gallon glass fermentation vessels with lids and three-piece

airlocks. Each fermentation vessel as incubated at approximately 72°F (Wyeast, 2022).

Figure 4.2.1: Sabco Brew-Magic^{TM} system

4.2.5   Mixed Culture Fermentations

The prepared wort was inoculated with an estimated 750 million cells/mL/plato. Lactic acid bacteria were initially inoculated at approximately 10 million cells/mL. These concentrations are approximate industry standard for a mixed cultured beer. For this research, simultaneous inoculation was used, with the intention of mimicking a spontaneous fermentation. Spontaneous fermentations have yeast and LAB present and active at the same time during fermentation.

L. plantarum and *S. cerevisiae* were monitored at seven time points, 0, 3, 6, 9, 12, 24 and 48 hours. Lactic acid bacteria typically have a 12-hour period of activity when the pH drops to its lowest level and the bacteria is producing lactic acid. The terminal pH of growth of *L. plantarum* in Berliner Wiese and other sour beers is approximately 3.2 (Tonsmeire & Humbard, 2015). At this pH, lactic acid bacteria growth is slowed and cell replication almost stops as the pH inhibits bacterial replication in wort. Often there are sufficient nutrients for lactic acid bacteria to continue to grow but the self-imposed acidification halts growth (Papadimitriou et al., 2016). As the pH becomes inhibitory for bacteria, yeast then can take advantage of nutrients in wort as yeast are much more tolerant of low pH. The 48-hour time point was chosen as the final time point in this experiment as primary fermentation was completed. Primary fermentation is defined as the time when yeast are the most active and the process can last anywhere from 36 hours to 1 week (Palmer, 2017; Bartlett, 2020). Up to 70% of the alcohol in the finished product is produced during primary fermentation. For an initial wort specific gravity of 1.038, primary fermentation was considered complete when the specific gravity fell below 1.030 (WMA, 2014). It should be noted that brewers need to be aware of style-specific primary fermentation times so

that pitch rates can be adjusted, to avoid under or over-pitching. Under-pitching can result in stalled fermentations and over-pitching can cause unwanted metabolites in the finished product.

4.2.6 Wort Cellometer X2 Evaluation

At each time point, 1.5 mL of sample was removed from the 1-gallon fermentation vessel. The yeast and LAB concentration was determined with Cellometer X2 prior to plating which enabled targeted inoculation of spread plating. The sample was diluted 10:1 in peptone water. Diluted samples were stained 1:1 with the mixed culture dye created in Section 4.2.7. Samples were evaluated in quadruplet (2 slides, 4 chambers) using the Cellometer X2.

4.2.7 Fluorescent Stain Preparation

A mixed culture dye was used to simultaneously stain yeast and LAB. Syto BC and Acridine orange were combined 2:1 (Syto BC: Acridine Orange). Syto BC was prepared following Section 2.2.4. Acridine orange was provided by Nexcelom Biosciences. Syto BC working solution was created by diluting stock Syto BC 1:100 in DI water. Syto BC was then combined with Acridine orange in a microcentrifuge tube in a 2:1 ratio and vortexed. Mixed culture dye solution was kept at ambient temperature for the duration of the experiment. Working solutions of the mixed culture dye was prepare fresh for each trial.

4.2.8 Plate Counting Method

After the yeast and LAB concentration were determined with the Cellometer X2, plates were prepared in triplicate with BD Difco™ acidified potato dextrose agar (APDA) for yeast and BD Difico™ Lactobacilli De Man, Rogosa and Sharpe Agar (MRS) for lactic acid bacteria. Yeast were incubated for 48-72 hours until distinct colonies were grown. LAB were incubated

24-48 hours until distinct colonies were present. 10% tartaric acid was the agent used to select against LAB on APDA plates. *L. plantarum* grows more quickly and outcompetes yeast on MRS plates. Yeast was not visible after 48 hours on the MRS plates.

4.2.9   Statistical Analysis

For brewing trails, colony forming units CFU/mL and cell counts were recorded.. The log values were compared to their counterpart using ANOVA in JMP Pro 15.2.0 (466311). A p-value of less than 0.5 was considered not statistically significant.

Brewing trial data was also plotted over time. The concentration of yeast and LAB for the X2 were plotted with their plated counterparts.

4.3   Results

4.3.1   Mixed Culture Fermentation of Wort Tracked

The validated mixed culture method from Chapter 3 was used to evaluate wort culture inoculated with *L. plantarum* and *S. cerevisiae*. Yeast samples ranged from 5.93-7.38 log and LAB ranged from 8.88 to 8.12 log. Table 4.3.1 shows the change in concentration at each time point (0, 3, 6, 9, 12, 24, and 48) for each trial.

Table 4.3.1: Average Population [Log(CFU/mL)] of *S. cerevisiae* and *L. plantarum* for over 48-Hours According to Plate Counts and Image based Cytometry

Media Type		Avg. Starting Gravity (0 Hours)		1.0364	Avg. Starting pH (0 Hours)		5.66						
Berliner Wiesse		Avg. Ending Gravity (48 Hours)		1.0214	Avg. Ending pH (12 Hours)		3.37						
Grain/Wheat Wort													
S. Cerevisiae													
Hour	0		3		6		9		12		24		48	
Method	X2	Plate	X2	Plate	X2	Plate	X2	Plate	X2	Plate	X2	Plate	X2	Plate
Brew 1	6.76	7.05	6.78	7.24	6.81	7.00	6.8	7.01	6.38	6.44	5.93	6.26	5.54	6.03
Brew 2	7.07	7.06	7.37	7.17	7.59	7.28	7.71	7.38	7.35	7.26	6.85	6.39	6.47	6.30
Brew 3	6.57	6.44	7.21	6.75	7.21	6.75	7.28	6.86	7.07	6.90	6.92	6.44	6.68	6.35
Overall Average	6.80	6.85	7.12	7.05	7.20	7.01	7.26	7.08	6.93	6.87	6.57	6.36	6.23	6.23
L. Plantarum													
Hour	0		3		6		9		12		24		48	
Method	X2	Plate	X2	Plate	X2	Plate	X2	Plate	X2	Plate	X2	Plate	X2	Plate
Brew 1	8.12	8.21	8.19	8.24	8.47	8.52	8.76	8.79	8.84	8.87	8.66	8.88	8.52	8.57
Brew 2	8.20	8.15	8.22	8.30	8.49	8.49	8.51	8.51	8.43	8.46	8.44	8.52	8.50	8.49
Brew 3	8.18	8.09	8.35	8.27	8.42	8.47	8.47	8.48	8.40	8.46	8.57	8.46	8.55	8.59
Overall Average	8.17	8.15	8.25	8.27	8.46	8.49	8.58	8.59	8.55	8.60	8.56	8.62	8.52	8.55

On average, the number of *S. cerevisiae* increased for the first 9 hours of the incubation to a peak concentration of about 7.26 log(CFU/mL) for Cellometer X2 and 7.08 Log(CFU/mL) for APDA plated. In the following 12 to 24 hours a decrease in concentration was recorded. At the end of 24 hours the average concentration was 6.57 log(CFU/mL) for Cellometer X2 and 6.35 log(CFU/mL) for APDA plated. Finally, yeast tapered off at a final concentration of 6.48 log(CFU/mL).

On average the number of *L. plantarum* increased for the first 12 hours and then plateaued following the 24 and 48 hour time points. *L. plantarum* started at a concentration of 8.17 log(CFU/mL) for Cellometer X2 and 8.14 log(CFU/mL) for MRS plated. The concentration increased to an average of 8.55 log(CFU/mL) for Cellometer X2 and 8.60 log(CFU/mL) for MRS plated. The concentration leveled off after 12 hours. This corresponds with known *lactobacillus* activity that usually decreasing after 12 hours. The final concentration after 48

hours with 8.51 log(CFU/mL) for Cellometer X2 and 8.55 log(CFU/mL) for plated. The corresponding trends for yeast and lactic acid bacteria shown in Figure 4.3.1 and Figure 4.3.2.

Figure 4.3.1: Comparison of Methods, Cellometer X2 and APDA plated, Concentration [log (CFU/mL)] of *S. cerevisiae* in Beer Wort Media

Figure Key: The first letter Refers to the microorganism. The second character refers to the trial or "A" for average. The last letter refers the to the method with P= Plated and C= Cellometer X2
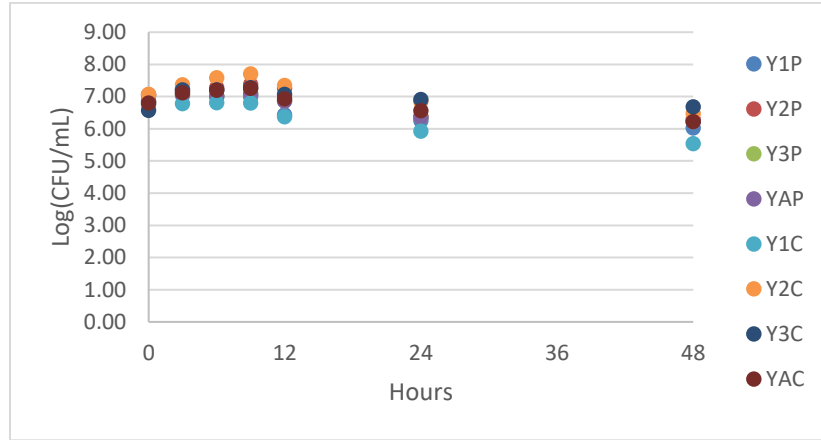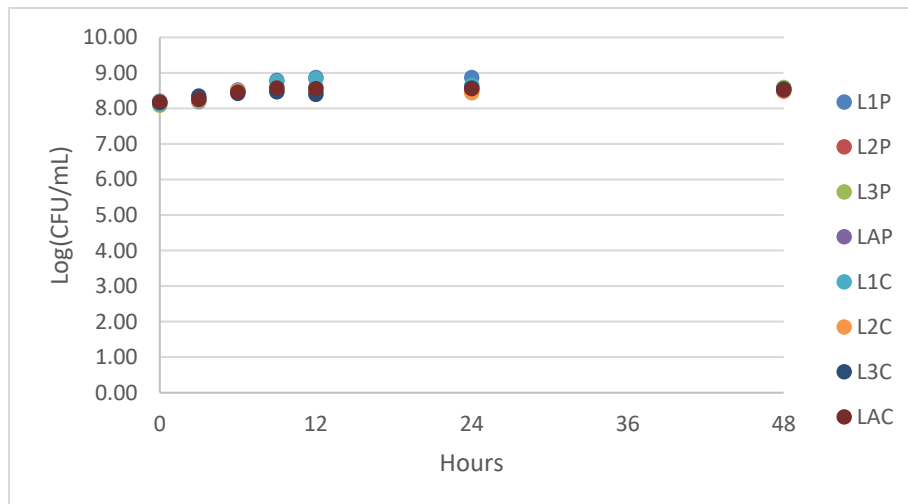

Figure 4.3.2: Comparison of Methods, Cellometer X2 and MRS plated, Concentration [log(CFU/mL)] of *L. plantarum* in Beer Wort Media

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Figure Key: The first letter Refers to the microorganism. The second character refers to the trial or "A" for average. The last letter refers the to the method with P= Plated and C= Cellometer X2

4.3.2 ANOVA Method Comparison

Before running an ANOVA, a boxplot was created for yeast and lactic acid bacteria. One Cellometer X2 point, the 48 hour 5.54 log(CFU/mL) was excluded as an outlier.

An ANOVA was conducted for the mixed culture wort fermentation between the Cellometer X2 and the microorganism respective plating methods. Concentrations were converted to log(CFU/mL) and averaged across all time points to determine an experimental average for plates and Cellometer X2. An ANOVA with a p-value of greater than 0.05 was consider not significantly different. The p-value for *S. cerevisiae* was 0.7296. The p value *L. plantarum* was 0.8128 (Table 4.3.2 and Table 4.3.3).

Table 4.3.2: ANOVA comparing Methods, APDA Plated & Cellometer X2, for

S. cerevisiae in a Beer Wort Media

Trial	Plating Log(CFU/ml)	Cellometer X2 Log(CFU/ml)	Difference	P-Value
1	6.72 ± 0.47	6.43 ± 0.43	0.29	
2	6.98 ± 0.40	7.20 ± 0.41	-0.22	0.7296
3	6.64 ± 0.26	6.99 ± 0.21	-0.35	

Table 4.3.3: ANOVA comparing Methods, APDA Plated & Cellometer X2, for

L. plantarum in a Beer Wort Media

Trial	Plating Log(CFU/ml)	Cellometer X2 Log(CFU/ml)	Difference	P-Value
1	8.51 ± 0.25	8.51 ± 0.22	0.00	
2	8.38 ± 0.12	8.40 ± 0.12	-0.02	0.8128
3	8.40 ± 0.12	8.42 ± 0.17	-0.02	

The ANOVA for *S. cerevisiae* and *L. plantarum* both displayed a p-value of greater than F0.05. This confirms no significant difference between the novel mixed culture method and their respective traditional plating method. Both evaluation techniques confirm trends from the previous work described in this thesis.

4.4   Discussion

The goal of the final mixed culture brewing application was to compare methods and analyze the difference between the Cellometer X2 and plated for *L. plantarum* and *S. cerevisiae* mixed culture fermentation of beer wort to simulate industry practices. No significant difference between the methods highlights the successful use of the mixed culture image-based cytometry for commercial brewing applications.

For these trials a mix of milled wheat and grain malt were mashed to create wort for inoculation and fermentation. The wheat and grain malt ratios were based on a Berliner Weisse recipe. Berliner Weisse is a German sour beer that dates back to the 16th century. It is a low alcohol, sour beer. It is traditionally fermented with *S. cerevisiae* spp. and lactic acid bacteria. The organism traditionally used to brew Berliner Weisse, aligns well with mixed culture validation organisms, so, this technique can be used successfully by sour beer brewers. It should

be noted, Berliner Weisse is traditionally not brewed with hops. Lactic acid bacteria are "hop sensitive" and do not reproduce well in hopped beer (Schurr et al., 2015). Hops contain a water-extractable antimicrobial compounds that can disrupt cell membranes. Hops also can cause a reduction in pH which inhibits LAB cell growth (Simpson, 1993). For these reason hops were omitted from the research beer recipe.

The brew trials were prepared at 0, 3, 6, 9, 12, 24, 48 hours. The first 12 hours is the period of greatest lactic acid bacteria activity. After 12 hours, a reduction in pH occurs in the fermenting wort, generally lowering from a pH of mid-5 to low 3. At low pH, lactic acid bacteria are inhibited and can no longer grow. This was observed throughout each brew trial as in the first 12 hours *L. plantarum* experienced highest growth. After 12 hours*, L. plantarum* counts plateaued.

S. cerevisiae experienced the greatest reproduction in the first 9 hours after inoculation, followed by a slight decline at every time point through 48 hours. This may be explained by a lower pH putting strain on the yeast or by the ending of primary fermentation. Primary fermentation can take anywhere from 36 hours (Palmer, 2017) to 72 hours (Bartlett, 2020). Some brewers define the end of primary fermentation as when the specific gravity falls below 1.030 (WMA, 2014). Additionally, because this was a low alcohol beer, there could be less fermentable sugars causing slower metabolizing past 9 hour time point. Even at a low pH, if fermentable sugars are present, yeast will continue to lower the specific gravity as they can better tolerate low pH and increasing alcohol concentration. This can result in some sour beer fermentation spanning many months. Fermentations were followed for 48 hours due to the specific gravity falling below 1.030, indicating that primary fermentation was complete.

An ANOVA was conducted comparing Cellometer X2 and plating for each microorganism. The p-value was greater than 0.05 for each experimental trial indicating a strong agreement between the two enumeration techniques.

4.5   Conclusion

The final goal of this research project was achieved in this study. The Cellometer X2 derived concentrations for both yeast and LAB at each time point that compared well with counts from plating microorganism on selective media during the course of beer (wort) fermentation. In addition, at each time point the difference in log(CFU/mL) was less than 0.5. Each fermentation trial had a p-value of greater than 0.05 confirming no significant difference between the methods. By shifting to image-based cytometry brewers can rapidly and precisely determine pitch concentration during fermentation concentration in less time, with improved efficiency and with fewer materials resources.

CHAPTER 5 THESIS SUMMARY

5.1   Thesis Conclusion

This thesis explored the ability of the Cellometer X2 to precisely determine

concentrations of yeast and lactic acid bacteria. To begin, monocultures methods were found not

to be significantly different (p-value >0.05) from each microorganism respective plating method.

Further, a strong R^2 value was observed for both microorganisms (>0.99) indicating strong

ability to determine concentrations precisely. This study confirmed early research conducted

using the Cellometer X2 for monoculture enumeration. Next, the mixed culture method

validation experiments results supported no statistical difference between the mixed culture

method and respective traditional plating methods. The first two range validations evaluated

varying one microorganism concentration while keep the other concentration constant. The goal

of these studies were evaluate the Cellometer's ability to precisely enumerate each

microorganism and determine the range of the method when one microorganism concentration

varied. A strong R^2 value (>0.99) was noted between 5 – 7 log(CFU/mL) for *S. cerevisiae* and 6

– 8 log(CFU/mL) for *L. plantarum*. Outside of these ranges an increased standard deviation,

decreased regression, and decrease p-value were noted. These concentration ranges encompass

pitch rates commonly used by brewers. The ratio validation experiment further confirmed the

efficacy of the newly develop method. No significance was noted and a strong R^2 (>0.99) was

also observed. Finally, the last section applied the validated method from Chapter 3 to a beer

wort matrix during a sour beer fermentation – a "real life" test of the methodology. The wort was

tracked over the course of 48 hours which was the length of primary fermentation and the

timeframe for brewers to adjust pitch rate. The Cellometer X2 mixed culture method was able to

track concentrations that were not significantly different from respective plating methods over the course of each trail.

The successful validation of mixed culture containing one *lactobacillus* strain and *S. Cerevisiae* can have a major impact on brewers and fermented beverage producers. With the mixed culture method brewers can quickly access the concentration of mixed cultures. The ability to rapidly analyze starters enables increased consistency and quality of fermented products while identifying problems such as over-pitching or under-pitching. If over-pitching is an issue brewers can dilute starter cultures to an appropriate concentrations that can be evaluated in 15 minutes with use of the novel mixed culture method. Under pitching may be identified before fermentation.

Future research using image-based cytometry can be expanded to include various types of microorganisms or inclusion of a viability assessment if more fluorescent channel become available. The mixed culture method could be expanded to gram negative bacteria by the inclusion of a distinct nucleic dye and additional fluorescent channel. Furthermore, finish product yeast and lactic acid bacteria concentrations can be evaluated. This research could contribute to the explanation of changes in fermented food and beverage quality.

This thesis research has validated a viable alternative to traditional plating methods for the evaluation of mixed cultures containing *lactobacillus* and *S. cerevisiae*. The newly developed image-based cytometry method can evaluate the total enumeration of each microorganism in a complex food matrix in as little as 15 minutes. The image-based method is not significantly different from accepted plating methods. Further exploration into viability and expanding the

microorganism library should be done to make the technology more appealing to producers of other fermented food products.

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