THE PENNSYLVANIA STATE UNIVERSITY
SCHREYER HONORS COLLEGE

DEPARTMENT OF FOOD SCIENCE

EXAMINATION OF INTERACTIONS BETWEEN SACCHAROMYCES AND BRETTANOMYCES YEASTS IN PRIMARY FERMENTATION OF BEER

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ABSTRACT

This study was conducted to evaluate the different methods of pitching mixed *Saccharomyces cervisiae* and *Brettanomyces bruxellensis* yeast cultures into simple un-hopped wort with the objective to find whether the order of inoculation significantly affected the production of volatile compounds in beer. Treatments regarding pitching of yeast were selected to control for single *S. cervisiae* and *B. bruxellensis* fermentations as well as mixed culture fermentations: one treatment undergoing simultaneous inoculation of both strains, one treatment undergoing a single *B. bruxellensis* inoculation and allowed to ferment for 96 hours before undergoing a second inoculation of *S. cervisiae*, while yet a third underwent initial inoculation with *S. cervisiae* and subsequent inoculation at 96 hours of *B. bruxellensis*. Worts into which the yeasts were pitched were made from dry malt extract (DME) and reached an initial specific gravity of 1.055 (12.5°P). Daily time points throughout the fermentation were run through GC/MS to determine relative amounts of 4-ethylphenol and 4-ethylguaiacol, two compounds that are accepted as being indicative of *Brettanomyes* fermentation. At the end of the fermentation trials, sensory panelists looked at each of the treatments characteristic of "Brett flavor" including vinegar, tropical fruit, spice, horse or barnyard, and Band-Aid® or medicinal aromas and flavors. The results of this study gave evidence that the order of pitching of yeasts may contribute to overall chemical and sensory differences, particularly an increase in positive Brett flavors.

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ACKNOWLEDGEMENTS

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To my parents for their unwavering confidence and sometimes misplaced faith in me that I would at some point come out of Penn State alive and with a degree.

To Rochelle Onwulata, Curtis Rainey, Sarah Leslie, Sarah Brown, Rachel Schaeffer, Carly Zwilcher, Margie Guy, Steve Bookbinder, Darcy McKinley-Lester, Jennifer Rumble, Laura Dzwonczyk, Kir O'Hanlon, Arlene Contreras, Ansley Sawyer, Kenny Vogel, the Penn State Ultimate Frisbee team, and particularly to Claire and Mark Cochrane: thank you for everything not only during this thesis, this semester, or this senior year but for making my time at Penn State rich and worthwhile both academically and personally.

Chapter 1 Introduction

1.1 Problem Statement

The role of *Brettanomyces* spp. as a spoilage yeast is well understood within the wine industry, but its role as a producer of favorable compounds in certain styles of beer is less understood despite its recent growth in popularity within the North American craft beer industry. The interactions within a mixed culture of *Saccharomyces cervisiae*, the traditional yeast species for beer and wine production, and *Brettanomyces bruxellensis* is of interest to the brewer or vintner looking to bring out the citrus, spice, and earth notes due the presence of 4-ethylphenol (4EP) and 4-ethylguaiacol (4EG) in low quantities without breaching the thresholds of at which these compounds yield horsey and band-aid aromas. The question comes down to whether the mixed culture should be added to the wort all at once, or if one strain of yeast ought to be pitched and allowed to condition the wort for a short period of time before the second yeast is pitched.

While problematic in its precision, sensory evaluation is a useful tool in identifying patterns for further investigation through chemical analysis (Boutou 2007). By pairing sensory scores with analytical data, a clearer picture can be drawn of what is happening inside the bottle during the fermentation process.

1.2 Hypothesis

The order and timing of yeast pitching has the potential to affect the rate of flavor and aroma compound production and concentrations in a finished beer. While some beers brewed with *Brettanomyces* spp. are held and matured for months in ways similar to wine, the flavor development in primary fermentation plays a large role in the final outcome of the beer's attributes. This study investigates the initial changes in wort character after it has been inoculated with single yeast culture controls and three different mixed culture treatments. It is hypothesized that staggering the pitch times of monocultures in a mixed yeast fermentation will play a significant role in the beer by the end of the primary fermentation time of ten days.

1.3 Objectives

The objective of this experiment was to determine whether the order of yeast inoculation in a mixed culture plays a significant role in the final flavor profile of beer and the composition and concentration of volatile phenols produced in the beer at the end of primary fermentation. Typical beers brewed with *Brettanomyces* spp. are often allowed to age for months in the bottle or held in casks due to the slow fermentation of *Brettanomyces* spp. but the condition of the yeast environment in early stages of fermentation play a role in the attenuation of the yeasts, the amount of acid produced, the rate of growth, and the consumption by one strain of yeast of metabolites produced by the other.

Chapter 2 Review of Literature

Considered a wild yeast, *Brettanomyces* spp. are crucial in the production of lambic, flanders red ales, and other sour and specialty style Belgian beers (Yakobson 2010). *Brettanomyces* spp. have received a fair amount of research attention from the wine industry due to their well-recognized role as spoilage organisms. Unlike *Saccharomyces*, *Brettanomyces* spp. are superattenuating, require very little sugar and therefore are capable of slow and long fermentation, evening dry (i.e. low sugar) media. In addition to maltose and glucose, *Brettanomyces* spp. are capable of fermenting certain sugars like dextrin when more easily metabolizable sugars are unavailable, metabolic activity that *Saccharomyces* spp are incapable of doing. The yeast seeps into cooperage and becomes nearly impossible to remove from wooden fermentation vessels once the vessel is inoculated. Some vintners and brewers sidestep the issue of residual contamination in cooperage by using metal or other non-porous fermentation vessels, but in cases where Brett character is a desired quality, the incomplete eradication of *Brettanomyces* spp. is a potential source for positive signature aroma in finished product. Particularly in wooden barrels, which allow small amounts of oxygen to slowly seep into the beer matrix, *Brettanomyces* spp. will continue their slow growth virtually indefinitely (Sparrow 2005).

Before the general understanding of the microbiological component of brewing, fermentations were essentially all wild. This isn't to say that brewers were preparing wort and simply hoping for the best for each batch. The environment and traditional practices of both vintners and brewers played a large role in the process.

Often times the pressed grape skins are taken out to the vineyard and used as fertilizer and in doing so inoculate the grapes which are currently growing with the yeasts on the used

grape skin surfaces. Over generations of repeated success, these wild cultures become a part of the identity of the brewer or vintner, practically embedded in the *terroir* (Sparrow 2007).

Likewise, the porous barrels which are filed and set to age, the wooden walls and floors of old farm houses, and the even air itself are all filled with wild yeasts when brewing with wild cultures. The more often the brewer produced beer, moving cultures around, splashing sugar in the form of wort onto surfaces, all encouraged the continued ecosystem which made spontaneous fermentation in open vessels possible in Belgium. *Brettanomyces* ssp. as stated earlier is quite capable of infiltrating any porous surface, from the barrels to the brewer's mash paddle to the beams in the old farmhouse style breweries. Additionally, the orchards surrounding the monasteries of these traditional Belgian breweries created a safe environment in which *Brettanomyces* spp. and other oxidative yeasts can thrive and travel through the air, essentially inoculating anything left open and exposed (Sparrow 2007).

The earliest published chronicling of *Brettanomyces* spp. came from N. Hjelte Claussen through the Institute of Brewing in 1904 (Yakobson 2010). Clausson, who was the laboratory director at Carlsberg in Copenhagen, described newly isolated yeasts that he had drawn from English stock ales in slow secondary fermentation, calling it *Brettanomyces* which translates directly to "British fungus" (Sparrow 2007). After its discovery, however, it wasn't until 1940 when M.T.J. Custers re-identified yeast found on the skins of French wine grapes as a strain of *Brettanomyces* and not *Mycotorula intermedia* as previously identified by Krumbholz and Tauschanoff in 1933 (Rodrigues and others 2001). Custers investigated the relatively new yeast and came back to present seventeen different strains of *Brettanomyces*, all but one drawn from beers samples, which he believed were unique to English and Belgian brewing (Yakobson 2010; Oelofse 2008).

Brettanomyces spp. are in fact pervasive to most environments worldwide, residing on the rinds of fruit and in wood (Oelofse 2008). Because they produce β-galactosidase,

Brettanomyces spp. are capable of digesting cellobiose, a hydrocarbon produced when the insides of wine and beer barrels are torched and therefore are quite at home in the cracks and crevices of cooperage (Sparrow 2007).

Brettanomyces is capable of decarboxylating trans p-coumaric acid, yielding vinylphenols (Pizarro et. al 2006). Of particular significance to the characteristic Brett flavor of beer are 4-vinylphenol (4VP), 4-vinylguaiacol (4VG), 4-ethylphenol (4EP), and 4-ethylguaicol (4EG) (figure 1) (Boutou 2007). These compounds give way to a number of different flavors and aromas in beer brewed with *Brettanomyces* spp. but the particular flavor profile is a result of the concentrations of these particular compounds.

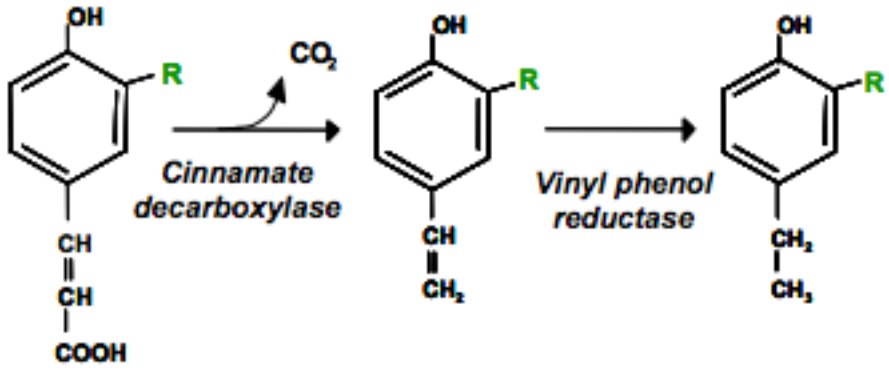


figure 1: The mechanism for the decarboxylation and reduction unique to *Brettanomyces* spp. When the R group is a hydrogen, the initial *p*-coutaric acid yields 4-VP and 4-EP. When the R group is an aldehyde, the initial ferulic acid is metabolized to 4-VG and 4-EG. (Oelofso 2008).

While a number of other brewing and wild yeasts and bacteria such as *Saccharomyces* spp. and *Lactobacillus* spp. are capable of converting *p*-coumeric and ferulic acids, present in the wort after the mash process, into vinyl phenols, the vinyl reductase mechanism yielding 4EP and 4EG is a unique contribution of *Brettanomyces* spp. (Fugelsang, Edwards 2007). Because of this, breweries and wineries will use 4EP as an indicator of *Brettanomyces* infection. *Brettanomyces*

will often benefit from the growth of other microbes in the wort or must and their contributions of vinyl phenols and organic acids.

Table 1: Aroma threshold values of volatile phenols (taken from Oelofse 2008)

Compound	Aroma Threshold (μg/L)	Aroma Descriptor
4-vinyl phenol	440/600*	phenol, medicinal
4-vinyl guaiacol	33/100*	clove-like
4-ethyl phenol	30-60	horsey, barnyard
4-ethyl guaiacol	20	spicy, clove

**In water, these compounds are perceived at lower concentrations but in a beer or red wine system the competing flavor compounds mask the aromas, raising the perception threshold.*

The range of aromas and flavors available to brewers through the use of *Brettanomyces* spp. is vast, but it requires the striking of a delicate balance. While the minimum aroma thresholds (table 1) seem low, compounds such as 4-EP are capable of transitioning quickly from pleasant earthy notes to an overwhelming antiseptic smell and taste. This is due simply to the concentration of the volatile phenols reaching a point at which they are perceived as phenolic off-flavors (POF)

As the North American craft beer industry expands, more brewers are interested in experimenting with less traditional yeasts and bacteria in their fermentations. Breweries such as New Belgium (Fort Collins, CO), Goose Island (Chicago, IL), Lost Abbey (San Marcos, CA), Portland Brewing Company (Portland, OR), and a number of others are boasting beers brewed with a mixed culture or are putting out all-brett beers to an audience of American beer consumers who seem interested in the new and varying flavor profile that can be found in beers brewed with *Brettanomyces* spp (Fromson, 2012). American brewers are taking a turn at their own interpretations of Belgian Trappist classics, brewed spontaneously in the same way for centuries, and applying modern techniques of quality control and consistancy.

While the wine industry has focused on keeping *Brettanomyces* spp. out of their process, regarding it as a spoilage and working to isolate and eliminate it when possible, the applictions of *Brettanomyces* spp. in beer are vastly underresearched. What is understood about *Brettanomyces* spp. in beer has been discovered through experimentation by the brewers themselves (Yakobson 2010). The interest of this study is to evaluate the pitching of mixed cultures in order to get an idea of how one might optimize volatile phenols without overdoing it and stepping into off-flavor ranges (Heresztyn 1986).

Chapter 3 Examination of Interactions Between Saccharomyces and Brettanomyces Fermentations

3.1 Materials and Methods

3.1.1 Selection of Yeasts

Strains of Saccharomyces (WLP002) and Brettanomyces (WLP645) were obtained from White Labs Yeast (Sand Diego, CA, USA). Yeasts were stored at refrigeration temperatures (4°C). Before pitching, yeast was brought to ambient temperature (18°C). WLP002 was chosen for its clean fermentation characteristics including a mild flavor profile. WLP645 was chosen for its known establishment of Brett flavor characteristics by the end of primary fermentation, whereas other strains of *Brettanomyces* take weeks or even months to fully develop the compounds of interest to this study.

3.1.2 Preparation of Wort

For the fermentation trials, an unhopped wort made from dry malt extract (DME) was prepared. 1.4kg of Munton's Extra Light DME (San Diego, Sufolk, UK) were dissolved in three gallons of water and boiled to create a sterile wort with a specific gravity of 1.050 (12.5°Plato). The wort was not hopped. The wort was then cooled to 21°C using a copper immersion chiller before separation into three 3.8L batches for pitching.

3.1.3 Fermentation Trials

11.4L prepared and cooled wort was divided into two separate 5.7L batches. Wort was poured from a height of about two feet in to increase aeration of each batch. Brettanomces bruxellensis was pitched into one batch while Saccharomyces cervisae was pitched into the second. One third of each of these beers were mixed together in a third container to yield three equal 3.8L batches of beer, two of single culture and one of mixed culture (figure 2).

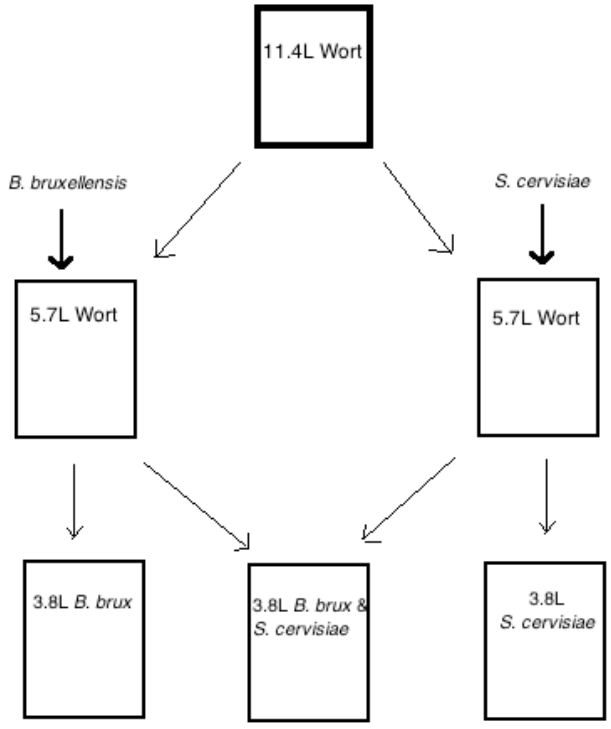


figure 2: Schematic of preparation of the various wort treatments in first round of fermentation trials.

Additional agitation of the buckets was performed after pitching to break up any undissolved yeast and to aid in aeration. Each batch of beers was partitioned out into five 650mL bottles, filled to the base of the neck to allow room for fermentation activity and foaming. Each

bottle was capped with a rubber stopper and fermentation lock and stored at ambient temperature (18°C). Sample aliquots (1mL) were withdrawn twice per day for10 days and stored at -20°C before being thawed and analyzed by GC/MS in order to detect 4EP, 4EG, 4VP, and 4VG.

A second round of fermentation trials began with the same structure as the first round of trials. After 96 hours of allowing single culture fermentative activity, each of the single culture bottles was inoculated with a 3mL starter of the second yeast species. The second fermentation trial followed the aliquot schedule and environmental conditions as the first trial.

3.1.4 Chemical Analysis

pH and specific gravity of the starting wort and final beers were measured by a glass hydrometer (0.990 to 1.170) and a SevenGo portable pH meters (Mettler Toledo, Columbus OH).

The method for GC/MS analysis of the beer were modeled after a similar study analyzing wine samples for Brett characteristics (Boutou, 2006). GC/MS analysis was performed on a Agilent Technologies DB5 ms column 30 m × 0.25 mm I.D., with 0.25 μm film thickness and a EZ-Guard 10 m protective column of deactivated silica. The carrier gas was Helium programmed to flow at a constant linear speed of 45 cm s^{-1}during all the run (flow 1.5 mL min^{-1}). The injector was a standard split/splitless operated in splitless mode at 270 °C (gas shield at 5.90 min flow 15 mL min^{-1}) with a direct injection sample size of 1μL. The oven program started at an initial temperature of 50 °C for 2.0 min. Temperature was then increased at a rate of 3.0 °C min^{-1} to 190 °C, then at 50 °C min^{-1} to 320 °C, the oven was held to 320 °C for 1 min. Detection was achieved with a HP-5973Inert quadrupolar mass detector working with EI ionization (EI, source temperature 230 °C, temperature of quaduropole 150 °C, energy of constant ionisation 70 eV, multiplier of electrons 1600 V) and was operated in the SIM mode (SIM) on the selected ions

characteristic of each molecule (the quantification ions are detailed below). The analysis takes 52 min, and a sample was injected every 63 min.

3.1.5 Sensory Analysis

An untrained sensory panel of thirty people living in the State College, PA area, aged 21 to 30 years, was convened in order to evaluate samples of each control and treatment. Samples were given three-digit blinding codes and presented to panelists who were then asked to indicate with a dash on a 15cm qualitative scale line the intensity of the organoleptic qualities suggested. The aromas tested for were indicative of Brett character and were as follows: malt, vinegar, tropical fruit, horse or barnyard, clove or spice, and antiseptic or Band-Aid®. Results were adjusted to a 100-point scale and run through ANOVA testing to determine statistical significance between fermentation treatments.

Chapter 4 Results and Discussion

4.1 pH of Finished Beers

pH was measured for each of the finished beers (five 650mL bottles per treatment, six treatments in total). pH values were analyzed by ANOVA and Tukey testing to determine significance between groups (figure 3).

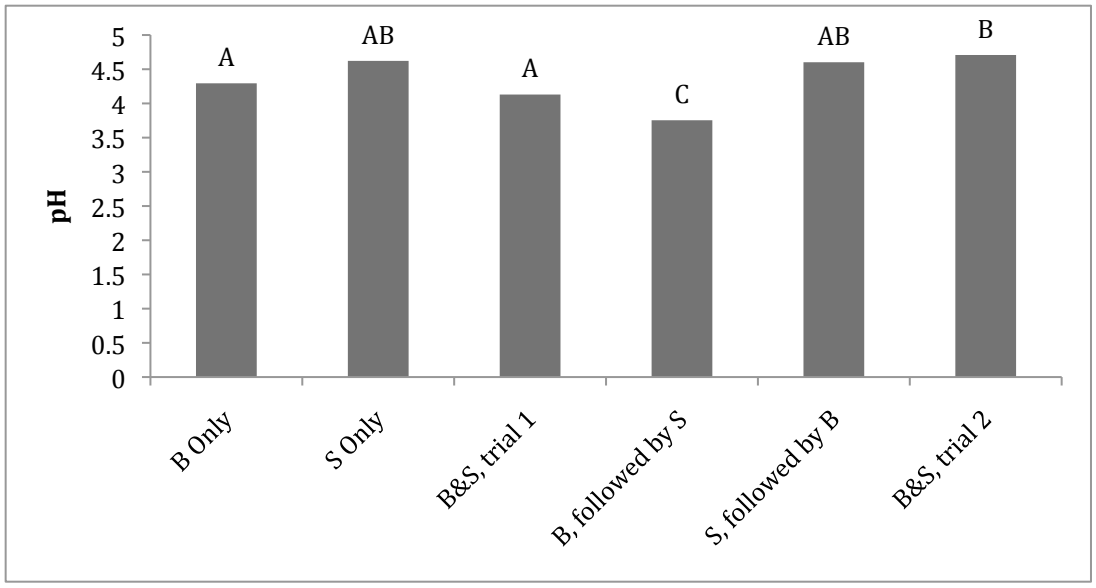


figure 3: pH values taken at the end of fermentation for each vessel.

ANOVA results indicate that by inoculating with *B. bruxellensis* followed by *S. cervisiae* after 96 hours in order to allow *B. bruxellensis* to enter active fermentation, the final pH of the resulting beer is significantly lower than samples of single culture, samples of mixed culture inoculated simultaneously, and mixed culture in which *S. cervisiae* was pitched first and allowed to ferment independently for 96 hours. One proposed explanation for this would be that the initial aeration of the wort before the containers were sealed for anaerobic fermentation allowed

B. bruxellensis to metabolize sugars aerobically, yielding some quantity of acetic acid before *S. cerviciae* was even added. It must be no coincidence, then, that the same trial came back from sensory evaluation with one of the highest overall perceived vinegar intensity, matched only in perceived vinegar by the *B. bruxellensis* single strain trial.

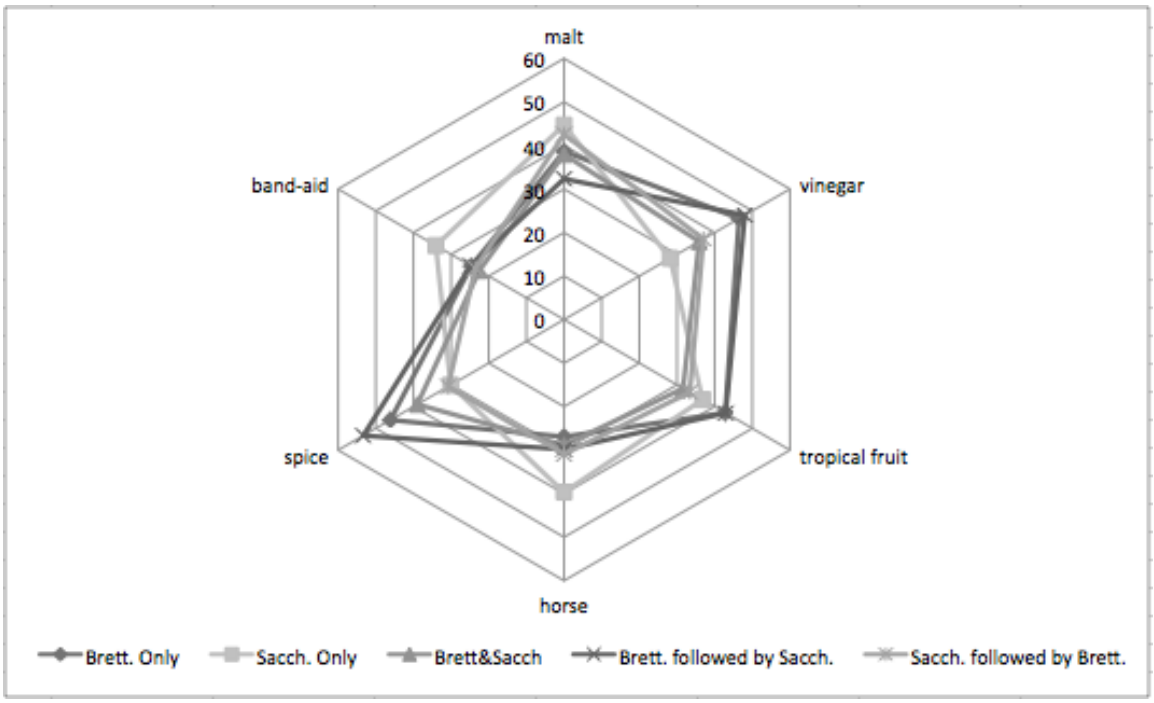


figure 4: Organoleptic qualities attributed to Brett flavor for each of the various pitching methods of *B. bruxellensis* and *S. cervisiae*.

By compiling all of the sensory data (figure 4), it is evident that all trials containing some degree of *B. bruxellensis* follow the same kind of geometric pattern, while the only unique shape to pull from the compiled data would be that of the treatment which underwent a single *S. cervisiae* inoculation. A radar chart is best suited for the demonstration of outliers in the data, of which there were none. In order to further investigate whether significant differences exist between treatments, analysis by ANOVA was performed (figure 5).

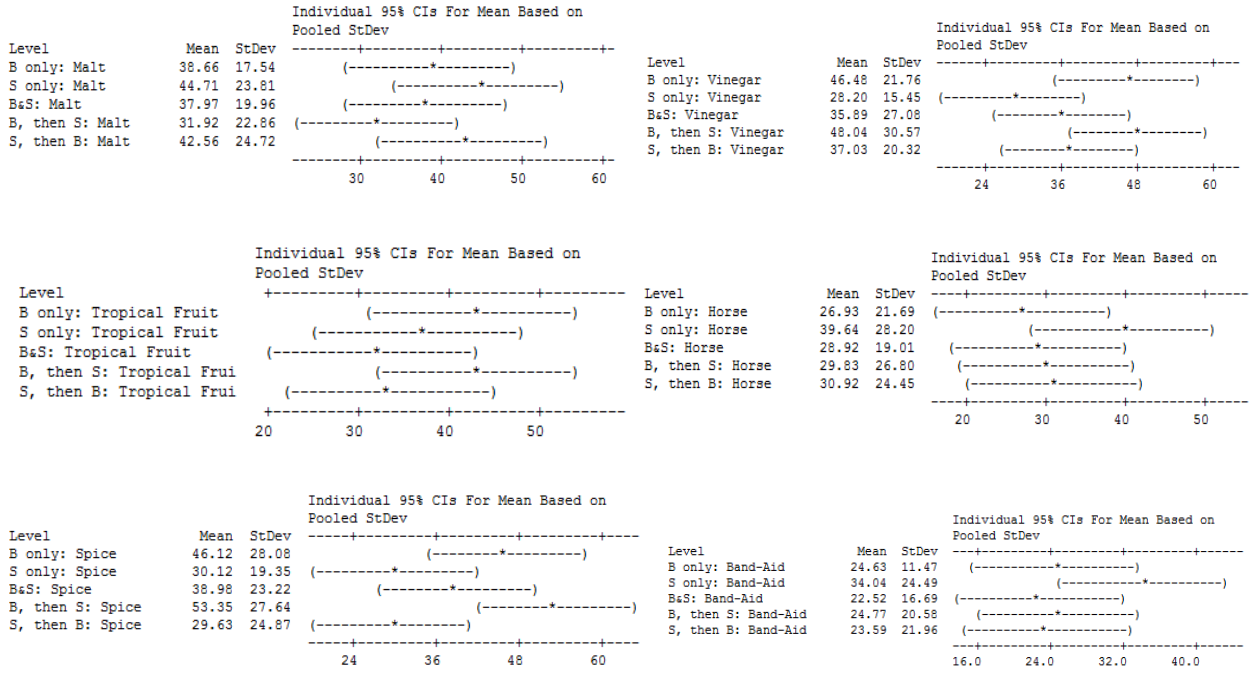


figure 5: ANOVA with Tukey Test for qualitative analysis of perceived organoleptic characteristics in each fermentation treatment. The results indicate a potential significance of the pitching order to the overall spice and vinegar qualities of the finished beer.

The brackets indicate the statistically significant range of noted perception of each flavor evaluated. Brackets which do not overlap vertically can be considered statistically significant in that attribute's perceived intensity. For instance, spice aroma in beer treated first with *B. bruxellensis* and then *S. cervisiae* appears to have a significantly greater spice character than beer treate first with *S. cervisiae* and then *B. bruxellensis*. Overlap between brackets from line to line within the same test shows that the differences between the average values collected were not themselves statistically significant. Therefore, the perceived intensity of spice character in beer treated with both yeast strains simultaneously is not significant from that which was treated with either strain first. This seems to be the case with all beer treatments when looking into the perceived intensity of both phenolic off flavors (barnyard and Band-Aid®). A simple explanation for this occurance might be that phenolic characteristics are not ordinary flavors an untrained

panelist might come across knowingly and may not have developed a sensitivity to the particular flavor or aroma.

With flavors more closely related to acid, such as tropical fruit or vinegar, it appears that while the differences between data are not significant, the trends seem to lean towards both high fruit intensity and high vinegar intensity in the *B. bruxellensis* single inoculation and the trial in which *B. bruxellensis* was inoculated first, followed by *S. cervisiae.* This is good news from the perspective of a brewer looking for citrus and sour notes in a finished beer: hints of pineapple and other tart flavors can be produced through yeast alone without the addition of citrus fruits to the fermentor or cask.

Percent alcohol by volume (ABV) was determined at the end of the fermentation cycle for each of the treatments (figure 6). While the range of values is not very large, the difference between the %ABV of the *B. bruxellensis* monoculture and the trial in which *S. cervisiae* was added after 96 hours of monoculture fermentation from *B. bruxellensis* indicates that the addition of a faster fermenting species to the beer to compete with the slower fermenting species introduced some kind of rate-limiting factor to the beer matrix, resulting in a lower %ABV compared to every other treatment in the trial.

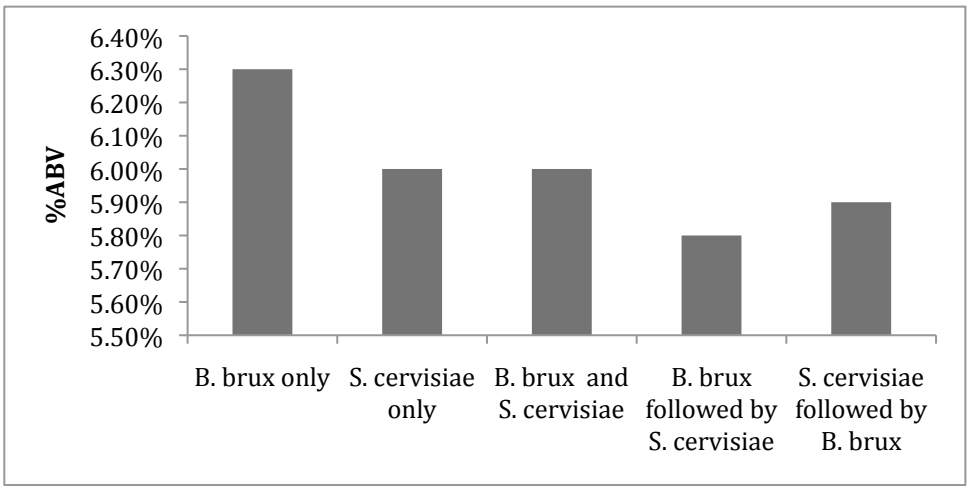


figure 6: Alcohol by volume (%ABV) of different treatments.

To support the qualitative and general analysis of the different treatments, GC/MS was performed with the intention of tracking the presence of 4EG and 4EP, which are attributed to Brett characteristics in beer and wine. Without first extracting the volatile phenols of interest, only 4EG was in a high enough concentration range to be detected in most of the samples throughout the trial (figure 7).

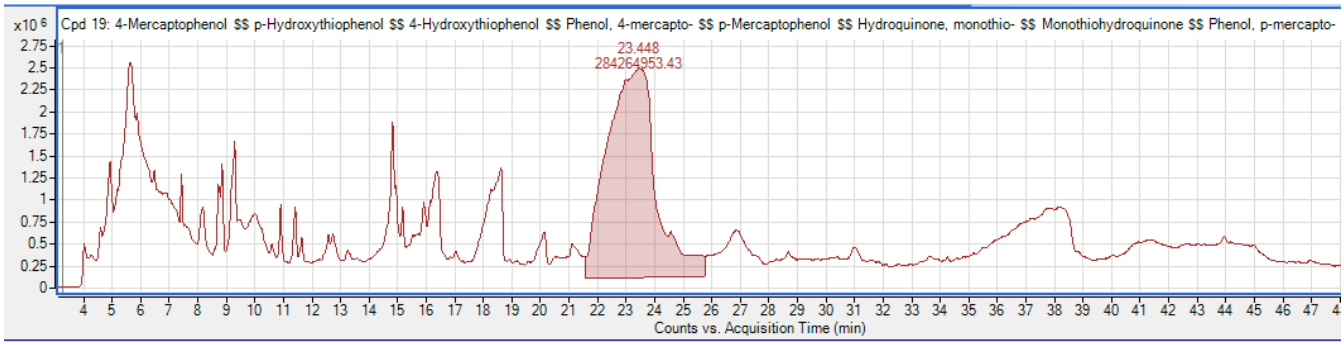
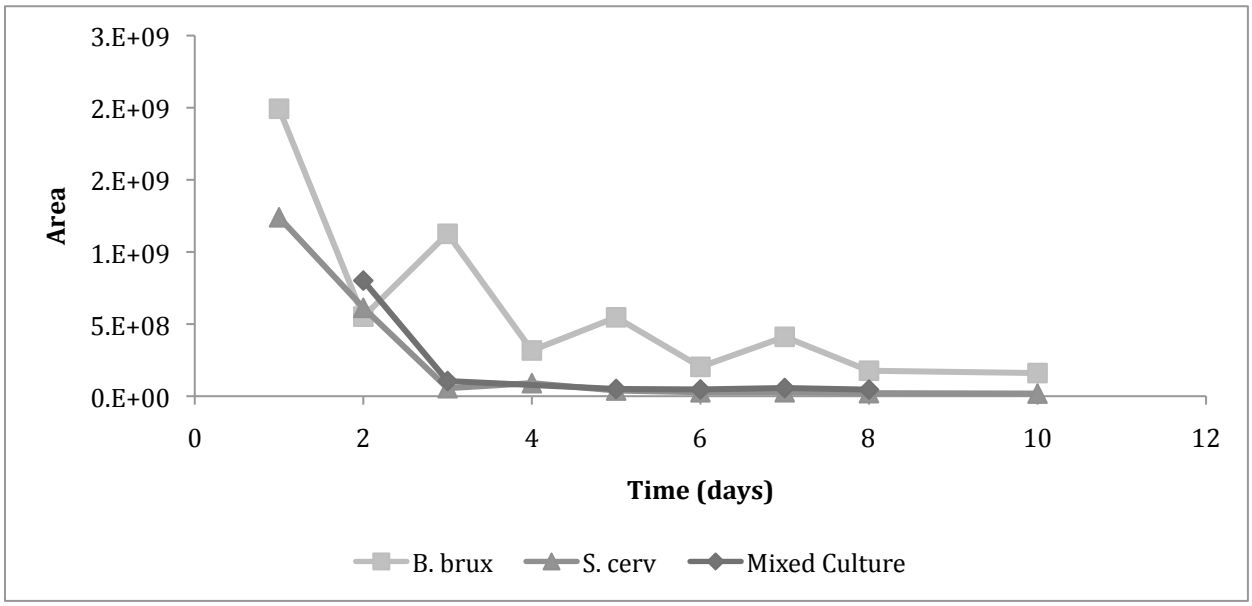


figure 7: A sample chromatogram readout shows the characteristic peak of interest tracked for all time points in the fermentation trials.

Ion fragmentation patterns matched the peak to similar phenolic compounds in the internal library. 4EG lends the fruit and spice notes more strongly detected in samples fermented with *B. bruxellensis* whereas 4EP lends itself to more of the barnyard aroma. Similar studies found that 4EG was detectable in far higher levels than 4EP in beers brewed with *Brettanomyces* spp (Yakobson 2010).



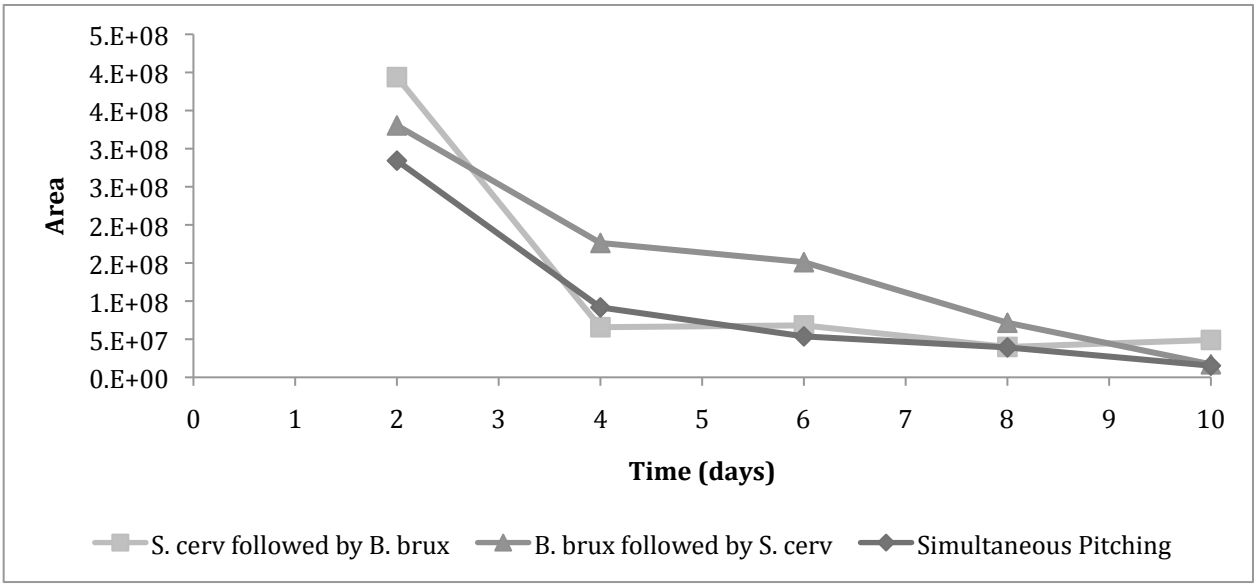


figure 8: Area under the curve for GC/MS analysis of each sample throughout fermentation. Only 4EG was at a level high enough for detection against the background noise of the beer matrix.

4EG concentrations were tracked over time for each of the trials to show the pattern of concentration throughout the fermentation cycle (figure 8). The result of the GC/MS is of particular interest because of its strangeness. Each curve trends downwards throughout the process of the fermentation, regardless of the inoculation treatment. Perhaps of most interest is

the pattern observed in the curve of the beer containing only *B. bruxellensis*. The downward trending zig-zag line is puzzling but might indicate loss of volatiles each time the container was opened for sampling. In fermentations containing *B. bruxellensis*, it was expected that the concentration of 4EG and 4EP would increase over time as the yeasts fermented sugars and the secondary metabolites of *S. cervisiae* yeasts in mixed cultures. In future experiments, it is suggested that a means of sampling with minimal disturbance of headspace gases be devised in order to avoid similar errors.

Chapter 5 Conclusion

Sensory and basic chemical analysis results indicate that the order in which different species of yeast are pitched into a wort plays a significant role on the outcome of certain aromatic aspects the beer, while other characteristics lacked evidence to suggest that the order of yeast pitching played a very significant role. The rate of acid production and volatile compound production, particularly volatile phenols contributing to the overall flavor of the finished beer, was observed to be different when yeasts were pitched simultaneously or staggered. Further analysis would be recommended by GC/MC using techniques to isolate the compounds of interest in order to avoid the noise caused by the presence of other compounds present in the beer matrix. Additionally, a method of withdrawing aliquots while minimizing the disturbance of the headspace composition of the beer during fermentation by opening and depressurizing the system would be strongly recommended.

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Education

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Honors and Awards

Katey Lehman Award for Poetry, 1st place, March 2014

Jake Cranage Award in creative writing (poetry), 2nd place, March 2014

Katey Lehman Award for Poetry, 2nd place, March 2013

Jake Cranage Award in creative writing (poetry), honorable mention, March 2013

Travel grant from Penn State Global Programs and the Alliance for Education, Science, Engineering and Development in Africa, summer 2010

Dean's List

Association Memberships/Activities

Food Science Club

Penn State Women's Ultimate Frisbee

Related Experience

Hochschule Ostwestfallen-Lippe

Lemgo, Germany

May-August 2013

Undergraduate Researcher, Department of Beverage Technology

- Worked with graduate student Marko Stefan under the guidance of Dr. Jan Schneider
- Studied bacterial culture activity in *Wasserkefir* project, a non-alcoholic fruit beverage using HPLC throughout the course of the fermentation to monitor organic acid development.

Research Interests

My overarching interest is in fermented products and controlled rot with yeast, mold, and bacteria alike. As a system in a constant state of change, fermented products offer an enormous array of challenges and future projects as a chemist, a microbiologist, and an appreciator of quality in food and beverages. Specifically, I'm interested in utilizing the metabolites of yeasts and bacteria in order to come to a final flavor profile in a food or beverage rather than larger non-metabolizing components.

Publications and Papers

Penn State Kaliope, Spring 2014

- Katey Lehman Award for Poetry; 1st place: *Laundry Day, But Not Necessarily In That Order*
- John Cranage Award for Creative Writing (poetry); 2nd place: *Ink*

International Consortium for Indigenous Knowledge

- Article discussing research in Ghana and study abroad. Newsletter Fall 2010, Vol. 2, No. 3