Performance of *Brettanomyces* Yeast Strains in Primary and Secondary Beer Fermentations

by

Riley Humbert

A THESIS

submitted to

Oregon State University

Honors College

in partial fulfillment of
the requirements for the
degree of

Honors Baccalaureate of Science in Chemical Engineering
(Honors Associate)

Presented May 21, 2021
Commencement June 2021
AN ABSTRACT OF THE THESIS OF

Riley Humbert for the degree of Honors Baccalaureate of Science in Chemical Engineering presented on May 21, 2021. Title: Performance of *Brettanomyces* Yeast Strains in Primary and Secondary Beer Fermentations.

Abstract approved: ____________________________________________________________

Christopher Curtin

Recently, there has been an expansion of large-scale studies looking at strain variation of *Brettanomyces*. These studies have found variation between strains in terms of utilization of maltose and production of volatile phenols. While there is research going into this subject, there is a gap in knowledge regarding the differences in fermentation behavior and sensory impacts between primary and secondary fermentation behavior. This paper looked at the difference between primary and secondary fermentation performance of 10 yeasts and sought to determine if there were significant differences between strains based on origin and fermentation environment. It was found that traits like malt sugar consumption and fermentation rate, differences between origins could be seen. It was also found that there were strain by strain differences in behavior in primary and secondary fermentation. These findings give insight into predicting strain behavior in different settings, and add to the building body of knowledge surrounding strain selection of *Brettanomyces*.

Key Words: Brettanomyces, yeast, bottle conditioning, primary fermentation, secondary fermentation

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APPROVED:

Christopher Curtin, Mentor, representing Food Science and Technology

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

Riley Humbert, Author
CONTRIBUTION OF AUTHORS

Kevin Pigao carried out the primary fermentation experiment and prepared the samples for sensory analysis. Karli Van Simaeys and Michael Fechir ran sensory panels analyses and performed statistical analyses on the resulting data (Karli: primary fermentation, Michael: secondary fermentation). Lindsey Rubottom ran and analyzed HPLC analysis. Dr. Elizabeth Tomasino ran and analyzed the GC MS samples. Riley Humbert carried out the secondary fermentation experiment and prepared the samples for sensory analysis (secondary fermentation) as well as prepared samples from both experiments for GC MS and HPLC analysis. Riley Humbert coordinated testing and performed statistical analyses for all samples with the exception of sensory data and drafted the research manuscript. Dr. Tom Shellhammer contributed to study conception, experimental design and supervised sensory analyses. Dr. Chris Curtin conceived and designed the study, supervised fermentation experiments, participated in data analyses and acted as the Principal Investigator of this research.
Introduction

The *Brettanomyces* genus of yeast was originally encountered in the early 1900’s, identified as responsible for secondary fermentation of English stock ale that imparted characteristic ‘vinous’ flavors [1]. *Brettanomyces* yeasts have since been isolated from a wide variety of environments, such as grape skins, wine barrels, and bioethanol production as well as many fermented beverages like cider, wine, tequila, and beer. [2].

*Brettanomyces* flavor is typically characterized by a specific flavor profile created by volatile phenols. Unlike *Saccharomyces*, *Brettanomyces* is primarily positive for the phenolic acid decarboxylase (PAD+) enzyme and is able to convert vinyl phenolic acids into their ethyl derivatives [3]. These create flavors described as “animal”, “smoky”, and “leather” [4]. More often than not, these characteristics are undesirable and are viewed as a sign of spoilage. This is especially true in the wine industry where the presence of *Brettanomyces* is essentially never viewed positively.

In the context of beer production, however, *Brettanomyces* plays an important role in imparting signature flavors to certain beer styles. The phenolic compounds, esters, and other chemical compounds that it produces impart a wide variety of aromas ranging from medicinal, smoky, and band aid to floral, tropical, and fruity [5]. It is most commonly used in funkier beer styles such as lambic. In these spontaneously fermented beers the microorganism population shifts throughout fermentation [6]. *Brettanomyces* typically shows up near the end as a “secondary” fermentation yeast after *Saccharomyces* has fermented much of the sugar. In these beers, *Brettanomyces* is responsible for producing esters, phenolic compounds, ethyl lactate, and ethyl acetate.

Recently, there has been increased interest in using *Brettanomyces* as a secondary fermentation yeast in inoculated fermentations. There are many aspects of *Brettanomyces* that make it an appealing option for inoculated beers. It has the ability to metabolize many different sugars and can therefore superattenuate beers. It is also highly resilient to harsh conditions which gives it the ability to serves as a bottle conditioning yeast for beers with high ethanol content or low pH. Various characteristics between different strains have been investigated in a number of
different capacities. Colomer et al. looked at 84 *Brettanomyces* strains in terms of ethanol and acetic acid production, volatile compound production, and various other metrics regarding fermentation. A correlation has been found between the genomic background of the yeast and its phenotypic characteristics [7]. It was also found that beer strains tend to be more closely related while wine strains exhibit wide genetic divergence[8]. Strain variation has been found to significantly affect flavor metabolite production, carbon utilization, and phenol production [9]. Variances between strains has also been found in *S. cerevisiae*. Distinct genetic groupings between beer, wine, and wild strains have been found, and these differences affect ethanol production and sugar metabolism.

**Background**

Standard ale and lager beers are produced in very similar ways with differences in flavor and style coming from the strains of *Saccharomyces* strain, grain and hop content, and fermentation temperature/length. The general process of brewing is depicted in Figure 1 and begins with mashing, where grains like malted barley or wheat are combined with hot water, typically 144-158 °F [10]. During this process, carbohydrates such as fructose, glucose, and maltose as well as chemical compounds like hydroxycinnamic acids (HCAs) are extracted from the grains [11]. After this, the wort is boiled to sterilize it and to add ingredients that add to the flavor of the beer. At this point hops are typically added as well as other flavoring agents such as fruit or spices. After the boiling is finished, typically around an hour, the wort is left to cool to around room temperature. At this point the beer is inoculated with yeast.
Beer is typically fermented by yeast from the genus *Saccharomyces*. Lager beer is made using *S. pastorianus*, which is a hybrid of *S. cerevisiae* and *S. eubayanus* capable of fermenting at colder temperatures to produce a very clean beer [13]. Ale fermentations are performed with strains of *S. cerevisiae*, which vary substantially in their impact upon finished beer flavor [13][14].

The fermentation process takes roughly two weeks, but this can vary widely depending on the style of beer. During this time, the yeasts consume the sugars extracted during the malting process and convert them to ethanol, at the same time producing a variety of volatile compounds. These contribute significantly to the final flavor profile of a beer and vary depending on the yeast. These can include higher alcohols, esters, and volatile phenols [14].

The fermentation process looks very different when it comes traditional styles of beer, such as Belgian, Lambic, or Gueuze. These beers begin in a very similar manner, going through a mashing and boiling process. However, after being boiled, the wort is left in an open vat, called a coolship, where it is allowed to be inoculated
with microorganisms present in the air [6]. After cooling, these beers are stored in casks and allowed to slowly undergo a multi-stage fermentation. At different stages, the dominant microorganisms change, as the environment of the beer changes [6]. *Brettanomyces* yeasts, along with lactic and acetic acid bacteria, become dominant in the later stages of beer maturation after *Saccharomyces* has completed the primary alcoholic fermentation of the wort. Fermentation and aging of these beers can take around 2 years, but this time frame is also highly variable.

*Brettanomyces* species and sources of isolation

There have been many different species of *Brettanomyces* identified over the past century, creating a wide range of different identifiers that frequently referred to the same actual yeast. Recently, a number of these synonymous species names within the *Brettanomyces* genus have been revised based on molecular data and it is now commonly accepted that the genus is comprised of *B. bruxellensis*, *B. anomalus*, *B. custersianus*, *B. naardenensis*, and *B. nanus* [15] [16]. The phylogenetic tree shown in Figure 2 indicates the relative relatedness between these species. In lambic beers, the main species found in the latter half of fermentation is *B. bruxellensis* [6]. This species along with *B. anomalus* are the species most frequently encountered in the context of beer brewing [17]. In the context of winemaking, *B. bruxellensis* is the primary species focused upon [18].

![Figure 2: Phylogenetic tree of the five currently accepted *Brettanomyces* species. Reproduced from [19]](image)

In winemaking, *Brettanomyces* is typically viewed as a spoilage organism. Because of this, much of the research relating to its fermentation and growth characteristics are in terms of stopping its growth and production of aromatic compounds. This can prove to be quite a task as *Brettanomyces* has been found to
have limited nutritional requirements which allows it to survive well in many different environments and makes it difficult to get rid of [2]. It has been isolated from a wide variety of different environments, such as grape skins, soft drinks, and bioethanol production [18], as summarized in Table 1.

<table>
<thead>
<tr>
<th>Source</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Winemaking</strong></td>
<td>Dry wine [20]</td>
</tr>
<tr>
<td></td>
<td>Sherry [21]</td>
</tr>
<tr>
<td></td>
<td>Spontaneously fermented wine [22]</td>
</tr>
<tr>
<td></td>
<td>Grape berries [23]</td>
</tr>
<tr>
<td></td>
<td>Grape must [24]</td>
</tr>
<tr>
<td><strong>Brewing</strong></td>
<td>English stock ale [1]</td>
</tr>
<tr>
<td></td>
<td>Lambic ale [25]</td>
</tr>
<tr>
<td></td>
<td>Coolship ale [26]</td>
</tr>
<tr>
<td><strong>Other fermented foods/beverages</strong></td>
<td>Cider [27]</td>
</tr>
<tr>
<td></td>
<td>Kombucha [28]</td>
</tr>
<tr>
<td></td>
<td>Kefir [29]</td>
</tr>
<tr>
<td></td>
<td>Sourdough [30]</td>
</tr>
<tr>
<td><strong>Non-fermentation</strong></td>
<td>Soft drink [31]</td>
</tr>
</tbody>
</table>

**Brettanomyces flavor impacts on fermented beverages**

The phenolic compounds produced by *Brettanomyces* are the primary contributors to its characteristic flavor profile. *Brettanomyces* differs from *Saccharomyces* in its ability to convert vinylphenols into their ethyl derivatives. Hydroxycinnamic acids (HCAs) are precursors to these volatile phenols. The metabolic pathway by which HCAs are converted into volatile phenols is shown in Figure 3.
The primary HCAs that are found in wort are p-coumaric acid and ferulic acid. These mainly come from the cereal grains and malts used in brewing [5]. Increased levels of ferulic acid, p-coumaric acid, and other HCA precursors allow for higher final concentrations of volatile phenols.

Amongst brewing strains of *Saccharomyces*, a major distinguishing feature is whether they have active phenol decarboxylase and are able to make vinyl derivatives of these precursors, 4-vinyl phenol and 4-vinyl guaiacol. These contribute a spicy, clove, phenolic aroma to beers [3]. Strains that have this capacity are considered “phenolic off-flavor” positive (or POF+), even though they may be intentionally used to make styles such as hefeweizens or saisons where clove-like aroma is desirable. The majority of brewing strains are actually POF-.
By contrast, to date nearly all known *Brettanomyces* are POF+ and in fact are also positive for vinyl phenol reductase, an enzyme that allows them to reduce the vinyl derivatives into their ethyl derivatives [7]. Compounds like 4-ethylphenol or 4-ethylguaiacol produce woody, stable, leather aromas [4]. Table 2 below goes into more detail on the sensory characteristics of these different compounds. There are only a few other known yeast organisms that are able to transform HCAs into ethyl derivatives. *P. guilliermondii* is another known yeast that has been found to be a strong 4-ethylphenol producer however only certain strains were able to produce with similar efficiency to *Brettanomyces* [32]. This species is also typically isolated from wines and occasionally soy sauce fermentations [33].

<table>
<thead>
<tr>
<th>Compound</th>
<th>Sensory Characteristics</th>
<th>Threshold</th>
</tr>
</thead>
<tbody>
<tr>
<td>4-vinylphenol</td>
<td>Medicinal, phenolic, spicy</td>
<td>725 $\mu g$ L (in wine)[3]</td>
</tr>
<tr>
<td>4-vinylguaiacol</td>
<td>Clove, curry, Spice</td>
<td>426 $\mu g$ L (in wine)[34]</td>
</tr>
<tr>
<td>4-ethylphenol</td>
<td>Animal, stable, leather</td>
<td>130 $\mu g$ L (in water)[4]</td>
</tr>
<tr>
<td>4-ethylguaiacol</td>
<td>Clove, Woody, Spice, Smoky</td>
<td>25 $\mu g$ L (in water) [4]</td>
</tr>
</tbody>
</table>

Table 2. Sensory characteristics of the four main chemical compounds looked at in this paper. The threshold indicates the concentration at which each chemical compound can be detected by humans in a specified medium.

Under anaerobic conditions, *Brettanomyces* has also been found to produce isovaleric acid which amplifies the perception of volatile phenols and is characterized by a rancid, cheesy flavor [35]. Isovaleric acid can also serve to amplify the sensory perception of volatile phenols even at low concentrations [36].

Relative concentration of various esters also contribute to the overall flavor profile that *Brettanomyces* is capable of producing. *Brettanomyces* produces esters such as ethyl lactate or ethyl caproate, which can contribute to fruity, tropical aromas to beers [9]. *Brettanomyces* also has esterase enzymes that are responsible for the hydrolysis and subsequent decrease in concentration of acetate esters, such as isoamyl acetate or phenylethyl acetate [37]. This could decrease perception of some
aromas such as banana in the case of isoamyl acetate and roses in the case of phenylethyl acetate.

Finally, some *Brettanomyces* strains have been shown to have very active β-glycosidase, which can efficiently access cleave bound flavor compounds glycosidically bound precursors [38].

**Brettanomyces bruxellensis strain-level diversity**

Because of interest in controlling contributions from *Brettanomyces* in a range of fermentation settings, there has been an increase in research studying the genetic diversity among strains. Interestingly, more than one study has shown that strains grouped by genetic similarity often correspond to their original environmental niche [7], [39]. Additionally, it is proposed that hybridization events have led to genetic distinctions between strains [40]. These divisions are thought to have been driven by the challenges presented by different environments.

This was observed in a study done by Colomer et al. where 84 strains of *Brettanomyces* were sequenced. They found that strains isolated from beer made up the majority of two distinct groups that they labeled “farmhouse” and “lambic”. This is in line with other studies that have sequenced high volumes of strains and found genetic groupings that related to isolation origin [39], [40].

There are also phenotypic variations between strains from different origins. It has been seen in multiple studies that a higher tolerance for SO₂, a common wine preservative, is present frequently in wine strains [7], [39]. Farmhouse and lambic strains tend to be efficient at maltose utilization, but less efficient at utilizing sugars like cellobiose which is found more frequently in winemaking environments [7][8].
Figure 4: Phylogenetic tree of Brettanomyces isolates from different origins, based upon whole genome data (reproduced from Colomer et al., 2020). Red arrows indicate isolates used in this thesis research, blue arrows indicate inferred relatedness (from [40],[41]) to isolates used in this research. Original figure caption: Circular cladogram of 84 Brettanomyces species included in this study. Phylogenetic tree was produced by comparison of the predicted proteins in the whole genome of each strain. Strains used as reference genome are marked with (*) Old CRL isolates are marked with (#). Branches are colored according to species, tree nodes according to continent of origin, and metadata layer is colored according to substrate of isolation. Yeasts have been grouped in different genetic clusters according to its location in the tree and genetic cluster names are indicated in the external circle.
Application of *Brettanomyces* for primary fermentation and bottle-conditioning of beer

Recently, there has been increased interest in using *Brettanomyces* as an alternative primary fermentation yeast in inoculated fermentations [42]. There are many aspects of *Brettanomyces* that make it an appealing option for this purpose. They are able to superattenuate beers by utilizing maltose, maltotriose, and glycosidically bound sugars [7][8]. They are also highly resilient to stress conditions which gives them the ability to serve as a bottle conditioning yeast for beers with high ethanol content or low pH [43]. In addition to this, *Brettanomyces* yeasts are able to impart a wide range of flavors ranging from ‘floral’, ‘tropical’, and ‘fruity’ to “animal”, “smoky”, and “leather” [4][5]. These abilities make it a dynamic yeast option for brewers.

*Brettanomyces* is typically not used as a primary fermentation yeast. This is because it tends to be a slower fermenter [44]. However, certain strains are able to produce similar ethanol concentrations to *Saccharomyces* [9]. These fermentations produce elevated concentrations of volatile phenols and esters like ethyl caprylate, ethyl decanoate, and ethyl caproate [9]. Conversely, concentrations of isoamyl acetate and ethyl phenylethyl tend to decrease in primary fermentation [45]

*Brettanomyces* is typically used in the context of secondary fermentation for lambic beers. However it can also be applied as a bottle conditioning yeast for beers with ‘brett’ character. In these secondary fermentation settings, it has the ability to overattenuate the beer due to its ability to degrade complex sugars and its tolerance to very high ethanol concentrations [45][26].

Objectives
Various characteristics between different strains have been investigated in a number of different capacities. Colomer et al. looked at 84 *Brettanomyces* strains in terms of ethanol and acetic acid production, volatile compound production, and various other metrics regarding fermentation. Strain variation has been found to significantly affect flavor metabolite production, carbon utilization, and phenol production [10]. Variances between strains has been found in *S. cerevisiae* and studied much more
thoroughly [11], [12]. These provide a basis for research into differences between different strains of *B. bruxellensis*. There is a distinct lack of research into the behavior of *B. bruxellensis* in primary and secondary fermentation. This paper will build on this knowledge and fill gaps in knowledge surrounding primary and secondary fermentation behavior.
**Manuscript**

**Introduction**

The Brettanomyces genus of yeast was originally encountered in the early 1900’s, identified as responsible for secondary fermentation of English stock ale. Claussen (1904) noted that the yeast he isolated imparted ‘vinous’ flavors that were considered ‘essential for the style’ [1]. Brettanomyces yeasts have since been isolated from a wide variety of environments, such as grape skins, wine barrels, and bioethanol production as well as many fermented beverages like cider, wine, tequila, and beer [2]. The flavor impacts noted by Claussen more than a century ago have come to be recognized near universally as spoilage in wine [3], yet for cider [27] and beer [15] they may denote spoilage or represent an expected component of the style being produced.

Brettanomyces flavor is mainly characterized by contributions from the volatile phenols 4-ethylphenol, 4-ethylguaiacol and 4-ethylcatechol, which impart sensory descriptors such as “animal”, “smoky”, and “leather” [4]. The phenolic compounds produced by Brettanomyces are the primary contributors to its characteristic flavor profile. Brettanomyces differs from Saccharomyces in its ability to convert vinylphenols into their ethyl derivatives. Hydroxycinnamic acids (HCAs) are precursors to these volatile phenols. They are produced when HCA precursors are decarboxylated to their vinyl derivatives and then are reduced by the enzyme vinylphenol reductase to their ethyl derivatives. Amongst brewing strains of Saccharomyces, a major distinguishing feature is whether they have active phenol decarboxylase and are able to make the vinyl derivatives of these compounds. Strains with this ability are referred to as phenolic off flavor positive (POF+). By contrast, to date nearly all known Brettanomyces are POF+ and in fact are also positive for vinyl phenol reductase, an enzyme that allows them to reduce the vinyl derivatives into their ethyl derivatives [7].

Where *Brettanomyces* yeasts naturally play an important role in imparting signature flavors, such as ‘floral’, ‘tropical’, and ‘fruity’ aromas in Lambic beer [5], their contributions beyond volatile phenols have been studied. In these beers, *Brettanomyces* is responsible for producing esters such as ethyl lactate and ethyl acetate
and through glycosidase activity has been shown to release aroma compounds such as vanillin or citronellol from glycosidically bound precursors [37]. The potential for Brettanomyces to be harnessed for beer bioflavoring [47] has seen increased interest in using *Brettanomyces* as an alternative primary fermentation yeast. Many of these traits in primary fermentation can be applied to secondary fermentation and bottle conditioning as well [37]. There are many traits of *Brettanomyces* yeasts that make them appealing option for this purpose, despite their relatively slow fermentation rate [9]. They have the ability to superattenuate beers by assimilating maltose and matotriose[8]. They are also highly resilient to stress conditions which gives them the ability to serve as a bottle conditioning yeast for beers with high ethanol content or low pH [42].

Recently there have been multiple large-scale studies looking into strain variation of *Brettanomyces*. In these studies, various phenotypes relevant to industry such as utilization of maltose and production of volatile compounds have been found to vary between strains of *Brettanomyces* [7]. It has also been found that strains from the same origin tend to group together genetically [7] as well as phenotypically [9]. Strain variation has been found to significantly affect flavor metabolite production, carbon utilization, and phenol production [9]. Variances between strains has been found in *S. cerevisiae* and studied much more thoroughly [48], [49]. These provide a basis for research into differences between different strains and origins within *Brettanomyces*. There is, however, a gap in knowledge surrounding the differences in fermentation behavior and sensory impacts between primary and secondary fermentation behavior. This paper will build on this knowledge and attempt to closely examine the effect that strain has on fermentation behavior by examining strains from different known genetic groups and varying ecological origins.

**Materials and Methods**

**Chemicals**
Unless stated otherwise, all chemicals were obtained from Sigma-Aldrich (St. Louis, MO, USA).
Yeast Strains and Laboratory Media

Reference strains obtained from other collections were included in this study alongside two commercially utilized brewing yeast and three beer isolates of *Brettanomyces bruxellensis* (Table 3). The latter were isolated by streak plating from beer samples onto YPD agar (10 g/L yeast extract, 20 g/L peptone, 20 g/L D-glucose, 15 g/L agar) supplemented with 10 mg/L cycloheximide. Species identity was determined by polymerase chain reaction (PCR) amplification of the internal transcribed spacer ribosomal DNA region and Sanger sequencing [13].

**Table 3.** Genera and numerical identifiers for the 10 yeast strains used in this study. Origins indicate where the yeast was sourced and what medium it was isolated in.

<table>
<thead>
<tr>
<th>Species</th>
<th>Identifier</th>
<th>Other identifiers</th>
<th>Origin</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>S. cerevisiae</em></td>
<td>Y-208</td>
<td>London III</td>
<td>Beer (Wyeast)</td>
</tr>
<tr>
<td><em>B. bruxellensis</em></td>
<td>Y-1</td>
<td>WLP648</td>
<td>Beer (White-Labs)</td>
</tr>
<tr>
<td><em>B. bruxellensis</em></td>
<td>Y-13</td>
<td>CBS74</td>
<td>Beer</td>
</tr>
<tr>
<td><em>B. bruxellensis</em></td>
<td>Y-160</td>
<td></td>
<td>Beer (Sour ale - barrel)1</td>
</tr>
<tr>
<td><em>B. bruxellensis</em></td>
<td>Y-185</td>
<td></td>
<td>Beer (Farmhouse ale - bottle)1</td>
</tr>
<tr>
<td><em>B. bruxellensis</em></td>
<td>Y-190</td>
<td></td>
<td>Beer (Lambic ale - bottle)1</td>
</tr>
<tr>
<td><em>B. bruxellensis</em></td>
<td>Y-8</td>
<td>AWRI1613</td>
<td>Wine</td>
</tr>
<tr>
<td><em>B. bruxellensis</em></td>
<td>Y-16</td>
<td>AWRI1608</td>
<td>Wine</td>
</tr>
<tr>
<td><em>B. bruxellensis</em></td>
<td>Y-78</td>
<td>AWRI1499</td>
<td>Wine</td>
</tr>
<tr>
<td><em>B. bruxellensis</em></td>
<td>Y-63</td>
<td>CBS6055</td>
<td>Soft-drink</td>
</tr>
</tbody>
</table>

1: Isolated in this study

**Yeast Starter Culture Preparation**

Yeast strains were retrieved from cryogenic storage at -80 °C and streaked onto YPD agar. Starter cultures were prepared by transferring single colonies into 5ml YPD broth in 15 ml vented culture tubes (Techno Plastic Products, Trasadingen, Switzerland).

Starter cultures were incubated at 28 °C in a shaking incubator (Lab-Line Instruments, Melrose Park, USA) at 175 RPM until turbid, around 2 days for *Saccharomyces* and around 4 days for *Brettanomyces*, then cell density was assessed using a hemocytometer (VWR, Radnor, USA) and microscope (Leica, Wetzlar, Germany). For the primary fermentation experiment these starter cultures were used
to inoculate 200 mL of wort in 250 mL Erlenmeyer flasks enclosed with 3 plastic bungs and a one-way 15psi check-valve, which were incubated under the same conditions as initial starter cultures and enumerated by hemocytometer once turbid.

**Wort Preparation**

The wort used for both experiments was a lightly hopped wort comprised of 49% Premium Pilsner Malt (Rahr Malting), 49% Maris Otter Malt, and 2% Dark Crystal Malt (both Thomas Fawcett Malting). This was mashed in water supplemented with 56 ppm calcium (from CaCl2 and CaSO4) at 70.2 °C for 45 minutes with a 3:1 water to malt grist ratio. The resultant wort at 13.4 degrees Plato was boiled for 5 minutes then distributed into buckets and frozen. Before use, wort was thawed and mixed, then distributed into 2 L media bottles (VWR, Radnor, USA) and autoclaved.

**Primary Fermentation Experimental Set Up**

For each yeast strain, autoclaved wort (200 mL) was transferred into triplicate 250 mL media bottles and inoculated at a rate of 10^7 cells/mL. Each media bottle was enclosed with an air-tight lid fitted with a one-way 15psi check-valve, and incubated at 22°C in an immersion heated (VWR, Radnor, USA) water bath with recirculating cooling. Fermentations were agitated using stirring plates (Cimarec i, ThermoFisher, Waltham, USA) and 4-point stir-bars. Fermentation progress was monitored by weight-loss (VWR 10001E, VWR, Radnor, USA). Upon completion of fermentation (11 days for *S. cerevisiae*, 30 days for *B. bruxellensis*), vented lids were exchanged for standard media bottle lids and bottles were transferred to 4 °C to cold-settle for 16 days (*S. cerevisiae*) or 5-days (*B. bruxellensis*). On the day of sensory analyses, replicate beers for each yeast were sub-sampled, then carefully decanted to remove yeast slurry and blended together. Fermentation curves were created using the weight loss data, and were modeled using the grofit package in R [50][51].
Secondary Fermentation Experimental Set Up

5 gallons of the lightly hopped wort underwent a primary fermentation using *S. cerevisiae* (White Labs WLP095 Burlington Ale Yeast). This was allowed to ferment at room temperature for seven days until CO2 evolution ceased. This base beer was filtered (Seitz HS2000 and HS400, Pall, New York, USA), primed with 10 g/L of dextrose then bottled in standard 355 mL beer bottles. Bottled beer was pasteurized using a tunnel pasteurizer which was a custom manufactured cascading water pasteurizer utilizing a Yokogawa process logic controller.

For each yeast strain, autoclaved wort (200 mL) was transferred into triplicate 250 mL media bottles and inoculated at a rate of $10^6$ cells/mL. Each media bottle was enclosed with an air-tight lid fitted with a one-way 15psi check-valve, and incubated at 22°C in a large box incubator. Fermentations were agitated using stirring plates (Cimarec i, ThermoFisher, Waltham, USA) and 4-point stir-bars. Fermentation progress was monitored by weight-loss (VWR 10001E, VWR, Radnor, USA). Upon completion of fermentation (19 days for *S. cerevisiae*, 35 days for *B. bruxellensis*), vented lids were exchanged for standard media bottle lids and bottles were transferred to 4 °C to cold-settle for 35 days (*S. cerevisiae*) or 19 days (*B. bruxellensis*). On the day of sensory analyses, replicate beers for each yeast were sub-sampled, then carefully decanted to remove yeast slurry and blended together. Fermentation curves were created using the weight loss data, and were modeled using the grofit package in R [50][51].

Sensory Data Analysis

A sensory panel was recruited consisting of 12 individuals from Oregon State University (7 males and 5 females, age 22–56). The panelists were selected based on previous experience as part of a descriptive panel evaluating the aroma of beer. Training of panelists included Ultra-Flash Profiling and Projective Mapping (or Napping®) in order to assess the aroma of beer samples[52][53]. For sensory evaluation, a room with neutral stimulus was selected, keeping the conditions
consistent across sessions. By means of Ultra-Flash Profiling, a pool of 16 attributes (acidic/sour, animal, band-aid, barnyard, bready, brine, cheesy, earthy/woody, fruity, leather, phenolic, smoky, spicy, sweaty, and vegetal) was developed to best represent the aroma differences between the evaluated beer samples. The attribute ‘acidic/sour’ was included as a generic descriptor for the aroma of sour beer. To familiarize the panelists with this specific pool of descriptors, training was performed using food references or aroma references for each of the selected attributes. For training purposes, a set of four commercially available beers fermented or co-fermented with Brettanomyces yeast strains (Trappist Ale, Brasserie d’Orval, Belgium; Bauernhaus Ensemble, Upright Brewing, OR, USA; Touch of Brett: Citra, Alesong Brewing & Blending, OR, USA and VAT 92 Mono Blend, The Boon Brewery, Belgium) was subjected to Ultra-Flash Profiling and Projective Mapping. For Projective Mapping, panelists carried out spatial placement of the beer samples by utilizing a poster board (22” × 22”) prior to using a digital ballot (Compusense Cloud 21.0.7713.26683, Compusense, Canada). By applying general randomization, 50 mL of each sample were presented simultaneously in 250 mL beer sensory glasses labeled with three-digit random codes. Subsequently, the panelists complemented their spatial placement with a selection of attributes from the agreed-upon pool to describe the aroma of each sample by means of Check-All-That-Apply (CATA)[53]. The CATA results were summarized as overall frequencies for each attribute in decreasing order (Table S1). Based on these frequencies, the percentage of the maximum frequency among attributes was calculated. These results were evaluated by Cochran’s Q test, identifying significant differences in frequency among the samples (p ≤ 0.05). Attributes exhibiting neither significant differences among samples, nor a percentage of ≥ 25% of the maximum frequency among attributes were excluded from further evaluation. The remaining CATA results were evaluated by Correspondence Analysis. In this analysis, the physical proximity of the samples indicates an overall large similarity in all major aroma attributes (N = 9). In addition, a close proximity between samples and attributes indicates that the frequency at which these attributes were selected to describe the aroma of the respective samples was particularly high. The PM sensory results were evaluated by Multiple Factor Analysis and separated the
samples according to the spatial placement by the panelists based on overall
differences and similarities, whereas proximity between samples indicates large
similarities in the aroma.

**Compositional Analyses**

**GC MS Analysis**

**Chemicals**

The following standards were obtained from Millipore Sigma (St. Louis, MO,
USA), 2-methoxy-4-vinylphenol (≥ 98%, CAS#7786-61-0), 4-vinylphenol (10% by
weight, CAS#2628-17-3), 4-ethyl phenol (≥ 98%, CAS#123-07-9) and 4-ethyl
guaiacol (≥ 98%, CAS#2785-89-9). d4-Ethylphenol (98%, CAS#340256-40-8) was
purchased from CDN Isotopes (Pointe-Claire, QC, Canada). HPLC grade ethanol was
purchased from Pharmco-AAPER (Vancouver, WA, USA). Milli-Q water was
obtained from a Millipore Continental water system (XX). Citric acid (monohydrate)
was purchased from Macron Fine Chemicals (VWR International Holdings, Inc, XX)
and sodium chloride from EMD Millipore (Billerica, MA, USA).

**Sample Preparation**

Beer samples were degassed and stored at 4 °C prior to sample preparation 15 ml
culture tubes (Techno Plastic Products, Trasadingen, Switzerland). Each beer sample
(1.65mL) was added to 3.29 mL of matrix (1 g/L citric acid at pH 3.5) in a 20 mL
amber glass, PTFE lined screw cap vials, 22.5 x 75.5 mm (Restek Corporation,
Bellefonte, PA, USA). 50 µL of the internal standard was added to the vial. The
volumes are equivalent to a 3-fold dilution.

**Head-space Solid-Phase-Microextraction (HS-SPME)**

For the headspace-solid phase microextraction, an AOC-5000 plus autosampler
(Shimadzu). A 2 cm Stableflex DVB/CAR/PDMS combination solid phase
microextraction fiber (p/n 57348-U, 50/30 µm, 24 gauge) was used. The fiber was
initially conditioned for 1 hour at 220 °C in the GC injection port. Samples were
incubated for 10 minutes at 60 °C, with agitation at 500 rpm (5 sec on, 2 sec off). The
fiber was inserted into the vial headspace and samples was further extracted for 30
minutes with no agitation. The fiber was inserted into the GCMS injection port for 10
min at 220 °C, followed by further conditioning in an NDL heater for 10 min at 250 °C.

**Gas Chromatography Mass Spectrometry (GCMS)**

This method was adapted from a volatile phenol in wine method used by the Oberholster lab (UC-Davis). All samples were run using a Shimadzu QP2010 GCMS (Shimadzu, Columbia, Maryland, USA). Two columns connected in series with a defined PressFit connector (Restek Corporation, Bellefonte, PA, USA), Rtx-wax column (30 m X 0.25 mm ID X 0.5 µm film thickness, polyethylene glycol, Restek), and a Rxi-1MS column (15 m X 0.25 mm ID X 0.5 µm film thickness, 100% dimethyl polysiloxane, Restek) were used for separation. The carrier gas within the GC was helium at a velocity of 23.5 cm/s. The oven temperature began at 50°C for 5 minutes before being increased to 130°C at a rate of 20 °C/min. It was held there for 1 minute and then increased to 250°C at a rate of 2.50°C/min where it was held for 10 minutes. The total runtime was 60 min. GCMS transfer line was set to 250 °C. The interface temperature was set to 250°C and the IonSource temperature was set to 200°C. Spectra was collected using electron impact ionization (EI 80 eV), using scan mode from 7 – 65 minutes, with a scan range m/z of 33-30.0 at 0.20 sec events. Compounds were identified using the NIST11 spectral library and comparison retention times and mass spectra of pure standards.

**Table 4**: Quantitative parameters for the HS-SPME-GCMS analysis of volatile phenols.

<table>
<thead>
<tr>
<th>Analyte</th>
<th>ITS/D</th>
<th>Ret. Time (min)</th>
<th>Target Ion (m/z)</th>
<th>Confirming Ions (m/z)</th>
<th>Calibration Range (µg/L)</th>
<th>Std. Curve R²</th>
<th>LOD (µg/L)</th>
<th>LOQ (µg/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>4-ethyl phenol</td>
<td>1</td>
<td>39.5</td>
<td>111</td>
<td>136</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>4-ethyl guaiacol</td>
<td>1</td>
<td>35.7</td>
<td>137</td>
<td>152, 122</td>
<td>0-175</td>
<td>0.99</td>
<td>0.21</td>
<td>0.70</td>
</tr>
<tr>
<td>4-ethyl phenol</td>
<td>1</td>
<td>39.7</td>
<td>107</td>
<td>122, 77</td>
<td>0-175</td>
<td>0.99</td>
<td>0.65</td>
<td>2.18</td>
</tr>
<tr>
<td>4-vinyl guaiacol</td>
<td>1</td>
<td>40.7</td>
<td>150</td>
<td>135, 107</td>
<td>0-175</td>
<td>0.99</td>
<td>1.79</td>
<td>5.98</td>
</tr>
<tr>
<td>4-vinyl phenol</td>
<td>1</td>
<td>47.3</td>
<td>120</td>
<td>91</td>
<td>0-175</td>
<td>0.99</td>
<td>0.15</td>
<td>0.51</td>
</tr>
</tbody>
</table>
Samples were run in duplicate, and were quantified with stable isotope dilution analysis procedures [54].

Limit of detection (LOD) and limit of quantitation (LOQ) were calculated as in Miller & Miller [55]. Four beer samples were measured at 0, 12, 24 and 48 hours to determine stability and reproducibility. Accuracy of the analytical method was evaluated by calculating the recoveries of the standard addition [56].

High Performance Liquid Chromatography Analysis

Carbohydrate analysis (fructose, glucose, maltose, and maltotriose) was performed on Agilent 1200 series HPLC with a refractive index detector using a Rezex RSO Oligosaccharide Ag+ column. The method used for carbohydrate analysis was adapted from ASBC Methods of Analysis, Sugars and Syrups 18 by Kirkpatrick and Shellhammer in 2018[57]–[60]. The mobile phase was Milli-Q water with a flow rate of 0.3 mL/minute with the column held at 80˚C. The injection volume was 10uL per sample.

Fermentation Curve Fitting

Fermentation curves were fitted using the R package grofit [50] [51], in the statistical language, R [61]. Data was formatted in a csv file with replicates arranged horizontally. Replicates were plotted on the same graph and the aggregate data was used to non-linearly fit each curve. The four models used in the grofit analysis were Logistic, Gompertz, Modified Gompertz, and Richards. The package determined the best fit model for each yeast and reported the parameters A, μ, and λ. These correspond to maximum CO₂ evolved, maximum specific fermentation rate, and length of fermentation lag phase, respectively.

Statistical Analysis

Analysis of the residual sugar data was done using the aov function in RStudio followed by the Sidak method to return p values for each comparison. The
cld function is used to return letter groupings based on statistical significance between pairs. Data was entered in a csv file with replicate data for each sugar analyzed.

The Wilcoxon Rank Sum test was used to determine statistical differences between the beer and “other” samples. The lm function in R was used for linear regressions to compare variables within experiments and determine significance.

Results

Primary Fermentation Kinetics and Chemical Composition

Primary fermentation of wort proceeded at a significantly faster rate for *S. cerevisiae* than for any of the *B. bruxellensis* strains (Table 5). The fermentation parameters showed distinct groupings between strains in terms of the total CO₂ evolved. The highest producers were Y160, Y13, and Y185 with Y160 being the highest reaching 76.7 g/L. Y8 and Y190 were distinctly the lowest producing strains both having a total CO₂ evolution lower than 20 g/L. The remaining strains, including *Saccharomyces*, reached more intermediate values ranging from 51.8 to 33.8 g/L. The

<table>
<thead>
<tr>
<th>Sample</th>
<th>Origin</th>
<th>Maximum Specific Fermentation Rate [g/L-day]</th>
<th>Total CO₂ Evolution [g/L]</th>
</tr>
</thead>
<tbody>
<tr>
<td>208</td>
<td><em>S. cerevisiae</em></td>
<td>31.6 ± 9.9&lt;sup&gt;a&lt;/sup&gt;</td>
<td>47.7 ± 9.3&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>1</td>
<td>Beer</td>
<td>3.90 ± 0.48&lt;sup&gt;bc&lt;/sup&gt;</td>
<td>51.8 ± 2.4&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>13</td>
<td>Beer</td>
<td>3.90 ± 0.31&lt;sup&gt;bc&lt;/sup&gt;</td>
<td>69.3 ± 2.6&lt;sup&gt;ab&lt;/sup&gt;</td>
</tr>
<tr>
<td>160</td>
<td>Beer</td>
<td>2.94 ± 0.06&lt;sup&gt;bd&lt;/sup&gt;</td>
<td>76.7 ± 7.9&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>185</td>
<td>Beer</td>
<td>2.54 ± 0.19&lt;sup&gt;bd&lt;/sup&gt;</td>
<td>64.4 ± 3.5&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>190</td>
<td>Beer</td>
<td>2.13 ± 0.93&lt;sup&gt;bd&lt;/sup&gt;</td>
<td>19.6 ± 9.8&lt;sup&gt;e&lt;/sup&gt;</td>
</tr>
<tr>
<td>63</td>
<td>Soft Drink</td>
<td>2.82 ± 0.41&lt;sup&gt;bd&lt;/sup&gt;</td>
<td>36.2 ± 1.4&lt;sup&gt;d&lt;/sup&gt;</td>
</tr>
<tr>
<td>8</td>
<td>Wine</td>
<td>2.29 ± 2.2&lt;sup&gt;b&lt;/sup&gt;</td>
<td>15.1 ± 11&lt;sup&gt;g&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

Table 5. Primary fermentation parameters. Parameter values derived from non-linear curve fitting for each fermentation replicate (n=3). Values are mean +/- 95% confidence intervals derived through curve-fitting replicate fermentation data. Different letters within column denotes significance difference according to the 95% confidence interval.
maximum fermentation rate showed much less variability than the total CO$_2$ evolved with the only standout being *Saccharomyces* with a maximum specific fermentation rate around 10 times larger than the next fastest strain. The *Brettanomyces* strains remained between 3.9 and 2.13 g/L-day with variability that caused significant overlap between rate parameters.

The residual sugar concentrations (Table 6) displayed correlations with the fermentation parameters. A negative correlation is seen between total CO$_2$ evolved and residual maltotriose ($p = 0.0109$), maltose ($p = 0.0155$), and glucose ($p = 0.0354$) (Supplemental Figure 5). These correlations check out with the residual sugar data. Strains Y63, Y8, Y16, and Y190 have the highest amount of residual sugars and are all on the lower end of CO2 produced. The maximum fermentation parameter was also seen to correlate negatively with residual maltotriose concentrations ($p = 0.0401$) (Supplemental Figure 5). The general groupings in the sugar data were consistent for each different type of sugar except fructose where strain 78 had significantly higher residual sugar than the other strains. It is also of note that when the *Brettanomyces* strains were grouped by origin into “beer” and “other” the residual maltotriose and maltose concentrations displayed significant differences between the groups ($p = 0.0342$ and $p = 0.0366$ respectively).

Table 6. Primary fermentation residual sugar concentrations. Concentrations are given in g/L. Values are mean +/- standard deviation, different letters within column denotes significance difference according to sidak’s post-hoc test ($p<0.05$).
The primary fermentation data had quite a bit of variability in the volatile phenol data. For the total volatile phenols produced, Y16 and Y208 were the only significantly different strains with Y16 being the highest and Y208 being the lowest. For 4-vinyl guaiacol, and both ethyl compounds, there are consistent similarities between strains Y8, Y16, Y63, Y160, and Y190 as the higher producing strains. Y1, Y13, and Y185 regularly show up as the lower producing strains. Y208 is the lowest producing strains for all compounds except 4-vinyl phenol where Y185 was the lowest.

<table>
<thead>
<tr>
<th>Strain</th>
<th>Product</th>
<th>Concentration (µg/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>160</td>
<td>Beer</td>
<td>0.39 ± 0.04a</td>
</tr>
<tr>
<td>185</td>
<td>Beer</td>
<td>0 ± 0</td>
</tr>
<tr>
<td>190</td>
<td>Beer</td>
<td>17 ± 1.7b</td>
</tr>
<tr>
<td>63</td>
<td>Soft Drink</td>
<td>18 ± 1.0b</td>
</tr>
<tr>
<td>8</td>
<td>Wine</td>
<td>18 ± 0.7b</td>
</tr>
<tr>
<td>16</td>
<td>Wine</td>
<td>18 ± 0.3b</td>
</tr>
<tr>
<td>78</td>
<td>Wine</td>
<td>1.8 ± 1a</td>
</tr>
</tbody>
</table>

For 4-ethyl guaiacol, 4-ethyl phenol, and 4-vinyl guaiacol, the concentration data is shown in the graphs. The concentration values for each strain and product are indicated. The graphs also show the origin of the products: beer, wine, and soft drink.
Figure 5. Concentrations of volatile phenols in primary fermentation samples. Data was collected using a gas chromatograph. Bars represent the average concentration between each replicate with error bars showing standard deviation between replicates.

Primary Fermentation Sensory Analysis

As shown by CA (Supplementary Figure 3A), the Saccharomyces control sample Y208, which is located in proximity to the attribute bready in the upper right corner of the plot, shows a clear separation from all other beer samples, which was to be expected. This observation was verified by the MFA of the PM results (Supplementary Figure 3B), which also clearly separated sample Y208 from all other beers in the bottom right corner of the plot. This indicates that fermentation of the identical wort with Brettanomyces yeast strains compared to fermentation with Saccharomyces yeast leads to significantly different aroma properties, as is generally recognized. As a result, the Saccharomyces control sample Y208 was excluded from further evaluation of the differences among the beers fermented with Brettanomyces yeast strains of different origin [Figure 6].

There were clear differences among the samples fermented with Brettanomyces strains and some of the differentiation correlated with the origins of the strains. For example, among the wine-derived strains, the descriptors barnyard, earthy/woody, and brine were predominant. In contrast there was less consensus and greater variation among the beer-derived strains. For instance, the aroma of Y13 was mostly defined by the attributes animal and sweaty, whereas samples Y1 and Y160 primarily had an acidic/sour aroma. The aroma of sample Y185 was also described as cheesy. The strongest fruity aroma however was observed for sample Y63, which was fermented with a soft drink-derived strain. The MFA of the PM results, in total covering 66.32% of the variation in the dataset overall showed comparable results, displaying samples Y13, Y78, and Y185 as relatively similar in both statistical analyses. The same was observed for the samples Y16 and Y63 as well as Y8 and Y190. In contrast to the CA, however, the MFA separated sample Y1 from Y63 and Y160. This might have been a result of similar aroma qualities as detected by CATA (CA) but differences in aroma
intensity between these samples, which were only detected by PM (MFA) in the form of overall aroma differences.

Figure 6. Correspondence Analysis of the CATA sensory results (A) and Multiple Factor Analysis of the Projective Mapping sensory results (B) for the Brettanomyces main ferments excluding the Saccharomyces control.

Secondary Fermentation Kinetics and Chemical Composition

For the bottle conditioning experiment, the fermentation data showed moderate variation between strains, but the two model parameters typically clustered very closely together. For the maximum fermentation rate parameter $Y_{208}$ (Saccharomyces) was the quickest by far with $Y_1$, $Y_{13}$, and $Y_{160}$ trailing by around 0.45 g/L-day. The slowest two strains were $Y_8$ and $Y_{78}$ at 0.38 and 0.37 g/L-day respectively. The total CO$_2$ evolved definitively placed $Y_{190}$ and $Y_{63}$ as the lowest producing strains with $Y_{13}$ slightly outperforming. $Y_{16}$ and $Y_{160}$ were the top two strains with all of the other strains varying by about 2 g/L.

Table 7. Secondary fermentation parameters. Parameter values derived from non-linear curve fitting for each fermentation replicate (n=3). Values are mean +/- 95% confidence intervals derived through curve-fitting replicate fermentation data. Different letters within column denotes significance difference according to the 95% confidence interval.
For the residual sugar data, significant variation was seen in levels of residual maltose. Y63 had the largest residual maltose quantity and was significantly larger than the other strains for glucose and fructose as well. Y190 and Y8 had the next largest residual maltose levels. Y16 had no residual maltose and was significantly different from all the other strains. *Saccharomyces* was near the middle of the data in terms of residual maltose with 4 strains leaving more behind and 5 leaving less behind. When the secondary strains were grouped based on isolation origin, it was found the maximum specific fermentation rate and residual maltotriose concentrations differed significantly ($p = 0.0158$ and $p = 0.0316$, respectively) between the “beer” and “other” groups.

Table 8. Secondary fermentation residual sugar concentrations. Concentrations are given in g/L. Values are mean +/- standard deviation, different letters within column denotes significance difference according to sidak’s post-hoc test ($p<0.05$).
In the bottle conditioning experiment, strains 63 and 8 display significant differences from the others in terms of total volatile phenols as well as ethyl derivatives of the volatile phenols. 8 had significantly higher amounts of ethyl phenol than all other strains, and 63 had elevated amounts of 4-ethyl guaiacol and 4-vinyl guaiacol. There was some difference between the other Brettanomyces strains, but for the most part these remaining strains were closely grouped by the amount of volatile phenols they produced. The main differences were seen in the 4-vinyl guaiacol group where 1, 13, and 16 were the lowest producing Brettanomyces strains. 185, 190, 8, and 160 are the mid-range for this compound. Saccharomyces was significantly different from the other strains in terms of both of the ethyl volatile phenols and overall production of volatile phenols, but was grouped with other strains for the vinyl derivatives. There was no apparent correlation between the fermentation rate parameter and the total amount of volatile phenols produced nor between the total amount of their vinyl or ethyl derivatives (Supplemental Figure 6).

<p>| | | | | | |</p>
<table>
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</thead>
<tbody>
<tr>
<td>190</td>
<td>Beer</td>
<td>4.7 ± 0.29&lt;sup&gt;a&lt;/sup&gt;</td>
<td>2.4 ± 0.24&lt;sup&gt;c&lt;/sup&gt;</td>
<td>0 ± 0&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0 ± 0&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>63</td>
<td>Soft Drink</td>
<td>5.1 ± 0.26&lt;sup&gt;a&lt;/sup&gt;</td>
<td>3.4 ± 0.13&lt;sup&gt;d&lt;/sup&gt;</td>
<td>0.84 ± 0.15&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.76 ± 0.18&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>8</td>
<td>Wine</td>
<td>18 ± 23&lt;sup&gt;a&lt;/sup&gt;</td>
<td>2.4 ± 0.1&lt;sup&gt;c&lt;/sup&gt;</td>
<td>0 ± 0&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0 ± 0&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>16</td>
<td>Wine</td>
<td>5.5 ± 0.4&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0 ± 0&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0 ± 0&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0 ± 0&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>78</td>
<td>Wine</td>
<td>5.6 ± 0.1&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.87 ± 0.11&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>0 ± 0&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0 ± 0&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
</tbody>
</table>
Secondary Fermentation Sensory Analysis

Similar to the sensory results of the primary ferments, CA and MFA of the secondary ferments revealed a clear separation of the *Saccharomyces* control Y208 from the other samples. As a result, an additional CA (Figure 8A) and MFA (Figure 8B) were performed excluding the control sample.

This CA showed overall the same amount of aroma variation between the different *Brettanomyces* strains compared to the primary ferments. Furthermore, there was no clear separation between the strains of different origins. Among the strains originating from beer, for example, Y185 led to a vegetal and sweaty aroma, whereas Y1 and Y13 were characterized as acidic/sour and to a lesser extend also earthy/woody. In contrast, strain Y190 resulted in a complex phenolic, spicy, and bready aroma.
Similarly, the aroma properties produced by the strains originating from wine or soft drink were spread across a wide spectrum. Sample Y8, which was the high 4-ethyl phenol and 4-ethyl guaiacol producer, was separated from all other samples exhibiting a barnyard aroma. After primary and secondary fermentation, several samples showed a comparable amount of similarities (e.g. Y13 and Y78) or similar aroma properties (e.g. Y185). However, this was not the case for all Brettanomyces strains. As examples, strain Y8 resulted in an earthy/woody and bready aroma after primary fermentation but resulted in a dominant barnyard aroma after secondary fermentation. Similarly, strain Y63 produced a fruity aroma during primary fermentation but a brine and slightly vegetal aroma during secondary fermentation. The MFA of the PM results, which covered 45.53% of the total variation, mostly verified these findings further emphasizing strain Y8 as being separated from all other strains. As an exception, samples Y63 and Y185 were identified as relatively similar by CA but different in comparison by MFA, which might have been a result of similar aroma qualities as detected by CATA (CA) but differences in aroma intensity between these samples, which were only detected by PM (MFA) in the form of overall aroma differences.

**Figure 8.** Correspondence Analysis of the CATA sensory results (A) and Multiple Factor Analysis of the Projective Mapping sensory results (B) for the Brettanomyces secondary ferments excluding the Saccharomyces control.
Similar to the results of the primary ferments, secondary fermentations with the analyzed *Brettanomyces* strains produced significantly different aroma results compared to the *Saccharomyces* control, which were comparable in scale between both experiments. Furthermore, the variations in aroma characteristics caused by the different *Brettanomyces* strains were of similar magnitude for primary and secondary ferments but, although comparable for most *Brettanomyces* strains, the aroma properties were qualitatively different between the two fermentation types in some cases, and panelists noted that the aromas from the secondary fermentations were more subtle than those derived from the primary fermentations. Aroma differences between primary and secondary fermentation types might have been a result of metabolites from the *Saccharomyces* primary fermentation that may have influenced the metabolism of the *Brettanomyces* strains used for secondary fermentation. This potential interaction was absent during the trial involving only the primary fermentations.

**Discussion**

*Brettanomyces* has typically operated in the capacity of a secondary fermentation yeast. It is intentionally used in farmhouse style beers, and typically makes its appearance after the majority of the easily consumable sugars have been metabolized and there are higher concentrations of ethanol [2][6]. *Brettanomyces* has also been known to superattenuate beers through its ability to assimilate more complex sugars that *Saccharomyces* is unable to [15][43]. Results from this study indicate that these unique abilities as highly attenuating secondary fermentation yeasts are strain dependent. There were some differences in ability to ferment between secondary and primary fermentation. These differences in fermentation were seen for a number of different strains, but there were many strains that behaved relatively similarly between the two experiments. The fermentation data shows that strains Y160, Y185, Y13, and Y1 are consistently high performers between the two
experiments with the higher specific fermentation rates and CO₂ evolution. This trend holds up for the sensory data where the strains continue to be placed in relatively similar locations between experiments. There are certain differences in the volatile phenol data, for example Y160 is a relatively high producer of 4-ethyl and 4-vinyl guaiacol in the primary fermentation, but ranks much lower in the secondary fermentation. The volatile phenol data is distinctly different between the primary and secondary fermentation experiments for many strains. In primary, there are 4 strains that are relatively high producers, Y8, Y16, Y160, and Y190, but in secondary fermentation Y8 and Y63 are the primary high producing strains with the other strains producing very similar amounts of volatile phenols. However, the secondary fermentations produced consistently higher amounts of volatile phenols while the primary fermentations had a wide range, but overall lower amounts. This could be due to the fact that in secondary fermentation, the yeast are being pitched into a fermented beer medium where *Saccharomyces* has already had the chance to produce vinyl volatile compounds. This gives the secondary fermentations a head start on producing ethyl compounds.

Different levels of artificial selection in *Brettanomyces* strains could also be an explanation for the strain differences found. Beer isolated *Brettanomyces* strains have been found to have more convergence than wine strains which is likely due to artificial selection through years of “intentional” use in farmhouse beer environments [7]. Conversely, wine strains have been combated through various techniques which has led to divergences where strains developed different survival mechanisms. There is also evidence for strain evolution based on origin in *S. cerevisiae* [49][48]. *Saccharomyces* has been used much more intentionally in a number of different fermentation contexts. Because of this, there are distinct phenotypic differences between strains isolated from different origins such as beer strains were found to be better at growing on maltotriose than wine strains or wine strains being more SO₂ tolerant [48]. This study found similar correlations among certain traits. Primary fermentation data showed differences between beer and “other” strains in residual maltose and maltotriose concentrations. This is corroborated by other experiments that have found that beer strains are more efficient at consuming maltotriose and
other complex sugars [62]. The secondary fermentations showed that fermentation rate for the beer strains was significantly higher than the “other” strains. These results agree with previous studies which have found poor attenuation of wort by wine isolated strains [9]. Interestingly, the wine strains evolved much more CO₂ in the secondary fermentation. There were two strains that consistently behaved more closely to the other group than their own. These were Y190 and Y78. Y190 frequently behaved more similarly to the “other” strains than the beer strains in both primary and secondary fermentation results. Y78 fermented better than the other wine strains and had sensory characteristics similar to the beer strains. The splitting of strains with the flipping of Y78 and Y190 can also be seen in the primary sensory correspondence analysis where the “other” strains and Y190 group on the upper left diagonal of the plot, and Y78 and the beer strains group on the lower right diagonal.

Volatile phenol production did not seem to correlate with strain origin. This is similar to findings from Tyrawa et al. who found differences in ester production but not in volatile phenol production [9]. Strains from both origin groups produced sensory compounds to varying degrees with no discernable pattern.

Research into the effects that fermentation environment and isolation origin of yeast play in fermentation outcomes has been increasing in recent years. However, there is still much to be uncovered. This study has broadened the current knowledge base to include information about secondary fermentation behavior, and how it differs from primary fermentation. This study could be broadened by investigating a wider range of yeast strains, larger scale fermentations, or by gathering more data about the chemical make up of the final fermentations.

Final Conclusions and Future Directions

This thesis has found distinct differences between beer and “other” strains for fermentation behavior characteristics like maltose and maltotriose assimilation as well as CO₂ evolution. Beer strains were more efficient at utilizing more complex sugars and fermenting. There was no discernable pattern that could be found for
volatile phenol concentrations. These results have implications for brewers who are interested in using *Brettanomyces* intentionally in their brewing. The lack of correlation between fermentation and volatile phenol production allows for a variety of fermentation abilities and volatile phenol concentrations.

While this thesis was able to explore certain aspects of the comparison between primary and secondary fermentation, there is room to expand on what has been looked at. One aspect that would be interesting to explore would be looking into primed and unprimed secondary fermentations. Future researchers could also branch out past *B. bruxellensis* to get a wider view of the entire genera. Certain papers have begun to explore the genera on a genetic level and through microarray growth experiments, but there is limited information on the behavior of these strains in a true fermentation setting. This study did not find a correlation between volatile phenol production and isolation origin, however, looking further into the chemical composition of samples could turn up correlations between fatty acids or esters that are known to be produced by *Brettanomyces*. 
References


10.1002/jib.565.


[60] “ASBC Method of Analysis: Sugars and Syrups 18 Fermentable carbohydrates by cation exchange HPLC.”


Appendix

\[ \text{CO}_2 \text{ evolved [g/L]} \]

\[ \text{Time (days)} \]

![Graphs showing \( \text{CO}_2 \) evolved over time for different samples Y1, Y8, Y13, Y16, Y63, Y78, Y160, Y185, and Y190.](#)
Supplemental Figure 1: Growth curves for the primary fermentation experiment generated using the R package grofit [50]
**Supplemental Figure 2**: Growth curves for the secondary fermentation experiment generated using the R package grofit [50]
Supplementary Table 1. Frequency of the aroma attributes selected by the panelists (N=12) during CATA sensory evaluation of the beer samples fermented with different strains of Brettanomyces or a Saccharomyces control yeast.

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*Significant differences among samples according to Cochran’s Q test (p = 0.05)

Supplementary Figure 3. Correspondance Analysis of the CATA sensory results (A) and Multiple Factor Analysis of the Projective Mapping sensory results (B) for the Brettanomyces main ferments including the Saccharomyces control.
Supplementary Table 2. Frequency of the aroma attributes selected by the panelists (N=12) during CATA sensory evaluation of the beer samples obtained by secondary fermentation with different strains of *Brettanomyces* or a *Saccharomyces* control yeast.

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*Significant differences among samples according to Cochran’s Q test (p = 0.05)

Supplementary Figure 4. Correspondance Analysis of the CATA sensory results (A)
and Multiple Factor Analysis of the Projective Mapping sensory results (B) for the *Brettanomyces* secondary ferments including the *Saccharomyces* control.

**Supplementary Figure 5**: Scatterplot matrix for the primary fermentations. R value is displayed on each panel. Red overlay indicates statistical significance (p < 0.05).
Supplementary Figure 6: Scatterplot matrix for the secondary fermentations. R value is displayed on each panel. Red overlay indicates statistical significance (p < 0.05).