Effects of Yeast Type and Environmental Factors on Kombucha Fermentation

by
Julianna Winding

A THESIS

submitted to

Oregon State University

Honors College

in partial fulfillment of
the requirements for the
degree of

Honors Baccalaureate of Science in Public Health
(Honors Scholar)

Presented March 14, 2024
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AN ABSTRACT OF THE THESIS OF

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Abstract approved: _____

Chris Curtin

Kombucha is a fermented sweet tea beverage of Asian origin which has become popular in the West due to its distinct flavor and possible health benefits. Kombucha is fermented by inoculating sweet tea with yeast and bacteria until a pellicle, referred to as a SCOBY (symbiotic culture of bacteria and yeast) forms.

Examining studies that have been conducted on SCOBY growth, discrepancies exist between how much pellicle, if any, has been produced by yeast types alongside a variety of environmental factors, such as sucrose concentration, surface area available for oxygenation, tea type used for inoculation, and initial pH adjustment with acetic acid. To determine the significance of these environmental factors as well as yeast type on pellicle growth and pH drop, a full factorial experimental design for 14 day fermentations was created to examine the significance of said environmental factors with the usage of *Brettanomyces bruxellensis (B. brucellensis)* or *Zygosaccharomyces bisporus (Z. bisporus)* with *Komagataeibacter intermedius.*

Final pellicle mass and pH samples taken at days 0, 3, 7, 10, and 14 were statistically analyzed using a linear model to determine the significance of cofactors. It was found that sucrose concentration and initial pH adjustment with acetic acid were significant for acidification, while sucrose concentration, initial pH adjustment with acetic acid and tea type were all significant for final pellicle mass. Yeast type between *Brettanomyces bruxellensis* and *Zygosaccharomyces bisporus* was not found to be statistically significant for pH drop or pellicle mass.

Keywords: Kombucha, Biofilm, SCOBY, Pellicle, Environmental Factors

Corresponding e-mail address: windingj@oregonstate.edu

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APPROVED:

_____

Chris Curtin, Mentor, representing Department of Food Science and Technology

_____

Si Hong Park, Committee Member, representing Department of Food Science and Technology

_____

Alyssa Thibodeau, Committee Member, representing Department of Food Science and Technology

_____

Toni Doolen, Dean, Oregon State University Honors College

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.

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Julianna Winding, Author

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I would like to thank my mentor Dr. Christopher Curtin as well as Alyssa Thibodeau for all the time and effort they have put forth in supporting me throughout the entire process of creating and executing this thesis. They have gifted me with so much knowledge and encouragement for which I am beyond grateful. Additionally, I would like to thank all the members of the Curtin Lab for their support and community throughout this process.

CONTRIBUTION OF AUTHORS

Dr. Christopher Curtin and Julianna Winding were involved in the creation of the experimental design.

Julianna Winding set-up fermentations and was responsible for necessary samplings and subsequent fermentation observation (enumeration, pH measurement, SCOBY sampling, optical density reading, etc.). Statistical analysis was performed by Alyssa Thibodeau.

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1. INTRODUCTION

Kombucha is a fermented sweet tea beverage of Asian origin which has become popular in the West due to its distinct flavor and possible health benefits (Coelho et al., 2020). Although traditionally fermented with black tea, a variety of teas can be used to make kombucha (Rodrigues et al., 2018). Considered to be a 'functional beverage', kombucha boasts antioxidant, anti-inflammatory, and anticancer properties (Kitwetcharoen et al., 2023). Due to its rising popularity, the beverage is being bottled and manufactured on an industrial scale, demonstrating a need for further research and innovation in its production (Kim & Adhikari., 2020).

The chemical components of kombucha include several organic acids, amino acids, ethanol, sugars, lipids, caffeine, carbon dioxide, and assorted bacterial metabolites (Bishop et al., 2022). During the fermentation process depicted in Figure 1, sucrose is broken down by yeast and converted into monosaccharides, namely glucose and fructose (Tran et al., 2021). Acetic acid bacteria (AAB) work to metabolize the products that are broken down by yeast, such as glucose, fructose, and ethanol (Landis et al., 2022). AAB themselves are not capable of utilizing sucrose efficiently prior to its breakdown in fermentation (Landis et al., 2022). Oxygen plays a vital role in the fermentation process, as the production of acetic acids and gluconic acids is carried out by AAB's oxidative metabolism (Tran et al., 2022). Many have suspected that the forming of SCOBY by AAB, which are obligate aerobes, is to control access to oxygen (Iguchi et al., 2000). Overall, the kombucha fermentation system displays both cooperation and competition among microbes existing within a single system (May et al., 2019).

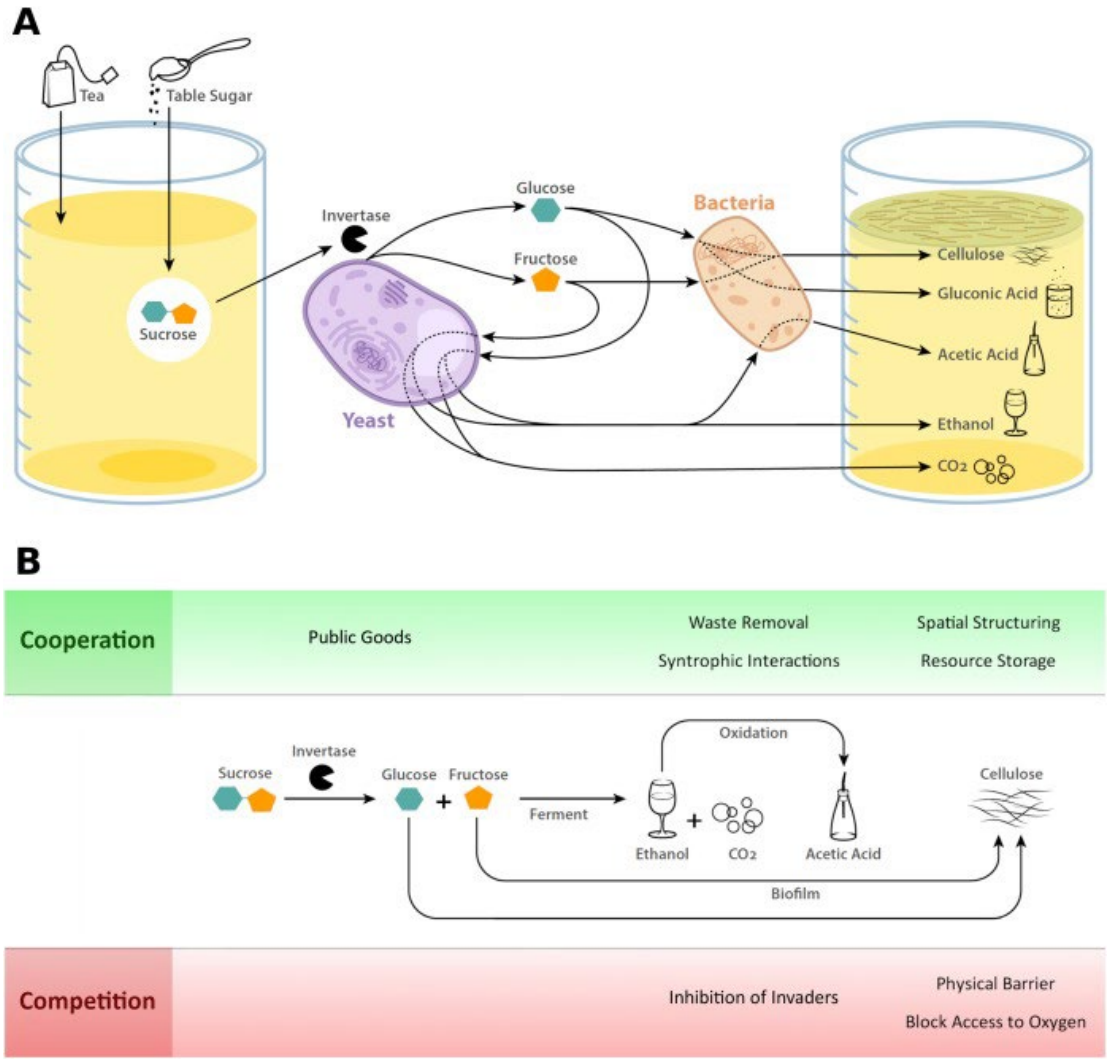

Figure 1. Kombucha fermentation model depicting metabolic interactions between yeast and acetic acid bacteria. Reproduced from May et al. (2019)

Of the several components of kombucha fermentation, acidification marked by pH change can be considered one of the most important (Navarro 2021). The more acids produced by AAB during the fermentation process, the more acidic the kombucha becomes, and the lower the final pH value (Villarreal-Soto et al., 2018). Additionally, pellicle growth is another reliable component of demonstrating fermentation at work (Villarreal-Soto et al., 2018). In additional to acetic acid, AAB oxidate glucose into gluconic acid (Villarreal-Soto et al., 2018). This acid is metabolized through

glycolysis into uridine diphosphate glucose, a well-documented cellulose precursor (Ross et al., 1991). Uridine diphosphate glucose is further polymerized by AAB into β-1, 4-glucan chains, and cellulose is produced (Ross et al., 1991). This form of cellulose production is extremely favorable by bacteria in stable conditions because it can utilize many different carbon sources such as ethanol, sucrose, and glucose (Villarreal-Soto et al., 2018).

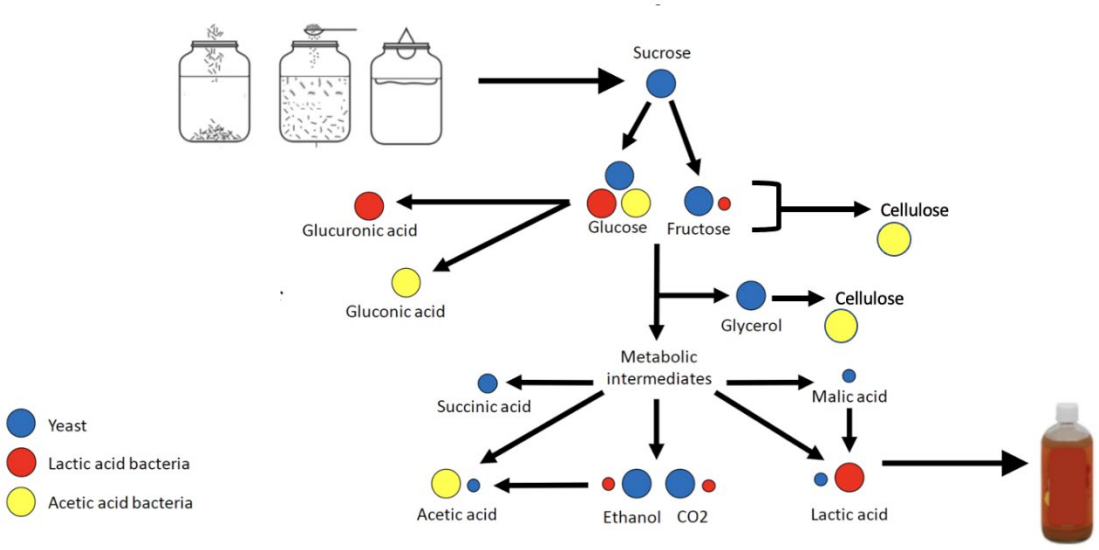

Figure 2. Division of metabolic labor between yeast, lactic acid bacteria and acetic acid bacteria, during kombucha fermentation. Reproduced from Harrison, (2023)

Colloquially, it is speculated that the role of yeast in sucrose metabolization could be interchangeable and is overall more or less nonspecific (Landis et al., 2022). Although the most utilized industrial bacteria and yeast are *Komagataeibacter rhaeticus* and *Brettanomyces bruxellensis*, a wider variety of yeast has been found in kombucha as is shown in Table 2 (Landis et al., 2022).

Table 1. List of key yeast species mentioned in studies of kombucha SCOBY.

Yeast species	**Publications**
Brettanomyces bruxellensis	(Landis et al., 2022; Mukadam et al., 2016; Teoh et al., 2004)
Candida stellata	(Teoh et al., 2004)
Pichia membranifaciens	(Coton et al., 2017; Jarrell et al., 2000; Landis et al., 2022; Mukadam et al., 2016)
Saccharomyces cerevisiae	(Coton et al., 2017; Landis et al., 2022; Mukadam et al., 2016)
Saccharomyces ludwigii	(Jarrell et al., 2000; Landis et al., 2022; Mukadam et al., 2016)
Schizosaccharomyces pombe	(Jarrell et al., 2000; Landis et al. 2022; Mukadam et al., 2016; Teoh et al. 2004)
Torulaspora delbrueckii	(Teoh et al. 2004)
Zygosaccharomyces bailii	(Coton et al., 2017; Landis et al. 2022; Mukadam et al., 2016; Teoh et al. 2004)
Zygosaccharomyces bisporus	(Landis et al., 2022)
Zygosaccharomyces rouxii	(Landis et al., 2022)

Table 2. List of key bacteria species mentioned in studies of kombucha SCOBY.

Bacteria Species	**Publications**
Acetobacter tropicalis	(Coton et al., 2017; Landis et al. 2022)
Gluconobacter oxydans	(Coton et al., 2017; Landis et al. 2022)
Komagataeibacter intermedius	(Dos Santos et al., 2015; Gaggìa et al., 2019; Reva et al., 2015)
Komagataeibacter rhaeticus	(Landis et al. 2022; Machado et al., 2016; Semjonovs et al., 2017; Gaggìa et al., 2019)
Komagataeibacter saccharivorans	(De Filippis et al., 2018; Reva et al., 2015)
Komagataeibacter xylinus	(De Filippis et al., 2018; Reva et al., 2015; Tran et al., 2022)
Lactobacillus nagelii	(Coton et al., 2017; Landis et al. 2022)

Nonetheless, it appears that some yeast may be more suited for fermentation than others, although there have been few studies focused on this (Teoh et al., 2003). For example, contrasting the study of Landis et al. (2022) and Navarro (2021) demonstrate possible effects of yeast type as well as several environmental factors on fermentation (Table 3).

Table 3. Comparison of environmental conditions used in core prior studies of de-novo Kombucha SCOBY formation.

Factors	Landis et al. (2022)	Navarro (2021)
Tea type	Green	Green & black
Sucrose %	10%	2%
Adjusted pH with acetic acid	No	Yes
Surface area for oxygenation	High	Low

Both studies utilize *Komagataeibacter intermedius, Zygosaccharomyces bisporus*, and *Brettanomyces bruxellenis* for their kombucha fermentation. However, Landis et al. observed little to no pellicle growth in their fermentation with *Zygosaccharomyces bisporus* and *Komagataeibacter intermedius*, while Navarro observed very strong pellicle growth with the same combination. The need for further study was apparent of whether the discrepancy in pellicle growth was due to the combination of *Zygosaccharomyces bisporus* and *Komagataeibacter* itself or could perhaps instead be attributed the difference in environmental factors in the two studies. Thus, this study was designed to mimic the fermentation environment of both studies, utilizing both *Zygosaccharomyces bisporus and Brettanomyces bruzellensis* alongside *Komagataeibacter intermedius.*

2. MATERIALS AND METHODS

2.1 Chemicals, laboratory media and microorganisms

All chemical reagents were bought from Sigma Aldrich (St Louis, MO, USA) unless otherwise specified.

Acetic acid (AA) stock solutions were prepared as 10 g/L, stock solution using 95% ethanol as solvent.

Yeast peptone dextrose (YPD) agar for plating were made using 10 g/L of yeast extract, 20 g/L of peptone, 20 g/L D-(+) glucose, and 15 g/L agar with no additional reagents. Ingredients were combined, dissolved in deionized water, and heated until broken down. The mixture was then autoclaved at 250°C at 15 PSI for 15 minutes. Medium 13 (M13) for plating of acetic acid bacteria was made using 25 g/L D-Mannitol, 5 g/L yeast extract, 3 g/L peptone, 15 g/L agar with no additional reagents. Ingredients were combined, dissolved in deionized water, and heated until broken down. The mixture was then autoclaved at 250°C at 15 PSI for 15 minutes. Wallerstein Laboratory Nutrient (WLN) agar with chloramphenicol (CHL) for plating of yeast were made using 80.26 g/L of WL nutrient agar and 10mg/L CHL. WLN was added to deionized water and heated until dissolved. The mixture was then autoclaved at 250°C at 15 PSI for 15 minutes. After autoclaving, CHL was pipetted into the agar once it had cooled to 50°C. Lastly the mixture was gently mixed.

2.2 Growing up of yeast and bacteria

All bacteria and yeast isolates used were supplied from cryogenic storage (-80°C). *Komagataeibacter intermedius* (B14) was grown up on solid M17 + 2% EtOH at 30 °C for 5 days until colonies were visible. *Brettanomyces bruxellensis* (Y459) and *Zygosaccharomyces bisporus* (Y460) were grown on solid YPD at 27°C for 5 days until colonies were visible.

2.3 Starter culture preparations and inoculation of experiments

Both yeasts and bacteria were inoculated using single loops to isolate single colonies. They were put into 5 mL of YPD and M13 respectfully, using 15 mL bioreactor tubes. Liquid re-inoculations were performed in 300mL flasks for yeast and 15 mL bioreactor tubes for bacteria following the initial inoculation with fresh media and ~ 100ul of culture to ensure there would be enough cultures for all the vessels. Cultures were then incubated at 27°C with agitation at 250 rpm until visually turbid.

2.4 Optical density (OD) readings

Optical densities (OD_{600nm}) of starter cultures were determined using low-volume cuvettes (Globe Scientific, Mahwah, New Jersey) and a SpectraMax M2 spectrophotometer (Molecular Devices, San Jose, CA, USA). After determining the quantity of starter culture needed for inoculation of the experiment, the starter cultures were centrifuged for 10 minutes at 3900 rpm. Then, the supernatant was decanted leaving only the pellet. PBS was added to the cells and vortexed until fully homogenized. This process was repeated three times until cells were fully washed of media. After, cells were resuspended in sterile DI water to a normalized optical density and inoculated into the respective media to a target optical density in the experimental tubes (Table 4). The actual inoculated cell densities are also provided, based upon simultaneous enumeration of the starter cultures used for inoculation.

Table 4. Inoculation densities of bacteria and yeast used in experiment.

	Komagataeibacter intermedius (B14)	***Brettanomyces bruxellensis* (Y459)**	***Zygosaccharomyces bisporus* (Y460)**
OD600 normalized	1	10	5
Final OD600 in experiment	0.003	0.17	0.17

Inoculation Densities	$4x10^2$ CFU/mL	$5x10^6$ cells/mL	$7x10^6$ cells/mL

2.5 Preparation of sweetened tea media

Several versions of the sweetened tea media were made, as shown in Table 5 to encompass all combinations of environmental variables. The sweet tea was made using either 25 g/L green tea or a combination of 12.5 g/L of green tea and 12.5 g/L black tea. The tea was steeped in deionized water at 100°C for 10 minutes. D-(+)-sucrose (Avantor, Radnor, Pennsylvania) was added at either 2% or 10%. The mixture was then autoclaved at 250°C at 15 PSI for 15 minutes. After autoclaving and cooling, 2% (v/v) acetic acid was added as necessary. The media was transferred to tubes or flasks for fermentation to provide either low or high surface area for oxygenation.

Table 5. Combinations of sucrose %, tea type, and acetic acid utilized within the experimental design.

Combination	Sucrose %	Tea Type	Acetic Acid Added
1	10%	Green and black	yes
2	2%	Green and black	yes
3	2%	Green and black	no
4	10%	Green and black	no
5	2%	Green	yes
6	10%	Green	yes
7	10%	Green	no

8	2%	Green	no

2.6 Sampling of pH and pellicle

pH samples were taken for all vessels on days 0, 3, 7, 10, and 14 of the experiment. 1000ul of media were pipetted into 2 mL Eppendorf tubes and frozen at -20°C. Samples were then defrosted at room temperature for approximately 1.5 hours and pH was measured using a pH probe. Pellicle mass was measure on day 14. After pH samples were taken, pellicles were placed strainer caps over 15 mL bioreactor tubes for approximately 5 minutes to strain any liquid media and ensure only the pellicle remained. The mass of the pellicles was then taken using a scale.

2.7 Statistical analysis

All statistical analyses and data visualizations were performed in the statistical programming language R v 2023.12.1+402 (RCore Team 2024) using R studio-64 v (RStudio Team 20204). Visualizations of data were generated using the ggplot2 package (Wickham 2009). Statistical analyses were determined using a linear model and Akaike and Bayesian Information Criterion.

3. RESULTS

The combinations of yeast and bacteria were co-inoculated into sweetened tea media to determine whether yeast type alongside environmental factors would affect the pH drop and final pellicle mass of the SCOBY. Because acidification is a key component of fermentation, pH was monitored over the course of the experiment to detect

changes. As seen in figure 2, pH change was much higher when acetic acid was not added. Additionally, there is a noticeable difference in average pH change between the two sucrose concentrations. Those with 10% sucrose tended to have a bigger pH drop. An additional significant set of covariables, as seen in Table 6, were black tea and 10% sucrose, the combination of which also led to a larger pH drop. All other variables (tea type, high or low surface area, yeast type) were not significant at the chosen alpha of 0.05.

Table 6. RStudio statistical analysis p values for pH drop: This table contains the P value outputs of the linear model used to perform statistical analysis, with 95% confidence level. Only statistically significant variables for pH drop were included, with a P value of less than 0.05. This data output was based on the difference between the final pH values taken day 14, and the initial pH values taken on day 0 of the experiment. Of note in this table is the only covariables that were found to be statistically significant, which are green and black tea with 10% sucrose concentration.

Variable	**P value**
Acetic acid adjustment	2e-16
Sucrose concentration	2e-16
Tea type x sucrose concentration	1.1e-9

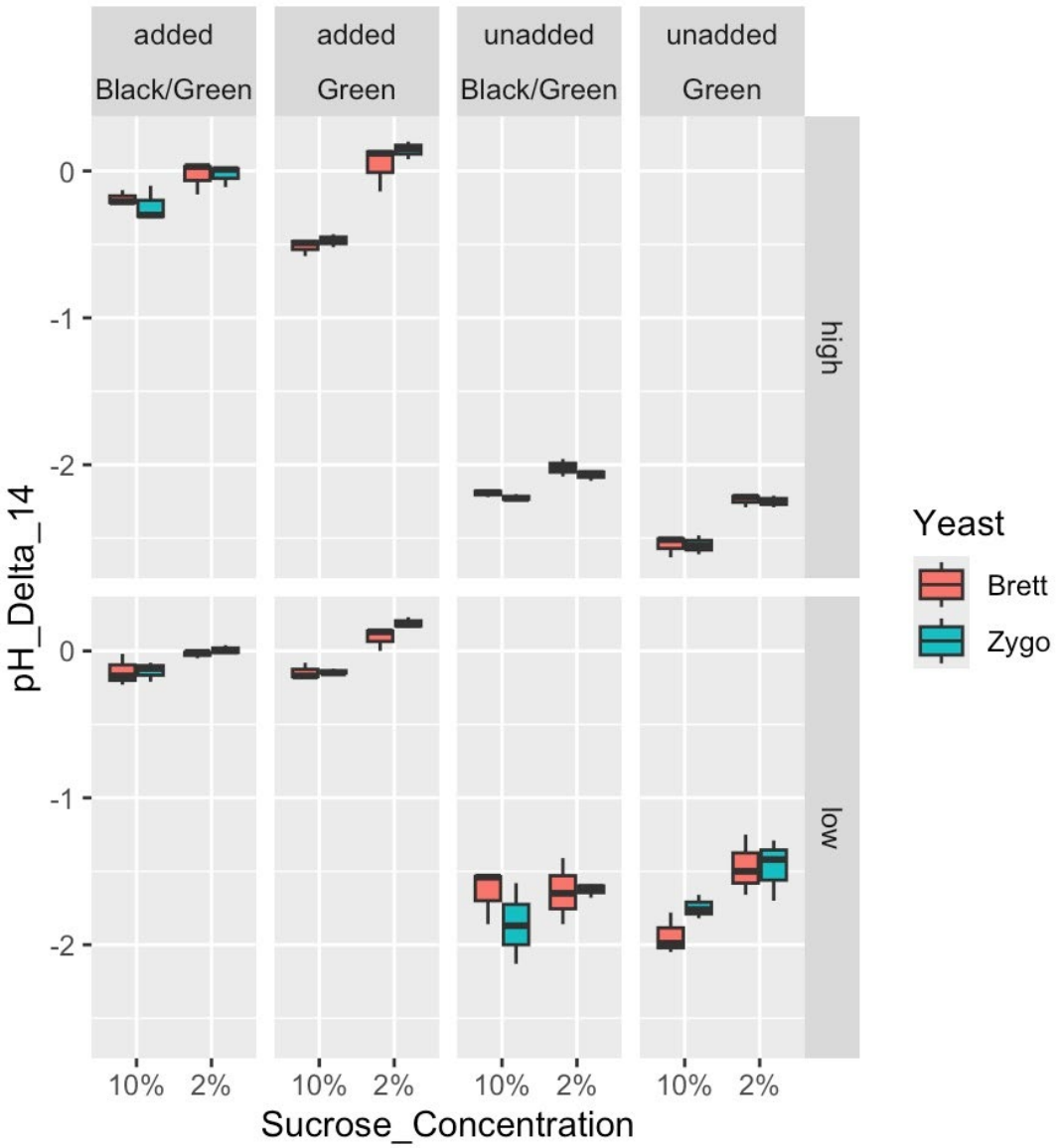

Figure 2. Visualization of pH drop with consideration of variables. The top line represents whether acetic acid was added for initial pH adjustment. The second line represents whether black teas was included in the sweet tea. The bar on the right with "low" and "high" refers to surface area for oxygenation.

In Figure 3, difference in pellicle growth can be seen between high and low surface for oxygenation. This is because, as seen in Table 7, surface area for oxygenation was determined to be statistically significant. Additionally, there are visible differences in

the amount of pellicle growth between 2% and 10% sucrose, as well as between the black and green tea mix and only green tea, both differences supported as statistically significant. Again, yeast type was not significant at the chosen alpha of 0.05.

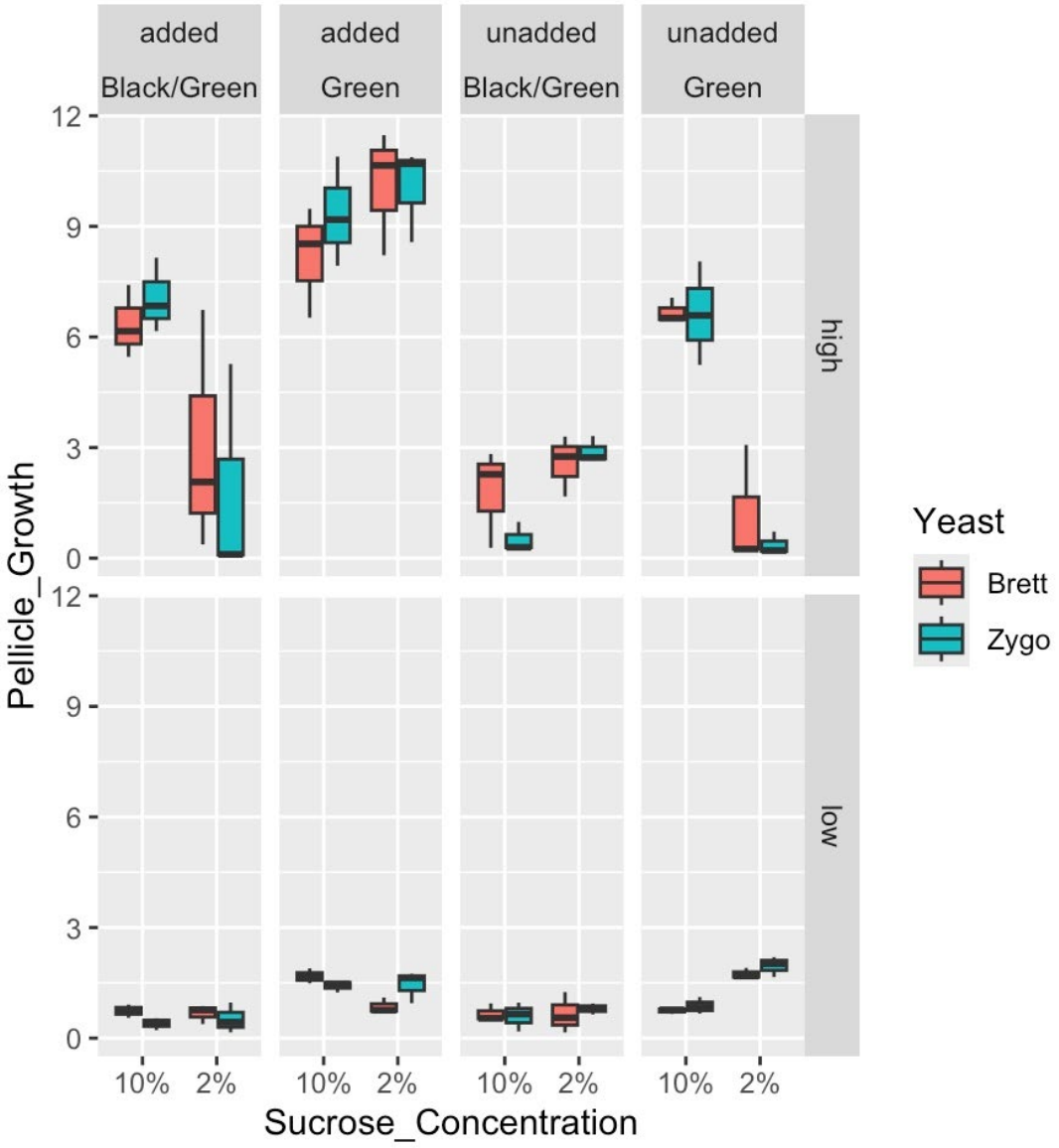

Figure 3. Visualization of pellicle growth with consideration of variables. The top line represents whether acetic acid was added for initial pH adjustment. The second line represents whether black teas was included in the sweet tea. The bar on the right with "low" and "high" refers to surface area for oxygenation.

Table 7. RStudio statistical analysis p values for pellicle mass: This table contains the P value outputs of the liner model used to perform statistical analysis, with 95% alpha significance level. Only statistically significant variables for pellicle mass were included, with a P value of less than 0.001. This data output was based on the final pellicle masses, measure on day 14 of the experiment.

Variable	**P-Value**
Acetic acid adjustment	6.77e-14
Tea type	3.84e-10
Sucrose concentration	0.000235
Surface area for oxygenation	1.25e-12

4. DISCUSSION

While the cooperation of a system of kombucha is vital to fermentation, it is equally necessary to inspect the importance of the environmental factors and yeast types that come into play, as seen in Table 8. In addition to differences in many environmental factors, Landis et al. 2022 and Navarro 2021 also used different strains of *Z. bisporus* and *B. bruxellensis.*

Table 8. Specific yeast strains used by Landis, Navarro, and this study.

	Z. bisporus strains	*B. bruxellensis* strains
Landis	IHD2Y1, DI2Y1, LC2Y1, LCKY1, N2Y1	OY1, UOU2Y1, NH2Y1, DCC2Y1
Navarro	Y460	Y459
This study	Y460	Y459

However, despite the differences in strain type in Landis' study, they still observed standardized behavior of yeast throughout their experiment. This implies that their yeast is still comparable to Navarro and this study despite the individual strain differences.

The statistical analysis performed found that initial pH adjustment with acetic acid was significant for both final pellicle mass and pH drop. However, despite adding 2% v/v acetic acid as outlined by Navarro's methods, initial pH values in this experiment, roughly ~2.8, were much lower than Navarro's initial pH of ~3.88. The lower initial pH in this experiment could have interfered with how much the pH would have dropped if it had been adjusted to Navarro's value, meaning we would have saw a bigger value for the difference between the initial and final pH samples.

Sucrose concentration was also found to be statistically significant for both final pellicle mass and pH drop. This aligns with what has been found in previous literature on kombucha fermentation (Goh, 2012). With higher amounts of sucrose added for fermentation, the system is able to make more glucose and fructose, and eventually cellulose and acids.

Surface area for oxygenation was found to be statistically significant for final pellicle mass, but not pH change. Specifically, a larger surface area for oxygenation was statistically significant for being important to more pellicle growth. This aligns with

results of other studies, as more oxygen is potentially beneficial for fermentation and its indicators (Tran et al., 2022).

The types of tea used were statistically significant for final pellicle mass, but not pH change. Of note is that the only significant covariable combination found from the statistical analysis performed was the interaction between black tea with high sucrose. This could be potentially significant due to an interaction between sucrose and the higher caffeine content of black tea compared to green tea. Caffeine offers yeast and bacteria nitrogen which is necessary for the fermentation process (Ngo 2023).

5. CONCLUSION

This thesis further establishes the notion that the role of yeast in kombucha fermentation is relatively nonspecific, between genus as well as species through the investigation of yeast type and environmental factors on kombucha fermentation. The literature review suggests that the variety of yeast commonly utilized in kombucha demonstrates the interchangeability of types of yeast within fermentation.
Additionally, this thesis suggests that environmental factors such as tea type, pH adjustment, and sucrose concentration have notable effects on kombucha fermentation.
The complex microbial communities in kombucha fermentation are slightly elusive despite the popularity of the beverage and would benefit from further research, especially concerning yeast type and environmental factors.

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