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WPI

North Country Hard Cider Yeast Investigation

A Major Qualifying Project Report

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In partial fulfillment of the requirements for the

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Sponsored by:

North Country Hard Cider Company

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Abstract

This project worked in collaboration with North Country Hard Cider Company, a small batch cidery started in 2014, to investigate the yeast, bacteria, and fungi present at different stages of the brewing process. These organisms were identified through DNA isolation, amplification, purification, and sequencing. The yeasts found in this study are not known to have an ill-effect on the cider brewing process and any inconsistencies in brewing likely rise from bacteria present in samples. This information can be used for further process development and improving product consistency at North Country Hard Cider Company.

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- Ron Dixon and Silas Gordon, sponsor liaisons. Thank you giving us the opportunity to work with your company and on this project.
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Introduction

North Country Hard Cider Company (North Country) is a hard cider brewery located in Rollinsford, New Hampshire. The brewery was established in 2014 by Ron Dixon and Silas Gordon, who were looking to create an all-natural product with no preservatives or added sugars. Between 2014 and 2018, the brewery gained popularity and grew significantly in size. Even though they have gained popularity, in the spirit of continuous improvement, the brewers are continuously analyzing their product and identifying areas of possible improvement.

One of the areas of improvement identified by North Country involves the consistency of fermentation timing across batches of cider, as well as the validation for North Country's problems and solutions. More specifically, North Country anecdotally noticed that the amount of time it takes to ferment their juice varies more in the months that are farther away from the fall; apple season. Although North Country created a solution to combat varying fermentation times, there was no data to support that there was a problem with varying fermentation times, nor that the solution would work.

When attempting to create a solution, North Country knew that fermentation was dependant on yeast, and they were aware of the type and amount of pitched yeast that they added to the batches of juice. However, they were unaware of the natural yeasts that were already occurring in the juice and how these natural yeasts were affecting their process. To combat the unknown yeasts present, North Country slightly changed their process to remove any living organisms before fermentation. However, they have no supporting evidence to prove the change has solved their problem by stabilizing fermentation times.

This project identified yeasts, bacteria, and fungi naturally occuring in the juice that North Country ferments, and provides validation for both their problem and solution. In order to accomplish these goals samples were taken throughout multiple stages of the cider brewing process, yeasts were grown and identified from these samples, and data from the excel sheets of individual batch information was analyzed.

Background

Yeast

Yeast is a single cell fungus that is commonly used in baking for its leavening properties and in brewing for its ability to turn sugars into alcohols. It has been used in food production since 7000 BC. The reaction takes glucose or another simple sugar and creates carbon dioxide and ethanol. Both products of the reaction are the reason for the many industrial uses of yeast. With over 1,500 different species, each has unique characteristics and are used in a variety of applications.

Yeast can be found in many places in the natural world, including, but not limited to, flowers, fruits, plant leaves, soil, digestive tracts of warm blooded animals, and skin. Yeast can also cause infections of the human body, so it can be both harmful and helpful to humans. The first human to ever recognize yeast was Anton van Leeuwenhoek in 1680. By the mid-1800's, yeast was being made commercially and became an essential, easy to acquire ingredient in the food industry. (Nature 537, 2016)

Yeast in North Country Hard Cider Production

North Country utilizes a species of yeast called Saccharomyces cerevisiae, Safale US-05.(Fermentis.) This strain contains the emulsifier E49. It ferments at an ideal temperature between 15°C and 22°C. The balanced chemical reaction of fermentation is a single step process given by the following:

$$C_6H_{12}O_6 \Longrightarrow 2(CH_3CH_2OH) + 2(CO_2)$$

This is the most common species of yeast in the brewing and food industry; it comes packaged as a dry dormant yeast, in order to extend its life span. Usually, this yeast would flocculate, or clump together in the solution. However, the emulsifier E49 is added and used as a deflocculating agent in the dry yeast, allowing the yeast to disperse evenly throughout the solution, and has no negative effect on the brewing process. This agent allows for the dispersion of the yeast into the entire solution. From its product page, US-05 is described as an "American ale yeast producing well balanced beers with low diacetyl and a very clean, crisp

end palate. [It] forms a firm foam head and presents a very good ability to stay in suspension during fermentation." (Fermentis). This allows North Country to create distinct flavors in each of their ciders, while maintaining a consistent base flavor every time, assuming this is the only yeast that is present during fermentation.

Process

North Country's process for creating hard cider begins in upstate New York at Cooper's Farm. Here, apples are grown and stored in cold storage facilities meant to prevent aging. These cold storage facilities allow for apples to be sold to customers year round. Once each storage room is filled with apples, nitrogen is pumped into the room, removing all oxygen. As oxygen is the main cause of aging for apples, storing apples in this room extends the amount of time it takes for the apples to age. However, some yeasts can survive at low temperatures with little oxygen, making these cold storage facilities at Cooper's Farm a possible source of unwanted lager yeast.

As apples are needed by customers, Cooper's Farm will open one cold storage room at a time. All of the apples from the opened room will then be shipped out to customers. For North Country, the apples from a cold storage room at Cooper's Farm are shipped to another location, Gile's Farm, which is located 30 miles north of North Country in Maine. Here, the apples can be stored short term before being pressed into a juiced liquid form. The juice press at Gile's Farm has been in operation for many years, but is thoroughly cleaned at the beginning of every season and incrementally after each pressing. Once juiced, all product goes through a UV filter to remove any bacteria, but not yeast, before being sent to North Country. Theoretically, yeast that is not removed when when the juicer is cleaned can grow and be transported with the juice. Additionally, any yeast that is located in the UV filter can grow and be transported with the juice as well.

Initially, all yeast present in the juice took part in the fermentation process. However, after a recent amendment to their process, the juice is now run through their industrial centrifuge as a first step to their process taking place in Rollinsford, NH. Centrifuging the juice removes all of the oxygen and particulates inside of it, and thus kills all of the yeast present up to this point. After centrifuging, food grade oxygen is added back into the juice. There is a possibility that some yeasts could be added through this process, however, it is assumed that this is not the case because food grade oxygen and sterilized equipment are used.

To start the fermentation process, North Country adds a set amount of yeast to the oxygenated juice. Having a set amount of yeast present in the juice before fermentation allows for relatively consistent fermentation times. Fermentation is completed on a batch-to-batch basis in separate 7 & 15 bbl tanks (one US bbl is equivalent to 42 gallons). Although fermentation times are relatively consistent, the sugar content is monitored by measuring specific gravity of the juice. When the juice reaches a desired sugar content, the temperature is lowered to 32°F or "cold crashed," which stops all fermentation. At this point, it is centrifuged again to remove any unwanted products, and then stored in an on-site walk-in refrigerator until it is ready to be carbonated and packaged for distribution. North Country's ingredient list strictly consists of apple juice and any additional flavors they may choose to add for that batch. For instance, North Country has used squash and cranberries in previous ciders. There are no added sugars or preservatives, which makes North Country Hard Cider a unique cidery. (Dixon, R 2017)

Given that North Country has not analyzed the yeasts present in their products at different points in the process, we tasked ourselves in tracking these yeasts and other organisms at different stages in the process and at different storage times during the year. Knowing what yeasts are present at each step in the process helps the brewers understand what is affecting the quality, taste, and process parameters of the cider in a scientific and organized manner. We also researched and provided information about what the expected outcomes of these fungi and yeasts are.

We also determined the overall validity of efforts to counteract the effects of changing yeasts due to storage times. Overall, we will give a better, scientific understanding of the effects of different yeasts on the process and products of North Country Hard Cider.

Problem Statement

Although there are potentially many different types of yeast that may make their way into the cider making process, there is only one that exists and takes part in every single batch. US-05 is the "pitched," or added, yeast that is consistent across every fermentation. Normally, this would allow for unchanging fermentation times and tastes for every batch. However, because secondary organisms are introduced somewhere along in the process, times and tastes vary greatly depending on the time of year and other factors. One of this project's goals

is to track these different factors across batches and map different causes of changing dependent variables.

Another goal involves validating the usage of a centrifuge to the fresh juice before it is fermented. This step was added recently and requires scientific data to determine its usefulness and provide a better understanding of what exists in the juice. Samples are taken at multiple points in the process in order to accurately compare the presence of different yeasts. Comparing this to the corresponding data for each batch affords us a better understanding of how each yeast affects measurable factors like fermentation time, and allows North Country to improve their process. The methods for gathering and comparing this data are included in the following section.

Methodology

This section contains the procedures used to identify different yeasts strains present in samples. Samples were collected as liquids or solids at different stages of the cider-making process.

Sample Collection

Samples were kept in a cooler during transport from North Country to the Gateway Laboratory. The samples were kept refrigerated until use. The samples were left at room temperature for at least 24 hours to allow yeast to begin to reproduce before plating.

Yeast Strain Identification

These procedures are adapted from *North Country Hard Cider Yeast Investigation.*
Materials

- Micropipettes (2 - 20 µL and 200 - 1000 µL)
- YPD Agar plates
- Celltreat Sterilized 10 microliter Inoculating Loop
- Falcon 14 mL Polypropylene Round-Bottom Tube
- 1.5ml Microcentrifuge Tubes
- Eppendoff Minispin Centrifuge
- YPD Liquid Broth
- Sunrise Science Products YEP Broth
- Sigma-Aldrich D-(+)-Glucose Powder (Glucose Powder)
- 50 mM EDTA (pH 8.0)
- Zymolyase, resuspended with Nuclease-Free Water to a final concentration of 75 units/µl
- Ice Bath
- Isopropanol (Room Temperature)
- 70% Ethanol (Room Temperature)
- 31.1 nM ITS1 Primer
- 33.7 nM ITS4 Primer
- OneTaq® 2X Master Mix with Standard Buffer
- Denuclearized Water

- Bio Rad Dyad Peltier Thermal Cycler
- Plastic Bag
- A Plasmid Editor Program
- PCR Tubes

Culturing Yeast Procedure

These procedures are adapted from *North Country Hard Cider Yeast Investigation.*

Materials

- Falcon Tubes
- YPD Plates
- Pipetter
- Pipette: Tips and long tubes
- YPD solution

Plating Procedure

1. Obtain Falcon Tubes and samples, transfer 5 ml into each falcon tube.
2. Place samples inside the incubator, distribute evenly along the wheel, retrieve after 24 hours.
3. Create 1x, 10x, 100x, 1000x solutions of each sample.
4. Plate each solution evenly using beads(find scientific name)
5. Incubate plates for minimum 24 hours
6. Identify a singular cell mass, pick with pipette label colony extraction point
7. Place 5 ml of YPD solution in Falcon tube
8. Place cell colony pipette in Falcon tube
9. Incubate for 24 hours
10. Plate yeast solution

Plate Preparation

1. Use a pipette to remove 5 uL of sample. Replace sample cap immediately to prevent contamination.
2. Open YPD agar plate only enough to streak the plate according to the pattern below. The initial inoculation area (1) should yield the heaviest yeast growth, followed by dense growth in area 2, weak growth in area 3, and single colony growth in area 4.

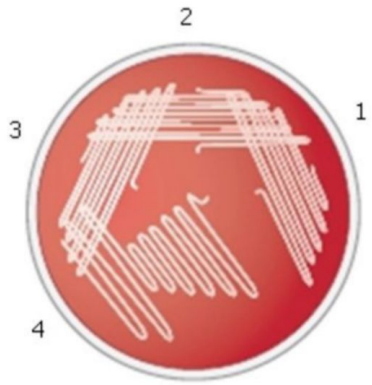

Figure 1: Plate Streak Pattern

3. Replace plate lid and leave plate to grow at room temperature until visible colonies form.

Yeast Colony Transplant/Preparation

During the growth process, some unexpected colonies grew. These colonies were removed from the original plate using a clean pipette tip and replated using the same streaking process as above, in order to isolate the species better.

DNA Isolation and Purification

These procedures are adapted from New England BioLaboratories.

1. Make YPD broth by mixing 15 g YEP, 10 g dextrose, and 500 mL DI water in a glass bottle. Autoclave bottle.

2. Fill 14 mL polypropylene round-bottom tubes with 5 mL YPD.
3. Pull single colonies from plates using a pipet tip and mix them into the tube.
4. Culture colony in a tube of YPD broth for 20 hours.
5. Add 1 mL of a culture grown for 20 hours to a 1.5 mL microcentrifuge tube.
6. Centrifuge at 13,400 rpm for 2 minutes to pellet the cells and remove the supernatant.
7. Resuspend cells in 293 µl of 50 mM EDTA.
8. Add 10 µl of 75 units/µl zymolyase, and gently pipette 4 times to mix.
9. Incubate the sample at 37°C for 60 minutes to digest the cell wall. Cool to room temperature.
10. Centrifuge at 13,400 rpm for 2 minutes and remove the supernatant.
11. Add 300µl of Nuclei Lysis Solution to the cell pellet and gently pipette to mix.
12. Add 100µl of Protein Precipitation Solution and vortex vigorously at high speed for 20 seconds.
13. Let the sample sit on ice for 5 minutes.
14. Centrifuge at 13,400 rpm for 3 minutes.
15. Transfer the supernatant containing the DNA to a clean 1.5ml microcentrifuge tube filled with 300µl of room temperature isopropanol.
16. Gently mix by inversion until the thread-like strands of DNA form a visible mass.
17. Centrifuge at 13,400 rpm for 2 minutes.
18. Decant the supernatant and drain the tube on clean absorbent paper. Add 300 µl of room temperature 70% ethanol and gently inverted the tube several times to wash the DNA pellet.
19. Centrifuge at 13,400 rpm for 2 minutes. Carefully aspirate all of the ethanol.
20. Drain the tube on clean absorbent paper and allow the pellet to air-dry for 10–15 minutes.
21. Add 50 µl of DNA Rehydration Solution.
22. Add 1.5µl of RNase Solution to the purified DNA sample. Vortex the sample for 1 second.
23. Centrifuge briefly in a microcentrifuge for 5 seconds to collect the liquid and incubate at 37°C for 15 minutes.
24. After incubating at 65 C for 1 hour, use the DNA Nanodrop to ensure there is at least 200 nanograms of DNA.
25. Store the DNA at 2–8°C until prepared for PCR.

PCR

These procedures were adapted from Promega.

1. Prepare primers through rehydration.
2. Add 311µl nuclease-free water to the 16S vial, making 100 mM solution. This primer is used to isolate the base sequence (5'-3') TCCGTAGGTGAACCTGCGG.
3. Add 337µl nuclease-free water to the 16S, making 100 mM solution. This primer is used to isolate the base sequence (5'-3') TCCTCCGCTTATTGATATGC.
4. Make a 10 mM solution of each primer by combining 10 µl in 90 µl of nuclease-free water.
5. Add 25 µl Master Mix, 2.5 µl of each primer, 2 µl of DNA sample, and 18 µl of nuclease free water to a sterile, thin-walled PCR tube to create a 50 µl reaction and gently mix.
6. Transfer PCR tubes to a PCR machine and thermocycle using the conditions below:

Table 1: Thermocycle Conditions

Step	**Temperature (°C)**	**Time (s)**
Initial Denaturation	94	30
Melting (30 Cycles) Annealing (30 Cycles) Extension (30 Cycles)	94 50 53	15 15 120
Final Extension	53	300
Hold	10	Until samples are removed

7. DPN1 digestion 1 uL at 37C for 1 hour
8. Clean DNA
9. Send samples and primers to Eton BioSciences DNA Sequencing service for DNA sequencing. An example of information entered when ordering sequencing is shown below.

Auto Fill	ID	*Sample Name	Sample Conc. (ng/µl)	*Sample Size (Insert + Vector)	*Primer Name	Order Primer	Primer Conc. (µM)	*Primer Source
		Clear	Clear	Clear	Clear	(Optional)	Clear	
	1	1	200	1 kb	16S-27F		10 µM	Providing Primer
	2	1	200	1 kb	16S-1492R		10 µM	Providing Primer
	3	2	200	1 kb	16S-27F		10 µM	Providing Primer
	4	2	200	1 kb	16S-1492R		10 µM	Providing Primer
	5	3	200	1 kb	16S-27F		10 µM	Providing Primer
	6	3	200	1 kb	16S-1492R		10 µM	Providing Primer
	7	4	200	1 kb	16S-27F		10 µM	Providing Primer
	8	4	200	1 kb	16S-1492R		10 µM	Providing Primer
	9	5	200	1 kb	16S-27F		10 µM	Providing Primer
	10	5	200	1 kb	16S-1492R		10 µM	Providing Primer
	11	6	200	1 kb	16S-27F		10 µM	Providing Primer
	12	6	200	1 kb	16S-1492R		10 µM	Providing Primer
	13	7	200	1 kb	16S-27F		10 µM	Providing Primer
	14	7	200	1 kb	16S-1492R		10 µM	Providing Primer
	15	8	200	1 kb	16S-27F		10 µM	Providing Primer
	16	8	200	1 kb	16S-1492R		10 µM	Providing Primer
	17	9	200	1 kb	16S-27F		10 µM	Providing Primer
	18	9	200	1 kb	16S-1492R		10 µM	Providing Primer
	19	10	200	1 kb	16S-27F		10 µM	Providing Primer
	20	10	200	1 kb	16S-1492R		10 µM	Providing Primer

Figure 2: Example Sequencing Form

Genotyping

The Eton BioScience genotyping results included the DNA sequence (.seq file) and the chromatogram file (.ab1 file) results for each sample (reverse and forward). The sequences needed to be analyzed in order to determine the species. The following steps were taken when analyzing the

DNA sequence:

1. Downloaded A Plasmid Editor program created by M. Wayne Davis using the following link: http://biologylabs.utah.edu/jorgensen/wayned/ape/

2. Opened the .ab1 files. An example of a partial .ab1 file is shown below.

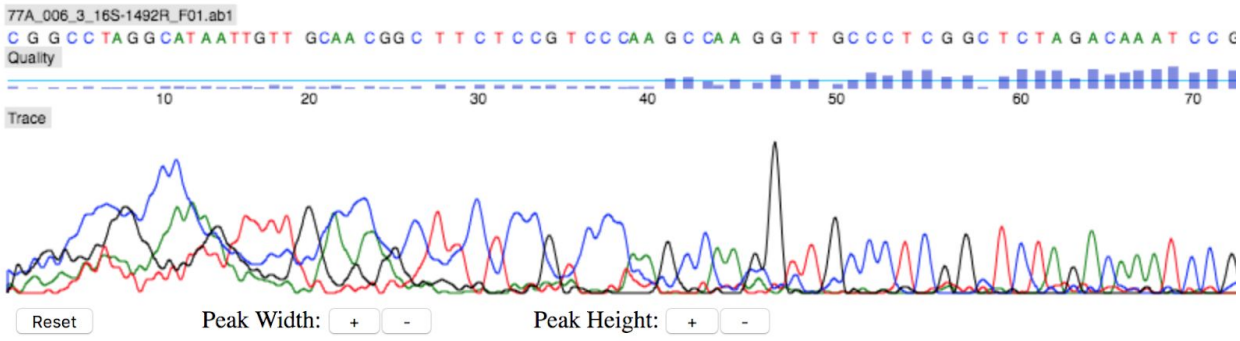

Figure 3: Example DNA Sequencing Results

3. Removed part of the sequence that did not have high accuracy.
4. Copied the shorter sequence from the .seq file.
5. Pasted this sequence into the U.S National Library of Medicine's Basic Local Alignment Nucleotide BLAST Tool (https://blast.ncbi.nlm.nih.gov/Blast.cgi)
6. Determined the species of the sequence, if results were found, using the query cover, E value, and identification percent.

Researching Types of Yeast Found

Once the DNA was sequenced and identified, we researched each yeast and how that yeast has been found to affect cider or alcohol fermentation. If no information on its' effect on fermentation was available, we would use the information to hypothesize what effect it may have on fermentation.

Batch Fermentation Time Patterns over Time

North Country keeps a record of processes and fermentation times for each batch they create. These records are kept in Excel spreadsheets, an example of such is shown below.

Batch # 150 Batch name : Jonagold (FV4)

Fermentation Monitoring

Date	Time	Temp	Plato	SG read	pH	TA	Days	Temp (C)	SG corr	FSU	Notes
10/24/16	11:08 AM	68.0	12.6	1.0511	3.50	0.5		20	1.0511		220 Jonagold 500g US-05
10/26/16	4:29 PM	68.0	10.9	1.0439			2	20	1.0439	360	
10/27/16	12:42 PM	68.0	9.6	1.0385			3	20	1.0385	544	
10/28/16	11:47 AM	68.0	8.5	1.0339			4	20	1.0339	456	
10/29/16	5:19 PM	68.0	7.3	1.0290			5	20	1.0290	493	
10/30/16	5:01 PM	68.0	6.5	1.0257			6	20	1.0257	326	
10/31/16	8:27 AM	68.0	6.0	1.0237			7	20	1.0237	203	
11/01/16	8:20 AM	68.0	5.2	1.0205			8	20	1.0205	323	
11/02/16	10:07 AM	68.0	4.4	1.0173			9	20	1.0173	321	Cool to 65F
11/03/16	4:18 PM	68.0	3.6	1.0141			10	20	1.0141	319	Crashed to 32F
11/04/16	8:20 AM	68.0	3.3	1.0129			11	20	1.0129	119	Racked to Polyconical using centrifuge @ 4.5gpm
11/09/16	10:05 AM	68.0		1.0000			16	20	1.0000	258	70 lbs back pressure w/B149
11/14/16	10:20 AM	68.0		1.0000			21	20	1.0000	0	Dumped 23 gallons of sludge
		68.0		1.0000			-42667	20	1.0000	0	Racked to IBC, set out to warm. Lots of diacetyl
11/25/16	11:48 AM	68.0	3.1	1.0121			32	20	1.0121	0	Warmed and added yeast from ongoing fermentat.
		68.0		1.0000			-42667	20	1.0000	0	Started to Move and less diacetyl
12/30/16	1:47 PM	68.0	2.6	1.0101			67	20	1.0101	0	Centrifuge @ 4-4.5gpm
03/03/17	10:33 AM	68.0	2.5	1.0097			130	20	1.0097	1	Cloudy
03/13/17											Blend w/B189 for FS made 3- 250 gallon batches
											100 gal B150/150 gal B 189

Temperatures in degrees F or C : F

Figure 4: Example Batch Data Spreadsheet

We groomed the data into a table containing only the fermentation start date and the total fermentation time, in days. The data was groomed to this amount because the spreadsheets did not consistently track data in the same manner. For example, not every sheet noted that the batch was crashed, but it may have noted when the batch was cooled or racked. This data was then plotted in excel and analyzed using excel's built in regression tools.

Results and Analysis

Yeast Identification

We identified two yeasts and 14 bacteria and fungi in the samples. A full table of each species identified can be found in the Appendix.

While Safale US-05 is the pitched yeast used in the brewing process, there is usually another yeast present. It is a wild yeast which we identified as *Hanseniaspora uvarum (see Figure 5 below)*. This is a spoilage yeast, which would normally cause problems. However, the metabolites created by *S. cerevisiae* during fermentation induces the lack of cultivability in *H. uvarum*. This effect does not require contact and spreads throughout the entire volume of the tank. It has also been shown in research that *H. uvarum* can reverse flocculation in solution, having a positive effect on the brewing process (Sosa et al., 2008).

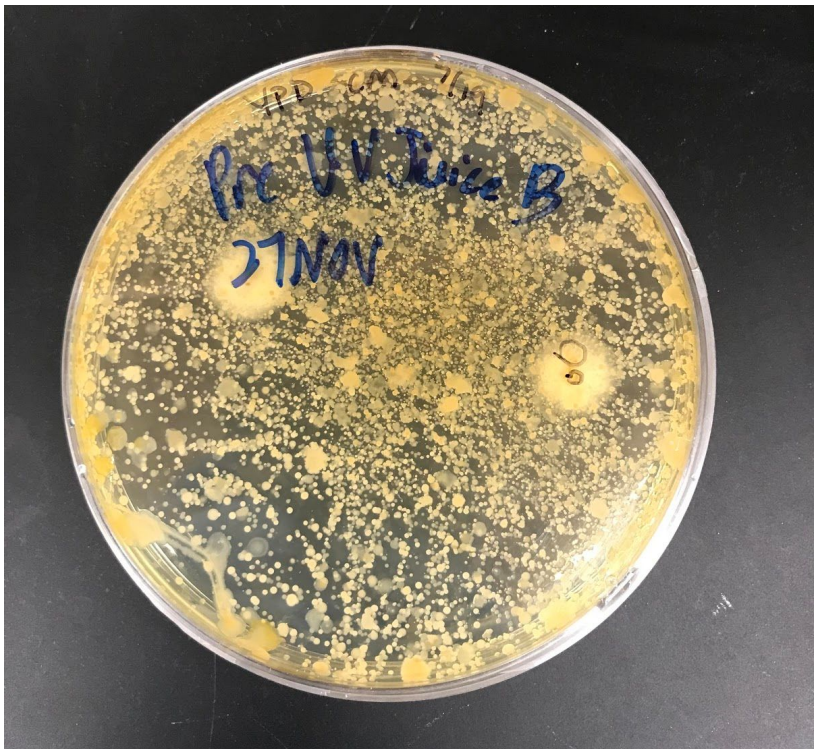

Figure 5: Pre-UV Juice B Plate

In our initial batches, multiple fungi were found in the samples before UV sanitization. These include *Galactomyces candidum* and *Geotrichum candidum* (see Figure 6 below), both of which have no effect on the final product because they are killed during UV treatment. The other initial batch included *Pichia fermentans* (see Figure 7 below), which is a potential

spoilage yeast that competes with *Saccharomyces* yeasts. Under the optimal storage conditions, spoilage potential is limited (Bokulich et al., 2013). In conjunction with the centrifugation of the final product, oxygen control and the overall cleanliness of the process at North Country eliminates any effects of this yeast.

Figure 6: Rolled Apple Plate

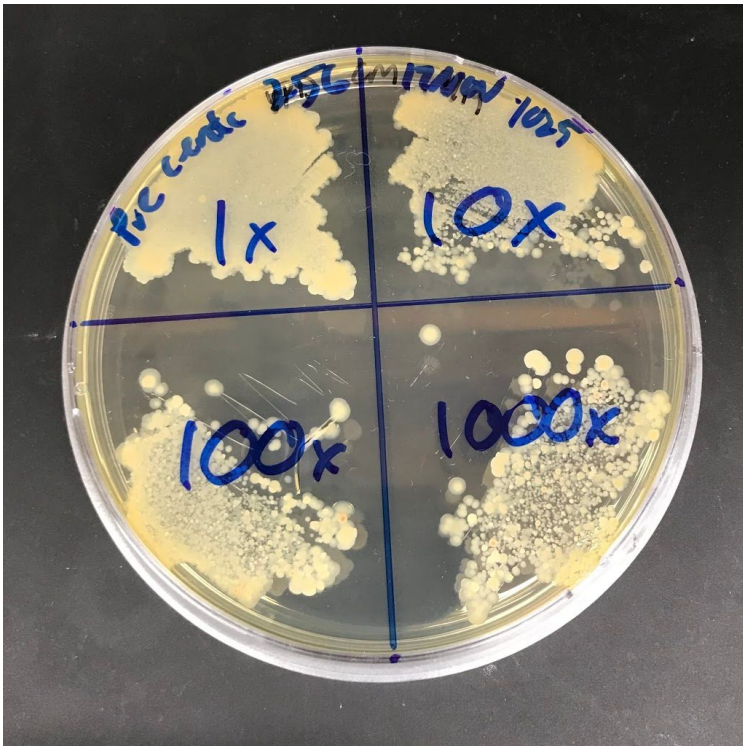

Figure 7: Batch 256 Pre-Centrifuge Post Fermentation

In all samples of fresh juice, both pre- and post-centrifugation, different types of bacteria were found. These included *Bacillus subtilis, Bacillus megaterium, Micrococcus luteus, and Pseudomonas fluorescens* (see Figure 8 below). All of these are usually found in soil or similar habitats, and most are harmless. However, *P. fluorescens* is a common contaminant in food which can cause spoilage by producing enzymes that degrade lipids and proteins. With that being said, when present on wild fruit, this bacteria can stop the spore production of different fungi which might cause the fruit to die or stunt its growth. (Note: when sequencing, the sample containing *P. fluorescens* returned a 7% query coverage. This means only 7% of our sample matched the genome reference. There is a high probability this is not the bacteria in the sample.) All forms of bacteria should have been killed in any UV treatment before delivery to North Country. This is an area of concern and should be addressed.

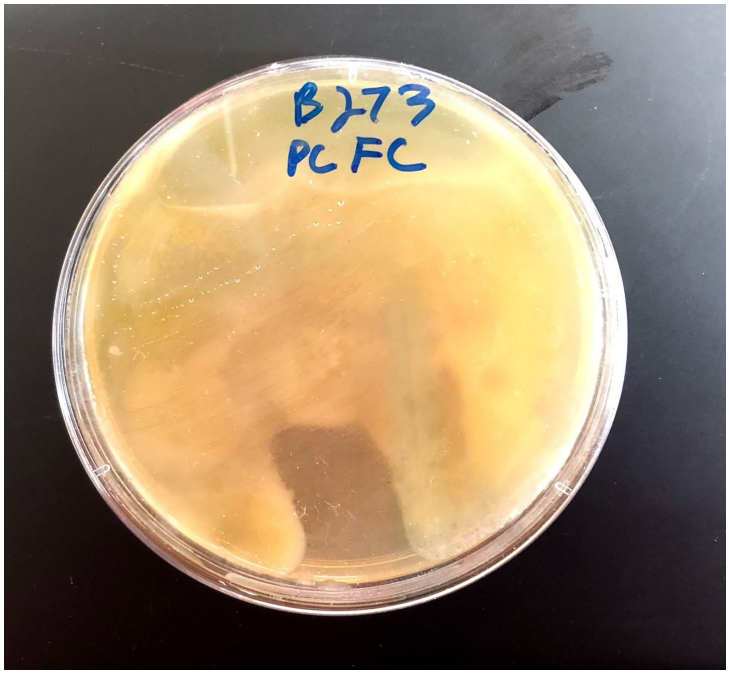

Figure 8: Batch 273, Pre-Centrifuge Fresh Juice

Oenococcus oeni was the primary result, found in two of the 4 successful samples of post-fermentation post-centrifuge product. This is a non-harmful bacteria that is mainly found in wines, which is responsible for the desired flavors that winemakers look for. Both this and *Lactobacillus nagelii* take part is malolactic fermentation, possibly causing unwanted flavors in the final cider product. The fourth sample contained *S. cerevisiae*, the only expected result as it is the pitched yeast in the process (See Figures 9-11 below). It should be noted that all final samples containing bacteria or leftover *S. cerevisiae* were taken from the cold room after fermentation and centrifugation. This could be a source of contamination. All yeasts and the locations in the process can be found in Appendix X.

Figure 9: Batch 271 Post-Centrifuge Post-Fermentation Cold Room

Figure 10: Batch 272 Post-Centrifuge Post-Fermentation Cold Room

Figure 11: Batch 273 Post-Centrifuge Post-Fermentation Cold Room

Over the course of the project, 30 colonies were picked from plates, and only a total of 12 samples were able to be identified. There are multiple reasons why this might be occurring, one the wizard genomic purification kit uses Zymolyase which is an enzyme specifically built to break down the cell wall of yeasts. While it has the ability to perform this action on other types of cells, it does not always work. This will stop the process of DNA purification. The other main blocker for identifying each sample is the primers used during the PCR reaction. The primers are specific to a section of DNA, so if primer does not attach to an identifying part of the DNA the reaction will not create an identifiable sample. Both of these are limiting factors when identifying each sample, and can be avoided by testing each sample multiple times with different enzymes and primers.

Fermentation Time Analysis

The plots in Figure 12 show how long fermentation took to occur in each batch between October 2014 and January 2018. These come from all different flavors of cider. The plots start during the fall, when cider production begins for the year.

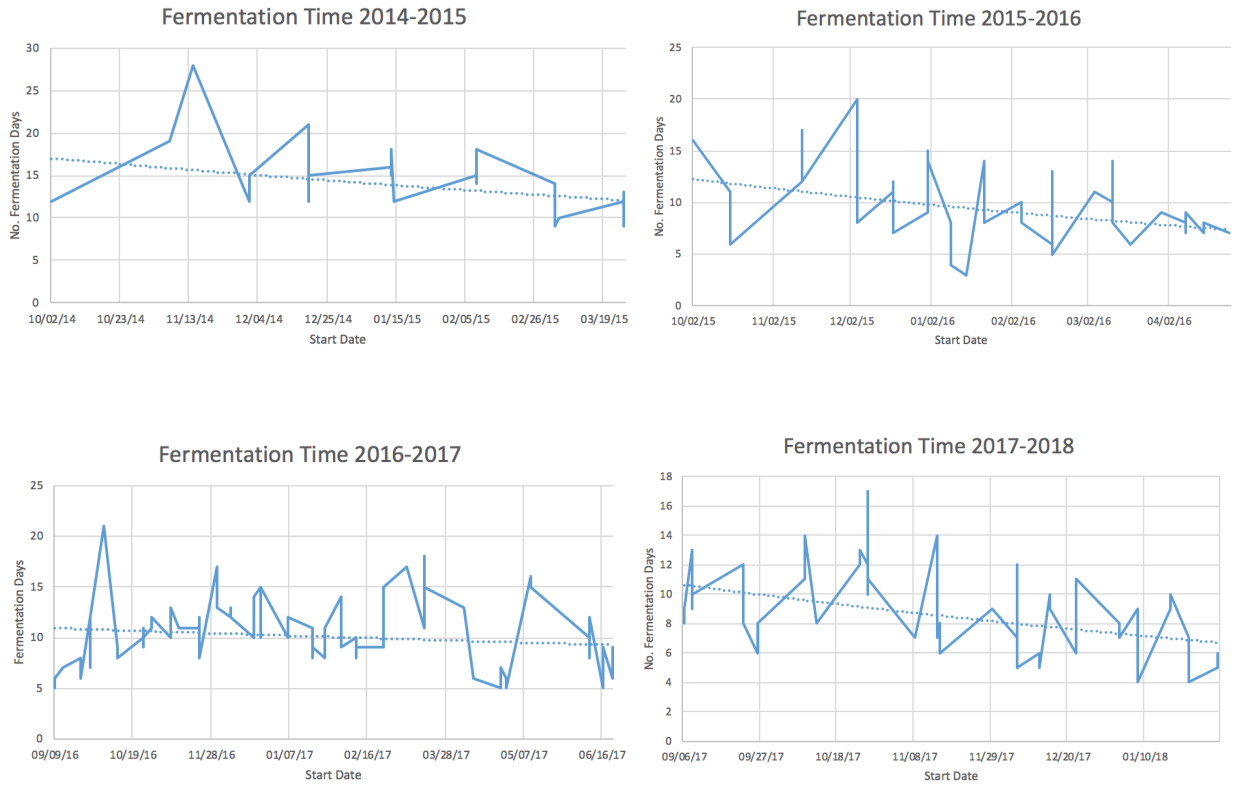

Figure 12: Fermentation Times in Number of Days 2014 - 2018

Each year shows that fermentation time decreases after apple season, or with increasing apple storage time. Fermentation times in 2014-2015 and 2015-2016 seem to indicate that fermentation time is more consistent further from apple season each year. However, 2016-2017 and 2017-2018 indicate that there is no change in consistency throughout the year.

Conclusions and Recommendations

While an original hypothesis of this project was the presence of a lager yeast causing shortened fermentation times and unwanted tastes, no lager yeasts were found during sample testing. This could be due to a number of factors, including centrifugation or the relatively warm weather during which the samples were taken. It has been noted through analysis of fermentation times that as the season progresses, overall times shorten. Without testing batches throughout the entire year, it is impossible to know if any lager yeasts have an effect on fermentation. What was found were many types of bacteria, each of which has the ability to cause the noted taste differences in the cider. With that being said, the best course of action is to sanitize more thoroughly and directly after receiving fresh juice. Sanitization methods might include additional UV treatment of juice after initial centrifugation or a different method for equipment sterilization. This should prevent any unwanted side reactions, including malolactic fermentation. As noted in the results above, the cold room is a possible source of contamination and should be thoroughly sanitized. While many bacteria grow at temperatures between 20-30^{o}C, they can survive at much lower temperatures.

We also recommend that North Country standardize batch data entry formats. Standardizing batch data is important because it allows for complete record keeping that can be used for future analysis, especially with these large quantities of data. Future analysis could include comparison of batch data amongst singular flavors of cider or during the same month of different years. Standardizing data would involve determining how data will be entered into the existing standard batch spreadsheets. We recommend having a standard notation for recording when batches are cooled, when they are crashed, and when they are racked. We also recommend using a nomenclature for naming the files that includes the batch number and flavor in the same format each time. Ideally, this would be done to existing spreadsheets as well as future sheets. We understand that this may be tedious, but it will greatly enhance future analyses.

Discussion

This section serves as a reflection on the techniques and processes used during this project as well as a reflection on our results and conclusions.

Performing regression and trend analysis should have been done at the beginning of the project to more fully understand the problem we were asked to solve. This would have led us to possibly try and collect samples from only one type of cider, giving a more accurate representation of the results.

Until we sat down and laid out our schedules, we had difficulties finding time to do lab work. It is difficult to have proper workflow and continuity when one person comes in and performs a task and must leave the process for the next person to continue. We eventually developed a schedule of when each person was available to be in the lab and a standard for keeping a tidy lab notebook, which helped immensely.

It also would have been helpful to collect more samples to do DNA sequencing on. There were some samples whose organisms we were not able to quantify and more samples would have given us more points to compare. Additionally, more data, say over the course of a year, would have allowed us to understand fully how the species present in the cider change based on how long from apple season it is. Additionally, it was difficult to gather enough samples to sequence. We did not start collecting samples until November, and if we had known that we would need to go to North Country more often, we should have started collecting samples sooner. The weather impeded us as well. More than once we planned to drive up to North Country to gather samples but because of the snow and ice we were forced to postpone the trip up.

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Appendix

Appendix A: Sample List

Label	Batch	Location	Description
Sample B03	N/A	Pre-UV Juice	White
Sample E03	N/A	Pre-UV Juice	Fuzzy, White
Sample A04	N/A	Apple Skin	White, Matte
Sample G04	256	Pre-Centrifuge Post-Fermentation	Beige, Opaque
Sample 8B	264	Pre-Centrifuge Post-Fermentation	White
Sample 2A	271	Post-Centrifuge Ferm Cold Room	White
Sample 11B	271	Post-Centrifuge Fresh Juice	Glossy White/ Peach
Sample 5A R	272	Post-Centrifuge Fermentation Cold Room	White
Sample 4A R	273	Post-Centrifuge Fermentation Cold Room	White, Matte
Sample 12A	273	Pre-Centrifuge Fresh Juice	White
Sample 5B	273	Pre-Centrifuge Fresh Juice	Orange
Sample 1A	277	Pre-Centrifuge Fresh Juice	White, Matte
Sample 3A	277	Pre-Centrifuge Fresh Juice	White, Matte
Sample 8A	277	Post-Centrifuge Fresh Juice	Yellow
Sample 9A	277	Post-Centrifuge Fermentation Cold Room	White
Sample 4B	277	Post-Centrifuge Fresh Juice	Glossy White

Appendix B: DNA Sequencing Data

Sample	Species	Query Cover
Sample B03	Hanseniaspora uvarum	99%
Sample E03	Galactomyces candidum	99%
Sample A04	Geotrichum candidum	91%

Sample G04	Pichia fermentans	99%
Sample 1A	No Match	
Sample 2A	Oenococcus oeni	92%
Sample 3A	No Match	
Sample 4A F	Saccharomyces cerevisiae	71%
Sample 4A R	Lactobacillus nagelii	85%
Sample 5A F	Saccharomyces cerevisiae	58%
Sample 5A R	Oenococcus oeni	98%
Sample 8A	Micrococcus luteus	98%
Sample 9A	Saccharomyces cerevisiae	80%
Sample 12A	Bacillus subtilis	93%
Sample 4B	Pseudomonas fluorescens	7%
Sample 5B	Bacillus megaterium	98%
Sample 8B	No Match	
Sample 11B	Failed	

Appendix C: Fermentation Time Data

Fermentation start date	Fermentation days	Fermentation start date	Fermentation days	Fermentation start date	Fermentation days
10/02/14	12	09/13/16	7	04/11/17	6
10/02/14	12	09/13/16	7	04/11/17	6
11/14/14	28	09/22/16	8	04/11/17	6
11/07/14	19	09/22/16	6	04/11/17	6
12/01/14	12	09/27/16	12	04/11/17	6
12/01/14	15	09/27/16	12	04/25/17	5
11/30/14	13	09/27/16	12	04/25/17	5
12/19/14	21	09/27/16	7	04/25/17	7
12/19/14	16	09/27/16	8	04/25/17	7
12/19/14	12	09/27/16	12	04/28/17	6
12/19/14	15	10/04/16	21	04/28/17	5
01/13/15	16	10/04/16	21	05/10/17	16
01/13/15	15	10/11/16	9	05/10/17	16
01/13/15	18	10/11/16	8	05/10/17	15
02/08/15	15	10/11/16	9	06/09/17	10
01/14/15	12	10/11/16	9	06/09/17	8
02/08/15	14	10/11/16	8	06/09/17	8
02/08/15	14	10/24/16	10	06/09/17	8
02/08/15	14	10/24/16	11	06/09/17	9
02/08/15	18	10/24/16	9	06/09/17	12
03/04/15	14	10/24/16	9	06/16/17	5
03/05/15	10	10/24/16	10	06/16/17	9
03/05/15	10	10/28/16	11	06/21/17	6
03/04/15	9	10/28/16	12	06/21/17	7
03/25/15	12	11/07/16	10	06/21/17	9

03/25/15	13	11/07/16	13	09/06/17	8
03/25/15	9	11/07/16	13	09/06/17	9
10/02/15	16	11/07/16	11	09/08/17	13
10/16/15	11	11/07/16	13	09/08/17	9
10/16/15	11	11/11/16	11	09/08/17	10
10/16/15	6	11/11/16	11	09/08/17	11
11/13/15	12	11/22/16	11	09/08/17	10
11/13/15	13	11/22/16	12	09/22/17	12
11/13/15	15	11/22/16	12	09/22/17	8
11/13/15	17	11/22/16	11	09/26/17	6
11/13/15	12	11/22/16	8	09/26/17	6
12/04/15	20	12/01/16	17	09/26/17	6
12/04/15	17	12/01/16	13	09/26/17	6
12/04/15	9	12/08/16	12	09/26/17	8
12/04/15	8	12/08/16	12	10/09/17	11
12/04/15	8	12/08/16	13	10/09/17	12
12/18/15	11	12/08/16	13	10/09/17	12
12/18/15	10	12/08/16	12	10/09/17	14
12/18/15	10	12/20/16	10	10/09/17	14
12/18/15	12	12/20/16	14	10/12/17	8
12/18/15	7	12/23/16	15	10/24/17	12
12/31/15	9	12/23/16	12	10/24/17	12
12/31/15	9	12/08/16	12	10/24/17	13
12/31/15	10	12/20/16	10	10/26/17	12
12/31/15	15	12/20/16	14	10/26/17	12
12/31/15	14	12/23/16	15	10/26/17	12
01/09/16	8	12/23/16	12	10/26/17	17
01/09/16	4	12/23/16	10	10/26/17	17
01/15/16	3	12/23/16	10	10/26/17	10

01/22/16	14	12/23/16	15	10/26/17	11
01/22/16	8	01/06/17	10	11/08/17	7
01/22/16	8	01/06/17	12	11/08/17	7
02/05/16	10	01/06/17	12	11/08/17	7
02/05/16	8	01/19/17	11	11/14/17	14
02/17/16	6	01/19/17	8	11/14/17	7
02/17/16	6	01/19/17	9	11/14/17	7
02/17/16	13	01/19/17	9	11/15/17	8
02/17/16	13	01/25/17	8	11/15/17	6
02/17/16	5	01/25/17	10	11/29/17	9
03/04/16	11	01/25/17	11	11/29/17	9
03/04/16	11	02/02/17	14	11/29/17	9
03/11/16	10	02/02/17	13	12/06/17	7
03/11/16	11	02/02/17	12	12/06/17	12
03/11/16	14	02/02/17	12	12/06/17	10
03/11/16	10	02/02/17	9	12/06/17	10
03/11/16	10	02/10/17	10	12/06/17	5
03/11/16	8	02/10/17	8	12/12/17	6
03/18/16	6	02/10/17	9	12/12/17	5
03/18/16	6	02/24/17	9	12/12/17	5
03/30/16	9	02/24/17	9	12/15/17	10
04/08/16	8	02/24/17	11	12/15/17	9
04/08/16	7	02/24/17	11	12/22/17	6
04/08/16	7	02/24/17	15	12/22/17	6
04/08/16	7	03/08/17	17	12/22/17	11
04/08/16	9	03/08/17	17	01/03/18	8
04/15/16	7	03/08/17	17	01/03/18	7
04/15/16	8	03/17/17	11	01/08/18	9
04/25/16	7	03/17/17	18	01/08/18	4

04/25/16	7	03/17/17	18	01/17/18	9
04/25/16	7	03/17/17	15	01/17/18	9
09/09/16	5	04/06/17	13	01/17/18	10
09/09/16	6	04/06/17	13	01/22/18	7
09/13/16	7	04/06/17	13	01/22/18	4