Processing of Dairy Waste Through Fermentation and Filtration to Produce Value-Added Products

Graduate School of Cornell University

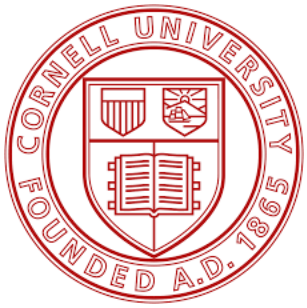
Degree Requirement for Master of Professional Science

in Food Science and Technology

By

Justin Fisk Marcus

May 2019



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This paper was reviewed by:

Dr. Sam Alcaine (Advisor)

BIOGRAPHICAL SKETCH

Justin Fisk Marcus, son of Randy and Terry Marcus, was born in Ithaca, New York. He received a Bachelor of Science from Elon University in 2015, where he focused on Plant Science and Agriculture. Upon graduation Justin moved back to Ithaca to work for Agricultural Consulting Services, a company advising dairy farms in the New England region how to improve crop yield and quality. After a year he took a position with Wegmans Food Market working in quality assurance for their corporate bakery. This job led Justin to make a career pivot, and to apply to Cornell University's Master of Professional Science program for a degree in Food Science and Technology, to increase his understanding of Food Science. He began his course work at Cornell University in August 2018 under Dr. Sam Alcaine, studying lactose-fermenting yeast strains to create value added products through upcycling of dairy by-products.

ACKNOWLEDGEMENTS

I would like to thank my advisor, Dr. Sam Alcaine, for his mentoring and dedication to my success. Cornell has been an amazing institution to study at, and I cherish the community I have become a member of, and my time spent here.

To the Cornell Community, I treasure the many connections I have made to students, faculty and alumni. Your wealth of knowledge, wisdom and compassion have helped me focus my priorities and achieve this degree. Our lab technicians Timothy DeMarsh and Sarah Kozack-Weaver have been invaluable for guidance and education in scientific practice. Viviana Rivera Flores, your positive attitude and hard work is inspiring, and I hope to collaborate with you again in the future. To all members of the Alcaine Research Group, it has been an honor to work with each of you. I extend my warmest regards to the faculty of the Food Science department.

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UPCYCLING OF WHEY PERMEATE THROUGH LACTOSE-FERMENTING YEAST UNDER ANAEROBIC AND AEROBIC CONDITIONS

ABSTRACT:

Several manufactured dairy products accrue large amounts of whey by-products, much of which is discarded as waste. One of the by-products is whey permeate, a powder containing lactose after the whey is deproteinated. The goal of this study was to determine yeast species that could utilize lactose from whey permeate, to produce ethanol or organic acids for a value-added product. Whey permeate was fermented under anaerobic and aerobic conditions for 34 days, using yeast and mold species obtained from Cornell University's Food Safety, the Alcaine Research Group, and Omega Labs. The yeast species: *Kluyveromyces marxianus*, *Kluyveromyces lactis, Dekkera anomala, Brettanomyces claussenii, Brettanomyces bruxellensis*; and mold species: *Mucor genevensis* and *Aureobasidium pullulans* were used. Measurements of organic acids, ethanol, sugar profiles, density, pH, and cell concentration were taken. Of these organisms, *K. marxianus* had the largest utilization of lactose and production of ethanol under anaerobic conditions (day 20: non-detectable levels of lactose, 4.52 ± 0.02%/Vol.). *Dekkera anomala* produced the most acetic acid under anaerobic conditions (day 34: 9.18 ± 3.38g/L). *A. pullulans* utilized the most lactic acid in whey permeate under aerobic conditions, increasing the pH of the fermentate (day 0: 0.83 ± 0.04 g/L, pH: 6.53 ± 0.05; day 34: 0.26 ± 0.21 g/L, pH: 7.91 ± 0.51). Fermentation of a whey permeate could be used industrially to make value-added beverages high in organic acids (acetic), a low alcohol tonic (ethanol), or in waste processing to increase pH, bringing new products or disposal method to increase profits, sustainability and efficiency for the dairy industry.

Introduction:

With an increase in Greek yogurt (GY), lactose free and ultra-filtered dairy products in the United States, there has been an excess of lactose containing whey by-products (*11*). Erickson's study found for every 1kg of GY, 2-3kg of acid whey was produced, with 771,000 metric tons of GY produced in New York State (NYS) alone in 2015. Acid whey is a by-product from Greek yogurt production, with a pH between 4.21-4.48 and a lactose content of 3.5% (*26*, 32). Many of these whey by-products will undergo further processing to remove protein, which once extracted, will be disposed of through land application or waste water treatment (*26*, *38*). Disposal and further processing can be costly for manufacturers, motivating governments, scientists and industry to develop solutions.

Beverage sales in America have been trending upwards, increasing by more than $2 billion in 2017 (*37*). Consumer preferences are shifting toward healthier, functional beverages that supply both micro and macro nutrients, or that have probiotics present (*33*). Fermented dairy products have been shown to aid in digestion, organ function (*1*). Furthermore, research suggests that organic acids such as acetic and lactic acid produced by microbes have health benefits, making fermented beverages like kombucha appealing to consumers (*33*). Large consumer products companies, like Coca-Cola, are interested in appealing to these consumer trends, by decreasing sugar content and increasing nutritional benefits, and acquiring companies whose products align with those trends as exemplified by their purchase of MOJO kombucha (*37*). Another rising beverage trend in the United States is consumption of low-alcohol beverages (*13*). These beverages have lower sugar content and fewer adverse health effects with a reduced ethanol content (*1*). Both of these trends stem from a consumer driven desire of being more health conscious in beverage selection, and growing interest in fermented beverages (*13*, *37*).

Fermentation of whey by-products could provide beverages of interest to consumers with high organic acid content, or low-alcoholic tonics, both value-added product for dairy manufacturers.

This study aimed to evaluate the fermentation of whey permeate, a powdered by-product from the deproteination of whey that is high in lactose, by a group of yeast and molds and the subsequent production of organic acids and ethanol, that are the base components for the preciously consumer trends (*36*, *40*). Under anaerobic conditions, yeast will produce ethanol from digestible sugars, as is commonly done in the brewing and wine industry; while under aerobic fermentations, the production of organic acids such as acetic and lactic acid, seen in products like kombucha or kefir (*6*, *27*). Molds will have similar outputs, and can sustain growth in highly acidic environments (*6*). Both of these conditions were studied in order to determine which species were most adapted for lactose utilization from whey permeate, and what concentrations of ethanol or organic acids could be produced. With our findings we are able to make suggestions as to which of the species studied would be best utilized to ferment value-added products from whey by-products.

Methods and Materials:

Microorganisms:

Yeasts that could potentially hydrolyze lactose were selected. The following species were selected for this experiment: *Aureobasidium pullulans* (mold), *Kluyveromyces lactis* and *Kluyveromyces marxianus* (both yeasts). These spoilage organisms isolated from commercial dairy products were provided by Cornell University's Food Safety Lab (Ithaca, NY). Commercial cultures of *Brettanomyces claussenii* and *Brettanomyces bruxellensis* (both yeasts) were obtained from Omega Yeast Labs (St. Louis, MO). *Dekkera anomala* (yeast) was obtained from ARS Culture Collection (NRRL), USDA. Also included was *Mucor genevensis* (mold), a

common dairy spoilage organism, which was isolate from commercial dairy products by the Alcaine Research Group (Cornell University, Ithaca, NY),

Experimental Design:

Both anaerobic and aerobic fermentations were conducted in triplicate and ran for 34 days. Each replicate was initiated on a separate day. Each species was grown up from a single colony in 12° Plato Dry Malt Extract broth. Hemocytometer counts were performed daily until a desired cell concentration sufficient to achieve a final inoculation level of ~6 x 10^6 CFU/mL. Cultures were then stored at 4 °C until inoculation. New cultures were propagated for each replicate.

Fermentation substrate was made in batches of 2L of MilliQ water, 200g of Great Lakes, Deproteinized Whey Powder (whey permeate; Great Lakes, Adams, NY), 2g of diammonium phosphate and 0.8g of Fermaid K (Lallemand; Fredericia, Denmark). This solution was sterilized using 0.45um PES filter, and 500mL aliquots were transferred to vessels appropriate for either anaerobic or aerobic fermentation, as outlined below. Each of the substrates received a pure culture inoculum containing ~6 x 10^6 CFU/mL of species outlined.

Anaerobic fermentations were conducted in airtight 500mL bottles, submerged in a water bath kept at 30°C with no agitation. For each fermentate, samples were taken aseptically by means of a sterile 5" hypodermic needle that was left inserted into the bottle for the duration of the experiment; single-use syringes were then used to aspirate samples through the needle.

Aerobic fermentations were conducted on 500mL volumes of substrate in 1L flasks covered with aluminum foil. The flasks were kept at 30°C with agitation of 185RPM. Samples were aspirated using a sterile stripette next to a flame.

Data Collection:

pH readings for the anaerobic fermentation were taken every 4 hours using an iCinac (AMS Alliance; Rome, Italy) equipped with InLab Smart Pro-ISM probes (Mettler Toledo; Columbus, OH). At the time of inoculation, a sterile probe was inserted into each fermentate through a cap fitted with a silicone septum. This probe stayed in the fermentate throughout the experiment. For the aerobic fermentations, pH samples were aspirated from the fermentates ±3hrs from inoculation time on days 0, 1, 2, 3, 4, 6, 8, 10, 12, 14, 16, 18, 20, 27 and 34.

Density was measured on the same schedule for the anaerobic and aerobic fermentations ± 3hrs from inoculation time. Measurements were taken on days 0, 1, 2, 3, 4, 6, 8, 10, 12, 14, 16, 18, 20, 27 and 34. Density was measured using an Anton Paar DMA^{TM} 35 (Graz, Austria) densitometer.

Cell counts were enumerated through serially dilutions and plating. Fermentates were serially diluted in Phosphate Buffered Saline (PBS). Desired dilutions were then plated onto Potato Dextrose Agar + Chloramphenicol (PDA-cam) in duplicate and incubated at 30^{o}C until visible growth occurred. Colony counting was performed using a Chemopharm® Color QCount Model 530 (Advanced Instruments, Inc., Norwood, MA). Species were plated on days 0, 2, 4, 6, 8, 10, 12, 14, 16, 18, 20, 27 and 34.

Organic acids and ethanol concentrations were measured by Cornell University's Wine & Cider Analyses Laboratory (Geneva, NY). Organic acids were measured using High Performance Liquid Chromatography. Ethanol was measured using Gas Chromatography-Flame Ion Detection (GC-FID). Analysis was done on samples from days 0, 20 and 34.

Sugar analysis was performed by Eurofin labs (Ithaca, NY). Measurements of fructose, glucose, sucrose, maltose, lactose, galactose and total sugar were taken using High-Performance

Anion-Exchange Chromatography coupled with Pulse Electrochemical Detection. Samples from each fermentation and uninoculated substrate from day 27 were analyzed. Only measurements of lactose, glucose and galactose are reported in this study, as the other sugars measured had no detectable values.

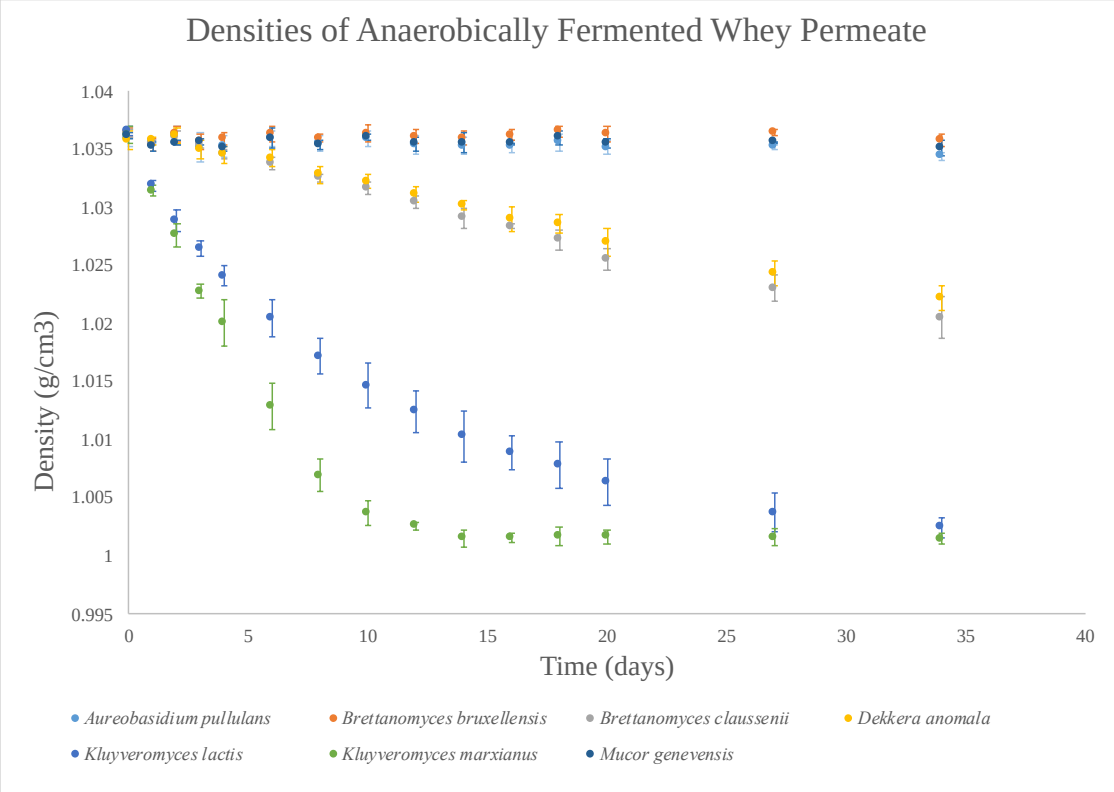
Results:

Density:

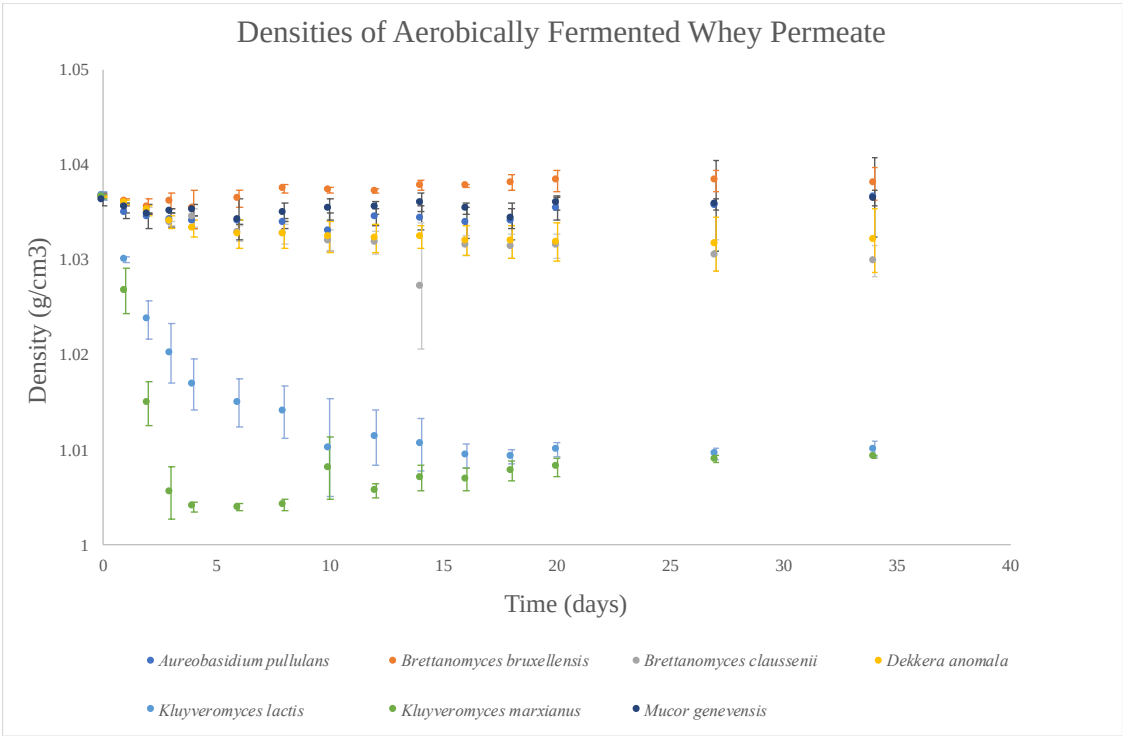
A common measurement in the brewing industry to test for sugar utilization is to measure the density throughout the fermentation (*9*). When microbes hydrolyze the sugars to smaller, less dense or volatile molecules, the density of the liquid will decrease (*9*). The variation of decreasing densities is likely attributed to the species' ability to hydrolyze lactose or available organic acids, and the ability to continue metabolic processes in anaerobic or aerobic environments, and/or the organism's ability to absorb lactose through their cell wall.

In both anaerobic and aerobic conditions, *K. marxianus* fermentate's density decreased consistently throughout the fermentation, and had the lowest final density on day (D) 34 of species measured (anaerobic D0: 1.037 ± 0.0, D34: 1.002 ± 0.0; aerobic D0: 1.037 ± 0.0, D34: 1.009 ± 0.0g/cm^3). Under aerobic conditions *K. marxianus* had measurements that increased in density after D8 (1.004 ± 0.0g/cm^3), however the lowest density was seen on D34 (1.0093 ± 0.0 g/cm^3).

The density decline in the fermentate containing *K. lactis* was slower than that by *K. marxianus*, however by D34 the densities were similar for each species anaerobically and aerobically (*K. lactis* D34 anaerobic: 1.002 ± 0.0; aerobic: 1.010 ± 0.0g/cm^3). Both species had a lower final density under anaerobic conditions [GRAPH 1.1, 1.2].



[GRAPH 1.1: Error bars represent standard deviation, calculated from three replicates for each species, except *M. genevensis*, which had two viable replicates].



[GRAPH 1.2: Error bars represent standard deviation, calculated from 3 replicates for each species].

Both fermentates of *B. claussenii* and *D. anomala* saw similar declines in density under aerobic and anaerobic conditions, however *B. claussenii* averaged a lower density by D34 in each condition (anaerobic: *B. claussenii* 1.021 ± 0.0, *D. anomala* 1.022 ± 0.0; aerobic: *B. claussenii* 1.030 ± 0.0, *D. anomala* 1.032 ± 0.0g/cm^3). *Brettanomyces claussenii* and *D. anomala* both had lower final densities under anaerobic conditions compared to aerobic [GRAPH 1.1, 1.2].

B. bruxellensis' fermentate had a decline in density under anaerobic conditions (D0: 1.036 ± 0.0, D34: 1.036 ± 0.0g/cm^3). A drop in acetic and lactic acid was seen, without utilization of lactose [TABLE 1.1, 1.3, 1.4]. Under aerobic conditions, lactose was utilized, however a minimal increase in density was observed (D0: 1.037 ± 0.0, D34: 1.038 ± 0.0g/cm^3) [TABLE 1.2]. Under both conditions *B. bruxellensis* had the highest final density reading compared to other species (D34 anaerobic: 1.0358 ± 0.0; aerobic: 1.0380 ± 0.0g/cm^3).

Minimal changes in fermentate density decreased for *A. pullulans* under aerobic conditions (density D0: 1.037 ± 0.0, D34: 1.034 ± 0.0g/cm^3), however no change was seen under anaerobic conditions (density D0: 1.037 ± 0.0, D34: 1.037 ± 0.0g/cm^3).

Mucor genevensis had minimal changes in fermentate density anaerobically (D0: 1.036 ± 0.0, D34: 1.035 ± 0.0g/cm^3), with a minimal increase in density in aerobic conditions (D0: 1.0363 ± 0.0, D34: 1.0365 ± 0.0g/cm^3). No utilization of lactose was seen from this organism [TABLE 1.1, 1.2]. Under anaerobic conditions, lactic acid was utilized, and in aerobic conditions, lactic and acetic acid were utilized [TABLE 1.4, 1.7, 1.8].

Sugar Utilization:

Each fermentate started with an average of 7.233g Lactose (Uninoculated), and a non-detectable (N.D., <0.1g sugar/100g sample) levels of glucose and galactose [TABLE 1.1, 1.2].

K. marxianus utilized lactose most efficiently of species studied, with an N.D. value of each sugar measured by D27, both anaerobically and aerobically [TABLE 1.1, 1.2]. *K. lactis* performed similarly under aerobic conditions, however small amounts of lactose were present under anaerobic conditions (0.2 ± 0.28g/100g).

D. anomala and *B. claussenii* had similar sugar utilization under both conditions [TABLE 1.1, 1.2]. Both species utilized more lactose under anaerobic conditions (anaerobic: *B. claussenii* 3.87 ± 0.5, *D. anomala* 3.97 ± 0.5; aerobic: *B. claussenii* 5.40 ± 0.5, *D. anomala* 5.50 ± 0.6 g/100g remaining lactose).

Brettanomyces bruxellensis did not utilize lactose under anaerobic conditions, however it did show decreases in lactose concentrations aerobically (anaerobic: 7.4 ± 0.0; aerobic: 6.5 ± 1.7g/100g) [TABLE 1.1, 1.2]. Glucose and galactose were present in the aerobic fermentate (glucose: 0.37 ± 0.5; galactose: 0.97 ± 0.39g/100g), while N.D. levels were in the anaerobic fermentate [TABLE 1.1, 1.2].

M. genevensis did not utilize lactose in anaerobic or aerobic conditions [TABLE 1.1, 1.2].

A. pullulans had utilization of lactose under anaerobic and aerobic conditions (anaerobic: 6.63 ± 0.2; aerobic: 7.53 ± 0.7g/100g).

Anaerobic Fermentation - Sugar Profiles				
	Remaining Lactose (g/100g)	Remaining Glucose (g/100g)	Remaining Galactose (g/100g)	Remaining Total Sugars (g/100g)
A. pullulans	6.633 ± 0.17	N.D.	0.23 ± 0.05	6.87 ± 0.21
B. bruxellensis	7.4 ± 0	N.D.	N.D.	7.4 ± 0
B. claussenii	3.87 ± 0.5	N.D.	0.37 ± 0.24	4.23 ± 0.29
D. anomala	3.97 ± 0.45	N.D.	0.47 ± 0.25	4.43 ± 0.21
K. lactis	0.2 ± 0.28	N.D.	N.D.	0.2 ± 0.28
K. marxianus	N.D.	N.D.	N.D.	N.D.
M. genevensis	7.2 ± 0	N.D.	N.D.	7.2 ± 0
Uninoculated	7.23 ± 0.9	N.D.	N.D.	7.23 ± 0.09

[TABLE 1.11: Samples were taken from D27. Each number represents averages and standard deviations calculated from three replicates for all species besides *M. genevensis*, which had two viable replicates. Measurements are in grams sugar/100g sample]

Aerobic Fermentation - Sugar Profiles				
	Remaining Lactose (g/100g)	Remaining Glucose (g/100g)	Remaining Galactose (g/100g)	Remaining Total Sugars (g/100g)
A. pullulans	7.53 ± 0.71	N.D	0.7 ± 0.09	7.63 ± 0.62
B. bruxellensis	6.5 ± 1.72	0.37 ± 0.52	0.97 ± 0.39	7.27 ± 0.77
B. claussenii	5.4 ± 0.5	0.03 ± 0.05	0.03 ± 0.05	5.5 ± 0.59
D. anomala	5.5 ± 0.62	N.D	N.D	5.5 ± 0.62
K. lactis	N.D	N.D	N.D	N.D
K. marxianus	N.D	N.D	N.D	0.00
M. genevensis	7.5 ± 0.1	N.D	N.D	7.5 ± 0.1
Uninoculated	7.23 ± 0.09	N.D	N.D	7.23 ± 0.09

[TABLE 1.12: Samples were taken from D27. Each value is from averages and standard deviations calculated from three replicates for all species besides *M. genevensis*, which had two viable replicates. Measurements are in grams sugar/100g sample].

Changes in Fermentate pH:
Anaerobic:

Both *Kluyveromyces* species decreased the fermentate's pH within 36 hours under anaerobic conditions [GRAPH 1.3]. The pH of *K. marxianus'* fermentate continued to drop from until D10, then began to increase through D34 [GRAPH1.3]. A decrease in lactic was seen from

D20 to D34 (D20: 0.81 ± 0.03; D34: 0.47 ± 0.07g/L lactic acid), while acetic acid content increased (D20: 1.17 ± 0.17; D34: 1.39 ± 0.07g/L acetic acid) [TABLE 1.3, 1.4].

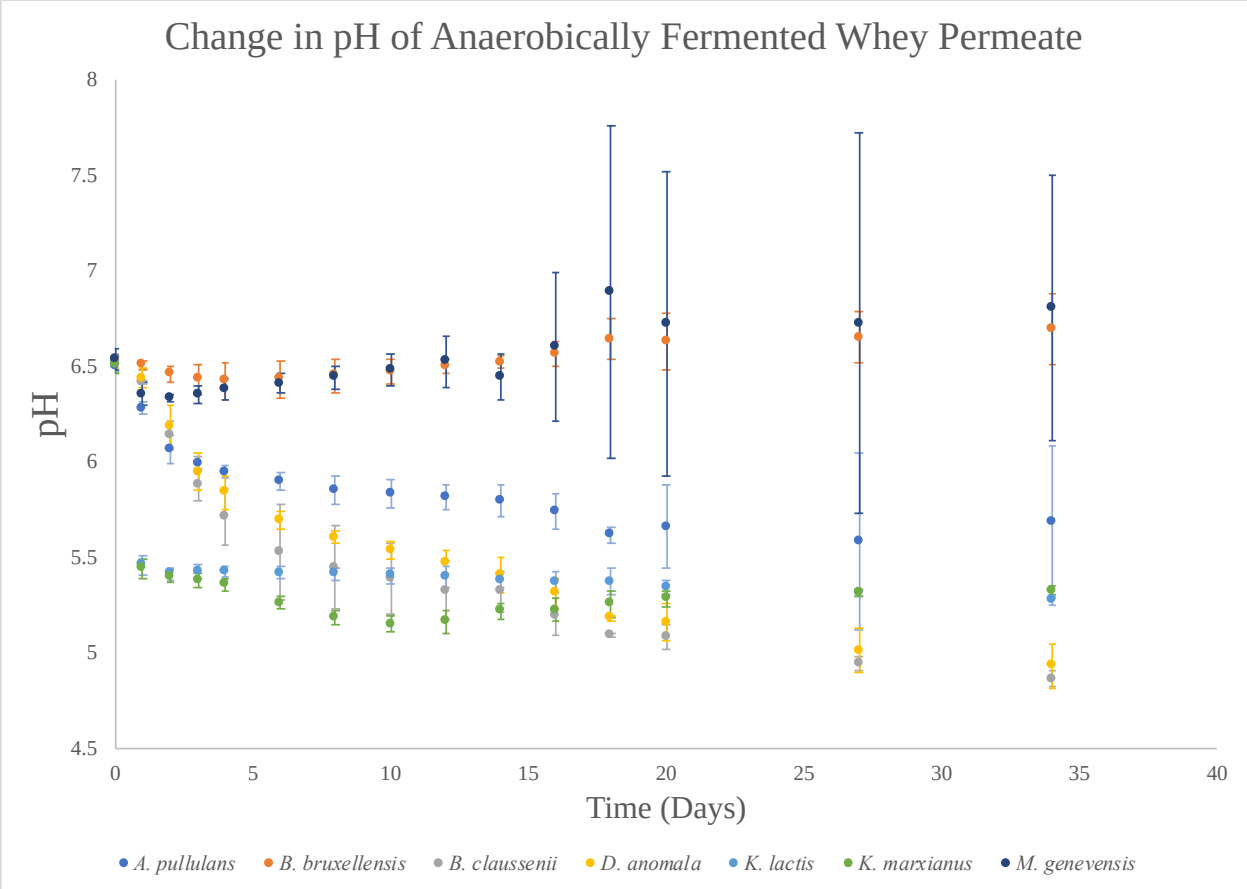
Kluyveromyces lactis continued to drop in pH D16, where minimal changes occurred for the duration of the fermentation [GRAPH 1.3]. Ethanol and acetic acid continued to increase from D20 to D34 (ethanol D20: 3.72 ± 0.50, D34: 4.33 ± 0.08%/Vol; acetic acid D20: 0.60 ± 0.18; D34: 0.79 ± 0.08g/L) [TABLE 1.3, 1.11].

Brettanomyces claussenii and *D. anomala* saw a constant decline through D34, ending in a pH of 4.86 ± 0.04 and 4.93 ± 0.11 respectively. Both species saw increases in organic acid and ethanol content [TABLES 1.3, 1.4, 1.11].

Brettanomyces bruxellensis saw minimal changes in pH, however the final pH was higher than the initial fermentate [GRAPH 1.3]

Aureobasidium pullulans' fermentate decreased from 6.50 ± 0.03 through D19 to 5.57 ± 0.16, after which increases were seen until D34 with a final pH of 5.68 ± 0.40 [GRAPH 1.3]. Acetic and lactic acid concentrations increased from D0 to D20, and decreased from D20 to D34 in correlation with these changes in pH (acetic acid D0: 0.09 ± 0.13; D20: 0.56 ± 0.17; D34: 0.45 ± 0.19g/L; lactic acid: D0: 0.85 ± 0.05; D20: 0.1.27 ± 0.25; D34: 1.09 ± 0.28g/L) [TABLE 1.3, 1.4].

Mucor genevensis had increases in pH from D0 to D34 [GRAPH 1.3]. Lactic acid levels decreased from D0 (0.75 ± 0.04g/L), to N.D. levels at D34 [TABLES 1.4].



[GRAPH 1.3: Changes in the anaerobically fermented whey permeate were recorded every 4 hours for each replicate. Data is displayed from D0, 1, 2, 3, 4, 6, 8, 10, 12, 14, 16, 18, 20, 27 and 34. Error bars represent standard deviation, calculated from three replicates for each species besides *M. genevensis*, which had two viable replicates].

Aerobic:

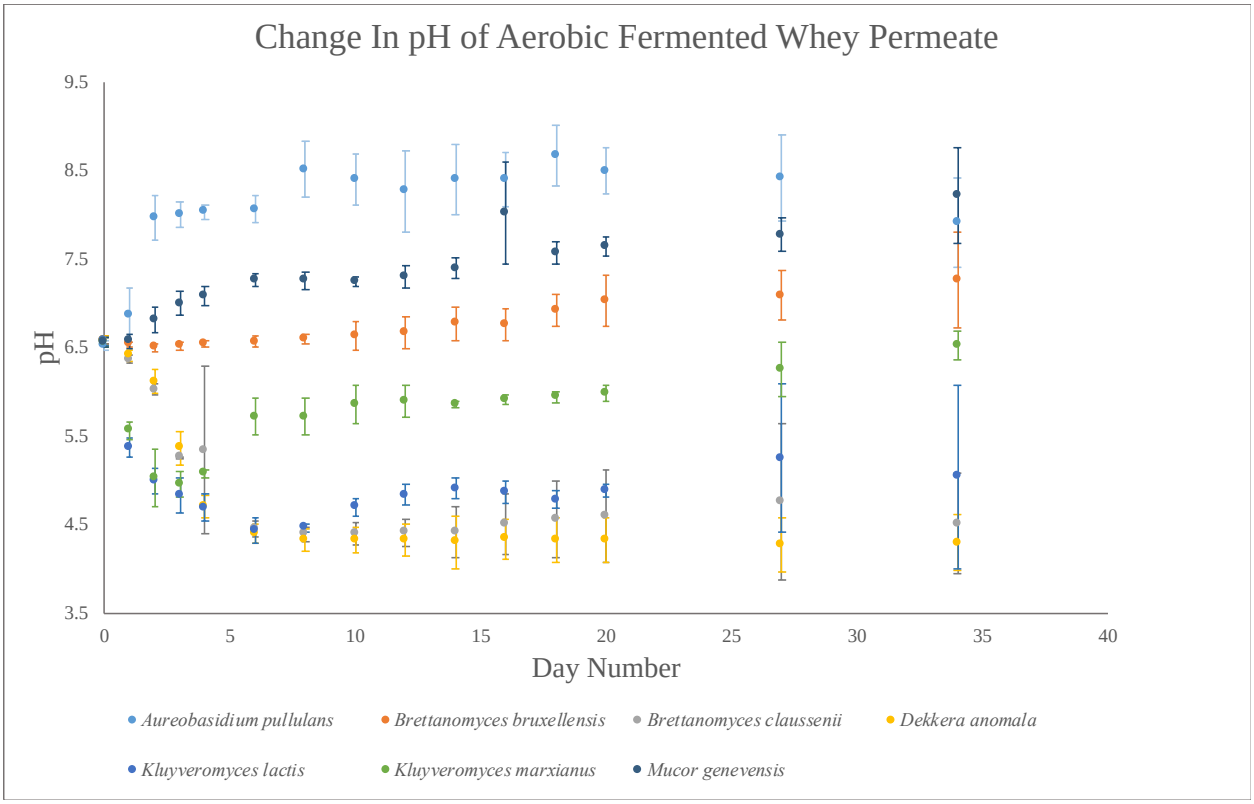
Kluyveromyces marxianus' fermentate pH decreased from 6.56 ± 0.04 to 4.95 ± 0.15 on D3, and increased to 6.51 ±0.16 by D34 [GRAPH 1.4]. *Kluyveromyces lactis'* fermentate pH continued to decrease from 6.57 ± 0.04 until D6 to a pH of 4.43 ± 0.14, where it began to increase to 5.04 ± 1.03 by D34. Acetic and lactic acid contents for both species decreased from D0 to D34 (*K. marxianus* acetic acid D0: 0.52 ± 0.26; D34: 0.34 ± 0.17; lactic acid D0: 0.86 ± 0.01; D34: 0.06 ± 0.08; *K. lactis* D0: 0.76 ± 0.24; D34: 2.68 ± 1.81; lactic acid D0: 0.86 ± 0.01; D34: N.D. g/L) [TABLE 1.7, 1.8].

The pH of the fermentate for *B. claussenii* and *D. anomala* decreased from 6.58 to a pH 4.51 ± 0.57 for *B. claussenii*, and 4.29 ± 0.31 for *D. anomala*'s fermentates [GRAPH 1.4]. Acetic acid content increased from D0 to D34 for both species (*B. claussenii* D0: 0.69 ± 0.51, D34: 7.75 ± 4.37; *D. anomala* D0: 0.86 ± 0.34, D34: 9.18 ± 3.38g/L) [TABLE 1.7].

Brettanomyces bruxellensis' fermentate pH increased from 6.58 ± 0.05 to 7.26 ± 0.54 by D34 in *B. bruxellensis'* fermentate [GRAPH 1.4]. Concentrations of acetic and lactic acid decreased (acetic D0: 0.40 ± 0.08, D34: 0.03 ± 0.05; lactic D0: 0.84 ± 0.01, D34: 0.29 ± 0.41g/L) [TABLE 1.7, 1.8].

Aureobasidium pullulans increased from a pH of 6.52 ± 0.05 to 8.66 ± 0.35 by D18, and then declined to 7.91 ± 0.51 by D34 [GRAPH 1.4]. A decrease in lactic acid was seen (D0: 0.83 ± 0.04; D34: 0.27 ± 0.21g/L) [TABLE 1.8].

Mucor genevensis increased fermentate pH from 6.56 ± 0.05 to 8.01 ± 0.57 by D16, until D34 at 8.21 ± 0.54 [GRAPH 1.4]. Decreases in acetic and lactic acid concentrations were seen by *M. genevensis* (acetic D0: 0.50 ± 0.24, D34: 0.40 ± 0.15; lactic D0: 0.86 ± 0.01, D34: 0.37 ± 0.29g/L) [TABLE 1.7, 1.8].



[GRAPH 1.4: Error bars represent standard deviation, calculated from three replicates for each species].

Organic Acid Production/Utilization (acetic, lactic, tartaric, malic):
Anaerobic*:*

K. marxianus, K. lactis, D. anomala, B. claussenii, A. pullulans and *M. genevensis* all saw increases in acetic acid anaerobic conditions; while *B. bruxellensis* saw a decline in acetic acid concentration [TABLE 1.3].

Anaerobic Fermentation - Acetic Acid Production							
Time (days)	*A. pullulans* (g/L)	*B. bruxellensis* (g/L)	*B. claussenii* (g/L)	*D. anomala* (g/L)	*K. lactis* (g/L)	*K. marxianus* (g/L)	*M. genevensis* (g/L)
0	0.09 ± 0.13	0.24 ± 0.05	0.52 ± 0.37	0.21 ± 0.02	0.28 ± 0.13	0.66 ± 0.55	0.28 ± 0.06
20	0.56 ± 0.17	0.07 ± 0.1	0.66 ± 0.37	0.54 ± 0.06	0.6 ± 0.18	1.17 ± 0.17	0.56 ± 0.14
34	0.45 ± 0.19	0.08 ± 0.11	0.9 ± 0.27	0.73 ± 0.24	0.79 ± 0.08	1.39 ± 0.19	0.66 ± 0.39

[TABLE 1.3: Measurements are shown grams organic acid/L of sample. Standard deviations were calculated from three replicates for all species besides *M. genevensis*, which had two viable replicates].

Lactic acid concentrations increased in *K. lactis, B. claussenii, D. anomala* and *A. pullulans* fermentates, while *K. marxianus, B. bruxellensis* and *M. genevensis* saw a decrease in lactic acid [TABLE 1.4].

Anaerobic Fermentation - Lactic Acid Production							
Time (days)	*A. pullulans* (g/L)	*B. bruxellensis* (g/L)	*B. claussenii* (g/L)	*D. anomala* (g/L)	*K. lactis* (g/L)	*K. marxianus* (g/L)	*M. genevensis* (g/L)
0	0.85 ± 0.05	0.78 ± 0.06	0.78 ± 0.07	0.81 ± 0.03	0.8 ± 0.03	0.79 ± 0.05	0.75 ± 0.04
20	1.27 ± 0.25	0.7 ± 0.2	1.37 ± 0.24	1.21 ± 0.19	0.84 ± 0.05	0.81 ± 0.03	N.D.
34	1.09 ± 0.28	0.57 ± 0.29	1.33 ± 0.15	1.25 ± 0.11	0.85 ± 0.11	0.47 ± 0.07	N.D.

[TABLE 1.4: Measurements are shown grams organic acid/L of sample. Standard deviations were calculated from three replicates for all species besides *M. genevensis*, which had two viable replicates].

K. lactis, K. marxianus, B. claussenii, D. anomala and *M. genevensis* all had detectable levels of tartaric acid, suggesting these species are capable of tartaric acid production as N.D. levels were seen in initial fermentate [TABLE 1.5]. *Kluyveromyces marxianus, K. lactis* and *D. anomala* saw decreases in tartaric acid from D20 to D34, suggesting these species may have utilized this acid. *Aureobasidium* pullulans and *B. bruxellensis* did not have detectable levels of tartaric acid in their fermentates throughout this study [TABLE 1.5].

Anaerobic Fermentation - Tartaric Acid Production							
Time (days)	*A. pullulans* (g/L)	*B. bruxellensis* (g/L)	*B. claussenii* (g/L)	*D. anomala* (g/L)	*K. lactis* (g/L)	*K. marxianus* (g/L)	*M. genevensis* (g/L)
0	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.
20	N.D.	N.D.	0.01 ± 0.01	0.01 ± 0.01	0.63 ± 0.07	0.17 ± 0.1	0.02 ± 0.03
34	N.D.	N.D.	0.02 ± 0.03	N.D.	0.44 ± 0.05	N.D.	0.28 ± 0

[TABLE 1.5: Measurements are shown grams organic acid/L of sample. Standard deviations were calculated from three replicates for all species besides *M. genevensis*, which had two viable replicates].

No malic acid was produced by any species in this study under anaerobic conditions [TABLE 1.6].

Anaerobic Fermentation - Malic Acid Production							
Time (days)	*A. pullulans* (g/L)	*B. bruxellensis* (g/L)	*B. claussenii* (g/L)	*D. anomala* (g/L)	*K. lactis* (g/L)	*K. marxianus* (g/L)	*M. genevensis* (g/L)
0	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.
20	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.
34	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.

[TABLE 1.6: Measurements are shown grams organic acid/L of sample. No malic acid was measured in analysis. Standard deviations were calculated from three replicates for all species besides *M. genevensis*, which had two viable replicates].

***Aerobic**:*

Acetic acid levels in fermentates increased in *K. lactis*, *B. claussenii*, *D. anomala* and *A. pullulans* [TABLE 1.7]. Concentrations of acetic acid in fermentates decreased with *K. marxianus*, *B. bruxellensis* and *M. genevensis*. Both *A. pullulans* and *M. genevensis* had decreases in acetic acid from D0 to D20, and increased in concentration from D20 to D34.

Aerobic Fermentation - Acetic Acid Production							
Time (days)	*A. pullulans* (g/L)	*B. bruxellensis* (g/L)	*B. claussenii* (g/L)	*D. anomala* (g/L)	*K. lactis* (g/L)	*K. marxianus* (g/L)	*M. genevensis* (g/L)
0	0.25 ± 0.18	0.4 ± 0.08	0.69 ± 0.51	0.86 ± 0.34	0.76 ± 0.24	0.52 ± 0.26	0.5 ± 0.24
20	0.29 ± 0.02	N.D.	5.88 ± 3.24	8.49 ± 3.03	0.75 ± 0.34	0.48 ± 0.28	0.26 ± 0.01
34	0.25 ± 0.07	0.03 ± 0.05	7.75 ± 4.37	9.18 ± 3.38	2.68 ± 1.81	0.34 ± 0.17	0.4 ± 0.15

[TABLE 1.7: Measurements are shown grams organic acid/L of sample. Standard deviations were calculated from three replicates for all species].

All species saw decreases in lactic acid composition under aerobic conditions, suggesting utilization of this acid [TABLE 1.8].

Aerobic Fermentation - Lactic Acid Production							
Time (days)	*A. pullulans* (g/L)	*B. bruxellensis* (g/L)	*B. claussenii* (g/L)	*D. anomala* (g/L)	*K. lactis* (g/L)	*K. marxianus* (g/L)	*M. genevensis* (g/L)
0	0.83 ± 0.04	0.84 ± 0.01	0.85 ± 0.03	0.87 ± 0.02	0.86 ± 0.01	0.86 ± 0.01	0.86 ± 0.01
20	0.26 ± 0.2	0.66 ± 0.29	0.4 ± 0.08	0.34 ± 0.05	N.D.	0.13 ± 0.18	0.67 ± 0.08
34	0.26 ± 0.21	0.29 ± 0.41	0.31 ± 0.23	0.19 ± 0.14	N.D.	0.06 ± 0.08	0.37 ± 0.29

[TABLE 1.8: Measurements are shown grams organic acid/L of sample. Standard deviations were calculated from three replicates for all species].

Brettanomyces claussenii, D. anomala, B. bruxellensis and *A. pullulans* all saw increases in tartaric acid content in the fermentate [TABLE 1.9]. *M. genevensis* and *K. lactis* did not have detectable levels of tartaric acid in their fermentate.

Aerobic Fermentation - Tartaric Acid Production							
Time (days)	*A. pullulans* (g/L)	*B. bruxellensis* (g/L)	*B. claussenii* (g/L)	*D. anomala* (g/L)	*K. lactis* (g/L)	*K. marxianus* (g/L)	*M. genevensis* (g/L)
0	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.
20	0.23 ± 0.17	0.04 ± 0.05	0.31 ± 0.31	0.44 ± 0.46	N.D.	0.04 ± 0.05	N.D.
34	0.39 ± 0.28	0.16 ± 0.22	0.65 ± 0.55	0.83 ± 0.62	N.D.	N.D.	N.D.

[TABLE 1.9: Measurements are shown grams organic acid/L of sample. Standard deviations were calculated from three replicates for all species].

No malic acid was measured in the initial substrate or after fermentation from each of the fermentation [TABLE 1.10].

Aerobic Fermentation - Malic Acid Production							
Time (days)	*A. pullulans* (g/L)	*B. bruxellensis* (g/L)	*B. claussenii* (g/L)	*D. anomala* (g/L)	*K. lactis* (g/L)	*K. marxianus* (g/L)	*M. genevensis* (g/L)
0	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.
20	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.
34	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.

[TABLE 1.10: Measurements are shown grams organic acid/L of sample. No malic acid was measured in analysis. Standard deviations were calculated from three replicates for all species].

Ethanol Production:
Anaerobic:

Both *K. marxianus* and *K. lactis* had the highest levels of ethanol production in this study [TABLE 1.11]. *K. marxianus* percent ethanol was the highest at D20 (4.52 ± 0.02%/Vol.), which decreased by D34 (4.47 ± 0.01%/Vol.). Even with this decreases, *K. marxianus* still had a higher percent ethanol than other fermentates on D34. Both of these species had more ethanol production from D0 to D20 than from D20 to D34. *K. lactis* did not have a decrease in ethanol concentration from D20 (3.72 ± 0.50%/Vol.) to D34 (4.33 ± 0.08%/Vol.).

Dekkera anomala and *B. claussenii* ethanol production increased from N.D levels on D0 to D34 (*D. anomala* D34 1.63 ± 0.18; *B. claussenii* D34 1.81 ± 0.36%/Vol.) [TABLE 1.11].

Aureobasidium pullulans had detectable levels of ethanol on D20 and D34 (D20 0.02 ± 0.03; D34 0.02 ± 0.02 %/Vol.), while *M. genevensis* only had detectable levels on D20 (0.03 ± 0.03%/Vol.). *Brettanomyces bruxellensis* had no detectable levels of ethanol [TABLE 1.11].

Anaerobic Fermentation - Ethanol Production							
Time (days)	*A. pullulans* (%/Vol.)	*B. bruxellensis* (%/Vol.)	*B. claussenii* (%/Vol.)	*D. anomala* (%/Vol.)	*K. lactis* (%/Vol.)	*K. marxianus* (%/Vol.)	*M. genevensis* (%/Vol.)
0	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.
20	0.02 ± 0.02	N.D.	1.27 ± 0.21	1.04 ± 0.13	3.72 ± 0.5	4.52 ± 0.02	0.03 ± 0.03
34	0.02 ± 0.03	N.D.	1.81 ± 0.36	1.63 ± 0.18	4.33 ± 0.08	4.47 ± 0.01	N.D.

[TABLE 1.11: Ethanol values are presented in percent ethanol/volume of solution. Standard deviation was calculated from three replicates for each species besides *M. genevensis*, which had two viable replicates].

Aerobic:

The highest percent ethanol of species tested in fermentate was seen from *K. marxianus* and *K. lactis* on D20 (*K. marxianus* 0.68 ± 0.48; *K. lactis* 0.53 ± 0.33%/Vol.); however both fermentates declined in percent ethanol by D34 (*K. marxianus* 0.027 ± 0.038; *K. lactis* 0.043 ± 0.061%/Vol.) [TABLE 1.12].

Dekkera anomala and *B. claussenii* continued to produce ethanol from N.D. levels on D0 to D34 (*D. anomala* 0.201 ± 0.149; *B. claussenii* 0.198 ± 0.238%/Vol.); having the highest final percentages of ethanol of fermentates at D34 [TABLE 1.12].

B. bruxellensis, *M. genevensis* and *A. pullulans* did not see any ethanol production [TABLE 1.12]. A minimal concentration of ethanol was detected on D0 in the *A. pullulans'* fermentate (0.01 ± 0.01%/Vol.), however none was seen on D20 or D34.

Aerobic Fermentation - Ethanol Production							
Time (days)	*A. pullulans* (%/Vol.)	*B. bruxellensis* (%/Vol.)	*B. claussenii* (%/Vol.)	*D. anomala* (%/Vol.)	*K. lactis* (%/Vol.)	*K. marxianus* (%/Vol.)	*M. genevensis* (%/Vol.)
0	0.01 ± 0.01	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.
20	N.D.	N.D.	0.15 ± 0.18	0.12 ± 0.09	0.53 ± 0.33	0.68 ± 0.48	N.D.
34	N.D.	N.D.	0.2 ± 0.24	0.2 ± 0.15	0.04 ± 0.06	0.03 ± 0.04	N.D.

[TABLE 1.12: Ethanol values are presented in percent ethanol/volume of solution. Standard deviation was calculated from three replicates for each species].

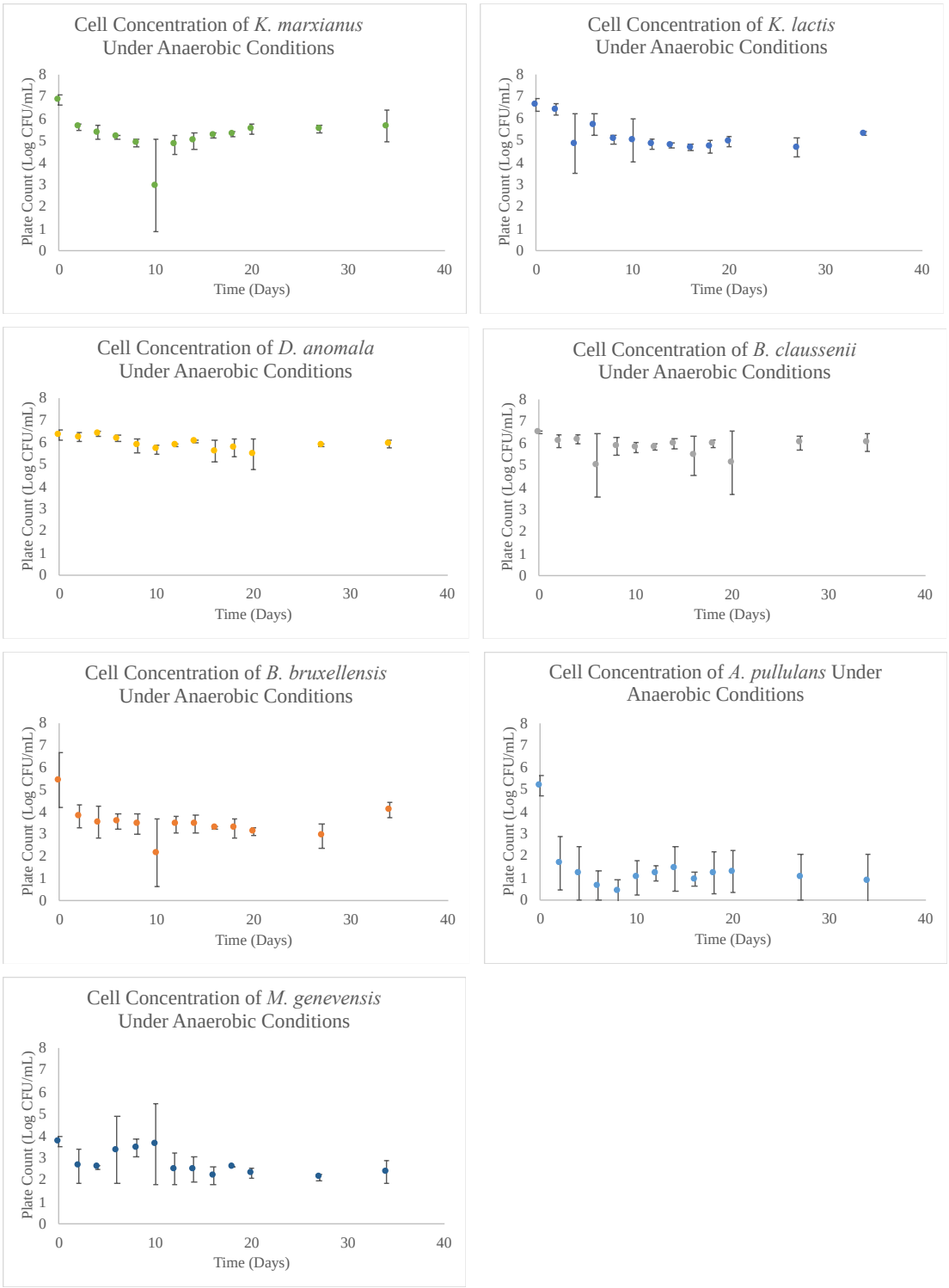
Cell Count:
Anaerobic:

Under anaerobic conditions, each species saw declines in cell count from the initial inoculation [GRAPH 1.5]. It is possible that because anaerobic fermentates were not agitated, cells settled out of the fermentate, and when aspirating a homogenized sample was not taken. *Kluyveromyces marxianus, K. lactis, B. bruxellensis, A. pullulans* and *M. genevensis* all decreased in cell concentrations by one or more log reductions (*K. marxianus* D0 6.82 ± 0.23, D34 5.65 ± 0.72; *K. lactis* D0 6.60 ± 0.30, D34 5.31 ± 0.10; *B. bruxellensis* D0 5.41 ± 1.23, D34 4.06 ± 0.34; *A. pullulans* D0 5.17 ± 0.45, D34 0.86 ± 1.22; *M. genevensis* D0 3.73 ± 0.24, D34 2.36 ± 0.53 Log CFU/mL), while *B. claussenii* and *D. anomala* had less than a one log reduction in cell concentration (*B. claussenii* D0 6.49 ± 0.06, D34 6.01 ± 0.41; *D. anomala* D0 6.31 ± 0.21, D34 5.91 ± 0.15) [GRAPH 1.5].

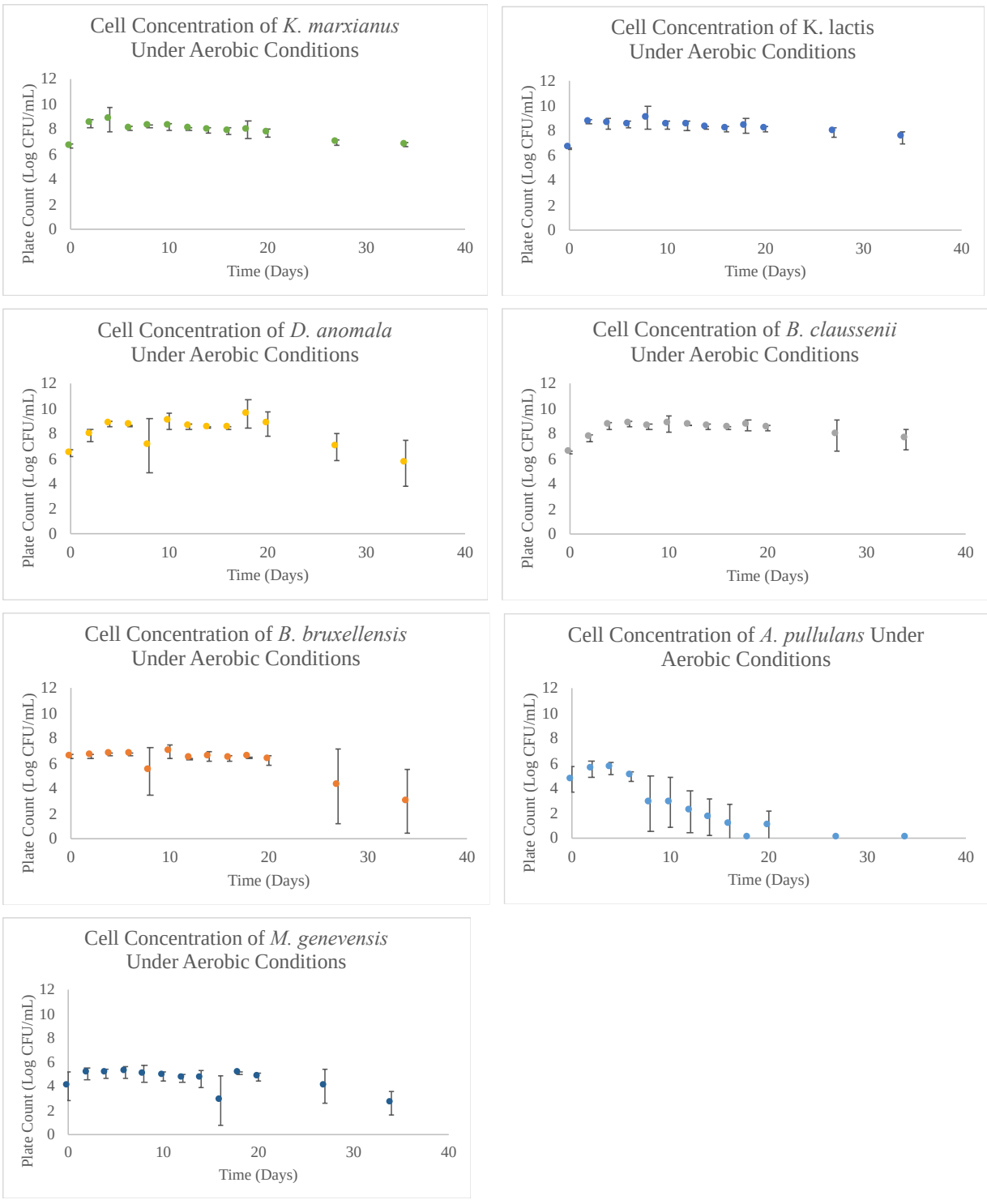
Aerobic:

Under aerobic conditions species varied in increasing or decreasing their cell concentration [GRAPH 1.6]. *Kluyveromyces marxianus, K. lactis* and *B. claussenii* all increased in cell count by D34 (*K. marxianus* D0 6.61 ± 0.16, D34 6.73 ± 0.14; *K. lactis* D0 6.57 ± 0.03, D34 7.40 ± 0.44; *B. claussenii* D0 6.51 ± 0.10, D34 7.52 ± 0.85LogCFU/mL) [GRAPH 1.6]. *D. anomala, B. bruxellensis, A. pullulans* and *M. genevensis* all had final cell count lower than the initial inoculation (*D. anomala* D0 6.41 ± 0.25, D34 5.66 ± 1.84; *B. bruxellensis* D0 6.52 ± 0.12,

D34 2.96 ± 2.52; *A. pullulans* D0 4.67 ± 1.03, D34 0.00 ± 0.00; *M. genevensis* D0 3.94 ± 1.19, D34 2.60 ± 0.98 Log CFU/mL) [GRAPH 1.6].



[GRAPH 1.5: Error bars represent standard deviation, calculated from three replicates for each species besides *M. genevensis*, which had two viable replicates].



[GRAPH 1.6: Error bars represent standard deviation was calculated from three replicates for each species].

Discussion:
Yeast species:
Kluyveromyces Species:

The species that most effectively utilized lactose was *K. marxianus* under anaerobic and aerobic conditions, followed by *K. lactis* [TABLE 1.1, 1.2]. The *Kluyveromyces* species have been observed to thrive in dairy manufacturing and as spoilage organisms because of their ability to hydrolyze lactose through the expression of β-galactosidase and lactose permease (*31, 35, 39*). Both organisms utilized the most lactose at the fastest rate of species tested, shown by declines in lactose concentration and density [GRAPH 1.1, 1.2]. Under aerobic conditions, both *Kluyveromyces* species had a faster decline in density than in anaerobic conditions, and lower final densities. Both organisms saw an increase in cell concentration by D2, which remained higher than initial inoculation concentration in aerobic conditions. *Kluyveromyces marxianus* also saw an increase in density after D6, which could correlate to an increase of biomass in the fermentate [GRAPH 1.2, 1.6]. Cell concentration and lactose utilization was increased in aerobic environments, suggesting these yeasts prefer aerobic conditions for lactose hydrolysis [GRAPH 1.6, TABLE 1.2].

Both *Kluyveromyces* species reached a lower pH value under aerobic conditions than anaerobic (Lowest pH reached: *K. marxianus* D10 anaerobic: 5.15 ± 0.04, D3 aerobic: 4.95 ± 0.15; *K. lactis* D34 anaerobic: 5.27 ± 0.08, D6 aerobic: 4.43 ± 0.14). Under aerobic conditions, the pH increased from that point until day 34 (*K. marxianus* D34 anaerobic: 5.32 ± 0.02, aerobic: 6.51 ± 0.16; *K. lactis* D34 anaerobic: 5.27 ± 0.08, aerobic: 5.04 ± 1.03); while in anaerobic conditions the initial drop to that pH was maintained until the end of the fermentation [GRAPH 1.3, 1.4]. These changes in pH may correlate to the production of acetic acid and ethanol in anaerobic conditions (*K. marxianus* D34 anaerobic: 1.39 ± 0.19g/L acetic acid, 4.47 ±

0.01%/Vol. ethanol; *K. lactis* D34 anaerobic: 0.79 ± 0.08g/L acetic acid, 4.33 ± 0.08%/Vol. ethanol), while lactic acid was utilized in aerobic conditions (*K. marxianus* D34 aerobic: 0.07 ± 0.08g/L lactic acid; *K. lactis* D34 aerobic: N.D. g/L lactic acid) [TABLE 1.3, 1.8, 1.11].

Research suggests that the *Kluyveromyces* species have optimal lactose utilization between a pH of 5-6, and a temperature of 35ºC (*23*). Under anaerobic conditions, both species stayed in this pH range, and continued lactose utilization and organic acid production throughout the experiment [GRAPH 1.3, TABLE 1.4, 1.5, 1.6]. In aerobic conditions, *K. marxianus* stayed in this pH range of 5-6, while *K. lactis* held a lower pH from D3 (4.83 ± 0.15) to D20 (4.88 ± 0.09). Lactose utilization and organic acid production continued for both species, suggesting that *K. lactis* may have a larger range in pH for optimal cellular function [GRAPH 1.4, TABLE 1.7, 1.8, 1.9].

Under anaerobic conditions, *Kluyveromyces marxianus* had the highest acetic acid production of species in anaerobic conditions, while *K. lactis* had the highest tartaric acid production among species (*K. marxianus* acetic acid 1.39 ± 0.19; *K. lactis* tartaric acid 0.44 ± 0.05g/L) [TABLE 1.3, 1.5]. Under aerobic conditions, both species saw the largest decreases in lactic acid concentrations by D34 (*K. marxianus* 0.06 ± 0.08; *K. lactis* N.D. g/L), suggesting both organisms utilize this acid under aerobic conditions [TABLE 1.8]. Under aerobic conditions, both *B. claussenii* and *D. anomala* produced more acetic acid than each *Kluyveromyces* species [TABLE 1.8].

Under anaerobic conditions, the production of ethanol in fermentation can be inhibitory for cell growth and survival for unicellular species (*29*). A decrease in cell counts were observed under anaerobic conditions for *K. marxianus* and *K. lactis*, which may be attributed to their

ethanol production [TABLE 1.11]. Not unexpectedly, less ethanol production was seen in aerobic conditions, and both species increased in cell concentration [TABLE 1.12, GRAPH 1.6].

Brettanomyces Species and D. anomala:

Brettanomyces and *Dekkera* genera are genetic teleomorphs, and are facultative anaerobes (*12, 22*). *Brettanomyces claussenii* is used in brewing operations, and *D. anomala* is viewed as spoilage organisms in wine production (*15*, *17*). *Brettanomyces spp.* and *Dekkera spp*. have often been used in alcoholic fermentations due to their ability to survive and continue metabolic activities in acidic and low oxygen environments (*16, 17*). The ability to continue metabolic activity in an acidic environment from these species was seen as both continued organic acid production, lactose utilization and had stable cell concentrations in anaerobic and aerobic conditions, while having the lowest final pH of fermentates [GRAPH 1.5, 1.6, TABLE 1.1-1.12] (*17*).

Both *B. claussenii* and *D. anomala* species produced the most acetic acid under aerobic conditions (D34 *B. claussenii* 7.75 ± 4.37; *D. anomala* 9.18 ± 3.38g/L), giving a possible reason as to why these species had the lowest fermentate pH on D34 (*B. claussenii* 4.51 ± 0.57; *D. anomala* 4.29 ± 0.31) [TABLE 1.7, GRAPH 1.4]. Both species saw declines in lactic acid under aerobic conditions, and increases in anaerobic conditions [TABLE 1.4, 1.8]. Tartaric acid production was seen under both conditions; however, more was produced in aerobic conditions [TABLE 1.5, 1.9].

Brettanomyces claussenii was more efficient on average than *D. anomala* at utilizing lactose under both conditions [TABLE 1.1, 1.2]. *Dekkera anomala* and *B. claussenii* both had small amounts of galactose present in anaerobic conditions, suggesting glucose is preferred once lactose is hydrolyzed [TABLE 1.1, 1.2]. *Brettanomyces claussenii* and *D. anomala* were less

effective at lactose utilization than the *Kluyveromyces spp.*, however both were more efficient than other species in this study [TABLE 1.1, 1.2]. Longer fermentations would need to be conducted to see if total utilization of lactose is possible.

Brettanomyces bruxellensis, had no lactose utilization under anaerobic and limited under aerobic conditions [TABLE 1.1, 1.2]. In both anaerobic and aerobic conditions, *B. bruxellensis* was able to utilize acetic and lactic acid, which is supported by the increase in fermentate pH [GRAPH 1.3, 1.4, TABLE 1.2, 1.3, 1.7, 1.8]. Production of tartaric acid was seen in aerobic conditions [TABLE 1.5]. Cell concentrations decreased in anaerobic and aerobic conditions [GRAPH 1.5, 1.6]. It is likely this *Brettanomyces* species is not as well suited for fermentation of dairy products in comparison to the other *Brettanomyces* and *Dekkera* species in this study.

Mold species
M. genevensis and A. pullulans:

Mucor species have been shown to utilize lactose, and are considered spoilage organisms in dairy facilities (*18, 24, 35*). Research suggests that lactose utilization and enzyme production in *M. circinelloides* and *M. miehei* can be affected by temperature and pH (*21, 30*). Both species showed optimal lactose utilization at 60^{o}C in a pH range of 4-6 (*21, 30*). It is possible that our fermentation at 30^{o}C was not warm enough to promote lactose utilization for *M. genevensis* [TABLE 1.1, 1.2].

Mucor genevensis is a dimorphic fungi, growing as a mold in aerobic conditions, and as a yeast in anaerobic conditions (*4, 7*). It is possible when expressing different morphologies, the organism produces different enzymes, changing its metabolic capabilities. This study did not examine gene, and further experiments would be needed to support this hypothesis.

Mucor genevensis had several fluctuations in cell count throughout the fermentation [GRAPH 1.5]. This may be attributed to the dimorphic lifecycle (*7*). More research will need to

be conducted on this species' lifecycle to draw further conclusions on how these conditions effect cell growth and which morphology is more suited for cell growth.

Under anaerobic conditions, *M. genevensis* utilized all lactic acid in the fermentate having N.D values on D20 and D34, the most of any species, while under aerobic conditions, *M. genevensis* utilized the least lactic acid of species tested [TABLE 1.4, 1.8]. Acetic acid was produced under anaerobic conditions, and utilized under aerobic conditions [TABLE 1.3, 1.7]. Tartaric acid was seen in anaerobic conditions, but not in aerobic conditions [TABLE 1.5, 1.9]. The pH of the fermentate increased in both conditions, however a much larger increase was seen in an anaerobic environment, likely attributed to the utilization of lactic acid [GRAPH 1.3, 1.4]. Given the differences in organic acid utilization under anaerobic vs. aerobic conditions, it is likely the environmental condition impacts the species' metabolic activities, which may correlate to the expressed morphology.

The data suggests *A. pullulans* prefers glucose to galactose, as residual galactose was detected under anaerobic and aerobic conditions, while glucose levels were not [TABLE 1.1, 1.2].

Aureobasidium pullulans has been seen to prefer acidic environments below a pH of 4 (*1*). Studies have shown that given this preference, adaptation of *A. pullulans* to a lactose substrate can be achieved in an aerobic environment with a pH below 5 (*2, 28*). This study did not optimize the substrate, pH or environmental conditions specifically for each yeast, which could suggest why lactose utilization was limited under anaerobic and aerobic conditions. More research would need to be conducted to test of adaption to whey permeate is possible.

Aureobasidium pullulans has been shown to prefer aerobic environments for cellular functions and growth (*10*, *19*). This study agreed with previous research in that higher cell concentrations were seen in aerobic conditions compared to anaerobic [GRAPH 1.5, 1.6].

Conclusion:

From this study, we have hypothesized several alternative fermentation methods for whey by-products using the organisms studied under anaerobic or aerobic conditions.

Under anaerobic conditions, the *Kluyveromyces* species would be best suited for producing alcoholic beverage from whey permeate, as it had the most ethanol production [TABLE 1.11], though both *B. claussenii* and *D. anomala* could also be used to product low-alcohol variants with residual sugar. These beverages could potentially provide economic gains for dairy manufacturers, and could compete in the rising market of low alcohol beverages (*13*). This product could also decrease the costs accrued from waste disposal by creating a demand for upcycling whey permeate.

The U.S. Tax and Trade Bureau require that if a fermented beverage has greater than 0.5% alcohol at any point of production or post bottling, the beverage must be labeled and regulated as an alcoholic beverage (*5*). *Brettanomyces claussenii* and *D. anomala* were capable of producing the most acetic acid of species studied, while maintaining low ethanol percentages (<0.5%) under aerobic conditions [TABLES 1.7, 1.12]. This organic acid has become a popular nutrient in drinking vinegars and kombucha, which expect to see large increases in sales, forecasted to be a $3.8 billion industry by 2023 (*8*). With the rise of kombucha and functional beverages, this product would be poised for success in the growing market of functional probiotic beverages. Further research would need to be done post bottling of this product to

ensure ethanol levels do not exceed 0.5%, however these species should be considered as viable options for a whey permeate fermented beverage.

Both *A. pullulans* and *M. genevensis* saw increases in pH under aerobic conditions and decreases in lactic acid content [GRAPH 1.4, TABLE 1.8]. Whey permeate has similar lactose composition to acid whey, however the pH of the whey permeate substrate in this study was ~6.5 [GRAPH 1.3, 1.4], while acid whey has been measured to have a pH between 4.21-4.48 (*20*, *26*). This pH limits disposal methods, governed under Solid Waste in NYS (*38*). Inoculating acid whey with *A. pullulans* or *M. genevensis* should be tested, as it might increase the pH to neutral level. If these species were able to neutralize acid whey, they could be used as part of a waste processing system, to increase the pH to a level that would allow for more acid whey to be deposited into soil, or addition to animal feed. This simple process could increase the quantity of acid whey disposal/acre of land, decrease the amount of energy that waste treatment plants use processing acid whey, thus lowering costs of dairy manufacturers.

Several species were seen to produce tartaric acid in anaerobic or aerobic conditions. Tartaric acid is often found to by synthesized by plants, particularly grapes in the wine industry (*16*). Few microbes are known to produce tartaric acid; however, some yeast strains have been seen to synthesize it (*16*). Under anaerobic conditions, *K. marxianus, K. lactis, B. claussenii, D. anomala* and *M. genevensis* had detectable levels of tartaric acid; and in aerobic conditions, *K. marxianus, B. claussenii, D. anomala, B. bruxellensis* and *A. pullulans* had detectable levels [TABLES 1.5, 1.9]. This acid is commonly used in the food industry as a leavening agent and has been suggested to have nutritional benefits (*14*). It should be noted that these yeasts are capable of tartaric acid production from a lactose source. Optimizing the biological synthesis of

this acid will need to be studied, as a higher production could be of economic benefit to the dairy industry.

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CHAPTER 2:
CLARIFICATION OF ACID WHEY WITH ACTIVE CARBON

ABSTRACT:

Acid whey, a by-product from Greek yogurt production, is regulated as a solid waste in New York State. It is yellow, murky and pungent, with a high concentration of lactose and an acidic pH (4.21-4.48); all traits making it difficult to process. Active carbon has been used in the beverage industry to remove color, turbidity and strong flavors of beverages, making a more appealing product to a wider range of consumers. Research has shown that acid whey can be fermented to produce organic acids or ethanol, which can be used to make low alcohol tonics or kombucha style beverages. This study looked at clarifying acid whey through active carbon exposure. Measuring colorimetry (L*a*b* values), a significant correlation was seen in a reduction of color from a* and b* values with increases in carbon concentration (CC; *P-value* a*: 0.00; b*: 0.01) and time of exposure (*P-value* a*: 0.03; b*: 0.01). No significant correlation was seen in changes of pH (*P-value* CC: 0.25; time: 0.66), turbidity (*P-value* CC: 0.54; time: 0.18) or L* (*P-value* CC: 0.27; time: 0.50) to carbon concentration or time of exposure. Utilizing fermentation of acid whey and active carbon exposure, a process could be designed for production of an upcycled fermented commercial beverage. This product could replace the current disposal methods and upcycle large quantities of this by-product.

Introduction:

Acid whey is the liquid by-product from manufacturing Greek yogurt. It has a strong dairy smell, and a murky yellow color with a pH between 4.21-4.48 (*5*). These attributes make it an off-putting liquid, that is processed as waste. Acid whey is currently disposed of either through land application, addition to animal feed or through waste water treatment, and is regulated as solid waste by New York State (NYS) (*9*). The cost of removal and disposal of acid whey can become costly for dairy operations, and with the rise in Greek yogurt production in NYS there is increasing need to address alternatives for removal (*4*).

Research on the composition of acid whey indicated that there are several mineral components that could make this the base for a value-added product of the dairy industry such as calcium, lactose, magnesium, and other minerals (*5*). Due to the lactose composition in acid whey, yeast have been used in fermentation to produce ethanol and organic acids (*3*). Organic acids have been shown to have positive health effects, and which have increased kombucha sales, a product that is rich in these acids (*1*, *7*). Low alcohol tonics have also become popular in the United States with a rise in fermented beverages (*8*).

A challenge with whey-based beverages unpleasant dairy flavors and color. A common technique used in the brewing industry is to treat malt beverages with active carbon to remove color, turbidity and flavor to clarify the final product (*2*). This study used active carbon exposure to see if the yellow color and turbidity of acid whey could be reduced. Using this method, acid whey could be fermented, clarified and flavored to create a healthy value-added product.

Experimental Design:

Acid whey was obtained from a local commercial producer and made sterile through autoclaving. 250mL of acid whey was exposed to either 0.0 g/L, 0.01 g/L, 0.05 g/L, 0.1 g/L, or 0.5 g/L of active carbon for either 30, 60, 120, or 240 minutes. Each carbon concentration (CC) and time of exposure was repeated in triplicate.

Once exposure began, samples were placed at 4^{o}C without agitation. Once exposure time was completed, samples were centrifuged for 25 minutes at 4000 RPM. Samples were then poured off to leave residual active carbon in the cuvette. Treated acid whey was made sterile through a 45μm PES filter and held at 4^{o}C until data collection.

Sample Processing:

Each sample was tested for pH, colorimetry and turbidity. pH was measured using an AMS Alliance *iCinac*, with InLab Smart Pro-ISM probes (Mettler Toledo, Columbus, OH). Colorimetry was taken using a Konica Minolta Chroma Meter CR-400 (Ramsey, NJ), measuring L*a*b* values. Turbidity was taken using a HACH 2100P TURBIDIMETER 46500-00 (Loveland, CO) and reported NTU values.

Statistics:

One-way regression analysis using ANOVA were run on pH, turbidity, L*, a* and b* as response values comparing time and CC as explanatory values. Microsoft Excel 2016 data analysis add-in was used as the statistical processor.

Results:

pH:

Changes in pH were not seen to be correlated to concentration of carbon added or time of carbon exposure (*Significance F*: 0.47; CC *P-value*: 0.25; Time *P-value*: 0.66) [TABLE 2.1].

pH of Acid Whey Treated With Active Carbon				
Carbon Concentration	30 Minutes	60 Minutes	120 Minutes	240 Minutes
0.000	4.36 ± 0.07	4.33 ± 0.06	4.32 ± 0.08	4.33 ± 0.05
0.001	4.33 ± 0.01	4.32 ± 0.05	4.29 ± 0.05	4.32 ± 0.04
0.005	4.31 ± 0.01	4.33 ± 0.05	4.29 ± 0.05	4.32 ± 0.04
0.010	4.32 ± 0.01	4.32 ± 0.05	4.29 ± 0.05	4.32 ± 0.04
0.050	4.31 ± 0.01	4.32 ± 0.05	4.27 ± 0.06	4.31 ± 0.04

[TABLE 2.1: pH values are reported with ± standard deviation].

Turbidity:

Changes in turbidity due to exposure time or concentration of carbon were not seen to be statistically significant (*Significance F*: 0.15; CC *P-value*: 0.31; Time *P-value*: 0.10) [TABLE 2.2].

Turbidity in NTU of Acid Whey Treated With Active Carbon				
Carbon Concentration	30 Minutes	60 Minutes	120 Minutes	240 Minutes
0.000	7.11 ± 4.74	2.12 ± 0.38	4.49 ± 2.23	7.35 ± 2.36
0.001	5.52 ± 2.62	2.13 ± 0.39	4.51 ± 1.98	6.4 ± 1.64
0.005	6.39 ± 1.73	2.16 ± 0.29	4.5 ± 2.06	5.54 ± 1.06
0.010	6.28 ± 2.52	2.85 ± 1.11	5.89 ± 3.6	5.33 ± 0.92
0.050	4.02 ± 1.53	2.41 ± 0.83	4.49 ± 3.48	5.14 ± 1.16

[TABLE 2.2: NTU values are reported with ± standard deviation].

Colorimetry:

For each exposure and concentration of active carbon, no significant correlation was seen in change of L* to CC or time of exposure (*Significance F* = 0.20; CC *P-value*: 0.66; Time *P-value*: 0.09) [TABLE 2.3].

L* value of Acid Whey Treated With Active Carbon				
Carbon Concentration	30 Minutes	60 Minutes	120 Minutes	240 Minutes
0.000	58.47 ± 2.34	56.09 ± 1.89	55.6 ± 0.24	59.08 ± 0.91
0.001	59.37 ± 2.87	48.2 ± 0.42	58.64 ± 2.94	55.89 ± 0.52
0.005	58.69 ± 2.66	57.88 ± 0.5	55.72 ± 1.33	57.16 ± 0.33
0.010	58.24 ± 3.73	57.05 ± 2.22	55.61 ± 1.45	55.52 ± 1.06
0.050	58.31 ± 1.71	59.38 ± 1.36	58.02 ± 0.32	56.17 ± 0.19

[TABLE 2.3: L* values are reported with ± standard deviation].

Both regression models for a* or b* as response variables, and carbon and time as explanatory variables had significant correlations (a* *Significance F*: 2.6×10^{-4}, CC *P-value*: 1.9×10^{-4}, Time *P-value*: 0.03; b* *Significance F* = 5.4×10^{-6}, CC *P-value*: 2.9×10^{-6}, Time *P-value*: 0.02) [TABLES 2.4, 2.5]. According to R^2 value, 62.13% of a*, and 75.99% of b* response can be explained through changes in time and carbon concentration by this model.

a* value of Acid Whey Treated With Active Carbon				
Carbon Concentration	30 Minutes	60 Minutes	120 Minutes	240 Minutes
0.000	-2.17 ± 0.59	-1.54 ± 0.21	-1.58 ± 0.53	-2.94 ± 0.66
0.001	-2.28 ± 0.37	-1.17 ± 0.33	-1.74 ± 0.55	-2.42 ± 0.46
0.005	-2.07 ± 0.38	-1.44 ± 0.24	-1.46 ± 0.52	-2.29 ± 0.6
0.010	-2.07 ± 0.62	-1.25 ± 0.08	-1.41 ± 0.57	-1.95 ± 0.33
0.050	-1.15 ± 0.18	-0.84 ± 0.05	-0.82 ± 0.24	-0.99 ± 0.08

[TABLE 2.4: a* values are reported with ± standard deviation].

b* value of Acid Whey Treated With Active Carbon				
Carbon Concentration	30 Minutes	60 Minutes	120 Minutes	240 Minutes
0.000	4.18 ± 0.91	2.47 ± 0.54	2.88 ± 1.16	5.9 ± 1.33
0.001	4.13 ± 0.52	1.99 ± 0.38	2.88 ± 0.87	4.71 ± 0.76
0.005	3.74 ± 0.33	1.94 ± 0.64	2.3 ± 1.08	4.14 ± 1.11
0.010	1.67 ± 1.06	1.5 ± 0.34	2.2 ± 1.35	3.38 ± 0.74
0.050	1.37 ± 0.19	0.13 ± 0.17	0.43 ± 0.53	0.94 ± 0.19

[TABLE 2.5: b* values are reported with ± standard deviation].

Discussion:

Using the L*a*b* colorimetry scale, a value for L* (lightness as a positive number, darkness as a negative number), a* (red as a positive number, green as a negative number) and b* (yellow as a positive number, blue as a negative number) were obtained. Using this scale, our research suggests that treating acid whey with carbon can clarify the product, as reductions in a* and b* color values were seen to correlate with CC and time of exposure [TABLE 2.4, 2.5].

Previous research has shown that the lactose in acid whey can be fermented by yeast strains that are capable of utilizing lactose (*3*). A combination of microbial fermentation flowed by clarification could be a process used to upcycle acid whey into a neutral-bases for commercial beverages.

Further research will need to be conducted to test sensory perception to track consumer preference in smell, taste and color of active carbon treated acid whey. This treatment may reduce strong dairy flavor or smells, that make the raw material unappealing to consumers, however sensory analysis was not conducted in this study.

Longer exposure times should be studied. These experiments measured up to four hours for exposure to active carbon, which did not see significant changes in turbidity, pH or L* [TABLES 2.1, 2.2, 2.3]. The exposure time used did see significant changes in a* and b*

[TABLE 2.4, 2.5], and it may be assumed that longer exposure time or increased CC may render a reduction in turbidity, pH or L* of acid whey.

Conclusion:

Active carbon filtration was not seen to correlate to changes in pH, turbidity or L* of acid whey regardless of the concentration or duration of exposure. Significant changes were seen in a* and b* color values of acid whey, which correlated to CC and time of exposure. More research will need to be conducted on these variables in larger volumes; however, it appears that this method could be employed to clarify acid whey.

While this method will need to be refined and optimized before it can be employed commercially, the results of this study suggest that it could be used as a treatment to clarify acid whey before or after fermentation or processing for an upcycled beverage.

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