BIOTECHNOLOGICAL APPROACHES FOR COMBATING FOOD WASTE IN THE DAIRY INDUSTRY

A Dissertation

Presented to the Faculty of the Graduate School

Of Cornell University

In Partial Fulfillment of the Requirements for the Degree of

Doctor of Philosophy

by

Marie Rose Lawton

December 2021

BIOTECHNOLOGICAL APPROACHES FOR COMBATING FOOD WASTE IN THE DAIRY INDUSTRY

Marie Rose Lawton, Ph.D.

Cornell University, 2021

Sustainability of the current global food system needs to be revaluated. Food waste, particularly that coming from the dairy industry accounts for a large portion of the food system's global warming potential. The large amounts of energy and resources required to power this food system call for efforts to reduce the burden of emissions from the dairy industry. At the consumer and industrial level, waste in the dairy industry occurs through by-products and food loss due to spoilage or contamination. Previous efforts have been introduced and studied to combat this waste and loss through biopreservation. The present work aims to expand on these opportunities by studying some biotechnological approaches for preservation of dairy products to reduce waste. First, enzymes endogenous to barley were studied for their ability to hydrolyze the lactose in dairy by-products making it available as fermentable sugars. Measurement of residual glucose levels in acid whey mashes containing barley indicated successful hydrolysis of the lactose. This led to successful fermentation of acid whey with the yeast *Saccharomyces cerevisiae* to produce ~3.2% ABV ethanol. Next, lactic acid bacteria were evaluated for their bioprotection ability to enhance the food safety of fresh-style cheese from *Listeria monocytogenes*. Application of several species of lactic acid bacteria into a lab-scale fresh cheese model revealed that they had an insignificant effect on reducing or limiting listerial growth at multiple inoculation levels (2 or 4 log CFU/g *L. monocytogenes*) and two temperatures (6°C or

21°C). Further evaluation of these species or incorporation with additional control strategies is necessary in order to implement this approach. Building upon the enzymatic hydrolysis of acid whey, a lactose-utilizing yeast, *Brettanomyces claussenii* was then evaluated for its potential to valorize lactose-containing dairy by-products by producing acetic acid. Optimization of these fermentations was done through a screening design and response surface methodology to determine the best fermentation conditions for acetic acid production with this yeast. Additionally, a molecular biology and bioinformatic approach was used to probe the genomic mechanism responsible for the lactose metabolism of this yeast. Cloning of two putative genes from *B. claussenii* into *S. cerevisiae* confirmed identity of a β-galactosidase (*LAC4*) and lactose permease (*LAC12*) gene. The information gained from these approaches allows for the potential of additional applications to reduce food waste in the dairy industry. Reutilization of by-products to produce value-added goods or enhancement of food safety through bioprotection schemes can provide powerful opportunities to increase sustainability in the dairy industry.

BIOGRAPHICAL SKETCH

Marie is from Massachusetts where she received her bachelor's degree in Food Science from the University of Massachusetts Amherst in 2016. During that time, she participated in undergraduate research focusing on small farm food safety practices under the advisement of Amanda Kinchla. Additionally, she worked part time as Quality Assurance Technician at a local apple cider production facility. Following these experiences and after graduation from the University of Massachusetts, Marie pursued her Ph.D. in Food Science at Cornell under the advisement of Dr. Samuel Alcaine. Following completion of her Ph.D., Marie will work in Research & Development at a biotech company in Boston, MA.

ACKNOWLEDGMENTS

I would like to thank my advisor, Dr. Samuel D. Alcaine, for all his encouraging support, advice, and guidance over the past 5 years. In addition, I would like to thank Drs. Sam Nugen, and Randy Worobo for their support and encouragement as my committee members. Thank you to Amanda Kinchla for her guidance and unwavering support during my undergraduate years at UMass Amherst. And of course, thank you to my family and friends – those new and old – this work would not have been possible without your support.

This work was supported by AFRI Predoctoral Fellowship [Grant # 2020-67034-31743] from the USDA National Institute of Food and Agriculture. Additionally, this project was funded, in part by, the New York State Department of Environmental Conservation (Project Number 76370/A001), the National Dairy Council, and the Innovation Center for US Dairy.

TABLE OF CONTENTS

CHAPTER 1

INTRODUCTION

1.1 Global Sustainability of the Current Food System

The current global food system accounts for 26-34% of the total greenhouse gas emissions [1,2]. Roughly two thirds of these emissions are attributed directly to agriculture, i.e., land use, crop and livestock production while the remaining comes from the industrial food supply chain, i.e., processing and transport, whereas specifically in more industrialized countries this ratio is divided more evenly, tipping towards the majority from industrial [1,3,4]. With attention to this large contribution to global emissions and climate change, the sustainability of the food system needs to be revaluated.

Studies from the past decade have shown that approximately a third (1.3 billion tons) of the food meant for human consumption is wasted on the global level, and 35-36 million tons of this comes from the United States alone [5–7]. Food waste is defined through multiple categories: surplus – occurring due to overproduction from a variety of factors, waste – occurring at the retail and consumer end of the food system, and loss – occurring within the industrial processing step of food production [5,8]. While a majority of waste occurs with the consumer – in Europe and North America, the Food and Agriculture Organization estimates 95-115 kg/year per capita [5] – a fair share also occurs at the industrial level [9,10]. Generally, waste can occur due to spoilage at the consumer and industrial level, issues with supply and demand, processing malfunctions, transportation delays, or for a variety of other reasons [8]. A 2018 study looking at food waste data from Europe, determined that 15% of the total food system global warming potential was directly related to food waste, the majority being during production [11].

1.1.2 Food Waste in the Dairy Industry

When considered by commodity, almost 5% of this food waste is in the dairy sector [9,10]. The dairy industry as a whole contributes to a large portion of overall greenhouse gas emissions. In 2007, it was found that milk products (production, processing, and transportation) accounted for 2.7% of total global emissions [12]. When considering the large amount of energy and resources put into this system and the large impact that food waste in general has on global warming, 5% of total food waste coming from dairy products is distressing. While efforts to sustain emissions from the dairy industry on the agricultural side have been implemented and are improving [13], an all hands on deck approach is the key to keeping up with society's current situation of climate change.

Aside from waste at the consumer and industrial level through the routes mentioned above, a large portion of the waste in the dairy industry is seen amid by-products that come from manufacturing of dairy products such as cheese and yogurt, mainly in the form of liquid whey. Many efforts have been established to recycle and even valorize this by-product; concentration and drying to obtain its protein for protein powders, and further processing to whey protein concentrates and isolates [14,15]. However, this strategy primarily applies to whey coming from cheese production, considered sweet whey. Over the past two decades, consumption of Greek-Style and strained yogurts has been popular among consumers for their high protein content and texture properties [16,17]. This production process, and its protein-concentrating straining step, generates a large amount of liquid by-product, termed acid whey. Approximately 2-3 kg of acid whey are produced for every 1 kg of yogurt [18]. Unlike sweet whey, acid whey, acidic due to yogurt cultures, does not have the same potential for protein powder recovery due to its low pH and protein content. Owing to this acidity as well as a large lactose and mineral content [16], traditional disposal of acid whey is regulated due to a large biological oxygen demand caused by

these components leading to potential for polluting waterways [19]. Repurposing of this by-product has been endeavored through application to fields as fertilizer, incorporation into animal feed, and feeding to anaerobic digestors [19,20]. However, these utilization schemes are still limited. Revisiting the substantial climate impact from food waste and the dairy industry in general, the constituents that are present in acid whey present as valuable resources for reutilization.

In addition to edible waste considered as by-products within the dairy industry, a portion of inedible waste can arise from food safety concerns and product recalls. Food products can be recalled for three levels of causes: (i) serious injury or death – i.e., contamination with a food pathogen; (ii) may cause injury or illness – i.e., physical contaminants in food; (iii) unlikely to cause injury or illness – i.e., mislabeled packaging [21]. While there is limited data for amount of food recalled annually, one study collected data within a 22-year period (1994-2015) and reported that 313 million kg of meat and poultry products were recalled during this time frame. Of these recalls, 44% were due to contamination with microbial pathogens [22]. Within the dairy industry, fresh-style cheeses provide the pathogen *L. monocytogenes* an environment to thrive and has resulted in previous outbreaks and deaths from contaminated products [23–25]. In the United States, the Food and Drug Administration (FDA) has established a zero-tolerance policy for *L. monoctogenes* [21,26]. This means that any product of concern, must be recalled and destroyed. From 2017-2020, *L. monocytogenes* accounted for 12-30% of total FDA recalls [27]. While ensuring food free from biological hazards is extremely important and is addressed through current policies in place, more food processing hurdles to increase food safety would allow for less food waste.

1.2 Opportunities for Improving Sustainability

As our society reaches for a more sustainable future, the food and agriculture industry needs to address this waste. A study from 2018 suggested that if current food waste were to be reduced by half, the projected global burden based on current emissions could be lowered by as much as 6-16% [28]. Although this proposed reduction is a large feat, attacking the issue from multiple angles could be beneficial and is necessary.

Harnessing the power of beneficial microorganisms through biopreservation is a well-established option for extending food shelf life. This form of food preservation began millenniums ago, emerging from the natural fermentation of dairy, fruits, and grains to create products we still consume today [29].

Among fermented dairy products, microorganisms that are beneficial to these fermentations, are those that metabolize lactose, mainly lactic acid bacteria, and yeasts such as *Kluyveromyces* found in yogurt, cheese, kefir, and other products [29,30]. These organisms produce a variety of metabolic end products such as organic acids that have a beneficial effect on the food properties from a food spoilage and safety perspective [31–34].

1.2.1 Prospects for the Dairy Industry

Employing these microorganisms for their metabolites is an advantageous strategy for reducing food waste. Regarding food safety, lactic acid bacteria have been studied for their protective effect against *L. monocytogenes*, primarily for meat applications but with more recent work focusing on dairy products, i.e., fresh cheese [34–38]. These studies will be explored in more detail throughout Chapter 3.

In terms of valorizing dairy by-products, a particular species of yeast, *B. claussenii*, has been relatively over-looked for its lactose-metabolizing ability [39]. This yeast could provide options for bioremediation of dairy by-products and contribute to the balance of sustainability in

the dairy sector. An in-depth look at these waste reutilization opportunities will be explored and investigated further in Chapters 4, 5, and 6.

As a whole, the goal of this research is to explore and substantiate potential approaches to increasing sustainability in the dairy industry through use of biotechnological methods. This research aims to disseminate practical findings that will be beneficial to the establishment and use of these approaches industrially. Increased adoption of practices that further enhance food safety and valorize by-products, predominantly through the use of microorganisms, is a prospective tactic to combat food waste in the dairy industry.

REFERENCES

1. Crippa M, Solazzo E, Guizzardi D, Monforti-Ferrario F, Tubiello FN, Leip A: **Food systems are responsible for a third of global anthropogenic GHG emissions**. *Nat Food* 2021, **2**:198–209.
2. Poore J, Nemecek T: **Reducing food's environmental impacts through producers and consumers**. *Science* 2018, **360**:987–992.
3. Loken B *et al*.: *Bending the Curve: the Restorative Planet-Based Diets*. 2020.
4. Clark MA, Domingo NGG, Colgan K, Thakrar SK, Tilman D, Lynch J, Azevedo IL, Hill JD: **Global food system emissions could preclude achieving the 1.5° and 2°C climate change targets**. *Science* 2020, **370**:705–708.
5. FAO: **Global food losses and food waste - Extent, causes and prevention**. 2011,
6. Bräutigam KR, Jörissen J, Priefer C: **The extent of food waste generation across EU-27: Different calculation methods and the reliability of their results**. *Waste Manag Res* 2014, **32**:683–694.
7. Ishangulyyev R, Kim S, Lee SH: **Understanding food loss and waste-why are we losing and wasting food?** *Foods* 2019.
8. Huang CH, Liu SM, Hsu NY: **Understanding global food surplus and food waste to tackle economic and environmental sustainability**. *Sustainability* 2020, **12**:1–18.
9. Rao M, Bast A, de Boer A: **Valorized Food Processing By-Products in the EU: Finding the Balance between Safety, Nutrition, and Sustainability**. *Sustainability* 2021, **13**:4428.
10. Caldeira C, De Laurentiis V, Corrado S, van Holsteijn F, Sala S: **Quantification of food waste per product group along the food supply chain in the European Union: a mass flow analysis**. *Resour Conserv Recycl* 2019, **149**:479–488.
11. Scherhaufer S, Moates G, Hartikainen H, Waldron K, Obersteiner G: **Environmental impacts of food waste in Europe**. *Waste Manag* 2018, **77**:98–113.
12. FAO: *Greenhouse gas emissions from the dairy sector*. 2010. Rome.
13. Gerber P, Vellinga T, Opio C, Steinfeld H: **Productivity gains and greenhouse gas emissions intensity in dairy systems**. *Livest Sci* 2011, **139**:100–108.
14. Smithers GW: **Whey-ing up the options–Yesterday, today and tomorrow**. *Int Dairy J* 2015, **48**:2–14.
15. Smithers GW: **Whey and whey proteins-From "gutter-to-gold."** *Int Dairy J* 2008, **18**:695–704.
16. Menchik P, Zuber T, Zuber A, Moraru CI: **Composition of coproduct streams from dairy processing: Acid whey and milk permeate**. *J Dairy Sci* 2019, **102**:3978–3984.
17. Menchik P, Moraru CI: **Nonthermal concentration of liquid foods by a combination of reverse osmosis and forward osmosis. Acid whey: A case study**. *J Food Eng* 2019, **253**:40–48.
18. Erickson BE: **Acid whey: Is the waste product an untapped goldmine**. *Chem Eng News* 2017, **95**:26–30.
19. Mawson AJ: **Bioconversions for whey utilization and waste abatement**. *Bioresour Technol* 1994, **47**:195–203.
20. Prazeres AR, Carvalho F, Rivas J: **Cheese whey management: A review**. *J Environ Manage* 2012, **110**:48–68.
21. Ivanek R, Gröhn YT, Tauer LW, Wiedmann M: **The cost and benefit of listeria monocytogenes food safety measures**. *Crit Rev Food Sci Nutr* 2004, **44**:513–523.

22. Gorton A, Stasiewicz MJ: **Twenty-two years of u.s. meat and poultry product recalls: Implications for food safety and food waste**. *J Food Prot* 2017, **80**:674–684.
23. Lin C-M, Zhang L, Doyle MP, Swaminathan B: **Comparison of media and sampling locations for isolation of Listeria monocytogenes in queso fresco cheese**. *J Food Prot* 2006, **69**:2151–2156.
24. Ibarra-Sánchez LA, Van Tassell ML, Miller MJ: **Invited review: Hispanic-style cheeses and their association with Listeria monocytogenes**. *J Dairy Sci* 2017, **100**:2421–2432.
25. CDC: **National Outbreak Reporting System**. 2020. Atlanta, GA.
26. Shank FR, Elliot EL, Wachsmuth IK, Losikoff ME: **US position on Listeria monocytogenes in foods**. *Food Control* 1996, **7**:229–234.
27. FMI: *FDA Recalls 2017-2020*. 2021.
28. Springmann M, Clark M, Mason-D'Croz D, Wiebe K, Bodirsky BL, Lassaletta L, de Vries W, Vermeulen SJ, Herrero M, Carlson KM, et al.: **Options for keeping the food system within environmental limits**. *Nature* 2018, **562**:519–525.
29. Paul Ross R, Morgan S, Hill C: **Preservation and fermentation: Past, present and future**. *Int J Food Microbiol* 2002, **79**:3–16.
30. Marsh AJ, Hill C, Ross RP, Cotter PD: **Fermented beverages with health-promoting potential: Past and future perspectives**. *Trends Food Sci Technol* 2014, **38**:113–124.
31. Hsieh HH, Wang SY, Chen TL, Huang YL, Chen MJ: **Effects of cow's and goat's milk as fermentation media on the microbial ecology of sugary kefir grains**. *Int J Food Microbiol* 2012, **157**:73–81.
32. Hati S, Das S, Mandal S: *Technological Advancement of Functional Fermented Dairy Beverages*. Elsevier Inc.; 2019.
33. Laureys D, De Vuyst L: **Microbial species diversity, community dynamics, and metabolite kinetics of water Kefir fermentation**. *Appl Environ Microbiol* 2014, **80**:2564–2572.
34. Coelho MC, Silva CCG, Ribeiro SC, Dapkevicius MLNE, Rosa HJD: **Control of Listeria monocytogenes in fresh cheese using protective lactic acid bacteria**. *Int J Food Microbiol* 2014, **191**:53–59.
35. Nielsen JW, Dickson JS, Crouse JD: **Use of bacteriocin produced by Pediococcus acidilactici to inhibit Listeria monocytogenes associated with fresh meat**. *Appl Environ Microbiol* 1990, **56**:2142–2145.
36. Vera Pingitore E, Todorov SD, Sesma F, Gombossy de Melo Franco BD: **Application of bacteriocinogenic Enterococcus mundtii CRL35 and Enterococcus faecium ST88Ch in the control of Listeria monocytogenes in fresh Minas cheese**. *Food Microbiol* 2012, **32**:38–47.
37. Vermeiren L, Devlieghere F, Debevere J: **Evaluation of meat born lactic acid bacteria as protective cultures for the biopreservation of cooked meat products**. *Int J Food Microbiol* 2004, **96**:149–164.
38. Pucci MJ, Vedamuthu ER, Kunka BS, Vandenbergh PA: **Inhibition of Listeria monocytogenes by using bacteriocin PA-1 produced by Pediococcus acidilactici PAC 1.0**. *Appl Environ Microbiol* 1988, **54**:2349–2353.
39. Lawton MR, deRiancho DL, Alcaine SD: **Lactose utilization by Brettanomyces claussenii expands potential for valorization of dairy by-products to functional beverages through fermentation**. *Curr Opin Food Sci* 2021.

CHAPTER 2

Leveraging endogenous barley enzymes to turn lactose-containing dairy by-products into fermentable adjuncts for *Saccharomyces cerevisiae*-based ethanol fermentations.[1]

2.1 Abstract

Acid whey, a by-product of strained yogurt production, represents a disposal challenge for the dairy industry. Utilization schemes are currently limited, however, acid whey contains valuable components that could be used to create value-added products. One potential scheme would be the fermentation of acid whey into an alcoholic beverage. Sour beers are gaining popularity and acid whey, sour to begin with, could provide a new product opportunity. However, the main sugar of acid whey, lactose, cannot be fermented by the traditional brewer's yeast *Saccharomyces cerevisiae*. It has been reported that barley contains enzymes capable of hydrolyzing lactose to glucose and galactose, which are fermentable by *S. cerevisiae*. In this study we investiage whether a barley-based mash results in detectable hydrolysis of lactose into sugars fermentable by *S. cerevisiae.* We demonstrate the ability to hydrolyze lactose in acid whey using a barley-based mash, resulting in the average release of 3.70 g/L glucose. Additionally, the subsequent liquid was fermented by *S. cerevisiae* to an average ethanol concentration of 3.23 % ABV. This work demonstrates the ability to hydrolyze the lactose in acid whey using barley and the opportunity to utilize acid whey as a fermentable sugar source in beer production.

2.2 Introduction

---

[1]Lawton MR, Alcaine SD: **Leveraging endogenous barley enzymes to turn lactose-containing dairy by-products into fermentable adjuncts for Saccharomyces cerevisiae-based ethanol fermentations**. *J Dairy Sci* 2019, **102**:2044–2050.

Acid whey is a byproduct of strained-yogurt products such as Greek yogurt and skyr, and represents a significant disposal challenge for the dairy industry. Estimates are that over 2.3 million metric tons of Greek yogurt acid whey are produced per year [1]. The main component of acid whey is lactose, a disaccharide made up of glucose and galactose linked by a beta-1,4 glycosidic bond, which is present at ~3.0-6.0 % [2]. The high heat treatment of the milk in yogurt production, which is done to denature the whey protein and capture it in the yogurt body thus improving consistency [3], results in very low residual protein. Acid whey contains ~ 6.4 g/L lactic acid [4], resulting in a lower pH and increased mineral solubility, and thus it is high in minerals including calcium (1.23 g/L), magnesium (0.11 g/L), and phosphate (2.0 g/L) in comparison to sweet whey [4,5]. All these components contribute to a significant biochemical oxygen demand (BOD) of 30-50 g/L and a chemical oxygen demand (COD) of 60-80 g/L, making disposal environmentally problematic [6]. The high lactic acid content of acid whey makes its utilization in dry powders, which is common for other dairy by-products, difficult due to the impact on crystallization [4]. Currently, the main acid whey utilization schemes involve: i) direct application to agricultural fields; ii) use in animal feed; and iii) as a feed to anaerobic digestors and waste water treatment sites [7,8]. These alternatives for acid whey utilization, however, pose an economic burden as there is little value returned from them and producers are typically responsible for the transportation costs. Additional venues for utilization of acid whey are needed.

Incorporation of acid whey into the production of alcoholic beverages, like beer, represents a possible value-added outlet. Sour beers, i.e. beers produced with lactic acid bacteria in the mash to drop the pH of the wort prior to fermentation, are gaining popularity and there is interest in improving their production process [9]. Acid whey could be used to replace some, or

all of the the water introduced during the mash process, thus providing the sour base. Some brewers are known to use Greek yogurt as the source of the lactic acid bacterial cultures containing *Lactobacillus bulgaricus* and *Streptococcus thermophilus* [10], thus utilizing acid whey from strained yogurt could simplify this process.

One potential drawback of utilizing acid whey in the brewing process is the remaining lactose. Traditional brewer's yeast, *Saccharomyces cerevisiae*, cannot utilize lactose. There are non-*Saccharomyces* yeast strains, like *Kluveryomyces marxianus* [8,11,12], that have been used to convert lactose to ethanol, but they are not used in beer brewing and are considered spoilage organisms in wines, potentially producing undesirable off notes. Another option is the addition of lactases to break down the lactose in glucose and galactose, that can then be utilized by *S. cerevisiae* [8,13,14]. It is the latter avenue, the use of enzymes to hydrolyze lactose in simple sugars for fermentation, that we explore in this study.

Enzymes are an added cost for production. In beer production, malted barley not only provides the starch that will be converted into ethanol, but also provides the enzymes – alpha and beta-amylase, that breakdown these complex starches into simple sugars for *S. cerevisiae* to ferment into ethanol [15]. Though not evaluated in beer production, beta-galactosidase (beta-gal) and beta-glucosidase (beta-gluc) endogenous to barley with activity against lactose have been identified [16]. The beta-gluc has been shown to develop during barley seed maturation, and both enzymes remain active during germination [17,18]. The barley beta-gal is reported to have optimum activity around pH 4.0 and the optimal pH of the beta-gluc is reported as 4.5-5.0 [16]. These optimums fall within the pH of acid whey, which has been reported to range from 3.5 to 5.1 [2]. In this study, we aimed to: i) evaluate if beta-glycosidases present in barley, and related grains, are sufficient to hydrolyze lactose in a mash; ii) evaluate lactose hydrolysis in a acid

whey-based raw barley mash; and iii) demonstrate that ethanol can be produced from acid whey using a barley mash. Hydrolysis of lactose into glucose and galactose using barley beta-glycosidases in a mash would allow for the incorporation of acid whey as both a natural acidulant and as fermentable adjunct sugar source in beer production.

2.3 Materials & Methods

Materials

Barley Grits (Meal) and Whole Grain Cracked Rye was purchased from Bob's Red Mill Natural Foods (Milwaukie, OR, U.S.A.). Rahr Unmalted Wheat was purchased from Northern Brewer (Roseville, MN, U.S.A.). Barley meal was used as purchased, rye and wheat were ground in a coffee grinder for 30 one-second pulses. D-Lactose was purchased from Sigma Aldrich (St. Louis, MO, U.S.A.). D-Galactose was purchased from VWR (Radnor, PA, U.S.A.). Acid whey was obtained from a local Greek yogurt manufacturer in upstate New York. Yeast used in the study was *Saccharomyces cerevisiae* var. bayanus (Uvaferm 43) purchased from Lallemand (Ontario, Canada). Reagents and equipment for the enzymatic glucose assay were purchased from Randox Laboratories Ltd. (Kearneysville, WV, U.S.A.).

Unmalted Barley Mash with Lactose

A mash containing 250 mL of 10 % (wt/wt) lactose solution and 65.9 g of barley meal was shaken constantly at 40 or 50 °C for 3 hours. A control mash consisting of barley meal and water, with no source of lactose added, was used to determine the amount of free glucose in the grain or released from barley amylase activity. Samples were taken at 0, 10, 60, 120, and 180 minutes and heated to 70 °C for 5 minutes to stop further enzyme activity. Samples were then centrifuged at 10,000 rpm for 5 minutes and the supernatant was collected as the wort for glucose analysis.

Mash with Unmalted Wheat and Rye

A mash containing 250 mL of 10 % (wt/wt) lactose solution and 65.9 g of rye or wheat was shaken constantly at 40 °C for 3 hours. A control mash consisting of 250 mL water and 65.9 g of rye or wheat with no source of lactose added was used. Samples were taken at 0, 10, 60, 120, and 180 minutes and heated to 70 °C for 5 minutes to stop further enzyme activity. Samples were then centrifuged at 10,000 rpm for 5 minutes and the supernatant was collected as the wort for glucose analysis.

Mash with Acid Whey

A mash containing 250 mL of acid whey and 65.9 g of barley meal was shaken constantly at 40 or 50 °C for 3 hours. A control mash consisting of barley meal and water, with no source of lactose added, was used to determine the amount of free glucose in the grain or released from barley amylase activity. Samples were taken at 0, 10, 60, 120, and 180 minutes and heated to 70 °C for 5 minutes to stop further enzyme activity. Samples were then centrifuged at 10,000 rpm for 5 minutes and the supernatant was collected as the wort for glucose analysis.

Enzyme Deactivation

Barley meal was autoclaved at 121 °C for 20 minutes to deactivate any lactose-hydrolyzing enzymes. This barley (65.9 g) was then used in a mash containing 250 mL of 10 % (wt/wt) lactose solution. A control mash consisted of 65.9 g untreated barley meal and 10 % (wt/wt) lactose solution. Samples were taken at 0 and 180 minutes and heated to 70 °C to stop further enzyme activity. Samples were then centrifuged at 10,000 rpm for 5 minutes and the supernatant was collected as the wort for glucose analysis.

Fermentation

A mash was prepared from a 10 % (wt/wt) lactose solution or acid whey and barley meal. The mash was shaken constantly at 50 °C for 8 hours. The controls consisted of a mash containing water and barley meal that incubated for 8 hours or a mash containing a 10 % (wt/wt) lactose solution and barley meal that did not incubate. Without filtering, each mash was inoculated with 0.25 g of *S. cerevisiae* and incubated at 25 °C for 14 days. Samples for glucose and ethanol analysis were taken before and after fermentation.

Glucose, Ethanol, and Titratable Acidity Analysis

Levels of glucose in the wort samples were analyzed with the Randox Glucose/Fructose UV Method on the Randox Monaco Analyser (Randox Laboratories Ltd., Kearneysville, WV, U.S.A.). Samples were analyzed according to the manufacturer's instructions. Levels of ethanol in the samples were analyzed via high-pressure liquid chromatography (HPLC) with a Rezex ROA-Organic Acid H + (8 %) column and a refractive index detector.

Statistical Analysis

Triplicate samples were taken for analysis at each time point and each experiment was repeated three times on separate occasions. A student's T-test was conducted to determine differences between mean glucose levels in the treatment and the control at 0 and 180 minutes during mash experiments or 0 and 14 days for fermentation experiments.

2.4 Results & Discussion

Lactose Hydrolysis in a Raw Barley Mash

Lactose is not considered an adjunct sugar by brewers because it is not hydrolyzed during the mash and is subsequently unfermentable by *S. cervisiae*. Our first question was whether or not we could detect beta-gal/beta-gluc activity through an increase in glucose levels during a mash. In a typical mash, alpha-amylase is responsible for glucose release from barley starch

molecules, but its optimum temperature is greater than 60 °C [19], well above the reported optimum of 40 °C [16] of the beta-gal/beta-gluc. Initial studies with malted barley mash at 40 °C (data not shown) showed no increase in glucose levels. We suspect that the high temperatures during the kilning of malted barley heat-inactivate the beta-gal and beta-gluc enzymes. Base malts are typically produced by initially drying the germinated barley at temperatures of 40-50 °C, and subsequently heating the malt to 80-85 °C [20]. The lactose hydrolyzing enzymes that are associated with barley are reported to lose 70 % of activity after 10 minutes at 60 °C [16], which is well below typical malting temperatures. Since these lactose hydrolyzing enzymes were characterized in unmalted barley [16], we proceeded to do a mash solely with barley meal. Alpha amylase activity is quite low in unmalted barley [21], thus background glucose levels would be lower in the mash and beers produced with raw barley. Raw barley as a portion [22,23] or complete [24] make up of the grain in a mash has been reported, so its use by a brewer would be feasible for the production of a beer. We thus performed mashes with 100 % barley meal at 40 and 50 °C, and monitored glucose levels over time via an enzymatic assay to detect any lactose hydrolysis. These values were compared to the control treatment, with no added lactose to account for any potential background glucose released from the barley. As seen in Figure 1, at both 40 and 50 °C there was an increase in glucose concentrations over time. At 3 hours the concentration of glucose in the sample was higher ($P<0.05$) than the control for both temperatures. The concentration of glucose at 3 hours in the 50 °C experiment reached an average of 10.16 g/L and was also higher ($P<0.05$) than at 40 °C. It is interesting that higher activity was observed at 50 °C, a temperature greater than the 40 °C optimum reported [16]. Enzyme levels and activity have been shown to vary between barley varieties [25]. While the variety of the barley used in these experiments was not reported by the

manufacturer, it was sourced from different continents more than 25 years later than the original studies by Simos and Georgatsos, and thus it is likely that our barley is not the same variety as in their study. Furthermore, Simos and Georgatsos did not look at the activity of these enzymes under mashing conditions, where the viscosity of the mash may impart some degree of heat protection to the enzymes. These two differences, barley variey and experimental conditons, likely explain the observed difference in beta-gal/beta-gluc activity. Future research into the differences in beta-gal/beta-gluc levels and activity between modern barley varieties would be an important next step in implementation and optimization of this process.

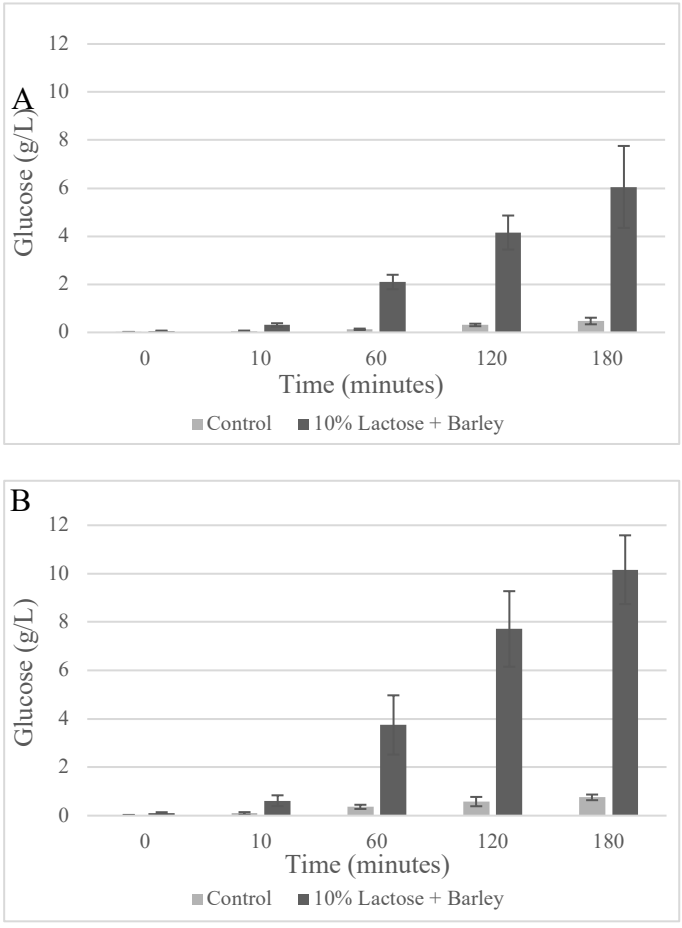

Figure 1: Hydrolysis of 10 % (wt/wt) lactose solution by barley over time at 40 °C (A) and 50 °C (B)

Limited Lactose Hydrolysis in Other Raw Grains

Gelman (1969) also reported isolating lactose-hydrolyzing enzymes from wheat and rye, as well as barley [26]. Like raw barley, both raw wheat [27] and raw rye [23], have also been used as adjunct grains in brewing. We were curious whether these raw grains could also be used as an enzyme source for lactose hydrolysis in a mash. We then performed a mash containing either wheat or rye in a 10 % (wt/wt) lactose solution at 40 °C for 3 hours. Low levels of lactose hydrolysis were observed over time (Figure 2). In the rye mash, glucose concentrations reached 3.17 g/L after 3 hours and in the wheat mash, glucose concentrations reached 4.10 g/L after 3 hours. Final glucose levels in rye were higher ($P<0.05$) than the control, while the final glucose levels in the wheat were not ($P>0.05$). In both cases there appeared to be much more pronounced background glucose, as seen in the control, compared to the raw barley, and the final glucose levels were less than those observed in the raw barley mash at the same temperature. We thus decided to continue further evaluations with only the raw barley.

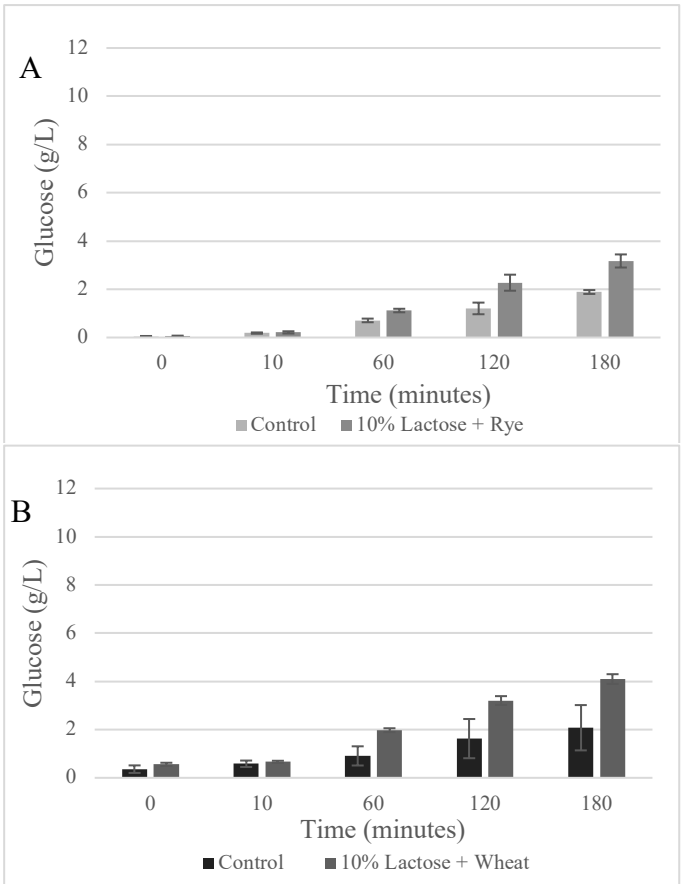

Figure 2: Hydrolysis of 10 % (wt/wt) lactose solution over time by rye (A) and wheat (B) at 50 °C.

Heat Inactivation of Lactose Hydrolyzing Enzymes in Raw Barley

We had originally speculated that we did not see lactose hydrolysis in the early malted barley experiments due to heat inactivation of the enzymes during the malting. The drying step during malting, known as kilning, can reach temperatures in excess of 200 °C. To confirm that a heat sensitive enzyme was responsible for the observed hydrolysis in the raw barley mash, we autoclaved the barley meal at 121 °C for 20 minutes. We then proceeded to compare 3 hour mash glucose levels between the heat treated barley meal and a control, non-heat treated barley meal (Figure 3). No increase in glucose concentration over time was detected, when the autoclaved barley was incubated in a mash with 10 % (wt/wt) lactose solution, where we saw a final level of 7.76 g/L glucose detected in the control. This supports the claim that a heat-sensitive enzyme is responsible for lactose hydrolysis in the raw barley mash. Germination is the first step in malting barley, resulting in green malt, and research indicates that both beta-gal/beta-gluc are present during this step [18]. Future work, confirming lactose hydrolysis in green malt, and investigating when in the subsequent malting process and at what temperature the enzymes are inactivated could result in a novel malting regime that enables the production of barley malt that possesses high levels of both lactose and starch hydrolyzing enzymes.

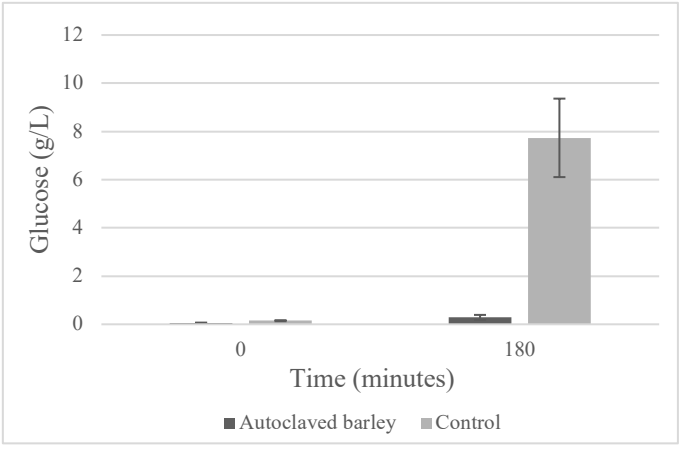

Figure 3: Deactivation of lactose-hydrolyzing enzymes after autoclaving at 121 °C for 20 minutes.

Hydrolysis of Acid Whey Derived Lactose in a Raw Barley Mash

In the production of sour beers, acid whey can potentially act as a natural acidulant and as a fermentable sugar source. Acid whey typically has a pH < 4.5, a lactose content of 3.0 to 3.5 %, and calcium content greater than 1.2 mg/g [28], which is in contrast to our initial mash containing 10 % lactose and no added calcium. These factors, pH, mineral content, and lactose concentration, could all impact enzyme activity. To evaluate the potential of acid whey in this process, we performed a mash with acid whey containing raw barley at both 40 and 50 °C, and measured glucose levels over time. These values were compared to the control treatment, which contained no added lactose. As seen in Figure 4, at both 40 and 50 °C there was an increase in glucose levels over time. At 3 hours for both temperatures, the concentration of glucose in the sample was higher ($P<0.05$) than the control. At 50 °C the glucose levels reached on average 4.70 g/L after 3 hours. There was no difference ($P>0.05$) in glucose concentrations between 40 and 50 °C. The glucose levels were less than those observed with the mash containing 10 % lactose, but still show that hydrolysis of lactose via a raw barley mash is possible and that acid whey could serve as both a fermentable adjunct sugar source in beer production.

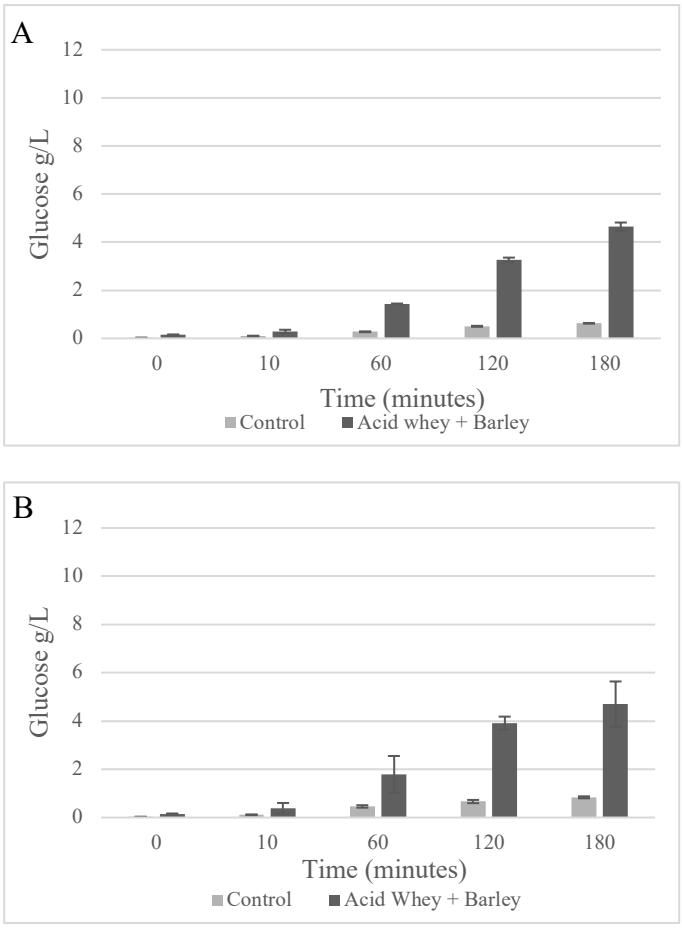

Figure 4: Hydrolysis of lactose by barley in acid whey over time at 40 °C (A) and 50 °C (B).

Ethanol from Raw Barley Hydrolyzed Lactose

S. cerevisiae can utilize glucose and galactose, once released from lactose, in the production of ethanol, though galactose is not used as efficiently as glucose in this metabolic process [29]. We wanted to confirm that ethanol could successfully be produced by *S. cerevisiae* from a raw barley mash containing lactose, either added directly or from acid whey. We performed 4 mashes: i) a control 8 hr mash (50 °C) with barley meal and no added lactose; ii) an 8 hr mash (50 °C) with barley meal and acid whey as the lactose source; iii) an 8 hr mash (50 °C) with barley meal and a 10 % (wt/wt) lactose solution; and iv) barley meal with 10 % (wt/wt) lactose solution that was immediately fermented with no mash at 50 °C. Our expectation was that alcohol production would be observed with the mashes containing lactose, and minimal alcohol production in the control mash and in the mixture that did not incubate at 50 °C, since there would be low background glucose, and without a mash there would not have been the conditions for lactose hydrolysis. Indeed, after 14 days of fermentation, the barley mash containing 10 % (wt/wt) lactose reached an ethanol concentration of on average 5.82 % ABV and the mash containing acid whey reached an ethanol concentration of on average 3.23 % ABV as seen in Figure 5. The control mash, with no added lactose, only reach an ethanol concentration of on average 1.06 % ABV after 14 days. These results confirm that lactose and acid whey, and potentially other lactose-containing dairy by-products, could be used as fermentable adjunct sugar in beer production if the mash includes raw barley and is held for sufficient time and temperature for lactose hydrolysis to occur.

Interestingly, the fermentation of the 10 % (wt/wt) lactose solution and barley meal which did not undergo a mash step reached on average an ethanol concentration of 3.37 % ABV, which was unexpected. After the mash, there was no heat inactivation step, so this result

suggests that while the fermentation temperature of 25 °C is well below the optimum for the beta-gal/beta-gluc, it is sufficient for some enzymatic activity resulting in lactose hydrolysis and subsequent fermentation by *S. cerevisiae.* While fermenting on grains is not as common in beer production as it is in distilled spirit production, this result indicates that adding raw barley to the fermentation tank, as is done with many fruits added to beers, could represent another method for hydrolyzing lactose into a fermentable sugar for ethanol production.

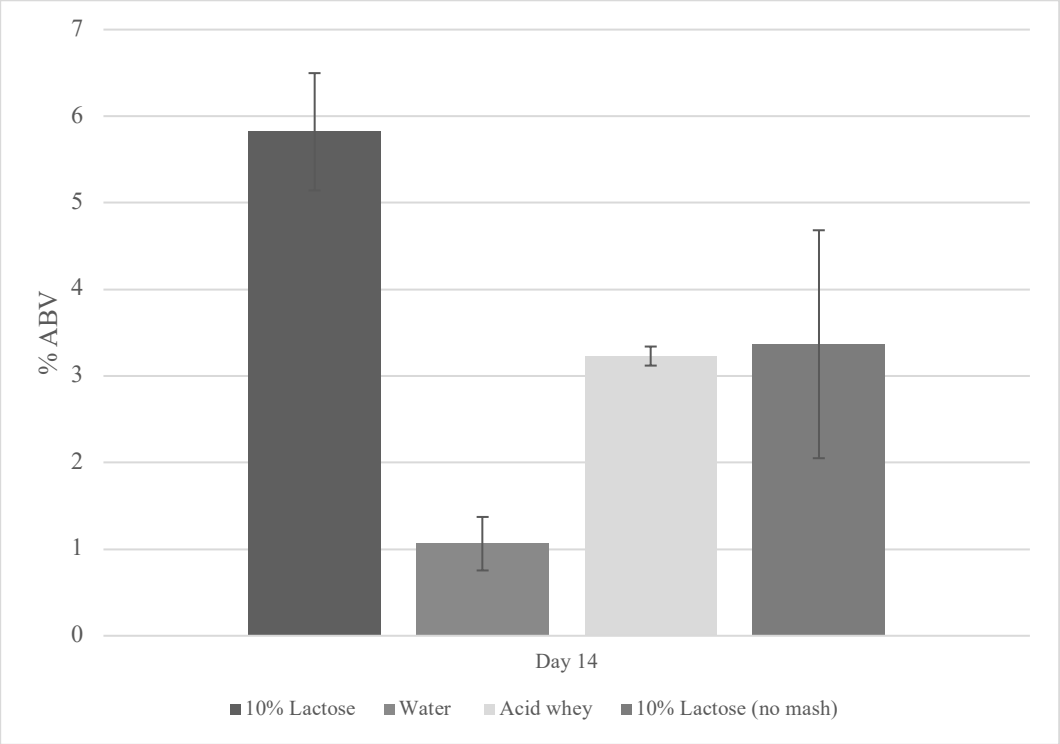

Figure 5: Final ethanol concentration (% ABV) from barley hydrolyzed lactose fermented with *Saccharomyces cerevisiae* after 14 days.

2.5 Conclusions

In this work the lactose hydrolyzing activity of barley meal was investigated in a lactose solution and acid whey. Analysis of glucose levels in lactose-supplemented barley meal mash showed increasing concentration over time, indicating lactose hydrolysis. Supplementation of mash with 10 % (wt/wt) lactose led to glucose levels of 10.16 g/L on average after 3 hours at 50

°C and lower levels at 40 °C. The acid whey mash took place at 40 or 50 °C in order to retain the activity of the lactose hydrolyzing enzymes, which were seen to be deactivated after autoclaving at 121 °C. Lower lactose hydrolyzing activity was seen in the other grains, rye and wheat. Furthermore, the use of acid whey, with a lactose concentration of ~ 33 g/L, in a mash resulted in glucose concentrations of 4.70 g/L after 3 hours. Additionally, a yeast fermentation of acid whey and barley meal mashed for 8 hours produced ethanol concentrations of on average 3.23 % ABV and on average 3.37 % ABV when no mash step occurred, indicating that an alcoholic beverage can be produced directly from acid whey without supplementation of sugar for fermentation. This provides an opportunity to create new beers and beer styles with acid whey as a base for fermentation.

Acknowledgments

This project was funded by the New York State Department of Environmental Conservation, Project Number 76370/A001. Thank you to the New York State Wine Analytical Lab for help with analysis of samples.

REFERENCES

1. Erickson BE: **Acid whey: Is the waste product an untapped goldmine**. *Chem Eng News* 2017, **95**:26–30.
2. Smithers GW: **Whey-ing up the options–Yesterday, today and tomorrow**. *Int Dairy J* 2015, **48**:2–14.
3. Shah NP, Chandan RC: **Manufacturing yogurt and fermented milks**. *Chandan, Rc, white, HC, Kilara, A, Hui, YH Heal benefits yogurt fermented milks* 2006,
4. Bansal N, Bhandari B: **Functional milk proteins: Production and utilization—whey-based ingredients**. In *Advanced dairy chemistry*. . Springer; 2016:67–98.
5. Smith S, Smith TJ, Drake MA: **Flavor and flavor stability of cheese, rennet, and acid wheys**. *J Dairy Sci* 2016, **99**:3434–3444.
6. Domingues L, Dantas MM, Lima N, Teixeira JA: **Continuous ethanol fermentation of lactose by a recombinant flocculating Saccharomyces cerevisiae strain**. 1999,
7. Prazeres AR, Carvalho F, Rivas J: **Cheese whey management: A review**. *J Environ Manage* 2012, **110**:48–68.
8. Mawson AJ: **Bioconversions for whey utilization and waste abatement**. *Bioresour Technol* 1994, **47**:195–203.
9. Osburn K, Amaral J, Metcalf SR, Nickens DM, Rogers CM, Sausen C, Caputo R, Miller J, Li H, Tennessen JM: **Primary souring: a novel bacteria-free method for sour beer production**. *Food Microbiol* 2018, **70**:76–84.
10. Burke S, Edmunds B, Love B: **Kettle souring: Three brewers talk methods and practices**. In *Prog. Craft Brewers Conf.* . BrewExpo America, Portland, OR; 2015:31.
11. Kosikowski F V, Wzorek W: **Whey wine from concentrates of reconstituted acid whey powder**. *J Dairy Sci* 1977, **60**:1982–1986.
12. Risner D, Shayevitz A, Haapala K, Meunier-Goddik L, Hughes P: **Fermentation and distillation of cheese whey: Carbon dioxide-equivalent emissions and water use in the production of whey spirits and white whiskey**. *J Dairy Sci* 2018, **101**:2963–2973.
13. O'Leary VS, Green R, Sullivan BC, Holsinger VH: **Alcohol production by selected yeast strains in lactase-hydrolyzed acid whey**. *Biotechnol Bioeng* 1977, **19**:1019–1035.
14. O'leary VS, Sutton C, Bencivengo M, Sullivan B, Holsinger VH: **Influence of lactose hydrolysis and solids concentration on alcohol production by yeast in acid whey ultrafiltrate**. *Biotechnol Bioeng* 1977, **19**:1689–1702.
15. MacGregor AW, Bazin SL, Macri LJ, Babb JC: **Modelling the contribution ofAlpha-Amylase, beta-amylase and limit dextrinase to starch degradation during mashing**. *J Cereal Sci* 1999, **29**:161–169.
16. Simos G, Georgatsos JG: **Lactose-hydrolyzing β-glycosidases of barley meal**. *Biochim Biophys Acta (BBA)-General Subj* 1988, **967**:17–24.
17. Giannakouros T, Karagiorgos A, Simos G: **Expression of β-galactosidase multiple forms during barley (Hordeum vulgare) seed germination. Separation and characterization of enzyme isoforms**. *Physiol Plant* 1991, **82**:413–418.
18. Simos G, Panagiotidis CA, Skoumbas A, Choli D, Ouzounis C, Georgatsos JG: **Barley β-glucosidase: Expression during seed germination and maturation and partial amino acids sequences**. *Biochim Biophys Acta (BBA)-General Subj* 1994, **1199**:52–58.
19. Bertoft E, Andtfolk C, Kulp S: **Effect of pH, temperature, and calcium ions on barley malt α-amylase isoenzymes**. *J Inst Brew* 1984, **90**:298–302.
20. Kunze W: **Malt Production: Barley steeping, barley germination, malt kilning**.

Technol Brew Malting, 2nd ed Versuchs-und Lehranstalt fur Brauerei Berlin 1999,
21. Georg-Kraemer JE, Mundstock EC, Cavalli-Molina S: **Developmental expression of amylases during barley malting**. *J Cereal Sci* 2001, **33**:279–288.
22. Hudson JR, MacWilliam IC, Birtwistle SE: **Beer from unmalted barley**. *J Inst Brew* 1963, **69**:308–309.
23. Koszyk PF, Lewis MJ: **Unmalted grains as maltsters' adjuvant and brewers' adjunct**. *J Am Soc Brew Chem* 1977, **35**:77–81.
24. Enevoldsen BS: **I. Conditions of mashing and carbohydrate composition of the wort**. *J Inst Brew* 1970, **76**:546–552.
25. Gunkel J, Voetz M, Rath F: **Effect of the malting barley variety (Hordeum vulgare L) on fermentability 1**. *J Inst Brew* 2002, **108**:355–361.
26. Gelman AL: **Some β-glycosidases in barley and other cereals**. *J Sci Food Agric* 1969, **20**:209–212.
27. Depraetere SA, Delvaux F, Coghe S, Delvaux FR: **Wheat variety and barley malt properties: influence on haze intensity and foam stability of wheat beer**. *J Inst Brew* 2004, **110**:200–206.
28. Menchik P, Zuber T, Zuber A, Moraru CI: **Composition of coproduct streams from dairy processing: Acid whey and milk permeate**. *J Dairy Sci* 2019, **102**:3978–3984.
29. Lee K, Hong M, Jung S, Ha S, Yu BJ, Koo HM, Park SM, Seo J, Kweon D, Park JC: **Improved galactose fermentation of Saccharomyces cerevisiae through inverse metabolic engineering**. *Biotechnol Bioeng* 2011, **108**:621–631.

CHAPTER 3

Short Communication: Evaluation of commercial meat cultures to inhibit *Listeria monocytogenes* in fresh cheese laboratory model[2]

3.1 Abstract

Control of *Listeria monocytogenes* in queso fresco and other fresh cheeses continues to be a challenge in the United States. These cheese types are a particular challenge due to their high moisture and high pH, which provides favorable conditions for *L. monocytogenes* growth. Protective cultures, i.e. viable strains of lactic acid bacteria that inhibit other microorganisms, have been investigated in several foods, such as meat, as an alternative, clean-label control strategy for *L. monocytogenes*. The efficacy of protective cultures, however, can vary based on food matrix. In this study we were interested in whether protective cultures used in meats to control *L. monocytogenes*, could be applied to control the pathogen in queso fresco. Four commercially available bacterial cultures used for the control of *L. monocytogenes* in meat were selected: i) *Lactobacillus curvatus*; ii) *L. sakei;* iii) *Pediococcus acidilactici*; and iv) *Leuconostoc carnosum*. These cultures were incorporated into batches of queso fresco during the manufacturing, and evaluated for their ability to inhibit the growth of surface applied *L. monocytogenes* at levels of 1 x 10^2 and 1 x 10^4 CFU/g. The queso fresco was stored at 6 and 21 °C for up to 21 days. After 14 days, *Listeria* was able to grow to 1 x 10^7 CFU/g on the cheese. Our data shows that these cultures did not significantly inhibit the growth of *Listeria*

---

[2]Lawton MR, Jencarelli KG, Kozak SM, Alcaine SD: **Evaluation of commercial meat cultures to inhibit Listeria monocytogenes in a fresh cheese laboratory model**. *J Dairy Sci* 2020, **103**:1269–1275.

monocytogenes in queso fresco. The results from this study highlight the complexity of antagonistic bacterial interactions, and their potential variability across food matrices. Protective cultures represent an important, clean-label tool for the control of *L. monocytogenes* in foods, though each strain must be evaluated in the food environment they are intended to be used to ensure their efficacy.

3.2 Introduction

L. monocytogenes is prevalent in the environment and has regularly been isolated from food manufacturing facilities [1,2]. This organism is an important cause of foodborne outbreaks and can be introduced into ready-to-eat (RTE) food during post-processing if proper hygienic practices are not in place [1]. *L. monocytogenes* can readily grow in certain RTE dairy products such as fresh cheeses due to a low salt, high moisture, and near neutral pH environment [3]. There have been 18 deaths linked to *L. monocytogenes* in cheese between 1998 and 2017 according to the Centers for Disease Control (CDC) National Outbreak Reporting System (NORS). In particular, Latin/Hispanic style cheeses (e.g. queso fresco) have been the source of many outbreaks [4]. There is growing demand for these types of cheeses, with the United States Department of Agriculture (USDA) reporting the production of over 264 million pounds of Hispanic Cheese in 2017 [5]. The increasing popularity and consumption of these cheese highlights the need for improved strategies to control or prevent the contamination and growth of *L. monocytogenes* in these food products.

The use of lactic acid bacteria (LAB) in food products has been investigated as a clean label, bio-preservation method against spoilage and pathogenic microorganisms [6]. LAB are naturally present in traditionally fermented foods and have been shown to have antagonistic interactions with other microorganisms also present in the food either through direct competition

for nutrients and/or production of antimicrobial products such as bacteriocins or organic acids [6–8]. This ability to inhibit undesirable microorganisms in a food, has led to some of these LAB strains being described as protective cultures.

Protective cultures have become increasingly used in food products as specific controls for *L. monocytogenes*, particularly in meat products. Several strains of LAB from the species of *Lactobacillus curvatus*, *L. sakei*, *Pediococcus acidilactici*, and *Leuconostoc carnosum*, have all been evaluated for their antilisterial properties in various meat applications including frankfurters, cooked ham, fermented sausages, chicken cold cuts, and cold smoked salmon and have been shown to be effective in reducing or preventing the growth of *L. monocytogenes* without noticeable sensory defects to products [9–13]. Some of the species, such as strains of *P. acidilactici*, *L. sakei*, and *L. curvatus*, have been shown to produce bacteriocins with antilisterial activity [8,10,14,15].

With the wealth of research on these protective cultures, it is perhaps not surprising that strains of *L. curvatus*, *L. sakei*, *P. acidilactici*, and *L. carnosum* are commercially available for use in high risk RTE meat applications. Like many of these meats, controlling post-processing contamination of high risk cheese, like queso fresco, by *L. monocytogenes* is a challenge [16,17]. Interestingly, there has been little published work on investigating the ability of these meat cultures to protect high risk cheese. In this study we took four commercial protective cultures available for the control of *L. monocytogenes* in meats, each representing one of the four species: *L. curvatus*, *L. sakei*, *P. acidilactici*, and *L. carnosum;* and evaluated their ability to inhibit the growth of surface applied *L. monocytogenes* during storage at 6 °C to reflect average consumer refrigeration temperature and 21 °C to reflect accelerated shelf life.

3.3 Materials & Methods

***LAB strains and culture conditions*:** Strains of each culture, *L. curvatus*, *L. sakei*, *P. acidilactici*, and *L. carnosum*, were obtained from a commercial supplier. Each strain was propagated on MRS agar (Becton, Dickinson, and Company, Franklin Lakes, NJ) at 30 (*L. curvatus*, *L. sakei,* and *L. carnosum*) or 40 °C (*P. acidilactici*) for 48 hours from a frozen glycerol stock. Individual colonies of each strain were inoculated into 5 mL of MRS broth (Becton, Dickinson, and Company, Franklin Lakes, NJ) and incubated at 30 or 40 °C for 24 hours. One mL of each culture (OD = 1.5) was centrifuged at 1538 x g for 4 min and the resulting pellet was re-suspended in 1 mL ultra-high temperature pasteurized skim milk (Parmalat USA Corp., Grand Rapids, MI). The suspension was then transferred to a culture tube containing 9 mL of UHT milk. Cultures were then further incubated at 30 or 40 °C for 48 hours to achieve a final concentration of 7-8 log CFU/mL which was confirmed with dilution and plating on MRS agar during each experiment.

L. monocytogenes inoculum: Five strains of *L. monocytogenes* representing 4 outbreak strains linked to fresh cheese (FSL R9-5621, FSL R9-5623, FSL R9-5624, and FSL R9-5625) and one internal lab strain (FSL X1-0001), were obtained from Dr. Wiedmann's Food Safety Lab at Cornell University and can be accessed through www.foodmicrobetracker.com. Each strain was propagated on BHI agar (Becton, Dickinson, and Company, Franklin Lakes, NJ) at 37 °C for 24 hours. An individual colony from each strain was then used to separately inoculate 5 mL of BHI broth (Becton, Dickinson, and Company, Franklin Lakes, NJ). The cultures were grown overnight with shaking (200 rpm) at 37 °C. Then, each culture was diluted 1:10 and 1:100 and both dilutions were grown at 30 and 37 °C with shaking (200 rpm) for approximately 8 hours. The cultures closest to OD_{600} = 1.00 (9 log CFU/mL based on previous standard curves for these

strains) were used to create a cocktail containing approximately equal amounts of each strain. The cocktail was then serially diluted to achieve the desired inoculum concentration of either 2 log CFU/g or 4 log CFU/g. The initial inoculum cocktail was also diluted and spread-plated on BHI and MOX agar for enumeration to confirm the starting concentration. Plates were incubated at 30 °C for 48 hours.

Cheese manufacturing: Cheese was prepared at lab scale as follows with guidance from previously established protocols [18,19]. For each treatment, 600 mL of non-homogenized whole milk was brought to 35 °C in 1 L sterile bottles. To each bottle, the following were added: 804 µl of a 32-33 % $CaCl_2$ solution (Dairy Connection Inc., Madison, WI), 6 mL of a 7-8 log CFU/mL LAB culture, and 90 µl of double strength rennet (Chy-Max Extra, Chr. Hansen, Milwaukee, WI) brought to a final volume of 6 mL in deionized water. The bottles were swirled to mix and poured into individual plastic cheese vats. The vats were then incubated for 45 minutes in a 35 °C water bath. The curd was cut and the vats were incubated for 15 mins at 35 °C with gradual increase to a final temperature of 40 °C. Sixty mL of whey was taken out of each vat and replaced with 60 mL of an aqueous NaCl solution (0.16 g/mL) and stirred to mix. The vats were incubated at 40 °C for another 20 minutes. The whey was then drained from the vats with cheesecloth for 1 hour. The curd was then scooped into 12-well plates (Corning Inc., Corning, NY), 6 g of curd in each well. The curd was then pressed for 2 hours giving a final cheese with a weight of ~4 g. The pH of each treatment was measured using an InLab Surface pH probe (Mettler Toledo, LLC., Columbus, OH) and the moisture content was measured using a Smart Turbo Moisture Analyzer (CEM Corporation, Matthews, NC). Each measurement was taken in duplicate for each treatment and each batch. Cheeses were prepared in duplicate for each time point and treatment in at least 3 replicates on separate days.

Listeria Challenge: After pressing, cheeses (4 g) were surface inoculated with 100 µl of the prepared *L. monocytogenes* cocktail achieving a final concentration of either 2 log CFU/g or 4 log CFU/g. Positive controls, (inoculated with *L. monocytogenes* only) and negative controls (no inoculum) were included. Each set of cheeses were incubated at both 21 °C and 6 °C for 4 or 21 days, respectively.

Microbiological Analysis: Samples were enumerated on days 0, 1, 2, and 4 (21 °C incubation) or days 0, 1, 7, 14, and 21 (6 °C incubation) for *L. monocytogenes* and LAB. Each cheese (4 g) was placed in a sterile Whirl-pak filter bag (Nasco, Fort Atkinson, WI) and diluted 1 to 10 with sterile phosphate buffered saline (PBS). Samples were then homogenized using a stomacher set at normal speed for 60 seconds, and then serially diluted in PBS. Appropriate dilutions were spread-plated on MRS and MOX agar (Becton, Dickinson, and Company, Franklin Lakes, NJ) for enumeration. Plates were incubated at 30 °C for 48 hours. A total LAB count was counted on MRS agar and *Listeria* (round, concave, black colonies) were counted on MOX agar.

Statistical Analysis: All statistical analyses were performed using R (version 3.5.2; R Development Core Team, Vienna, Austria). Analysis of variance and Tukey's honest significant difference test were performed individually for each time point to compare the log differences in *Listeria* counts between all LAB treatments and the positive control.

3.4 Results & Discussion

Total MRS counts on Day 0 and Day 1 were 5-6 log CFU/g while *L. monocytogenes* counts were either 2 log or 4 log CFU/g, indicating that the LAB strains were present and viable in the cheese. For each treatment, levels of *L. monocytogenes* increased throughout the incubation period, resulting in a final concentration of ~7-8 log CFU/g for each temperature and inoculation level. For the higher *L. monocytogenes* inoculation level of 4 log CFU/g at 21 °C

(Figure 1A), no treatment resulted in any significant difference ($p>0.05$) in *L. monocytogenes* levels compared to the control for all time points. At 6 °C (Figure 1B) at this inoculation level, results of the samples treated with *L. curvatus* and *L. sakei* showed a significant difference ($p<0.05$) in *L. monocytogenes* counts on Day 14. However, these counts were only ~0.5 log CFU/g lower than the control and did not continue to be significant through 21 days of storage.

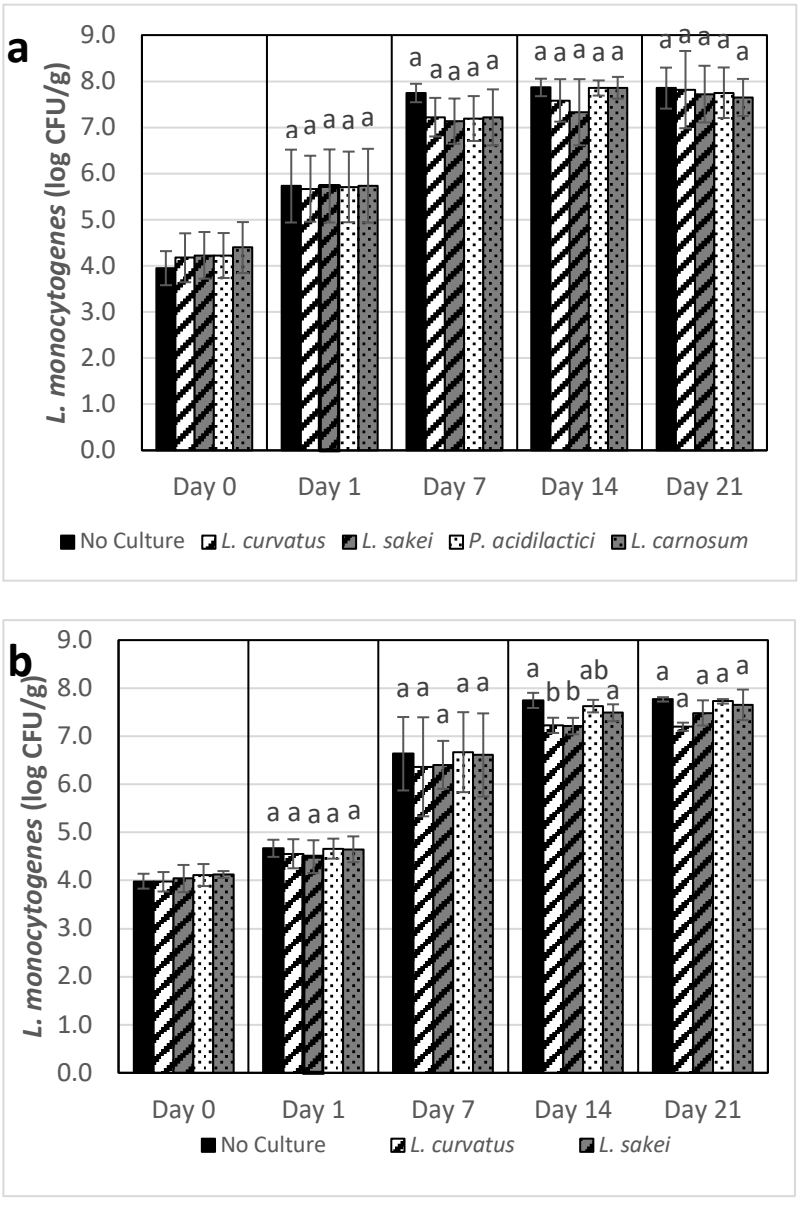

Figure 1. *L. monocytogenes* counts in a laboratory model of queso fresco prepared with protective cultures and initial *L. monocytogenes* load of 10^4 CFU/g at (a) 21°C and (b) 6°C. Bars

with different letters indicate significant difference ($p<0.05$) between protective culture treatments on the same day.

When the cheese was inoculated with a lower concentration of *L. monocytogenes* (2 log CFU/g), at 21 °C (Figure 2A), the *L. monocytogenes* count of the samples treated with *L. curvatus* displayed a significant difference ($p<0.05$) from all other treatments and the control on Day 1. However, the difference seen here was only ~0.5 log CFU/g. All treatments showed similar *L. monocytogenes* counts to the control with no significant differences throughout incubation at 21 °C. At 6 °C (Figure 2B), *L. curvatus* showed counts ~0.5 log CFU/g lower than the control ($p<0.05$) on Days 7, however, by Day 21 there was no significant difference ($p>0.05$) in the levels.

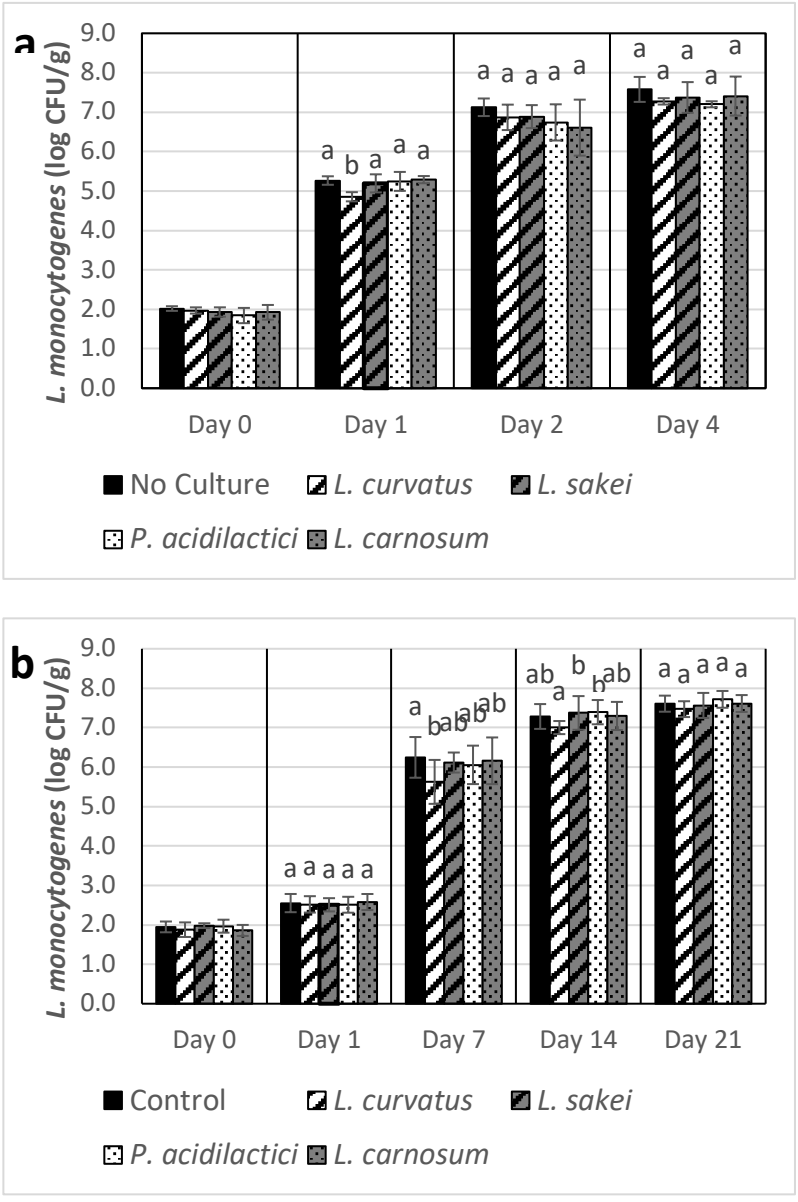

Figure 2. *L. monocytogenes* counts in a laboratory model of queso fresco prepared with protective cultures and initial *L. monocytogenes* load of 10^2 CFU/g at (a) 21°C and (b) 6°C. Bars with different letters indicate significant difference ($p<0.05$) between protective culture treatments on the same day.

The pH of the queso fresco, regardless of treatment, was slightly less than a neutral pH of 7.0 (Table 1). Typical queso fresco cheeses made in the United States have a pH above 6.0, and traditionally the pH can range from 5.3 – 6.5, however, there is no standard of identity for queso fresco regulated by the FDA [2,20,21]. The negative control and treatments containing LAB were of similar pH even with the addition of LAB, indicating that the cultures did not produce sufficient amounts of acid to affect pH during the cheese make. The moisture content of queso fresco is typically high relative to other cheeses and can range from 41-59% moisture [21]. This variability in moisture content is due to preference of the manufacturer and the consumer [22,23]. The queso fresco samples from this study were on the higher end of this range (Table 1). Although there was variability within the moisture contents among treatments, the low standard deviation between microbial counts for each treatment and the positive control suggest that moisture content did not influence microbial growth in this experiment.

Table 1. Composition of Queso Fresco Samples

Culture Treatment*	**Moisture Content (%)**	**pH**
No Culture	57.19 ± 3.02	6.82 ± 0.29
L. curvatus	57.72 ± 1.45	6.68 ± 0.16
L. sakei	58.76 ± 2.22	6.71 ± 0.16
P. acidilactici	57.95 ± 2.79	6.68 ± 0.19
L. carnosum	58.51 ± 2.37	6.72 ± 0.24
Negative control	58.30 ± 2.44	6.74 ± 0.24

*Indicates the protective culture added to the queso fresco. Negative control had no protective culture added and was not challenged with *L. monocytogenes.*

These results suggest that the selected LAB are unable to inhibit the growth of *L. monocytogenes* in a fresh cheese matrix. Although *L. monocytogenes* counts were reduced compared to the control on certain days, the difference was only ~0.5 log CFU/g. This reduction is not significant from a biological standpoint and would not be effective as a control strategy. Each of these species of LAB have previously been shown to have antagonistic activity towards

L. monocytogenes in media and in meat matrices [8,9,11,12]. However, there are certain factors that could contribute to the contrasting antagonistic behavior of the LAB seen here in queso fresco. The effectiveness of a strain depends on its ability to adapt to the food matrix and relies on aspects such as pH, moisture, and water activity [24]. In a study that reported antagonistic activity of *L. sakei* against *L. monocytogenes* in cooked ham, the ham had a pH close to 6.0 which is lower than that of the queso fresco used in this study [12]. Additionally, antimicrobial compounds produced by the LAB, such as bacteriocins, can have limitations depending on the food matrix. For example, binding and interactions to certain components in the food such as lipids can occur rendering the bacteriocin ineffective [24]. A previous study investigating other protective cultures in queso fresco also had unpromising results regarding the inhibition of *L. monocytogenes*, showing that after 21 days *L. monocytogenes* counts in a cheese containing a strain of *Lactococcus lactis* were only 0.7 log CFU/g lower than the control [25].

There have been studies performed using other fresh cheeses that have shown successful inhibition of *L. monocytogenes* by LAB. For example, several strains of *Enterococcus* spp. and a bacteriocin-producing *L. lactis* strain were evaluated for their antagonistic activity towards *L. monocytogenes* in Pico cheese, a type of Latin-style fresh cheese. In this study several combinations of different strains gave a 5-log CFU/g reduction of *L. monocytogenes* after 7 days and no sensory differences were detected when cheese inoculated with these strains was given to consumers [26]. Another study found a bacteriostatic effect against *L. monocytogenes* when several strains of *Enterococcus* spp. were applied in fresh Minas cheese. These cultures resulted in a 3-log inhibition in final *L. monocytogenes* levels and a decrease in pH to 5.2 after 12 days [27]. These studies suggest cultures may be more effective when multiple strains are used in combination or if they produce enough acid to lower the pH.

Protective cultures have been effective at inhibition of *L. monocytogenes* in other cheese types. A study of the antagonistic activity of a bacteriocin-producing *Lactococcus lactis* strain against *L. monocytogenes* in cottage cheese saw an initial 1-log CFU/mL reduction of *L. monocytogenes* after 24 hours, and after 5 days the levels of *L. monocytogenes* were reduced to below detectable limits. Although also considered a fresh cheese, cottage cheese traditionally has a starter culture for acidification and therefore has a lower pH (5.2) than queso fresco [28]. These cheeses, although similar to queso fresco, may be better suited for protection from *L. monocytogenes* by protective cultures due to compositional characteristics such as moisture content, salt concentration, which impact water activity, and pH. These parameters are known to play an important role as hurdles for *L. monocytogenes* outgrowth [29], and potential more so when used in combination with hurdles like protective cultures. The salt, moisture, and pH of queso fresco is reported to vary widely [30], and the composition of the queso fresco in this study (Table 1) was at the high end of those ranges for both moisture and pH, likely representing a worst case scenario for inhibiting *L. monocytogenes*.

3.5 Conclusions

Control of *L. monocytogenes* contamination continues to be a challenge in queso fresco and other Latin-style fresh cheeses due to their high pH and high moisture content. When working with these types of cheeses, control strategies such as protective cultures may not provide a high level of protection; therefore, good manufacturing practices should continue to be a focus in order to prevent contamination. Compositional traits such as moisture levels, salt content, and pH are also critical for *L. monocytogenes* control and future research is needed to understand the interplay between those factors and protective cultures in order to more effectively combine these hurdles to control *L. monocytogenes* in high risk cheeses. Certain

cultures, such as the ones tested here, may be suited for protection against *L. monocytogenes* in applications such as meat, however, their efficacy is clearly challenged under the queso fresco conditions in this study.

Acknowledgments

The authors would like to thank the National Dairy Council and the Innovation Center for US Dairy for their support of this project. The authors would also like to thank the Cornell Food Safety Lab for guidance during the cheese manufacturing.

REFERENCES

1. Kabuki DY, Kuaye AY, Wiedmann M, Boor KJ: **Molecular subtyping and tracking of Listeria monocytogenes in Latin-style fresh-cheese processing plants**. *J Dairy Sci* 2004, **87**:2803–2812.
2. Ibarra-Sánchez LA, Van Tassell ML, Miller MJ: **Invited review: Hispanic-style cheeses and their association with Listeria monocytogenes**. *J Dairy Sci* 2017, **100**:2421–2432.
3. Lin C-M, Zhang L, Doyle MP, Swaminathan B: **Comparison of media and sampling locations for isolation of Listeria monocytogenes in queso fresco cheese**. *J Food Prot* 2006, **69**:2151–2156.
4. (NORS): **Centers for Disease Control and Prevention**. *Natl Outbreak Report Syst* 2020,
5. USDA: **Dairy Products 2017 Summary**. *Natl Agric Stat Serv* 2018,
6. Devlieghere F, Vermeiren L, Debevere J: **New preservation technologies: Possibilities and limitations**. *Int Dairy J* 2004, **14**:273–285.
7. Vogel RF, Pohle BS, Tichaczek PS, Hammes WP: **The Competitive Advantage of Lactobacillus curvatus LTH 1174 in Sausage Fermentations is Caused by Formation of Curvacin A**. *Syst Appl Microbiol* 1993, **16**:457–462.
8. Tichaczek PS, Nissen-Meyer J, Nes IF, Vogel RF, Hammes WP: **Characterization of the bacteriocins curvacin A from Lactobacillus curvatus LTH1174 and sakacin P from L. sake LTH673**. *Syst Appl Microbiol* 1992, **15**:460–468.
9. Amézquita A, Brashears MM: **Competitive inhibition of Listeria monocytogenes in ready-to-eat meat products by lactic acid bacteria**. *J Food Prot* 2002, **65**:316–325.
10. Katla T, Møretrø T, Aasen IM, Holck A, Axelsson L, Naterstad K: **Inhibition of Listeria monocytogenes in cold smoked salmon by addition of sakacin P and/or live Lactobacillus sakei cultures**. *Food Microbiol* 2001, **18**:431–439.
11. Budde BB, Hornbæk T, Jacobsen T, Barkholt V, Koch AG: **Leuconostoc carnosum 4010 has the potential for use as a protective culture for vacuum-packed meats: Culture isolation, bacteriocin identification, and meat application experiments**. *Int J Food Microbiol* 2003, **83**:171–184.
12. Vermeiren L, Devlieghere F, Debevere J: **Evaluation of meat born lactic acid bacteria as protective cultures for the biopreservation of cooked meat products**. *Int J Food Microbiol* 2004, **96**:149–164.
13. Benkerroum N, Daoudi A, Hamraoui T, Ghalfi H, Thiry C, Duroy M, Evrart P, Roblain D, Thonart P: **Lyophilized preparations of bacteriocinogenic Lactobacillus curvatus and Lactococcus lactis subsp. lactis as potential protective adjuncts to control Listeria monocytogenes in dry-fermented sausages**. *J Appl Microbiol* 2005, **98**:56–63.
14. Pucci MJ, Vedamuthu ER, Kunka BS, Vandenbergh PA: **Inhibition of Listeria monocytogenes by using bacteriocin PA-1 produced by Pediococcus acidilactici PAC 1.0**. *Appl Environ Microbiol* 1988, **54**:2349–2353.
15. Nielsen JW, Dickson JS, Crouse JD: **Use of bacteriocin produced by Pediococcus acidilactici to inhibit Listeria monocytogenes associated with fresh meat**. *Appl Environ Microbiol* 1990, **56**:2142–2145.
16. Moreno-Enriquez RI, Garcia-Galaz A, Acedo-Felix E, Gonzalez-Rios H, Call JE, Luchansky JB, Diaz-Cinco ME: **Prevalence, types, and geographical distribution of Listeria monocytogenes from a survey of retail Queso Fresco and associated cheese processing plants and dairy farms in Sonora, Mexico**. *J Food Prot* 2007, **70**:2596–2601.

17. Jackson KA, Biggerstaff M, Tobin-D'Angelo M, Sweat D, Klos R, Nosari J, Garrison O, Boothe E, Saathoff-Huber L, Hainstock L: **Multistate outbreak of Listeria monocytogenes associated with Mexican-style cheese made from pasteurized milk among pregnant, Hispanic women**. *J Food Prot* 2011, **74**:949–953.
18. Van Tassell ML, Ibarra-Sánchez LA, Takhar SR, Amaya-Llano SL, Miller MJ: **Use of a miniature laboratory fresh cheese model for investigating antimicrobial activities**. *J Dairy Sci* 2015, **98**:8515–8524.
19. Henderson LO, Cabrera-Villamizar LA, Skeens J, Kent D, Murphy S, Wiedmann M, Guariglia-Oropeza V: **Environmental conditions and serotype affect Listeria monocytogenes susceptibility to phage treatment in a laboratory cheese model**. *J Dairy Sci* 2019, **102**:9674–9688.
20. Van Hekken D, Tunick M, Renye Jr J, Vallejo-Cordoba BG: **Characterization of queso fresco cheeses manufactured in Mexico and the United States**. *J Dairy Sci* 2006, **89**:313.
21. Clark S, Costello M, Drake M, Bodyfelt F: *The sensory evaluation of dairy products*. Springer; 2009.
22. Clark S, Warner H, Luedecke L: **Acceptability of Queso Fresco cheese by traditional and nontraditional consumers**. *Food Sci Technol Int* 2001, **7**:165–170.
23. Tunick MH, Van Hekken DL: **Rheology and texture of commercial queso fresco cheeses made from raw and pasteurized milk**. *J Food Qual* 2010, **33**:204–215.
24. Holzapfel WH, Geisen R, Schillinger U: **Biological preservation of foods with reference to protective cultures, bacteriocins and food-grade enzymes**. *Int J Food Microbiol* 1995, **24**:343–362.
25. Lourenço A, Kamnetz MB, Gadotti C, Diez-Gonzalez F: **Antimicrobial treatments to control Listeria monocytogenes in queso fresco**. *Food Microbiol* 2017, **64**:47–55.
26. Coelho MC, Silva CCG, Ribeiro SC, Dapkevicius MLNE, Rosa HJD: **Control of Listeria monocytogenes in fresh cheese using protective lactic acid bacteria**. *Int J Food Microbiol* 2014, **191**:53–59.
27. Vera Pingitore E, Todorov SD, Sesma F, Gombossy de Melo Franco BD: **Application of bacteriocinogenic Enterococcus mundtii CRL35 and Enterococcus faecium ST88Ch in the control of Listeria monocytogenes in fresh Minas cheese**. *Food Microbiol* 2012, **32**:38–47.
28. McAuliffe O, Hill C, Ross RP: **Inhibition of Listeria monocytogenes in cottage cheese manufactured with a lacticin 3147-producing starter culture**. *J Appl Microbiol* 1999, **86**:251–256.
29. Schvartzman MS, Belessi C, Butler F, Skandamis PN, Jordan KN: **Effect of pH and water activity on the growth limits of Listeria monocytogenes in a cheese matrix at two contamination levels**. *J Food Prot* 2011, **74**:1805–1813.
30. Guo L, Van Hekken DL, Tomasula PM, Shieh J, Tunick MH: **Effect of salt on the chemical, functional, and rheological properties of Queso Fresco during storage**. *Int Dairy J* 2011, **21**:352–357.

CHAPTER 4

Lactose utilization by *Brettanomyces claussenii* expands potential for valorization of dairy by-products to functional beverages through fermentation[3]

4.1 Abstract

Consumer interest in functional beverages has been increasing recently and has led to expansion in the market. Fermented beverages containing acetic acid such as kombucha are often produced using alternative microorganisms such as lactic and acetic acid bacteria as well as non-*Saccharomyces* yeasts. These organisms are used to produce health-promoting compounds which make these products popular with consumers. One such alternative yeast, *Brettanomyces claussenii*, has been gaining recognition for its biotechnological potential. Its capabilities include fermentation of non-traditional substrates and production of valuable by-products. Specifically, this species has the ability to utilize lactose and aerobically produce acetic acid. Harnessing the potential of this yeast leads to opportunities to upcycle lactose-containing dairy by-products to acetic acid fermented beverages.

4.2 Introduction

The rise in demand for functional, health promoting products within the past few decades has taken the beverage industry along for the ride [1–4]. A consumer shift towards foods that are beneficial and have the potential to prevent disease has expanded opportunities for the fermented beverage industry in particular [4]. In the United States, expansion into this area is easily

---

[3]Lawton MR, deRiancho DL, Alcaine SD: **Lactose utilization by Brettanomyces claussenii expands potential for valorization of dairy by-products to functional beverages through fermentation**. *Curr Opin Food Sci* 2021,

observable as startups are focusing on the health promoting qualities of foods and large corporations are seeking to acquire these product lines. Fermented beverages, such as kefir and kombucha, have been present in various cultures for centuries and are frequently acknowledged for their health benefits [4,5]. The functional and fermented beverage markets have been increasing globally in recent years due to a growing consumer interest in pursuing physical and mental well-being through their food and beverage choices. In addition to acetic acid-containing beverages, the functional beer category has been growing by advancing health properties of beverages such as low alcohol beer [6,7]. New research investigating the health attributes of these beverages and recent technological advances in these areas are expanding the marketplace options to keep up with consumer demand [1,8]. The spotlight on fermented beverages arose from the aforementioned health benefits offered by beneficial microorganisms present in these drinks and the compounds they produce during fermentation, such as organic acids. Many of these beverages, especially those which are dairy-based, are abundant with lactic acid bacteria, traditionally considered to be probiotic. In addition, these dairy-based beverages are often lactose-free after production due to the consumption of lactose by the microorganisms. Approximately 70% of adults in the world suffer from lactose intolerance and often turn to lactose-free and non-dairy beverages to reach their calcium and other dietary intake needs [9,10]. This being said, the benefits offered by these fermented drinks are vast, with some properties having been studied more rigorously than others. In regard to the microorganisms present within fermented beverages, cultures can vary from pure, such as a single probiotic lactic acid bacteria strain, to a complex symbiotic relationship between many bacteria and yeasts for example those found in traditional kombucha and kefir [1,4,8,11]. Aside from lactic acid bacteria, acetic acid bacteria also participate in the production of fermented drinks; working in combination with

other organisms to produce additional flavor and health-promoting compounds. A variety of yeasts in addition to conventional *S. cerevisiae* are often found within these symbiotic communities. Alternative yeasts have also been studied as options for producing reduced alcohol beverages [7]. *Brettanomyces* spp. fall into this category of yeasts and are often found in the microbiome of certain fermented beverages, such as kombucha and water kefir [4,8]. As a non-*Saccharomyces* yeast, this genus has several divergent characteristics that give it an advantage in non-traditional fermentations.

With this organism in mind, in this review we summarize relevant characteristics of the genus, highlighting one species, *B. claussenii*, in particular, as it relates to the beverage industry. Within this review we aim to spotlight *B. claussenii* and its biotechnological potential for production of novel fermented beverages from untraditional substrates, such as agricultural by-products, particularly those containing lactose. We focus on its ability to ferment lactose and highlight its potential application for bioremediation of lactose containing by-products. Wholesome reviews for *Brettanomyces* spp. exist and cover many aspects that we will not cover here, however, they usually focus on *B. bruxellensis*. We start this review by covering the genus as a whole and illustrating its environment. Next, we introduce fermentation characteristics and potential for waste product applications. Then we review the current research into the genomics of this organism and the mechanism behind its biotechnological significance. The goal of this overview is to highlight the potential for *B. claussenii* in the functional beverage marketplace and aid in describing its application to lactose-containing waste streams in order to foster sustainability.

4.3 History of *Brettanomyces* and background on environmental isolation

Brettanomyces spp. have been isolated from a variety of sources, mainly alcoholic fermentations such as beer, wine or cider; but they have also been isolated from kombucha, soft drinks, and fruit juices, as well as from some dairy products such as fermented milk, yogurt, and cheese [4,12–15]. Isolation sources specific to *B. claussenii* can be seen in Table 1. There are five established species for *Brettanomyces: B. bruxellensis*, *B. claussenii*, *B. custersianus*, *B. naardenensis*, and *B. nanus*. A sixth strain (*B. acidodurans*) has been identified and proposed, but has not been formally accepted due to large genetic differences observed [14,16]. The teleomorph name for this genus is *Dekkera* and there have been many controversies over correct naming [13]. Additionally, synonyms for the species *B. claussenii* exist including *B./D. anomala* and *B./D. anomalus*. For this paper we will refer to the genus as *Brettanomyces*, to include *Dekkera*, and we will refer to the aforementioned species as *B. claussenii*.

Table 1. Environmental isolation sources of *B. claussenii* related to food production. References prior to 2013 (except for Jayabalan *et al.* 2008) are compiled from Blackburn 2006 [15], Kurtzman 2011 [14], and Steensels *et al.* 2015 [13]. References for which full text could not be found to confirm source are cited as Blackburn 2006 [15] and/or Steensels *et al.* 2015 [13].

Isolation source	Reference and year
Alcoholic beverages:	
Wine	Osterwalder 1912 [13]
	Janke 1924 [13]
Sherry	Ibeas *et al.* 1996 [77]
	Esteve-Zarzoso *et al.* 2001 [78]
Cider	Legakis 1961 [13]
	Morrisey *et al.* 2004 [79]
	Coton 2006 *et al.* [80]
	Gray *et al.* 2011 [81]
Tequila	Lachance 1995 [13,15]
Beer	Custers 1940 [13]
	Walters 1943 [82]
	Gilliland 1962 [83]
	Lee & Jong 1985 [13]
	Verachtert 1992 [13]
Dairy:	
Fermented milk (airag, Amasi, kefir, koumiss)	Tanaka 1996 [84]
	Wydes 1997 [85]
	Gadaga 2000 [86]
	Marsh 2013 [87]
	Wang 2018 [88]
	Tang 2019 [89]
Cheese (Blue-veined, ewe's milk, feta, Harzer)	Engel & Rosch 1995 [15]
	Cosentino *et al.* 2001 [90]
	Fadda *et al.* 2001 [91]
	Viljoen *et al.* 2003 [92]
Other:	
Kombucha	Jayabalan *et al.* 2008 [93]
	Coton *et al.* 2017 [94]
Cassava flour	Okagbue 1990 [95]
Sourdough	Comasio *et al.* 2020 [96]
Carbonated beverage	Smith 1984 [97]
	Gray *et al.* 2011 [81]

In the wine industry, *Brettanomyces* spp. are a ubiquitous spoilage organism; the main species that is routinely isolated is *B. bruxellensis*. In wine, it is undesirable due to the off-putting flavor and aroma compounds it can produce in the final product. This microorganism has been shown to exist in harsh, minimal environments where other species such as *S. cerevisiae* cannot survive, i.e. the end of a wine fermentation where sugars are marginal, pH is low, and ethanol levels are high [12]. Certain species of *Brettanomyces* have even been shown to be able to grow with ethanol as the sole carbon source [17]. To remedy the spoilage concern, *B. bruxellensis* is often controlled in wine through the addition of sulfur dioxide. Although this preservative can reduce levels in wine, *B. bruxellensis* can still be viable and go undetected if it is unculturable [18]. In cider, *Brettanomyces* spp. have also commonly been isolated as spoilage organisms that produce phenolic off flavors through enzymatic activity of phenolic acid decarboxylase and vinylphenol reductase [13,19]. In contrast to the wine and cider industry, the global craft beer industry, has slowly climbed aboard the *Brettanomyces* flavor train – introducing new fermentations to product lines using the yeast for sour and "bretty" style beers similar to historic beers such as lambic and geuze [20,21]. The same compounds produced by *Brettanomyces* that cause spoilage in wine and cider, produce desirable compounds during the second fermentation characteristic of beer styles such as lambic, geuze, and coolship ales [22,23]. As a genus, this yeast has had a history in and out of the limelight. It was originally isolated in the 1800s in a brewery in Denmark and first recorded in the literature in 1904 [24]. The brewers characterized it as producing flavors that were distinctive of some of their beers. Traditional lambic beers are spontaneously inoculated and left to ferment around one year where they are inhabited by different phases of bacterial and yeast species [25]. *Brettanomyce* spp. take up residence in the fermentation towards the end of the process, after six months, and after *S.*

cerevisiae has diminished [25,26]. In a study of American coolship ales, *B. bruxellensis* was seen as the dominant, and sometimes only, yeast present after six months of fermentation [27].

Table 2. Characteristics of *Brettanomyces* growth in various growth conditions and carbon sources. Compiled from Geros *et al.* 2000 [98], Ciani *et al.* 2003 [99], Freer *et al.* 2003 [37], Silva *et al.* 2004 [17], Brandam *et al.* 2008 [35], Blomqvist *et al.* 2010 [100], Kurtzman *et al.* 2011 [14], and Cibrario *et al.* 2020 [63]. Growth range based on data generalized from *B. bruxellensis* and *B. claussenii*. + = positive growth, v = variable growth

	Growth on various substrates				
Species	Glucose	Galactose	Sucrose	Maltose	Lactose
B. bruxellensis	+	v	v	v	v
B. claussenii	+	+	+	v	v
	Growth range				
	Temperature (°C)			pH	
	15 – 37			3.0 – 6.5	

Some of the flavor compounds *Brettanomyces* spp. produce are unique to this genus and can be desirable as mentioned previously. The positive flavor attributes are often described as fruity or floral and are due to specific enzymatic activity occurring within this yeast. *Brettanomyces* spp. possess enzymes that are able to release flavor compounds from certain plant components, which produce these different flavor and aroma attributes [13,28]. This includes an ability to hydrolyze monoterpenes from hops to produce flavor compounds through beta-glucosidase activity [29–31]. The craft beer industry is on the rise and always looking for new technology options to create novel beverages. *Brettanomyces* has previously been used in this domain and is the most studied non-*Saccharomyces* alternative yeast option [29,32].

4.4 *Brettanomyces* is of biotechnological potential

Brettanomyces spp. have been introduced to various substrates to produce bioethanol and ferment waste streams. This genus has been studied for its use in creating ethanol from renewable sources due to its ability to survive low pH and ferment various non-traditional substrates [12,33]. It has even been isolated as a contaminant from industrial ethanol

fermentations [34]. As can be seen in Table 2, *Brettanomyces* spp. can grow on a variety of substrates and throughout a wide range of cultivation conditions in regard to temperature and pH. Ability to grow on these various substrates is variable and strain dependent [14]. *Brettanomyces* spp. are able to ferment glucose to ethanol under anaerobic conditions. Some species also display the Crabtree effect, similar to *S. cerevisiae*, where fermentation of glucose to ethanol occurs even in aerobic conditions when sugar is abundant [13]. This trait is not shared by all species within the genus. Additionally, under aerobic conditions, some species of *Brettanomyces* can produce high levels of acetic acid [13]. Since *Brettanomyces* spp. are considered wine spoilage organisms, production of acetic acid, an undesirable trait in wine, has been well studied. When considering the fermented beverage industry as a whole, ethanol and acetic acid are valuable metabolic end products. There are several studies that evaluate production of ethanol or acetic acid with *B. claussenii* and *B. bruxellensis* on glucose. Brandam *et al.* saw production of 16-18 g/L ethanol by *B. bruxellensis* on 50 g/L glucose, the range varying depending on temperature [35]. Additionally, Galafassi *et al.* evaluated the production of ethanol from glucose and agricultural waste streams and found that *B. bruxellensis* gave promising ethanol yields of 0.44 gram ethanol per gram of glucose [33]. As for acetic acid, *Brettanomyces* spp. have been shown to produce high levels under aerobic conditions, as previously mentioned. Freer *et al.* evaluated several species of *Brettanomyces*, including *B. bruxellensis* and *B. claussenii*, and saw production of over 30 g/L acetic acid from 100 g/L of glucose [36,37]. The creation of these end products by *Brettanomyces* spp. inspires encouraging perspectives for utilization of this organism industrially, especially for the transformation and bioremediation of waste streams. Ethanol and acetic acid are valued components of fermented beverages. Use of this organism for production

of these value-added components could be promising for agricultural waste streams and the functional beverage industry.

4.5 Lactose-containing by-products as prospective substrates for *B. claussenii* fermentation

Possible substrate options for fermentation include dairy effluents such as whey and whey permeate. By-products from dairy processes (e.g. cheese and yogurt production) are in surplus worldwide due to the large ratio of by-product to product [38,39]. Acid whey in particular, a waste stream from Greek yogurt production, currently poses a large disposal issue for the dairy industry in the United States due to its low pH and high biological oxygen demand making it unsuitable for direct disposal [40]. Sweet whey, from cheese production, is largely used in production of whey powders for its protein content. Lower in protein due to the manufacturing process of Greek yogurt, acid whey still contains some valuable components such as vitamins, minerals, and sugar [38,41]. The main sugar present in acid whey, as well as cheese whey, lactose, is a potential substrate for fermentation with *Brettanomyces* spp.

The use of whey for beverages dates back to ancient Greece and has been used for centuries as a therapeutic treatment for common ailments [42]. Over the past few decades, advances of its use in beverages have been increasing [41–44]. Whey has been used as a base in beverages with additional flavors added, but often, the dairy taste, low pH, and other intrinsic flavors can be off-putting to consumers [44]. Fermentation of whey using bacteria and yeast to produce alcoholic and acetic acid-containing beverages is an option to not only overcome this problem but add functional properties to products. Whey has been fermented with lactic acid bacteria to produce potentially probiotic products [1,44]. It has also been fermented using non-traditional methods to produce ethanol or acetic acid containing products. With lactose as the main component, this is done in several ways: use of a non-*Saccharomyces* yeast [45–50], initial

hydrolysis of lactose and subsequent fermentation with *S. cerevisiae* [51,52], or through recombinant methods [53,54]. Currently, most lactose-containing fermentations focus on the use of *Kluyveromyces* spp. for ethanol production. This alternative yeast has the ability to utilize lactose, unlike *S. cerevisiae*. Subsequent fermentation with acetic acid bacteria leads to production of vinegar-type beverages from whey [44].

Presently, there are very few studies that focus on lactose as a substrate for ethanol or acetic acid production from *Brettanomyces*, despite the knowledge that the species *B. claussenii* does have the ability to consume lactose. In 1983, Sandhu & Waraich demonstrated growth of *B. claussenii* on cheese whey and production of beta-galactosidase by the yeast [45]. In 1986, Bothast *et al.*, published on the use of *B. claussenii* and several other yeast species for production of ethanol on varying amounts of lactose. The study concluded that the species was not effective at producing ethanol from lactose [46]. More recently, *B. claussenii* has been shown to produce ethanol and acetic acid from synthetic lactose media and dairy effluents (M.R. Lawton *et al.*, abstract 473, American Dairy Science Association Annual Meeting, Knoxville, TN, 2018; V. K. Rivera Flores *et al.*, abstract M52, American Dairy Science Association Annual Meeting, Virtual, 2020) [55]. This creates a potential opportunity to produce value-added goods from dairy by-products and expand in the fermented functional beverages category.

Fermented, vinegar-type beverages such as kombucha have historically been promoted and investigated for their health benefits. These benefits include reduction of cholesterol, hypoglycemic properties, and antimicrobial effects, including against *H. pylori* [5,56]. Preliminary studies, including some in humans, have also investigated the health benefits attributed to vinegar itself [57]. Some largely studied functional properties of vinegar include reduction of blood pressure, treatment of hyperglycemia, reduced cholesterol, assistance with

weight loss, and demonstration of antimicrobial activities [58–60]. Many of these properties are recognized as the action of acetic acid in the vinegar or kombucha [56,60]. Beverages containing acetic acid would fall into the functional beverage category. Additional opportunity exists for creating low alcoholic beverages with acetic acid content.

4.6 Genomic studies and insight into mechanism behind lactose utilization of *B. claussenii*

Due to its spoilage history, much of the current genomic research of *Brettanomyces* has focused on spoilage prevention or investigation into genomic characteristics of spoilage [22]. As mentioned previously, there are five established species of *Brettanomyces* [14]; within the literature, *B. bruxellensis* is the most highly studied of the genus, due to routine isolation from spoiled beverages and industrial fermentations. This species is also of interest industrially for bioremediation and in the craft beer sector as mentioned in the previous sections. Genetic studies of the genus have varied within similarity between species, in terms of phylogeny. According to Roach & Borneman, most recent studies with 18S ribosomal RNA analysis include *B. naardenensis* and *B. nanus* grouped separately from the others; they confirmed this with whole genome analysis. In addition, whole genome sequence availability is somewhat limited for the genus to *B. bruxellensis*; only a single or very few assemblies exist for the other species and are usually not annotated [61].

Existing genetic studies identify genes potentially involved in some aspects of metabolism, preferentially production of spoilage compounds, but they often do not confirm gene activity with phenotype assays. This is true of lactose-hydrolyzing activity for the genus. Of the five established species, *B. claussenii* is often the species that is identified as having the phenotype with the ability to grow on and ferment lactose [14]. There are some reports of *B. bruxellensis* metabolizing lactose [28,62,63] while classical definitions say it cannot [14]. The sixth proposed

species, *B. acidodurans*, is indicated to ferment lactose but it has not officially been placed in the genus and there is little existing information on this species [14,16]. *B. nanus* is defined as variable for growth on lactose [14]. In terms of genetic analysis for lactose metabolism, several studies have identified genes through genome sequencing that could potentially allow growth on or fermentation of lactose [28,61,64–66]. A putative beta-galactosidase has been mentioned in the literature through whole genome sequencing analyses of *B. bruxellensis* [66,67] as well as in *B. nanus* [61]. Genomic studies looking at *B. bruxellensis* conclude that within the species, strains are very divergent and can have contrasting phenotypes [28,62,66,68,69]. Additionally, a beta-glucosidase has been indicated in *B. bruxellensis* and *B. claussenii* [28,30,65,66]. While not traditionally thought of as a lactase, beta-glucosidases can have activity towards lactose albeit with lower beta-galactosidase activity [70–74]. Vervoort *et al.* identified, isolated, and characterized a beta-glucosidase in *B. anomalus*. This study indicated that the enzyme had strong activity towards producing flavor compounds in fruit beers and fruit milk [30]. The potential for lactose hydrolysis activity of this enzyme was not indicated. Through investigation of the differences in genotype and phenotype of several strains of *B. bruxellensis*, Crauwels *et al.* found several strains that showed lactose metabolic activity. However, of the seven lactose positive strains indicated, two of them lacked the putative beta-galactosidase as well as the initial beta-glucosidase identified in other strains, but were found to have a homolog beta-glucosidase [28]. This leaves the question open as to how some species and strains of *Brettanomyces* are able to utilize lactose while others are not. Figure 1 demonstrates the pathway lactose would take to ethanol and acetic acid production in *B. claussenii* strains. Within *Kluyveromyces*, a yeast genus known to ferment lactose, a beta-galactosidase and lactose permease have been identified and activity confirmed through transformation into *S. cerevisiae* to confer a lactose positive

phenotype [75,76]. This gene has even been used for bioremediation studies of lactose in whey [51]. More in-depth studies of the genomes of *Brettanomyces* spp. are needed to expand the knowledge of metabolic activity within this genus and to investigate the differences in lactose hydrolysis activity, specifically at strain and species level.

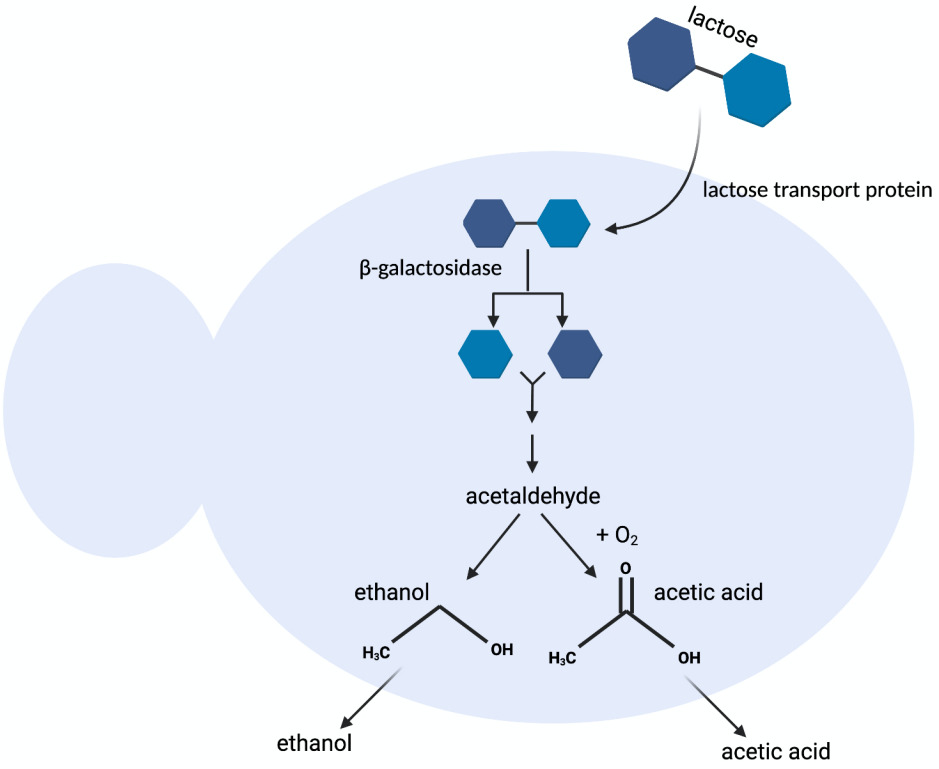

Figure 1. Pathway for ethanol/acetic acid production in lactose utilizing strains of *Brettanomyces claussenii*. Proposed utilization of lactose would involve transport into the cell through a lactose transport protein and cleavage by a beta-galactosidase. Subsequent aerobic or anaerobic fermentation of glucose and galactose would lead to production of acetic acid and/or ethanol. Diagram adapted from Steensels *et al.* 2015 [13] and Galafassi *et al.* 2013 [101].

4.7 Conclusion

The existing research points toward the opportunity to harness the lactose hydrolysis power of *B. claussenii* for novel dairy-based fermented beverages. This yeast can survive in harsh conditions such as the low pH environment of acid whey, produce beneficial by-products (acetic acid), and impart unique flavor compounds. Fermentation with this yeast could produce

fermented beverages, alcoholic and non-alcoholic, with beneficial properties stemming from production of acetic acid. Additionally, its ability to ferment lactose provides a unique substrate source. Opportunity arises for valorization of dairy waste streams to expand the dairy industry into the fermented functional beverage market. Further research into the mechanism behind and characterization of lactose utilization in this yeast will provide information necessary for expanding on the opportunity that it provides.

Acknowledgements

The authors would like to thank the Alcaine Research Group in their support and revision of this manuscript. Figure 1 and graphical abstract created using biorender.com. Funding: This work is supported by AFRI Predoctoral Fellowship [Grant # 2020-67034-31743] from the USDA National Institute of Food and Agriculture.

REFERENCES

1. Turkmen N, Akal C, Özer B: **Probiotic dairy-based beverages: A review**. *J Funct Foods* 2019, **53**:62–75.
2. *Nazir M, Arif S, Khan RS, Nazir W, Khalid N, Maqsood S: **Opportunities and challenges for functional and medicinal beverages: Current and future trends**. *Trends Food Sci Technol* 2019, **88**:513–526.
   This review covers the current status of the functional beverage market including current products and global market size.
3. Nazhand A, Souto EB, Lucarini M, Souto SB, Durazzo A, Santini A: **Ready to Use Therapeutical Beverages: Focus on Functional Beverages Containing Probiotics, Prebiotics and Synbiotics**. *Beverages* 2020, **6**:26.
4. Marsh AJ, Hill C, Ross RP, Cotter PD: **Fermented beverages with health-promoting potential: Past and future perspectives**. *Trends Food Sci Technol* 2014, **38**:113–124.
5. Jayabalan R, Malbaša R V, Lončar ES, Vitas JS, Sathishkumar M: **A review on kombucha tea—microbiology, composition, fermentation, beneficial effects, toxicity, and tea fungus**. *Compr Rev Food Sci Food Saf* 2014, **13**:538–550.
6. Habschied K, Živković A, Krstanović V, Mastanjević K: **Functional beer—A review on possibilities**. *Beverages* 2020, **6**:51.
7. Bellut K, Arendt EK: **Chance and challenge: Non-saccharomyces yeasts in nonalcoholic and low alcohol beer brewing–A review**. *J Am Soc Brew Chem* 2019, **77**:77–91.
8. Tolun A, Altintas Z: **Medicinal properties and functional components of beverages**. In *Functional and Medicinal Beverages*. . Elsevier; 2019:235–284.
9. Ugidos-Rodríguez S, Matallana-González MC, Sánchez-Mata MC: **Lactose malabsorption and intolerance: a review**. *Food Funct* 2018, **9**:4056–4068.
10. Hodges JK, Cao S, Cladis DP, Weaver CM: **Lactose intolerance and bone health: the challenge of ensuring adequate calcium intake**. *Nutrients* 2019, **11**:718.
11. Ashaolu TJ: **A review on selection of fermentative microorganisms for functional foods and beverages: the production and future perspectives**. *Int J Food Sci Technol* 2019, **54**:2511–2519.
12. **Smith BD, Divol B: **Brettanomyces bruxellensis, a survivalist prepared for the wine apocalypse and other beverages**. *Food Microbiol* 2016, **59**:161–175.
    This paper thoroughly reviews key aspects of *Brettanomyces*, specifically *B. bruxellensis*. It thoroughly reviews genetic studies of the species and discusses certain genetic traits in the context of wine spoilage.
13. **Steensels J, Daenen L, Malcorps P, Derdelinckx G, Verachtert H, Verstrepen KJ: **Brettanomyces yeasts - From spoilage organisms to valuable contributors to industrial fermentations**. *Int J Food Microbiol* 2015, **206**:24–38.
    This paper also thoroughly reviews the genus of *Brettanomyces*, specifically *B. bruxellensis*. It covers the history of the genus as well as application to industrial fermentations.
14. Kurtzman C, Fell JW, Boekhout T: *The yeasts: a taxonomic study*. Elsevier; 2011.
15. de W Blackburn C: *Food spoilage microorganisms*. Woodhead Publishing; 2006.
16. Péter G, Dlauchy D, Tóbiás A, Fülöp L, Podgoršek M, Čadež N: **Brettanomyces acidodurans sp. nov., a new acetic acid producing yeast species from olive oil**. *Antonie Van Leeuwenhoek* 2017, **110**:657–664.

17. Silva P, Cardoso H, Gerós H: **Studies on the wine spoilage capacity of Brettanomyces/Dekkera spp.** *Am J Enol Vitic* 2004, **55**:65–72.
18. Du Toit WJ, Pretorius IS, Lonvaud-Funel A: **The effect of sulphur dioxide and oxygen on the viability and culturability of a strain of Acetobacter pasteurianus and a strain of Brettanomyces bruxellensis isolated from wine**. *J Appl Microbiol* 2005, **98**:862–871.
19. Buron N, Coton M, Legendre P, Ledauphin JÔ, Kientz-Bouchart V, Guichard H, Barillier D, Coton E: **Implications of Lactobacillus collinoides and Brettanomyces/Dekkera anomala in phenolic off-flavour defects of ciders**. *Int J Food Microbiol* 2012, **153**:159–165.
20. Tonsmeire M: *American sour beer: Innovative techniques for mixed fermentations*. Brewers Publications; 2014.
21. **Serra Colomer M, Funch B, Forster J: **The raise of Brettanomyces yeast species for beer production**. *Curr Opin Biotechnol* 2019, **56**:30–35.
This review highlights the increasing use of Brettanomyces industrially in the craft beer sector and provides key advantages and differences in its use as compared to *S. cerevisiae.*
22. Curtin CD, Pretorius IS: **Genomic insights into the evolution of industrial yeast species Brettanomyces bruxellensis**. *FEMS Yeast Res* 2014, **14**:997–1005.
23. Vanderhaegen B, Neven H, Coghe S, Verstrepen KJ, Verachtert H, Derdelinckx G: **Evolution of chemical and sensory properties during aging of top-fermented beer**. *J Agric Food Chem* 2003, **51**:6782–6790.
24. Claussen NH: **On a method for the application of Hansen's pure yeast system in the manufacturing of well-conditioned English stock beers**. *J Inst Brew* 1904, **10**:308–331.
25. Steensels J, Verstrepen KJ: **Taming wild yeast: potential of conventional and nonconventional yeasts in industrial fermentations**. *Annu Rev Microbiol* 2014, **68**:61–80.
26. Spitaels F, Wieme AD, Janssens M, Aerts M, Daniel HM, Van Landschoot A, De Vuyst L, Vandamme P: **The microbial diversity of traditional spontaneously fermented lambic beer**. *PLoS One* 2014, **9**.
27. Bokulich NA, Bamforth CW, Mills DA: **Brewhouse-resident microbiota are responsible for multi-stage fermentation of American coolship ale**. *PLoS One* 2012, **7**.
28. *Crauwels S, Van Assche A, de Jonge R, Borneman AR, Verreth C, Troels P, De Samblanx G, Marchal K, Van de Peer Y, Willems KA, et al.: **Comparative phenomics and targeted use of genomics reveals variation in carbon and nitrogen assimilation among different Brettanomyces bruxellensis strains**. *Appl Microbiol Biotechnol* 2015, **99**:9123–9134.
This study of various *B. bruxellensis* strains delivers a comparison between genotypic and phenotypic information of strains and also provides insight into potential lactose metabolism genes.
29. Iattici F, Catallo M, Solieri L: **Designing new yeasts for craft brewing: When natural biodiversity meets biotechnology**. *Beverages* 2020, **6**:1–20.
30. *Vervoort Y, Herrera-Malaver B, Mertens S, Guadalupe Medina V, Duitama J, Michiels L, Derdelinckx G, Voordeckers K, Verstrepen KJ: **Characterization of the recombinant Brettanomyces anomalus β-glucosidase and its potential for bioflavouring**. *J Appl Microbiol* 2016, **121**:721–733.
This paper is one of the only studies to investigate the genome of *B. claussenii*. A beta-glucosidase is confirmed and characterized.

31. Colomer MS, Funch B, Solodovnikova N, Hobley TJ, Förster J: **Biotransformation of hop derived compounds by Brettanomyces yeast strains**. *J Inst Brew* 2020,
32. *Basso RF, Alcarde AR, Portugal CB: **Could non-Saccharomyces yeasts contribute on innovative brewing fermentations?** *Food Res Int* 2016, **86**:112–120.
This review provides an in depth overview of the use of non-traditional yeasts, including *Brettanomyces*, in the brewing industry.
33. Galafassi S, Merico A, Pizza F, Hellborg L, Molinari F, Piškur J, Compagno C: **Dekkera/Brettanomyces yeasts for ethanol production from renewable sources under oxygen-limited and low-pH conditions**. *J Ind Microbiol Biotechnol* 2011, **38**:1079–1088.
34. Blomqvist J, Passoth V: **Dekkera bruxellensis-spoilage yeast with biotechnological potential, and a model for yeast evolution, physiology and competitiveness**. *FEMS Yeast Res* 2015, **15**:1–9.
35. Brandam C, Castro-Martínez C, Délia ML, Ramón-Portugal F, Strehaiano P: **Effect of temperature on Brettanomyces bruxellensis: Metabolic and kinetic aspects**. *Can J Microbiol* 2008, **54**:11–18.
36. Freer SN: **Acetic acid production by Dekkera/Brettanomyces yeasts**. *World J Microbiol Biotechnol* 2002, **18**:271–275.
37. Freer SN, Dien B, Matsuda S: **Production of acetic acid by Dekkera/Brettanomyces yeasts under conditions of constant pH**. *World J Microbiol Biotechnol* 2003, **19**:101–105.
38. Menchik P, Zuber T, Zuber A, Moraru CI: **Composition of coproduct streams from dairy processing: Acid whey and milk permeate**. *J Dairy Sci* 2019, **102**:3978–3984.
39. Rao M, Bast A, de Boer A: **Valorized Food Processing By-Products in the EU: Finding the Balance between Safety, Nutrition, and Sustainability**. *Sustainability* 2021, **13**:4428.
40. Smithers GW: **Whey-ing up the options–Yesterday, today and tomorrow**. *Int Dairy J* 2015, **48**:2–14.
41. Jelen P: **Whey Processing: Utilization and Products**. *Encycl Dairy Sci Second Ed* 2011, doi:10.1016/B978-0-12-374407-4.00495-7.
42. Jelen P: **Whey-based functional beverages**. In *Functional and speciality beverage technology*. . Elsevier; 2009:259–280.
43. Holsinger VH, Posati LP, DeVilbiss ED: **Whey beverages: a review**. *J Dairy Sci* 1974, **57**:849–859.
44. **Zotta T, Solieri L, Iacumin L, Picozzi C, Gullo M: **Valorization of cheese whey using microbial fermentations**. *Appl Microbiol Biotechnol* 2020, **104**:2749–2764.
This paper reviews the current technology and approaches to up-cycling whey through fermentation.
45. Sandhu DK, Waraich MK: **Conversion of cheese whey to single-cell protein**. *Biotechnol Bioeng* 1983, **25**:797–808.
46. Bothast RJ, Kurtzman CP, Saltarelli MD, Slininger PJ: **Ethanol Production by 107 Strains of Yeasts on 5 , 10 , and 20 % Lactose**. *Biotechnol Lett* 1986, **8**:593–596.
47. Parrondo J, Herrero M, García LA, Díaz M: **A Note—Production of Vinegar from Whey**. *J Inst Brew* 2003, **109**:356–358.
48. Tamura Y: **Manufacture of the vinegar from cheese whey**. *Milk Sci* 2000, **49**:15–20.
49. Lustrato G, Salimei E, Alfano G, Belli C, Fantuz F, Grazia L, Ranalli G: **Cheese whey**

recycling in traditional dairy food chain: effects of vinegar from whey in dairy cow nutrition. *Acetic Acid Bact* 2013,
50. Risner D, Shayevitz A, Haapala K, Meunier-Goddik L, Hughes P: **Fermentation and distillation of cheese whey: Carbon dioxide-equivalent emissions and water use in the production of whey spirits and white whiskey**. *J Dairy Sci* 2018, **101**:2963–2973.
51. Guimarães PMR, Teixeira JA, Domingues L: **Fermentation of lactose to bio-ethanol by yeasts as part of integrated solutions for the valorisation of cheese whey**. *Biotechnol Adv* 2010, **28**:375–384.
52. Lawton MR, Alcaine SD: **Leveraging endogenous barley enzymes to turn lactose-containing dairy by-products into fermentable adjuncts for Saccharomyces cerevisiae-based ethanol fermentations**. *J Dairy Sci* 2019, **102**:2044–2050.
53. Domingues L, Dantas MM, Lima N, Teixeira JA: **Continuous ethanol fermentation of lactose by a recombinant flocculating Saccharomyces cerevisiae strain**. 1999,
54. Domingues L, Guimarães PMR, Oliveira C: **Metabolic engineering of Saccharomyces cerevisiae for lactose/whey fermentation. Bioeng Bugs 1: 164–171**. 2010,
55. *Marcus JF, DeMarsh TA, Alcaine SD: **Upcycling of Whey Permeate through Yeast- and Mold-Driven Fermentations under Anoxic and Oxic Conditions**. *Fermentation* 2021, **7**:16.
This paper demonstrates the utilization of *B. claussenii* for fermentation of lactose containing dairy by-products to valuable end products of ethanol and acetic acid.
56. Greenwalt CJ, Steinkraus KH, Ledford RA: **Kombucha, the fermented tea: microbiology, composition, and claimed health effects**. *J Food Prot* 2000, **63**:976–981.
57. Ho CW, Lazim AM, Fazry S, Zaki UKHH, Lim SJ: **Varieties, production, composition and health benefits of vinegars: A review**. *Food Chem* 2017, **221**:1621–1630.
58. Budak NH, Aykin E, Seydim AC, Greene AK, Guzel-Seydim ZB: **Functional properties of vinegar**. *J Food Sci* 2014, **79**:R757–R764.
59. Petsiou EI, Mitrou PI, Raptis SA, Dimitriadis GD: **Effect and mechanisms of action of vinegar on glucose metabolism, lipid profile, and body weight**. *Nutr Rev* 2014, **72**:651–661.
60. Chen H, Chen T, Giudici P, Chen F: **Vinegar functions on health: Constituents, sources, and formation mechanisms**. *Compr Rev Food Sci Food Saf* 2016, **15**:1124–1138.
61. *Roach MJ, Borneman AR: **New genome assemblies reveal patterns of domestication and adaptation across Brettanomyces (Dekkera) species**. *BMC Genomics* 2020, **21**:1–14.
This paper reviews current genomic information on *Brettanomyces* while providing the most inclusive analysis of the genus in terms of whole genome availability.
62. Conterno L, Joseph CML, Arvik TJ, Henick-Kling T, Bisson LF: **Genetic and physiological characterization of Brettanomyces bruxellensis strains isolated from wines**. *Am J Enol Vitic* 2006, **57**:139–147.
63. Cibrario A, Miot-Sertier C, Paulin M, Bullier B, Riquier L, Perello MC, de Revel G, Albertin W, Masneuf-Pomarède I, Ballestra P, et al.: **Brettanomyces bruxellensis phenotypic diversity, tolerance to wine stress and wine spoilage ability**. *Food Microbiol* 2020, **87**.
64. Curtin CD, Borneman AR, Chambers PJ, Pretorius IS: **De-novo assembly and analysis of the heterozygous triploid genome of the wine spoilage yeast dekkera bruxellensis**

AWRI1499. *PLoS One* 2012, **7**:1–10.
65. Woolfit M, Rozpędowska E, Piškur J, Wolfe KH: **Genome survey sequencing of the wine spoilage yeast Dekkera (Brettanomyces) bruxellensis**. *Eukaryot Cell* 2007, **6**:721–733.
66. Crauwels S, Zhu B, Steensels J, Busschaert P, De Samblanx G, Marchal K, Willems KA, Verstrepen KJ, Lievens B: **Assessing genetic diversity among Brettanomyces yeasts by DNA fingerprinting and whole-genome sequencing**. *Appl Environ Microbiol* 2014, **80**:4398–4413.
67. Valdes J, Tapia P, Cepeda V, Varela J, Godoy L, Cubillos FA, Silva E, Martinez C, Ganga MA: **Draft genome sequence and transcriptome analysis of the wine spoilage yeast Dekkera bruxellensis LAMAP2480 provides insights into genetic diversity, metabolism and survival**. *FEMS Microbiol Lett* 2014, **361**:104–106.
68. Avramova M, Cibrario A, Peltier E, Coton M, Coton E, Schacherer J, Spano G, Capozzi V, Blaiotta G, Salin F, et al.: **Brettanomyces bruxellensis population survey reveals a diploid-triploid complex structured according to substrate of isolation and geographical distribution**. *Sci Rep* 2018, **8**:1–13.
69. Curtin CD, Bellon JR, Henschke PA, Godden PW, De Barros Lopes MA: **Genetic diversity of Dekkera bruxellensis yeasts isolated from Australian wineries**. *FEMS Yeast Res* 2007, **7**:471–481.
70. Simos G, Georgatsos JG: **Lactose-hydrolyzing β-glycosidases of barley meal**. *Biochim Biophys Acta (BBA)-General Subj* 1988, **967**:17–24.
71. Bhatti HN, Batool S, Afzal N: **Production and characterization of a novel (beta)-glucosidase from Fusarium solani**. *Int J Agric Biol* 2013, **15**.
72. Li B, Wang Z, Li S, Donelan W, Wang X, Cui T, Tang D: **Preparation of lactose-free pasteurized milk with a recombinant thermostable β-glucosidase from Pyrococcus furiosus**. *BMC Biotechnol* 2013, **13**:73.
73. Akiyama K, Takase M, Horikoshi K, Okonogi S: **Production of galactooligosaccharides from lactose using a β-glucosidase from Thermus sp. Z-1**. *Biosci Biotechnol Biochem* 2001, **65**:438–441.
74. Wołosowska S, Synowiecki J: **Thermostable β-glucosidase with a broad substrate specifity suitable for processing of lactose-containing products**. *Food Chem* 2004, **85**:181–187.
75. Dickson RC, Barr K: **Characterization of lactose transport in Kluyveromyces lactis**. *J Bacteriol* 1983, **154**:1245–1251.
76. Sreekrishna K, Dickson RC: **Construction of strains of Saccharomyces cerevisiae that grow on lactose**. *Proc Natl Acad Sci* 1985, **82**:7909–7913.
77. Ibeas JI, Lozano I, Perdigones F, Jimenez J: **Detection of Dekkera-Brettanomyces strains in sherry by a nested PCR method**. *Appl Environ Microbiol* 1996, **62**:998–1003.
78. Esteve-Zarzoso B, Peris-Torán MJ, Garcıa-Maiquez E, Uruburu F, Querol A: **Yeast population dynamics during the fermentation and biological aging of sherry wines**. *Appl Environ Microbiol* 2001, **67**:2056–2061.
79. Morrissey WF, Davenport B, Querol A, Dobson ADW: **The role of indigenous yeasts in traditional Irish cider fermentations**. *J Appl Microbiol* 2004, **97**:647–655.
80. Coton E, Coton M, Levert D, Casaregola S, Sohier D: **Yeast ecology in French cider and black olive natural fermentations**. *Int J Food Microbiol* 2006, **108**:130–135.
81. Gray SR, Rawsthorne H, Dirks B, Phister TG: **Detection and enumeration of Dekkera**

anomala in beer, cola, and cider using real-time PCR. *Lett Appl Microbiol* 2011, **52**:352–359.
82. Walters LS: **II. THE DISTINGUISHING CHARACTERS OF TORULOPSIS CYLINDRICA NOV. SP., A YEAST CAUSING A TURBIDITY AND AN UNPLEASANT TASTE AND SMELL IN BEER**. *J Inst Brew* 1943, **49**:253–256.
83. Gilliland RB: **The Brettanomyces. II. Taxonomic significance of slow fermentations and description of a new species**. *J Inst Brew* 1962, **68**:51–59.
84. Tanaka Y, Mori N, Kitamoto Y: **Microbial flora of" Airag", a traditional fermented milk of Inner-Mongolia in China**. *Anim Sci Technol* 1996,
85. Wydes MT, Spillmann H, Puhan Z: **Investigation of the yeast flora in dairy products: a case study of kefyr**. *Food Technol Biotechnol* 1997, **35**:299–304.
86. Gadaga TH, Mutukumira AN, Narvhus JA: **Enumeration and identification of yeasts isolated from Zimbabwean traditional fermented milk**. *Int Dairy J* 2000, **10**:459–466.
87. Marsh AJ, O'Sullivan O, Hill C, Ross RP, Cotter PD: **Sequencing-based analysis of the bacterial and fungal composition of kefir grains and milks from multiple sources**. *PLoS One* 2013, **8**:e69371.
88. Wang L, Gao E, Hu M, Oladejo A, Gong X, Wang J, Zhong H: **Isolation, identification and screening of high-quality yeast strains for the production of milk beer**. *Int J Dairy Technol* 2018, **71**:944–953.
89. Tang H, Ma H, Hou Q, Li W, Xu H, Liu W, Sun Z, Menghe B: **Profiling of Koumiss Microbiota and Organic Acids and their Effects on Koumiss Taste**. 2019,
90. Cosentino S, Fadda ME, Deplano M, Mulargia AF, Palmas F: **Yeasts associated with Sardinian ewe's dairy products**. *Int J Food Microbiol* 2001, **69**:53–58.
91. Fadda ME, Cosentino S, Deplano M, Palmas F: **Yeast populations in Sardinian feta cheese**. *Int J Food Microbiol* 2001, **69**:153–156.
92. Viljoen BC, Knox AM, De Jager PH, Lourens-Hattingh A: **Development of yeast populations during processing and ripening of blue veined cheese**. *Food Technol Biotechnol* 2003, **41**:291–297.
93. Jayabalan R, Marimuthu S, Thangaraj P, Sathishkumar M, Binupriya AR, Swaminathan K, Yun SE: **Preservation of Kombucha Tea Effect of Temperature on Tea Components and Free Radical Scavenging Properties**. *J Agric Food Chem* 2008, **56**:9064–9071.
94. Coton M, Pawtowski A, Taminiau B, Burgaud G, Deniel F, Coulloumme-Labarthe L, Fall A, Daube G, Coton E: **Unraveling microbial ecology of industrial-scale Kombucha fermentations by metabarcoding and culture-based methods**. *FEMS Microbiol Ecol* 2017, **93**.
95. Okagbue RN: **Identification of yeasts and aerobic spore forming bacteria from cassava flour**. *Food Microbiol* 1990, **7**:27–32.
96. Comasio A, Verce M, Van Kerrebroeck S, De Vuyst L: **Diverse microbial composition of sourdoughs from different origins**. *Front Microbiol* 2020, **11**:1212.
97. Smith MT, Van Grinsven AM: **Dekkera anomala sp. nov., the teleomorph of Brettanomyces anomalus, recovered from spoiled soft drinks**. *Antonie Van Leeuwenhoek* 1984, **50**:143–148.
98. Gerós H, Cássio F, Leão C: **Utilization and transport of acetic acid in Dekkera anomala and their implications on the survival of the yeast in acidic environments**. *J Food Prot* 2000, **63**:96–101.

99. Ciani M, Maccarelli F, Fatichenti F: **Growth and fermentation behaviour of Brettanomyces/Dekkera yeasts under different conditions of aerobiosis**. *World J Microbiol Biotechnol* 2003, **19**:419–422.
100. Blomqvist J, Eberhard T, Schnürer J, Passoth V: **Fermentation characteristics of Dekkera bruxellensis strains**. *Appl Microbiol Biotechnol* 2010, **87**:1487–1497.
101. Galafassi S, Capusoni C, Moktaduzzaman M, Compagno C: **Utilization of nitrate abolishes the "Custers effect" in Dekkera bruxellensis and determines a different pattern of fermentation products**. *J Ind Microbiol Biotechnol* 2013, **40**:297–303.

CHAPTER 5

Optimization of acetic acid production from lactose by fermentation with *Brettanomyces claussenii* using response surface methodology to direct utilization of acid whey and other dairy by-products

5.1 Introduction

Over the past decade, consumer consumption of Greek yogurt has increased (add references). This rise in popularity has led to increased production and an abundance of the by-product, acid whey. For every 1 kg of yogurt produced, there are 2-3 kg of acid whey. In 2015, it was reported that 2.1 million tons of acid whey were generated [1]. This liquid surplus, normally mined for whey protein, is a burden to processors due to its high biological oxygen demand [2]. Traditional disposal is costly due to need for neutralization. Current utilization schemes include application to agricultural fields, incorporation into animal feed, or feeding to an anaerobic digestor [3,4]. This issue places weight on the dairy processor financially, as they must pay to discard their acid whey. Disposal of the by-product remains an environmental and financial issue as the current re-use schemes do not make a large impact on utilization [5]. However, this waste stream, deemed invaluable for protein powder production because of its minor protein content from the straining process and low pH due to yogurt cultures, is a rich source of other valuable components such as vitamins, minerals mainly calcium, and sugar. Acid whey contains approximately 3-6 % lactose, a disaccharide made of glucose and galactose [5].

Recently, consumers are becoming increasingly aware of their health and have shifted their focus to functional beverages (i.e. beverages that offer health benefits), such as kombucha or other fermented beverages [6]. These products can contain probiotics, organic acids, or other health promoting compounds and are placed in the category of wellness tonics. In kombucha,

one of the main reasons for its health benefits is due to the organic acid content, of which a large portion is acetic acid [7–9]. The vitamin, mineral, and although slight, protein content, of acid whey makes it a potential substrate for the base of a functional beverage. Historically, whey has been used as a base for beverages, alcoholic and non-alcoholic [10,11]. As mentioned previously, the main sugar source in acid whey is lactose, a disaccharide made up of glucose and galactose. Fermentation of this carbon source is not possible with traditional fermentation yeast (*Saccharomyces cerevisiae*) as this organism does not possess a beta-galactosidase for hydrolysis of the sugar [2,12]. However, many non-alcoholic fermented beverages (kombucha, kefir) are produced with alternative microorganisms including yeasts such as other *Saccharomyces spp*., *Kluyveromyces spp*., *Brettanomyces spp*., and others [8]. Fermentation of lactose from by-products specifically has been studied using *K. lactis* or *K. marxianus*, species well established in fermenting lactose, or with recombinant *Saccharomyces* strains or enzymatic lactose hydrolysis methods [13,14].

Another genus of alternative yeast, *Brettanomyces*, has recently gained popularity in the craft beer industry, flavoring styles of beers such as the American Sour and the American Brett [15]. *Brettanomyces* yeasts were originally isolated from beer in the 1900s and have long been associated with wine and beer spoilage but they are also responsible for some of the characteristic flavors of traditional beers such as Lambics and Geuze [16–18]. The species *B. claussenii* has the ability to ferment lactose [19] and has been shown to produce acetic acid from glucose in aerobic conditions, and produces ethanol in anaerobic fermentation [20]. *B. claussenii* is also called *B. anomala* or *Dekkera anomala*, indicating its teleomorph form and the terms are often used interchangeably [20]. Within this study, the term *B. claussenii* will be used to also refer to *B. anomala* and *D. anomala* and the term *Brettanomyces* will be used to also refer to

Dekkera. When referring to a specific strain or study, the correct naming will be used from the source it was obtained from.

Previous studies have focused on acetic acid production of *B. claussenii* from glucose. An aerobic fermentation of *B. claussenii* in 5% glucose medium resulted in an acetic acid content of 1.41 g/L after 60 hours [21]. In a 100 g/L glucose medium, *D. anomala* produced 10.2 g/L acetic acid with 24.4 g/L residual glucose. When held under constant pH (6.5), the strain produced 29.3 g/L acetic acid with no residual glucose after 70 hours [22,23]. The acetic acid content of beverages such as kombucha can vary with ranges reported as 5.6-11 g/L with a fermentation time between 15-30 days [7,24–26]. In kombucha fermentation, yeasts convert sucrose to ethanol and then ethanol is converted to acetic acid by acetic acid bacteria, similar to vinegar production but in symbiosis [7]. Fermentation with *B. claussenii* has the potential to simplify vinegar production, allowing for the production of novel, dairy-based acetic acid beverages and utilization of dairy by-products.

B. claussenii can tolerate harsh environments, including low pH settings such as acid whey [27,28]. However, very little research on the lactose utilization by this yeast is available and the existing studies do not focus on acetic acid production [28]. Further understanding of the lactose utilization within the yeast *B. claussenii* is needed to establish and improve upon its use for dairy by-product valorization.

Statistical design of experiments (DoE) and response surface methodology (RSM) are popular and established tools used to optimize factors in biological experiments [29–31]. These methods can be used to identify the effect of several factors on a response while limiting the time and resources needed to conduct the experiments. Sequential design of experiments can include a

factor screening experiment (fraction factorial design), optimization of significant factors (response surface design), and further validation of optimization results.

The specific goal of this study is to optimize the production of acetic acid from lactose-containing dairy by-products. To do this, an initial screening of yeast strains was performed to identify potential candidates. Then, a 30-day aerobic fermentation was evaluated to understand the fermentation profile. Subsequently, factors hypothesized to be relevant for acetic acid production were identified and screened using a factorial design. Finally, response surface methodology was used, and a central composite design was developed to optimize the factors identified as significant towards acetic acid production from lactose. Understanding and optimizing factors affecting acetic acid production from lactose will allow for the design of fermentations to transform dairy by-products, such as acid whey, into value-added goods. This redirection of dairy by-products to create value-added beverages would provide an opportunity for the dairy industry to expand into the functional beverage market. Potential for expansion to other dairy waste streams such as whey permeate is also possible.

5.2 Materials & Methods

Yeast strains

All yeast strains used in this study can be seen in Table 1. Our lab strain of *B. claussenii* (TD-0007) was obtained from Omega Yeast (OYL-201) and was used for all subsequent experiments following the strain evaluation. *Brettanomyces*/*Dekkera* strains used during evaluation were obtained from the USDA ARS NRRL library. *K. lactis* (FSL B9-0069) was obtained from the Food Safety Lab. Sources of each strain and descriptions of isolation can be seen in Table S1.

Yeast inoculum preparation

Malt Extract Broth (MEB) was used for initial propagation of yeast. A frozen glycerol stock of yeast was streaked onto Malt Extract (MEA) or Potato Dextrose Agar (PDA) and incubated at 30 °C for 5-7 days. An individual colony was then used to inoculate 5 ml tubes of MEB and was incubated at 30 °C for 48 hours with agitation (200 rpm). This culture was then diluted 1:20 into fresh MEB in a flask and incubated at 30°C for 72 hours (200 rpm) so that the culture reached ~1x10^8 CFU/ml. Yeast concentration was confirmed with hemocytometer counts using 0.1% methylene blue to stain cells for viability detection. This culture was used as the starter inoculum for experiments.

Fermentation medium

Yeast Nitrogen Base with ammonium phosphate and complete amino acids (YNBaa) was used as the base medium. When buffered, a sodium phosphate/citric acid buffer in the correct proportions for the desired pH was used. When unbuffered medium was used, the pH was adjusted to pH 4.0 using 0.1 N sodium hydroxide. Lactose was added (YNBaa-L) at the concentration required for each experimental condition (40 g/L, 70g/L, or 100g/L).

Statistical Methods & Experimental Design

For the strain evaluation, an Analysis of Variance (ANOVA) combined with Tukey's Honest Significant Difference Test was used to determine differences in acetic acid production, acetic acid yield, and efficiency between various strains of *B. claussenii/D. anomala*. The experiments were repeated in three biological replicates. A two-level fraction factorial design (2^{6-1}) was used to screen fermentation factors (temperature, agitation, lactose concentration, initial pH, initial yeast concentration, time) for significance. Each factor was set to a high and a low level. Due to physical constraints, the factorial design was broken into whole plots by temperature. Because of the repeatability offered by the whole plots and to cut down on number of trials, the experiment

was only run in one replicate. Eight randomized experiments were run within each whole plot giving a total of 32 experiments. The significant factors were then further evaluated and optimized using response surface methodology. A central composite design with three levels (α=1) was used to evaluate the chosen factors agitation, initial yeast concentration, and initial pH. Three center points were added for a total of 17 combinations in the design, which was repeated in two biological replicates, giving a total of 51 experiments. The resulting responses of acetic acid concentration, ethanol concentration, acetic acid yield, and acetic acid efficiency were fit to a second-order model. All statistical analysis previously mentioned as well as RSM predicted values, optimization predictions, and response surface graphs were created using JMP software.

Fermentation Set Up

Fermentations were carried out in shake flasks, incubated in a chamber with humidity set at 65% relative humidity to minimize evaporation. For these flask fermentation experiments, liquid volume was always at a ratio of 20% of the flask volume to allow for consistent oxygenation. For each experiment and condition, the required amount of starter was calculated to achieve the desired inoculation concentration. For volumes greater than 0.2 ml, cells were spun down (4,000 rpm for 4 min) and supernatant discarded once before resuspension in the fermentation medium. Appropriate yeast concentration was then inoculated into a flask with appropriate media conditions and secured with a foam stopper. Flasks were then incubated at the correct conditions for the appropriate amount of time. The inoculum was serial diluted in PBS and spread plated on Potato Dextrose Agar (PDA) to confirm concentration.

Strain Evaluation

Seven strains of *B. claussenii*/*D. anomala* (Table 1) were collected and evaluated for their potential to produce acetic acid from lactose. *B. bruxellensis* was included as a species unable to utilize lactose but known to produce acetic acid and *K. lactis* was included as a control known to utilize lactose. Starters of each yeast strain were inoculated into 50 ml of YNBaa-L (40 g/L lactose, pH 4) at a concentration of $3x10^6$ CFU/mL. Flasks were incubated at 30°C for 14 days with samples taken on Days 0, 7, and 14 in duplicate for lactose and acetic acid analysis. Day 14 samples were also analyzed for pH. The experiment was repeated in 3 biological replicates.

Table 1. Yeast strains used in this study.

Strain	Description	Source
B. claussenii OYL-201	commercial yeast	Omega Labs
D. anomala Y-1414	isolated from lambic beer	USDA NRRL
D. anomala Y-1415	isolated from stout	USDA NRRL
D. anomala Y-17520	isolated from cider	USDA NRRL
D. anomala Y-17521	isolated from apple	USDA NRRL
D. anomala Y-17522	isolated from soft drink	USDA NRRL
D. anomala YB-4241	isolated from Australian beer	USDA NRRL
B. bruxellensis Y-1411	isolated from lambic beer	USDA NRRL
K. lactis B9-0069	isolated from cheese	Wiedmann Lab

30-day Fermentation

A fermentation of *B. claussenii* OYL-201 was carried out to further understand acetic acid production from lactose. A starter of OYL-201 was inoculated into 200 ml of YNBaa-4%L buffered to pH 4.4 at a concentration of $3x10^6$ CFU/mL. The fermentation was incubated aerobically (200 rpm) at 30°C for 30 days. Samples were taken every two days in duplicate for lactose, acetic acid, ethanol, and microbiological analysis. Control flasks spiked with known amounts of acetic acid and lactose were included and sampled at appropriate conditions to be used to normalize the experimental values for evaporation. The experiment was repeated in 3 biological replicates.

Factor Screen

A two-level fraction factorial design (2^{6-1}) was used to evaluate six factors: X_1: Temperature of Incubation (°C), X_2: Agitation Level (rpm), X_3: Yeast Inoculation Level (Log CFU/mL), X_4: Initial Lactose Concentration (g/L), X_5: Initial pH, and X_6: Time of Fermentation (days). This design resulted in 32 total combinations. The factor and settings can be seen in Table 2. Media was prepared according to the appropriate pH and lactose concentration (YNBaa-4%L, pH 4.1; YNBaa-10%L, pH 4.1; YNBaa-4%L, pH 6.5; YNBaa-10%L, pH 6.5). Starters of each yeast strain were inoculated into 50 ml of the appropriate YNBaa-L at the appropriate concentration for each combination. Flasks were incubated at 25 or 35°C with the appropriate shaking (50 or 200 rpm) for 2 or 10 days with samples taken on the final day in duplicate for lactose, acetic acid, and ethanol analysis. Control flasks spiked with a known amounts of acetic acid, lactose, and ethanol were included and sampled at appropriate conditions to be used to normalize the experimental values for evaporation.

Table 2. Factors with their low (-1) and high (+1) settings for 2^{6-1} fraction factorial design.

Factor											
X1 (°C)		X2 (rpm)		X3 (Log CFU/mL)		X4 (g/L)		X5 (pH)		X6 (days)	
-1	+1	-1	+1	-1	+1	-1	+1	-1	+1	-1	+1
25	35	50	200	5	8	40	100	4.1	6.5	2	10

Optimization

Significant factors chosen from the factor screen were further evaluated and optimized using response surface methodology. Each factor (X_1: Agitation (rpm), X_2: Yeast Inoculation Level (Log CFU/mL), and X_3: Initial pH) was set to three levels and three center points were added. Each factor and settings can be seen in Table 3. The design of 17 combinations was repeated in three biological replicates, giving a total of 51 experiments. Media was prepared according to the

appropriate pH at 7% lactose concentration (YNBaa-7%L, pH 4.1; YNBaa-7%L, pH 5.3; YNBaa-7%L, pH 6.5). Starters of each yeast strain were inoculated into 50 ml of the appropriate YNBaa-L at the appropriate concentration for each combination. Flasks were incubated at 25°C with the appropriate shaking (50 or 200 rpm) for 14 days with samples taken on the final day in duplicate for lactose, acetic acid, and ethanol analysis. Control flasks spiked with a known amounts of acetic acid and lactose were included and sampled at appropriate conditions to be used to normalize the experimental values for evaporation. The experiment was repeated in two biological replicates.

Table 3. Factors with their low (-1), middle (0), and high (+1) settings for central composite design.

Factor								
X1 (rpm)			X2 (Log CFU/mL)			X3 (pH)		
-1	0	+1	-1	0	+1	-1	0	+1
50	125	200	5	6.5	8	4.1	5.3	6.5

Sampling and analysis

Samples for sugar, acid, and ethanol analysis were centrifuged (4,000 rpm, 4 min) at time of collection and supernatant was removed and sent to the Cornell Craft Beverage Analytical Laboratory (Geneva, NY) for HPLC analysis. HPLC was performed with a Prominence HPLC system (Shimadzu, Kyoto, Japan) equipped with a 300 x 7.8 mm Rezex™ ROA-Organic Acid H+ Column (Phenomenex, Torrance, CA), a Photodiode Array Detector, model SPD-M20A (Shimadzu, Kyoto, Japan), and a Refractive Index Detector, model RID-10A (Shimadzu, Kyoto, Japan). A mobile solution of 0.005 N H2SO4 was used and 20uL of sample that had been filtered through a 0.2 micron filter was injected at a flow rate of 0.5 mL/min. The limits of detection are as follows: lactose 0.003 g/L, glucose 0.004 g/L, galactose 0.005 g/L, acetic acid 0.001 g/L, and ethanol 0.009 % v/v. Results that fell below these detection limits are indicated as “nd” in table

format or as zero for graphical and statistical analysis. For microbiological analysis of certain time points, samples were serial diluted and spread plated on PDA. The pH of samples was measured using an iCinac.

Calculations

Assuming the following carbon balance equation [32], the ratio of moles lactose to moles acetic acid produced was assumed to be 1 mole lactose: 4 moles acetic acid.

$$eq.1\ C_{12}H_{22}O_{11}\ (lactose) \rightarrow\ C_6H_{12}O_6\ (glucose) + C_6H_{12}O_6\ (galactose)$$

$$eq.2\ 2C_6H_{12}O_6 \rightarrow 4C_2H_4O_2(acetic\ acid) + 4CO_2\ [+ 4NADH + 4NAD(P)H]$$

Assuming this ratio, the maximum achievable acetic acid was calculated to be 0.7017 g acetic acid / g lactose. For the experimental values, the maximum achievable acetic acid was calculated as follows:

$$eq.3\ Maximum\ Achievable\ Acetic\ Acid\ \left({}^{g}/_{L}\right) = [Sugar]_0\ \times\ Theoretical\ Yield$$

To determine the yield of the fermentations, the following equation was used:

$$eq.4\ Yield\ \left({}^{g}/_{g}\right) = \frac{Acetic\ acid\ produced\ (g)}{Initial\ lactose\ (g)}$$

Additionally, to determine the efficiency of the fermentations, the following equation was used:

$$eq.5\ Efficiency\ (\%) = \left(\frac{Acetic\ acid\ produced\ (g)}{Lactose\ consumed\ (g)} \div 0.7017\right) \times 100$$

5.4 Results & Discussion

Strain Evaluation

Seven strains of *B. claussenii*/*D. anomala* were evaluated for their potential to produce acetic acid from lactose (Figure 1). All strains except for one (YB-4241) appeared to utilize lactose during the aerobic fermentations. Upon further investigation, it is not clear whether strain YB-4241 is *D. anomala*. It is indicated as so in the ARS NRRL catalogue, however, in a 2002

publication from the ARS, this strain is referred to as *D. intermedia*, a synonym for *B. bruxellensis*. This strain is also identified as ATCC 56865, CBS 1947, and IFO 1591. On the ATCC website, strain 56865 is listed as *B. bruxellensis*. However, in *The Yeasts: A Taxonomic Study*, this strain is identified as *D. anomala*, originally being classified as *Torulopsis cylindrica* (Maudy). Further genome analysis of this strain should be investigated to determine species

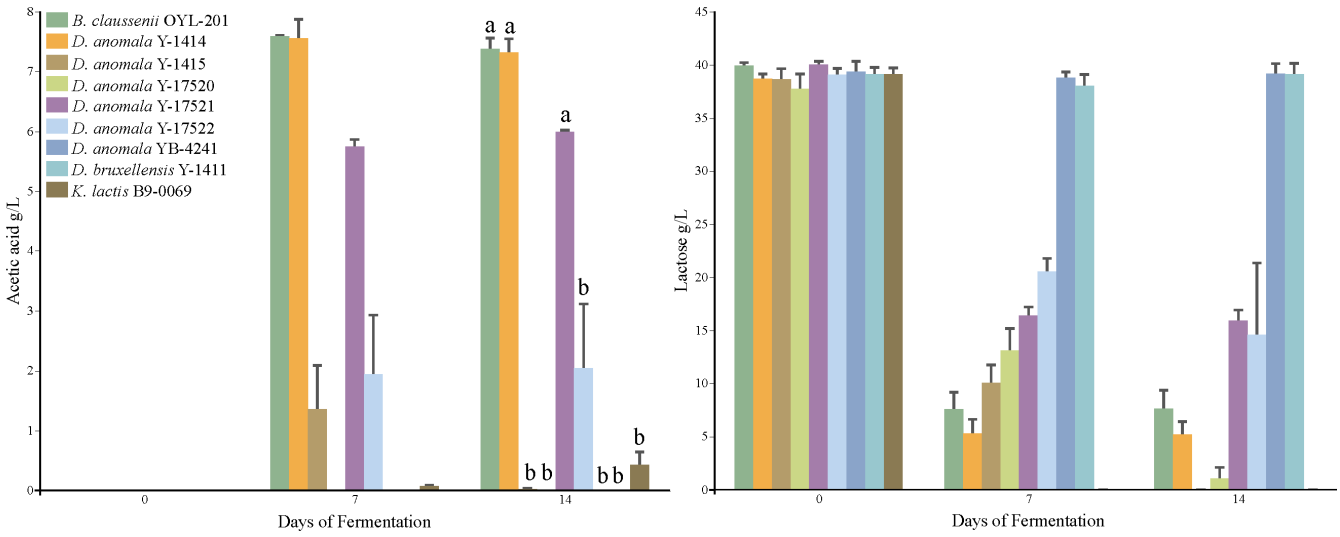

Figure 1. Acetic acid production and lactose consumption (a) and ethanol production (b) by various strains of yeast. Error bars indicate standard deviation of 3 biological replicates. Day 14 acetic acid bars not connected by the same letter are significantly different ($p<0.05$).

Of the seven strains, only five produced acetic acid. When analyzing the final amount of acetic acid produced at the end of the fermentation (Day 14), three strains, OYL-201, Y-1414, and Y-17521, produced the most acetic acid, 7.38±0.26 g/L, 7.31±0.4 g/L, and 5.99±0.04 g/L respectively. These three strains utilized approximately 60%, 87%, and 61% of the lactose respectively. When considering the yield (Table 4) of acetic acid (g of acetic acid produced per g of lactose) strains Y-1414, OYL-201, and Y-17521 also had the highest yields of 0.19±0.01 g/g, 0.18±0.01 g/g, and 0.15±0.00 g/g respectively. This yield resulted in an efficiency (Table 4) of

31.17±1.83 %, 32.61±0.89 %, and 35.53±2.5 % respectively for each strain. Interestingly, strain Y-1415, appeared to consume the acetic acid that it had produced between Day 7 and Day 14. This strain of *D. anomala* was also the only strain that completely utilized all the lactose by Day 14. Genomic analysis of these strains using PCR and Sanger sequencing, confirmed ITS (ITS4 and ITS5 primers) identity of each strain as *B. claussenii*/*D. anomala*, however, probing of a putative beta-galactosidase throughout each strain, showed absence in YB-4241 (Lawton, in preparation). The inability of strain YB-4241 to use lactose and the absence of this gene points towards its identity as being *B. bruxellensis*. Nevertheless, as stated earlier, further genomic analysis of this strain should be investigated. Phenotypic and genotypic variation among strains of *Brettanomyces* spp. is common as has been previously studied through genomic and metabolic testing [33–35].

Table 4. Product yield and fermentation efficiency for yeast strains at Day 14.

Strain	Yield (g/g)	Efficiency (%)
B. claussenii OYL-201	0.18±0.01a	32.61±0.89a
D. anomala Y-1414	0.19±0.01a	31.17±1.83a
D. anomala Y-1415	0.00±0.00b	0.08±0.13b
D. anomala Y-17520	0.00±0.00b	0.00±0.00b
D. anomala Y-17521	0.15±0.00a	35.52±2.5a
D. anomala Y-17522	0.05±0.05b	16.65±15.14ab
D. anomala YB-4241	0.00±0.00b	0.00±0.00b
D. bruxellensis Y-1411	0.00±0.00b	0.00±0.00b
K. lactis B9-0069	0.01±0.01b	1.55±1.33b

Values represent mean of 3 replicates ± standard deviation.
[a]Yield is defined as g acetic acid produced/g initial lactose
[b]Efficiency is defined as g of acetic acid produced/theoretical yield x 100%

Analysis of the pH of fermentations on Day 14 (Figure 2) revealed a significant drop in pH of fermentation medium with strains that produced acetic acid. The top acetic acid producers resulted in pH levels of 2.7±0.03 (OYL-201), 2.68±0.03 (Y-1414), and 2.39±0.04 (Y-17521) at

Day 14. The largest pH drop was seen by strain Y-17520 at pH 2.03±0.03 which is interesting since this strain did not display any acetic acid production. Although *Brettanomyces* has a high tolerance to acid and low pH [20,28], this acidification could have resulted in slower growth, reduced acetic acid production, and an inability for the yeast to completely consume the carbon source.

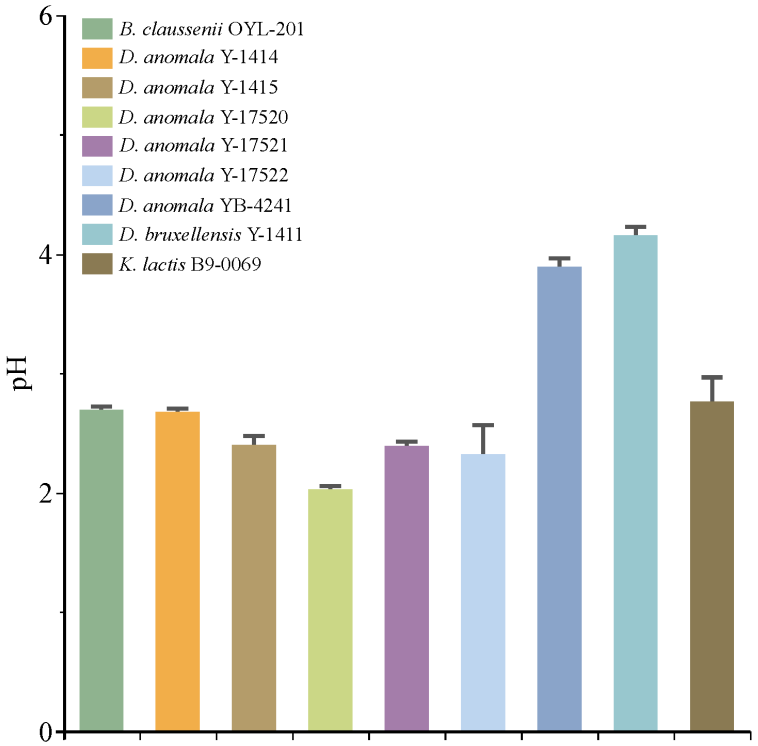

Figure 2. pH profile for Day 14 of aerobic fermentation of various yeast strains. Error bars represent ± standard deviation of 3 biological replicates.

Considering these acetic acid production results, *B. claussenii* OYL-201 was chosen to move forward with for further experiments as it was statistically grouped with strain Y-1414 and Y-17521 for final amount of acetic acid, acetic acid yield, and efficiency. Additionally, this is our laboratory strain and has been used for other experiments within our group.

30-day Fermentation

To better understand how *B. claussenii* behaves in lactose fermentations, a 30-day fermentation was set up with samples taken every 2 days (Figure 3). Thirty days was chosen as previously all the lactose had not been utilized as seen in the strain evaluation experiment. Additionally, during this experiment, a buffered media was used due to observance of the pH falling below pH 3 by Day 14 in a non-buffered media. It was hypothesized that this could be a limiting factor for yeast growth and complete carbon utilization. From this experiment, it was seen that all the lactose was completely utilized by Day 4. Acetic acid production began at Day 2 and plateaued by Day 6 at 9.28±0.87 g/L. Ethanol was also analyzed during this experiment and at Day 4, the ethanol concentration peaked at 1.57±0.06 % v/v but quickly decreased throughout the rest of the sampling period, reaching below 0.5% after Day 14. For beverage production, this is important as non-alcoholic beverages must be below 0.5 % v/v due to labeling restrictions. The decrease in ethanol concentration is most likely due to evaporation. During a separate experiment (Factor Screen), an uninoculated control containing 1 %v/v ethanol was sampled at Day 0 and after 2 days of incubation. By Day 2, 31.84±11.01% of the ethanol had been lost to evaporation. *B. claussenii* has been shown to utilize ethanol as a carbon source and produce acetic acid [22,23]. However, from the ethanol measurements throughout this 30-day experiment, there appeared to be an initial 35.05±2.22% loss from Day 4 to Day 6 and a roughly 9-25% loss between every time point thereafter. The loss calculated here was consistent with the uninoculated control, indicating that drop in ethanol concentration was most likely due to evaporation. Additionally, after the lactose was utilized, there did not appear to be an additional increase in acetic acid.

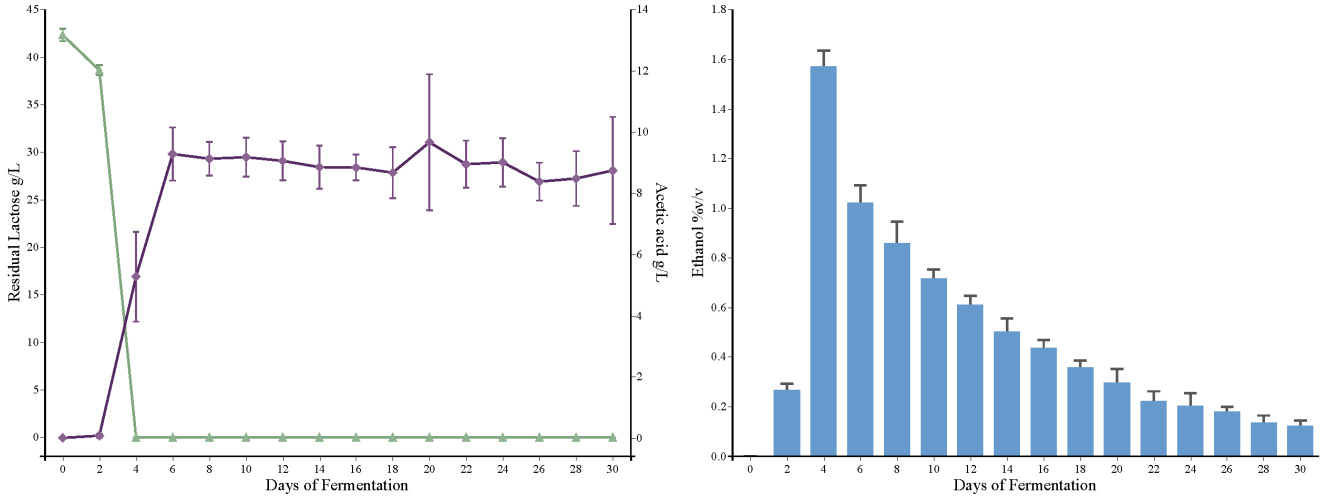

Figure 3. Residual lactose (▲), acetic acid (◆), and ethanol production by B. claussenii OYL-201 during a 30-day fermentation of synthetic lactose media. Error bars represent ± standard deviation of 3 biological replicates.

Considering the amount of acetic acid produced on Day 6, the yield was calculated to be 0.22±0.02 g of acetic acid produced per g of lactose. This yield is comparable to acetic acid yields obtained for aerobic fermentations of *B. claussenii* with glucose media [22,23]. The efficiency of acetic acid production during this fermentation was calculated to be 31.23±2.63 %. Considering that some ethanol was also produced, the efficiency for ethanol production from lactose used (based on the maximum amount of ethanol produced at Day 4) was calculated to be 54.49±2.67% (theoretical yield of ethanol from lactose is 0.538 g/g). The combined efficiency considering the maximum concentration for each of the two products was calculated to be 85.72±0.01%. Considering that all the lactose had been utilized, this efficiency corresponds well with similar known biological processes in *S. cerevisiae*, where ~15% of carbon use can be assumed for biomass and respiration [32]. From these results, a basis of understanding for *B. claussenii* behavior in lactose fermentations was established.

Factor Screen

According to the literature, *B. claussenii* has a broad growth range and production of acetic acid has only been evaluated for other carbon sources such as glucose. To optimize acetic acid production from lactose, statistical design of experiments was used to identify important fermentation factors and understand how they influence the production of acetic acid. A two-level fraction factorial design (2^{6-1}) was used to evaluate six factors: X_1: Temperature of Incubation (°C), X_2: Agitation Level (rpm), X_3: Yeast Inoculation Level (Log CFU/mL), X_4: Initial Lactose Concentration (g/L), X_5: Initial pH, and X_6: Time of Fermentation (days). A high (+1) and low (-1) level (Table x) were chosen for each factor by considering the growth range of *B. claussenii* in various conditions and identifying optimum fermentation settings studied in the literature. Initial lactose concentrations of 100 g/L (+1) and 40 g/L (-1) were chosen based on the range of lactose concentrations in dairy by-products such as acid whey and whey permeate [5,36,37]. The pH range was chosen based on these dairy by-products as well. The full design and responses can be seen in Table 3. Using acetic acid production, the yield was calculated to use as a response (Table 5).

Several main effects and interactions were identified as significant for acetic acid yield or ethanol concentration set as the response of the model. A model maximizing acetic acid yield as the response (Table 6) presented the main effect of Initial pH ($p<0.05$) as significant. Additionally, the 2-way interactions of Temperature and Agitation ($p<0.05$), Temperature and Yeast Inoculation Level ($p<0.01$), Temperature and Time ($p<0.01$), Agitation and Yeast Inoculation Level ($p<0.05$), and Agitation and Initial pH ($p<0.05$) were considered significant in this model. When considering the model for minimizing ethanol as the response (Table 7), the main effects Yeast Inoculation Level ($p<0.01$) and Initial pH ($p<0.05$) were determined as significant. The 2-way interaction of Agitation and Time ($p<0.05$) was also determined as

Table 5. Fraction factorial design, response values, and calculated yield and efficiency.

Run	X1	X2	X3	X4	X5	X6	Lactose (Residual g/L)	Acetic acid (g/L)	Ethanol (%)	Yield[a] (g/g)	Efficiency[b] (%)
1	25	50	8	40	4	10	0.00	1.30	1.57	0.04	5.35
2	25	200	5	40	4	2	37.57	0.00	0.00	0.00	0.00
3	25	200	8	100	6.5	10	49.57	12.60	0.82	0.12	32.03
4	25	200	8	40	6.5	2	36.34	0.00	0.00	0.00	0.00
5	25	50	5	100	4	2	94.20	0.00	0.00	0.00	0.00
6	25	50	8	100	4	2	103.94	0.48	0.28	0.01	0.00
7	25	200	5	100	6.5	10	80.15	4.11	0.32	0.04	22.98
8	25	50	5	40	6.5	10	33.40	0.27	0.16	0.01	3.44
9	35	50	8	40	6.5	2	41.35	0.28	0.00	0.01	0.00
10	35	200	8	40	4	2	21.97	1.22	0.71	0.04	21.34
11	35	50	5	40	4	10	25.28	0.00	0.00	0.00	0.00
12	35	50	8	100	6.5	10	75.19	0.90	0.36	0.01	4.75
13	35	200	8	40	4	10	14.63	1.37	0.23	0.05	12.59
14	35	200	5	100	6.5	2	107.84	0.00	0.00	0.00	0.00
15	35	50	5	100	4	10	90.76	0.00	0.10	0.00	0.00
16	35	200	5	100	6.5	2	84.95	0.00	0.00	0.00	0.00
17	25	50	8	40	6.5	2	34.79	0.17	0.00	0.00	2.68
18	25	200	5	100	4	10	50.08	9.99	0.77	0.10	29.43
19	25	200	8	40	4	10	7.01	11.41	0.30	0.28	48.16
20	25	200	8	100	6.5	10	47.14	12.15	0.88	0.12	30.26
21	25	50	8	100	6.5	2	104.35	0.23	0.00	0.00	0.00
22	25	200	5	40	6.5	10	28.76	0.69	0.23	0.02	6.45
23	25	50	5	100	4	2	97.70	0.00	0.00	0.00	0.00
24	25	50	5	40	4	2	37.12	0.00	0.00	0.00	0.00
25	35	200	8	100	4	10	63.24	1.86	0.12	0.02	8.09
26	35	200	5	100	4	2	91.26	0.00	0.00	0.00	0.00
27	35	50	5	40	6.5	10	39.12	0.00	0.00	0.00	0.00
28	35	200	8	40	4	2	18.05	2.24	0.67	0.06	17.21
29	35	50	8	100	4	10	52.24	0.81	1.45	0.01	2.63
30	35	50	8	100	6.5	2	94.95	0.29	0.00	0.00	8.75
31	35	200	5	40	6.5	2	38.70	0.00	0.00	0.00	0.00
32	35	50	5	40	6.5	10	40.80	0.00	0.00	0.00	0.00

[a]Yield is defined as g acetic acid produced/g initial lactose
[b]Efficiency is defined as g of acetic acid produced/theoretical yield × 100%

significant for the ethanol model. The combination resulting in the highest acetic acid concentration (12.6 g/L), Run 3, had an acetic acid yield of 0.12 g/g and an efficiency of 32.03%.

However, the combination resulting in the highest acetic acid yield (0.28 g/g), Run 19, had an efficiency of 48.16% even though only 11.41 g/L of acetic acid was produced. These combinations had ethanol concentrations of 0.82% and 0.3% and lactose consumption of 53.08% and 82.82% respectively. The higher efficiency (Run 19) occurred at pH 4.1 and 40 g/L lactose as compared to Run 3 which was at pH 6.5 and had 100 g/L lactose.

Table 6. Coefficients, t values, and significance levels for 2-level fraction factorial design model for maximizing acetic acid yield as response.

Term	Estimate	Std Error	t Ratio	Prob>|t|	Significance
Temperature	0.0202	0.0085	2.3837	0.0914	
Agitation	0.0056	0.0085	0.6522	0.5316	
Yeast Inoculation Level	-0.0010	0.0078	-0.1318	0.8980	
Initial Lactose	-0.0041	0.0059	-0.6933	0.5060	
Initial pH	0.0149	0.0057	2.6031	0.0293	*
Time	-0.0040	0.0103	-0.3907	0.7046	
Temp*Agitation	-0.0251	0.0096	-2.6141	0.0279	*
Temp*Yeast	-0.0241	0.0071	-3.4003	0.0076	**
Temp*Lactose	-0.0017	0.0056	-0.3011	0.7707	
Temp*pH	0.0012	0.0075	0.1664	0.8717	
Temp*Time	-0.0291	0.0067	-4.3455	0.0019	**
Agitation*Yeast	0.0189	0.0058	3.2464	0.0112	*
Agitation*Lactose	-0.0109	0.0063	-1.7354	0.1172	
Agitation*pH	-0.0231	0.0075	-3.0813	0.0119	*
Agitation*Time	0.0170	0.0093	1.8238	0.0982	
Yeast*Lactose	-0.0055	0.0065	-0.8428	0.4192	
Yeast*pH	-0.0198	0.0094	-2.0975	0.0637	
Yeast*Time	0.0183	0.0082	2.2464	0.0517	
Lactose*pH	0.0069	0.0061	1.1299	0.2850	
Lactose*Time	0.0099	0.0065	1.5299	0.1620	
pH*Time	-0.0043	0.0061	-0.7006	0.5031	

***Statistically significant at 99.9% probability level ($p<0.001$)
**Statistically significant at 99% probability level ($p<0.01$)
*Statistically significant at 95% probability level ($p<0.05$)

Considering these results, factors were ranked based on their p-value for the models describing maximizing yield and minimizing ethanol as the response. Any main effects that were significant ($p<0.05$) were chosen for further optimization (Yeast Inoculation Level and Initial pH). Agitation

was chosen to move forward with optimization since it had a significant interaction effect with

Table 7. Coefficients, t values, and significance levels for 2-level fraction factorial design model for minimizing ethanol as response.

Term	Estimate	Std Error	t Ratio	Prob>|t|	Significance
Temperature	-0.1630	0.0947	-1.7210	0.1855	
Agitation	-0.0787	0.0798	-0.9866	0.3516	
Yeast Inoculation Level	0.2808	0.0716	3.9202	0.0037	**
Initial Lactose	0.0474	0.0554	0.8555	0.4157	
Initial pH	-0.1430	0.0536	-2.6666	0.0271	*
Time	0.1617	0.0959	1.6863	0.1259	
Temp*Agitation	-0.0251	0.0895	-0.2807	0.7855	
Temp*Yeast	0.0980	0.0651	1.5043	0.1677	
Temp*Lactose	-0.1134	0.0523	-2.1697	0.0608	
Temp*pH	-0.0138	0.0698	-0.1979	0.8479	
Temp*Time	-0.1393	0.0622	-2.2412	0.0531	
Agitation*Yeast	-0.0935	0.0546	-1.7112	0.1245	
Agitation*Lactose	0.0649	0.0584	1.1111	0.2968	
Agitation*pH	0.1176	0.0688	1.7099	0.1207	
Agitation*Time	-0.2580	0.0828	-3.1153	0.0116	*
Yeast*Lactose	-0.0232	0.0596	-0.3892	0.7058	
Yeast*pH	-0.0473	0.0876	-0.5398	0.6024	
Yeast*Time	0.0585	0.0761	0.7680	0.4631	
Lactose*pH	0.0005	0.0560	0.0097	0.9925	
Lactose*Time	0.0343	0.0602	0.5692	0.5842	
pH*Time	0.0051	0.0575	0.0883	0.9318	

***Statistically significant at 99.9% probability level ($p<0.001$)
**Statistically significant at 99% probability level ($p<0.01$)
*Statistically significant at 95% probability level ($p<0.05$)

both significant main effects. As can be seen in Table 5, most of the factor combinations still had residual lactose by the end of the fermentation time, either two or ten days. From this it was decided that the fermentation needed to go longer, and a set time of 14 days was decided for the optimization experiment. A set temperature of 25°C was chosen by using a trend profiler tool in JMP and reviewing the response for each combination. Responses for maximizing acetic acid yield and minimizing ethanol trended towards this temperature. Since initial lactose

concentration didn't appear to have any significant interaction effects, initial lactose concentration was set to the center point of the range of dairy by-products (70 g/L) to move forward with optimization.

Optimization

Significant factors chosen from the factor screen were further evaluated and optimized using response surface methodology. Each factor (X_1: Agitation (rpm), X_2: Yeast Inoculation Level (Log CFU/mL), and X_3: Initial pH) was set to three levels (-1, 0, +1) and three center point runs were added for a total of 17 combinations (Table 8). Final acetic acid concentration, final ethanol concentration, acetic acid yield, and acetic acid efficiency were evaluated as the responses for the model.

Table 8. Central composite design, measured residual lactose, acetic acid, and ethanol, response values, and predicted yield and efficiency.
Values represent mean of 3 replicates ± standard deviation.
[a]Yield is defined as g acetic acid produced/g initial lactose
[b]Efficiency is defined as g of acetic acid produced/theoretical yield x 100%

							Response		Predicted Values	
Run	X1	X2	X3	Lactose (Residual g/L)	Acetic acid (g/L)	Ethanol (%)	Yield (g/g)	Efficiency (%)	Yield (g/g)	Efficiency (%)
1	50	6.5	5.3	0.00±0.00	1.57±0.22	2.44±0.02	0.02±0.00	3.40±0.47	0.03	3.81
2	50	5	6.5	43.95±4.44	0.62±0.00	0.53±0.04	0.01±0.00	3.46±0.61	0.00	4.44
3	125	6.5	5.3	13.61±1.58	9.91±1.21	0.83±0.01	0.15±0.02	27.25±4.16	0.15	27.48
4	50	8	4.1	0.00±0.00	1.75±0.41	1.97±0.16	0.03±0.01	4.02±0.94	0.02	3.66
5	200	5	6.5	46.05±2.99	4.38±1.14	0.23±0.05	0.06±0.02	26.80±10.15	0.07	26.72
6	200	5	4.1	20.34±4.42	9.01±1.85	0.51±0.06	0.15±0.03	30.62±3.07	0.14	31.65
7	125	6.5	4.1	19.82±0.68	9.14±0.6	0.56±0.04	0.15±0.01	30.85±2.53	0.14	31.16
8	200	8	4.1	27.63±0.68	10.61±0.91	0.29±0.04	0.17±0.01	43.97±4.67	0.18	42.56
9	125	6.5	5.3	15.15±4.12	10.10±1.46	0.97±0,03	0.15±0.02	28.82±6.51	0.15	27.48
10	200	8	6.5	25.91±3.75	11.73±1.67	0.37±0.04	0.17±0.02	38.37±8.69	0.16	37.49
11	125	5	5.3	12.25±1.05	9.35±2.33	1.06±0.24	0.14±0.04	25.08±6.73	0.14	22.72
12	50	8	6.5	18.59±2.78	1.82±0.31	2.04±0.01	0.03±0.00	5.09±1.15	0.03	3.62
13	200	6.5	5.3	21.90±1.16	10.11±0.84	0.53±0.06	0.15±0.01	33.06±3.61	0.15	34.40
14	50	5	4.1	11.10±1.85	1.39±0.12	2.48±0.2	0.02±0.00	3.89±0.49	0.03	4.32

15	12 5	8	5. 3	0.00±0.0 0	10.88±1 .21	0.87±0. 12	0.17±0. 02	23.66±2. 63	0.17	27.77
16	12 5	6. 5	6. 5	32.16±1. 91	7.21±0. 34	0.21±0. 01	0.10±0. 00	27.24±0. 09	0.11	28.68
17	12 5	6. 5	5. 3	16.12±0. 02	10.01±1 .31	0.84±0. 09	0.15±0. 02	29.86±2. 36	0.15	27.48

Overall, the acetic acid concentrations for the design ranged from the lowest of 0.62±0.00 g/L to the highest of 11.73±1.67 g/L. The ethanol concentrations ranged from 0.21±0.01 % v/v reaching all the way to 2.48±0.20 % v/v. Values for yield and efficiency were calculated based on the experimental acetic acid and residual lactose data (Table 8). These values ranged from 0.01 g/g to 0.17 g/g and the efficiency ranged from 3.4±0.47% to 43.97±4.67%. were in good correlation to the predicted values from the model. All responses and predicted values from the models can be seen in Table 8.

Yield and efficiency were used as responses to fit a quadratic model. Independently, the equations of the models were as follows:

$$Yield\ \left({}^{g}/_{g}\right) = -0.138 + 0.003X_1 - 0.082X_2 + 0.116X_3 - 0.00001X_1^2 + 0.004X_2^2 - 0.015X_3^2 + 0.0001X_1X_2 - 0.0001X_1X_3 + 0.006X_3X_2$$

$$Efficiency\ (\%) = -10.196 + 0.483X_1 + 11.483X_2 - 17.101X_3 - 0.001X_1^2 - 0.993X_2^2 + 1.693X_3^2 + 0.026X_1X_2 - 0.014X_1X_3 - 0.020X_3X_2$$

The model for yield had an R^2 of 0.95 and an ANOVA test showed high significance ($p<0.0001$) that this model fit the data well and that the model terms could explain 95% of variation in the model. For maximizing yield, all model terms except for the quadratic effect of Yeast Inoculation Level (X_2^2) and the interaction effect of Agitation and pH (X_1*X_3) were significant. The model for efficiency as the response had an R^2 of 0.93 and an ANOVA test also showed high significance ($p<0.0001$) that the model fit the data well and that 93% of the

variation in the model could be accounted for by the model terms. This model had less significant parameters. Only the main effects of Agitation (X_1) and Yeast Inoculation Level (X_2) had significant p-values. The interaction effect of Agitation and Yeast Inoculation Level (X_1*X_2) and the quadratic effect of Agitation (X_1^2) were also significant.

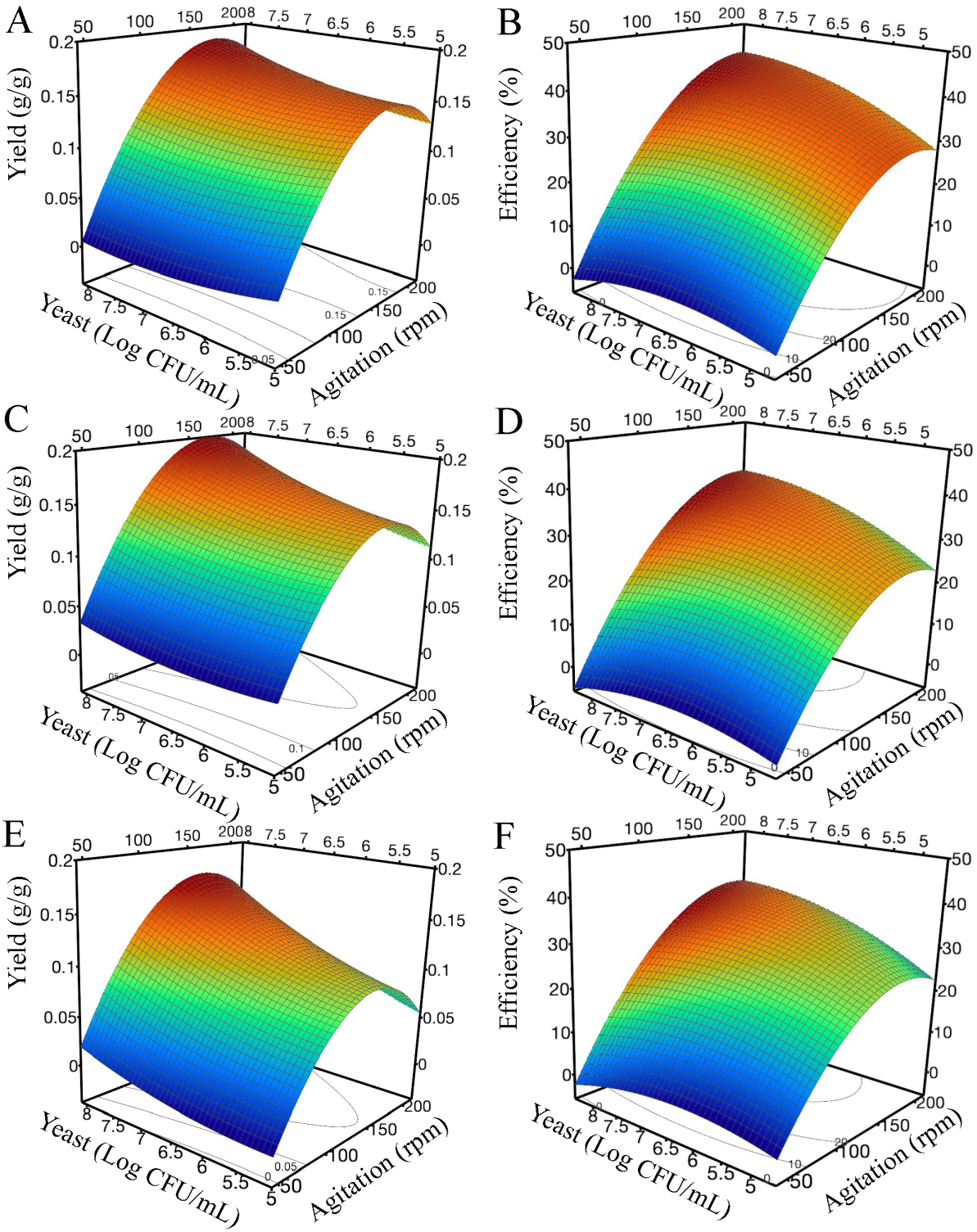

Figure 4. Response surface graphs for the model of maximizing acetic acid yield at fixed pH levels of 4.1 (a), 5.3 (c), and 6.5 (e) and for model of maximizing efficiency at fixed pH levels of 4.1 (b), 5.3 (d), and 6.5 (f).

In order to find the optimum level for each factor, both of these responses were considered individually and together, and a desirability function was used to optimize overall. The optimum factor settings to maximize yield were determined to be an agitation of 171 rpm, yeast inoculation level of 8 Log CFU/mL and a pH of 5.0. This gave a desirability of 0.98 and a predicted yield of 0.2 g/g. To maximize efficiency, the optimum factor settings were an agitation of 200 rpm, yeast inoculation level of 8 Log CFU/mL, and a pH of 4.1. This gave a desirability of 0.84 and the predicted efficiency was 42.56%. Together, the optimum factor settings were an agitation of 193 rpm, yeast inoculation level of 8 Log CFU/mL, and a pH of 4.1 (desirability = 0.87). This calculated a predicted yield of 0.18 g/g and a predicted efficiency of 42.23%. Additionally, the factors were optimized using the combined models to consider pH levels of various dairy by-products, setting the pH to the fixed factor levels of 4.1, 5.3, and 6.5. At a fixed pH of 5.3, the optimum setting for agitation was determined to be 185 rpm and yeast inoculation level was 8 Log CFU/mL (desirability = 0.84, predicted yield = 0.20 g/g, predicted efficiency = 36.95%). At a fixed pH of 6.5, the optimum agitation setting was 181 rpm and the yeast inoculation level was 8 Log CFU/mL (desirability = 0.78, predicted yield = 0.17 g/g, predicted efficiency = 36.9%). The response surface graph for each model separately at each pH level can be seen in Figure 4.

Overall, agitation had a significant effect on yield and efficiency. Higher agitation, which results in more oxygenation of the fermentation media, shifts yeast in aerobic fermentation to produce a higher ratio of acetic acid over ethanol. The optimum settings to maximize efficiency

and shift yeast towards acetic acid production, trended towards the upper limit of the design space (200 rpm). Seeing this, further oxygenation may result in increased yeast efficiency and acetic acid. Scaling up and providing oxygen through aeration may have a better impact than further increasing agitation. Changing the pH settings to simulate dairy by-products did not have a large influence on the other factor settings. Additionally, yeast inoculation level had more a linear relationship to response, increased concentration resulted in higher yields and efficiency. Considering the optimized factor settings, a validation study will be performed to confirm the model. Validation at the same scale and using the base medium of YNBaa-L will be performed for each pH setting. Upon confirmation, scale up will include performing the fermentations at the appropriate settings in actual dairy by-products (acid whey, milk permeate, and whey permeate).

5.5 Conclusions

Overall, these findings demonstrate valuable information for the valorization of dairy waste streams. From scale up of lactose fermentations with the yeast *B. clausseni*, positive impacts can be had on the dairy industry through expansion of the market with development of value-added beverages. Additionally, an understanding the kinetics and thermostability and pH tolerance of the specific enzyme responsible for lactose metabolism in *B. clausseni* could allow for further optimization of fermentations. Understanding how *B. claussenii* performs in lactose to acetic acid fermentations presents new opportunities and venues for dairy by-products.

REFERENCES

1. Erickson BE: **Acid whey: Is the waste product an untapped goldmine**. *Chem Eng News* 2017, **95**:26–30.
2. Domingues L, Dantas MM, Lima N, Teixeira JA: **Continuous ethanol fermentation of lactose by a recombinant flocculating Saccharomyces cerevisiae strain**. 1999,
3. Mawson AJ: **Bioconversions for whey utilization and waste abatement**. *Bioresour Technol* 1994, **47**:195–203.
4. Prazeres AR, Carvalho F, Rivas J: **Cheese whey management: A review**. *J Environ Manage* 2012, **110**:48–68.
5. Menchik P, Zuber T, Zuber A, Moraru CI: **Composition of coproduct streams from dairy processing: Acid whey and milk permeate**. *J Dairy Sci* 2019, **102**:3978–3984.
6. Sloan E: **Top 10 Food Trends of 2021**. *Food Technol Mag* 2021, **75**.
7. Villarreal-Soto SA, Beaufort S, Bouajila J, Souchard J, Taillandier P: **Understanding kombucha tea fermentation: a review**. *J Food Sci* 2018, **83**:580–588.
8. Jayabalan R, Malbaša R V, Lončar ES, Vitas JS, Sathishkumar M: **A review on kombucha tea—microbiology, composition, fermentation, beneficial effects, toxicity, and tea fungus**. *Compr Rev Food Sci Food Saf* 2014, **13**:538–550.
9. Kumar V, Joshi VK: **Kombucha : Technology, Microbiology, Production, Composition and Therapeutic Value** . *Int J Food Ferment Technol* 2016, **6**:13.
10. Jelen P: **Whey Processing: Utilization and Products**. *Encycl Dairy Sci Second Ed* 2011, doi:10.1016/B978-0-12-374407-4.00495-7.
11. Holsinger VH, Posati LP, DeVilbiss ED: **Whey beverages: a review**. *J Dairy Sci* 1974, **57**:849–859.
12. Domingues L, Guimarães PMR, Oliveira C: **Metabolic engineering of Saccharomyces cerevisiae for lactose/whey fermentation. Bioeng Bugs 1: 164–171**. 2010,
13. Guimarães PMR, Teixeira JA, Domingues L: **Fermentation of lactose to bio-ethanol by yeasts as part of integrated solutions for the valorisation of cheese whey**. *Biotechnol Adv* 2010, **28**:375–384.
14. O'leary VS, Sutton C, Bencivengo M, Sullivan B, Holsinger VH: **Influence of lactose hydrolysis and solids concentration on alcohol production by yeast in acid whey ultrafiltrate**. *Biotechnol Bioeng* 1977, **19**:1689–1702.
15. Tonsmeire M: *American sour beer: Innovative techniques for mixed fermentations*. Brewers Publications; 2014.
16. Claussen NH: **On a method for the application of Hansen's pure yeast system in the manufacturing of well-conditioned English stock beers**. *J Inst Brew* 1904, **10**:308–331.
17. Steensels J, Verstrepen KJ: **Taming wild yeast: potential of conventional and nonconventional yeasts in industrial fermentations**. *Annu Rev Microbiol* 2014, **68**:61–80.
18. de W Blackburn C: *Food spoilage microorganisms*. Woodhead Publishing; 2006.
19. Kurtzman C, Fell JW, Boekhout T: *The yeasts: a taxonomic study*. Elsevier; 2011.

20. Steensels J, Daenen L, Malcorps P, Derdelinckx G, Verachtert H, Verstrepen KJ: **Brettanomyces yeasts - From spoilage organisms to valuable contributors to industrial fermentations**. *Int J Food Microbiol* 2015, **206**:24–38.
21. Castro-Martinez, C.; Escudero-Abarca, B.I.; Gomez Rodriguez, J.; Hayward-Jones, P.M.; Aguilar-Uscanga MG: **Production in Brettanomyces Strains**. *J Food Process Eng* 2005, **28**:133–143.
22. Freer SN, Dien B, Matsuda S: **Production of acetic acid by Dekkera/Brettanomyces yeasts under conditions of constant pH**. *World J Microbiol Biotechnol* 2003, **19**:101–105.
23. Freer SN: **Acetic acid production by Dekkera/Brettanomyces yeasts**. *World J Microbiol Biotechnol* 2002, **18**:271–275.
24. Jayabalan R, Marimuthu S, Thangaraj P, Sathishkumar M, Binupriya AR, Swaminathan K, Yun SE: **Preservation of Kombucha Tea Effect of Temperature on Tea Components and Free Radical Scavenging Properties**. *J Agric Food Chem* 2008, **56**:9064–9071.
25. Blanc PJ: **Characterization of the tea fungus metabolites**. *Biotechnol Lett* 1996, **18**:139–142.
26. Chen C, Liu BY: **Changes in major components of tea fungus metabolites during prolonged fermentation**. *J Appl Microbiol* 2000, **89**:834–839.
27. Blomqvist J, Passoth V: **Dekkera bruxellensis-spoilage yeast with biotechnological potential, and a model for yeast evolution, physiology and competitiveness**. *FEMS Yeast Res* 2015, **15**:1–9.
28. Lawton MR, deRiancho DL, Alcaine SD: **Lactose utilization by Brettanomyces claussenii expands potential for valorization of dairy by-products to functional beverages through fermentation**. *Curr Opin Food Sci* 2021,
29. Beigbeder J-B, Dantas JM de M, Lavoie J-M: **Optimization of Yeast, Sugar and Nutrient Concentrations for High Ethanol Production Rate Using Industrial Sugar Beet Molasses and Response Surface Methodology**. *Fermentation* 2021, **7**:86.
30. Sarchami T, Johnson E, Rehmann L: **Optimization of fermentation condition favoring butanol production from glycerol by Clostridium pasteurianum DSM 525**. *Bioresour Technol* 2016, **208**:73–80.
31. El-Gendy NS, Madian HR, Amr SSA: **Erratum: Design and optimization of a process for sugarcane molasses fermentation by Saccharomyces cerevisiae using response surface methodology (International Journal of Microbiology)**. *Int J Microbiol* 2014, **2014**.
32. Waterhouse AL, Sacks GL, Jeffery DW: *Understanding wine chemistry*. John Wiley & Sons; 2016.
33. Crauwels S, Van Assche A, de Jonge R, Borneman AR, Verreth C, Troels P, De Samblanx G, Marchal K, Van de Peer Y, Willems KA, et al.: **Comparative phenomics and targeted use of genomics reveals variation in carbon and nitrogen assimilation among different Brettanomyces bruxellensis strains**. *Appl Microbiol Biotechnol* 2015, **99**:9123–9134.
34. Crauwels S, Zhu B, Steensels J, Busschaert P, De Samblanx G, Marchal K, Willems KA, Verstrepen KJ, Lievens B: **Assessing genetic diversity among Brettanomyces yeasts by DNA fingerprinting and whole-genome sequencing**. *Appl Environ Microbiol* 2014, **80**:4398–4413.

35. Conterno L, Joseph CML, Arvik TJ, Henick-Kling T, Bisson LF: **Genetic and physiological characterization of Brettanomyces bruxellensis strains isolated from wines**. *Am J Enol Vitic* 2006, **57**:139–147.
36. Marcus JF, DeMarsh TA, Alcaine SD: **Upcycling of Whey Permeate through Yeast- and Mold-Driven Fermentations under Anoxic and Oxic Conditions**. *Fermentation* 2021, **7**:16.
37. Beucler J, Drake M, Foegeding EA: **Design of a beverage from whey permeate**. *J Food Sci* 2005, **70**:S277–S285.

CHAPTER 6

Exploration of lactose metabolism genes within *Brettanomyces claussenii*: a bioinformatic and molecular biology approach

6.1 Introduction

Dairy by-products contribute to a large volume of waste. The upcycling of their residual nutrients has been a topic widely studied [1]. Opportunities to transform the lactose in these waste streams into value-added goods has attracted the attention of researchers studying fermentation [2,3]. Conversion of lactose to value-added end products (i.e., ethanol and acetic acid) through fermentation requires the use of a yeast with innate lactose hydrolysis activity or through an initial enzymatic hydrolysis step.

Production of fermented beverages, alcoholic and non-alcoholic, stems from a variety of microorganisms. Traditionally, *S. cerevisiae* has been domesticated to produce ethanol-containing products such as beer. Other fermented beverages such as kombucha or kefir, contain a community of various microorganisms [4]. In terms of fermented dairy beverages and products, such as kefir and yogurt, the breakdown of lactose into fermentable sugars is essential for fermentation. This conversion into simpler sugars is typically achieved by β-galactosidase (lactase); an enzyme capable of cleaving lactose, a β-glucoside, into glucose and galactose. This enzyme is available commercially, commonly produced from microbial sources such as *Kluyveromyces* or *Aspergillus* species. Functionality of β-galactosidase is typically dependent on the environment in which it is expected to perform. Low pH substrates often benefit from an enzyme derived from bacterial origin while more neutral substrates require the use of yeast or fungal enzymes [5].

In the context of sustainable food systems, the potential for β-galactosidase activity to "upcycle" food waste in the dairy industry is an exciting opportunity to develop more sustainable systems. Fermentation of lactose substrates can occur through direct use of these β-galactosidase containing yeasts. For example, the yeast *Kluyveromyces* has gained a share of research interest for its capacity to produce ethanol and bioethanol from lactose-containing waste streams such as whey [3,6,7]. For organisms lacking β-galactosidase activity (i.e., *S. cerevisiae*); genetic modification would be required to include lactose in their fermentation capacities [2].

One such yeast in which its ability to utilize lactose has been relatively understudied is *Brettanomyces claussenii*. The mechanism behind the ability of *B. claussenii* to hydrolyze lactose is unknown however it is classified as being able to consume and ferment the sugar [8]. The genome of *Brettanomyces* is relatively understudied, with *B. bruxellensis* being the most prominent in the literature. Studies have identified a putative β-galactosidase within the genome of *B. bruxellensis* [9–12], yet no such gene has been identified within the genome of *B. claussenii*. Further analysis of the *Brettanomyces* genome is needed to understand the mechanism of lactose utilization. This ability is divergent from the rest of the genus as well as from other closely related organisms [8]. The most closely related microorganism with the ability to utilize lactose is *Kluyveromyces*. As mentioned previously, this genus has several species with an identified β-galactosidase as well as a lactose transport gene that have been well characterized [13]. Identification of the lactose utilization gene(s) within *B. claussenii* will allow for a better understanding of the way in which it exploits lactose. Understanding this gene lays the groundwork for future studies to optimize strains for lactose utilization through both genetic editing and natural selection.

The objective of this study was to explore the genome of *B. claussenii* to identify a mechanism for lactose consumption. A bioinformatic approach was used to identify potential candidates. Further investigation included expression into *S. cerevisiae* in order to validate the function of identified genes. The results from this study will help characterize the lactose utilization activity of *B. claussenii* and help to understand the mechanism of its metabolism. Further analysis including comparison to other closely related organisms can help uncover the evolution of this phenotype in *B. claussenii*. Identification of new enzymes for lactose hydrolysis can expand the capabilities for utilization of dairy by-products.

6.2 Materials & Methods

Media

Powdered media was obtained from BD Difco or Sunrise Science. Media was prepared as follows unless otherwise indicated. Non-transformed (wild-type) yeast strains were cultivated on either Yeast Extract-Peptone-Dextrose (YPD - 2% bacto peptone, 1% yeast extract, 2% glucose) or Potato Dextrose (PDA – 0.4% potato extract, 2% glucose) medium. All *E. coli* cultivation was done on LB medium with ampicillin added as a selective marker (LBA). Minimal medium (0.67% Yeast Nitrogen Base (YNB) without amino acids, 2% carbon source) was prepared for a glucose carbon source (SD) and lactose carbon source (YNB-L) and used for cultivation of transformed strains and lactose growth tests. For cultivation of *B. claussenii* and *K. lactis* during comparison tests of beta-galactosidase activity, minimal medium (0.67% Yeast Nitrogen Base with amino acids (SC - 0.2%) (YNBaa), 2% carbon source) was prepared for a glucose carbon source (YNBaa-G) and lactose carbon source (YNBaa-L). When used as plates, agar was added at 20 g/L for all mediums.

Yeast Strains, Plasmids, and Primers

For PCR screen in various strains, yeast was obtained from the USDA ARS NRRL library. Specific strains and isolation sources can be seen in Table S1. For cloning gene amplification, our lab strain of *B. claussenii* (TD-0007) obtained from Omega Yeast (OYL-201) was used. Plasmids p425GPD and p416GPD [14] stored in *E. coli* (Table S2), competent *E. coli* for transformation and *S. cerevisiae* strains used for transformation (PGY453 and PGY456 – described in Table S1) were obtained from the Gibney Lab. *K. lactis* (FSL B9-0069) used for β-galactosidase activity comparison was obtained from the Wiedmann Food Safety Lab. Primers seen in Table S3 were designed using Primer3 available through Geneious software. Primers were ordered from IDT.

Bioinformatic analysis of B. claussenii genome

The genomic DNA of *B. claussenii* OYL-201 was obtained from the Gibney Lab. Four whole genomes of *B. claussenii* (GenBank: GCA_001005505.1, GCA_001754015.1, GCA_012295365.1, and GCA_018344455.1) and nucleotide sequences for β-galactosidase genes from *B. bruxellensis* (GenBank: VUG18697.1), *B. nanus* (GenBank: QPG74631.1), *K. marxianus* (GenBank: QLH93952.1), and *K. lactis* (GenBank: QLH93951.1) were obtained from the NCBI database. Using the BLAST function in Geneious, the *B. bruxellensis* β-galactosidase nucleotide sequence (GenBank: VUG18697.1) was searched against the B. *claussenii* OYL-201 genome for a match. The sequence from *B. bruxellensis* was used as this is the most closely related organism to *B. claussenii*. The region of the match was then submitted to NCBI OrfFinder to detect a coding region. The putative sequence (3,051 bp) can be seen in Table S4. The putative sequence was then searched for in the other *B. claussenii* genomes using the BLAST function in Geneious. Additionally, the translated amino acid sequence for the putative β-galactosidase was compared against the β-galactosidase protein sequences from the other

related organisms using Blosum62 alignment in the Geneious software. The same process was repeated to find a putative lactose permease in the *B. claussenii* OYL-201 genome using a reference sequence of a lactose permease from *K. marxianus* (GenBank: QGN17576.1) obtained from the NCBI database. The putative sequence (1,662 bp) can be seen in Table S4. The translated amino acid sequence was compared against amino acid sequences of lactose permeases from *B. bruxellensis* (GenBank: VUG19339.1), *B. nanus* (GenBank: QPG72701.1), *K. lactis* (GenBank: CAA30053.1), and *K. marxianus* (GenBank: QGN17576.1). All accession numbers and descriptions of sequences obtained from the NCBI database can be seen in Table S5.

Screening of yeast strains for putative β-galactosidase

Using the putative β-galactosidase (*LAC4*) and lactose permease (*LAC12*) nucleotide sequences, primers were designed to amplify an internal part of each gene within the genomic DNA of several *B. claussenii* strains to determine gene presence/absence. Primers 1 and 3 were used to amplify *LAC4* and primers 10 and 11 were used for *LAC12* (Table S3). Single colonies of each strain were selected from PDA plates and genomic DNA was purified using a LiAc/1%SDS protocol according to the method of Looke *et al.*, and described in Burns *et al.* [15,16]. The PCR reaction contained 1µl of genomic DNA, 25µl of 2X PCR Master Mix (Thermo Scientific), 1µl each of 10uM forward and reverse primer, and 22µl of water to bring the reaction volume to 50µl. The thermocycler was programmed as follows: 95°C for 3 min (1 cycle), 95°C for 30 s (35 cycles), 54°C for 30 s (35 cycles), 72°C for 90 s (35 cycles), 72°C for 5 min (1 cycle), hold at 4°C. The PCR products were visualized using gel electrophoresis and 2X GelRed as a fluorescent aid to identify if a band of the expected size formed (primers 1 & 2 – 1280 bp, primers 10 & 11 – bp). The resulting PCR product from each strain was sent for Sanger

sequencing at the Cornell Biotechnology Resource Center (BRC) using the same primers previously stated to confirm sequence.

Construction of plasmids

Using the putative gene sequences, primers were designed to amplify the entire gene from start to stop codon and add an overhang on either end corresponding to the target region of the plasmid: p425GPD for *LAC4* and p416GPD for *LAC12*. The PCR reaction contained 1µl of genomic DNA, 10µl of 5X Phusion HF buffer, 1µl of 10mM dNTPS, 2.5 ul each of 10uM forward and reverse primer, 0.5 ul of Phusion DNA polymerase and 32.5µl of water to bring the reaction volume to 50µl. The thermocycler was programmed as follows: 98°C for 30 sec (1 cycle), 98°C for 5 s (30 cycles), 70°C for 20 s (30 cycles), 72°C for 60 s (30 cycles), 72°C for 60 s (*LAC4*) or 40 s (*LAC12*) (1 cycle), 72°C for 5 min (1 cycle), hold at 4°C. Each plasmid was cut in two places with restriction enzymes SpeI and HindIII. Cut plasmids and corresponding amplified genes were then combined using Gibson assembly [17] and transformed into competent Top10 *E. coli* for storage and amplification. Several positive *E. coli* clones were picked from transformant plates (LBA) and screened for correct construct using PCR with primers specific to the β-galactosidase (Primers 1 & 2 for *LAC4*, primers 10 & 11 for *LAC12*). A confirmed positive clone was then used to amplify the plasmid. Plasmid was purified from an overnight culture of the positive *E. coli* strain using an Omega Bio-tek E.Z.N.A.® Plasmid DNA Mini kit (Omega Bio-tek Inc.). The purified product was sent for Sanger sequencing at the Cornell BRC with primers covering the entire sequence including plasmid overhangs and spaced approximately every 400-500 bp. Primers used for sequencing the p425GPD-*LAC4* construct included 1, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 28, and 29. Primers used for sequencing the p416GPD-*LAC12* construct included 15, 16, 17, 18, 19, 20, 21, 22, 23, 28, and 29. The sanger

sequencing results were cleaned and a consensus sequence for each gene was created using Geneious. The consensus sequence was then aligned with the putative *B. claussenii* OYL-201 *LAC4* and *LAC12* sequences and checked for any base pair mismatches.

Transformation of B. claussenii putative β-galactosidase into S. cerevisiae

The p425GPD-*LAC4* and p416GPD-*LAC12* constructs were transformed into *S. cerevisiae* using a protocol from the Gibney Lab. *S. cerevisiae* strains used for transformation can be seen in Table S1. Briefly, an overnight culture (YPD) was diluted 100-fold and allowed to grow for 5-6 hours. At this time the optical density was read to confirm cells were at approximately $1x10^7$ CFU/mL (OD_{600} = 0.3). Cells were then harvested through centrifugation and washed with water and subsequently LiAc solution (100 mM Lithium Acetate, 10 mM Tris, 1 mM EDTA). The cell pellet was then resuspended in the LiAc solution and carrier DNA was added. The construct DNA was added at 100-300 ng. The reaction was incubated at 30°C for 30 min with PEG solution (100 mM Lithium Acetate, 10 mM Tris, 1 mM EDTA, 40% PEG_{3350}). Then 10% DMSO was added, and the cells were heat shocked at 37°C for 15 min. Cells were pelleted and resuspended in water and immediately plated on SD agar. Plates were incubated at 30°C for 3-4 days. The experimental strain received both p425GPD-*LAC4* and p416GPD-*LAC12*. Controls consisted of creating the following transformant strains: p425GPD (empty) + p416GPD (empty), p425GPD-*LAC4* + p416GPD (empty), and p425GPD (empty) + p416GPD-*LAC12*. Additionally, non-transformed yeast and a transformation including just one plasmid was plated to confirm selective media. All transformant strains are described in Table S6. Positive transformants were picked in three replicates and grown overnight in SD broth. The overnight tube was then isolation streaked onto SD for saving.

Detection of β-galactosidase activity in transformed S. cerevisiae

Positive transformants were tested for β-galactosidase activity using the Fisher Scientific Yeast β -galactosidase Assay Kit. The assay was performed according to kit instructions for "Microcentrifuge Protocol". Test cultures were obtained by inoculating an individual colony into 5 ml SD broth and incubating for 16-24 hours. Optical densities (660 nm) of the final cultures were recorded and β -galactosidase activity was calculated using the following equation where t= time of reaction (2 min) and V= volume of culture used (0.35 ml):

$$\beta - galactosidase\ activity = \frac{1{,}000 \times A_{420}}{t \times V \times OD_{660}}$$

A_{420} values that fell below the linear range of the assay (0.02) were reported as 0. Values are reported as averages of 3 biological replicates (3 individual transformants or colonies).

Growth of transformants in lactose media

Positive transformants were tested for their ability to grow in media with lactose as the sole carbon source. An individual colony was inoculated into 5 ml of SD broth and incubated at 30°C rotating at 70 rpm for 16-24 hours. The OD_{600} of the culture was measured, and 1 ml of cells was harvested and washed one time with YNB-L. Cleaned cells were then resuspended in 1 ml of 1X PBS. The appropriate amount of resuspended culture was then inoculated into 5 ml of fresh YNB-L to achieve an OD_{600} of 0.05. Resuspended culture was also inoculated into 5 ml SD and 5 ml YNB (no sugar) for growth comparison. The new culture was incubated at 30°C with agitation for up to 6 days. The OD_{600} of the tube was measured after 48 and 144 hours against a blank of the appropriate uninoculated media.

Statistical Analysis

All experiments involving testing of transformed *S. cerevisiae* strains were repeated in 3 replicates. A Student's t-Test was used to detect differences between the treatment and control.

6.3 Results

Bioinformatic analysis of B. claussenii OYL-201 genome

Regions of similarity between a deposited nucleotide sequence of an annotated β-galactosidase (*LAC4*) from *B. bruxellensis* available on NCBI (GenBank: VUG18697.1) (Friedrich & Schacherer) and the genome sequence of *B. claussenii* OYL-201 were searched for using the tblastn function of Geneious. The search resulted in a high coverage match (98.64%) with 75.5% pairwise identity. Aligning the *LAC4* sequence of *B. bruxellensis* with the matching genome region of OYL-201 allowed for identification of the entire coding region for a putative β-galactosidase within *B. claussenii*. NCBI ORFfinder was used to help identify the coding region from start codon to stop codon. The full sequence (3,051 bp) of the putative *B. claussenii LAC4* gene can be seen in Table S4. The final nucleotide sequence was searched for similarity in the genomes of all *B. claussenii* genomes available from NCBI (GenBank: GCA_001005505.1, GCA_001754015.1, GCA_012295365.1, and GCA_018344455.1). A match resulted for all genomes except for GCA_001005505.1. Alignment of the amino acid sequences for all of these matching strains resulted in 100% pairwise identity. A single nucleotide difference was detected in strain NRRL Y-17522 however this did not result in any amino acid shift. A Geneious Blosum62 alignment of the translated *B. claussenii* OYL-201 *LAC4* sequence with existing *LAC4* amino acid sequences from *B. bruxellensis* (GenBank: VUG18697.1), *B. nanus* (GenBank: QPG74631.1), *K. lactis* (GenBank: GLH93951.1), and *K. marxianus* (GenBank: QLH9352.1) resulted in pairwise identities of 74.8%, 61.8%, 57.4%, and 57.5% respectively. A similar analysis using a reference sequence of a lactose permease from *K. marxianus* (GenBank: QGN17576.1) obtained from the NCBI database was repeated to identify regions of similarity in the *B. claussenii* OYL-201 genome to find a putative lactose permease. The search resulted in a high coverage match (79.63%) with 71.6% pairwise identity. Alignment of the matching region

with the OYL-201 genome was performed as described above to find the full sequence of the lactose permease. The *LAC12* gene was located 2,054 bp upstream of the putative *LAC4* gene. The full sequence (1,662 bp) of the putative *B. claussenii LAC12* gene can be seen in Table S4. The final *LAC12* nucleotide sequence was searched for similarity in the genomes of all *B. claussenii* genomes previously stated (GenBank: GCA_001005505.1, GCA_001754015.1, GCA_012295365.1, and GCA_018344455.1). A high coverage (100%) match resulted for all genomes except for GCA_001005505.1 (highest coverage match: 9.51%, pairwise identity: 68.9%). Alignment of the amino acid sequences for the matches in the other four strains resulted in the following pairwise identities: GCA_001754015.1 – 99.1%, GCA_012295365.1 – 100%, and GCA_018344455.1 – 99.5%. The variation in pairwise identities was a result of five amino acid changes overall. The translated *LAC12* amino acid sequence was compared against amino acid sequences of lactose permeases from *B. bruxellensis* (GenBank: VUG19339.1), *B. nanus* (GenBank: QPG72701.1), *K. lactis* (GenBank: CAA30053.1), and *K. marxianus* (GenBank: QGN17576.1), resulting in 38.9%, 71.2%, 61.2%, and 64.6% pairwise identities respectively.

Screening of yeast strains

Internal primers (Table S3) designed for the *B. claussenii* OYL-201 *LAC4* putative gene were used in a PCR screen of various *B. claussenii* strains (Table S1). Amplification with these primers resulted in a band of the expected size (1280 bp) for all but one strain (YB-4241) as can be seen in Figure S1. Interestingly, seen from previous experiments, this strain did not appear to have the ability to utilize lactose and is identified as both *D. anomala* and *B. bruxellensis* across different sources in a contradictory manner (Lawton, in preparation).

Transformation of putative β-galactosidase into S. cerevisiae

Transformation of *S. cerevisiae* with the constructs resulted in positive clones on SD plates for all expected transformations (ML1-4). ML5 did not have any colonies on SD plates indicating the suitability of this selective media.

Detection of β-galactosidase activity in transformed S. cerevisiae

As can be seen in Figure 1, all transformed *S. cerevisiae* strains that received the *LAC4* gene (ML1 and ML3), showed positive β-galactosidase activity. Strains that did not receive this gene and only received *LAC12* (ML4) or empty plasmids (ML2) did not show any β-galactosidase activity. These results confirmed the successful transformation and expression of the *LAC4* gene.

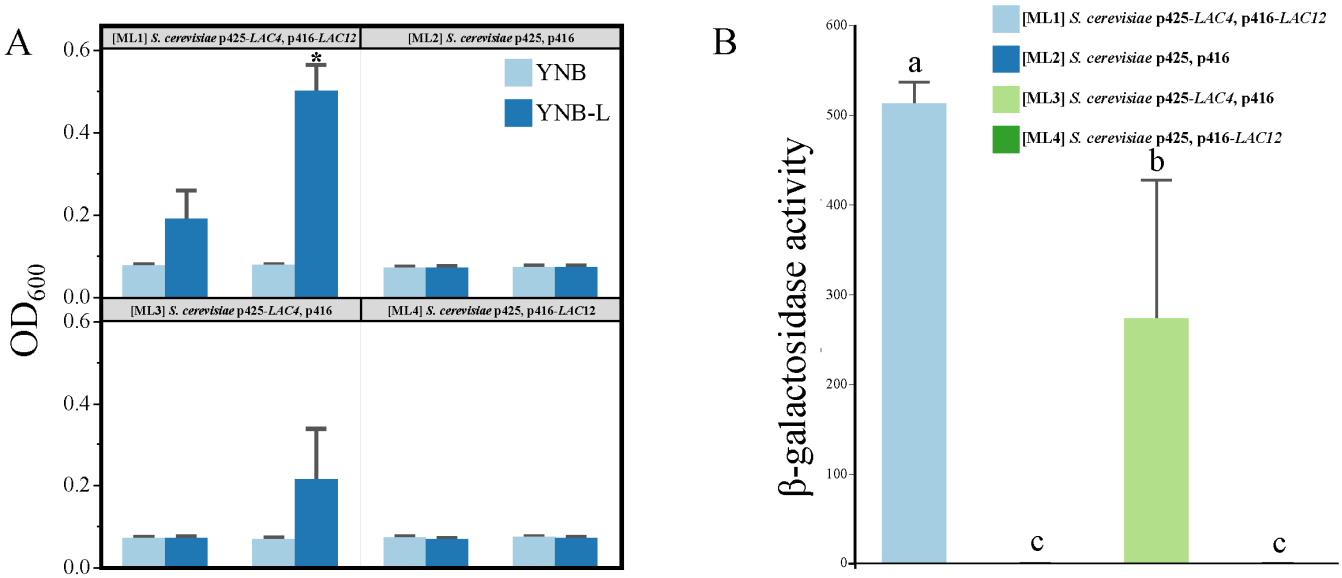

Figure 1. (a) Growth of transformed *S. cerevisiae* strains in YNB and YNB-L at 48 and 144 hours. Error bars represent standard deviation of 3 biological replicates. Bars within strains at a timepoint with a * indicate OD_{600} for media type is significantly different ($p<0.05$). (b) β-galactosidase activity of transformed *S. cerevisiae* strains as determined by assay kit. Error bars represent standard deviation of 3 biological replicates. Bars not connected by the same letter are significantly different ($p<0.05$).

Growth in lactose media

Positive transformants were tested for their ability to grow in media with lactose as the sole carbon source. The expected phenotype for each transformed *S. cerevisiae* can be seen in Table

S6. ML1, the double knockout strain that received both *LAC4* and *LAC12* was expected to grow in lactose media, confirming the activity of the identified genes. In Figure 1, ML1 had slightly higher growth in YNB-L at 48 hours than it did in YNB. At 144 hours, this growth increased and became statistically significant ($p<0.05$). Although not statistically significant ($p>0.05$), strain ML3 showed a slight increase in the YNB-L media at 144 hours. This could be due to dead cells rupturing and releasing the enzyme for hydrolysis or potentially lactose diffusion across the membrane without the use of a transport protein. However, the YNB-L 144-hour timepoint for ML1 was still higher and significantly different from ML3. This points toward the ability of this transformed strain to use lactose, due to the incorporation of *LAC4* and *LAC12*.

6.4 Discussion

Successful transformation of the putative β-galactosidase *LAC4* and lactose permease *LAC12* from *B. claussenii* into *S. cerevisiae* and detection of growth on lactose, confirms the identity of these genes responsible for lactose utilization in *B. claussenii*. Identification of these genes is a key step in the understanding of lactose metabolism in this yeast and the evolutionary progress of this phenotype.

Of the most closely related species to *B. claussenii*, *B. bruxellensis*, high similarity existed between the *LAC4* region (74.8%), however for the *LAC12* region, there was only 38.9% pairwise identity, with higher similarities occurring between the *B. claussenii LAC4* and *LAC4* sequences from *Kluyveromyces*. Interestingly, the *B. claussenii LAC4* also had high similarity with the *LAC4* from *B. nanus* (61.8%) and had a much higher similarity for the *LAC12* from *B. nanus* (71.2%). Further analysis should be done but this could point towards loss of lactose metabolism activity through mutations in the lactose permease gene within the *Brettanomyces* genus. Further analysis of more genes from different species and strains could help identify

where this gene was lost or gained across the genus. Within this genus, many studies have shown differences in phenotype and genotype across species and strains [9,12,18].

Although β-galactosidase, or lactase, is an enzyme commonly responsible for lactose hydrolysis, additional genes may still be responsible for lactose utilization within *B. claussenii*. Supplementary analysis and confirmation of these genes could be studied with a gene knockout to determine if loss of lactose metabolism occurs. If lactose hydrolysis activity is still present after gene knockout, other genes, such as a β-glucosidase may also be responsible for lactose metabolism. A β-glucosidase has been identified in *B. claussenii* and studies have shown that β-glucosidases can have hydrolysis activity towards lactose [19–22]. Moreover, a clone screening of a genomic library created from *B. claussenii* genomic DNA can be used to identify all possible genes conferring lactose metabolism.

Further protein purification and thermostability and pH stability studies of the beta-galactosidase enzyme, can lead to important information for the use of this enzyme commercially. Lactose fermentations with *B. claussenii* could also benefit from this information, to target the ideal settings for optimal enzyme activity and lactose usage. Commercial use of the lactose-hydrolyzing *S. cerevisiae* recombinant would require further strain optimization including integration of the lactose metabolism genes into the yeast chromosome. This has been done previously with lactose metabolism genes from *Kluyveromyces* [2,23].

REFERENCES

1. Zotta T, Solieri L, Iacumin L, Picozzi C, Gullo M: **Valorization of cheese whey using microbial fermentations**. *Appl Microbiol Biotechnol* 2020, **104**:2749–2764.
2. Domingues L, Guimarães PMR, Oliveira C: **Metabolic engineering of Saccharomyces cerevisiae for lactose/whey fermentation. Bioeng Bugs 1: 164–171**. 2010,
3. Guimarães PMR, Teixeira JA, Domingues L: **Fermentation of lactose to bio-ethanol by yeasts as part of integrated solutions for the valorisation of cheese whey**. *Biotechnol Adv* 2010, **28**:375–384.
4. Marsh AJ, Hill C, Ross RP, Cotter PD: **Fermented beverages with health-promoting potential: Past and future perspectives**. *Trends Food Sci Technol* 2014, **38**:113–124.
5. Saqib S, Akram A, Halim SA, Tassaduq R: **Sources of β-galactosidase and its applications in food industry**. *3 Biotech* 2017, **7**:79.
6. Risner D, Shayevitz A, Haapala K, Meunier-Goddik L, Hughes P: **Fermentation and distillation of cheese whey: Carbon dioxide-equivalent emissions and water use in the production of whey spirits and white whiskey**. *J Dairy Sci* 2018, **101**:2963–2973.
7. Lane MM, Morrissey JP: **Kluyveromyces marxianus: A yeast emerging from its sister's shadow**. *Fungal Biol Rev* 2010, **24**:17–26.
8. de W Blackburn C: *Food spoilage microorganisms*. Woodhead Publishing; 2006.
9. Crauwels S, Van Assche A, de Jonge R, Borneman AR, Verreth C, Troels P, De Samblanx G, Marchal K, Van de Peer Y, Willems KA, et al.: **Comparative phenomics and targeted use of genomics reveals variation in carbon and nitrogen assimilation among different Brettanomyces bruxellensis strains**. *Appl Microbiol Biotechnol* 2015, **99**:9123–9134.
10. Crauwels S, Zhu B, Steensels J, Busschaert P, De Samblanx G, Marchal K, Willems KA, Verstrepen KJ, Lievens B: **Assessing genetic diversity among Brettanomyces yeasts by DNA fingerprinting and whole-genome sequencing**. *Appl Environ Microbiol* 2014, **80**:4398–4413.
11. Woolfit M, Rozpędowska E, Piškur J, Wolfe KH: **Genome survey sequencing of the wine spoilage yeast Dekkera (Brettanomyces) bruxellensis**. *Eukaryot Cell* 2007, **6**:721–733.
12. Conterno L, Joseph CML, Arvik TJ, Henick-Kling T, Bisson LF: **Genetic and physiological characterization of Brettanomyces bruxellensis strains isolated from wines**. *Am J Enol Vitic* 2006, **57**:139–147.
13. Dickson RC, Barr K: **Characterization of lactose transport in Kluyveromyces lactis**. *J Bacteriol* 1983, **154**:1245–1251.
14. Mumberg D, Müller R, Funk M: **Yeast vectors for the controlled expression of heterologous proteins in different genetic backgrounds**. *Gene* 1995, **156**:119–122.
15. Lõoke M, Kristjuhan K, Kristjuhan A: **Extraction of genomic DNA from yeasts for PCR-based applications**. *Biotechniques* 2017, **62**:v.
16. Burns LT, Sislak CD, Gibbon NL, Saylor NR, Seymour MR, Shaner LM, Gibney PA: **Improved Functional Assays and Risk Assessment for STA1+ Strains of Saccharomyces cerevisiae**. *J Am Soc Brew Chem* 2021, **79**:167–180.
17. Gibson DG, Young L, Chuang R-Y, Venter JC, Hutchison CA, Smith HO: **Enzymatic assembly of DNA molecules up to several hundred kilobases**. *Nat Methods* 2009, **6**:343–345.
18. Roach MJ, Borneman AR: **New genome assemblies reveal patterns of domestication**

and adaptation across Brettanomyces (Dekkera) species. *BMC Genomics* 2020, **21**:1–14.
19. Vervoort Y, Herrera-Malaver B, Mertens S, Guadalupe Medina V, Duitama J, Michiels L, Derdelinckx G, Voordeckers K, Verstrepen KJ: **Characterization of the recombinant Brettanomyces anomalus β-glucosidase and its potential for bioflavouring**. *J Appl Microbiol* 2016, **121**:721–733.
20. Simos G, Georgatsos JG: **Lactose-hydrolyzing β-glycosidases of barley meal**. *Biochim Biophys Acta (BBA)-General Subj* 1988, **967**:17–24.
21. Bhatti HN, Batool S, Afzal N: **Production and characterization of a novel (beta)-glucosidase from Fusarium solani**. *Int J Agric Biol* 2013, **15**.
22. Li B, Wang Z, Li S, Donelan W, Wang X, Cui T, Tang D: **Preparation of lactose-free pasteurized milk with a recombinant thermostable β-glucosidase from Pyrococcus furiosus**. *BMC Biotechnol* 2013, **13**:73.
23. Sreekrishna K, Dickson RC: **Construction of strains of Saccharomyces cerevisiae that grow on lactose**. *Proc Natl Acad Sci U S A* 1985, **82**:7909–7913.

APPENDIX A

SUPPLEMENTARY TABLES AND FIGURES FOR CHAPTER 6

Table S1. Yeast strains used in this study.

Strain	Description	Source
D. anomala Y-17522	isolated from soft drink	USDA NRRL
D. anomala Y-1414	isolated from lambic beer	USDA NRRL
D. anomala Y-1415	isolated from stout	USDA NRRL
D. anomala Y-17520	isolated from cider	USDA NRRL
D. anomala Y-17521	isolated from apple	USDA NRRL
D. anomala YB-4241	isolated from Australian beer	USDA NRRL
B. claussenii OYL-201	commercial yeast	Omega Labs
B. bruxellensis Y-1411	isolated from lambic beer	USDA NRRL
K. lactis B9-0069	isolated from cheese	Wiedmann Lab
S. cerevisiae PGY453	leu2Δ0/leu2Δ0 ura3Δ0/ura3Δ0	Gibney Lab

Table S2. Plasmids used in this study

Plasmid	Description	Reference
p425GPD	2μ, $LEU2^+$, amp^r	Mumberg *et al.* [14]
p416GPD	2μ, $URA3^+$, amp^r	Mumberg *et al.* [14]
p425GPD-*LAC4*	*LAC4* inserted at SpeI/HindIII (2μ, $LEU2^+$, amp^r)	this study
p416GPD-*LAC12*	*LAC12* inserted at SpeI/HindIII (2μ, $URA3^+$, amp^r)	this study

Table S3. Primers used in this study

No.	Target	Primer Name	Sequence (5'-3')
1	*LAC4* (*B. claussenii*)	MRL_1_BC_BetGal1_F	ATGTTCCAAATCCGCCGACT
2	*LAC4* (*B. claussenii*)	MRL_2_BC_BetGal1_R	CATCGCCTCAAGAGTTGGGT
3	*LAC4* (*B. claussenii*)	MRL_25_BC_BetGal1S_R	AGTCGGCGGATTTGGAACAT
4	*LAC4* (*B. claussenii*)	MRL_26_BC_BetGal1S_F	GCAGTAGAAGAGGCCAAGCA
5	*LAC4* (*B. claussenii*)	RL_1_BC_BetGal1S_F	TGAATGGCGTATGTCGGGAA
6	*LAC4* (*B. claussenii*)	RL_2_BC_BetGal1S_R	GCACGGCATCAAACTGTCC
7	*LAC4* (*B. claussenii*)	RL_3_BC_BetGal1S_F	AGGGGGTTAACAGACATGATCA
8	*LAC4* (*B. claussenii*)	RL_4_BC_BetGal1S_R	ACCCATCTGTACTCCGTGTG
9	*LAC4* (*B. claussenii*)	RL_5_BC_BetGal1S_F	ACCCAACTCTTGAGGCGATG
10	*LAC4* (*B. claussenii*)	RL_6_BC_BetGal1S_R	CCATGATTTGCCCACTCCCA
11	*LAC4* (*B. claussenii*)	RL_7_BC_BetGal1S_F	TGGATATTCAGACGACAGGTGT
12	*LAC4* (*B. claussenii*)	RL_8_BC_BetGal1S_R	AGCGAGACGTTTCCTCATACT
13	*LAC4* (*B. claussenii*)	RL_9_BC_BetGal1S_F	CAAACCAATTGCGTATTCATACCA
14	*LAC4* (*B. claussenii*)	RL_10_BC_BetGal1S_R	AGACCAAATTTCTGGGAAAGCT
15	*LAC12* (*B. claussenii*)	MRL_6_BC_LacPer1_F	GCCTTCCTTGGATTTGGTGC
16	*LAC12* (*B. claussenii*)	MRL_7_BC_LacPer1_R	GTGCTGTCGTTGGTGCAATT
17	*LAC12* (*B. claussenii*)	MRL_8_BC_LacPer1S_F	TCCAACCCTATCATGAGCGA
18	*LAC12* (*B. claussenii*)	MRL_9_BC_LacPer1S_R	CGCTTGATGTCAGGCCATTG
19	*LAC12* (*B. claussenii*)	MRL_21_BC_LacPer1_F	ATGACTACCGAAGATAAAGTCGTT
20	*LAC12* (*B. claussenii*)	MRL_27_BC_LacPer1S_R	CAATTGCACCAACGACAGCA
21	*LAC12* (*B. claussenii*)	MRL_28_BC_LacPer1S_F	GCACCAAATCCAAGGAAGGC

22	*LAC12* (*B. claussenii*)	MRL_29_BC_LacPer1S_R	CAATGGCCTGACATCAAGCG
23	*LAC12* (*B. claussenii*)	MRL_30_BC_LacPer1S_F	TGGGTGCTTTCGCTCATGAT
24	*LAC12* / p416GPD	MRL_10_BC_p416SpeILacPer1C_F	CACCAGAACTTAGTTTCGACGGATTCTAGAACTAGTATGACTACCGAAGATAAAGTCGTT
25	*LAC12* / p416GPD	MRL_11_BC_p416HindIIILacPer1C_R	CTAATTACATGACTCGAGGTCGACGGTATCGATAAGCTTTCAGACAGATTCAGCAGTGC
26	*LAC4* / p425GPD	MRL_15_BC_p426SpeIBetGal1C_F	CCAGAACTTAGTTTCGACGGATTCTAGAACTAGTATGGTTACAGCTGTTCCACTTCATA
27	*LAC4* / p425GPD	MRL_16_BC_p426HindIIIBetGal1C_R	TGACTCGAGGTCGACGGTATCGATAAGCTTTCAGGTAAAGTTGATATCAAATGTGAAATC
28	p416/p425 GPD promoter	MRL_23_p416GPD_S_F	CGGTAGGTATTGATTGTAATTCTG
29	p416/p425 CYC terminator	MRL_24_p426CYC_S_R	GCGTGAATGTAAGCGTGAC

Table S4. Genes identified in this study

Gene	Sequence (5'-3')
LAC4 (*B. clausseni i*)	ATGGTTACAGCTGTTCCACTTCATATTCGCAATCCTCGTTGCATTCAAG AAAATCGACTTCCCACAAGGGCTTATACATATGACCCTGATATTTTCTC TTCCTTGAATGGGCAGTGGTTCTTCAAACTATTCAGTAATCCTTTGGAA TCTCCAGATCTCACCAGCTTCAACTTATCCTCTGCAGAAATTTGGGACA CCATCAAAGTCCCATCTCATTGGCAATTAGAAAACAACGGTAAATACG GATCTCCTGGATACCAGAATGTTCAGTACCCATATCAACTTGATGTTCC AAATCCGCCGACTATGAACCCAACTGGTGTATATTTCCGCAGCTTTCAT GTCGAGCAAAGAGATTCAAATATAAATTATCGGATCAGGTTTGAAGGT GTGGATAATTGTTTTGAAGTTTACGTGAATCAGAATTATGTGGGCATG AGTAAGGGTTCAAGAAATGCCTCTGAATTTATTATCGATGATTACTTAG TTGACGGAGAAAATTTTATTTCCGTGAAGGTTTATAAGTGGTCTGATTC

ATCTTACTTGGAAGATCAGGATGAATGGCGTATGTCGGGAATTTTCAG
GGATGTCTCTCTTGTAAAGTTGCCAAAGAGTCACATTGAGAACTTTCA
AGTTATTCCTTCTTTTGATGAAAATTACGAAGATGCTACTCTAGAATTA
AAATTGGATGTTCTAGGACAGTTTGATGCCGTGCATTTCACACTCTATG
ACTGTGAAGATCCACATAGGGCGATTGAACCTAAAGATTTACTTGGTG
CCAGAGATGAACCAACAACAAAACAAATAAAAGAAGTTACAATATCT
GCAGAGAAAGTAAAAGGACCAATTGAGGTTAAAATTAATAGCCCAAG
ACATTGGACAGCTGAGGATCCTTATCTCTATAAGTATAAGTTGGACTTG
ATCCACGGGGGTGTGGTTTTACATACAATCCATAGTCATGTTGGCTTTC
GTCAGGTCGAGTTGCTAAAGGGTAATATCAAAGTGAATGGTCAAAGAG
TCTTATTTAAGGGGGTTAACAGACATGATCATCATCCATTGTACGGTAG
ATCTGTTCCGTTGGAATTTGTTTTAAGAGATTTGGTTATCATGAAAAAA
TACAACGTCAACGCAGTTAGGACCTCTCATTACCCTGATAATCCAAGA
ATATACGATTTCTTTGATCGTCTCGGATTCTATGTTATTGATGAAGCTG
ATCTAGAGACACACGGAGTACAGATGGGTTATACTGTTTACAATGATA
TTAAAGTAGAGTTTCCCGAGACCAAACAGAAAAATTATGATCCAAATG
TTTGCTATTTATCCAGCAATCCAGAGTATACAAATGCCTATTTGGACAG
AGCTTCTCAACTAGTTTTAAGGGATATCAATCATCCTTCAATCATTCTA
TGGTCATTGGGTAACGAGTCTGGCTATGGAACCAATCATCAAGAAATG
GCAAAATTAGTTCGCAAGTTAGATCCTTCGAGATTGATTCATTATGAG
GGAGACGCTAATGCGATATCTGTCGATACCTATAGCTTTATGTACCCA
ACTCTTGAGGCGATGGAAATCTGGAGAAAAGATCACACAAAAAGTAA
TGGTGAATTTGAGAAGCCTTTAATTTTGTGCGAGTATGCACATGCAATG
GGTAATGGTCCTGGAAATTTGAAAGAATATCAAGATCTATTCTATTCA
AATGACTTTTATCAGGGTGGTTTTATCTGGGAGTGGGCAAATCATGGT
ATCAAAACAACAAGTAAAGAAAACGGTCGGATGGAAGATATCTATGC
CTATGGTGGTGATTTTGGAGAGGAAGTGCATGACGGTGTTTTTATCATG
GATGGCCTTTTGAATTCAGAGCATAACCCAACACCTGGCATTATAGAG
CTAAAGAAGACGTATGAGCCTGTTCTTATTGATGTTTCAGAGTCTCAAA
TTACCATTGAAAATAAAAACAACTTCAAAACTACTGATTATTTGGATTT
CATCAACAAGGATGACAATAGCATTATACCTGTTCCTTCTTTAAAACCT
GATCAGAAAATAACCTTGGATATTCAGACGACAGGTGTGTCTGCTATT
CTTAAAAAAGATTATGGAATTTTAAAAGCTGGTTATGAAATTGCATGG
GGTCAGGTCTCCCCCAAGATAAGGATTCCAATTTCTCAAAAAGCACCA
AAAGAAGAGATTAAGTATGAGGAAACGTCTCGCTTTTTGATAGCAATA
TCAAAGACATTGCTTTTGAAATTTGATAAGATGTTGGGAATGATTGTG
GATTTGAGGATTGGAGGAAAGTCTTTGAGCAATAAGTTTGATGGCTCA
ACAATAACATTCTGGAGGCCTCCTACAAACAATGATGATGGTAAGGAT
ACAAAGTATTGGAAAGATTTCAATATGCATTTGGTCAAGCAAAATGTT
AGAGATATTGAAGTTAAGAAGGACTGTGAGGATTGCTTGGTGACGATT
ATCGTGAAGTCTCGTATAGGACCTCTCGTCTTTGACTGGGGATTTAACA
CAGTCCAGGAGTATAAATTTAGTGCAAACCAATTGCGTATTCATACCA
TTATGAAAAATACAGGAAGATACCAACCAAAATATCTTCCTCGACTTG
GATATCAATTCTGGCTTGGCGAAAATTATGATCATTTTGAATGGTACGG
CCGTGGTCCTGGCGAATCATATCCTGATAAAAAGCTTTCCCAGAAATTT
GGTCTATACTCATCAGAGAAGATTTCCAAGTTTGTCTACGACTATCCGC

	AGGAGAATGGTAATCACACAGACACTCACTATGCTAAAATAAGTTACA AAGGCGGTAATGGTGCTCTACTCATTTCTGAAAGCAACAAGAAATTTA ATTTCAAGATTAGTGATGAATATGCAGTAGAAGAGGCCAAGCACCCAA ATGACGTGAAGCACTATGGAAAATACTATCTTAGGCTTGATGATTCTA TGGAAGGAGTTGGTTCAGAGGCATGTGGACCACCAGTTCTTGATAAGT ATAGAGTTAAGATGAAAGATTATGATTTCACATTTGATATCAACTTTAC CTGA
LAC12 (*B. clausseni i*)	ATGACTACCGAAGATAAAGTCGTTGAAACTCCTGACGATGGTCAGGAT GTTATGCTAAAAGGCAATCCCTCTGATGATGAGGGTATGAACATGGAT AACTTAAATCTTCCTGGTGCCTTCTCCAAACAGTACTTACACTTATTCT TGATTTGCCTTATTGTTTACTTTTGTTCCACAATGCAAGGTTTTGATGGA TCATTGATGGGTTCTCTTTATACGCAAAGTGATTATTTGAACTATTACC ATCTTGATGTGAACTCTTCAACTGGTACTGGTTTGGTGTTTTCCATTTAC AACATTGGTCAGATTACAGGTGCCTTCTTTGTGTGGCTAATGGATTGGA AAGGAAGAAAGCTTTCTATCTGGGTTGGATGTTTGGGTGCTGTCGTTG GTGCAATTGTTACCGCAGTTACATCAACCAAAGGAGGATTGATTGGTG GTCGTTGGCTTATGTCATTTTGTGCCACCATTGCAAATACTGCTGCCCC AAATTATTGTATCGAGGTTTCCCCACCGCACATAAGAGGTCGTGTTGCT GGATTATATAACACATTATGGTATGTTGGCTCAATTGTGGCTTCATTCA CAGCTTATGGTTGTAATGAGCATTTATCGGGCACCAATAAAGCATTTA AGATACCTCTTTGGGTGCAAATTGGTTTCCCTGGTTTGGTTGTCCTCTT GGGTTGGATTATTCCAGAGTCACCAAGATGGCTCATTGGTGTTGGCCG TTATGATGAGGCTCGTCGCTTCCTTGTGAAGTATCATTGCAATGGAAAT GAAAATGATCCATTAGTTGATCTCGAGATGTCTGAAATAGAAGATTCA TTTCAACAAATGAAGCTTTCTGATCCAAAAACTGCGCTTGATGTCAGG CCATTGTTTAAGAAAAGATCAGATCGCTACAGACTAGGATTAATGATT GCCATGGGTTGGTTTGGACAATTTTCAGGTAACAACGTTTGCTCATACT ACTTGCCTACAATGTTGACGCAAGTTGGAATGAGTTCTCATTCCTTGGA TGTCCTTATGAACGGAGTCTACTCTATTGTTTCTTGGGTTGCTTCTATTT TGGGTGCTTTCGCTCATGATAGGGTTGGAAGAAGAAAGATGTTCATGG TGTCTACTCTTGGCTCTGCACTTGCCTTGGTGTGTCTAGCTATTTGTACC GCTCGTTTCCAGGCTACAGGAGCCAATTCAGCAGCAAATGGTACTTTA GTTTTTATTTATTTCTTTGGTGTGATCTTTTCATTTGCCTTTACCCCTATG CAACCAATATATCCTGGTGAGGTTGCTTCTAACTTGATCAGATCTAAGG CACAATTTGTTCAACAAATTGTTTCTGGAGTCGCACAGTTTGTTAATCA ATTTGCTTCTCCAAAGGCCATGCAAAACATCAAATATTGGTTCTACGTC TTTTATGCCTTTTTTGATGTGTTTGAGTTTGTCATAGTTTATTTCTTCTTC GTTGAAACTAAGGGAAAGACTTTGGAAGAACTTGATTTCATTTTTGAG GCACCAAATCCAAGGAAGGCTTCCGTTGATCCTGATTTCCTTTCTAGCA CCAGACTTGCTTCTGGATTTGAGGCTGAAAACAAGAAGGAGCAGCTAT TGAATCCGAAACCCGATGTTGAACATTTGAGCACTGCTGAATCTGTCT GA

Table S5. Sequences obtained from NCBI database

GenBank Accession	Organism	Strain	Description

GCA_001005505.1	*B. anomalus*	YV396	Whole genome
GCA_001754015.1	*B. anomalus*	CBS 7654	Whole genome
GCA_012295365.1	*B. anomalus*	CRL-49	Whole genome
GCA_018344455.1	*B. anomalus*	NRRL Y-17522	Whole genome
VUG18697.1	*B. bruxellensis*	-	*LAC4*
QPG74631.1	*B. nanus*	CBS 1945	*LAC4*
QLH93951.1	*K. lactis*	VKM Y-1339	*LAC4*
QLH93952.1	*K. marxianus*		*LAC4*
VUG19339.1	*B. bruxellensis*		*LAC12*
QPG72701.1	*B. nanus*	CBS 1945	*LAC12*
CAA30053.1	*K. lactis*		*LAC12*
QGN17576.1	*K. marxianus*		*LAC 12*

Table S6. Transformant strains generated from this study

Strain	Description	Expected Phenotype
S. cerevisiae PGY453-ML1	p425GPD-*LAC4* + p416GPD-*LAC12*	SD + / Lac +
S. cerevisiae PGY453-ML2	p425GPD + p416GPD	SD + / Lac -
S. cerevisiae PGY453-ML3	p425GPD-*LAC4* + p416GPD	SD + / Lac -
S. cerevisiae PGY453-ML4	p425GPD + p416GPD-*LAC12*	SD + / Lac -
S. cerevisiae PGY453-ML5	p425GPD	SD - / Lac -

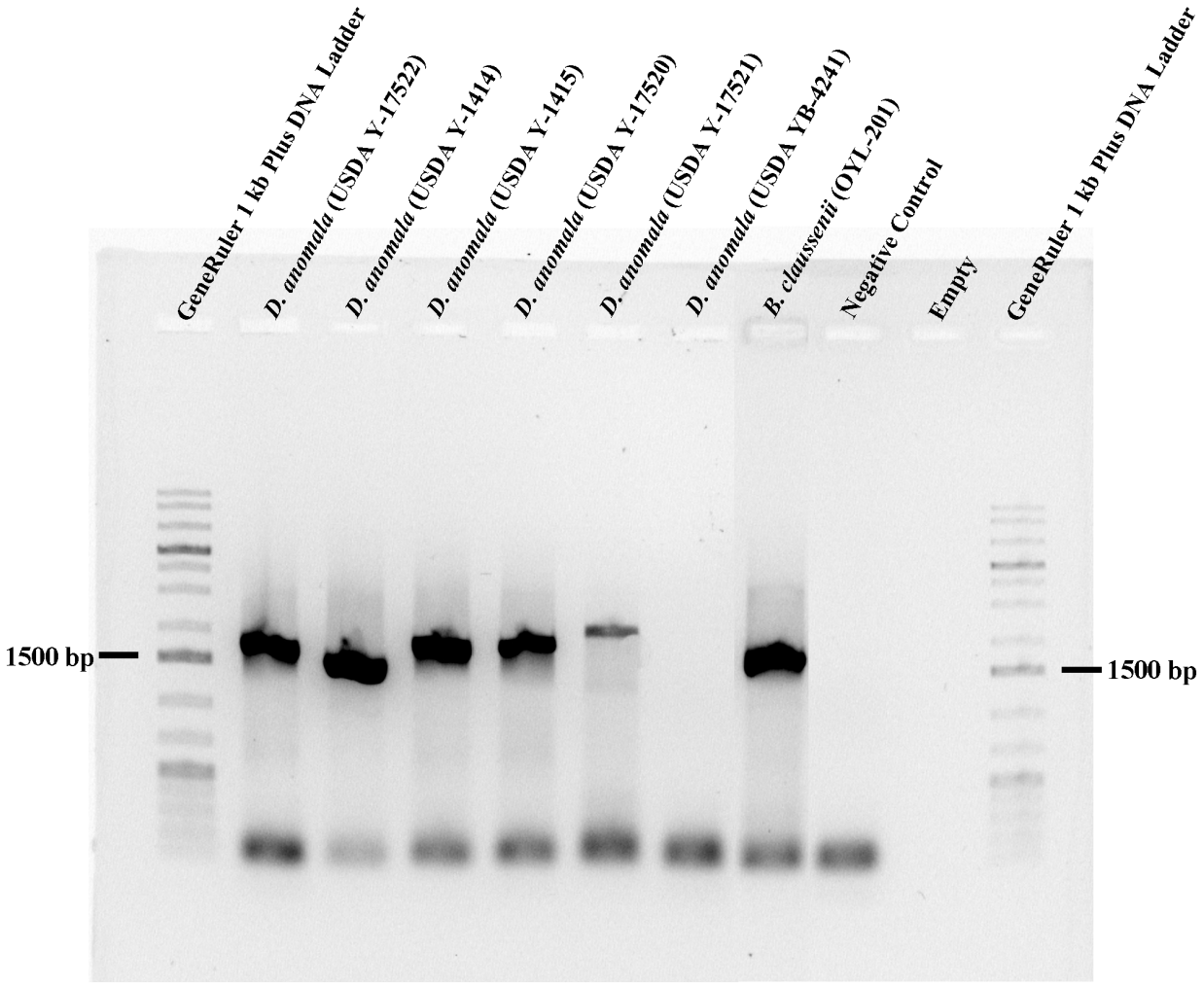

Figure S1. DNA gel of PCR with primers 1 and 2 for beta-galactosidase screen in various strains of *B. claussenii*/*D. anomala*