CONTROLLING BACTERIAL CONTAMINANTS IN SUGARCANE ETHANOL FERMENTATIONS

BY

MARY ANGELA DAUM

THESIS

Submitted in partial fulfillment of the requirements
for the degree of Master of Science in Food Science and Human Nutrition
with a concentration in Food Science
in the Graduate College of the
University of Illinois at Urbana-Champaign, 2016

Urbana, Illinois

Adviser:

Associate Professor Michael J. Miller

ABSTRACT

Bioethanol from sugarcane has a lower carbon footprint than petroleum based fuels but the industry is plagued by various economic pressures, including product loss due to contamination of fermentation facilities. *Lactobacillus* species and other lactic acid producing bacteria from the feedstock and environment are important bacterial contaminants. Further studies focused on process optimization and validated the use of antibiotics and acid treatment to reduce ethanol losses due to bacterial contamination. Monensin, penicillin and virginiamycin have been shown to reduce bacterial counts in bioethanol but increased incidence of antibiotic resistant bacteria means alternative approaches are needed. To test efficacy of the antimicrobials hen egg white lysozyme, nisin and the combination of nisin and penicillin against representative contaminants a model system was developed. To mimic the typical Brazilian conditions, the model incorporates high initial yeast inoculum, fermentation times of twelve hours or less and final ethanol concentration of 5.5 to 6 % weight/volume. The impact of penicillin and nisin on bacteria isolated from sugarcane juice or adapted by successive passes in sugarcane juice in fermentation of sugarcane juice by *Saccharomyces cerevisiae* JAY291 was measured by counting the bacteria and yeast. In addition, ethanol and lactic acid titers were measured and by HPLC analysis of the fermentate. Plate counts revealed that the combination of 250 ppm of nisin and 2 ppm penicillin was the most effective treatment against *Lactobacillus paraplantarum*, as well as against a mix of *L. paraplantarum* and four other bacterial species. Combining nisin with penicillin could decrease the amount of penicillin used to control contamination and mitigate the risk of antibiotic resistant bacteria proliferating. This model system can be employed for testing other novel antibacterial measures such as bacteriophages, or engineered phage endolysins and bacteriocins.

DEDICATION AND ACKNOWLEDGEMENTS

This is for my brother Ben. He assured me that one day I would write a book, although this is not what either of us had in mind. Every new flavor of Mountain Dew® or Doritos® makes me wish he was still here to give his two cents on whether food scientists are on the right track or going off the rails. Lots of folks provided essential support during this interesting two years. My family and friends provided food, distractions and laughs. My mom and dad were consistently unconditionally supportive. Samantha Gardiner kept me from leaving before the first year was over, ensured I had some social life and helped with my crazy dog. Finishing this thesis would not have happened without the sage advice, practical input, statistical analysis, humor and reality checks provided by Max Van Tassell. He also provided critical feedback on experiment design, controls and life in general. Jeni Hoeflinger masterfully kept the lab running and in top shape, assisted with numerous questions and protocols and graciously permitted the use of custom growth curve software. Eric Fritz helped me though course work, was also usually in lab nights and weekends, and reminded me to go with the flow. Josh Warren contributed tons of plates, media and his hard work kept the lab stocked and clean. Josh was also instrumental in evaluating strain sensitivity to nisin and did countless spot on lawn assays. Christine Liu provided snacks, humor, assistance with sequencing prep and reminded me that I should really start writing. Luis Ibarra-Sanchez let me borrow his bench for plates and sugarcane filtration and was a quiet calm presence even when I was crunching potato chips and muttering when trying to analyze data. Suneet Takhar worked on sugarcane media prep, adaptation of bacterial strains in sugarcane juice and bioscreen operation. Jun-Seob Kim provided troubleshooting with HPLC. My committee members Dr. Jin and Dr. White provided valuable input and feedback. Last but not least I'd like to thank Dr. Miller for his support and guidance.

TABLE OF CONTENTS

CHAPTER 1 INTRODUCTION

1.1 Problem statement

Once upon a time, ethanol from carbohydrate rich feedstocks was put forth as sustainable alternative for finite petroleum based fuels. In 2015 the United States of America led world production of bioethanol followed closely by Brazil[1,2] Corn provides the main substrate for bioethanol in the US and sugarcane serves the same function in Brazil. Numerous pressures challenge the bioethanol industry, including product loss due to bacterial contamination of fermenters. Surveys of fermenter and substrate populations revealed that a significant portion of bacterial contaminants belonged to the *Lactobacillaceae* [3–6].

The family *Lactobacillaceae* contains several genera of bacteria which produce lactic acid from carbohydrates, and these bacteria are often referred to as lactic acid bacteria (LAB). LAB are Gram-positive with low G-C ratios[7,8]. Plants are often the natural habitat of LAB. Tolerance of low pH, high ethanol and higher osmotic pressures make them well suited for growth in ethanol fermentations. These characteristics, combined with the variety and diversity of contaminating species make them difficult to control in large scale ethanol fermentations which do not take place under aseptic conditions[9,10].

1.2 Objectives and results

The overall objectives of this research were to 1) create a model system to study contaminants of sugarcane bioethanol; 2) evaluate potential antimicrobials against growing cells; and 3) test promising antimicrobials in sugarcane ethanol fermentations by *Saccharomyces cerevisiae*. The model described in Chapter 3 incorporates key aspects of the Brazilian sugarcane

ethanol process including; yeast inoculum of ten percent vol/vol, fermentation of sugarcane juice in 12 hours, more than 5 % final ethanol concentration with a yield of greater than 50 percent. Based on review of the literature and experimental data, antimicrobials were testing by pipetting a small amount of antimicrobial on to a freshly spread lawn of early log phase bacterial cells. This spot on lawn assay provided insight into the relative spectrum of nisin, hen egg white lysozyme (lysozyme), and mutanolysin against growing bacteria which are the most problematic in ethanol contaminations. The bacteriocin nisin impacted the largest number of LAB tested without inhibiting the growth of *S. cerevisiae*. The efficacy of nisin alone and combined with penicillin was evaluated in the model system first in *S. cerevisiae* fermentations contaminated with a single LAB and then in fermentations contaminated with a mix of 5 LAB. Nisin resulted in a reduction of log CFU/ml of *Lactobacillus paraplantarum* and in combination with penicillin reduced the total CFU/ml of the mix of five LAB contaminants. This model system could be used to study the impact of other alternative antimicrobials including bacteriophage endolysins, engineered lytic enzymes or bacteriocins.

1.3 References

1. Renewable Fuels Association » Industry Statistics. Renew. Fuels Assoc.

2. The Brazilian Sugarcane Industry Association (UNICA). Sugarcane Industry in Brazil. Sao Paulo Brazil: UNICA; 2015 Dec.

3. Costa OYA, Souto BM, Tupinambá DD, Bergmann JC, Kyaw CM, Kruger RH, et al. Microbial diversity in sugarcane ethanol production in a Brazilian distillery using a culture-independent method. J. Ind. Microbiol. Biotechnol. 2015;42:73–84.

4. Lucena BT, dos Santos BM, Moreira JL, Moreira APB, Nunes AC, Azevedo V, et al. Diversity of lactic acid bacteria of the bioethanol process. BMC Microbiol. 2010;10:298.

5. Li Q, Heist EP, Moe LA. Bacterial Community Structure and Dynamics During Corn-Based Bioethanol Fermentation. Microb. Ecol. 2016;71:409–21.

6. Skinner K, Leathers TD. Bacterial contaminants of fuel ethanol production. J. Ind. Microbiol. Biotechnol. 2004;31:401–8.

7. Holzapfel WH, Wood BJB, editors. Lactic Acid Bacteria: Biodiversity and Taxonomy. Chichester, UK: John Wiley & Sons, Ltd; 2014.

8. Boone DR, Castenholz RW, Garrity GM. Bergey's manual of systematic bacteriology. New York: Springer; 2001.

9. Amorim H V., Lopes ML, de Castro Oliveira JV, Buckeridge MS, Goldman GH. Scientific challenges of bioethanol production in Brazil. Appl. Microbiol. Biotechnol. 2011;91:1267–75.

10. Liu M, Bischoff KM, Gill JJ, Mire-Criscione MD, Berry JD, Young R, et al. Bacteriophage application restores ethanol fermentation characteristics disrupted by *Lactobacillus fermentum*. Biotechnol. Biofuels. 2015;8.

CHAPTER 2 REVIEW OF LITERATURE

2.1 Bioethanol overview

In the search for a renewable replacement for finite petroleum resources, many countries are turning to fermentation of plant sugars or starch rich plants like sugarcane and corn [11]to produce ethanol for fuel. The same techniques implemented for centuries to produce beer, wine and distilled spirits have been scaled up to manufacture quantities of ethanol sufficient to be used directly as fuel or as a fuel supplement. Government programs aimed at achieving energy independence in both the United States of America (US) [12] and Brazil subsidized the fledgling ethanol industry and to some extent, related infrastructure[13]. In 2015, the number of operating ethanol plants was 199 in the US [12]and Brazil had more than 300 plants[2]. In Brazil most facilities are designed to produce both sugar and ethanol[13]. The US ethanol plants produce ethanol and lesser amounts of co-products. Co-products in the US include dried distillers grain solids (DDGS) and corn oil[14,15]. Brazil plants may produce spent yeast as a coproduct. Both spent yeast and DDGS are used primarily for animal feed so the amount of allowable antibiotic residues present is regulated[16,17]. This regulation is an important consideration to keep in mind when determining the best method to control bacterial contamination of bioethanol plants.

In scaling up the processes typically used for fermented beverages, costly steps related to maintaining aseptic conditions, such as pasteurization of the substrate, were omitted since the ethanol produced is meant to be consumed by vehicles rather than people[6]. Corn and sugarcane plants undergo minimal processing prior to being used as fermentation substrate[18]. These plants are the natural habitats of diverse communities of microbes, including wild yeast and lactic acid producing bacteria (LAB)[5,19,20]. The same types of microbes which enabled our ancestors to

enjoy the products of spontaneously fermented fruits like wine and cider and vegetables such as pickles, sauerkraut, kim chi still inhabit the same ecological niches[21]. These niches include plants such as sugarcane and corn. The lack of a sterilization equipment in ethanol facilities combined with the use of minimally processed plants results in an ongoing microbial contamination which decreases ethanol production efficiency[6,22,23].

2.2 Contaminants common in bioethanol facilities

Given the non-aseptic conditions of typical bioethanol fermentation, it is unsurprising that contaminants include all three kingdoms. A recent survey of sugarcane bioethanol plants chronicled a variety of archaea[3]. While some organisms are inhibited by the high ethanol, high sugar, and low pH, wild yeast [19] and bacteria [4–6,24,25] are commonly identified as contaminants. Focusing on the bacterial contaminants, members of the *Lactobacillaceae* family are consistently the predominant family of bacteria identified in ethanol fermentations from both corn[5,6,26–28] and sugarcane [3,4,25]. An important point to note is that many of these papers isolate contaminants by plating on de Mann, Rogosa, Sharpe (MRS) plates with cycloheximide to inhibit yeast growth. MRS was developed specifically to cultivate lactobacilli[29], so it is not surprising that Lactobacillus and other LAB were frequently isolated. In the past two years, culture independent studies have expanded the information available on diversity of contaminants. In Brazil, samples of substrate at different points in the ethanol fermentation process of one facility revealed Proteobacteria were the most abundant in the juice from the mill and the must (the clarified, concentrated then cooled juice, pH 5-6). Reads from Firmicutes, the phyla containing the *Lactobacillaceae* family, outnumbered all other bacteria in the fresh sugarcane juice; the clarified juice (after undergoing heat treatment of 105°C and addition of calcium compounds to precipitate out solids pH > 7.0 then adjusted to 5-6); the concentrated juice (heated to 115°C then cooled);

and the wine (the product of fermentation, after centrifugation of the yeast pH 4)[3]. Studies of nine [28] and five [5] dry mill corn ethanol plants in the United States provided insight into the larger bacterial community in ethanol facilities. The study by Li et al., evaluated up to 5 types of samples from 5 different facilities[30]. Three samples were at different stages in the fermentation, early, mid and late and another sample was from the yeast propagation tank. At a given facility, the communities in the fermentation samples were similar to each other but distinct from the yeast propagation community[5]. The five facilities had very different communities with no identifiable commonality linking them[5]. Two of the facilities had predominantly Firmicutes but the other three had predominantly Proteobacteria.

2.3 *Lactobacillaceae* family contaminations

Of the Firmicutes, the predominant family found in the Li et al., study was *Lactobacillaceae*[5]. *Lactobacillaceae* includes the many genera of LAB, including *Lactobacillus, Leuconostoc, Pediococcus, Enterococcus, Aerococcus, Oenococcus, Lactococcus* and *Weissella.* The same characteristics which make many species from these genera ideal for fermented products, including acid and ethanol tolerance [31], fermentative metabolism and the ability to thrive even with the presence of higher than average solutes commonly used to reduce water activity like salt and sugar, enable LAB to thrive in ethanol fermentations. Lactic acid bacteria may impact fermentation through production of lactic acid, acetic acid, exopolysaccharides which foul equipment[32], forming biofilms[28,33,34], competing for key nutrients[35]; and causing flocculation of *S. cerevisiae* which impairs centrifugation[19,36,37]. Many studies aiming to characterize ethanol facility contaminants focus on members of the genera *Lactobacillus.* This is likely due to them being the predominant genera isolated, particularly in the culture dependent

studies mentioned previously. *Lactobacillus fermentum* and *Lactobacillus plantarum* are two of the most frequently studied.

Lactobacillus fermentum

L. fermentum is one of the most oft evaluated ethanol fermentation contaminants. A recent survey of corn ethanol facility microbiome found 62 out of 768 sequenced were *L. fermentum* isolates[28]. Further, 50 of those 62 caused a ten g/L reduction in ethanol with simulated contaminated corn mash fermentations[28]. While those numbers are impressive, not all *L. fermentum* species are effective at reducing ethanol, as one study found when evaluating the reaction of 66 different *S. cerevisiae* strains to common stressors in the Brazilian sugarcane fermentation process [37]. When using an inoculum of 10^6 CFU/ml of a *L. fermentum* strain isolated from a distillery and 10^8 CFU/ml of *S. cerevisiae* strains there was no difference in the fermentation profiles with or without the bacteria. However, this lack of effect may also be due to the low inoculation level of bacteria. Using a wheat mash model system to study the impact of inoculation level of LAB on final ethanol production by *S. cerevisiae* showed that the amount of ethanol inhibition by *L. fermentum* increased as the CFU/ml of LAB increased[38]. The 1997 Narendranath et al., study evaluated *L. paracasei*, *L. rhamnosus*, *L. plantarum* as well as *L. fermentum*[39]. A later study by Narendranath and Power, also looked at the relationship between yeast inoculation rates and the impact of LAB contaminants[40].

L. plantarum

Because it is often one of the most abundant species isolated in ethanol plants[25,28], *L. plantarum* has also been studied extensively. Many studies of bioethanol contaminants consider at least two contaminant strains. *L. fermentum* or *L. brevis* are often compared to *L. plantarum.* A recent study by Peng et al., concluded that *L. plantarum* was more likely to reduce ethanol, at least in glucose

based media[38]. This was attributed to higher amounts of lactic acid produced by *L. plantarum* compared to *L. brevis.* A similar conclusion was reached by researchers in Brazil based on initial lab assays[34]. However, when industrial sugarcane bioethanol conditions were simulated, the homofermentative *L. plantarum* was not as detrimental as the heterofermentative *L. fermentum*.

Heterofermentative and Homofermentative metabolism as a consideration

The Rich et al. 2015 study suggested a link between heterofermentative fermentation profiles and impact on ethanol. The two species with the most isolates which reduced ethanol by 10 g/L or more when corn mash was the substrate were *L. fermentum* and *L. mucosae*[28]. Both species are obligately heterofermentative[8]. Heterofermentative metabolism of carbohydrates by LAB means that in addition to lactic acid, CO_2, acetic acid and ethanol are produced [21]. The Rich et al group evaluated ethanol inhibition based on a model developed by Bischoff et al. The LAB and *S. cerevisiae* were co-cultured in corn mash with initial inoculations of 10^7 for each[39]. One heterofermentative bacteria, W*eissella confusa*, and two homofermentative species, *Lactobacillus delbrueckii* and *Lactobacillus amylovorus* did not thrive in the simulated fermentation conditions and 10^2 or fewer were present at the end of fermentation (Bischoff et al., 2008). Only the two heterofermentative *L. fermentum* strains and a *Lactobacillus brevis* caused significant reductions of ethanol in corn mash[42]. Under the exact same conditions, but with YP glucose instead of corn mash, no reduction of ethanol resulted[42]. These three strains produced more than 1 g/L of acetic acid in addition to 2.5 – 4.9 g/L of lactic acid. Acetic acid has a pKa of 4.76 and lactic acid has a pKa of 3.86[43]. The primary mechanism of microbial inhibition of weak organic acids, such as lactic and acetic acid depends on free diffusion of the un-dissociated (uncharged) acid through the cell membrane of the target microbe. Once inside the higher pH of the cytoplasm causes the acid to dissociate, lowering the pH of the cytoplasm

and requiring the microbe to redirect ATP from cell growth to pumping the extra protons out of the cell [44]. In *S. cerevisiae* this diversion of ATP typically results in inhibition by extending the lag phase while the cell works to maintain the cytoplasmic pH[44]. Because only the un-dissociated form can freely diffuse into the cell, the pH of the medium and the pKa of the acid are key considerations. Sugarcane ethanol fermentation typically starts at pH 5.5- 6 and ends with a final pH of 4.0[3,9,45]. At a pH of 4.76 the acetic acid will be equal portions dissociated and un-dissociated forms but lactic acid will have more dissociated form because 4.76 is significantly higher than its pKa of 3.86. Keeping in mind that pH is a log scale, the amount of acetic acid needed to inhibit *S. cerevisiae* will be considerably less than the amount of lactic acid. Various studies have found differing amounts of lactic acid [41,46,47] needed to reduce ethanol production by *S. cerevisiae* and in the corn ethanol the level which is considered likely cause a serious impact is 8 g/L[38] . *L. plantarum*, a homofermentative LAB, grows quickly and produces 2 moles of lactic acid per mole of glucose and under lab conditions in sugarcane media, *L. plantarum* inhibited ethanol production[34]. However, in a fermentation simulating the normal conditions in Brazil, high yeast inoculum and 12 hour fermentation time and 5 cell recycles, *L. plantarum* did not inhibit ethanol production but the heterofermentative *L. fermentum* resulted in a notable decrease in ethanol yield[36]. The authors suggest that the heterofermentative ability to quickly consume fructose while the *S. cerevisiae* consumes the glucose may provide heterofermentative species an advantage[36]. This would not be a consideration in corn derived ethanol which is glucose based due to the saccharification of corn starch. In addition to the inhibition by the un-dissociated form of the acetic acid produced by heterofermentative organisms, acetic acid may also trigger apotosis in *S. cerevisiae* [45,46].

It is important to note that the acid wash step which normally is applied in Brazil was not included in that experiment. It is not inconceivable that the *L. fermentum* would have survived the acid wash step and the cell recycling would select for more acid tolerant strains. Looking at the populations in four facilities at different points of the season, Lucena et al., found diversity decreased as the season progressed and *L. fermentum* along with *L. vini* were two of the species still remaining at the end of the season[50]. In fact, at three of the four plants those two species accounted for more than 2/3 of the species identified in the period ranging from 60 – 180 days into the season[50]. The fourth facility did not have any *L. fermentum* at any point of the sampling period and was the only facility which used molasses as the sole feedstock[50].

Which bacteria causes the most inhibition of ethanol production appears to be highly species specific and conditions such as bacterial inoculation level, yeast inoculation level, total fermentation time, and medium composition are all important considerations. Other factors to consider are the source of the yeast and bacteria. Basso et al. used both industrial yeast and contaminants isolated from molasses from an ethanol plant [34]. But other studies pair bacteria isolated from acutely contaminated ethanol facilities with lab/wild type *S. cerevisiae* strains[10,28,39] or common bakers' yeast[41]. To study the impact of contamination in sugarcane bioethanol fermentation it is important that lab conditions closely mimic distinctive features of the Brazilian process

2.4 Sugarcane bioethanol fermentation process in Brazil

The Brazilian process of bioethanol fermentation from sugarcane has several distinguishing features. These features directly impact contamination diversity and treatments. Characteristic features of Brazilian bioethanol fermentation from sugarcane include: substrate with

readily fermentable sugars; short fermentation times, high yeast inoculation level; acid wash between fermentations; yeast cell recycle; minimal residual sugar and ethanol yields from 7-11 percent w/vol. Seventy five to eighty five percent of the distilleries utilize a fed batch process[51–53]. The fed batch model with recycling of the yeast is often referred to as the Melle-Boinot process [54–56]. The Frenchman Firmin Boinot patented a process in the 1930s which entailed the use of large yeast inoculum, cell recycle and acid treatment to reduce contamination between cycles[57]. High yeast inoculation levels decrease fermentation time which make it more difficult for bacteria to grow to levels which inhibit ethanol production. Fermentation is primarily but not strictly anaerobic, with agitation and carried out at 30-33 °C. The heat of the environment and generated by the fermentation often results in temperatures higher than the targeted temperature of 30 °C.

Portuguese settlers introduced sugarcane to Brazil in the early 1500s [58]. Today, Brazil is the largest grower of sugarcane in the world[55]. Most of the sugarcane facilities in Brazil are designed to produce both sugar and ethanol depending on market demand for each [52,59]. Sugarcane degrades quickly after harvest, which means unlike corn, production of sugar or ethanol occurs almost exclusively during the harvest season which can range from 180 to 240 days depending on the weather and the region. Approximately 90 percent of Brazilian sugarcane is produced in the South-Central region. In the South-Central region, which includes the state of Sao Paulo. The harvest season is typically from April through December [37,59]. Lesser amounts are produced in the northeast of Brazil and that season runs from September through March[59,60] (UNICA 2015). The desired temperature for fermentation is 30 °C but may regularly reach 32°C or higher depending on the weather and plant design. The fast fermentation with a large initial inoculum generates heat[9,37,48,61].

During the season most sugarcane distilleries run 24 hours a day. Since fermentation times range from 6 to 12 hours[9,23,45] two or more fermentations may be completed per day. As a result the yeast may be recycled 400 or more times in a season. After less than 0.5 % of sugars remain in the fermenter, the yeast are centrifuged and the wine is separated. Following centrifugation the yeast cream is treated with sulfuric acid diluted with water to a pH ranging from 1.8 – 2.5 for 1-2 hours[61]. The acid wash step is done under aerobic conditions due to the mechanism of sulfuric acid which kills microorganisms[25,46,62]. Wild yeasts and contaminating bacteria are reduced during this acid treatment, and some of the fermenting *S. cerevisiae* are also impacted[19,63,64]. While a broad spectrum, including both Gram-negative and Gram-positive bacteria are reduced by the sulfuric acid wash[25,37,62], some persist and contamination remains a problem[9,23,36].

The key to complete fermentation is a short time is a high yeast inoculation level and yeast that are adapted to the Brazilian conditions. Typically inoculation levels are 8 to 12% (w/vol) percent of the fermentation. The practice of starting the season with bakery yeast exclusively was discontinued as long term studies utilizing karyotyping of *S. cerevisiae* strains demonstrated that the bakery yeast was quickly displaced and did not survive the season. Indigenous yeast which consistently predominated at the end of the season were isolated and studied further[45,56,61]. At the beginning of the season, the first inoculation is predominantly baker's yeast with a much smaller proportion of the industrial strains with proven track records such as CAT-1 or PE-2[9,13,23]. As the season progresses the most competitive strains survive, which are often a mix of the industrial strains and some indigenous *S. cerevisiae* with stress response regulation suited for this environment[45,65].

The fermentation substrate, molasses and/or concentrated sugarcane juice, is added to the yeast inoculum gradually. Both sugarcane juice and molasses are predominantly sucrose. Small amounts of the component monosaccharides glucose and fructose are also typically present. Since *S. cerevisiae* naturally produce invertase[61], the saccharification step of corn ethanol production is not needed for sugarcane fermentation. While sugars are readily available, sugarcane juice needs to be concentrated via evaporation and contains limited amounts of nitrogen and essential growth factors[66–68]. It may also contain trace metals like aluminum which inhibit *S. cerevisiae* at high enough levels[9,66,68]. The sugars in molasses are already concentrated during the sugar crystallization process, and contain higher amounts of nutrients but contain different components from the refining steps which may detrimentally impact the yeast[66,69,70]. The distinctive characteristics of molasses may also translate in different diversity of contaminating bacteria as show in the Trapiche facility in the Lucena et al., study[4]. Many distilleries use a combination of sugarcane juice and molasses as the must which serves as the fermentation substrate depending on the economic conditions[13,23,56]. The must is adjusted to a target pH of 5.5[9,61].

Seventy-five to eighty-five percent of the distilleries operate on a fed batch process[9,23]. The tanks used in Brazil range from 0.5 to 3.0 million liters[9,13,23]. Filling the fermenter often takes four to six hours.[61]. The total sugars added ranges from 16 to 22 % but to minimize osmotic stress, the feeding rate is managed so that the level in the tank is typically kept around 6% [37,66,71]. Elements of the fed batch process which mitigate contamination include the acid wash, fast fermentation time and cleaning of the fermenters between batches. Continuous fermentation facilities in Brazil have greater issues with contamination compared to fed batch fermentations [23] which is consistent with other industrial continuous fermentations[72][22,38]. This can be due in part to the lack of downtime which allows regular cleaning of the fermenters.

2.5 Approaches to controlling bacterial contamination in bioethanol

Bioethanol producers employ several approaches to combat bacterial contamination including: acid washing the yeast[62,73], optimization of facility and process design[51], antibiotics[16,74], and less common approaches such as bacteriophages[10], bacteriocins and lytic enzymes are being explored[75]. The acid wash step employed in fed batch fermentation in Brazil is described above. Penicillin, virginiamycin and monensin which have been shown to kill Gram-positive bacteria are the primary antibiotics used by the bioethanol industry[16,76,77]. As antibiotic resistance continues to increase, research into alternative methods has included bacteriophages, lysozyme and other lytic enzymes, engineered yeast strains and bacteriocins.

Antibiotics may be used either to address a severe infection[23,33] or proactively by ethanol producers[6,26]. The beta-lactam penicillin is widely used in both the US and Brazil. The typical dose ranges from 1-2 ppm per dose[16,33,72]. Acid penicillin or penicillin G may be used. Penicillin degrades quickly in fermentation conditions[16,77] which is results in less risk of residue in co-products but also limits the bacteriocidal effect. One study suggests adding pulses of penicillin in different amounts will counter the risk of loss of efficacy and also may have less risk of increasing antibiotic resistant bacteria[60]. Virginiamycin is an antibiotic which inhibits protein synthesis. Early studies looked at the impact of virginiamicin on wheat mash[77]. Virginiamycin is used particularly in corn ethanol fermentations at concentrations up to 6 ppm[26]. It is more stable which increases antibacterial activity but also increases residue in DDGS[26,31,32]. There is also some evidence that higher than recommended levels negatively impact *S. cerevisiae*[61,62] which could be a more of a problem with the cell recycling model used in Brazil. Monesin, an ionophore, is an alternative to penicillin deployed in Brazil [16,51]. It is soluble in ethanol but not in water and may be used in doses up to 3 ppm[16].

Nisin, a lantibiotic antimicrobial peptide has been proven effective against LAB in food applications and is generally recognized as safe in certain food and beverages. Lantibiotics contain uncommon amino acids and have unique structures with multiple rings. This small cationic peptide is produced by *Lactococcus lactis* and is considered a class I lantibiotic because it undergoes several post translational modifications prior to reaching the active form. Nisin has been explored as a means of controlling beer contaminants. A few studies have done preliminary investigations on the efficacy of nisin in biofuel in lab conditions[41,64,78]. One study determined the MIC of nisin for *L. fermentum, L. plantarum* and *W. confusa* in MRS broth and evaluated the impact of nisin on CO2 production by *S. cerevisiae* during fermentation. Another study used co-cultures of LAB and *S. cerevisiae* to study the effect of nisin. These assays used a YP glucose substrate and lasted 72 hours[41], so are not readily translatable to conditions of bioethanol production in Brazil. Another study looked at nisin in sugarcane but did not use co-cultures of yeast and bacteria[64].

Bacteriophages and phage endolysins have also been explored as possible weapons against Gram-positive contaminants[10,79]. Bacteriophages isolated from ethanol plant *L. fermentum* isolates reduced contaminant levels in corn mash using an MOI of 10 when initial inoculums of yeast and bacteria were at 10^7 CFU/ml [10]. Of course, this requires the producer to have bacteriophages specific for the contaminant prior to infection. Enzymes from bacteriophages show promise as an alternative treatment including LysA and LysA2 because they have a suitable target range [80,81].

2.6 Future Directions

In the future other methods to control ethanol contaminants could include engineering yeast to express antimicrobials, including bacteriocins[41] or endolysins[80,82]. Since nisin is stable

and active at lower pH, adding it to the yeast wash step may allow decreased use of sulfuric acid and or shorting wash times. Finally, outcompeting deleterious contaminants by using ethanol facility probiotic cultures could be explored.

2.7 References

1. USDA. THE ECONOMIC FEASIBILITY OF ETHANOL PRODUCTION FROM SUGAR IN THE UNITED STATES. 2006.

2. Renewable Fuels Association. Fueling a High Octane Future, 2016 Ethanol Industrial Outlook. Washington, DC: Renewable Fuels Association; 2016.

3. Andrietta MGS, Andrietta SR, Steckelberg C, Stupiello ENA. Bioethanol - Brazil, 30 years of Proálcool. Int. Sugar J. 2007;109:195–200.

4. The Brazilian Sugarcane Industry Association (UNICA). Sugarcane Industry in Brazil. Sao Paulo Brazil: UNICA; 2015 Dec.

5. Wang Z, Dunn JB, Han J, Wang MQ. Influence of corn oil recovery on life-cycle greenhouse gas emissions of corn ethanol and corn oil biodiesel. Biotechnol. Biofuels. 2015;8.

6. Lyons TP. Ethanol around the world: rapid growth in policies, technology and production. In: Jacques KA, Ingledew WM, editors. Alcohol Textb. Ref. Beverage Fuel Ind. Alcohol Ind. 4. ed. Nottingham: Nottingham Univ. Press; 2003. p. 1–7.

7. Stroppa CT, Andrietta MGS, Andrietta SR, Steckelberg C, Serra GE. Use of penicillin and monensin to control bacterial contamination of Brazilian alcohol fermentations. Int. Sugar J. 2000;102:78–82.

8. De Alwis H, Heller DN. Multiclass, multiresidue method for the detection of antibiotic residues in distillers grains by liquid chromatography and ion trap tandem mass spectrometry. J. Chromatogr. A. 2010;1217:3076–84.

9. Skinner K, Leathers TD. Bacterial contaminants of fuel ethanol production. J. Ind. Microbiol. Biotechnol. 2004;31:401–8.

10. Amorim HV, Gryschek M, Lopes ML. The success and sustainability of the Brazilian sugarcane−fuel ethanol industry. Sustain. Sugar Sugar Ethanol Ind. Washington, D.C.: American Chemical Society; 2010. p. 73–82.

11. de Souza RB, dos Santos BM, de Fátima Rodrigues de Souza R, da Silva PKN, Lucena BTL, de Morais MA. The consequences of *Lactobacillus vini* and *Dekkera bruxellensis* as

contaminants of the sugarcane-based ethanol fermentation. J. Ind. Microbiol. Biotechnol. 2012;39:1645–50.

12. Schwan RF, Mendonça AT, da Silva Jr JJ, Rodrigues V, Wheals AE. Microbiology and physiology of Cachaça (Aguardente) fermentations. Antonie Van Leeuwenhoek. 2001;79:89–96.

13. Li Q, Heist EP, Moe LA. Bacterial Community Structure and Dynamics During Corn-Based Bioethanol Fermentation. Microb. Ecol. 2016;71:409–21.

14. Montville TJ, Matthews KR, Kniel KE. Food microbiology: an introduction. 3rd ed. Washington, DC: ASM Press; 2012.

15. Narendranath N V. Bacterial contamination and control in ethanol production. Alcohol Textb. Ref. Beverage Fuel Ind. Alcohol Ind. 4th ed. Nottingham: Nottingham University Press; 2003. p. 287–97.
16. GODOY A, AMORIM H V., LOPES ML, OLIVEIRA AJ. Continuous and batch fermentation processes : Advantages and disadvantages of these processes in the Brazilian ethanol production. Int. Sugar J. Informa Healthcare; 110.

17. Costa OYA, Souto BM, Tupinambá DD, Bergmann JC, Kyaw CM, Kruger RH, et al. Microbial diversity in sugarcane ethanol production in a Brazilian distillery using a culture-independent method. J. Ind. Microbiol. Biotechnol. 2015;42:73–84.

18. Beckner M, Ivey ML, Phister TG. Microbial contamination of fuel ethanol fermentations: Bioethanol contamination. Lett. Appl. Microbiol. 2011;53:387–94.

19. Lucena BT, dos Santos BM, Moreira JL, Moreira APB, Nunes AC, Azevedo V, et al. Diversity of lactic acid bacteria of the bioethanol process. BMC Microbiol. 2010;10:298.

20. Gallo CR. Determinação da microbiota bacteriana de mosto e de dornas na fermentação alcoolica. [Campinas:]: Faculdade de Engenharia de Alimentos; 1990.

21. Bischoff KM, Skinner-Nemec KA, Leathers TD. Antimicrobial susceptibility of Lactobacillus species isolated from commercial ethanol plants. J. Ind. Microbiol. Biotechnol. 2007;34:739–44.

22. Schell DJ, Dowe N, Ibsen KN, Riley CJ, Ruth MF, Lumpkin RE. Contaminant occurrence, identification and control in a pilot-scale corn fiber to ethanol conversion process. Bioresour. Technol. 2007;98:2942–8.

23. Rich JO, Leathers TD, Bischoff KM, Anderson AM, Nunnally MS. Biofilm formation and ethanol inhibition by bacterial contaminants of biofuel fermentation. Bioresour. Technol. 2015;196:347–54.

24. De MAN JC, Rogosa M, Sharpe ME. A MEDIUM FOR THE CULTIVATION OF LACTOBACILLI. J. Appl. Bacteriol. 1960;23:130–5.

25. Li Q, Heist EP, Moe LA. Bacterial Community Structure and Dynamics During Corn-Based Bioethanol Fermentation. Microb. Ecol. 2016;71:409–21.

26. R. Shane Gold a, M.M. Meagher a'b, R. Hutkins a and T. Conway c. Ethanol tolerance and carbohydrate metabolism in lactobacilli*. J. Ind. Microbiol. 1992;

27. Malang SK, Maina NH, Schwab C, Tenkanen M, Lacroix C. Characterization of exopolysaccharide and ropy capsular polysaccharide formation by *Weissella*. Food Microbiol. 2015;46:418–27.

28. Rich JO, Leathers TD, Nunnally MS, Bischoff KM. Rapid evaluation of the antibiotic susceptibility of fuel ethanol contaminant biofilms. Bioresour. Technol. 2011;102:1124–30.

29. Murphree C a., Heist EP, Moe L a. Antibiotic Resistance Among Cultured Bacterial Isolates from Bioethanol Fermentation Facilities Across the United States. Curr. Microbiol. 2014;69:277–85.

30. Oliva-Neto P de, Yokoya F. Susceptibility of *Saccharomyces cerevisiae* and lactic acid bacteria from the alcohol industry to several antimicrobial compounds. Braz. J. Microbiol. 2001;32:10–4.

31. Basso TO, Gomes FS, Lopes ML, de Amorim HV, Eggleston G, Basso LC. Homo- and heterofermentative lactobacilli differently affect sugarcane-based fuel ethanol fermentation. Antonie Van Leeuwenhoek. 2014;105:169–77.

32. Ramos CL, Duarte WF, Freire AL, Dias DR, Eleutherio ECA, Schwan RF. Evaluation of stress tolerance and fermentative behavior of indigenous *Saccharomyces cerevisiae*. Braz. J. Microbiol. 2013;44:935–44.

33. Narendranath N V., Hynes SH, Thomas KC, Ingledew WM. Effects of lactobacilli on yeast-catalyzed ethanol fermentations. Appl. Environ. Microbiol. 1997;63:4158–63.

34. Narendranath NV, Hynes SH, Thomas KC, Ingledew WM. Effects of lactobacilli on yeast-catalyzed ethanol fermentations. 1997;63(11):4158-4163. Appl. Environ. Microbiol. 1997;63:4158–63.

35. Narendranath N V., Power R. Effect of yeast inoculation rate on the metabolism of contaminating lactobacilli during fermentation of corn mash. J. Ind. Microbiol. Biotechnol. 2004;31:581–4.

36. Peng J, Zhang L, Gu Z-H, Ding Z-Y, Shi G-Y. The role of nisin in fuel ethanol production with *Saccharomyces cerevisiae*: Nisin and fuel ethanol production. Lett. Appl. Microbiol. 2012;55:128–34.

37. Boone DR, Castenholz RW, Garrity GM. Bergey's manual of systematic bacteriology. New York: Springer; 2001.

38. Bischoff KM, Liu S, Leathers TD, Worthington RE, Rich JO. Modeling bacterial contamination of fuel ethanol fermentation. Biotechnol. Bioeng. 2009;103:117–22.

39. Campbell MK, Farrell SO. Biochemistry. 6th ed. Australia ; United States: Thomson/Brooks/Cole; 2009.

40. Lambert RJ, Stratford M. Weak-acid preservatives: modelling microbial inhibition and response. J. Appl. Microbiol. 1999;86:157–64.

41. Basso LC, de Amorim H V., de Oliveira AJ, Lopes ML. Yeast selection for fuel ethanol production in Brazil. FEMS Yeast Res. 2008;8:1155–63.

42. Amorim H V., Lopes ML, de Castro Oliveira JV, Buckeridge MS, Goldman GH. Scientific challenges of bioethanol production in Brazil. Appl. Microbiol. Biotechnol. 2011;91:1267–75.

43. Dorta C, Oliva-Neto P, de -Abreu-Neto MS, Nicolau-Junior N, Nagashima AI. Synergism among lactic acid, sulfite, pH and ethanol in alcoholic fermentation of *Saccharomyces cerevisiae* (PE-2 and M-26). World J. Microbiol. Biotechnol. 2006;22:177–82.

44. Narendranath N V., Thomas KC, Ingledew WM. Effects of acetic acid and lactic acid on the growth of *Saccharomyces cerevisiae* in a minimal medium. J. Ind. Microbiol. Biotechnol. 2001;26:171–7.

45. Giannattasio S, Guaragnella N, Ždralević M, Marra E. Molecular mechanisms of *Saccharomyces cerevisiae* stress adaptation and programmed cell death in response to acetic acid. Front. Microbiol. 2013;4.

46. Ludovico P, Leão C, Sousa MJ, Côrte-Real M, Silva MT. *Saccharomyces cerevisiae* commits to a programmed cell death process in response to acetic acid. Microbiology. 2001;147:2409–15.

47. Lucena BT, dos Santos BM, Moreira JL, Moreira APB, Nunes AC, Azevedo V, et al. Diversity of lactic acid bacteria of the bioethanol process. BMC Microbiol. 2010;10:298.

48. Liu M, Bischoff KM, Gill JJ, Mire-Criscione MD, Berry JD, Young R, et al. Bacteriophage application restores ethanol fermentation characteristics disrupted by *Lactobacillus fermentum*. Biotechnol. Biofuels. 2015;8.

49. Godoy A, Amorim HV, Lopes ML, Oliveira AJ. Continuous and batch fermentation processes : Advantages and disadvantages of these processes in the Brazilian ethanol production. Int. Sugar J. 2008;110:175–81.

50. Amorim HV, Lopes ML, Oliveira JV de C, Buckeridge MS, Goldman GH. Scientific challenges of bioethanol production in Brazil. Appl. Microbiol. Biotechnol. 2011;91:1267–75.

51. Della-Bianca BE, Gombert AK. Stress tolerance and growth physiology of yeast strains from the Brazilian fuel ethanol industry. Antonie Van Leeuwenhoek. 2013;104:1083–95.

52. Basso LC, de Amorim HV, de Oliveira AJ, Lopes ML. Yeast selection for fuel ethanol production in Brazil. FEMS Yeast Res. 2008;8:1155–63.

53. Amorim HV, Lopes ML, de Castro Oliveira JV, Buckeridge MS, Goldman GH. Scientific challenges of bioethanol production in Brazil. Appl. Microbiol. Biotechnol. 2011;91:1267–75.

54. Lopes ML, Paulillo SC, Cherubin R, Godoy A, Amorim H V., Neto HB de A. Tailored yeast strains for ethanol production: The process driven selection. First. Piracicaba: Fermentec Tecnologias em Azucar e Alcool Ltda; 2015.

55. Boinot F. Alcoholic fermentation of sugar containing liquids. France; 1936. p. 3.

56. Amorim HV, Gryschek M, Lopes ML. The success and sustainability of the Brazilian sugarcane−fuel ethanol industry. Sustain. Sugar Sugar Ethanol Ind. [Internet]. Washington, D.C.: American Chemical Society; 2010 [cited 2016 Jan 16]. p. 73–82. Available from: http://pubs.acs.org/doi/pdf/10.1021/bk-2010-1058.ch005

57. The Brazilian Sugarcane Industry Association (UNICA). Sugarcane Industry in Brazil [Internet]. Sao Paulo Brazil: UNICA; 2015 Dec p. 20. Available from: http://sugarcane.org/resource-library?b_start:int=30

58. Andrietta MGS, Andrietta SR, Steckelberg C, Stupiello ENA. Bioethanol - Brazil, 30 years of Proálcool. Int. Sugar J. 2007;109:195–200.

59. Della-Bianca BE, Basso TO, Stambuk BU, Basso LC, Gombert AK. What do we know about the yeast strains from the Brazilian fuel ethanol industry? Appl. Microbiol. Biotechnol. 2013;97:979–91.

60. Chang IS, Kim BH, Shin PK. Use of sulfite and hydrogen peroxide to control bacterial contamination in ethanol fermentation. Appl. Environ. Microbiol. 1997;63:1–6.

61. Bassi APG, Silva JCG da, Reis VR, Ceccato-Antonini SR. Effects of single and combined cell treatments based on low pH and high concentrations of ethanol on the growth and fermentation of *Dekkera bruxellensis* and *Saccharomyces cerevisiae*. World J. Microbiol. Biotechnol. 2013;29:1661–76.

62. Franchi MA, Serra GE, Cristianini M. The Use of Biopreservatives in the Control of Bacterial Contaminants of Sugarcane Alcohol Fermentation. J. Food Sci. 2003;68:2310–5.

63. Brown NA, de Castro PA, de Castro Pimentel Figueiredo B, Savoldi M, Buckeridge MS, Lopes ML, et al. Transcriptional profiling of Brazilian *Saccharomyces cerevisiae* strains selected for semi-continuous fermentation of sugarcane must. FEMS Yeast Res. 2013;13:277–90.

64. Amorim H V., Basso LC, Lopes ML. Sugar cane juice and molasses, beet molasses and sweet sorghum: composition and usage. In: Ingledew WM, editor. Alcohol Textb. Ref. Beverage Fuel Ind. Alcohol Ind. 5. ed. Nottingham: Nottingham Univ. Press; 2009. p. 39–46.

65. Kim M, Day DF. Composition of sugar cane, energy cane, and sweet sorghum suitable for ethanol production at Louisiana sugar mills. J. Ind. Microbiol. Biotechnol. 2011;38:803–7.

66. Legendre BL. Varietal Differences in the Chemical Composition of Sugarcane. Elsevier; 1988. p. 176–85.

67. El-Gendy NS, Madian HR, Amr SSA. Design and Optimization of a Process for Sugarcane Molasses Fermentation by Saccharomyces cerevisiae Using Response Surface Methodology. Int. J. Microbiol. 2013;2013:1–9.

68. Converti A, Arni S, Sato S, de Carvalho JCM, Aquarone E. Simplified modeling of fed-batch alcoholic fermentation of sugarcane blackstrap molasses. Biotechnol. Bioeng. 2003;84:88–95.

69. Della-Bianca BE, Gombert AK. Stress tolerance and growth physiology of yeast strains from the Brazilian fuel ethanol industry. Antonie Van Leeuwenhoek. 2013;104:1083–95.

70. Bayrock DP, Thomas KC, Ingledew WM. Control of Lactobacillus contaminants in continuous fuel ethanol fermentations by constant or pulsed addition of penicillin G. Appl. Microbiol. Biotechnol. 2003;62:498–502.

71. Gallo CR. Determinação da microbiota bacteriana de mosto e de dornas na fermentação alcoolica [Internet]. [Campinas:]: Faculdade de Engenharia de Alimentos; 1990. Available from: http://www.bibliotecadigital.unicamp.br/document/?code=000038046

72. Islam M, Toledo R, Hamdy M. Stability of virginiamycin and penicillin during alcohol fermentation. Biomass Bioenergy. 1999;17:369–76.

73. Kim J-S, Daum MA, Oh E-J, Jin Y-S, Miller MJ. Submitted for Review: Yeast derived LysA2 can control bacterial contamination in ethanol fermentation. Biotechnol. Biofuels. 2016;

74. Bayrock DP, Thomas KC, Ingledew WM. Control of Lactobacillus contaminants in continuous fuel ethanol fermentations by constant or pulsed addition of penicillin G. Appl. Microbiol. Biotechnol. 2003;62:498–502.

75. Hynes SH, Kjarsgaard DM, Thomas KC, Ingledew WM. Use of virginiamycin to control the growth of lactic acid bacteria during alcohol fermentation. J. Ind. Microbiol. Biotechnol. 1997;18:284–91.

76. Müller-Auffermann K, Grijalva F, Jacob F, Hutzler M. Nisin-producing microorganisms and their implementation in brewers' wort: Nisin production in brewer's wort. J. Inst. Brew. 2015;121:320–31.

77. Cui F-X, Zhang R-M, Liu H-Q, Wang Y-F, Li H. Metabolic responses to *Lactobacillus plantarum* contamination or bacteriophage treatment in *Saccharomyces cerevisiae* using a GC–MS-based metabolomics approach. World J. Microbiol. Biotechnol. 2015;31:2003–13.

78. Khatibi PA, Roach DR, Donovan DM, Hughes SR, Bischoff KM. *Saccharomyces cerevisiae* expressing bacteriophage endolysins reduce *Lactobacillus* contamination during fermentation. Biotechnol. Biofuels. 2014;7:104.

79. Ribelles P, Rodríguez I, Suárez J. LysA2, the *Lactobacillus casei* bacteriophage A2 lysin is an endopeptidase active on a wide spectrum of lactic acid bacteria. Appl. Microbiol. Biotechnol. 2012;94:101–10.

80. Kim J-S, Daum MA, Oh E-J, Jin Y-S, Miller MJ. Submitted for Review: Yeast derived LysA2 can control bacterial contamination in ethanol fermentation. 2016;

CHAPTER 3 NISIN AND PENICILLIN REDUCE LACTIC ACID BACTERIA CONTAMINANTS IN MODEL SUGARCANE ETHANOL FERMENTATIONS

3.1 Abstract

Bioethanol from sugarcane has a lower carbon footprint than petroleum based fuels but the industry is plagued by various economic pressures, including product loss due to contamination of fermentation facilities. *Lactobacillus* species and other lactic acid producing bacteria from the feedstock and environment are important bacterial contaminants. Further studies focused on process optimization and validated the use of antibiotics and acid treatment to reduce ethanol losses due to bacterial contamination. Monensin, penicillin and virginiamycin have been shown to reduce bacterial counts in bioethanol but increased incidence of antibiotic resistant bacteria means alternative approaches are needed. To test efficacy of the antimicrobials hen egg white lysozyme, nisin and the combination of nisin and penicillin against representative contaminants a model system was developed. To mimic the typical Brazilian conditions, the model incorporates high initial yeast inoculum, fermentation times of twelve hours or less and final ethanol concentration of 5.5 to 6 % weight/volume. The impact of penicillin and nisin on bacteria isolated from sugarcane juice or adapted by successive passes in sugarcane juice in fermentation of sugarcane juice by *Saccharomyces cerevisiae* JAY291 was measured by counting the bacteria and yeast. In addition, ethanol and lactic acid titers were measured and by HPLC analysis of the fermentate. Plate counts revealed that the combination of 250 ppm of nisin and 2 ppm penicillin was the most effective treatment against *Lactobacillus plantarum*, as well as against a mix of *L. plantarum* and four other bacterial species. Combining nisin with penicillin could decrease the amount of penicillin used to control contamination and mitigate the risk of antibiotic resistant bacteria proliferating. This model system can be employed for testing

other novel antibacterial measures such as bacteriophages, or engineered phage endolysins and bacteriocins.

3.2 Introduction

Biofuel fermentations are particularly at risk from Lactic Acid Bacteria (LAB) contamination because the plants which provide the fermentation substrate are also natural habitats for LAB. Since the ethanol is for transportation rather than consumption, the fermentation substrate is rarely subjected to bacteriostatic or bactericidal treatment. Gram-positive bacteria from the *Lactobacillaceae* family including *Lactobacillus, Pediococcus, Leuconostoc, Enterococcus*, *Aerococcus* and *Weisella* have been implicated in ethanol inhibition contaminants[1–4]. These bacteria, which produce primarily lactic acid from carbohydrate sources, have adapted to conditions with comparatively low pH and oxygen and high salt and ethanol concentrations. Consequently, they have been a recurring problem in wine, beer and spirit fermentations [5–7]. The predominant genera of LAB identified is *Lactobacillus,* regardless of whether the fermentation is from corn [1, 4]sugarcane[3, 8] or lignocellulose[9]. Treatments to control bacterial contamination of fuel ethanol fermentation must include measures against lactobacilli and other LAB to be effective.

Currently, antibiotics, acid treatment of the yeast and cleaning of equipment are the most common measures employed in industry. Acid-washing stresses the fermenting yeast and can lead to decreased ethanol production efficiency [10]. Stopping production for additional cleaning leads to loss in productivity. US fuel ethanol plants use penicillin and virginiamycin [11, 12] while Brazilian plants are more likely to use monensin [13, 14] although the efficacy of the antibiotic treatments has been challenged. Additional complications from antibiotic use are

unacceptably high antibiotic residues in the dried distillers grain solids[15] or dried deactivated yeasts[16] sold as co-products by ethanol producers and increased development of antibiotic resistance in the contaminating strains[2].

In this study, a model system using sugarcane juice as the source of carbohydrates was developed. Supplementing the sugarcane juice with 0.6% yeast extract provided a source of nitrogen and amino acids. Due to the yeast cell recycling and sulfuric acid wash, dead yeast cells are a normal component of the fermenters in Brazil[14, 17]. The addition of the yeast extract improved bacterial and *S. cerevisiae* growth. Optimization of a pulsed fed batch method resulted in fermentations with final ethanol concentrations greater than 5% produced in 12 hours by the industrial derivative *S. cerevisiae* JAY291. In the spot on lawn assay which assessed antimicrobial action against growing LAB at the concentrations tested, nisin inhibited every lysozyme inhibited ten of the strains and nisin inhibited all of the strains. Due to its broad spectrum, nisin was tested in simulated contaminated fermentations of sugarcane juice first with a single LAB then with a Mix of five LAB. Nisin alone and in combination with penicillin resulted in reduced CFU/ml of LAB in conditions similar to Brazilian bioethanol fermentations.

3.3 Materials and methods

Strains and growth conditions

The LAB and *S. cerevisiae* strains used in this study are listed in Table 3.1 and Table 3.2 respectively. Stock cultures were preserved at -80°C in 12.5% glycerol (v/v). From frozen stock, cultures were inoculated in de Mann, Rogosa, Sharpe (MRS) broth (Difco, Sparks, MD) with the exception of: *Lactococcus lactis* grown anaerobically at 30° C in M17 (Oxoid) supplemented with 10 g/L of lactose and *Pediococcus damnosus* grown in B-MRS(1:1 filtered

Natural Light beer and MRS). *Aerococcus viridans* was incubated in 6 ml aerobically at 37 °C and the remaining strains were incubated in 3 ml of media anaerobically at 30°C.

Analysis of growth

Growth patterns of strains in different media were evaluated by measuring the optical density at 600 nm using a Bioscreen C (Growth Curves USA, Piscataway, NJ, USA). Strains were evaluated in: MRS, MRS-6, yeast peptone dextrose (YPD); YPD-6, Sugarcane juice diluted to 6% sugars (SC-6), and SC-6 with 0.6% yeast extract (YSC -6). YPD was prepared with 10 grams/L of yeast extract (BD Difco Sparks MD USA), 20 g/L of both Bacto peptones (BD Difco, Sparks MD USA) and dextrose (Sigma Aldrich St. Louis, MO). MRS-6 and YPD-6 were prepared and autoclaved without the additional sugars then the concentration of dextrose was adjusted using a separately autoclaved solution of 50% dextrose and water. SC-6 was prepared from clarified sugarcane juice by centrifuging the juice at 4 °C for 45 minutes, drawing off the juice from the pelleted solids, checking the total reducing sugars by HPLC, then diluting to 6% sugars with autoclaved pure water. YSC-6 was prepared the same as SC-6 but diluted with autoclaved water with 6 g/L yeast extract. The pH of all media used was adjusted to 5.5 using dilute HCl, then filtered using a 0.22 nm Millipore bottle-top filter. Strains were resuscitated from frozen stock and grown 24 hours in the conditions and media described above and passed twice. The third pass was a 1% inoculation into the growth medium being evaluated. The third pass was incubated anaerobically for 18 hours at 30° C. Fresh media of interest was inoculated from the 3rd pass and adjusted to an OD of 0.1 using an YSI 9500 photometer. Triplicate samples of 200 µl of each strain in each media tested and media only controls were pipetted into the well plate then loaded into the Bioscreen. The change in OD at 600 nm was measured every half hour for up to 72 hours anaerobically at 30° C with shaking. Regression analysis of growth curves

was done using the MATLAB script and protocol as previously described by Hoeflinger et al., 2015[18] except the threshold for growth was set at OD 0.5.

Isolation of bacteria from clarified sugarcane juice

Clarified sugarcane juice from Patout, Louisiana (pH 7.0 or higher) was plated on MRS or MRS with cycloheximide[19] (200 μg/ml) (MRS-C) plates with 1.5% agar, or directly plated on MRS. One hundred microliters of sugarcane juice diluted 1:9 in phosphate buffered saline (PBS) (MP Biomedicals, Santa Ana, CA, USA), or undiluted sugarcane juice was spread-plated then incubated at 30 °C in an anaerobic chamber for 24 to 72 hours. Morphology and acid production were checked. DNA was purified using the Qiagen DNeasy® Blood & Tissue kit (Qiagen N.V. Venlo, The Netherlands) according to the manufacturer's instructions for Gram-positive bacteria. The primers 8F and 1392R were used for PCR amplification of the 16-S rDNA region. Products were gel confirmed, cleaned with the Zymo DNA Clean & Concentrator Kit (Zymo Research) and then sent for Sanger sequencing. Sequences were checked and identity confirmed using NCBI BLAST-N, with 99% identity considered a match[20].

Adaptation of LAB in sugarcane

Select LAB were successively passed in YSC-6 to promote selection of isolates with faster growth in sugarcane juice based substrate. Strains were initially inoculated from frozen stock and resuscitated in MRS anaerobically at 30°C for 24 hours. Three ml of YSC-6 was inoculated with 1% of the initial MRS culture. Strains were incubated in YSC-6 for 24 hours at 30°C. After 24 hours the OD was recorded using the YSI 9500 photometer and 1% of culture in YSC-6 was used to inoculate 3 ml of fresh YSC-6. This was repeated daily for at least 7 days. After 7 days the growth was compared to the original strain using the above described Bioscreen

protocol. The adapted strains were streaked on MRS, and colonies selected inoculate fresh YSC-6, grown 24 hours then used to make glycerol stock of the adapted strains.

Sensitivity of growing bacteria to nisin, lysozyme or mutanolysin

A spot on lawn assay modified from the method described by Nelson et al., 2012 [21] and Redondo et al.,[22] was employed to test the impact of lysozyme, mutanolysin and nisin on growing cells. Bacterial strains were inoculated in fresh media from glycerol stock then passed two times. The third pass was used for the spot on lawn assay. When the cultures were between 0.2 to 0.6 OD at 600 nm, 300 µl of culture was pipetted onto a MRS plate with 1.5% agar, spread and then allowed to dry for up to one hour. Nisaplin (Dupont, St. Louis MO USA) containing 2.5% nisin was hydrated in dilute HCl at pH 2.0. Mutanolysin was hydrated using pure water. Hen egg white lysozyme solution from Sigma was used (50% glycerol in in 25 mM sodium acetate buffer, Sigma Aldrich St. Louis, MO 10 mg/ml). Dilutions of the antimicrobials were made 1:1 using pure water or dilute HCl for nisin and 2 µl of decreasing concentrations of antimicrobial were pipetted onto the plate with the freshly spread bacteria. Plates were incubated anaerobically at 30°C, except for *A. viridans* which was incubated aerobically at 37°C. Plates were incubated 24-72 hours depending on the growth rate of the strain and complete clearing was regarded as an indication of sensitivity. Water diluted to pH 2.0 with HCl was used as a negative control for nisin assays.

Fermentation in sugarcane media

Bacteria and *S. cerevisiae* JAY291 were resuscitated from frozen stock and passed one time in MRS as previously described. The second pass was into 3 ml of YSC-6 and incubation was anaerobic at 30° C for 16-18 hours. Bacteria were incubated anaerobically without agitation at 30°C. The first two passes were one 1% vol/vol inoculations and the remaining pass(es) were

10% vol/vol inoculation for both yeast and bacteria. The pH of all sugarcane juice and media except for MRS was adjusted to pH 5.5 with HCl and filtered using a bottle-top 0.22 nm pore Millipore bottle-top filter (Millipore Sigma, Billerica, MA USA). For JAY291 the third pass was into 300 ml of 10 g/L yeast extract, 20 g/L peptones and 60 g/L sucrose (YPS-6). JAY291 was incubated aerobically at 30°C at 150 rpm. The third pass for LAB was into YSC-10. The day of the fermentation, 100 ml of fresh YPS-6 is added to the yeast. A 10% inoculation of the LAB into YSC-6 was incubated anaerobically for 3 hours at 30°C to reach early log phase. Cells were harvested by centrifuged at 4000 x g for 20 minutes (bacteria) or 30 minutes (yeast). The yeast were re-suspended in water to wash and centrifuged again. LAB were re-suspended in YSC-6 in an amount sufficient to provide the target initial inoculation. JAY291 was re-suspended in 0.1 ml autoclaved water per ml of yeast in the pellet. Twenty ml test tubes autoclaved with stir bars were used as fermentation vessels. The initial volume was 3.0 ml comprised of YSC-6, 0.3 ml of yeast and LAB or antimicrobials as appropriate.

Nisin and penicillin were re-suspended in YSC-6 immediately prior to use and filtered through a 0.45 nm filter. Magnetic stir plates were used to agitate the mixtures containing yeast in an anaerobic chamber at 30°C for 12 hours. LAB only and media only controls were not stirred. At 0.5 and 2 hours after starting the fermentation, pulses of undiluted sugarcane juice were added to double the volume, resulting in a final volume of 12 ml. Initial samples and post fermentation samples were diluted in PBS for plating. LAB were counted from MRS-C plates and *S. cerevisiae* JAY291 was counted on MRS plates with 5 μg/ml penicillin G (MRS-P). Ten μl drops of the dilutions were plated as described previously [23] modified instead to use three dilutions per plate and three drops per dilution for duplicate plates of each sample. The LAB in the mix were distinguished based on colony morphology, which was further confirmed by

morphology in broth or by streaking representative colonies on MRS. Fermentate samples were analyzed via on an Agilent Technologies 1260 Infinity HPLC (Agilent, Santa Clara, CA, USA) using the Biorad HPX-87H (Biorad, Hercules CA, USA) organic acid column as described previously[24]. Duplicate plates from each sample were averaged for calculating the means. Analysis of variance with Tukey's was calculated using R statistical software. In the figures, bars with the same letter had P values > than 0.05 when compared and bars labeled with different letters had P values < 0.05 when compared.

3.4 Results

Sugarcane fermentation model system development

To study the impact of antimicrobials in sugarcane bioethanol fermentation, a model system was needed. Key components of the system included: *S. cerevisiae* and LAB which thrived on sugarcane juice, antimicrobials with a broad spectrum and fermentation conditions which produced more than 5% ethanol and utilization of 99.5% sugars within twelve hours.

Growth in sugarcane juice

Sugarcane juice contains sucrose and lesser amounts of the component monosaccharides glucose and fructose[25, 26]. There are trace amounts of minerals, lipids and gums, but amino acids and nitrogen are scarce. To grow well on sugarcane juice an organism needs to be able to easily utilize sucrose and survive in a nutrient limited medium. The initial yeast strain tested *S. cerevisiae* D452-2, is an auxotroph (MAT α leu2 his3 ura3 can1)[27] and grows very slowly on sugarcane juice even when the osmotic pressure is reduced by diluting the total sugars to 6% (SC-6) Figure 3.1(a). Adding 0.6% yeast extract to the sugarcane juice (YSC-6) improved growth by D452-2, Figure 3.1(a) but fermentation was still slow. As an alternative, *S. cerevisiae*

JAY291 was evaluated. JAY291 grew well in SC-6, Figure 3.1(b) and in YSC-6 doubling time decreased and maximum OD increased, Table 3.3.

Wild-type bacteria strains grew poorly in SC-6 as well, Figure 3.2(a). The addition of the 0.6% yeast extract (YSC-6) resulted in improved growth rates for all LAB strains evaluated and increased the maximum OD from 0.3 to 0.8 Figure 3.2(b). However, since this was half of the maximum OD of JAY291, further steps were pursued to obtain competitive contaminants.

Isolates from clarified sugarcane juice and adaptation of LAB strains

To strengthen the pool of contaminants, LAB were isolated from clarified sugarcane juice obtained from Louisiana. Isolates included *Enterococcus gallinarum, Weissella cibaria* and *Lactobacillus plantarum. L. plantarum*, one of our sugarcane isolates grew faster than our wild-type strains, Figure 3.3. Also, from the initial group of potential contaminants, wild-type strains from species shown to inhibit ethanol production by *S. cerevisiae* were screened for growth in ethanol. Based on good relative growth in MRS with 10% ethanol evaluated as described previously[28] *L. brevis, L. delbrueckii, L. fermentum, L. plantarum* and *W. confusa* were selected to adapt for better growth in the sugarcane juice supplemented with 0.6% g/L yeast extract with sugar concentrations of 6% (YSC-6) and 10% (YSC-10). *L. plantarum*, the sugarcane isolate, was also successively passed in YSC-6 and YSC-10. Nine successive passes of *L. paracasei* and 27 passes of *L. plantarum* produced strains which grew to a higher maximum OD in YSC-6. The *W. confusa* strain did not significantly change maximum OD but successive passes in YSC-10 and YSC-6 resulted in increased production of exopolysaccharides anaerobically at 30 °C. These polysaccharides visibly increased the viscosity of the media. In the thickest samples, vortexing did not disrupt the surface tension of the culture.

Spot on lawn antimicrobial assay:

With improved contaminants and a *S. cerevisiae* strain well suited for sugarcane fermentation, the next step was selection of the antimicrobial to test in fermentation conditions. Hen egg white lysozyme, mutanolysin and nisin were selected based on previously demonstrated ability of all three to inhibit growth of Gram-positive bacteria[6, 17, 29–31]. The spot on lawn assay[21] which demonstrates the impact of the antimicrobials against growing cells was employed to evaluate the spectrum and check whether the yeast was negatively impacted. The *S. cerevisiae* strains were not susceptible to nisin, lysozyme or mutanolysin at any of the concentrations evaluated, Table 3.4. All 19 LAB strains tested were susceptible to nisin. A higher concentration of nisin for complete clearing of strains from a separate hard cider study and our sugarcane isolates compared to the wild-type strains, Table 3.3. Only *Aerococcus viridans* was susceptible to mutanolysin at the concentrations tested. It was also sensitive to the lowest concentrations of nisin and lysozyme tested. Lysozyme also inhibited the growth of *O. oeni* and the *E. gallinarum* strains at the lowest concentration tested, 1,250 μg/ml. Eleven strains were insensitive to lysozyme at 10,000 μg/ml, the highest concentration of any antimicrobial tested. Since less than half of the bacteria tested were susceptible to lysozyme at these high doses and all were sensitive to nisin, nisin was the antimicrobial selected for further testing in simulated contaminated fermentations.

Sugarcane juice fermentations by *S. cerevisiae* JAY291 with and without LAB contaminants

Lab scale fermentations based on the Brazilian bioethanol process were used to evaluate how nisin compared with the commonly applied penicillin against LAB contaminants. The five conditions were: untreated (Unt); nisin 250 μg/ml (250N); penicillin 2 μg/ml (2P); nisin 125 μg/ml with 1 μg/ml penicillin (125N1P); and nisin 250 μg/ml with 2 μg/ml penicillin (250N2P).

Fermentations were performed in duplicate with triplicate samples of the contaminated (co-cultures of LAB and JAY291) treatments. Media only, yeast only and bacteria only controls were also performed. JAY291 was added to YSC-6 at 10% vol/vol of the initial YSC-6 volume, providing a CFU/ml of 10^8, with a single exception. Two pulses of undiluted sugarcane juice were timed to allow the fermentation to be complete in 12 H and minimize osmotic stress by not exceeding 6-7% of total sugars. The first batch of undiluted sugarcane juice had 12.5% total sugars and the second batch contained 12.9% total sugars. For the 12.5% total sugars, when the second pulse was added at 2 hours JAY291 completed fermentation in 12 hours. After 12 hours, 0.5% or less of sugars remained and ethanol concentrations produced by untreated, uncontaminated JAY291 samples were 55 g/L or greater. Based on the literature showing levels of bacteria often reach 10^7 to 10^8 in the fermentation tanks and in sugarcane juice[3], LAB were inoculated at 10^8 for the first four fermentations. Two fermentations were contaminated with the adapted *L. plantarum* at a concentration of 10^8 CFU/ml. Another other two fermentations were contaminated with cocktail of five LAB strains with a combined CFU/ml of 10^8. The five strains were the adapted *L. plantarum, W. confusa* and *L. paracasei* as well as the hard cider isolate *L. ghanensis* and wild-type *L. fermentum* (Mix). An additional two fermentations were done with *L. plantarum* at an initial concentration of 10^9 CFU/ml to test whether increased ethanol reduction would occur. All bacteria were added at early log phase. The impact of the fermentation conditions on JAY291 and the LAB was measured by determining CFU/ml of the strains and fermentation product concentrations via HPLC and CFU/ml for all fermentations.

<u>*S. cerevisiae* JAY291 CFU/ml for all fermentations.</u>

The average starting CFU/ml for 5 of the fermentations for JAY 291 was between 1.0 and $4.0 * 10^8$. On 3-30, the first fermentation with *L. plantarum* it was $8.8 * 10^7$. When the initial

LAB inoculation level was 10^8, after the twelve hour fermentations the final *S. cerevisiae* JAY291 CFU/ml was consistently between 1.0 and 3.0 x 10^8, there was no significant difference in the average CFU/ml of JAY291 due to the different treatments. There was also no significant difference in the *S. cerevisiae* JAY291 due to the presence or absence of contaminants among and between the fermentations with the initial LAB inoculum of 10^8.

L. plantarum starting and final CFU/ml

The CFU/ml of *L. plantarum* varied considerably across the different treatments and even between the co-cultures of JAY291 and *L. plantarum* only controls. The highest CFU/ml *L. plantarum* reached was 10^9 in the untreated bacteria only controls regardless of whether the initial inoculation was 10^8 or 10^9. When co-cultured with *S. cerevisiae* JAY291, the highest CFU/ml *L. plantarum* reached the end of fermentation was 10^8, even if the initial inoculation level was 10^9, Figure 3.4.

L. plantarum contaminated fermentations initial inoculation of 10^8 - *L. plantarum* CFU/ml and fermentation products

When initial *L. plantarum* inoculation was 10^8, in the bacteria only controls the combination of 250 μg/ml of nisin with 2 μg/ml of penicillin caused 4 log CFU/ml reduction and there were significant differences between the different treatments, Figure 3.5. The second largest reduction was from 125 μg/ml of nisin with 1 μg/ml of penicillin. Because there was no significant difference in the CFU/ml reduction in the bacteria only samples when comparing the first fermentation and the second fermentation the averages of the samples from both fermentations are combined in Figure 3.5.

By contrast, in the co-cultures of *S. cerevisiae* JAY291 and *L. plantarum*, there was a significant difference in reduction of the CFU/ml of *L. plantarum* by the treatments when

comparing the two fermentations. This can be seen in Figure 3.6 which shows the log CFU/ ml reduction of *L. plantarum* for the two fermentations separately for the co-cultured samples. This difference may be due to a lower initial inoculation level of *S. cerevisiae* JAY291 in fermentation 1 (8.8×10^7) compared to fermentation 2(2.0×10^8). As noted in the bacteria only samples, the greatest reduction was caused by the 250 μg/ml of nisin with 2 μg/ml of penicillin. In fermentation one it caused a 3 log CFU/ml reduction in fermentation 1 (lilac bars) but only 1.4 log reduction in fermentation 2 (purple bars). There were significant differences between this combination and the penicillin only treatment in both fermentations.

The log CFU/ml reduction data is supported by the HPLC results. A similar pattern was seen in lactate present. Both days the penicillin only samples had the highest amount of lactate but the nisin and combination treatments had the lowest amounts of lactate. Compared to the highest average ethanol concentration, which was in the JAY291 contaminated with *L. plantarum* treated with the highest combination of nisin and penicillin, the untreated contaminated samples contained 4% less ethanol. Interestingly, although the *L. plantarum* only controls did not contain ethanol, the yeast and bacteria samples frequently had higher concentrations of ethanol than then yeast only samples.

Mix contaminated fermentations - Total LAB CFU/ml and fermentation products

When *S. cerevisiae* JAY291 was contaminated with the Mix of 5 LAB, there was no significant difference between the two replicates in the impact of the treatments on the CFU/ml of the Mix. Figure 3.7 is based on the average log CFU/ml reduction of *L. plantarum* from the relevant samples for both days. The blue bars signify the log reduction per treatment in total CFU/ml of the Mix in the bacteria only controls. The orange bars indicate the log reduction per treatment in total CFU/ml of the Mix co-cultured with *S. cerevisiae* JAY291. As mentioned previously there were

no significant differences in the CFU/ml of the yeast which remained at 10^8. The presence of yeast correlated with a significant difference in the reduction of CFU/ml of the Mix for all treatments, except for nisin, Figure 3.7. However, in the bacteria only controls, nisin was not significantly different than the untreated samples. In the bacteria only samples, the log CFU/ml reduction was more than two times higher than the reductions in the co-cultures with yeast for penicillin and the 125 μg/ml nisin with 1 μg/ml penicillin. The largest reduction in CFU/ml of the Mix both in the presence and absence of *S. cerevisiae* JAY291 was caused by the 250 μg/ml nisin with 2 μg/ml penicillin. This data is reinforced by the HPLC results which reveal that lactate is the lowest in the 250 μg/ml nisin with 2 μg/ml penicillin samples, Figure 3.8. In the untreated co-cultures, the ethanol concentration was 2% lower than samples treated with 250 μg/ml nisin with 2 μg/ml penicillin, Figure 3.8.

Impact of treatments on CFU/ml of the individual strains in the Mix co-cultures

While the largest reduction of the total Mix co-cultured with JAY291 was only 1.5 CFU/ml, analysis of the individual strains showed that both *L. fermentum* and *L. ghanensis* experienced a reduction of more than 2 log in each treatment. *L. paracasei* was not reduced at all by 250 μg/ml nisin and minimally impacted by penicillin and the combinations, Figure 3.7. In the bacteria only controls it was apparent that in the nisin only treatment *W. confusa* was also growing well due to the increased viscosity of samples.

3.5 Discussion

Previous work demonstrated the impact of nisin on LAB ethanol contaminants in MRS[17] or in YPD media[32]. In this study, in conditions which mimic the typical Brazilian bioethanol process using sucrose from sugarcane juice as the substrate, nisin reduces the CFU/ml of *L. plantarum* an adapted sugarcane isolate which was minimally impacted by penicillin. In a

mixed population there were also reductions in *L. fermentum* and *L. ghanensis*. The combination of 250 μg/ml nisin with 2 μg/ml penicillin caused the greatest reduction of *L. plantarum* and of the Mix co-cultured with *S. cerevisiae*. Using 125μg/ml nisin with 1 μg /ml of penicillin caused a reduction in the Mix that was not significantly different than 2 μg /ml of penicillin and not significantly different than 250 ug/ml of nisin against *L. plantarum* as a single contaminant. This suggests that nisin could be used as alternative to penicillin, or that a combination could have the same reduction in CFU/ml with less penicillin than is currently used. Using a rotation of antimicrobials, including nisin, or combinations with smaller doses could mitigate the increasing risk of antibiotic or nisin resistant strains. Smaller doses of penicillin or perhaps virginiamycin and monensin would mean less antibiotic residue in co-products. Toxic effects of monensin impact horses and sheep and although it has been used as a growth promoter in cattle it is lethal in high doses[33]. Current market trends indicate a shift away from using antibiotics as growth promoters and recent study showed monensin did not significantly improve growth of cattle when fed with *S. cerevisiae* [34].

The absence of *S. cerevisiae* resulted in significantly higher reduction in bacterial CFU/ml. The effective treatments caused greater reduction in CFU/ml in the bacteria only controls compared to the co-cultures of bacteria and *S. cerevisiae* with the single bacteria or Mix of contaminants. Further support for this conclusion come from the greater impact of the antimicrobials in the first *L. plantarum* fermentation when the yeast inoculation was lower than the other three fermentations. There was a greater reduction of the *L. plantarum* when the initial *S cerevisiae* was high 10^7 instead of low 10^8, Figure 3.6. Since the inoculation level of *S. cerevisiae* in Brazilian bioethanol ranges from 10-15 % weight by volume[16, 35, 36], this is an important thing to consider when controlling LAB contamination. These results suggest that

adding antimicrobials to the must could be more effective than adding the same dose into the fermenter with the yeast. The effect does not appear to specific to nisin only or penicillin only, because the nisin was the more effective against *L. plantarum* only and penicillin was more effective against the Mix. While both inhibit cell wall synthesis, the mechanisms are different. Nisin binds to Lipid II and can also result in pore formation in the cell membrane[37, 38]. Penicillin's beta lactam ring binds to penicillin binding proteins blocking cell wall synthesis particularly at cell division[39]. When investigating the impact of sulfite and hydrogen peroxide on sugarcane ethanol contaminants Chang et al, saw a similar effect, and postulated that it might be due to reduction of reactive oxygen species due to the production of catalase by *S. cerevisiae*[40]. An opposite effect was seen with virginiamycin where it was effective in presence of yeast but after 48 hours in wheat mash, bacteria only samples grew, the antibiotic was less effective [41].

The different effects seen in the co-cultures of yeast and bacteria and the bacteria only controls emphasizes the importance of evaluating antimicrobials in conditions as close to the intended application. We included a mix of representative bacteria because the studies of ethanol contaminants show diverse communities[3, 8, 20]. The overall reduction in CFU/ml of the Mix was not significantly different for the nisin compared to the penicillin, but a look at the species results showed that the less sensitive species to each microbial took the place of the sensitive species. *L. paracasei* was not impacted by nisin but was reduced slightly by penicillin. In Brazil, where recycling of the yeast also results in recycling of the bacterial contaminants[42], an antibacterial strategy which uses different treatments with different mechanisms of action may be more effective against a community where one species or two species insensitive or adapted

to the treatment may predominate at the end of the season like *L. fermentum* and *L. vini* in the Lucena survey of Brazilian distilleries[3].

While the ethanol reduction by the contaminants selected did not reduce the untreated controls by more than a few percent, this model is still a viable method for testing antimicrobials and could optimized by using industrial contaminants, contaminants which reach 10^9 by the end of the fermentation in co-culture, or implementing a cell recycle step. Starting with an industrial grade *S. cerevisiae* at 10^8 CFU/ml, means that bacterial contaminants need to be industrial strength as well. Peng et al demonstrate ethanol inhibition by lactic acid bacteria when the LAB inoculum was 10^7 and the *S. cerevisiae* from baker's yeast inoculation was 10^6 [38] . Bischoff et al also did not get ethanol reduction of greater than 2% in YPD even with industrial bacterial contaminants, when the initial inoculum of yeast and bacteria were both 10^7 but did see reductions when the medium was corn mash instead of YPD[26]. They note that the final CFU/ml of LAB in the corn mash reached 10^9, a log higher than the final CFU/ml in YPD. The importance of the medium is shown in these two studies and that a higher yeast inoculation level decreases the impact of LAB was discussed by Narendranrath and Power [37]. It is possible that by decreasing the yeast inoculation level to 10^7 or altering the substrate, the LAB discussed in this study could reduce ethanol, but it both of those changes would make the experimental conditions less similar to industrial conditions in Brazil.

3.6 Conclusion

This model system with a 10% vol/vol *S. cerevisiae* JAY291 inoculation using sugarcane juice supplemented with 0.6% yeast extract in anaerobic agitated fermentations produced ethanol concentrations of 5.5% or greater and utilized 99.5% or more of sugars in twelve hours. This

mimics Brazilian bioethanol fermentations and can be used to study the impact of antibacterial treatments. Other treatments could include different amounts of nisin, combinations with monensin, muramidases like lysozyme, bacteriophage endolysins such as LysA2 or bacteriophage treatments.

The small cationic peptide nisin, reduced LAB contamination by a sugarcane isolate and in combination with penicillin decreased a Mix of LAB. Recent work showed that combining nisin improved the activity of penicillin against Gram-positive bacteria, including strains with multiple antibiotic resistance genes[45]. Nisin is stable and active at low pH, so adding it to the acid wash step may be a way to shorten the treatment time resulting in less stress on the yeast and increase the effectiveness of the acid wash. Another option to explore is evaluating whether nisin producing *Lactococcus lactis* could be added with the initial yeast inoculum to reduce the viability of the naturally present LAB. Because sugarcane juice is predominantly sucrose, the conditions are favorable for nisin production. In *Lc. lactis* nisin production and sucrose utilization have been shown to be genetically linked[46].

3.7 Tables and Figures

Table 3.1 Bacterial strains.

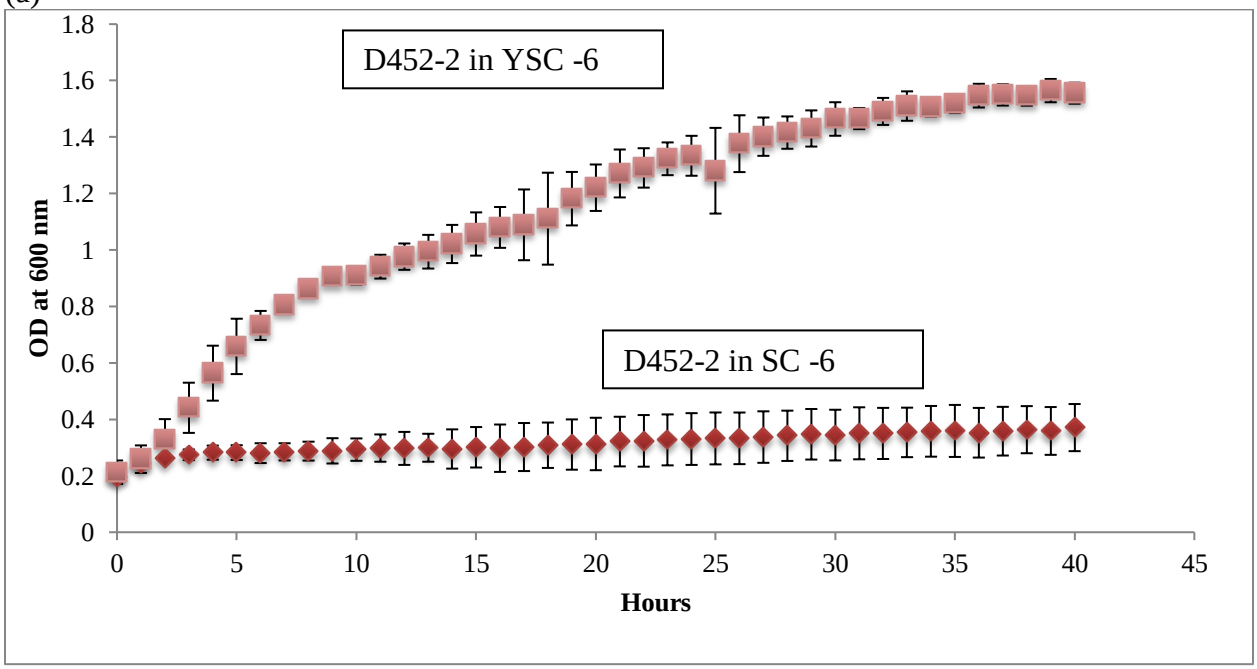
Strains	**Designation**	**Peptidoglycan Chemotype**[47]	**Lactic acid fermentation**[48, 49]	**Origin**
Aerococcus viridans	ATCC 11563	A1α direct	Obligately homofermentative	Air sample
Lactobacillus plantarum	MJM 461	A1γ mesoDpm	Facultatively heterofermentative	Sugarcane juice
Lactobacillus plantarum	MJM 494	A1γ mesoDpm	Facultatively heterofermentative	Adapted
Lactobacillus plantarum	ATCC 14917	A1γ mesoDpm	Facultatively heterofermentative	Pickled cabbage
Leuconostoc mesenteroides	ATCC 8293	A3αL-Lys-L-Ser-L-Ala	Obligately heterofermentative	Foods
Oenococcus oeni	MJM 485	A3αL-Lys-L-Ala(Ser)-L-Ser	Obligately heterofermentative	Hard Apple Cider
Weissella confusa	ATCC 10881	A3α L-Lys-Ala	Obligately heterofermentative	Adapted
Weissella confusa	MJM 493	A3α L-Lys-Ala	Obligately heterofermentative	Sugarcane
Weissella cibaria	MJM 489	A3α L-Lys-L-Ser-L-Ala	Obligately heterofermentative	Sugarcane juice
Enterococcus gallinarum gp	MJM 488	A4α L-Lys-D-Asp	Obligately homofermentative	Sugarcane juice
Enterococcus gallinarum	ATCC 49573	A4α L-Lys-D-Asp	Obligately homofermentative	Chicken intestine
Lactobacillus brevis	ATCC 14869	A4α L-Lys-D-Asp	Obligately heterofermentative	Human feces
Lactobacillus casei	ATCC 393	A4α L-Lys-D-Asp	Facultatively heterofermentative	Dairy
Lactobacillus delbrueckii	ATCC 9649	A4α L-Lys-D-Asp	Obligately homofermentative	Sour grain mash
Lactobacillus paracasei	ATCC 25598	A4α L-Lys-D-Asp	Obligately homofermentative	Milking machine
Lactobacillus paracasei	MJM 492	A4α L-Lys-D-Asp	Obligately homofermentative	Adapted
Lactobacillus rhamnosus	ATCC53103	A4α L-Lys-D-Asp	Facultatively heterofermentative	Human feces
Lactococcus lactis	ATCC 19257	A4α L-Lys-D-Asp	Obligately homofermentative	Dairy
Pediococcus acidilactici	MJM 231	A4α L-Lys-D-Asp	Obligately homofermentative	Goat rumen
Pediococcus damnosus	ATCC 29358	A4α L-Lys-D-Asp	Obligately homofermentative	Lager beer yeast
Lactobacillus fermentum	ATCC 9338	A4β L-Orn-D-Asp	Obligately heterofermentative	Not specified
Lactobacillus ghanensis	MJM 487	Undetermined	Homofermentative (in sugarcane juice)	Hard Apple Cider

Table 3.2 *Saccharomyces cerevisiae* strains

Strains	Designation	Description	References
Saccharomyces cerevisiae	D452-2	Lignocellulosic strain	[27]
Saccharomyces cerevisiae	JAY291	PE-2 Derivative	[50]

Figure 3.1. *S. cerevisiae* D452-2 (a) and JAY291 (b) growth curves in sugarcane juice pH 5.5 diluted to 6% total sugars (SC-6) with and without 0.6% yeast extract (YSC-6). (a) The original *S. cerevisiae* strain tested, D452-2, which grew poorly in sugarcane juice (SC-6) improved significantly with the addition of 0.6% yeast extract (YSC-6); (b) JAY291 grew well in SC-6 and improved in YSC-6

(a)



(b)

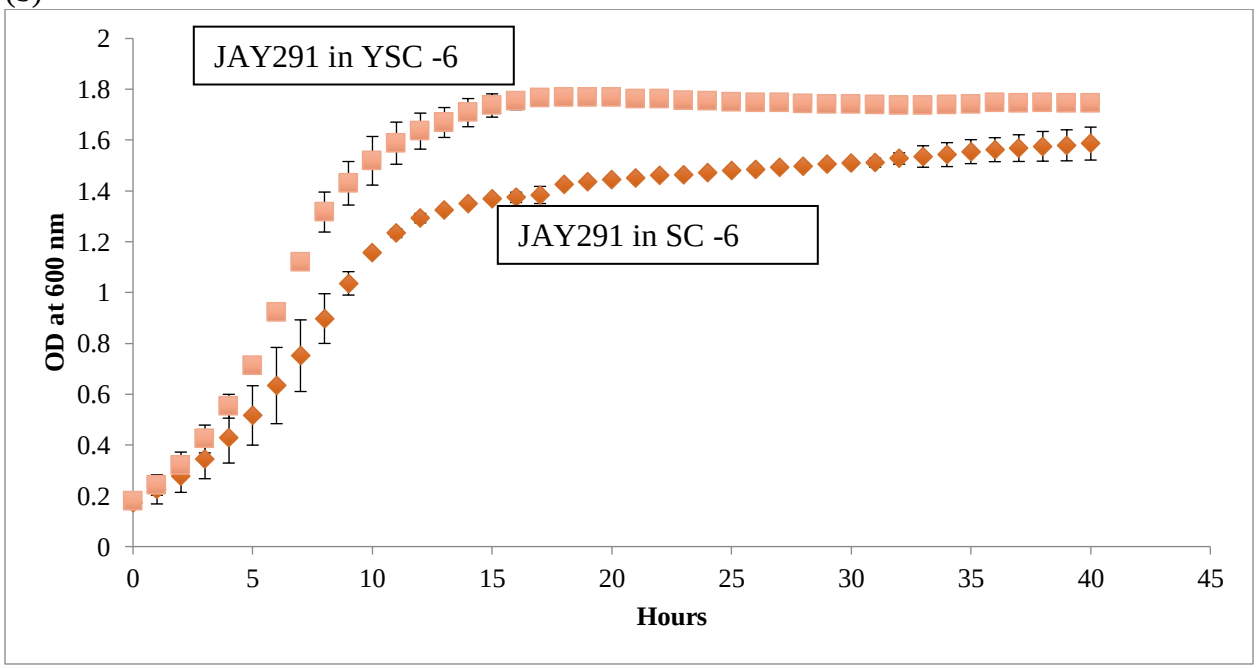


Table 3.3 Comparison of growth of *S. cerevisiae* JAY291 in different media. The addition of 0.6% yeast extract increased the maximum OD to levels approaching the max OD with YPD and decreased the doubling time compared to SC-6

Media	Lag Time (hours)	Max Specific Growth Rate (1/hours)	Doubling Time (hours)	Max OD	Median OD	Delta OD	Replicate	R^2	SSE	RMSE
SC-6	4.1	0.15	4.74	1.39	1.39	1.36	1	0.9967	0.0437	0.0279
SC-6	2.24	0.12	5.76	1.36	1.35	1.29	2	0.9968	0.0327	0.0240
YSC-6	3.04	0.19	3.7	1.63	1.63	1.59	1	0.9972	0.0472	0.0288
YSC-6	3.18	0.2	3.4	1.63	1.61	1.57	2	0.9992	0.0134	0.0155
YPD-6	2.45	0.18	3.89	1.65	1.65	1.58	1	0.9980	0.0314	0.0235
YPD-6	2.18	0.22	3.2	1.71	1.7	1.63	2	0.9977	0.0348	0.0247

Figure 3.2 Growth of LAB in sugarcane juice 6 percent sugars (SC-6); and with the addition of 0.6 % yeast extract (YSC-6). Wild-type LAB strains of the same species shown to inhibit ethanol barely grew in SC-6. The best growth was by *W. confusa* ATCC 10881, which was originally found on sugarcane and deposited with the ATCC. b) Similar to the results seen with *S. cerevisiae*, faster growth and higher maximum OD readings resulted when 0.6 yeast extract was added to the sugarcane juice. However, this maximum OD of these LAB is still half of the maximum OD of *S. cerevisiae* JAY291 in the same time frame.

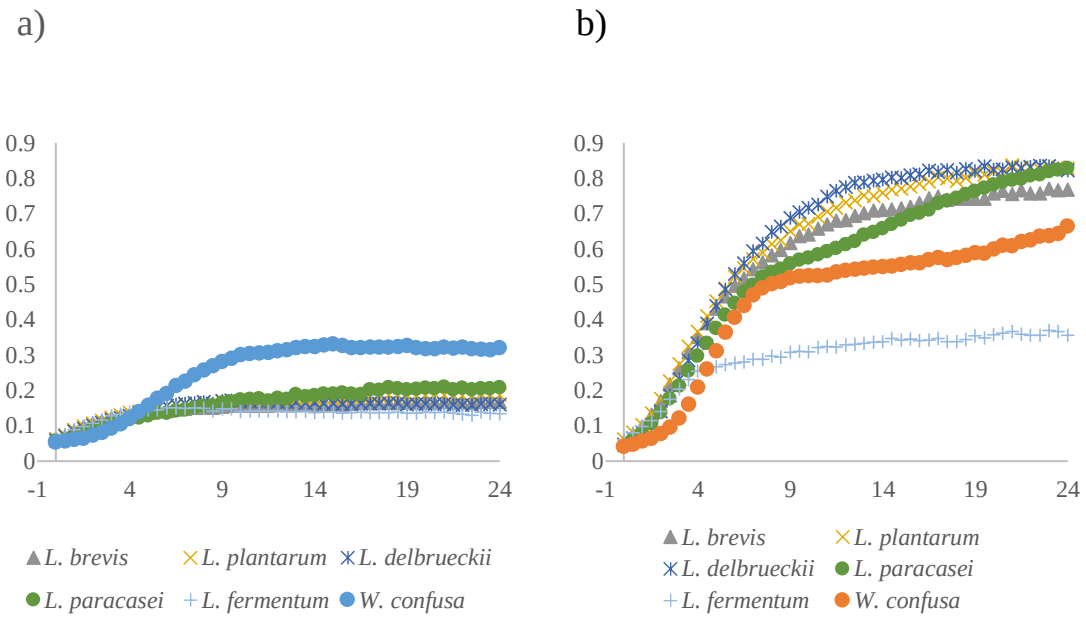


Figure 3.3 Growth of adapted strains in YSC-6 compared to wild-type strains: a) *W. confusa* b) *L. paracasei;* and c) *L. paraplantarum. (a*) *W. confusa* produced more exopolysaccharides after adaptation, but did not show an in OD. (b) *L. paracasei* successively passed in YSC-6 demonstrates higher maximum OD and faster growth compared to the wild-type *L. paracasei;* (c) *L. paraplantarum* isolated from clarified sugarcane juice (total sugars 12.5% and pH 7.6) grew to higher maximum OD after successive passes in YSC-6 (6 percent sugars, pH 5.5)

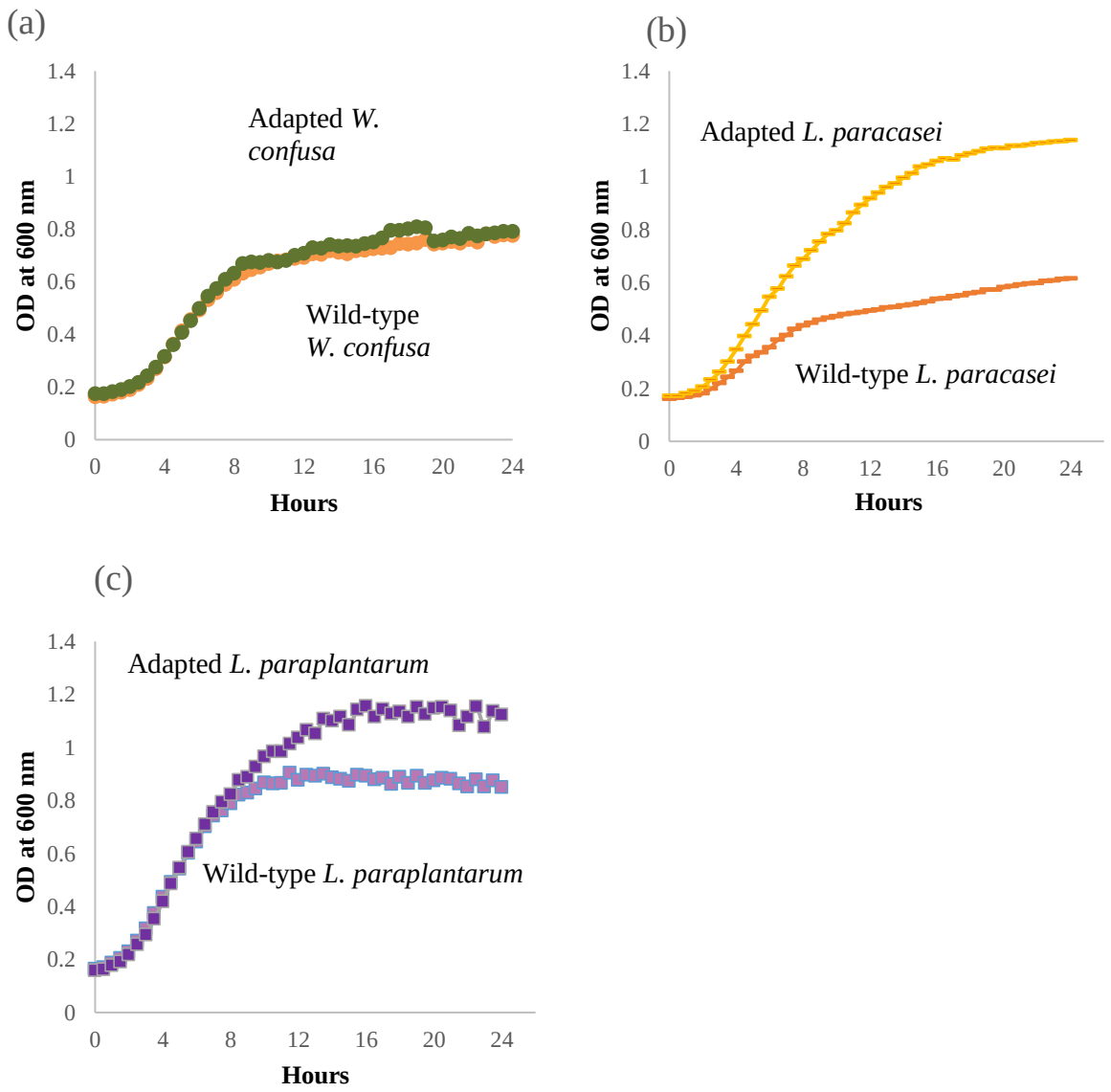


Table 3.4 Minimum inhibitory concentration (MIC) for lysozyme, mutanolysin and nisin in spot on lawn assay

Strains	**Designation**	**Lysozyme**	**Mutanolysin**	**Nisin**
Aerococcus viridans	ATCC 11563	< 1,250 μg/ml	250 μg/ml	< 4 μg/ml
Lactobacillus paraplantarum	MJM 461	>10,000 μg/ml	>500 μg/ml	32 μg/ml
Lactobacillus paraplantarum	MJM 494	>10,000 μg/ml	>500 μg/ml	32 μg/ml
Lactobacillus plantarum	ATCC 14917	2500 μg/ml	>500 μg/ml	16 μg/ml
Leuconostoc mesenteroides	ATCC 8293	>10,000 μg/ml	>500 μg/ml	32 μg/ml
Oenococcus oeni	MJM 485	< 1,250 μg/ml	>500 μg/ml	32 μg/ml
Weissella confusa	ATCC 10881	>10,000 μg/ml	>500 μg/ml	32 μg/ml
Weissella confusa	MJM 493	>10,000 μg/ml	>500 μg/ml	32 μg/ml
Weissella cibaria	MJM 489	10,000 μg/ml	>500 μg/ml	32 μg/ml
Enterococcus gallinarum	MJM 488	< 1,250 μg/ml	>500 μg/ml	32 μg/ml
Enterococcus gallinarum	ATCC 49573	>10,000 μg/ml	>500 μg/ml	16 μg/ml
Lactobacillus brevis	ATCC 14869	2500 μg/ml	>500 μg/ml	16 μg/ml
Lactobacillus casei	ATCC 393	10,000 μg/ml	>500 μg/ml	< 4 μg/ml
Lactobacillus delbrueckii	ATCC 9649	>10,000 μg/ml	>500 μg/ml	16 μg/ml
Lactobacillus paracasei	ATCC 25598	>10,000 μg/ml	>500 μg/ml	16 μg/ml
Lactobacillus paracasei	MJM 492	>10,000 μg/ml	>500 μg/ml	32 μg/ml
Lactobacillus rhamnosus	ATCC53103	>10,000 μg/ml	>500 μg/ml	32 μg/ml
Lactococcus lactis	ATCC 19257	>10,000 μg/ml	>500 μg/ml	16 μg/ml
Pediococcus acidilactici	MJM 231	>10,000 μg/ml	>500 μg/ml	8 μg/ml
Pediococcus damnosus	ATCC 29358	10,000 μg/ml	>500 μg/ml	< 4 μg/ml
Lactobacillus fermentum	ATCC 9338	5,000 μg/ml	>500 μg/ml	8 μg/ml
Lactobacillus ghanensis	MJM 487	5,000 μg/ml	>500 μg/ml	32 μg/ml
Saccharomyces cerevisiae	D452-2	>10,000 μg/ml	>500 μg/ml	>32 μg/ml
Saccharomyces cerevisiae	JAY291	>10,000 μg/ml	>500 μg/ml	>32 μg/ml

Figure 3.4 Comparison of final CFU/ml of L. paraplantarum alone and with S. cerevisiae
When the starting inoculation level of *L. paraplantarum* was 10^9, the CFU/ml at the end of fermentation decreased even without treatment in the presence of *S. cerevisiae*.

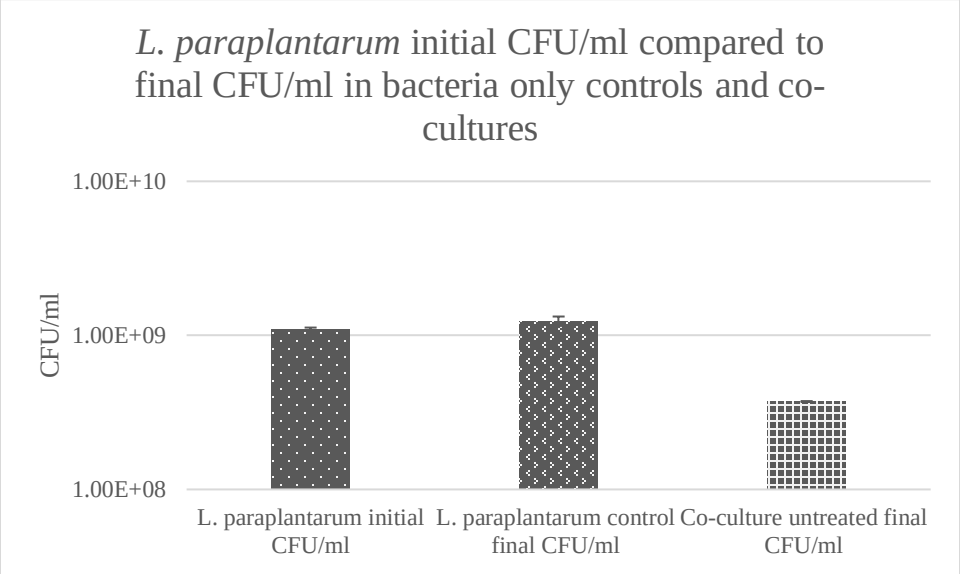


Figure 3.5 Bacteria only control CFU/ml for *L. paraplantarum* contaminated fermentations, impact of antimicrobials for *L .paraplantarum* bacteria only controls. In the bacteria only controls the penicillin alone and in combination with nisin resulted in average log CFU/ml reductions of *L. paraplantarum* ranging from 1.6 log to 4.6 log. In the bacteria only controls, the reduction by nisin was not significantly different from the untreated. Different letters above the treatment indicate that the P value was < 0.05 when comparing those treatments

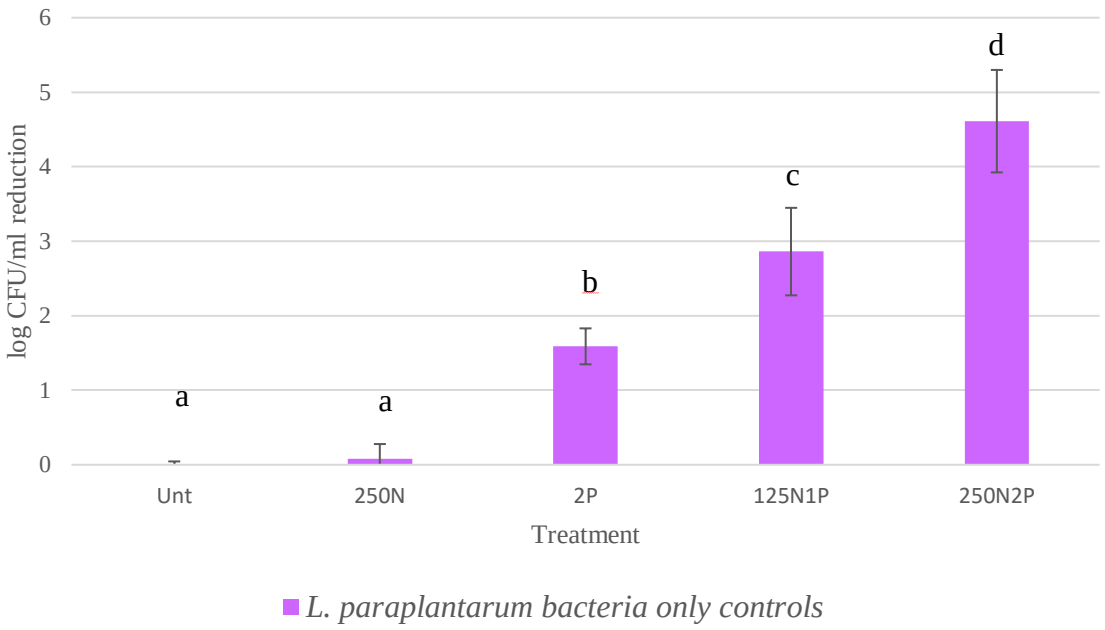


Figure 3.6 *L. paraplantarum* CFU/ml in co-culture with *S. cerevisiae* JAY291. In the two fermentations with *L. paraplantarum* as the only contaminant, a different impact of the treatments occurred in co-culture. In fermentation 1, with a *S. cerevisiae* initial inoculation of 8.8 x 10^7 (lilac bars), larger CFU/ml reductions occurred. Less impact of nisin and the combinations with nisin resulted in fermentation 2 (purple bars) when the yeast inoculation level was higher 2.1 x10^8. Different letters above the treatment indicate that the P value was < 0.05 between those treatments.

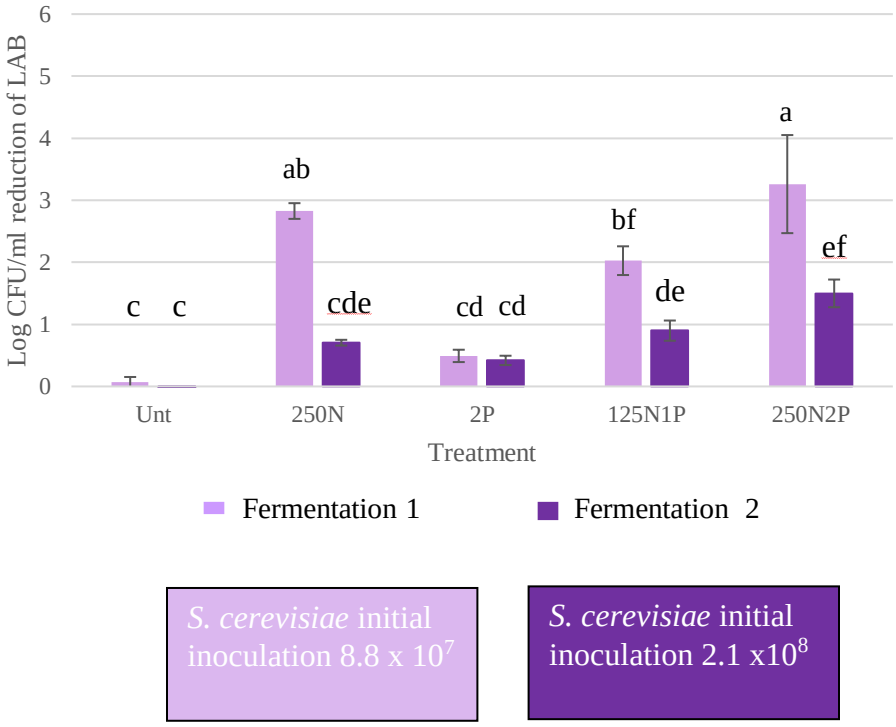


Figure 3.7 Log reduction of CFU/ml of Mix in Mix only controls (blue bars) and Mix co-cultured with *S. cerevisiae* (orange bars). With the Mix of 5 LAB as contaminants, there is a greater impact of the antibacterial treatments in the bacteria only controls (blue bars) compared to when the bacteria are cultured with *S. cerevisiae* (orange bars). Also against the Mix of 5 bacteria, the most reduction in total CFU/ml resulted from the penicillin and the combinations of nisin and penicillin. Different letters indicate a P value <0.05 between those treatments. Penicillin and penicillin/nisin mixtures have more effect in the Mix bacteria only controls compared to the co-cultures with *S. cerevisiae* JAY291

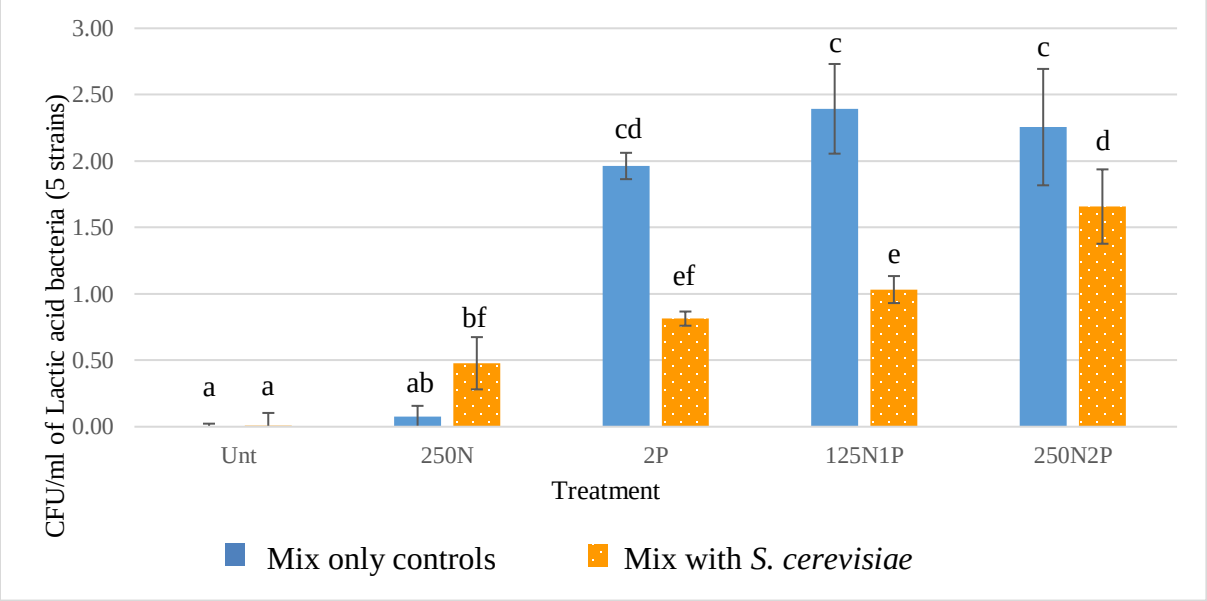


Figure 3.8 Reduction of CFU/ml of the Mix of bacteria in co-culture with *S. cerevisiae* by different treatments on the five separate strains. The Mix (green bars) contained 5 LAB, *L. fermentum* (blue bars), *L. ghanensis* (orange bars), *L. paracasei* (grey bars), *W. confusa* (yellow bars) and *L. paraplantarum* (purple bars). *L. fermentum and L. ghanensis* showed the most sensitivity to all treatments. Nisin did not reduce *L. paracasei* which overall was the least impacted by any of the treatments. Different letters above the strains indicate that the P value was < 0.05 when compared other strains receiving the same treatment.

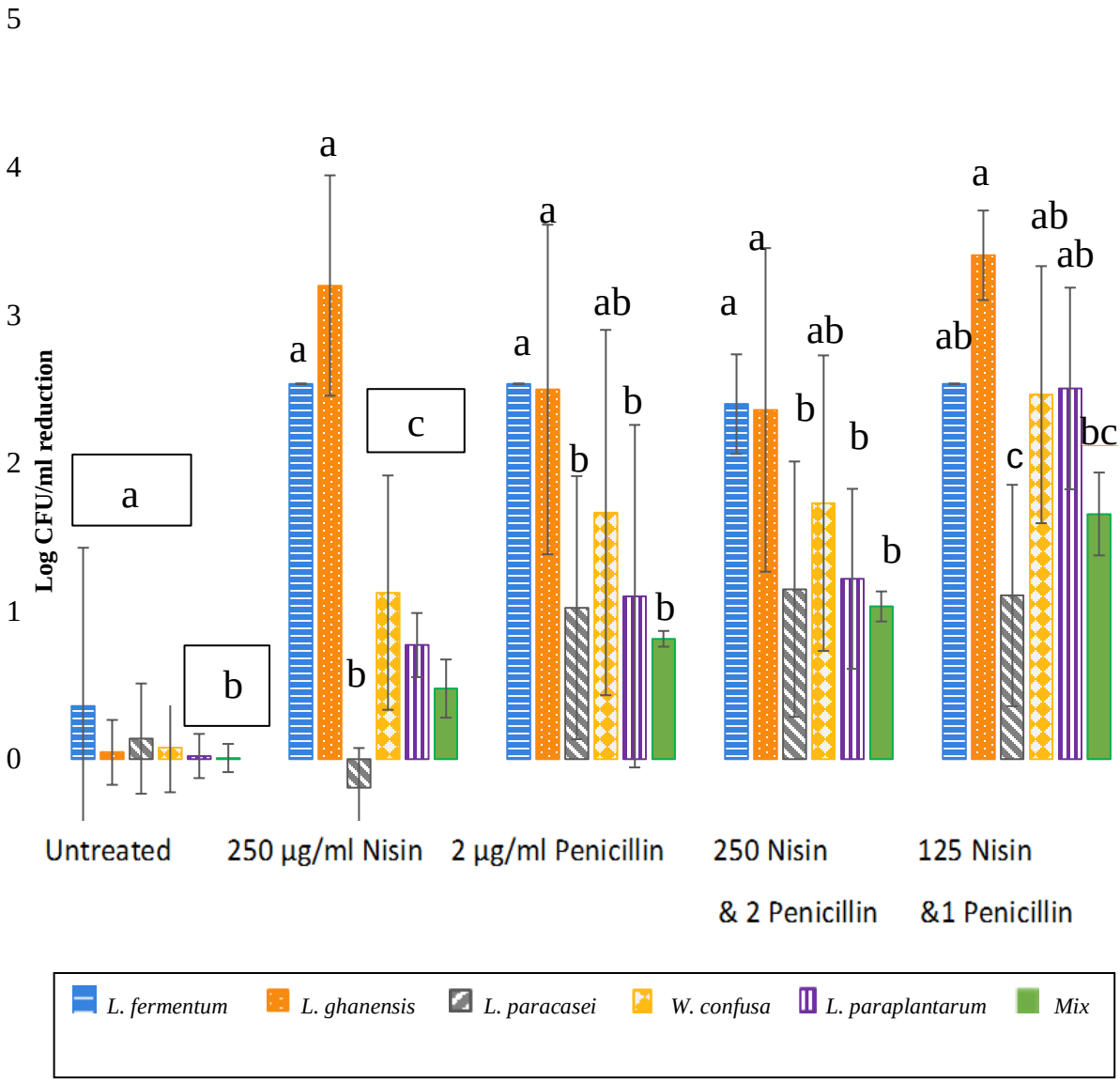
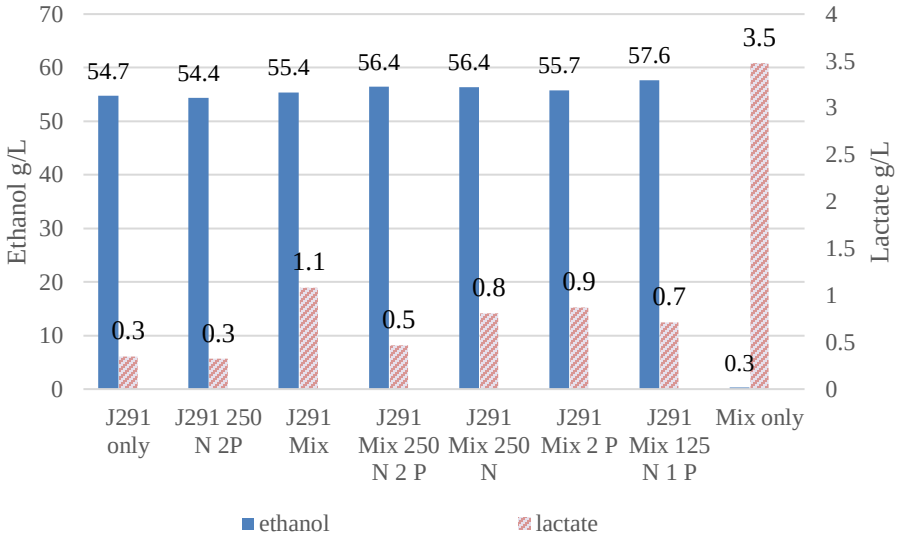


Figure 3.9 Mix contaminated fermentations, ethanol and lactate concentration after twelve hour fermentation. Ethanol and lactate concentrations from the fermentations showed ethanol concentrations of 54 g/L or higher produced by *S. cerevisiae* JAY291. The JAY291 untreated and treated with 250 µg/ml nisin and 2 µg/ml penicillin had slightly lower ethanol concentrations than the JAY291 contaminated with the Mix of bacteria, even though the Mix only control produced 0.26 g/L ethanol. The combination treatments had the highest ethanol concentrations and lowest lactate concentrations. With the highest combination treatment, the lactate present was only slightly higher than the JAY291 only controls.



3.8 References

1. Skinner KA, Leathers TD (2004) Bacterial contaminants of fuel ethanol production. J Ind Microbiol Biotechnol 31:401–408. doi: 10.1007/s10295-004-0159-0

2. Murphree C a., Heist EP, Moe L a. (2014) Antibiotic Resistance Among Cultured Bacterial Isolates from Bioethanol Fermentation Facilities Across the United States. Curr Microbiol 69:277–285. doi: 10.1007/s00284-014-0583-y

3. Lucena BT, dos Santos BM, Moreira JL, et al (2010) Diversity of lactic acid bacteria of the bioethanol process. BMC Microbiol 10:298. doi: 10.1186/1471-2180-10-298

4. Liu M, Bischoff KM, Gill JJ, et al (2015) Bacteriophage application restores ethanol fermentation characteristics disrupted by Lactobacillus fermentum. Biotechnol Biofuels. doi: 10.1186/s13068-015-0325-9

5. Simpson KL, Pettersson B, Priest FG Characterization of lactobacilli from Scotch malt whisky distilleries and description of Lactobacillus ferintoshensis sp. nov., a new species isolated from malt whisky fermentations. Microbiol Read Engl 147:1007–1016.

6. Guzzo F, Cappello MS, Azzolini M, et al (2011) The inhibitory effects of wine phenolics on lysozyme activity against lactic acid bacteria. Int J Food Microbiol. doi: 10.1016/j.ijfoodmicro.2011.05.023

7. Kelly D, O'Sullivan O, Mills S, et al (2012) Genome sequence of the phage clP1, which infects the beer spoilage bacterium Pediococcus damnosus. Gene 504:53–63. doi: 10.1016/j.gene.2012.04.085

8. Costa OYA, Souto BM, Tupinambá DD, et al (2015) Microbial diversity in sugarcane ethanol production in a Brazilian distillery using a culture-independent method. J Ind Microbiol Biotechnol 42:73–84. doi: 10.1007/s10295-014-1533-1

9. Schell DJ, Dowe N, Ibsen KN, et al (2007) Contaminant occurrence, identification and control in a pilot-scale corn fiber to ethanol conversion process. Bioresour Technol 98:2942–2948. doi: 10.1016/j.biortech.2006.10.002

10. Basso TO, de Kok S, Dario M, et al Engineering topology and kinetics of sucrose metabolism in Saccharomyces cerevisiae for improved ethanol yield. Metab Eng 13:694–703. doi: 10.1016/j.ymben.2011.09.005

11. Bischoff KM, Skinner-Nemec KA, Leathers TD (2007) Antimicrobial susceptibility of Lactobacillus species isolated from commercial ethanol plants. J Ind Microbiol Biotechnol 34:739–744. doi: 10.1007/s10295-007-0250-4

12. Rich JO, Leathers TD, Nunnally MS, Bischoff KM (2011) Rapid evaluation of the antibiotic susceptibility of fuel ethanol contaminant biofilms. Bioresour Technol 102:1124–1130. doi: 10.1016/j.biortech.2010.08.118

13. Neto P, Lima F, Silva K, et al (2014) Brazilian Archives of Biology and Technology. Braz Arch Biol Technol. doi: 10.1590/S1516-8913201401214

14. Stroppa C, Andrietta MGS, Andrietta SR, et al (2000) Use of penicillin and monensin to control bacterial contamination of brazilian alcohol fermentations. Int Sugar J 78–82.

15. De Alwis H, Heller DN (2010) Multiclass, multiresidue method for the detection of antibiotic residues in distillers grains by liquid chromatography and ion trap tandem mass spectrometry. J Chromatogr A 1217:3076–3084. doi: 10.1016/j.chroma.2010.02.081

16. Amorim H V., Lopes ML, de Castro Oliveira JV, et al (2011) Scientific challenges of bioethanol production in Brazil. Appl Microbiol Biotechnol 91:1267–1275. doi: 10.1007/s00253-011-3437-6

17. Franchi MA, Serra GE, Cristianini M (2003) The Use of Biopreservatives in the Control of Bacterial Contaminants of Sugarcane Alcohol Fermentation. J Food Sci 68:2310–2315. doi: 10.1111/j.1365-2621.2003.tb05764.x

18. Hoeflinger JL, Davis SR, Chow J, Miller MJ (2015) In Vitro Impact of Human Milk Oligosaccharides on Enterobacteriaceae Growth. J Agric Food Chem 63:3295–3302. doi: 10.1021/jf505721p

19. Leite AMO, Miguel MAL, Peixoto RS, et al (2015) Probiotic potential of selected lactic acid bacteria strains isolated from Brazilian kefir grains. J Dairy Sci 98:3622–3632. doi: 10.3168/jds.2014-9265

20. Li Q, Heist EP, Moe LA (2015) Bacterial Community Structure and Dynamics During Corn-Based Bioethanol Fermentation. Microb Ecol. doi: 10.1007/s00248-015-0673-9

21. Nelson DC, Schmelcher M, Rodriguez-Rubio L, et al (2012) Endolysins as Antimicrobials. Elsevier, pp 299–365

22. Redondo LM, Carrasco JMD, Redondo EA, et al (2015) Clostridium perfringens Type E Virulence Traits Involved in Gut Colonization. PLOS ONE 10:e0121305. doi: 10.1371/journal.pone.0121305

23. Chen CY, Nace GW, Irwin PL (2003) A 6 x 6 drop plate method for simultaneous colony counting and MPN enumeration of Campylobacter jejuni, Listeria monocytogenes, and Escherichia coli. J Microbiol Methods 55:475–479.

24. Rich JO, Leathers TD, Bischoff KM, et al (2015) Biofilm formation and ethanol inhibition by bacterial contaminants of biofuel fermentation. Bioresour Technol 196:347–354. doi: 10.1016/j.biortech.2015.07.071

25. Legendre BL (1988) Varietal Differences in the Chemical Composition of Sugarcane. Elsevier, pp 176–185

26. Kim M, Day DF (2011) Composition of sugar cane, energy cane, and sweet sorghum suitable for ethanol production at Louisiana sugar mills. J Ind Microbiol Biotechnol 38:803–807. doi: 10.1007/s10295-010-0812-8

27. Ha S-J, Kim SR, Kim H, et al (2013) Continuous co-fermentation of cellobiose and xylose by engineered Saccharomyces cerevisiae. Bioresour Technol 149:525–531. doi: 10.1016/j.biortech.2013.09.082

28. R. Shane Gold a, M.M. Meagher a'b, R. Hutkins a and T. Conway c (1992) Ethanol tolerance and carbohydrate metabolism in lactobacilli*. J. Ind. Microbiol.

29. Yokogawa K, Kawata S, Nishimura S, et al (1974) Mutanolysin, bacteriolytic agent for cariogenic Streptococci: partial purification and properties. Antimicrob Agents Chemother 6:156–165.

30. Gill AO, Holley RA (2000) Inhibition of bacterial growth on ham and bologna by lysozyme, nisin and EDTA. Food Res Int 33:83–90. doi: 10.1016/S0963-9969(99)00152-0

31. Seo HJ, Kitaoka M, Ohmiya K, Hayashi K (2003) Substrate specificity of the N,6-O-diacetylmuramidase from Streptomyces globisporus. J Biosci Bioeng 95:313–316. doi: 10.1016/S1389-1723(03)80037-3

32. Gao YC, Zhang G, Krentz S, et al (2002) Inhibition of spoilage lactic acid bacteria by lysozyme during wine alcoholic fermentation. Aust J Grape Wine Res 8:76–83. doi: 10.1111/j.1755-0238.2002.tb00214.x

33. Boden E (2005) Black's veterinary dictionary, 21st ed. A. & C. Black, London

34. Monnerat JPI dos S, Paulino PVR, Detmann E, et al (2013) Effects of Saccharomyces cerevisiae and monensin on digestion, ruminal parameters, and balance of nitrogenous compounds of beef cattle fed diets with different starch concentrations. Trop Anim Health Prod 45:1251–1257. doi: 10.1007/s11250-013-0356-9

35. Andrietta MGS, Andrietta SR, Steckelberg C, Stupiello ENA (2007) Bioethanol - Brazil, 30 years of Proálcool. Int Sugar J 109:195–200.

36. Della-Bianca BE, Basso TO, Stambuk BU, et al (2013) What do we know about the yeast strains from the Brazilian fuel ethanol industry? Appl Microbiol Biotechnol 97:979–991. doi: 10.1007/s00253-012-4631-x

37. Field D, Begley M, O'Connor PM, et al (2012) Bioengineered Nisin A Derivatives with Enhanced Activity against Both Gram Positive and Gram Negative Pathogens. PLoS ONE 7:e46884. doi: 10.1371/journal.pone.0046884

38. Branen JK, Davidson PM (2004) Enhancement of nisin, lysozyme, and monolaurin antimicrobial activities by ethylenediaminetetraacetic acid and lactoferrin. Int J Food Microbiol 90:63–74. doi: 10.1016/S0168-1605(03)00172-7

39. Islam M, Toledo R, Hamdy M. (1999) Stability of virginiamycin and penicillin during alcohol fermentation. Biomass Bioenergy 17:369–376. doi: 10.1016/S0961-9534(99)00052-5

40. Chang IS, Kim BH, Shin PK (1997) Use of sulfite and hydrogen peroxide to control bacterial contamination in ethanol fermentation. Appl Environ Microbiol 63:1–6.

41. Hynes SH, Kjarsgaard DM, Thomas KC, Ingledew WM (1997) Use of virginiamycin to control the growth of lactic acid bacteria during alcohol fermentation. J Ind Microbiol Biotechnol 18:284–291.

42. Oliva-Neto P de, Yokoya F (2001) Susceptibility of Saccharomyces cerevisiae and lactic acid bacteria from the alcohol industry to several antimicrobial compounds. Braz J Microbiol 32:10–14. doi: 10.1590/S1517-83822001000100003

43. Peng J, Zhang L, Gu Z-H, et al (2012) The role of nisin in fuel ethanol production with Saccharomyces cerevisiae: Nisin and fuel ethanol production. Lett Appl Microbiol 55:128–134. doi: 10.1111/j.1472-765X.2012.03275.x

44. Narendranath N V., Power R (2004) Effect of yeast inoculation rate on the metabolism of contaminating lactobacilli during fermentation of corn mash. J Ind Microbiol Biotechnol 31:581–584. doi: 10.1007/s10295-004-0191-0

45. Tong Z, Zhang Y, Ling J, et al (2014) An In Vitro Study on the Effects of Nisin on the Antibacterial Activities of 18 Antibiotics against Enterococcus faecalis. PLoS ONE 9:e89209. doi: 10.1371/journal.pone.0089209

46. Rauch PJ, Beerthuyzen MM, de Vos WM (1994) Distribution and evolution of nisin-sucrose elements in Lactococcus lactis. Appl Environ Microbiol 60:1798–1804.

47. (1973) Peptidoglycan types of bacterial cell walls and their taxonomic implications. Bacteriol Rev 37:258.

48. Holzapfel WH, Wood BJB (2014) Introduction to the LAB. In: Holzapfel WH, Wood BJB (eds) Lact. Acid Bact. John Wiley & Sons, Ltd, Chichester, UK, pp 1–12

49. Boone DR, Castenholz RW, Garrity GM (2001) Bergey's manual of systematic bacteriology. Springer, New York

50. Argueso JL, Carazzolle MF, Mieczkowski PA, et al (2009) Genome structure of a Saccharomyces cerevisiae strain widely used in bioethanol production. Genome Res 19:2258–2270. doi: 10.1101/gr.091777.109

CHAPTER 4 SUMMARY AND FUTURE DIRECTIONS

4.1 Summary and future directions

First generation biofuels like ethanol from corn and sugarcane have been established as viable alternatives to petroleum based fuels. In Brazil, more than 90 percent of passenger vehicles are flex fuel which can run on mixtures of ethanol and gasoline[2,9]. To remain competitive, bioethanol plants need to successfully control contaminants which reduce ethanol production. Alternative bioethanol production from non-food substrates also depends on plant material as the primary substrate and will likely encounter similar bacterial contamination problems[27,95]. Lactic acid bacteria have been linked to ethanol inhibition and the protocols developed in this work can be used to further understand the mechanisms of inhibition and antimicrobial strategies to minimize the impact of contamination. While several theories of why lactic acid bacteria inhibit ethanol production by *S. cerevisiae* have been suggested, there is a lack of agreement on the mechanism or mechanisms. This system could be used to take a closer look at the impact of heterofermentative vs homofermentative metabolism, competition for key nutrients and CFU/ml levels related to ethanol reduction in the conditions encountered in Brazil.

Nisin alone and in combination with penicillin reduced the levels of susceptible contaminating bacteria in sugarcane juice under conditions similar to Brazilian bioethanol distilleries. Further research on the use of nisin could include using purified nisin rather than Nisaplin® which has high sodium content; combining nisin and monensin, and adding the nisin to shorten the acid wash step.

Adding a cell recycling step to this model system could provide valuable insight on how levels of contamination change with cell recycle and what strains predominate. Further, adding

an acid wash with dilute sulfuric acid would enable future researchers to evaluate the efficacy of the acid wash and investigate LAB adaptation to the acid and fermentation conditions.

Finally, to truly tackle the problem of contamination in bioethanol by bacteria, more emphasis should be put on understanding the impact of antimicrobial strategies against a community rather than a single strain. Protocols to investigate this should look more to the model of food contamination control studies where cocktails of strains are used for validation of antimicrobial treatments rather than looking to the methods used to tackle human infections.

4.2 References

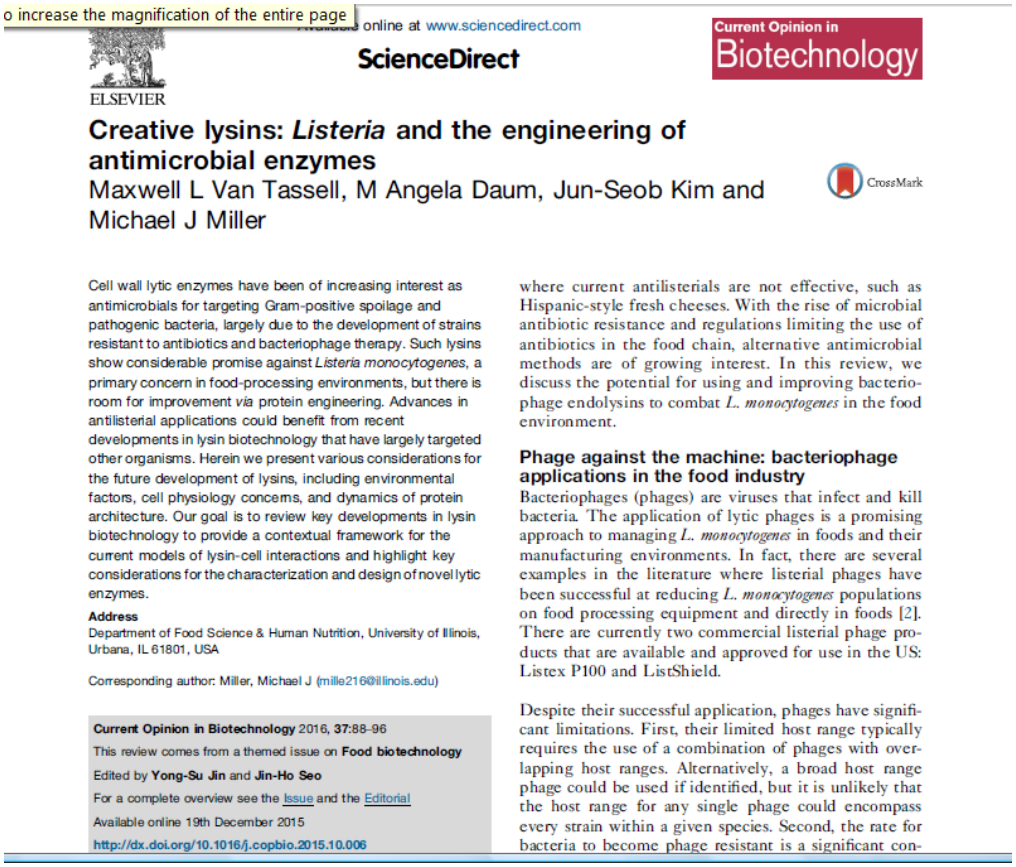
1. The Brazilian Sugarcane Industry Association (UNICA). Sugarcane Industry in Brazil. Sao Paulo Brazil: UNICA; 2015 Dec.

2. Amorim H V., Lopes ML, de Castro Oliveira JV, Buckeridge MS, Goldman GH. Scientific challenges of bioethanol production in Brazil. Appl. Microbiol. Biotechnol. 2011;91:1267–75.

3. Albers E, Johansson E, Franzén CJ, Larsson C. Selective suppression of bacterial contaminants by process conditions during lignocellulose based yeast fermentations. Biotechnol. Biofuels. 2011;4:59.

4. Schell DJ, Dowe N, Ibsen KN, Riley CJ, Ruth MF, Lumpkin RE. Contaminant occurrence, identification and control in a pilot-scale corn fiber to ethanol conversion process. Bioresour. Technol. 2007;98:2942–8.

APPENDIX A. CREATIVE LYSINS: *LISTERIA* AND THE ENGINEERING OF ANTIMICROBIAL ENZYMES



o increase the magnification of the entire page

online at www.sciencedirect.com

ScienceDirect

Current Opinion in Biotechnology

ELSEVIER

Creative lysins: *Listeria* and the engineering of antimicrobial enzymes

Maxwell L Van Tassell, M Angela Daum, Jun-Seob Kim and Michael J Miller

CrossMark

Cell wall lytic enzymes have been of increasing interest as antimicrobials for targeting Gram-positive spoilage and pathogenic bacteria, largely due to the development of strains resistant to antibiotics and bacteriophage therapy. Such lysins show considerable promise against *Listeria monocytogenes*, a primary concern in food-processing environments, but there is room for improvement *via* protein engineering. Advances in antilisterial applications could benefit from recent developments in lysin biotechnology that have largely targeted other organisms. Herein we present various considerations for the future development of lysins, including environmental factors, cell physiology concerns, and dynamics of protein architecture. Our goal is to review key developments in lysin biotechnology to provide a contextual framework for the current models of lysin-cell interactions and highlight key considerations for the characterization and design of novel lytic enzymes.

Address
Department of Food Science & Human Nutrition, University of Illinois, Urbana, IL 61801, USA

Corresponding author: Miller, Michael J (mille216@illinois.edu)

Current Opinion in Biotechnology 2016, **37**:88–96
This review comes from a themed issue on **Food biotechnology**
Edited by **Yong-Su Jin** and **Jin-Ho Seo**
For a complete overview see the Issue and the Editorial
Available online 19th December 2015
http://dx.doi.org/10.1016/j.copbio.2015.10.006

where current antilisterials are not effective, such as Hispanic-style fresh cheeses. With the rise of microbial antibiotic resistance and regulations limiting the use of antibiotics in the food chain, alternative antimicrobial methods are of growing interest. In this review, we discuss the potential for using and improving bacteriophage endolysins to combat *L. monocytogenes* in the food environment.

Phage against the machine: bacteriophage applications in the food industry

Bacteriophages (phages) are viruses that infect and kill bacteria. The application of lytic phages is a promising approach to managing *L. monocytogenes* in foods and their manufacturing environments. In fact, there are several examples in the literature where listerial phages have been successful at reducing *L. monocytogenes* populations on food processing equipment and directly in foods [2]. There are currently two commercial listerial phage products that are available and approved for use in the US: Listex P100 and ListShield.

Despite their successful application, phages have significant limitations. First, their limited host range typically requires the use of a combination of phages with overlapping host ranges. Alternatively, a broad host range phage could be used if identified, but it is unlikely that the host range for any single phage could encompass every strain within a given species. Second, the rate for bacteria to become phage resistant is a significant con-

Contribution: For this review article I reviewed current literature, contributed content and assisted with article scope and editing.

APPENDIX B. YEAST DERIVED LYSA2 CAN CONTROL BACTERIAL CONTAMINATION IN ETHANOL FERMENTATION

Yeast derived LysA2 can control bacterial contamination in ethanol fermentation.

Authors

Jun-Seob Kim[1,2], M. Angela Daum[1], Yong-Su Jin[1,2], Michael J. Miller[1,2*]

Affiliations

[1] Department of Food Science & Human Nutrition, University of Illinois, Urbana, IL 61801, USA.

[2] Institute for Genomic Biology, University of Illinois at Urbana-Champaign, Urbana 61801, Illinois

Contact info for corresponding author

*Email contact for corresponding author, Dr. Michael J. Miller: mille216@illinois.edu

Contribution: This manuscript was submitted to Biotechnology for Biofuels March 17, 2016 and is currently under review. JSK created the overall objectives and design with the advice of MJM and YSJ. Research and optimization of experiments was done by JSK and MAD. JSK performed the experiments, consolidated and analyzed the data. JSK and MAD wrote the manuscript with critical review provided by MJM.

APPENDIX C. DDW THE COLOR HOUSE NATURAL COLORING COMPETITION 2014-2015 WINNER MYSTICAL LEMON BERRY BLUSH

2015 Winners: Mary Angela Daum and Max Van Tassel

University of Illinois at Urbana-Champaign students Angie Daum and Max Van Tassel won the 2015 Student Competition with their idea to use the inherent color-changing properties of anthocyanins to create an ice-cream novelty for children called Mystical Lemon Berry Blush that changed colors from purple to pink.

Angie and Max were presented their awards at the University of Illinois reception at IFT by DDW associate and University of Illinois alumna, Jennifer Brown.

Contribution: I conceived the product, researched anthocyanins and the market, authored the report and formulated the gelato. Max provided critical input on the report and formulations, led the charge on the topping, designed the poster and killer product marketing and graphics.

DDW is a registered trademark of DDW the Color House.
http://www.ddwcolor.com/studentcompetition/student-competition-winners/ accessed April 30, 2016

APPENDIX D. EFFECT OF NISIN AND LYSOZYME ON FERMENTED CIDERS CONTAMINATED BY LACTIC ACID BACTERIA

Contributions: This abstract and poster was accepted for IFT 2016 and presented at the UIUC Undergraduate Research Symposium by an outstanding undergraduate researcher in our lab, Josh Warren. Since he was interested in researching fermented beverages I suggested a project researching the potential of nisin and hen egg white lysozyme to control in hard cider. His project was funded by an Undergraduate Research grant. With my guidance and initial references, he drafted the proposal. He designed and executed the experiments, coming to me for assistance, troubleshooting or questions on experimental direction. He plans on working on submitting a manuscript on his project this summer.