REPLICA PLATING AS AN EFFECTIVE MEANS TO IDENTIFY ANTIMICROBIAL SPOILAGE AGENTS

by

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ABSTRACT

During food processing, addition of preservation ingredients can prolong shelf life of food and thus limit waste. “Clean label” food preservatives are gaining popularity in the food manufacturing industry as consumer demands shift away from synthetic and chemical ingredients. Natural preservatives are antimicrobial substances derived from bacterial cell metabolism. This study utilized a library of microorganisms to screen for antimicrobial metabolites that inhibit food spoilage organisms. Replica plating was used to screen over 1,000 microbes from a variety of food and environmental sources against a panel of food spoilage organisms. The data was recorded and the top antimicrobial producer strains were tested further to identify the potential metabolite produced. GENEWIZ and the National Center for Biotechnical Information (NCBI) Blast database were used to identify potential producer strains using 16S sequencing and ensure the microorganisms are Generally Recognized as Safe (GRAS). The results of this study will be helpful in identifying novel microorganisms to be potentially used as natural antimicrobials in food.

DEDICATION

I want to thank my three older brothers for teaching me, throughout life, that as circumstances change, adjustment is made easier by leaning on others for support. I want to thank my grandfather, Dr. Robert Balmer, for encouraging me to follow his footsteps to higher education. This research could not have been possible without the many conversations and love shared between my mother, myself, and our adoration for food. To Anthony, my partner, I love you.

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Chapter 1

INTRODUCTION

Statement of Problem

One-third of food produced for human consumption is wasted globally, which amounts to about 1.3 billion tons per year. This means that enormous amounts of the resources used in food production are lost, and that greenhouse gas emissions caused by production of food that gets wasted are unnecessary. About 25% of all foods produced globally are lost due to microbial spoilage (Petruzzi, et al., 2017). Food waste is caused by a) production errors that create food products with the wrong weight, shape, appearance, or damaged packaging, all without affecting the safety, taste, or nutritional value of the food; b) microbial food spoilage in the production line and by consumers (Gustavsson, et al., 2011).

When a food product becomes spoiled, it becomes unsuitable for human consumption. Food spoilage can be initiated by a variety of factors including the type of food, how well it is packaged and stored, the quality of the raw materials, handling, distribution, and temperature.

Microbial food spoilage is caused by microorganisms that break down the food, producing acids and other waste products, and fungi (mainly mold and yeast) that can produce surface fuzz and slimes of many different colors (e.g., black, white, red, brown, and green). Microbial spoilage by molds and yeasts, such as the souring of milk and the growth of mold on bread and rotting of fruit and vegetables, are rarely harmful to humans, but bacterial spoilage is often more dangerous because the food does not always look bad, even if it is severely contaminated (Food and Agriculture Organization, 2011).

Some types of spoilage can be caused by pathogenic bacteria, which can have serious health consequences. *Clostridium perfringens*, which is the common cause of spoilage in meat and poultry, and *Bacillus cereus*, which is the common cause of spoilage of milk and cream, are both pathogenic. When exposed to unsuitable storage conditions these organisms can multiply rapidly and they can release toxins that will cause illness, even when cooked to proper temperatures.

The societal impact of food spoilage has been reduced through recent technological advancements such as utilizing antifungal microorganisms for use in food preservation (Li, et al.,

2020; Rather, et al., 2013; Magusson, et al.,2003). This increasingly popular technique is known as biopreservation, which involves the use of natural or added microorganisms, fermentates, or their metabolites to extend food shelf-life (Lianou, et al., 2016). Biopreservation is a relatively new concept and the vast library of probiotic bacteria already discovered is growing larger as research gains momentum (Minelli and Benini, 2008).

The food industry is continually creating new microbial habitats with the development of new food products, either by developing new products and reformulating traditional ones, or by chance, because of the composition of raw materials or in production. However, modern preference for fresh foods with extended shelf life and free of chemical preservatives leave foods more vulnerable to spoilage, as well as increasing the diversity of spoilage species. Abundant and diverse spoilage in food is a concern that requires extensive research to find novel solutions.

Purpose of the Research

The specific aim of this research was to discover anti-spoilage microbes. This was accomplished by:

1) screening at least 1,000 microorganisms for antimicrobial activity, and then

2) identifying the producer strains via 16S sequencing.

Chapter 2

LITERATURE REVIEW

The earliest reported methods of food preservation were sun drying and ice storage. Around 12000 BC, prehistoric people used the sun to dry copious quantities of foodstuffs. This prevents bacterial growth, because up to eighty percent of spoilage-causing moisture is removed (Kaloyereas, 1950; Rahman, M., S.,2007).

By 500 BC, Greeks and Romans discovered that immersing foodstuffs in fluids with a high pH (like honey) would preserve them and by 1400 AD people discovered that storing food in low pH solutions like a salt solution (brine) would preserve them (Stuart, 1986). When meat is submerged in a salt solution, the salt draws water from the meat and kills bacteria.

More recently, mechanical refrigeration, sterilization (canning), pasteurization, mechanical dehydration, vacuum packaging, irradiation, and biopreservation have been developed to keep food for extended periods (U. S. Food and Drug Administration, 2021).

Biopreservation, using natural microorganisms and their byproducts to preserve food, allows food processors to increase food safety by limiting exposure to synthetic preservation techniques, since repeated exposure to chemical preservatives may cause health concerns (MacDonald and Reitmeier, 2017). Furthermore, recent consumer trends indicate a desire for more natural foods as compared to chemically altered compounds (Third Wave Bioactives, 2019).

Food spoilage is a disagreeable change in food that affects taste, touch, or look as well as its potential safety for consumption. Food spoiling depends on the composition of food, the microorganisms present, and various physical, chemical, and biological factors. For example, species of the strain *Pseudomonas* are major causes of rancidity (Dousset, et al., 2016). In other cases, bacteria able to digest protein, known as proteolytic bacteria, break down the protein in meat and release odoriferous products such as putrescine and cadaverine.

Microbial spoilage of food is due to the growth and metabolism of certain bacteria, yeasts, or molds. Chemical spoilage of food may occur via nonmicrobial enzymatic action, oxidation, or non-enzymatic browning. Physical spoilage of food includes excessive water loss, increase in moisture of dry foods, freezer burn, and recrystallisation of frozen foods. However, there are

occasions when the cause and manifestation of spoilage is a combination of these different types of hazards, as shown in Figure 1 below (Blackburn, 2006).

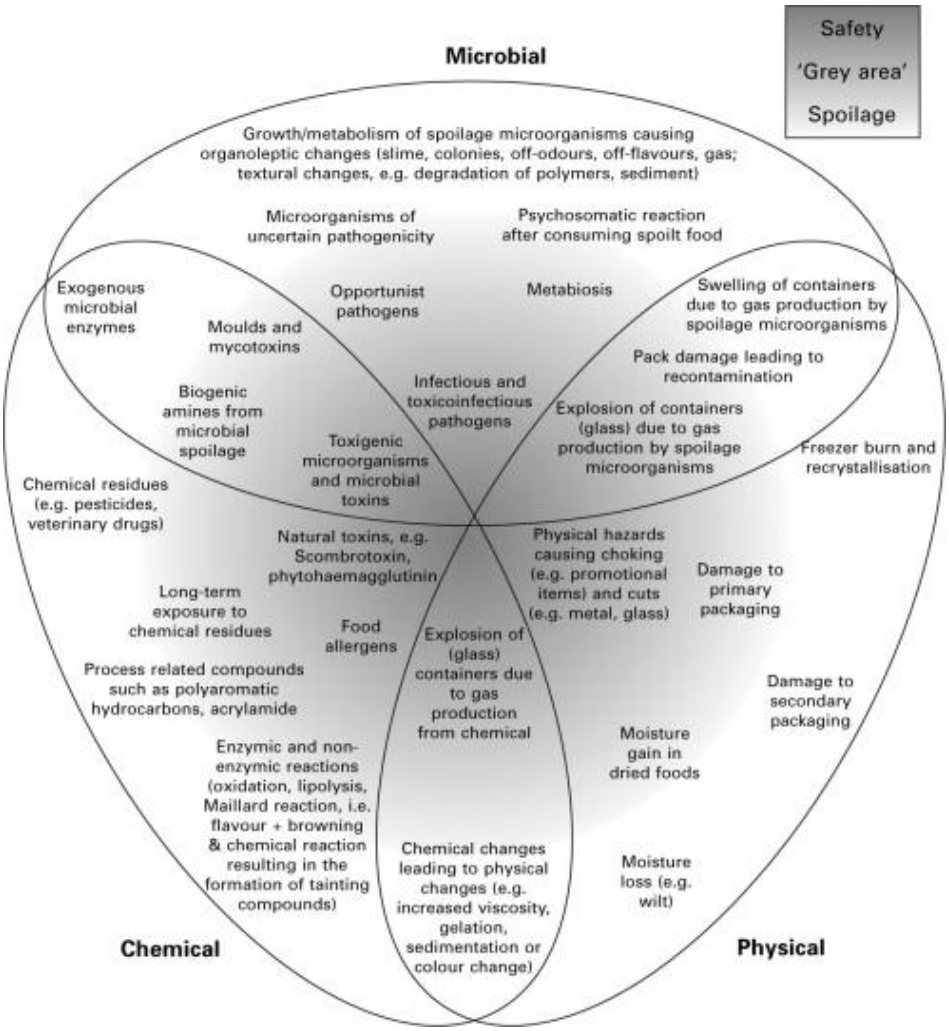

Figure 1: Continuum of potential food-related hazards and their consequences for consumers. (Blackburn, 2006).

For years, the exclusion or elimination of pathogens from food has been studied to prevent degradation and consumer illness. In most countries the legislation has tended to encourage this activity. However, from a microbiological-ecological point of view, research and legislation cannot be distinguished (Petruzzi et al., 2017). Legislation has not caught up to advances in research trends and regulations vary greatly among countries. Despite considerable efforts, microbiological safety assurance seems as remote as ever, even in advanced countries. Death,

suffering, economic losses, and civil claims on behalf of victims of foodborne diseases are matched by the economic losses caused by food spoilage (Petruzzi et al., 2017).

The USDA Economic Research Service estimates about 96 billion pounds of food, or more than a quarter of the 356 billion pounds of edible food available for human consumption in the United States, were lost to human use at three marketing stages: retail, foodservice, and consumers. Fresh fruits and vegetables (19.6%), fluid milk (18.1%), grain products (15.2%), and sweeteners (12.4%), mostly sugar and high-fructose corn syrup, accounted for two-thirds of these losses (Petruzzi et al., 2017). A significant portion of the food loss is due to spoilage by microorganisms, resulting in final products with an inadequate shape or appearance. About 25% of all foods produced globally are lost due to microbial spoilage (Bondi et al., 2014).

However, there is a growing interest in providing consumers with the option of buying surplus food at a discount. New companies like "Too Good to Go" are now connecting consumers to unsold food or soon-to-expire food that would otherwise be thrown away from local restaurants, cafes, bakeries, and grocery stores. Founded in Denmark in 2016, they have saved over 100 million meals across 17 countries, which is the equivalent to 550 million pounds of CO_2 gas emissions. They have also launched initiatives to change date labeling on food, produced free educational resources for schools, and inspired households to change food waste behavior. This company is now on the 2022 TIME magazine's list of 100 Most Influential Companies (Pearse, J., 2022).

The food industry is continually creating new microbial habitats, either by developing new products and reformulating traditional ones, or by chance, because of the composition of raw materials or in production (Argyri et al., 2014). Also, the modern consumers' preference for fresh foods with extended shelf life and products that are free of chemical pesticides leave foods more vulnerable to spoilage, as well as increasing the diversity of spoilage species (Blackburn, 2006; Brandelli, 2015). Food manufacturing companies are continuously looking for unique and safe ways to extend shelf life of food without compromising the sensory attributes of their products. Food preservation has long relied on salt, sugar, and vinegar, all of which contemporary consumers are shying away from to make room for low fat foods, sugar replacers, and less acidic alternatives. Consumers are also transitioning away from canned foods toward fresh food products. All these replacements open areas for microbial spoilage. Canned,

pasteurized, and high-salt, high-sugar foods all have historically been crucial to preservation for mitigating the challenge of long shelf-life. This consumer trend makes room for new market products to give consumers the fresh food they want without compromising the integrity of the product's shelf-life.

Biopreservation by lactic acid bacteria (LAB) is currently the principal method of natural food preservation (Imade, et al., 2021; FAO, 2011). It has been widely studied in fermented foods because of their ability to produce antifungal metabolites and because they are generally recognized as safe (GRAS). Behera et al. (2018) argue that use of probiotic bacteria is a useful strategy to obtain longer shelf life of foods. They also claim they have safer properties due to their ability to delay or prevent the growth of common contamination bacteria.

Salas et al. (2017) state that the food industry is driven by consumer demands and by large governing bodies. Specifically, "public authorities encourage the food industry to limit the use of chemical compounds and to develop natural methods for food preservation." Many grocery store chains are no longer supplying food products with certain ingredients on their labels. For example, Whole Foods Market does not allow food products containing many synthetic preservative products, such as potassium sorbate, butylated hydroxyanisole (BHA), and butylated hydroxytoluene (BHT), to be sold in their stores. Consumer and supplier trends indicate that this new wave of limiting exposure and ingestion of chemical compounds is increasingly common.

Current research typically involves various approaches related to screening for microbial growth used for biopreservation in the food industry. These approaches vary greatly depending on the considered species and strain-to-strain within a species. Screening methods range from spot-on-lawn assays to agar diffusion techniques. In spot-on-lawn assays, cultures of the tested strains are spotted on an agar plate and a second layer containing the fungal target is poured. In the agar diffusion method, the cultures are spotted on an agar layer inoculated with the fungal target. Both assays reveal inhibition after an inhibition halo appears around the samples post-incubation. Screening for microbial growth is similar in instances of fungal growth, mold growth, and bacterial growth. All microbes grow as cultures in nutrient broth where the microbes

can use the nutrients present to multiply. The main difference is that different microbes require different nutrients.

The research reported in this thesis focused on the agar diffusion method in search for bacteria demonstrating antimicrobial properties in screening of bacteria and yeast inhibition and acid production. Potential producers were characterized and identified via DNA sequencing of the 16S rDNA gene.

Chapter 3

METHODOLOGY

This research was conducted at the Technology Innovation Center (10437 W Innovation Drive, Milwaukee, WI) in the offices and laboratory of Third Wave Bioactives beginning in June 2021 and concluding May 2022.

Once the research goals had been established, a research plan flow chart was devised that included the steps shown in Figure 2 below.

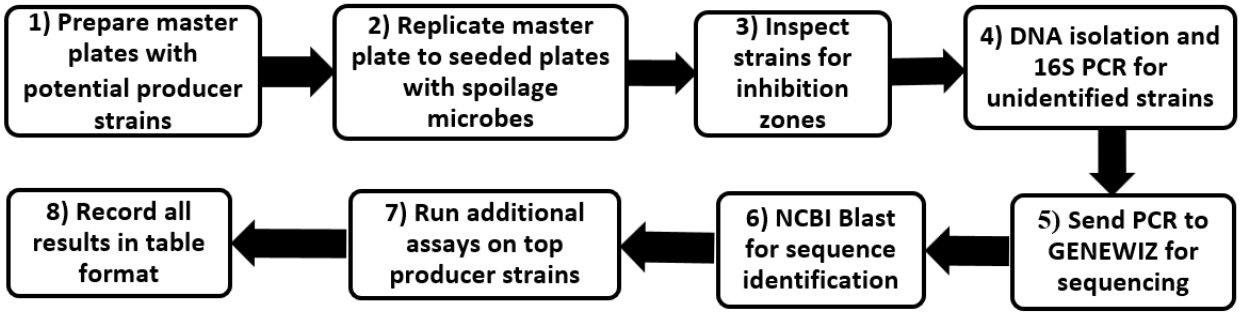

Figure 2: Research Design Flow chart

Preparation of Master Plates

The replica plating method was used to seed cultures on an agar-filled petri plate and, using a replica plating tool and velvet fabric squares, culture colonies were transferred onto new agar-filled petri plates by pressing the cultures onto the replica plates from the Master Plates.

De Man, Rogosa, Sharpe (MRS) agar was used for the growth of lactic acid bacteria (LAB). Tryptic Soy Agar (TSA) was used for the growth of Bacillus spp. The media was sterilized, then tempered in a water bath set between 50 and 60 ºC. Petri dishes were filled with 12 ml of tempered media and allowed to dry. Bacillus and LAB cultures were aseptically picked from frozen glycerol culture vials and inoculated on the surface of the agar plates. Depending on the

number of samples required for testing (9 or 12), the master plates were divided and marked with one of the patterns shown in Figure 3 below.

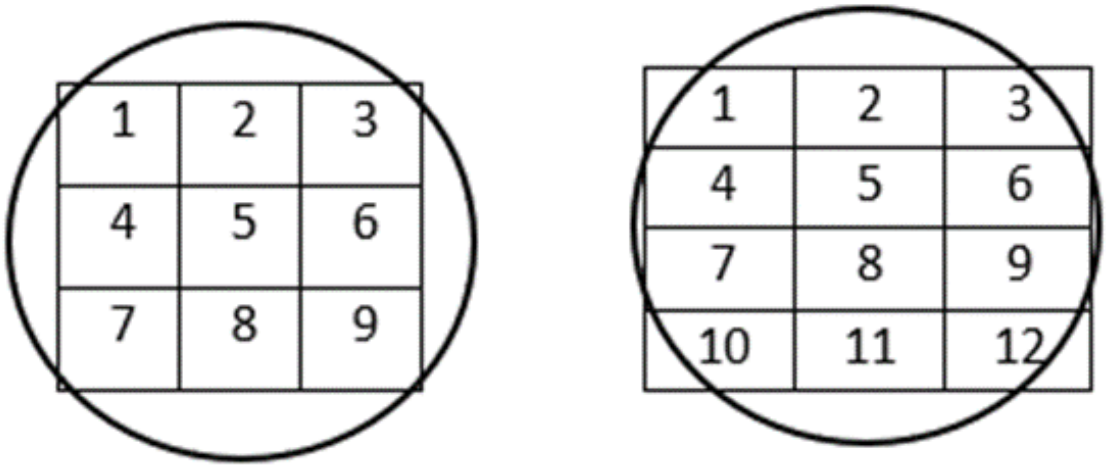

Figure 3: Master Plate Petri dish examples.
Either 9 or 12 samples can be placed on a single plate.

Cultures were allowed to dry on the plates for 20 minutes then inverted and incubated anaerobically for LAB or aerobically for Bacillus at 32°C overnight. Anaerobic conditions were created using an anerobic box with Anaero-Paks (Mitsubishi Gas Chemical Company Inc.) to create an oxygen-free environment.

Preparation of Indicator Plates

Yeast Indicator Plates

Four of the yeast indicators used in this study were obtained from the USDA-ARS culture collection: *Candida parapsilosis* (Y-619), *Rhodotorula mucilaginosa* (Y-632), *Saccharomyces cerevisiae* (Y-1545), *Zygosaccharomyces bailii* (Y-7239). A fifth yeast, *Pichia kudriavzevii* (Y-PK), was collected by Third Wave Bioactives. The day prior to replica plating, the yeast indicator cultures were grown in 5ml Potato Dextrose broth (PDB) at 28°C, shaking at 125 rotations per minute (rpm). Prior to use, indicators were diluted in PDB to a 0.5 McFarland using a densitometer. PDB soft agar was prepared by sterilizing half strength PDB plus 1% agar. The agar media was tempered in a water bath between 50 and 55°C then inoculated with 1% diluted

yeast indicator. Indicator plates were prepared with 12 mL seeded agar and allowed to dry prior to use.

Gram-Positive Indicator Plates

The day prior to replica plating, a *Micrococcus luteus* (B-287) culture was grown in 5ml Tryptic Soy Broth (TSB) at 32ºC, shaking at 125 rpm. Prior to use, the indicator organism was diluted in TSB to a 0.5 McFarland using a densitometer. TSA was prepared by sterilizing for one hour. The agar media was tempered in a water bath between 50 and 55ºC then inoculated with 1% diluted yeast indicator. Indicator plates were prepared with 12 mL seeded agar and allowed to dry prior to use.

Acid Indicator Plates

Calcium carbonate (0.5%) was added to MRS agar to serve as acid indicator plates. The agar was sterilized, tempered in a water bath between 50 and 55 °C, then 12 mL per plate was poured and allowed to dry.

Replication

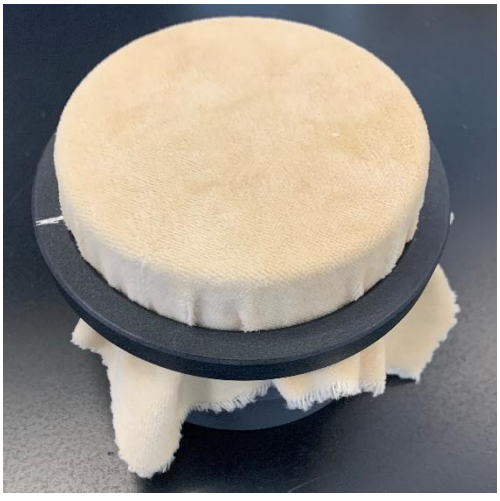

Figure 4: Picture of the Replica Plating tool and secured velvet square

Each master plate and all indicator plates were marked with a line on the edge to align with the line on the replica plating tool ring. A sterile velvet square was secured in place with the ring of the replica plating tool (Figure 4). The master plate was firmly pressed face-down onto the velvet, transferring the colonies onto the velvet surface. The indicator plates were then pressed down making sure to align the plate with the line on the ring. The indicator plates were replicated in the following order: Acid, *M. luteus*, then yeast.

This process was repeated with each master plate and new velvet square, cleaning between each new velvet square with ethanol. All replica plates were incubated at the appropriate incubation temperatures required by the indicators.

After 48 hours, each plate was visually assessed for signs of an inhibition clearing zone surrounding the colony on the indicator plates (Figure 5). Clearing zones were recorded as positive or negative under the indicator in which it was noted.

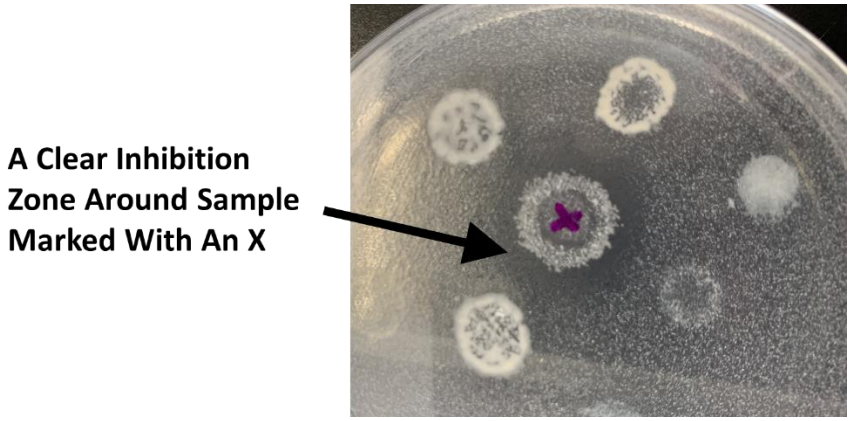

Figure 5: Example of a visual inhibition zone.

DNA Isolation

The procedures that accompany a High Pure PCR Template Preparation Kit for genomic DNA Isolation (manufactured by Roche Diagnostics GmbH in Germany) were followed with the following changes. At the sample lysis and DNA Binding step, researchers added 250 µl rather than 200 µl of bacteria or yeast cells. For bacteria, researchers added 10 mg/ml in 0.01 g/ml of PBS instead of 10 mM Tris-HCl. During the protocol for Washing and Elution, researchers used 450 µl instead of 500 µl of Inhibitor Removal Buffer and 450 µl instead of 500 µl of Wash Buffer.

DNA Identification: 16S rDNA Polymerase Chain Reaction (PCR)

Identification of bacterial strains was performed using a 16S rDNA PCR with primers 27F-YM (5'-AGAGTTTGATYMTGGCTCAG-3') and 1492R-Y (5'-TACCTTGTTAYGACTT-3'). The 20µl PCR reaction was composed of 2mM $MgCl_2$, 0.2mM dNTPs, 0.8 µM each primer (27F-YM and 1492R-Y), 1x PCR buffer, 2U Platinum Taq polymerase, Nuclease Free Water, and 2µL template DNA. The 16S PCR amplification was performed on an Applied Biosystems SimpliAmp Thermal Cycler (Thermo Scientific), with an initial denaturation at 95°C for four minutes, then 35 cycles of denaturation at 95°C (30 seconds), annealing at 50°C (30 seconds), and elongation at 72°C (2 minutes), with a final extension at 72°C for 7 minutes. The PCR products were visualized on a 1.5% agarose gel in 1X LB buffer then sequenced via Sanger sequencing (GENEWIZ). Sequences were run through the National Center for Biotechnology Information (NCBI) Blast 16S Ribosomal database for identification.

Extra Screening Steps

The strains that inhibited yeast growth most effectively were selected for further review. To further evaluate these strains, they were grown in 10 mL MRS broth at 32ºC for 24 hours, then inoculated at 1% into three separate 50 mL MRS broth shake flasks and incubated at 28ºC, 32ºC, and 37ºC shaking 125 rpm, one flask per incubation temperature. The pH of each flask was recorded, and 5 mL samples were taken every 4 hours beginning at 16 hours post inoculation and

ending at 36 hours. The timepoint samples gathered were filter sterilized and used in a broth assay.

Broth Assay

Clear, flat bottom, 96-well plates were inoculated with 100 µl PDB. Filter-sterilized samples were added to row A (100 µl). Serial dilutions were made by transferring 100 µl from row A to row B, mixing well, and so on down the plate. At the last row, 100 µl was discarded so all wells contained 100 µl total. Each well was inoculated with 2 µl of 0.5 McFarland standard indicator yeast Y-632 on one plate and Y-619 on the other. Plates were incubated at 28 ºC for 24 hours and read using an Epoch 2 Microplate Reader by Biotek. Absorbance was read at 600 nm to determine the optical density of the plates. Low optical density numbers confirmed inhibition of yeast due to the presence of samples in the wells.

Chapter 4

RESULTS

A total of 1,019 samples were analyzed. Sample sources included local lake water, soil, and foodstuffs from a variety of food manufacturers. The replica plating method yielded the results shown in Figure 7. 614 samples indicated microorganisms that were positive for acid production, 242 samples indicated inhibition of *Micrococcus luteus* (B-287) growth, 37 samples indicated inhibition of Y-619 growth, 53 samples indicated inhibition of Y-632 growth, 44 samples indicated inhibition of Y-1545 growth, 4 samples indicated inhibition of Y-7239 growth, and 49 samples indicated inhibition of Y-PK growth. Many samples indicated no inhibition activity at all during replica plating. This result indicates that many samples did not grow effectively or they did not produce antimicrobial compounds. The samples that did grow effectively and did inhibit spoilage organisms were selected for DNA identification.

Of all 1,019 samples screened, 591 samples received sequencing results from DNA identification that could be analyzed by the NCBI Blast Database to yield specific strain distinctions. Figure 6 shows the most abundant genus/species from the library. The most found species was *Lactococcus lactis*, with 73 screened samples identified. Figure 7 shows how often during screening each respective indicator organism showed signs of growth inhibition. The strain that was most often inhibited was B-287. The most difficult strain to inhibit was Y-7239. This strain also proved to be the most difficult organism to grow and often required more time in the incubator than the other fungal species.

Strains of microorganisms that inhibited yeast included *Leuconostoc citreum*, *Lactobacillus delbrueckii*, and *Weissella paramesenteroides*. *L.citreum* showed significant inhibition zones on replicate plate Y-632. This strain produced an organic acid compound and did not inhibit B-287. *Lactobacillus delbrueckii* showed significant inhibition zones on replicate plates Y-619, Y-632, Y-1545, and Y-7239. *L.delbrueckii* produced acid that reacted with $CaCO_3$ to produce inhibition zones on the replica plate. *L.delbrueckii* did not inhibit growth of B-287. *Weissella paramesenteroides* was found to inhibit Y-632 most effectively. *Weissella* is a genus of lactic acid bacteria consisting of species formerly in the *Leuconostoc paramesenteroides* group. Similar to

other LAB, they are commonly found in fermented foods but have also been isolated from environmental and human samples.

The *L.citreum* sample was isolated from sausage spoilage, the *L.delbrueckii* sample was isolated from raw milk obtained on a farm in Jefferson, WI, and the *W.paramesenteroides* was isolated from a farm in Johnson Creek, WI.

The results of the broth assay are shown in Figure 8. *L.citreum* (LC) and *L.delbrueckii* (LD) were tested against yeasts 619 and 632 using samples at different time points and different incubation temperatures.

Figure 6: Most found species in DNA identification.

Figure 7: Summary of inhibition in indicator organisms

LC / 619	37C 16HR	37C 20HR	37C 24HR	37C 36HR	32C 16HR	32C 20HR	32C 24HR	32C 36HR	28C 16HR	28C 20HR	28C 24HR	28C 36HR
50%	0.09	0.08	0.08	0.08	0.06	0.06	0.06	0.06	0.06	0.06	0.05	0.05
25%	0.20	0.16	0.19	0.09	0.14	0.12	0.13	0.14	0.13	0.13	0.12	0.12
12.50%	0.26	0.22	0.24	0.24	0.23	0.23	0.24	0.25	0.25	0.26	0.24	0.25
6.25%	0.27	0.27	0.30	0.29	0.28	0.33	0.33	0.32	0.37	0.30	0.33	0.31
pH	**4.6**	**4.55**	**4.51**	**4.48**	**4.31**	**4.31**	**4.3**	**4.3**	**4.33**	**4.3**	**4.3**	**4.31**

LD / 619	37C 16HR	37C 20HR	37C 24HR	37C 36HR	32C 16HR	32C 20HR	32C 24HR	32C 36HR	28C 16HR	28C 20HR	28C 24HR	28C 36HR
50%	0.07	0.06	0.06	0.06	0.12	0.10	0.08	0.06	0.14	0.13	0.11	0.09
25%	0.20	0.15	0.16	0.15	0.25	0.22	0.20	0.16	0.26	0.25	0.22	0.21
12.50%	0.31	0.26	0.29	0.27	0.33	0.35	0.30	0.29	0.39	0.36	0.35	0.31
6.25%	0.33	0.32	0.35	0.32	0.34	0.34	0.36	0.34	0.38	0.36	0.38	0.37
pH	**4.29**	**4.17**	**4.18**	**4.18**	**4.49**	**4.47**	**4.48**	**4.53**	**4.58**	**4.52**	**4.53**	**4.54**

LC / 632	37C 16HR	37C 20HR	37C 24HR	37C 36HR	32C 16HR	32C 20HR	32C 24HR	32C 36HR	28C 16HR	28C 20HR	28C 24HR	28C 36HR
50%	0.06	0.05	0.05	0.05	0.05	0.05	0.05	0.05	0.05	0.05	0.05	0.05
25%	0.09	0.08	0.08	0.08	0.07	0.07	0.07	0.07	0.08	0.07	0.08	0.07
12.50%	0.11	0.10	0.10	0.09	0.09	0.09	0.09	0.09	0.09	0.09	0.09	0.09
6.25%	0.12	0.11	0.11	0.11	0.11	0.11	0.11	0.11	0.11	0.11	0.11	0.11
pH	**4.6**	**4.55**	**4.51**	**4.48**	**4.31**	**4.31**	**4.3**	**4.3**	**4.33**	**4.3**	**4.3**	**4.31**

LD / 632	37C 16HR	37C 20HR	37C 24HR	37C 36HR	32C 16HR	32C 20HR	32C 24HR	32C 36HR	28C 16HR	28C 20HR	28C 24HR	28C 36HR
50%	0.05	0.05	0.05	0.06	0.06	0.05	0.05	0.05	0.07	0.06	0.06	0.05
25%	0.08	0.07	0.07	0.07	0.09	0.09	0.08	0.07	0.09	0.08	0.07	0.07
12.50%	0.09	0.09	0.09	0.09	0.10	0.10	0.09	0.10	0.11	0.11	0.10	0.10
6.25%	0.10	0.10	0.10	0.11	0.12	0.11	0.11	0.11	0.12	0.12	0.12	0.11
pH	**4.29**	**4.17**	**4.18**	**4.18**	**4.49**	**4.47**	**4.48**	**4.53**	**4.58**	**4.52**	**4.53**	**4.54**

Figure 8: Results of Broth Assay

Discussion

The goal of this research was to identify viable microbial and antifungal spoilage candidates. This was carried out by testing candidates against six known spoilage yeasts, one bacterial spoilage agent, and by screening for organic acid production. Ultimately this research will help to discover a strain of lactic acid bacteria that can be used as a natural antifungal food preservative.

Replica plating allowed research to progress quickly throughout the duration of the research window. Replica plating methods were used to screen, on average, 100 samples per week. After 10 weeks, the research parameter of screening 1,000 isolates had been reached and data analysis began. If each sample had to be individually screened, this research project could have taken twice as long to complete. The useful method of replica plating allowed dozens of cultures to be screened in one week. Cultures did not always grow effectively and human error called for retesting of samples.

If an organism evaluated within the scope of this research did not produce an organic acid but did inhibit the bacteria and one or all the yeast indicators, this organism would be considered an ideal candidate for further analysis. If, after DNA identification, the organism was identified as a known lactic acid bacterium, further testing via broth assay analysis would reveal the strength of inhibition within the sample's produced compounds. Strains with high inhibition results would be grown at a larger scale to observe pH, ideal growth temperatures, and growth density.

Within the guidelines of this research, DNA sequencing accurately identified half of the organisms in question. Contamination of samples may have limited the ability of the organisms to grow and inhibit spoilage and the ability of the PCR to amplify strain specifics, Also, cost of DNA identification limited the amount of samples identified within the time constraints of this research.

Leuconostoc citreum and *Lactobacillus delbrueckii* were identified as potential strains to function as a source of natural preservation in food products. The extra screening tests included pH analysis as an opportunity to observe change in the media, such as abundant growth or stasis of growth, temperature changes, and broth assays to assess the percent inhibition exhibited by each isolate. These measures were important to assess optimal temperatures and growth time of the species to fully understand the isolates and how they might function in food production.

Leuconostoc citreum is most often found in soil and in the sugars of food such as olives, cucumbers, processed sauerkraut, wine, and cheese. In an industrial application, *L.citreum* is attractive due to its ability to produce lactic acid and diacetyl. In sauerkraut, *L.citreum* converts glucose and fructose into lactic acid. This microorganism is a facultative anaerobe, meaning that it is an organism which can survive in the presence of oxygen, but can also survive in an atmosphere without oxygen via fermentation or anaerobic respiration.

Lactobacillus delbrueckii is the main bacterium used to produce yogurt and facilitate ripening in cheese. This is a homofermentive lactic acid bacteria and probiotic. Under the microscope, this is visually a gram-positive rod that is non-spore forming. This microorganism is aciduric, meaning that it requires a low pH to grow effectively and grows best anaerobically between 40-44 ºC. *L.delbrueckii* has the potential to work effectively in various products, mainly dairy products, because of its proven effectiveness in commercial yogurt products.

L. citreum and *L. delbrueckii* were used in broth assays following the completion of the objectives of this research. The assays revealed that both strains preferred to be grown at warmer incubation temperatures. At 37 ºC, *L.citreum* and *L.delbrueckii* both inhibited Y-632 best at 50 percent. The optical density was 0.05, and if these strains had been inhibited at percentages lower than 50, further testing would have been warranted.

Another LAB of significance identified in this study was Weissella, a facultative anaerobe with a mesophilic temperature range, typically between 20-45 ºC. It is gram positive and grows most effectively in complex media. Although this LAB shows effective antifungal activity in replica plating, previous research at Third Wave Bioactives showed filter sterilization, heat, and other methods of killing the live organism ultimately resulted in a significant reduction in antimicrobial activity. This limits the use of this strain in food systems because the strains are not alive when applied to food manufacturing.

Strain	**Isolation Location**	**Yeasts Inhibited***
Leuconostoc Citreum	Commercial Sausage Spoilage	Y-632
Lactobacillus Delbrueckii	WI Raw Dairy Milk	Y-619, Y-632, Y-1545, Y-7239
Weissella Paramesenteroides	WI Farmland Soil	Y-619, Y-632

*Determination of inhibition mechanism was outside of the parameters of this research

Table 1: Summary of Data

Chapter 5

CONCLUSIONS

Current research trends indicate large numbers of LAB strains can function as ingredients of food preservation. Novel LAB strains can be found in a wide array of sources, mainly in dairy and in plants. The ability to discover, ferment, and preserve the antimicrobial metabolites of LAB for natural food preservation is complex and can be time consuming. Replica plating on soft agar (half agar and half media broth) was a method that allowed growth of LAB and spoilage organisms together.

The limitations in this research rested in the relationship between how samples grow on various media. An unidentified sample may test well on one media culture but exhibit no growth on others. Replica plating should have shown accurate results; however, proper temperature control, media selection, and human error can skew data. Repeated replica plating for any samples considered for outlying factors mitigated these limitations. To learn if these organisms will work in food can only be discovered via testing in food samples.

Samples that showed yeast inhibition without organic acid production were likely strains that produced bacteriocin. Three strains were selected to undergo further testing via broth assay but did not show inhibition that was promising for further research. However, following the screening of 1,019 isolates, the data compiled will function as a roadmap for further research.

Third Wave Bioactives will continue to develop the results and apply results to run further tests in search of one or more food products to run a sensory analysis for possible commercialization.

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