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Use of Natural Antimicrobials to Control Spoilage in Marinara-Type Sauce

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Use of natural antimicrobials to control spoilage in marinara-type sauce

By

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Marinara-type sauces were created using three natural antimicrobials, as well as two combination treatments (natamycin, propionic acid, cultured dextrose, natamycin-propionic acid, and natamycin-cultured dextrose) and two controls (sodium benzoate-potassium sorbate, no preservatives). Samples were subjected to a shelf-life study at 20 C with both non-inoculated sauce and sauces that were either inoculated with *Zygosaccharomyces bailii* or a cocktail of thermophilic fermentative organisms. Natamycin and Natamycin-propionic acid treatments had fewer log colony counts (CFU/g) of yeast and lactic acid bacteria than the negative control after 42 days of storage and performed as well or better than the positive control throughout the storage period. No sensory differences were detected ($P>0.05$) between the natamycin treatment when compared to the industry standard (positive control), but the natamycin-propionic acid treatment was different ($P<0.05$). Results indicate that natamycin and/or natamycin-propionic acid could be used as a natural alternative in the formulation of marinara sauce.

DEDICATION

I would like to dedicate this research to my parents, Holly and Rocco Abessinio and my sisters Haley and Emma Abessinio for all of their love and support throughout my educational career.

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CHAPTER I

INTRODUCTION

1.1 Introduction

Spoilage of acidified salad dressings and sauces results from a variety of causes including oxidation, emulsion separation, chemical or biological hydrolysis of oils, and the growth of microorganisms that produce gas or off-flavors (Frazier 1967). Microbiological spoilage is commonly attributed to lactic acid bacteria and yeasts, but the growth of molds in these products is also common. Traditionally, acidified specialty products such as salad dressings and sauces are shelf stable or resistant to spoilage for several months at room temperature. Shelf-stability is achieved in these products by having a pH of 4.6 or less and a water activity (a_w) equal to or greater than 0.85 and the presence of chemical preservatives, such as sorbic and benzoic acid (Fellows 2009). Charlton and their colleagues (1934) reported the earliest report found to document the spoilage of acidified sauces and salad dressings due to *Lactobacillus fructivorans.*

Due to consumer perception of chemical preservatives, many food companies have considered investigating how to alter their current product formulations to align with the demand for more naturally preserved foods (Vermeulen and others 2007). The consumer's change of view on the use of ingredients that are not considered natural by the standards of the United States Department of Agriculture (USDA) is the result of a growing segment of 38% consumers (n=1197) who have begun to seek out natural or

organic foods as a perceived method of leading a healthier lifestyle as reported by the Organic Trade Association in 2009 (Sullivan and others 2012). The total sample of 1197 reflects the target population of U.S. households with at least one child under the age of 18, with the respondent being the individual in charge of making decisions at the grocery store and was performed through an online survey.

Acidified foods are formulated using organic acids, chemical preservatives and products of fermentation to reach a final pH of 4.6 or less. The pH of 4.6 indicates the required level of acidity that is necessary to inhibit the indicator pathogen *Clostridium botulinum.* While the prevention of pathogenic growth is the first priority, microbial spoilage will potentially dictate a premature end of shelf-life if ingredients are not included to inhibit the growth of lactic acid bacteria and yeasts. Smittle and Flowers (1982) and Kurtzman and others (1971) studied the microbiological spoilage of salad dressings and similar products and concluded that spoilage resulted from the growth of a select group of acid-tolerant microorganisms. Three organisms have been consistently isolated from these products and include the two lactobacilli, *Lactobacillus plantarum* and *Lactobacillus fructivorans* and the yeast *Zygosaccharomyces bailii.* While numerous other spoilage organisms have been isolated from these products, these organisms have demonstrated resistance to the antimicrobial effects of a low-pH food system.

The addition of antimicrobials to food products is necessary for the inhibition of pathogenic organisms and to lengthen shelf-life by inhibiting spoilage organisms. Davidson (2006) explains that naturally occurring antimicrobials can be derived from plant, animal, and microbial sources. The microbial-derived, natural antimicrobial natamycin has effectively inhibited yeast growth in wine and grape juice applications

(Siricururatana and others 2013; Thomas and others 2005) and dairy applications (Ollé Resa and others 2013; Davidson 2006). MicroGARD™, a naturally occurring microbial metabolite of either skim milk or dextrose, has been used to control Gram-negative bacteria (Lemay and others 2002; Al-Zoreky and others 1990). The product contains antimicrobial agents such as acetic and propionic acids and a proteinaceous inhibitor (Boudreaux and others 1988). Various propionates and active forms of propionic acid are highly effective and naturally derived method of food preservation that exhibits strong activity against acid-tolerant yeasts (Moon 1983).

The objective of this study is to determine the efficacy of a multiple hurdle antimicrobial system that consists of the cultured dextrose metabolite MicroGARD™, natamycin and potassium propionate on reducing the microbial load of lactic acid bacteria and the yeast *Zygosaccharomyces bailii* in a tomato-based marinara sauce, and to determine if combinations of these antimicrobials could be adapted for utilization in real-world applications in the production of natural products.

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CHAPTER II

REVIEW OF LITERATURE

2.1 Acid and Acidified Foods

The United States' Code of Federal Regulations (CFR) Title 21 defines an acid food as a food that has a natural pH of 4.6 or below. However, acidified foods are defined as low-acid foods to which acid(s) or acid food(s) are added. They have a water activity (A_w) greater than 0.85 and a finished equilibrium pH of 4.6 or below. These regulations ensure consumer safety from pathogenic microorganisms and their toxins, specifically *Clostridium botulinum.* Acidification of these foods cannot replace proper sanitation and care during manufacturing. The processor must adhere to high standards of cleanliness and production or Good Manufacturing Practices. Even with efficient acidification and sanitation, a food product can still be spoiled by bacteria, yeasts and molds. To prevent this spoilage, processors usually heat acid and acidified foods to approximately 82 °C or higher and package them hot or aseptically (21 CFR 110.80). This process kills yeasts and most mold spores in the products and in the container and cap. Commercial processors of foods such as beans, cucumbers, cabbage, artichokes, cauliflower, puddings, peppers, tropical fruits and fish are required to adhere to the guidelines for acid or acidified foods. However some common processed foods are not covered under the low-acid food regulations, including but not limited to: standardized and non-standardized food dressings and condiment sauces, alcoholic and carbonated beverages,

tomatoes and tomato products that have a finished equilibrium pH less than 4.7 (21 CFR 114; 21CFR 108.25).

Processors of dressings, sauces, marinades and other similar food products rely among other intrinsic factors than the presence of acids to prevent spoilage (Vermeulen and others 2007). Generally these products are considered "shelf-stable," with the implication of a shelf-life of several months at ambient or room temperature (20-23.5°C). Shelf stability can be achieved by a low pH, due to the presence of inorganic or organic acids, a low water activity due to higher solute concentrations, the presence of chemical preservatives, and a combination of these strategies. Among the most popular foods that fall into the categories of dressings and sauces are mayonnaise, mayonnaise-based sauces, tomato sauce, and other tomato-based sauces such as marinara sauce. Mayonnaise is defined by 21 CFR 25.1: "the semisolid emulsion of edible vegetable oil, vinegar, lemon juice and/or lime juice, egg yolk containing ingredients, with one or more of the following: salt, sweeteners, mustard, paprika, and other spices, monosodium glutamate." The finished product has a creamy yellow color with a final equilibrium pH range between 3.6 and 4.0, 0.29% to 0.5% acetic acid, 9.0 to 12% salt, 7.0 to 10% sugar and no less than 65% vegetable oil are present in the finished product (Smittle and Flowers 1982). Fialova and others (2007) state that mayonnaise is a relatively microbiologically stable product due to its high fat content (700-800 g kg^{-1}) and the inclusion of organic acids or acid ingredients. These acids contribute a desirable flavor to the product and are bactericidal to foodborne pathogens such as *Salmonella, Escherichia coli* O157:H7, *Listeria monocytogenes, Staphylococcus aureus* and *Yersinia enterocolitica*.

While no standard of identity exists for marinara sauce, it can be classified as a modified tomato concentrate by definition. As outlined in 21 CFR 155.191: tomato concentrates are the liquid obtained from mature red tomatoes (*Lycopersicum esculentum*) and or the peelings, pieces, or residues of such tomatoes. The resulting product must be preserved by sterilization, refrigeration or freezing. Optional ingredients may be added including salt, lemon juice/organic acids, sodium bicarbonate, water, spices and flavoring.

2.2 Physical Properties of Acidified Foods

The preservation of acidified foods is dependent on several intrinsic properties of the food system. The control of microbiological growth in high acid foods is dictated by the pH, water activity, the use of chemical preservatives, and in many cases through the primary packaging of the product. Often all of these measures in conjunction with thermal processing are used to provide a safe, stable product (Smith and Stratton 2006). Due to the often-harsh negative sensory changes that are caused by a single-control preservation system, multiple controls are often used simultaneously or hurdled together such as pH, Aw, and use of antimicrobials.

Smith and Stratton (2006) explain that acid foods rely on one or more organic food acids, such as citric, lactic, or acetic acid to achieve a pH of 4.6 or below. Acidified foods such as salad dressings and sauces utilize acetic acid (as vinegar) to achieve the desired acidity of the product. Water activity (A_w) can be defined as the ratio of the partial vapor pressure (p) of water associated with the food system and p_o, the vapor pressure of pure water at the same temperature (Bell 2007). Barron (2000) describes

water activity (A_w) as the measure of the available or free moisture in a food product. Water activity values of 0.85 or higher can promote the growth of potentially harmful bacteria such as *S. aureus.* This pathogen can grow at a water activity as low as 0.85 without any other inhibiting factors present. Therefore, 0.85 has been used as the safe cutoff level for pathogen growth (Smith and Stratton 2006). Most salad dressings and sauces are classified as moist foods with water activities above 0.85. Therefore, these foods require refrigeration or another hurdle to control the growth of microorganisms. Some sauces and dressings have high solute or oil contents and are considered intermediate moisture foods (Aw of 0.60 to 0.85). Since most dressings and sauces are moist foods, water activity must be controlled. There are two common methods that are used to control water activity in sauces and dressings. These methods include drying and adding solutes such as sugar or salt to bind water molecules. The method of drying is not applicable to dressings and sauces. Therefore, adding salt and sugar is necessary and water activity must be controlled through product formulations (Smith and Stratton 2006).

2.3 Preservation Technology

In addition to thermal processing, the most commonly used preservatives are sorbic and benzoic acids at concentrations of 0.05 to 0.2% as a preservation technique in acidified specialty products (Sperber 2009). Sperber also explains that both preservatives possess a high partition coefficient, which causes their inhibitory activity to decrease as the lipid content within a product is increased. Gould (1996) reported the lipophilicity of weak organic acids, and indicated that sorbic and benzoic acids were the most lipophilic acids. The lipid solubility of these acids in their undissociated forms gives them the

ability to cross the cell membrane and enter the cytoplasm within a cell (Booth 1985). Also imperative to the efficacy of these acids is their dissociation constants, which indicates the pH at which their activity begins to increase, with activity increasing as pH decreases. Benzoic and sorbic acids have p*Ka* values of 4.2 and 4.76, implying that when used as a preservative in a food system with an equilibrium pH of 4.2 or less, activity is greatly increased (Piper and others 2001; Gould 1996). Sorbic and benzoic acids are typically added to foods in their salt forms, potassium sorbate and sodium benzoate. The presence of the sodium and potassium ions in these compounds greatly increases water solubility and allows for the acid to remain relatively undissociated within the food system (Hettiarachchy and others 2007). The presence of these acids within the cytoplasm causes the cell to inefficiently use Adenosine Triphosphate (ATP) by transporting the excess hydrogen ions back out of the cell membrane (Eklund 1985). This increases the energy demand and causes the cell to discontinue growth by restricting the efficient generation of ATP (Gould 1996).

The use of the chemical preservatives sodium benzoate and potassium sorbate is considered an industry standard for salad dressings and sauces to inhibit yeast and mold growth (Smith and Stratton 2006). Some consumers have become progressively more concerned about foods containing chemical preservatives, and have the tendency to choose foods that they perceive to be natural and safe (Gutierrez-Larrainzar and others 2012; Lemay and others 2002; Siricururatana and others 2013). The current shift in consumer demand has required food companies to alter their formulations by removing compounds that are considered chemical preservatives and replace them with compounds that are considered natural antimicrobials. The pursuit for more natural antimicrobials has

resulted in food scientists investigating the inhibitory effects of compounds such as naturally produced organic acids, essential oils, bacteriocins as well as dried fermentation-based products for their use in food products (Lemay and others 2002).

The addition of antimicrobials to food products is necessary for the inhibition of pathogenic organisms, and to lengthen the shelf-life by inhibiting the growth of spoilage organisms. Davidson (2006) explains that naturally occurring antimicrobials can be derived from plant, animal, and microbial sources. These compounds can be used alone or in hurdle technology with other physical or chemical preservation methods. Many of these natural compounds used in conjunction with a traditional preservation method such as thermal processing can improve the compound's antimicrobial activity within the food system. While numerous studies have been performed on naturally occurring food antimicrobials, many have not received approval by regulatory agencies for their use as direct food additives. The microbial-derived compounds nisin and natamycin have been approved as food additives in over 40 countries and are considered GRAS (generally recognized as safe) products by the FDA and considered a natural preservative by the European Union (Ollé Resa and others 2013). While the list of available potential antimicrobial compounds is increasing, data pertaining to their antimicrobial spectrum and effectiveness in specific products is limited. Very few of these compounds have research supporting their activity in actual food systems. Among the many concerns with natural antimicrobials is the issue of potential development of resistance to the compounds by target organisms of interest. In addition, a drawback of many of these compounds is their adverse effects on the sensory properties of the foods. Thus, research must be performed to determine the level of activity of the compound in the food matrix,

as well as ensuring that sensory properties of the product are maintained or improved (Davidson 2006).

Natamycin or pimarcin is a polyene marcrolide antimycotic compound that is produced by the controlled fermentation of dextrose by the bacterium *Streptomyces natalensis.* (Ollé Resa and others 2013; Sriricururatana and others 2013; Davidson 2006). Ollé Resa and their colleagues (2013) explain that natamycin inhibits yeasts by specifically binding to ergosterol without permeating the plasma membrane. It prevents vacuolar fusion through this specific interaction with ergosterol. In unicellular eukaryotic organisms such as yeasts, ergosterol is the principal sterol present in their plasma membranes at concentrations of about 10 – 30% (mol/mol). Free ergosterol is located in the plasma membrane and is responsible for cell integrity. The ability of microorganisms to counteract stress conditions is correlated with their ergosterol content (Liu and others 2013). Therefore, natamycin is only active against yeasts and molds, not bacteria, protozoa and viruses.

The yeast-inhibiting effect of natamycin has been evaluated in several food systems, specifically in wine and dairy applications: A shelf-life study conducted by Siricururatana and others (2013) evaluated the effectiveness of antimicrobials for cold filled still and carbonated Concord and Niagara grape juices, which are conventionally preserved by chemical preservatives. Juices were inoculated with a spoilage yeast mixture of *Dekkera, Kluveromyces, Brettanomyces,* and *Zygosaccharomyces* at 10^2 and 10^4cfu/ml. The treatments included a negative control (no preservatives), a positive control (potassium sorbate and sodium benzoate), cultured dextrose (MicroGARD™ 200), dimethyldicarbonate (DMDC), natamycin, and a combination treatment of DMDC

and natamycin. The cultured dextrose treatment proved to be ineffective at levels tested in both types of juice. Whereas the most promising results were obtained from the DMDC and natamycin combination treatments in still Niagara juice and carbonated concord and Niagara juices. The combination treatment of DMDC and natamycin extended the shelf-life of the juices so that they had similar shelf-lives to that of the positive control (153 to 161 days).

von Staszewski and Jagus (2007) investigated the antimicrobial activity of MicroGARD™ individually and in combination with nisin against *Listeria innocua* in liquid cheese whey. MicroGARD™ displayed a similar effect to the untreated whey and did not reduce the initial load of *L. innocua* during storage at 7, 12, 20, and 25°C. Conversely, nisin exhibited an immediate partial bactericidal effect, followed by regrowth. A significant antagonistic effect was detected in the combination treatment of MicroGARD™ and nisin in all the systems that were evaluated. The combination of MicroGARD™ and nisin appears to be a practical means for extending the shelf life of liquid cheese whey due to its immediate and strong bactericidal, but short-term bacteriostatic effect.

Thomas and others (2005) studied the effects of the addition of natamycin on common spoilage yeasts in wine. Yeast spoilage has long plagued the wine industry worldwide as a major cause of economic loss. Yeast strains such as *Zygosaccharomyces bailii* and *Saccharomyces byanus* are known for their resistance to common chemical preservatives as well as sulfur dioxide, a common preservative compound added to wine. These strains have adapted to the harsh environmental conditions that are characteristic of wine such as low pH, high acid, and the presence of ethanol. The results of the study

indicated that natamycin could be used to reduce the levels of sulfur dioxide and chemical preservatives, and inhibit the growth of *Saccharomyces* and *Zygosaccharomyces* strains. This is potentially desirable to both consumers and producers in terms of public health and maintaining wine quality.

MicroGARD™ is described as a cultured dextrose or skim milk powder that is produced commercially to replace chemical preservatives by inhibiting spoilage organisms. MicroGARD™ is produced through the fermentation of dextrose or skim milk utilizing the bacteria *Propionibacteria freudenreichii* subsp. *shermanii,* with the product being standardized with either skim milk solids or maltodextrin. It has been demonstrated that MicroGARD™ has the ability to inhibit spoilage in a variety of foods by retarding or preventing the growth of Gram-negative psychrotrophs and some select yeast and molds (Sindt 2003; Buard and others 2003). The microbial metabolite contains various antimicrobial agents, with acetic acid and propionic acid being the most abundant (Buard and others 2003; Lemay and others 2002). MicoGARD™'s antibacterial activity functions similar to that of sorbic and benzoic acids, as the pH of the food system decreases the constituent acid's activity increases. Propionic and acetic acids have p*Ka* values of 4.87 and 4.76 in their undissociated forms and therefore readily diffuse through the cell membrane and increase the internal pH of the microorganism due to the increased hydrogen ion concentration. This is energetically unfavorable for the cell, using ATP to transport hydrogen ions back across the membrane instead of proliferating (Piper and others 2001; Gould 1996). The antimicrobial effect of MicroGARD™ is described in the following studies:

Lemay and others (2002) researched the inhibitory effect of MicroGARD™ 100, MicroGARD™ 300, nisin, Alta 2002, Perlac 1902, sodium lactate, and the essential oil of mustard on both pathogenic and spoilage microorganisms that were inoculated on an acidified chicken meat model (pH=5.0) and stored for two weeks at 22°C. The *Escherichia coli* population decreased to close to or below detection for all treatments, including the control during the 14 day storage period. Sodium lactate and the essential oil of mustard proved to be effective against *Brochothrix thermosphacta,* aerobic mesophilic and lactic acid bacteria. The other antimicrobials tested (MicroGARD™ 100, MicroGARD™300, nisin, Alta 2002 and Perlac 1902) had no significant effect on any of the target organisms when compared to the control. It was concluded that five of the antimicrobials were not effective at controlling pathogenic or spoilage organisms alone, but might have potential when combined with other preservative agents or methods using hurdle technology.

Al-Zoreky and others (1990) explored the antimicrobial activity of MicroGARD™ against food spoilage and pathogenic microorganisms. The fermented milk product which contains antimicrobial metabolites proved to be specifically inhibitory towards most Gram negative bacteria at a 1% concentration in growth media. Some yeasts were only partially suppressed by the antimicrobial. However, *Aspergillus niger* and a yeast common to yogurt spoilage were tolerant to concentrations of up to 5% MicroGARD™. Spoilage lactic acid bacteria such as *Lactobacillus plantarum* and *brevis* exhibited a stimulation effect at a low concentration (1%) of MicroGARD™. Total inhibition against the Gram-negative psychrotroph *L. plantarum* at pH 5.3 was achieved by using 3% MicroGARD™ against high microbial loads (10^6 to 10^7 cfu/ml), with

inhibition lasting more than 7 days at 30°C. At a 1% concentration, similar inhibition occurred when cells were further diluted to approximately 10^4cfu/ml. This demonstrates its usefulness as a shelf-life extender of acid foods. Compared with other food preservatives, diacetyl and propionate are more inhibitory at pH values below 7.0 than MicroGARD™. These results are likely due to weak acid components of MicroGARD™ acetic and propionic acids existing at a much lower concentration than directly adding propioniate to the food system.

Moon (1983) evaluated the effects of acetic, lactic, and propionic acids individually and as synergistic mixtures for their effectiveness at inhibiting the growth of acid tolerant yeasts. The in vitro study revealed that all yeasts could grow at relatively high concentrations of acetate and lactate (100 mmol/l) but were eventually inhibited as the concentrations continued to increase. Propionate possessed a greater inhibitory effect than lactate or acetate. Cellular growth rate was affected by high concentrations of all three acids. However, final yeast cell yields were not affected. A synergistic combination of propionate and acetate proved to be inhibitory to all yeast species. When propionate was used alone, higher concentrations were required to cause the same inhibitory effect as the propionate/acetate treatment at a lower concentration. Therefore, it may be advantageous to utilize propionic acid in foods with natural or added acetic acid present, as it may cause a reduction in yeast growth rate and increase storage stability.

2.4 Microbiological Spoilage

The low pH and high acid concentration in acidified specialty food products are effective at inhibiting bacterial sporeformers and other vegetative bacteria. However, several species of lactic acid bacteria, particularly *Lactobacillus fructivorans, L. brevis,*

L. buchneri, and *L. plantarum* are capable of growing in these environments and are responsible for approximately 25 % of the spoilage that occurs in these types of products (Sharpe and Pettipher 1983; Sperber 2009). The remaining 75% of spoilage in acidified food is due to yeasts, with 62 yeast species capable of spoiling these products (Sharpe and Pettipher, 1983). The most commonly isolated yeasts are *Saccharomyces cervisiae* with a frequency of 7.37% and *Zygosaccharomyces bailii* at 5.52% (Sperber 2009; Deak and Beuchat 1996). Due to its enhanced rate of growth in the presence of fructose, the organism was named. *Z. bailii. This yeast* is capable of tolerating low pH and high salt concentrations, as well as actively transporting weak acids to the exterior of the cell membrane, which greatly reduces the effectiveness of common preservatives.

Kurtzman and others (1971) studied the microbiological spoilage of mayonnaise and salad dressings, with the intent of isolating the microorganisms responsible and to identify the mode of action by which spoilage is caused in these products. *Saccharomyces bailii* (later renamed *Zygosaccharomyces bailii*) was isolated from two-thirds of the spoiled salad dressings that were investigated. The remaining samples were spoiled by *Lactobacillus fructivorans.* Conversely, one sample contained large counts of both *Z. bailii* and *L. plantarum*. It was determined that *Z. bailii* ferments glucose more readily than any other carbohydrate source. *L fructivorans* ferments monosaccharides more readily than sucrose, much like *Z. bailii*. The source of both microorganisms was determined to be attributed to contaminated ingredients and unsanitary manufacturing equipment and environment.

Smittle and Flowers (1982) conducted research that further substantiated and built off of the findings of Kurtzman and others (1971), specifically concentrating on acid

tolerant microorganisms that are involved in the spoilage of salad dressings. The data collected indicated that the spoilage of these products resulted from the growth of *Lactobacillus fructivorans* and *Saccharomyces bailii.* Aside from sharing a similar resistance to acidic conditions, these organisms also rapidly ferment fructose. Due to the deliberate hydrolysis of sucrose by acid or heat within products such as salad dressings or sauces, spoilage by these organisms is often delayed until sucrose is divided into its component parts of glucose and fructose. The research concluded that for any further studies, the addition of fructose to any growth media for enumeration would be advantageous for both organisms. This addition greatly improved the recovery of yeast colonies and decreased incubation time that was required for colony formation by lactobacilli.

Minimal research has been reported on the spoilage of food products by *Zygosaccharomyces bailii.* Thomas and Davenport (1985) profiled the characteristics and method by which spoilage occurs within various food products. It was reported that *Z. bailii* has proven to be exceptionally difficult to inhibit due to several chemical factors including the ability to grow in a pH range of 2-7, tolerance to organic acids up to 2.5% by volume; growth in high sugar habitats/products (up to 70% sucrose by volume), Tolerance of temperatures as high as 75°C, tolerance to both benzoic and sorbic acids at 1000 and 800 ppm respectively; as well as an ethanol tolerance up to 20% by volume. However, certain synergistic effects were determined that have been proven effective, such as reduced tolerance to acid in the presence of higher concentrations of sodium chloride. Several studies have observed the uptake and utilization of acetate by *Z. bailii* (Sousa and others 1996; 1998). However, there is minimal information available on how

Z. bailii acquires its resistance to weak acids (Piper and others 2001). This is in part due to the variability in cellular acid resistance among any *Z. bailii* culture, with a small proportion demonstrating remarkable acid resistance, which makes it difficult to assign numerical values on the weak acid resistance of the yeast (Steels and others 2000).

Spoilage characterized by bulging plastic bottles of tomato ketchup as a result of gas formation was studied by Bjorkroth and Korkeala (1997). Samples on Man-Rogosa-Sharpe agar produced microbial growth that was indicative of a species of *Lactobacillus* as the causative organism. Gel electrophoresis identified the strain as *Lactobacillus fructivorans* using morphological, physiological and biochemical characteristics of the organism. The spoilage level of *L. fructivorans* was determined as 10^5cfu/g, resulting in gas formation in samples that were incubated at between 15 to 30°C.

2.5 Summary

Acid and acidified foods encompass a vast variety of foods that have a natural or equilibria pH of 4.6 or lower (21 CFR 114). Among the most microbiologically stable and safe food products are acidified specialty products or condiments (Sperber 2009). Processors of dressings, sauces, marinades and other similar food products primarily rely among intrinsic factors and the presence of acids to prevent spoilage (Vermeulen and others 2007). Although these products are generally high in sugar and salt and have a low pH, spoilage still occurs due to psychrotrophic lactic acid bacteria, yeasts, and molds that can tolerate these product's seemingly harsh conditions (Sperber 2009). Organisms such as *Zygosaccharomyces bailii* and *Lactobacillus fructivorans* still cause the spoilage of many acidified specialty food products, sometimes causing economic loss (Sperber 2009). The most commonly used preservatives are sorbic and benzoic acids in acidified

specialty products are sorbic and benzoic acids (Sperber 2009). However, consumer demand for natural ingredients in processed foods has increased and the use of natural antimicrobial compounds from a variety of sources has become widely investigated (Ollé Resa and others 2013; Gould 1997). Acidified specialty food processors would value a natural preservative or preservation system that is inhibitory towards the stress-tolerant psychrotrophic organisms that cause product spoilage such as *Lactobacillus* and *Z. balii.*

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CHAPTER III

MATERIALS AND METHODS

3.1 Laboratory preparation of marinara sauces and preservatives

Marinara sauces were prepared in a laboratory-cooking vessel (Groen TDB720, Chicago, IL) in accordance with standard industry procedure and formulations. The marinara sauce was comprised of water (613.7 g kg^{-1}), tomato paste (279.5 g kg^{-1}), sucrose (10 g kg^{-1}), sodium chloride (5.2 g kg^{-1}), Italian spices (9.25 g kg^{-1}), citric acid (1.2 g kg^{-1}), potassium sorbate (0.9 g kg^{-1}), sodium benzoate (0.9 g kg^{-1}), and ascorbic acid (0.5 g kg^{-1}). Positive control samples were prepared using the aforementioned specifications. Conversely the negative control samples were prepared without the use of the chemical preservatives sodium benzoate and potassium sorbate.

Natural antimicrobials replaced traditional chemical preservatives in the preparation of the test treatments. The natural preservatives were added at a usage level in accordance with their manufacturer. The potassium propionate solution (Hawkins, Inc., Minneapolis, MN) was added at the suggested usage level of 0.5%. Natamycin as Natamax® SF (Danisco A/S, Copenhagen, Denmark) was added at 20 ppm. The cultured dextrose product MicroGARD™ 200 (Danisco A/S, Copenhagen, Denmark) was added at 1.5%. In the natamycin-MicroGARD™ combination treatment, the preservatives were added at 20 ppm and 1.5% /weight respectively. In the natamycin-potassium propionate combination treatment, the preservatives were added at 20 ppm and 0.5% by weight

respectively. All the preservatives added to the marinara sauces were added to the aqueous phase of the product mixture and thoroughly incorporated using a high-shear immersion hand blender KHB-1321 (Kitchen Aid Corp., St. Josephs, MI). Following adequate blending, the marinara sauces were subjected to heat treatment at 95°C for 5 minutes, in accordance with industry procedures (per industry correspondence). The sauces were then cooled and packaged in 25 g polypropylene pouches (VAK*3.0 R, Winpak, Winnepeg, CA) and sealed aerobically prior to storage at ambient temperature (~20-23.5°C).

3.2 Culture Preparation

3.2.1 Yogurt Culture

A frozen stock yogurt culture F-DVS YF-L901 (CHR Hansen, Milwaukee, WI), comprised of the fermentative organisms *Lactobacillus delbrueckii* subsp. *bulgaricus* and *Streptococcus thermophilus* were reconstituted in MRS broth (Oxoid Ltd. Hampshire, United Kingdom) and incubated for 24h at 36°C, per the manufacturer's instructions for reconstitution.

3.2.2 *Zygosaccharomyces bailii*

A stock culture of *Z. bailii* (Lindner) Guiliermond, teleomorph (ATCC® 66826™) (ATCC Manassas, VA) was rehydrated in 5 ml of sterile distilled water. The tube was stored at ambient temperature (~20-23.5°C) overnight. The culture was then propagated by transferring it to 9 ml tubes of yeast extract peptone dextrose broth (YEPD) (TEKnova Hollister, CA) and incubated overnight at 25°C.

3.3 Preparation of Growth Medias

de Man, Rogosa, Sharpe (MRS) agar (Remel, Hampshire, United Kingdom) and potato dextrose agar (PDA) (Oxoid, Hampshire, United Kingdom) agars were prepared for the enumeration of Lactobacilli and yeasts according to the manufacturer's instructions and autoclaved at 121° for 15 minutes in a Sterilmatic STM-E (Market-Forge Industries, Everett, MA). Upon cooling to 50°C, a 10% tartaric acid solution (Fisher Scientific, Fair Lawn, NJ) was used to adjust the pH of the PDA to 3.5 to make the media selective for fungi (Mislivec and others 1992).

3.4 Inoculation of Sauces

The yogurt mixture culture and *Z. bailii* were diluted to 4 log cfu ml^{-1}in sterile distilled water. 500 µl of both inoculums were added to 500g of each sauce treatment and then thoroughly mixed using a laboratory stomacher 400 (Seward, West Sussex, United Kingdom) for 30s. The inoculated sauce samples were then divided into 25 g portions in plastic pouches (VAK*3.0 R, Winpak, Winnepeg, CA) and aerobically sealed.

3.5 Analyses of Sauces

Samples were taken after every 14 days of storage until 42 days at ambient temperature (~20-23.5°C). On each day of analysis, the total count of lactobacilli and yeast, pH and water activity were determined. All plate counts, pH, and water activity measurements were performed in duplicate.

3.6 Microbiological Analysis

Sauces (25 g) were diluted in 225 ml of a 1% buffered peptone water solution (Oxoid, Hampshire, United Kingdom) and homogenized by hand shaking for 30s. Serial

decimal dilutions were made using 900 µl buffered peptone water and 100 µl of sample, the original sample being diluted to 10^{-4} of the original concentration. The count of lactobacilli and yeast were enumerated by the surface or spread plate method. 0.1 ml of diluted sample was transferred to the surface of the agar using a sterile pipette. The 0.1 ml sample was then spread on the surface of the respective agar mediums using a bent steel rod that was flame-sterilized between samples starting with the most dilute plate and proceeding to the least dilute plate in the series using aseptic technique throughout. The plates were dried for 15 min and then inverted (Swanson and others 1992). PDA plates were placed directly in a dual program incubator 818 (Cole-Parmer, Vernon Hills, IL) at 35° C for 48h. Conversely, the MRS plates were placed in Gas-Pak™ 150 anaerobic chambers (Becton Dickinson and Co. East Rutherford, NJ) with Humidity Sponge 3150 water desiccants (Control Co. Houston,TX) and AnaeroPouch-Anaero sachets (Mitsubishi Gas Chemical Co., Inc. Tokyo, Japan) to control humidity and achieve an environment suitable for anaerobic growth. The chambers were then placed in the incubator at 35°C for 48h. The plates were counted immediately following the incubation period. Only plates containing between 25 and 250 colonies were counted for lactobacilli and only plates containing between 15 and 150 colonies were counted for yeasts (Mislivec and others; Swanson and others 1992). Counts were then averaged across duplicates and converted to log CFU per gram for reporting (Swanson and others 1992).

3.7 Water Activity (a_w)

Approximately 5 g of sauce was placed into measurement cups, adequately covering the bottom of the cup's surface. The water activities were measured at room

temperature (~20-23.5°C) using an Aqualab 3TE water activity measurement device (Decagon Devices, Inc. Pullman, WA).

3.8 pH Measurement

The pH of the sauces were measured at ambient temperature using an Accumet Dual channel pH/ion meter (Accumet Research model AR25; Fisher Scientific, Pittsburgh, PA) that was equipped with a standardized glass electrode that was calibrated using buffer solutions with pHs of 4 and 7 (BDH Laboratory Supplies, Dorset United Kingdom) and placed directly into the sauce (Accumet Research ATC Probe; Accumet, Singapore)

3.9 Sensory Analysis

To determine if overall flavor differences existed between treatment samples and the positive control sample, a difference-from-control test was performed. Trained panelists (n=31) were presented with the control and test samples simultaneously in a random order with the control labeled accordingly. Panelists were asked to evaluate the control sample first before evaluating any of the randomly coded test samples as well as a randomly coded blind control. Panelists were asked to assess the degree of difference of each coded sample from the positive control sample on a 5-point hedonic scale (0=no difference; 1=slight difference; 2=moderate difference; 3=large difference; 4=very large difference) (Meilgaard and others, 2007).

3.10 Statistical Analyses

A randomized complete block design with a 4 (storage time) x 7 (antimicrobial treatment) factorial structure and three replications was utilized to determine the

antimicrobial activity of potassium propionate, natamycin and cultured dextrose and their combination treatments in acidified marinara sauce over a storage time of 42 d. The antimicrobial effect of each treatment was measured through the determination of the microbial load (log CFU g^{-1}) within the sauce samples after each storage time. The pH, water activity and sensory differences between marinara sauces were also analyzed. When significant differences ($P<0.05$) existed among treatments, the Duncan's Multiple Range Test was used to separate treatment means (Statistical Analysis Software, version 9.3, SAS Institute, Cary, NC). The sensory analysis data was analyzed using a randomized complete block design with panelists (n=31) as the block to determine if a significant difference ($P<0.05$) existed between the control and test samples (Meilgaard and others, 2007).

3.11 References

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CHAPTER IV

RESULTS AND DISCUSSION

4.1 Microbiological Analyses

4.1.1 Samples inoculated with *Zygosaccharomyces bailii*

After 0 days of storage, the negative control (NC) contained 6.1 log CFU/g of yeast, which was greater ($P<0.05$) than the yeast counts for propionic acid (PROPA) and the combination treatment of natamycin and propionic acid (NATAPA) which had colony counts of 2.4 and 3.1 log CFU/g respectively (Figure 4.1). The combination treatment of natamycin and propionic acid was effective at inhibiting yeast growth on day 14, which is indicated by a lower ($P<0.05$) CFU/g (1.6 log) when compared to all other treatments. After 28 days of storage, there were no differences ($P>0.05$) in colony counts between treatments. However the positive control (PC) appeared to be more effective than the other treatments, containing only 4.1 log CFU/g of yeast cells. On day 42, the treatment containing natamycin as an antimycotic agent had a lower ($P<0.05$) yeast count (CFU/g) than the negative control (NC) and MicroGARD™ (MICROG) treatments. Overall the results indicate that in the inoculated yeast samples, propionic acid (PROPA), the combination treatment of natamycin and propionic acid (NATAPA) and natamycin (NATA) were able to decrease the counts on days 0, 14 and 42 respectively when compared to the negative control (NC). In addition, the counts for propionic acid (PROPA), the natamycin and propionic acid combination (NATAPA) and natamycin

(NATA) were not different (P>0.05) from the positive control (PC) for the duration of the storage period. Overall, both treatments containing MicroGARD™ (MICROG and NATAMG) were not different (P>0.05) from the negative control (NC) (Table 4.1). Whereas when averaged over storage time, propionic acid (PROPA), natamycin (NATA) and the natamycin-propionic acid combination treatment (NATAPA) were not different (P>0.05) from the positive control (PC). This demonstrates that the NATA, PA, and NATAPA treatments could potentially be used as a natural alternative to benzoates and sorbates. It is likely that the treatments containing natamycin and potassium propionate were more effective antimicrobials than those containing MicroGARD™ because of differences in their inhibition mechanisms. Ollé Resa and their colleagues (2013) explain that natamycin inhibits yeasts by specifically binding to ergosterol without permeating the plasma membrane. Only unicellular eukaryotic organisms possess ergosterol in their plasma membrane, therefore prokaryotic bacteria would remain unaffected (Liu and others 2013). The ability of microorganisms to counteract stress conditions is correlated with their ergosterol content (Liu and others 2013). Yeasts and molds in the presence of natamycin are less likely to be able to resist the stressful conditions (low pH) of the marinara sauce. This is due to the correlation between the ergosterol concentration in the yeast's plasma membrane, with a direct relationship between stress-resistance and ergosterol content. Potassium propionate inhibits the growth of microorganisms by causing them to use ATP inefficiently due to the increased hydrogen ion concentration within the plasma membrane (Eklund 1985). The hurdling effects of both preservatives on the yeasts and molds present in the marinara sauce could explain the greater efficacy of those treatments when compared to the inhibition mechanisms of MicroGARD™.

Moon (1983) reported that 5% of propionic acid was effective at inhibiting acid tolerant yeasts. In addition, Ollé Resa and their colleagues (2013) determined that 20 ppm and 50 ppm of natamycin reduced the cell counts by 0.5 log CFU and 1.3 log CFU respectively of *Zygosaccharomyces rouxii* after 8 days of storage. *Z. rouxii* is an osmoresistant yeast with similar spoilage activities to that of *Z. bailii* (Pribylova and others 2007). Counts remained at this level for the remainder of the study (10 days).

4.1.2 Samples screened for yeasts and molds

The marinara sauce samples were screened for yeasts and molds with differences ($P<0.05$) in log cell counts (CFU/g) among treatments after zero days of storage (Figure 4.2). The positive control (PC) was the least effective in the inhibition of background yeasts and molds after zero days of storage. The ineffectiveness of the potassium sorbate and sodium benzoate in the positive control may be due to high pH. Sauces that are not completely homogeneous such as marinara sauce with particulates can often take up to 12 days to come to a pH equilibrium (Smith and Stratton 2006). On days 14 and 28, no differences ($P>0.05$) in log colony counts existed between all treatments screened for yeasts and molds. However, at the end of the storage period (42 days), natamycin (NATA) and the natamycin and MicroGARD™ combination (NATAMG) treatment contained fewer ($P<0.05$) log colony counts (CFU/g) than the negative control (NC). Overall for the duration of the experiment, there were no differences ($P>0.05$) between the treatment means (log CFU/g) (Table 4.2). However, natamycin (NATA) had an average 2.2 log colony count versus the 4.2 log colony count of the negative control (NC) and the 3.7 log count for the PC. Very similar to the samples inoculated with *Z. bailii*, natamycin was effective at controlling microorganism growth within the samples

screened for background yeasts and molds. Ollé Resa and their colleagues (2013) determined that 20 ppm and 50 ppm of natamycin reduced the cell counts by 0.5 log CFU and 1.3 log CFU respectively of *Z. rouxii* after 8 days of storage. Similarly, the natamycin-MicroGARD™ combination treatment (NATAMG) resulted in a 0.4 log (CFU/g) reduction over the 42 day storage period (Figure 4.2). The natamycin-MicroGARD™ (NATAMG) treatment was ineffective in reducing the cell count in those samples inoculated with *Z. bailii*. However, the combination displayed efficacy against the background microflora (Figures 4.1 and 4.2). MicroGARD's™ organic acid metabolites such as acetic and propionic acid hurdled with natamycin were more effective against the native yeasts and molds than the stress-resistant *Z. bailii*. Therefore, the natamycin-MicroGARD™ combination treatment could be an effective and practical natural preservation system against some spoilage microflora under acid food conditions.

4.1.3 Samples inoculated with yogurt culture

No differences ($P>0.05$) in log cell counts (CFU/g) were observed between the treatments through the first 14 days of storage (Table 4.3). On day 28, natamycin (NATA), the combination treatment of natamycin and propionic acid (NATAPA), and propionic acid (PROPA) treatments contained lower ($P<0.05$) log cell counts (CFU/g) than both negative and positive controls (PC and NC) and both treatments containing MicroGARD™ (MICROG and NATAMG). After 42 days of storage, the MicroGARD™ (MICROG), propionic acid (PROPA) and the negative control (NC) contained greater ($P<0.05$) log cell counts (CFU/g) than all other treatments and the positive control (PC). A 3.7 log reduction ($P<0.05$) in cell counts (CFU/g) was observed for the MicroGARD™ (MICROG) treatment after 14 days of storage. This reduction is likely due to organic acid

metabolite constituents of MicroGARD™ reaching equilibria pH within the food matrix of the marinara sauce (Smith and Stratton 2006). However, at some point between day 14 and day 28, the antimicrobial began to lose its efficacy. The loss in efficacy could be due to the antimicrobial effectiveness of MicroGARD™ as a function of pH. Al-Zoreky and others (1990) found that as pH increases, the effectiveness of MicroGARD™ as an inhibitory agent decreases. The pH of the marinara sauce containing MicroGARD™ was 4.43 on day 14 with the pH increasing for the remainder of the storage period to 4.56 on day 42 (Table 4.4). The negative control (NC) had similar counts with a reduction ($P<0.05$) after 14 days of storage, with counts increasing for the remainder of the storage period with a similar trend in increasing pH over the remainder of the storage period (Table 4.4). Greater ($P<0.05$) cell counts were observed on days 28 and 42 of storage when compared to 0 and 14 days of storage. The treatment containing natamycin produced a reduction ($P<0.05$) in log cell counts (CFU/g) after 28 days of storage. The cause for the reduction in this treatment is unclear due to the fact that natamycin has been reported to only be physiologically effective against eukaryotic unicellular organisms (Liu and others 2013). Yeasts are generally aerobic microorganisms. However, most species can grow under anaerobic conditions in the presence of a fermentable carbohydrate source such as the added sucrose and fructose that is present in the tomato paste (Sperber 2009). There is a possibility of some yeast species that adapted to grow in anaerobic conditions, *S. cervisiae* is one of the few yeasts that has the ability to grow rapidly under anaerobic conditions (Snoek and Steensma 2007). Assuming that this was the situation in the marinara sauce, the reduction could be due to a species of acid-tolerant, facultative anaerobic yeast being screened as a part of the anaerobic plate count

(APC). Thus the log count (CFU/g) of this yeast would be reduced by natamycin's ability to bind to the yeast's ergosterol in the plasma membrane, allowing for an increase in the hydrogen ion concentration within the cell (Ollé Resa and others 2013). Overall, the treatments containing natamycin and/or propionic acid (NATA, PROPA, NATAPA) and the positive control (PC) had lower ($P<0.05$) log colony counts (CFU/g) than treatments containing MicroGARD™ (MICROG, NATAMG) and the negative control for the duration of the experiment (Table 4.5).

4.1.4 Samples screened for lactic acid bacteria

No differences ($P>0.05$) in log colony counts (CFU/g) were observed between treatments after 0 and 14 days of storage (Table 4.6). After 28 days of storage, differences ($P<0.05$) in log cell counts (CFU/g) were observed between treatments screened for background lactic acid bacteria. MicroGARD™ (MICROG), natamycin (NATA), propionic acid (PROPA) and the positive control (PC) contained fewer ($P<0.05$) log cell counts (CFU/g) than the negative control (NC) and both combination treatments. After 42 days of storage, MicroGARD™ (MICROG), natamycin (NATA), the natamycin and propionic acid combination (NATAPA), propionic acid (PROPA), the natamycin-MicroGARD™ combination (NATAMG) and the positive control (PC) were inhibitory toward the growth of lactic acid bacteria when compared to the negative control (NC). However, only the natamycin-MicroGARD™ combination treatment (NATAMG) contained fewer ($P<0.05$) log colony counts than the negative control (NC). This difference is likely due to organic acid metabolite constituents such as propionic and acetic acids of MicroGARD™ reaching equilibria pH within the food matrix of the marinara sauce (Smith and Stratton 2006). In addition, the natamycin could have some

inhibitory effect against any eukaryotic background organisms that may have been present in the raw ingredients or in the environment during the microbiological analysis. The combination of inhibitory mechanisms of the MicroGARD™ and the natamycin were highly effective against the anaerobic organisms that were present in the non-inoculated samples. It is likely that some type of anaerobic, acid-tolerant yeast was present in the non-inoculated samples since approximately 25% of acidified specialty product spoilage is caused by lactic acid bacteria. Whereas yeasts are responsible for the remaining 75% of spoilage in these products (Sharpe and Pettipher 1983). Overall, there was no difference ($P>0.05$) between the treatments and controls for the duration of experiment (Table 4.7).

4.2 Water Activity

4.2.1 Inoculated Samples

There was no difference ($P>0.05$) in water activity between treatments on day 0 for inoculated samples (Table 4.8). Soluble solids such as sodium chloride, sucrose, citric acid, and ascorbic acid had not yet completely solubilized after 0 days of storage. Therefore, all treatments were essentially identical when water activity was measured. After 14 days of storage, water activity was greater ($P<0.05$) for natamycin (NATA) when compared to the combination of natamycin and propionic acid (NATAPA) and the propionic acid treatment (PROPA). On day 28, water activity was greater ($P<0.05$) for the negative control (NC) when compared to the positive control (PC). Water activity was the highest among the treatments for the negative control (NC) after 42 days of storage. The water activity was the greatest among all samples in the negative control (NC) because these samples allowed for relatively uninhibited growth and fermentation of

carbohydrates by the inoculated organisms. As the water activity increases, the pH decreases in the negative control (NC) over the storage period (Tables 4.4 and 4.8).

4.2.2 Non-inoculated Samples

On day 0, no difference ($P>0.05$) existed in water activity between treatments (Table 4.8). This lack of significance is likely due to the marinara sauce's components not yet reaching equilibrium. After 14 days of storage, the positive control (PC) had a lower ($P<0.05$) water activity than the propionic acid treatment (PROPA). No differences ($P>0.05$) existed in water activity between treatments after 28 days of storage. After 42 days of storage, the positive and negative controls (PC and NC) had higher ($P<0.05$) water activities than both combination treatments of natamycin and MicroGARD™ and propionic acid (NATAPA and NATAMG). The water activities of the combination treatments were likely lower because the growth of spoilage microorganisms were greater in the positive and negative controls (PC and NC) after 42 days of storage than the combination treatments (NATAPA and NATAMG) (Table 4.6). This indicates that the spoilage organisms may have digested the fermentable carbohydrates such as the added sucrose and converted it to their respective metabolic products.

4.3 pH Measurement

4.3.1 Inoculated Samples

On all days of storage (days 0, 14, 28 and 42), MicroGARD™ (MICROG) and the combination treatment of natamycin and MicroGARD™ (NATAMG) had the highest ($P<0.05$) pH when compared to all other treatments (Table 4.4). The treatments containing MicroGARD™ and natamycin (NATAMG, NATA, and MICROG) had

higher pH values since the preservatives themselves cannot immediately participate in donating hydrogen ions to the food system. The organic acid metabolites present in MicroGARD™ eventually may participate in such activities once an equilibrium has been reached within the marinara sauce. The very basic mechanism of natamycin indicates why treatments with natamycin had higher pH values than other treatments. Ollé Resa and their colleagues (2013) explain that natamycin inhibits yeasts by specifically binding to ergosterol without permeating the plasma membrane, which does not directly affect the intercellular pH. On days 14 and 28, the pH values for the positive control (PC) were lower ($P<0.05$) than MicroGARD™ (MICROG) and the natamycin-MicroGARD™ combination treatment (NATAMG) but had a higher ($P<0.05$) pH than all other treatments. On days 14, 28, and 42 the lowest ($P<0.05$) values measured for pH were the propionic acid (PROPA), the negative control (NC) and the combination treatment of natamycin and propionic acid (NATAPA). The propionic acid (PROPA) and natamycin-propionic acid combination (NATAPA) measured the lowest because propionic acid directly participates in the donation of hydrogen ions to the food system. The negative control (NC) exhibited similarly low pH values due to lactic acid produced by lactic acid bacteria.

4.3.2 Non-inoculated Samples

After 0, 14, 28 and 42 days of storage, both treatments containing MicroGARD™ (MICROG and NATAMG) had greater ($P<0.05$) pH values than all other treatments (Table 4.4). The treatments containing MicroGARD™ (NATAMG and MICROG) had higher pH values because the preservatives themselves cannot immediately participate in donating hydrogen ions to the food system. The organic acid metabolites present in

MicroGARD™ may eventually participate in such activities once an equilibrium has been reached within the marinara sauce. However, in this particular experiment, the preservative did not affect the pH. For the duration of the storage period (0, 14, 28, and 42 days), natamycin (NATA), the combination treatment of natamycin and propionic acid (NATAPA), propionic acid (PROPA) and the negative control (NC) all had lower ($P<0.05$) pH values than the positive control (PC). The treatments containing propionic acid (NATAPA and PROPA) had lower ($P<0.05$) pH values because propionic acid directly contributes hydrogen ions to the food matrix. The natamycin treatment (NATA) had a lower pH because the natamycin does not inhibit any lactic acid bacteria from growing and fermenting carbohydrates. After day 0, fermentation likely converted the digestible sugars into lactic acid resulting in a reduced pH on day 14 (Table 4.4). The negative control (NC) displayed similar pH values to those of the inoculated negative control. After 42 days of storage, the non-inoculated negative control measured the lowest pH value for the duration of the experiment at 4.05. This is likely due to ability of the background microorganisms to grow uninhibited, whereas the organisms that were added to the inoculated samples could have possibly been in competition with the background organisms. This would result in a hindrance of growth and less fermentative activity.

4.4 Sensory Analysis

No sensory differences existed ($P>0.05$) between the natamycin treatment (NATA) and the blind control treatment (PC) (Table 4.9). Davidson and others (2010) reported that sorbic acid imparted an off-flavor in fruit juice whereas natamycin did not. However, the natamycin-propionic acid combination treatment (NATAPA) and the

propionic acid treatment (PROPA) were different (P>0.05) than the blind control sample (PC) when compared to the control. It can be concluded that natamycin is not likely the component responsible for the difference in perceived flavor from the control sample. Propionic acid is often responsible for off-flavors and odors in fermented foods (Wilkes and others 2000). The differences perceived by the panelists are likely due to the added potassium propionate in the propionic acid (PROPA) and natamycin-propionic acid treatments (NATAPA). Panelists detected a sour taste in the PA and NATAPA samples. Further testing would need to be conducted to determine if the difference that was detected would negatively impact the consumer acceptability of the marinara sauce.

4.5 Conclusions

No differences in efficacy against microorganisms were detected between the preservative treatments and both controls in the non-inoculated samples for the duration of the study. Natamycin was found to have the lowest average log colony counts (CFU/g) in samples screened for both lactic acid bacteria and yeasts and molds in the non-inoculated samples, simulating the closest conditions to an industry situation. Overall in the inoculated samples, the natamycin-propionic acid treatment consistently inhibited lactic acid bacteria and yeast and mold growth the most effectively. When comparing the two treatments from a sensory standpoint, only natamycin could be used as preservative system on the basis of the results from this study. Further consumer testing would be required to draw any conclusions on the industry application of the natamycin-propionic acid treatment.

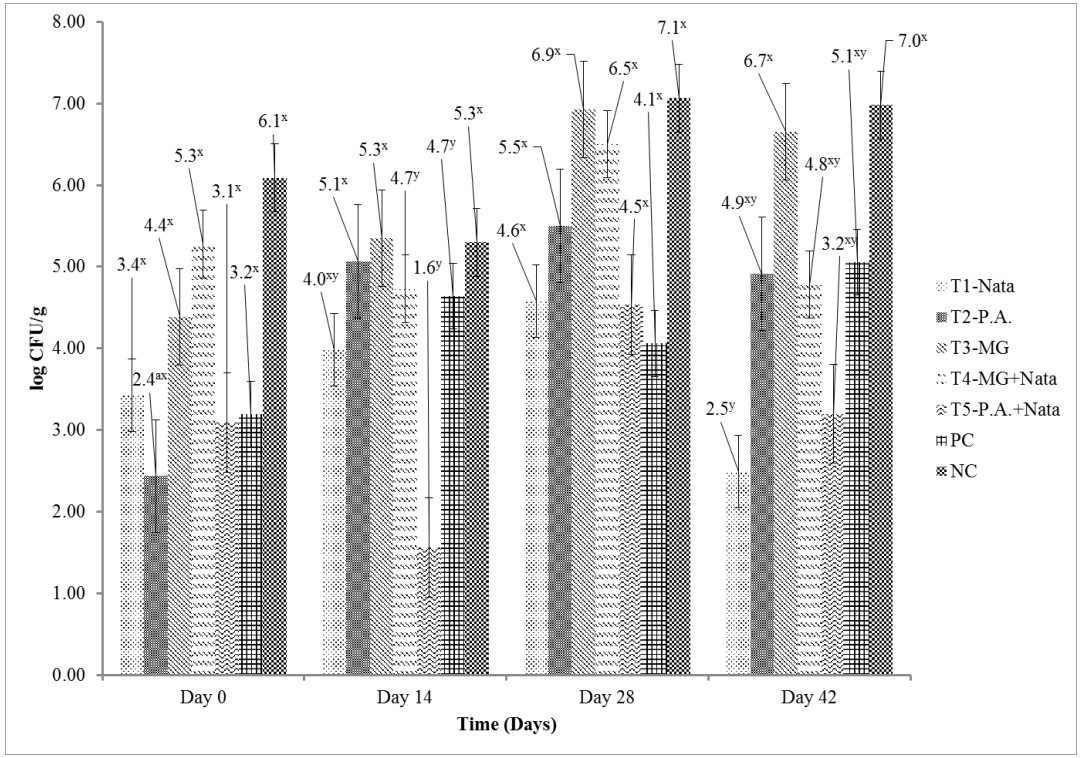

Figure 4.1 Use of clean label antimicrobials to inhibit the growth of *Zygosacchromyces bailli* in marinara sauce that was inoculated with 4 log CFU/g *Zygosacchromyces bailli*

T1-Nata: Natamycin, T2-P.A.: Propionic Acid, T3-MG: MicroGARD™, T4-MG+Nata: MicroGARD™ and Natamycin, T5-P.A. + Nata: Propionic Acid and Natamycin, PC: Positive Control, NC: Negative Control
x-y Means with the same letter between treatments are not different (P>0.05)

Table 4.1 The overall antimicrobial effect of synthetic and natural compounds on sauces inoculated with *Z. bailii* over a 42 day storage period.

Treatment	LS means	Groups				SEM
NC	6.4	A				
MICROG	5.8	A	B			
NATAMG	5.3	A	B	C		
PROPA	4.5		B	C	D	
PC	4.2			C	D	
NATA	3.6				D	
NATAPA	3.1				D	0.20

[A-D] Means with the same letter are not different (P>0.05)
SEM Standard Error Mean
MICROG: MicroGARD™, NATA: Natamycin, NATAMG: Natamycin + MicroGARD™, NATAPA: Natamycin + Propionic Acid, NC: Negative Control (No Preservatives), PC: Positive Control (Sodium Benzoate + Potassium Sorbate), PROPA: Propionic Acid)

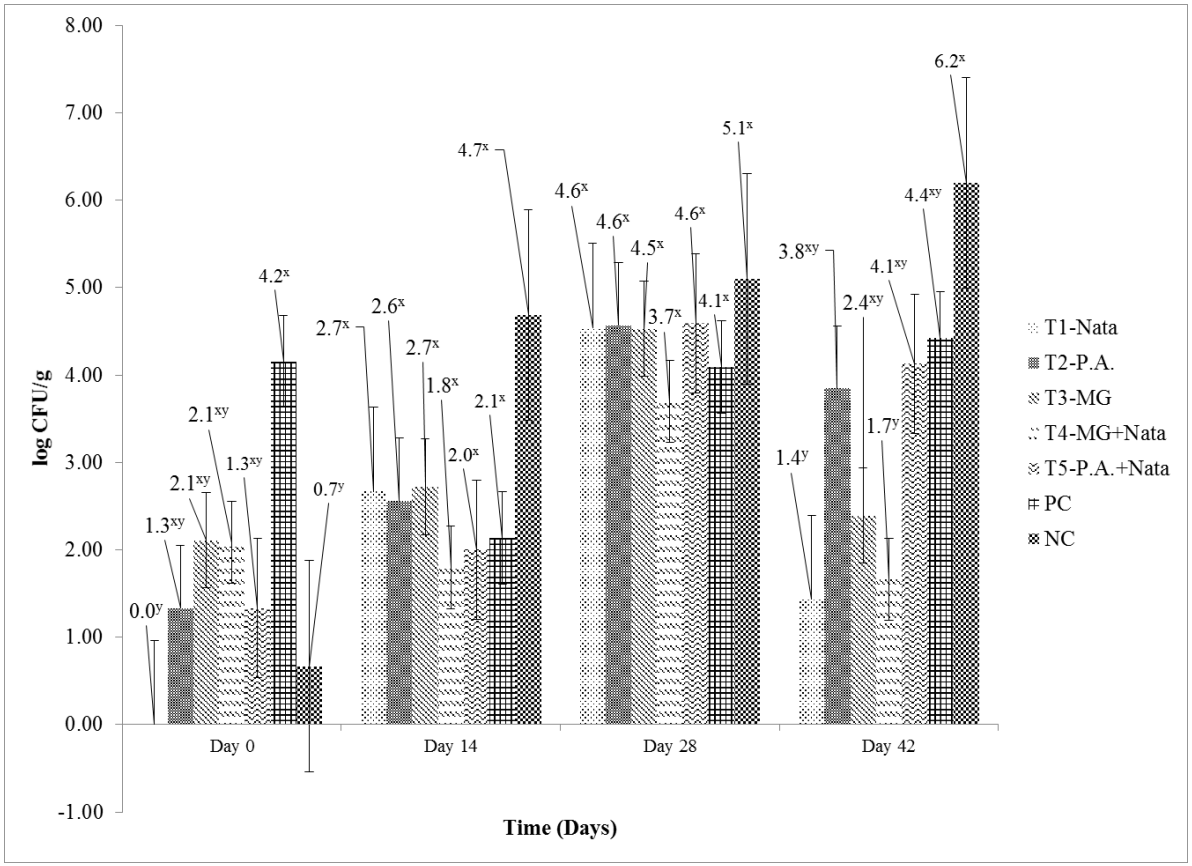

Figure 4.2 Use of clean label antimicrobials to control the growth of yeast and molds in non-inoculated marinara sauce.

T1-Nata: Natamycin, T2-P.A.: Propionic Acid, T3-MG: MicroGARD™, T4-MG+Nata: MicroGARD™ and Natamycin, T5-P.A. + Nata: Propionic Acid and Natamycin, PC: Positive Control, NC: Negative Control
x-y Means with the same letter between treatments are not different (P>0.05)

Table 4.2 The overall antimicrobial effect of synthetic and natural compounds on sauces screened for yeasts and mold over a 42 day storage period.

Treatment	LS means	Groups	SEM
NC	4.2	A	
PC	3.7	A	
PROPA	3.1	A	
NATAPA	3.0	A	
MICROG	2.9	A	
NATAMG	2.3	A	
NATA	2.2	A	0.28

[A] Means with the same letter are not different (P>0.05)
SEM Standard Error Mean
MICROG: MicroGARD™, NATA: Natamycin, NATAMG: Natamycin + MicroGARD™, NATAPA: Natamycin + Propionic Acid, NC: Negative Control (No Preservatives), PC: Positive Control (Sodium Benzoate + Potassium Sorbate), PROPA: Propionic Acid

Table 4.3 Antibacterial effect of natural antimicrobials on the growth and survival (log CFU/g) of the yogurt culture in marinara sauce (4 log CFU/g)

Attribute	Treatment	Day 0	Day 14	Day 28	Day 42	SEM
	MICROG	5.4^{ax}	1.7^{bx}	4.1^{abx}	6.9^{ax}	
	NATA	4.6^{ax}	3.4^{ax}	2.2^{abxy}	0.0^{bz}	
Standard Plate	NATAMG	4.7^{ax}	1.3^{ax}	3.7^{ax}	2.0^{ayz}	
Count (log CFU/g)	NATAPA	3.1^{ax}	2.5^{ax}	1.0^{axy}	2.1^{ayz}	
	NC	4.8^{ax}	1.1^{bx}	7.0^{ax}	5.9^{ax}	
	PC	3.2^{abx}	0.0^{bx}	4.1^{ax}	2.2^{abyz}	
	PROPA	4.0^{ax}	1.3^{abx}	0.0^{by}	3.9^{axy}	0.24

[a-c] Means within the same row with the same letter are not different (P>0.05)
[x-z] Means within the same column with same letter are not different (P>0.05)
SEM Standard Error Mean
MICROG: MicroGARD™, NATA: Natamycin, NATAMG: Natamycin + MicroGARD™, NATAPA: Natamycin + Propionic Acid, NC: Negative Control (No Preservatives), PC: Positive Control (Sodium Benzoate + Potassium Sorbate), PROPA: Propionic Acid).

Table 4.4 Effect of natural antimicrobials and spoilage organism growth on the pH of marinara sauce.

Attribute	Treatment	Day 0	Day 14	Day 28	Day 42	SEM
	MICROG	4.53^{ax}	4.43^{ax}	4.48^{ax}	4.56^{ax}	
	NATA	4.24^{ay}	4.14^{abz}	4.12^{abz}	4.08^{bz}	
	NATAMG	4.54^{ax}	4.46^{ax}	4.47^{ax}	4.48^{ax}	
pH	NATAPA	4.17^{ay}	4.10^{az}	4.06^{az}	4.10^{az}	
Inoculated	NC	4.17^{ay}	4.06^{az}	4.08^{az}	4.14^{ayz}	
	PC	4.35^{ay}	4.28^{ay}	4.24^{ay}	4.27^{ay}	
	PROPA	4.18^{ay}	4.09^{az}	4.06^{az}	4.08^{az}	0.01
	MICROG	4.56^{ax}	4.46^{ax}	4.46^{ax}	4.43^{bx}	
	NATA	4.25^{az}	4.14^{bz}	4.16^{ab}	4.14^{bz}	
	NATAMG	4.57^{ax}	4.47^{bx}	4.47^{bx}	4.47^{abx}	
pH	NATAPA	4.19^{az}	4.10^{bz}	4.08^{bz}	4.10^{bz}	
Non-Inoc.	NC	4.23^{az}	4.10^{bz}	4.08^{bz}	4.05^{bz}	
	PC	4.38^{ay}	4.25^{by}	4.27^{by}	4.28^{by}	
	PROPA	4.20^{az}	4.11^{bz}	4.07^{bz}	4.11^{bz}	0.01

$^{a-c}$ Means within the same row with the same letter are not different (P>0.05)
$^{w-z}$ Means within the same column with same letter are not different (P>0.05)
SEM Standard Error Mean
MICROG: MicroGARD™, NATA: Natamycin, NATAMG: Natamycin + MicroGARD™, NATAPA: Natamycin + Propionic Acid, NC: Negative Control (No Preservatives), PC: Positive Control (Sodium Benzoate + Potassium Sorbate), PROPA: Propionic Acid)

Table 4.5 The overall antibacterial effect of natural antimicrobials on sauces inoculated with the yogurt culture over a 42 day storage period.

Treatment	LS means	Groups		SEM
NC	4.7	A		
MICROG	4.5	A		
NATAMG	2.9	A	B	
NATA	2.6		B	
PC	2.4		B	
PROPA	2.3		B	
NATAPA	2.2		B	0.24

[A-B] Means with the same letter are not different (P>0.05)
SEM Standard Error Mean
MICROG: MicroGARD™, NATA: Natamycin, NATAMG: Natamycin + MicroGARD™, NATAPA: Natamycin + Propionic Acid, NC: Negative Control (No Preservatives), PC: Positive Control (Sodium Benzoate + Potassium Sorbate), PROPA: Propionic Acid

Table 4.6 Antibacterial effect of natural antimicrobials on the growth of lactobacillus cells (log CFU/g) in non-inoculated marinara sauce.

Attribute	Treatment	Day 0	Day 14	Day 28	Day 42	SEM
	MICROG	2.8^{ax}	3.6^{ax}	0.0^{ay}	1.0^{axy}	
	NATA	0.0^{ax}	3.4^{ax}	0.0^{ay}	1.5^{axy}	
Standard Plate	NATAMG	1.7^{abx}	2.3^{abx}	5.5^{ax}	0.0^{by}	
Count (log CFU/g)	NATAPA	1.7^{ax}	2.1^{ax}	1.7^{axy}	1.0^{axy}	
	NC	2.3^{ax}	3.8^{ax}	2.4^{axy}	4.1^{ax}	
	PC	2.3^{ax}	3.5^{ax}	1.3^{ay}	1.9^{axy}	
	PROPA	1.1^{ax}	2.9^{ax}	1.3^{ay}	1.6^{axy}	0.26

[a-c] Means within the same row with the same letter are not different (P>0.05)
[x-z] Means within the same column with same letter are not different (P>0.05)
SEM Standard Error Mean
MICROG: MicroGARD™, NATA: Natamycin, NATAMG: Natamycin + MicroGARD™, NATAPA: Natamycin + Propionic Acid, NC: Negative Control (No Preservatives), PC: Positive Control (Sodium Benzoate + Potassium Sorbate), PROPA: Propionic Acid)

Table 4.7 The overall antibacterial effect of natural antimicrobials on sauces screened for lactic acid bacteria over a 42 day storage period.

Treatment	LS means	Groups	SEM
NC	3.2	A	
NATAMG	2.4	A	
PC	2.3	A	
MICROG	1.9	A	
PROPA	1.7	A	
NATAPA	1.6	A	
NATA	1.2	A	0.26

[A] Means with the same letter are not different (P>0.05)
SEM Standard Error Mean
MICROG: MicroGARD™, NATA: Natamycin, NATAMG: Natamycin + MicroGARD™, NATAPA: Natamycin + Propionic Acid, NC: Negative Control (No Preservatives), PC: Positive Control (Sodium Benzoate + Potassium Sorbate), PROPA: Propionic Acid)

Table 4.8 Effect of natural antimicrobials and the growth of spoilage organisms on the water activity (Aw) of marinara sauce.

Attribute	Treatment	Day 0	Day 14	Day 28	Day 42	SEM
	MICROG	0.985^{ax}	0.985^{axy}	0.987^{axy}	0.986^{ayz}	
	NATA	0.985^{ax}	0.988^{ax}	0.986^{axy}	0.987^{axyz}	
	NATAMG	0.985^{ax}	0.985^{axy}	0.987^{axy}	0.984^{ayz}	
Aw	NATAPA	0.985^{ax}	0.983^{ay}	0.987^{axy}	0.982^{az}	
Inoculated	NC	0.985^{bx}	0.987^{abxy}	0.990^{ax}	0.991^{ax}	
	PC	0.986^{ax}	0.983^{axy}	0.985^{ay}	0.983^{ayz}	
	PROPA	0.984^{ax}	0.982^{ay}	0.986^{axy}	0.984^{ayz}	0.01
	MICROG	0.986^{ax}	0.986^{axy}	0.984^{axy}	0.985^{ayz}	
	NATA	0.984^{ax}	0.985^{axy}	0.988^{ax}	0.985^{axy}	
	NATAMG	0.983^{ax}	0.983^{axy}	0.985^{ax}	0.981^{ay}	
Aw	NATAPA	0.987^{ax}	0.986^{axy}	0.984^{abx}	0.981^{by}	
Non-Inoc.	NC	0.984^{ax}	0.985^{axy}	0.988^{ax}	0.987^{ax}	
	PC	0.986^{ax}	0.982^{ay}	0.985^{ax}	0.986^{ax}	
	PROPA	0.985^{ax}	0.987^{ax}	0.986^{ax}	0.984^{axy}	0.01

[a-c] Means within the same row with the same letter are not different (P>0.05)
[x-z] Means within the same column with same letter are not different (P>0.05)
SEM Standard Error Means
MICROG: MicroGARD™, NATA: Natamycin, NATAMG: Natamycin + MicroGARD™, NATAPA: Natamycin + Propionic Acid, NC: Negative Control (No Preservatives), PC: Positive Control (Sodium Benzoate + Potassium Sorbate), PROPA: Propionic Acid)

Table 4.9 Difference-from-control test results between marinara sauce with natural preservatives and the industry standard with synthetic antimicrobials (Positive Control).

Treatment	Mean	Groups		SEM
PROPA	2.2	A		
NATAPA	2.0	A		
PC	1.4		B	
NATA	1.0		B	0.29

[A-B]Means with the same letter are not different (P>0.05)

PROPA: Propionic Acid, NATAPA: Natamycin + Propionic Acid, NATA: Natamycin, PC: Positive Control.

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