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Characterization of the Ability of Yeast Probiotics and Paraprobiotics to Directly Interact with Gram-Positive and Gram-Negative Bacteria

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Characterization of the ability of yeast probiotics and paraprobiotics to directly interact with Gram-positive and Gram-negative bacteria

By

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Yeast probiotics and paraprobiotics, live and inactivated yeast cells, respectively, improve health and performance of livestock by stabilizing the intestinal microbial community. They have also been used for infection prevention and treatment. Despite much research already conducted, the mechanism of direct antagonism, or adhesion of bacteria to the probiotic/paraprobiotic, is under characterized. Additionally, it is unknown which probiotic/paraprobiotic is optimal to use for specific infections. The interactions between the yeast and certain pathogens were analyzed qualitatively with scanning electron microscopy (SEM) and quantitatively with membrane filtration assays. Gram-positive bacteria were found to exhibit specificity under SEM. Through membrane filtration, *Listeria monocytogenes* exhibited binding to all samples ($P<0.05$), while *Salmonella* Typhimurium exhibited binding ($P<0.001$) with all samples except with 2338. *Escherichia coli* O157:H7 only bound to the probiotics ($P<0.001$). With a better understanding of how specific yeast probiotics and paraprobiotics interact with bacteria, specific therapies can be administered to combat infections.

DEDICATION

I would like to dedicate this thesis and all the work required to obtain my Master's degree to all of my family, friends/co-workers, and professors who helped me along the way. Thank you, Mama and Papa, for supporting me throughout my undergraduate and graduate degree. Thank you to all of my co-workers, especially Lindsey Brown, Jessica Wilson, Zach Burcham, Joseph Bryant, Katie Heath, Reid Hargrove, Chase Robinson, and everyone else in the Biological Sciences department for sticking by me through this entire endeavor. If I did not have all of you all to lean on when times were rough, I wouldn't have made it. All of you made it the best experience I could have ever wanted. I will miss all of you so very dearly, and I wish all of you the best of successes in your own futures! Dr. Donaldson, you are the best mentor I could have ever dreamed of having, and without you, I never would have finished. I know I had many setbacks, many obstacles that I made for myself, but you brought me through each and every single one. I just wanted to thank you from the bottom of my heart, and I will always be appreciative of everything you did for me. You are the best!

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CHAPTER I

LITERATURE REVIEW

Animal Health

Importance of animal health

Morbidity and mortality associated with livestock disease accounts for a large economic burden worldwide. A major contributor to this economic burden is the cost associated with illness. Livestock health not only impacts the livelihood of farmers, but also veterinarians, food business owners who sell these animal products, and the food consumers. Additionally affected are the health care systems, the tourism industries, and the overall economy of the countries plagued with animal disease.

In 2001, a foot and mouth disease outbreak cost the United Kingdom an estimated $12 billion (Anderson, 2002). In 1997, a swine fever epidemic in the Netherlands resulted in the destruction of 11 million pigs and over $2 billion in loss (Horst et al., 1999). In 2007, an outbreak of avian influenza in Bangladesh cost the country's poultry sector an estimated $750 million (Samad, 2011). The monetary cost initially incurred due to the direct loss of livestock is quite substantial, but another considerable cost that must be taken into consideration is the economic impact on countries exterior to the disease. Once a disease outbreak is recognized, a domino effect of preventative measures are taken, such as the raising of border patrol awareness, development of contingency plans, stockpiling of resources, and even restricting trade (reviewed in Pearson, 2005).

One of the biggest influences on national, and sometimes international, communities is the prevalence of human cases of zoonotic diseases (reviewed in Pearson, 2005). Zoonotic transfer has the potential to cause human outbreaks, such as is seen with the avian influenza in 2003, Dengue fever in 2000, and rabies in 2005 (Koopmans et al., 2004; Rahman et al., 2002; Schneider et al., 2009). It is estimated that nearly 61% of known infectious organisms can be transferred zoonotically, and of that percentage, 31% are bacteria (Taylor et al., 2001)

Foodborne pathogens

One of the main sources of foodborne illnesses is the consumption of food products contaminated with bacteria. Products can become contaminated at multiple points, starting with animal harvest processes and carrying through to improper handling techniques by consumers during preparation and cooking. These illnesses account for nearly 128,000 hospitalizations and 3,000 deaths each year in the United States alone (Morris, 2011). Enteric bacterial pathogens display a wide array of virulence factors, which enable them to affect and/or colonize their host. Some pathogens interact with the intestinal tract or epithelium via adhesion or invasion, while others can secrete exotoxins or cytotoxins (Guerrant et al., 1999). Of all the pathogens that affect both human and animal health, the Centers for Disease Control and Prevention recognize the following bacteria that account for the majority of these infections: *Escherichia coli* O157:H7*, Salmonella, Campylobacter, Listeria monocytogenes, Staphylococcus aureus,* and *Clostridium perfringens*. These organisms are not only the most common food-borne pathogens, but they all have been linked to antibiotic resistance, which impacts treatment options for not only humans, but livestock as well (Teuber, 1999).

Escherichia coli

The bacterium *Escherichia coli* is a Gram-negative, facultative anaerobe, human commensal of the gastrointestinal tract and is one of the most commonly isolated bacterial pathogens in the food industry (Matic et al., 1997). Hundreds of different serotypes of *E. coli* exist, but few are considered pathogenic (Ray & Schaffer, 2011). These pathogenic serotypes of *E. coli* are categorized by their specific pathogenic mechanisms (e.g., toxins, adhesins, invasiveness, etc.), known as "virotypes" (Gonzalez Garcia, 2002). These virotypes are enterotoxigenic *E. coli* (ETEC), enteroinvasive *E. coli* (EIEC), enterohemorrhagic *E. coli* (EHEC), enteropathogenic *E. coli* (EPEC), and enteroaggregative *E. coli* (EAEC) (Levine, 1987). Enterohemorrhagic strains of *E. coli,* including the serotype O157:H7, are the causative agents of hemolytic uremic syndrome. *Escherichia coli* O157:H7 causes this disease by adhering to the epithelium/endothelium, inducing a lesion as well as releasing the Shiga toxin (Stx) that cleaves ribosomal RNA and disrupts protein synthesis of the target cell, thereby leading to cell death (Melton-Celsa & O'Brien, 1998). Despite the severity of pathogenesis in humans, ruminants have been known to be asymptomatic carriers of EHEC O157:H7 (Witold & Hovde, 2011). Ruminants do not express the receptor for the Shiga toxins, thus the bacteria do not cause disease in these animals (Pruimboom-Brees et al., 2000). The feces of ruminants are considered the primary source of O157:H7 contamination of food supplies and environments, but pigs, poultry, and dogs have been found to be sources as well (O'Brien et al., 2001; Van Donkersgoed et al., 2001; Witold & Hovde, 2011).

Salmonella

Salmonella are Gram-negative, non-spore-forming, nonencapsulated, motile bacteria that cause many enteric diseases in both humans and animals. There are over 2,000 different serotypes of *Salmonella*. Salmonellosis, a diarrheal infection that can result in fever and abdominal cramps is caused by various *Salmonella* subtypes such as *S. enterica* Typhimurium, *S. enterica* Enteritidis, and *S. enterica* Heidelberg (Brenner et al., 2000; Jones et al., 2008). Another serovar of importance to human and livestock health is *S. enterica* Typhi, which is the causative agent of typhoid fever, a disease endemic across the entire world (Rowe et al., 1997). These serovars are ubiquitous in nature and can affect a wide array of livestock, such as cattle, chickens, swine, and turkeys (CDC, 2006). *Salmonella* can colonize a multitude of sites within a host (e.g., small intestine, colon, and cecum) through the utilization of fimbriae or pili for attachment and internalization (Foley & Lynne, 2007).

The food industry has been widely affected by *Salmonella*. *Salmonella* Enteritidis frequently contaminates poultry products and eggs, while *S.* Typhimurium has been isolated from poultry and pork products (White et al., 2001). It has also been reported that other food sources of *Salmonella* include milk and other dairy products, pork, vegetables, and fruit (Helmick et al., 1994). A study conducted by the United States Department of Agriculture (USDA) estimated that 85-96% of the 4 million total cases of non-typhoid salmonellosis were foodborne, of which the total costs ranged between $0.6 and $3.5 billion annually, which marks salmonellosis as one of the most costly bacterial foodborne diseases to date (Busby et al., 1996).

Campylobacter

Campylobacter sp. are Gram-negative, microaerophilic, spiral-shaped bacteria that are commonly associated with human gastroenteritis and peptic ulcers (Drumm et al., 1987). The genus *Campylobacter* is comprised of 16 species, the most common being *C. jejuni, C. coli, C. lari, C. fetus,* and *C. upsaliensis* (Fouts et al., 2005). The majority of *Campylobacter* infections are from the intake of raw/undercooked foods, such as beef, pork, poultry, lamb, and various seafood (Nielsen et al., 1997). The USDA estimates around 1.3 – 1.7 million cases annually (7,000-9,000 hospitalizations and 100-500 deaths) are caused by foodborne *Campylobacter*, which results in an estimated cost of $0.6 - $1.0 billion annually (Busby et al., 1996). It is even more prevalent in Europe, where the European Union estimates around 9 million cases of campylobacteriosis occur annually, resulting in a cost of $2.4 billion per year (Bahrndorff et al., 2013).

The main virulence factors of *Campylobacter* sp. are its motility, invasiveness, catalase production, and its resistance to a number of antimicrobials (Bhavsar & Kapadnis, 2006). *Campylobacter* infects the host by adhering and invading intestinal epithelial cells (Fauchere et al., 1986). Once inside the epithelial cells, the bacteria will release toxins that can damage tissue, resulting in inflammation, and thereby gastroenteritis (Bhavsar & Kapadnis, 2006).

Listeria

The genus *Listeria* contains 7 different species, of which *L. monocytogenes* is the primary species associated with regard to foodborne illnesses (Collins et al., 1991). *Listeria monocytogenes* is a Gram-positive, facultative anaerobic, non-spore-forming bacterium that is the causative agent of listeriosis (Glaser et al., 2001). *Listeria* colonizes

its host is through the utilization of internalin proteins InlA and InlB, which facilitate internalization of the bacteria into target cells (Birmingham et al., 2007). *Listeria* utilizes other proteins as well, including listeriolysin O (LLO) and phospholipase A (PlcA), which promote its escape from phagocytic vacuoles, and actin assembly-inducing protein (ActA) and phospholipase B (PlcB), which are integral for its intracellular actin-based motility to promote cell-to-cell movement (Glaser et al., 2001).

There is a zero-tolerance policy for the presence of *L. monocytogenes* on ready-to-eat food (Chen et al., 2003). The USDA estimates that approximately $0.3 billion are lost annually due to illness and deaths caused by foodborne listeriosis (Busby et al., 1996); this number increases to between $2.3 - $22 billion when including the cost of industrial loss (Ivanek et al., 2005). Pregnancy-related listeriosis can lead to severe neonatal disease or potentially fetal death (CDC 2011). Listeriosis can also cause bovine abortions and stillbirths (Kirkbride, 1993). Although it is rare, cases of cutaneous listeriosis have been reported in veterinarians and farmers exposed to contaminated bovine products and also from amniotic fluid from livestock containing high concentrations (10^8 CFU/mL) of *Listeria* (McLauchlin & Low, 1994).

Staphylococcus

As a normal inhabitant of the human microbiota, *Staphylococcus* sp. are Gram-positive, non-motile, facultative anaerobic, coccus-shaped bacteria, and over 40 species have been identified (Layer et al., 2006). Of those species, many are nonpathogenic, but some do cause disease. *Staphylococcus aureus* is an opportunistic pathogen that has gained much attention due to the increased prevalence of antibiotic resistant strains (Lowy, 2000). Studies have shown that *S. aureus* can block components of the innate

immune system by secreting various proteins, such as extracellular complement-binding protein (Ecb) and extracellular fibrinogen-binding protein (Efb) (Jongerius et al., 2012). The Ecb protein interferes with the activation of the C3 complement cascade by binding to the C3d domain, while Efb also interferes with complement activation and also prevents the adhesion of neutrophils to fibrinogen (Jongerius et al., 2012). Many clinical strains of *S. aureus* express capsular polysaccharides that aid in evading the host immune system (Harris et al., 2002). *Staphylococcus aureus* also produces five different toxins that are capable of damaging cell membranes, four of which are hemolysins (i.e., α, β, γ, and δ) and one is a leucocidin (Nilsson et al., 1999).

Staphylococcus aureus is associated with high mortality and morbidity in humans who have hospital- or community-acquired infections, with severities ranging from non-life-threatening skin disorders to bacteremia compounded with diseases such as endocarditis and pneumonia (Klevens et al., 2007). Foodborne *S. aureus* infections result in approximately $1 billion annually (Busby et al., 1996). *Staphylococcus aureus* has also been found to infect a large array of animal species, such as household pets, horses, cattle, pigs, and poultry (Weese, 2010).

Clostridium

Clostridium is a genus containing nearly 100 species of Gram-positive, obligate anaerobic, spore-forming bacteria (Wells & Wilkins, 1995). The most common pathogenic strains are *C. botulinum, C. difficile, C. tetani, C. sordellii,* and *C. perfringens*. *Clostridium* sp. cause a variety of diseases (e.g., botulism and tetanus), but in 2000 the poultry industry lost an estimated $2 billion worldwide due to necrotic

enteritis with *C. perfringens* being one of the etiological agents (McReynolds et al., 2004).

Clostridium perfringens is characterized as expressing five different toxins (i.e., α, β, ε, ι, and θ), which are used to classify these bacteria into toxinotype A, B, C, D, or E based on the specific toxin(s) expressed (Rumah et al., 2013; Wells & Wilkins, 1995). Type A strains are commonly found as normal microflora within animals since they lack some of the more potent toxins that are produced by the other types, but have been linked to cases of necrotic enteritis in poultry, hemorrhagic diarrhea in dogs, and porcine clostridial enteritis in neonatal and weaned pigs (Keyburn et al., 2006; J. G. Songer & Uzal, 2005; Weese et al., 2001). Type B strains have been linked to dysentery, hemorrhagic enteritis, and enterotoxemia in newborn lambs, neonatal calves and foals, and sheep, respectively (Petit et al., 1999). Type C strains have been known to cause enterotoxemia in sheep, but also necrotic enteritis in piglets, calves, foals, and lambs (Petit et al., 1999). Type D strains have been linked to brain lesions in sheep and enterocolitis and enterotoxemia in goats (Uzal & Kelly, 1998). Type E has been diagnosed in calves and causes a severe local intestinal necrosis and systemic toxemia analogous to type C (J.G. Songer & Miskimins, 2004).

Clostridium perfringens causes disease by coordination of direct toxins (e.g., beta, alpha, and perfringolysin O) diffusing into the target cell (Sayeed et al., 2008). Alpha-toxins are able to lyse cell membrane lecithins, which leads to cell membrane disruption and eventually cell death, while theta toxins cause rapid tissue destruction (Wells & Wilkins, 1995). *Clostridial* sp. produce the highest number of toxins of any bacteria; therefore developing a disease prevention strategy is essential (Popoff & Bouvet, 2009).

Antibiotics and probiotics

Antibiotics are compounds that exhibit bactericidal or bacteriostatic properties. They have been used not only for the promotion of growth in food animals (Davies & Davies, 2010), but also have been used to treat and prevent infections (McEwen & Fedorka-Cray, 2002). However, resistance to these antibiotics became prevalent whenever these antibiotics themselves came into existence (Phillips et al., 2003). For instance before the introduction of penicillin as a therapeutic agent, bacterial penicillinases (β-lactamases) were identified, so once the antibiotic was broadly utilized, resistant strains of bacteria were selected for within the population (Davies & Davies, 2010). With this rising prevalence of antibiotic resistant bacteria, the shift away from the use of antibiotics has led to the need of alternative methods of treatment or new prevention strategies. Currently, probiotics are being used as preventative and therapeutic treatments for diarrheal diseases and allergic diseases as well as supplemental enhancements of vaccine-induce protective immunity (Vanderhoof & Young, 2004). There are also some studies that suggest uses for probiotics in cancer prevention, immune stimulation, allergy treatment and prevention, and respiratory disease reduction (Vanderhoof, 2001). With all of these possible benefits, the use of probiotics presents a favorable alternative to the use of antibiotics and may prove beneficial in reducing the prevalence of antibiotic resistance.

Probiotics

Probiotics, although not seen as an immediate substitution for antibiotics, are being considered as another possible answer to the emerging issue of antibiotic resistance. Probiotics are defined by the World Health Organization and the Food and

Agriculture Organization as "live organisms which when administered in adequate amounts confer a health benefit on the host" (Reid et al., 2003). The gastrointestinal microbiota is comprised of a gamut of microorganisms interacting with one another. This microbiota is vital to host health, as it aids in food digestion and the "development and optimal functioning of the immune system" (Hooper & Gordon, 2001). The microbiome of the gut also represents an actual physical barrier that inhibits the growth and colonization of pathogens, but once the gut microbiota is modified or altered, pathogens can rapidly proliferate and cause a substantial shift in the normal microbial flora (Martin et al., 2014). This shift in microbial community, also called dysbiosis, can be linked to diseases such as irritable bowel syndrome, celiac disease, and even colorectal cancer (Miquel et al., 2013). Probiotics confer a health benefit by stabilizing that microbial balance (Fuller, 1992).

Bacterial probiotics versus yeast probiotics

Probiotics can be bacterial or yeast. The best-characterized bacterial probiotics include various *Lactobacillus* species, *Bifidobacterium* species, *Streptococcus* species, and other lactic/non-lactic acid bacterial species (Vemuri et al., 2013). Bacterial probiotics are primarily used for preventing many illnesses and disorders, such as acidosis. Acidosis is a digestive disorder that results from an accumulation of lactic acid or volatile fatty acids (Nagaraja & Titgemeyer, 2007) and has been linked to a variety of health issues including laminitis, bloat, and liver abscesses (Enemark, 2009; J.E. Nocek, 1997). Research has shown that lactic acid-producing bacteria have the ability to maintain a steady level of lactic acid, which can reduce the risk of acidosis (Moran et al., 2006; J. E. Nocek et al., 2002). An increase in growth performance has been seen when

pigs were supplemented with lactic acid bacteria (Moran et al., 2006). However, the survival rate of bacterial probiotics within the gastrointestinal tract is estimated to be around 20-40% for most strains due to the acidity of the gut and bile salts encountered (Bezkorovainy, 2001). It is believed that bacterial probiotics would be able to increase their effect if they were to adhere to the mucosal cells, but it has been demonstrated that some probiotics when administered exogenously do not adhere to the mucosal cells and proceed directly into the feces (Bezkorovainy, 2001). *Bifidobacterium* has been reported to endure the gastrointestinal stresses and still confer a health benefit despite its inability to colonize (Fujiwara et al., 1997). It does so by producing a 100kDa protein which interferes with pathogenic *E. coli*'s adhesion to intestinal epithelial cells. To be administered as a prophylactic, bacterial probiotics must be ingested continually to ensure competition with pathogens within the intestines.

Certain yeast can also be utilized as probiotics. The most commonly used are *Saccharomyces boulardii* and *S. cerevisiae*. Research has demonstrated that *S. cerevisiae* has the capability to colonize and replicate within the digestive tract (Martins et al., 2005). Yeast probiotics are able to survive the low pH environment of the stomach (2.5 to 3.5) as well as the distal part of the gastrointestinal tract where local stresses, such as bile, limit most microbial growth (Czerucka et al., 2007).

Administration of the yeast probiotic *S. cerevisiae* has been reported to reduce mortality associated with infections with the pathogens *Salmonella* Typhimurium and *Clostridium difficile* in a mouse model (Martins et al., 2005). Additionally, yeast probiotics have been reported to help initiate microbial colonization within the rumen of young ruminants if administered within the first few days after birth (Frederique

Chaucheyras-Durand & Fonty, 2002). Yeast probiotics have also been reported to induce an increase in the digestion of fiber in the colon as well as regulate the microbial balance of the hindgut (Jouany et al., 2008; Medina et al., 2002). For example, yeast probiotics have been found to prevent the accumulation of lactate in horses, which can lead to acidosis (Kronfeld & Harris, 1997; Potter et al., 1992). Normally, horse feed is supplemented with large amounts of starch, but only a fraction is digested, which leaves undigested starch to enter the hindgut. This disturbs the microbial balance, which leads to accumulation of lactate. Yeast probiotics prevent this by initiating a healthy balance of microorganisms, which prevents the buildup of lactate. Many probiotic bacteria have been known to produce lactic acid (e.g., *Lactobacillus* sp., *Bifidobacterium* sp.), so yeast probiotics present the best option against acidosis since yeasts do not naturally produce lactic acid (Sauer et al., 2010).

Paraprobiotics

There has been an increased interest in cell wall components or non-viable microorganisms as potential probiotics. The increased interest in these microbial components is primarily due to the fact that providing live microorganisms has the increased risk of morbidity and mortality with individuals with weakened immune systems. These cell wall components, termed "paraprobiotics", are defined as components of microbial cells that confer a health benefit to the host. Paraprobiotics can be made from both bacteria and yeasts. Bacterial paraprobiotics are comprised of cell fractions of bacteria or inactivated cells (Taverniti & Guglielmetti, 2011). Yeast paraprobiotics are also comprised of cell wall fractions or inactivated cells, and their composition is comprised of β(1-3)-D-glucans, β(1-6)-D-glucans, chitin, and

mannoproteins, which elicit their protective effects by various mechanisms of action (F. Chaucheyras-Durand et al., 2008; Kollar et al., 1997).

Yeast paraprobiotics have been used to increase milk production in dairy ruminants, and various growth parameters in cattle, but these responses vary between yeast strain depending on the diet and physiological status of the animal (F. Chaucheyras-Durand et al., 2008). Another study proved that supplementation with yeast paraprobiotics resulted in positive weight gain and general productivity of lambs and dairy cows (Jenkins & Jenkins, 2014). Paraprobiotics have been demonstrated to provide similar results as probiotics in the induction of IL-8 and inhibition of pathogen binding to the human colon epithelial cell line Caco-2 (Besselink et al., 2008; Lopez et al., 2008; Ostad et al., 2009).

Mechanisms of action

Yeast probiotics and paraprobiotics confer benefits to the host by stabilizing the normal gut microflora via a multitude of proposed mechanisms, including host immune system modulation, active antimicrobial inhibition, and indirect mechanisms of actions on pathogens, host, or food components. However, these mechanisms tend to vary between strains (Hatoum et al., 2012; Ng et al., 2008; Oelschlaeger, 2010; Ohland & MacNaughton, 2010).

There is much evidence of yeast probiotics and paraprobiotics interacting with the host immune system in order to stimulate an immune response to combat pathogens. *Saccharomyces cerevisiae* has been documented to significantly increase concentrations of IgA and secretory components of immunoglobulins when orally administered to growing rats (Buts et al., 1990). Administration of these yeast cell wall paraprobiotics

enhances the immunomodulatory response, but does so via a different mechanism than that utilized by live microorganisms (reviewed in Auclair, 2001). Yeast cell wall components have a role in activating the complement system due to the inner yeast cell wall glucans (i.e., β-(1-3)-D-glucose) stimulating components of the mammalian immune system, such as the inflammatory response and the reticuloendothelial system (Pillemer et al., 1956). Glucans are believed to be immunostimulants since peripheral blood leukocytes and extravascular macrophages have a specific glucan receptor, which, when activated, can stimulate macrophages and the production of cytokines (Czop, 1986; Riggi & Di Luzio, 1961; Song & di Luzio, 1979).

Yeast probiotics and paraprobiotics have been demonstrated to affect host internal environments in order to disrupt pathogenic microbes' enzymatic activities. In rumen cannulated sheep that received live yeast supplements during diet changes, the rumen pH was reported to sustain an environment optimal for rumen function, including higher fibrolytic activities (Fonty & Chaucheyras-Durand, 2006). It has also been reported that *S. cerevisiae* was able to outnumber *Streptococcus bovis*, a lactate-producing bacterium, *in vitro* as well as limit the concentration of lactate produced by outcompeting for the utilization of sugars (Chaucheyras et al., 1996). The reduction of lactate accumulation and stabilization of rumen pH can lead to the decreased risk of pathogen colonization and to the promotion of resident microbes (F. Chaucheyras-Durand & Durand, 2010).

Yeast probiotics have also been found to exhibit a protective effect for the host against pathogenic bacteria. It is reported that *S. boulardii* demonstrated the capability to reduce the amount of available toxins secreted by *S. typhimurium* and *Shigella flexneri* and the capability to outcompete for adhesion sites within mice (Rodrigues et al., 1996).

Some yeast have also been shown to produce antimicrobial peptides, called bacteriocins, which hinder the growth of pathogens or hydrolyze their toxins (Woods & Bevan, 1968).

Direct antagonism

Yeast probiotics and paraprobiotics are well characterized in their abilities of stabilizing pH in the gastrointestinal tract, inhibiting the growth of lactate-producing bacteria, and modulating the host immune system. Though much has been done in terms of defining the mechanism by which probiotics and paraprobiotics confer benefits, little has been done to analyze the interaction, if any, that these products have with microbes encountered within the host. One characteristic of yeast is their ability to directly interact with bacteria. Certain serovars of *S. enterica* and *E. coli* have been found to bind to mannose on the surface of yeast cell walls due to the bacterial expression of mannose-specific adhesins (Sharon & Ofek, 1986). Type I fimbrinated *E. coli* was found to bind to mannose on yeast cell walls (Gedek, 1999; Sharon & Ofek, 1986). A study conducted at the Universidade Federal de Minas Gerais demonstrated in vitro and in vivo the adhesion of yeast probiotics, *Saccharomyces boulardii* and *Saccharomyces cerevisiae*, to enteropathogenic bacteria (Tiago et al., 2012). With gnotobiotic mice that were exposed to yeast probiotics prior to infection, Tiago demonstrated that the bacterial cells were attracted to the surface of the yeast cells as opposed to the intestinal epithelials (Tiago et al., 2012). This direct interaction between the probiotic/ paraprobiotic and pathogen (i.e. direct antagonism) presents a mechanism of rapid pathogen elimination from a host through defecation (Gedek, 1999; Normark et al., 1986). Although under-characterized, direct antagonism presents an important mechanism of action for illness prevention.

Conclusion

Probiotics and paraprobiotics present a great alternative for disease prevention and/or treatment, but their mechanisms of action are still under debate and are varied. Bacterial probiotics confer many health benefits, but are limited due to their inability to withstand certain stresses within the GI tract. Yeast probiotics and paraprobiotics are able to endure the stresses encountered, therefore have the potential to confer a wide array of health benefits to the host. This thesis focuses upon characterizing the mechanism of direct antagonism as a method of pathogen removal from the host.

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CHAPTER II

CHARACTERIZATION OF THE ABILITY OF YEAST PROBIOTICS AND PARAPROBIOTICS TO DIRECTLY INTERACT WITH GRAM-POSITIVE AND GRAM-NEGATIVE BACTERIA

Introduction

The use of antibiotics as a means for treating and preventing illness in livestock has impacted animal health and performance (Dunlop et al., 1998). Antibiotics are administered individually (e.g., cows, calves, sows) or supplemented in feed and water to whole groups of animals (e.g., poultry, pigs) (McEwen, 2006). However, the increased risk of antibiotics allowing for the proliferation of antibiotic resistant bacteria in a microbial population has led to an increase in the use of probiotics. Probiotics, as defined by The Food and Agriculture Organization of the United Nations (FAO) and the World Health Organization (WHO), are "live organisms which when administered in adequate amounts confer a health benefit on the host" (Nations, 2001). Since live microorganisms, present a potential risk of infection to the host, especially if immunocompromised, the FAO and WHO have constructed guidelines in order to properly identify and characterize organisms as potential probiotics. Common probiotics recognized by the FAO and WHO include *Lactobacillus* sp., *Bacillus* sp., *Enterococcus* sp., *Saccharomyces* sp., and *Aspergillus* sp. (Frederique Chaucheyras-Durand & Fonty, 2002; Reid et al., 2003). Of these, the yeast *Saccharomyces boulardii* and *S. cerevisiae* have been the most commonly

used among livestock (Duarte et al., 2012; Martins et al., 2005). Yeast probiotics that have been dried/fragmented into probiotic components have also been shown to confer a health benefit to a host (Middelbos et al., 2007). These products are referred to as "paraprobiotics" (Taverniti & Guglielmetti, 2011). Yeast cell walls are composed of β(1-3)-$_D$-glucans, β(1-6)-$_D$-glucans, chitin, and mannoproteins; interest has increased in yeast paraprobiotics since research has demonstrated that inactivated bacterial paraprobiotics can exert immunological effects on the host (Kollar et al., 1997; Taverniti & Guglielmetti, 2011).

Yeast probiotics have multiple mechanisms of action by which they confer a health benefit to the host, including direct binding to toxins produced by pathogens and also stimulating the host immune system. Additionally, probiotics have the potential to prevent colonization of bacteria to the mucosal surface of the intestine through either direct antagonism or through competitive inhibition (Shoaf-Sweeney & Hutkins, 2008). This inhibition is hypothesized to be due to the ability of certain pathogenic bacteria with mannose-binding fimbriae to bind mannoproteins within yeast cell walls (Ofek et al., 1977). By preventing the initial attachment to the intestinal epithelium and directly adhering to the bacteria themselves, the yeast-bacteria complex that is formed will then be removed via the digestive tract (Gedek, 1999). Despite previous research on the binding effects of pathogenic bacteria to yeast, it is not known whether strain specificity of adhesion within yeast products exists (Gedek, 1999; Korhonen et al., 1981; Martins et al., 2010). Therefore, the objective of this study was to characterize the binding relationship of multiple *Saccharomyces cerevisiae* probiotic and paraprobiotic products with Gram-positive and Gram-negative bacteria.

Materials and Methods

Bacterial and yeast strains and cultivation conditions

The bacterial strains used in this study were *Escherichia coli* O157:H7 (ATCC 43895), *Salmonella enterica* serovar Typhimurium (ATCC 13311), *Salmonella enterica* serovar Enteritidis (ATCC 13076), *Salmonella enterica* serovar Heidelberg (ATCC 8326), *Salmonella enterica* serovar Typhi (ATCC 6539), *Listeria monocytogenes* (F2365), and *Clostridium perfringens* (ATCC 13124). *Escherichia coli* O157:H7, *L. monocytogenes* F2365, and all *Salmonella* strains were grown in tryptic soy agar or broth (TSA/TSB) at 37°C. *Clostridium perfringens* was grown in clostridial reinforced medium (CRM; BD 218081) anaerobically at 37°C. The *Saccharomyces cerevisiae* yeast samples used in this study were the two live yeast probiotics (Batch 2775 and Procreatin 7) and the three yeast cell wall paraprobiotics (Cell Wall Yeast #2194, Safmannan A #2338, Safmannan #3711). All of the products were reconstituted in yeast peptone dextrose (YPD) media at 37°C at a concentration of 0.1g/mL (~$2x10^8$ CFU/mL). The concentrations of paraprobiotics were based on initial populations of the live yeast probiotics and weighed out similarly. Viability of the products was verified by plating aliquots on YPD agar. Where required, anaerobic conditions were achieved by using a Coy anaerobic chamber with a gas mix of 5% H_2 and 95% N_2 (Type B, Coy Laboratory Products INC.). Anaerotest strips and an oxygen sensor were used to monitor anaerobiosis throughout the student.

Scanning Electron Microscopy Adhesion Assay

Overnight cultures of *S. enterica* Typhimurium, *E. coli* O157:H7, and *L. monocytogenes* F2365 were cultured at 37°C with constant agitation in TSB. Overnight

cultures of *C. perfringens* were grown at 37°C anaerobically in CRM. Overnight cultures of the yeast probiotic samples Procreatin 7 and Batch 2775 and yeast paraprobiotic samples 2194, 2338, and 3711 were cultured overnight at 37°C in YPD broth.

A Thermonox coverslip (Thermonox #174934) was placed in each well of a 6-well culture plate. Overnight cultures of the yeast probiotics and paraprobiotics (2mL, ~4x10^8 CFU/ml) were added to coverslips and incubated at 37°C for ~16 h; coverslips were then washed three times with 1X phosphate buffered saline (PBS). Overnight bacterial cultures (5 mL) were pelleted for 5 min at 13,000 *x g* and resuspended in TSB at a concentration of 2 x 10^{10} CFU/mL, at which point 1 mL of bacteria was added to the yeast coverslips. The co-culture of yeast and bacteria was incubated for 4 h at 37°C, after which each coverslip was washed with 1X PBS three times. After extensive washings, 2-3mL of 2.5% glutaraldehyde in PO_4 fixative was added to each well. Each coverslip was rinsed with distilled water, post-fixed with 2% osmium tetroxide (OsO_4), rinsed again with distilled water, and then dehydrated in a graded ethanol series (Merritt, 2009). Each coverslip was critical point dried, mounted on aluminum stubs, and coated with 15nm platinum. The coverslips were then viewed under a JEOL JSM-6500F scanning electron microscope (SEM). Per coverslip, 40 yeast probiotic cells were counted, and of that count, the number of yeast cells found with bacteria bound was used in calculating the percent adherence per sample.

Membrane Filtration Adhesion Assay

Overnight bacterial and yeast cultures were prepared similarly as samples for SEM analysis. Yeast were cultured for 16 h at 37°C in 50 mL conical tubes, after which

50 uL (~1x10^6) was added to 4900µL of YPD, and co-cultured with 50 uL of bacteria (~1x10^8 CFU/mL). The yeast+bacteria (YB) co-culture was vortexed and incubated for 4 h at 37°C. For controls, 50 µL of the bacterial culture was added to 4950 µL of YPD and incubated for 4 h at 37°C. Following the 4 h incubation, 50µL of YB co-culture or bacteria only control was added to 1450 µL of PBS in microcentrifuge tubes. Membrane filters (3.0µm, Millipore SSWP09025) were first washed with 1500µL of PBS, then the bacteria or YB mix was vacuum filtered, followed by a wash with 2000µL of PBS. The resulting filtrate (5mL) was serially diluted in PBS and plated onto TSA. Viable bacterial colonies were counted on the plates following a 24 h incubation at 37°C. A minimum of three independent replicates was conducted.

Yeast Probiotic and Paraprobiotic Supernatant Effect Assay

Bacterial and yeast cultures were prepared similarly to the membrane filtration assay. Yeast (concentration needed) were cultivated for 16 h at 37°C and vacuum filtered using 3.0µm membrane filters. Fifty uL of the resulting filtrate was added to 4900uL of YPD and co-cultured with 50 uL of bacteria (1x10^8 CFU/mL). The supernatant+bacteria (SB) co-culture was then vortexed and incubated for 4 h at 37°C. For controls, 50 µL of the bacterial culture was added to 4950 µL of YPD and incubated for 4 h at 37°C. Following the 4 h incubation, 50µL of the SB co-culture was serially diluted in PBS and plated onto TSA. Viable bacterial colonies were counted on the plates following a 24 h incubation at 37°C. A minimum of three independent replicates was conducted.

Yeast Lysate Growth Analysis

All yeast probiotics and paraprobiotics were reconstituted at 0.2g into 5mL mineral salts medium (MSM) with no glucose, and vortexed. The medium per 1000 mL contained 9.0g Na_2HPHO_4, 1.5g KH_2PO_4, 1.0g NH_4Cl, 0.2g $MgSO_47{\cdot}H_20$, 0.02g $CaCl_2{\cdot}2H_20$, 1.2mg $FeNH_4$-citrate, and 2mL Hoagland's Solution, pH 6.9 (Schlegal, Kaltwasser, and Gottschalk, 1961. Arch Microbiology 38:209-222). Yeast products were lysed on ice using a sonicator (Fisherbrand Sonic Dismembrator Model 100, setting 3) for eight 1-min intervals, with 1 min cooling on ice between intervals. Yeast lysates were collected after centrifugation for 2 min at 12,000 *x g* and filtered using a syringe filter (Milliplex 0.2µm filter). Overnight (2 mL) cultures of all *Salmonella* strains (i.e., Typhimurium, Typhi, Enteritidis, and Heidelberg) were centrifuged at 13,000 *x g* for 2 min, washed twice with 1 mL MSM (no glucose), then resuspended in 2 mL of MSM (no glucose). For analysis of *Listeria monocytogenes,* overnight cultures were centrifuged, washed twice with 1 mL of glucose limited mineral media (GLMM) and resuspended in 2 mL of GLMM without glucose (Schneebeli & Egli, 2013). The yeast lysates were added to a 96-well plate in 20 µL increments to 2 µL of bacterial cells and 180 µL of MSM (no glucose); as a control, bacteria were added to MSM supplemented with 3% glucose. Growth of the bacteria was monitored using a PowerWave plate reader (BioTek), with OD_{600} collected every 1 h for 16 h. Growth was analyzed in a minimum of three replicates.

Statistical Analysis

The data from the SEM adhesion assay and the membrane filtration adhere assay were analyzed using the GLIMMIX Procedure using SAS (SAS Inst. Inc.; Cary, NC).

Significance was declared at $P < 0.05$. Within both assays, the four bacterial strains were co-incubated with five different yeast samples, the amount of adhesion was counted (n=50) per coverslip, and those data were tested for contrasts. When overall significant differences ($P < 0.05$) existed among the samples, Tukey Grouping for Least Squares Means option of SAS was used to separate the sample (SAS Inst. Inc.; Cary, NC). The membrane filtration adhesion assay included one sample per bacterium that did not contain any yeast sample (bacterial control).

Results

Direct binding of bacteria to yeast probiotics and paraprobiotics varies.

The SEM adhesion assay was used to qualitatively determine whether the yeast probiotics and paraprobiotics bound to *E. coli* O157:H7, *L. monocytogenes, S.* Typhimurium*, and C. perfringens* (Fig. 2.1). *C. perfringens* exhibited binding to the paraprobiotic Safmannan 3711 as compared its interaction to the other yeast products ($P < 0.001$; Fig. 2.2). O157:H7 bound exhibited the least amount of binding to Safmannan A 2338, but bound to all other yeast products with similar affinity. *S.* Typhimurium exhibited no preference to any of the yeast products with an average adherence of 92%. F2365 also exhibited no preference to any of the yeast products, but unlike *S.* Typhimurium, F2365, as well as O157:H7, displayed low percentages of binding, with averages of 6.43% and 10.59%, respectively.

Averaging the mean adhered of each Gram reactivity group from the SEM assay, the Gram-positive bacteria (*L. monocytogenes* and *C.* perfringens) were found to exhibit preferential binding with the probiotic Procreatin 7 and the paraprobiotics Safmannan A

2338 and Safmannan 3711 (Fig. 2.3). The Gram-negative bacteria (*E. coli* and *Salmonella*) demonstrated no preference to any yeast product ($P > 0.05$).

The membrane filtration adhesion assay was used to accurately quantify the binding efficiency of each yeast probiotic and paraprobiotic to each bacterium. The results of the membrane filtration adhesion assay varied from strain to strain of bacteria (Fig. 2.4). *Escherichia coli* O157:H7 bound ($P < 0.001$) to both probiotics, but did not exhibit binding to any of the paraprobiotics. *Listeria monocytogenes* F2365 bound with all yeast samples ($P < 0.05$). *Salmonella* Typhimurium bound well to all yeast samples ($P < 0.001$), except for the paraprobiotic Safmannan A 2338. While the ability of *Salmonella* Typhimurium to bind to yeast paraprobiotic 2338 was not different than the control, there was a significant difference of this binding efficiency when compared to all of the other yeast binding efficiencies ($P < 0.001$).

In order to confirm that the decrease in bacterial concentration in the membrane filtration adhesion assay was due to bacterial binding to the yeast samples and not due to extracellular components of the yeast impeding the viability of the bacteria, the supernatants of the yeast products were co-incubated with the bacteria. None of the supernatants exhibited a significant effect on bacterial growth (Fig. 2.5).

Growth of bacteria in MSM/GLMM containing yeast probiotic/paraprobiotic lysate

From the filtrate analysis, the paraprobiotic 2338 appeared to improve the growth of *S. enterica* Typhimurium. To determine whether this indicated that *Salmonella* was utilizing components of the product as a carbon source, the cytoplasmic components of the yeast products were collected and added to minimal media lacking carbon. Four serovars of *Salmonella* were analyzed. *Salmonella* Heidelberg and *S.* Enteritidis exhibited

the most growth with both yeast probiotic lysates ($P < 0.05$), *S.* Typhimurium exhibited growth with the Procreatin 7 lysate ($P < 0.05$), but *S.* Typhi exhibited no significant growth with either probiotic lysates (Fig. 2.6). All *Salmonella* strains exhibited a similar increase in growth with the yeast paraprobiotic lysates 2194 and 3711, but only *S.* Heidelberg and *S.* Enteritidis exhibited significant growth in Safmannan 2338 (Fig. 2.7).

To determine whether the impact on growth was limited to *Salmonella, L. monocytogenes* F2365 was also analyzed (Fig. 2.8). Yeast probiotic 2775 and paraprobiotic 2194 were selected due to their efficiency in binding in the membrane filtration adhesion assay. *Listeria monocytogenes* F2365 exhibited an increase in growth with the probiotic 2775 lysate ($P < 0.05$), but was not able to sustain growth in the presence of the paraprobiotic 2194 lysates after 8 h.

Discussion

In order to characterize the relationship between pathogenic bacteria and probiotics/paraprobiotics, we qualitatively and quantitatively analyzed the binding of *E. coli* O157:H7, *S.* Typhimurium, *L. monocytogenes*, and *C. perfringens* to either live yeast probiotics or yeast cell wall paraprobiotics. Previous probiotic studies have suggested a correlation between the administration of probiotics and the decrease in concentrations of *E. coli* O157:H7 in adult ruminants and *in vitro* in sheep fecal suspensions (F. Chaucheyras-Durand et al., 2006; Stephens et al., 2007; Tabe et al., 2008). This could be attributed to the probiotics'/paraprobiotics' ability to bind directly to bacteria, allowing for the yeast-bacteria cluster to be removed from the host (Gedek, 1999). Research into this mechanism of action has confirmed such binding interactions *in vitro*, but has

revealed specificity with binding potentials between various bacterial strains and yeast samples.

Using SEM, the interaction between yeast probiotics and bacteria was further analyzed. The Gram-positive bacterial strains exhibited significant binding to three of the five yeast products (one probiotic and two paraprobiotics). The Gram-negative bacterial strains displayed no preference in binding, indicating that the Gram-negative bacteria bound to all yeast samples similarly. This was to be expected as *Salmonella* Typhimurium and *Escherichia coli* have both been found to express mannose-specific adhesins that allow for direct interaction with yeast cell walls (Sharon & Ofek, 1986).

As the SEM was only used to qualitatively assess the binding interaction between yeast and bacteria, the membrane filtration adhesion assay was used to quantitatively assess the binding potentials of each bacterial strain to each yeast product. During processing in the SEM assay, the majority of yeast and bacteria that were unadhered to the coverslip were washed away, so the actual binding efficiencies could differ from what is displayed on the coverslip. The Gram-negative bacteria previously thought to exhibit no preference to any of the yeast samples as displayed in the SEM assay, bound well ($P < 0.05$) to both yeast probiotics and varied between the yeast paraprobiotics in the membrane filtration assay. *Salmonella* Typhimurium bound well to all paraprobiotics except for Safmannan A 2338 ($P < 0.01$). Although it was not different when compared to the control, the binding potential of *S. typhimurium* with paraprobiotic 2338 is significantly different when compared to the binding potentials of *S. typhimurium* with the other yeast samples. Since all of the yeast samples were of different strains of *S. cerevisiae*, this particular binding potential suggests strain specificity of *S.* Typhimurium

to the yeast samples. This was unexpected as *S.* Typhimurium bound well to all yeast samples (>86%; Fig. 2.2). This suggests that the components of the yeast paraprobiotic 2338 could have served as a source of nutrients, positively affecting the growth of *S. typhimurium*.

The live yeast probiotics exhibited the greatest amount of binding to all bacteria when compared to the control. On average, all of the yeast samples significantly bound to all of the bacteria as compared to the bacterial control. This result was expected since probiotics have all of their yeast cell wall components and surface-anchored proteins still intact and available for the bacteria to bind. The yeast paraprobiotics were dehydrated or fractionated during their individual processing procedures, and those cell wall components and surface proteins used for binding could have been damaged or denatured.

In order to pursue the impact that Safmannan A 2338 had on the viability of *S.* Typhimurium, four different strains of *Salmonella* were cultivated in MSM media supplemented with the lysate of the yeast samples. When cultivated in MSM media supplemented probiotic lysate after 6 – 8 h, *S.* Heidelberg and *S.* Enteritidis exhibited an increase in growth with both probiotics ($P < 0.05$), while *S.* Typhimurium only exhibited growth with the Procreatin 7 lysates ($P < 0.05$) and *S.* Typhi exhibiting no significant growth with either probiotic lysate. The yeast paraprobiotics were also subjected to the same lysis procedure even though some may have already been fractionated due to processing. When cultivated in the MSM+paraprobiotic lysates after 6 – 8 h, all *Salmonella* strains displayed a similar increase in growth with most of the yeast paraprobiotics. This may be a result of the paraprobiotics already being fractionated,

allowing for easier access to the cellular components needed for nutrients. When *Listeria monocytogenes* was also used in this assay, the yeast probiotic 2775 yielded an increase in growth while the yeast paraprobiotic 2194 could not sustain growth after 8 h. These results support the hypothesis that the cellular components of these yeasts improve the viability of certain bacteria by providing a source of nutrients, but the amount of growth is bacterial strain dependent as well as yeast strain dependent. Further research is needed to determine how various strains of yeast affect the growth of bacteria.

Conclusion

Pathogenic bacteria affect both humans and animals through a wide array of virulence factors. Yeast probiotics and paraprobiotics are being explored as not only a prophylactic use, but also therapeutic use in animal and human health. Though it is known that these products confer a health benefit to the host, limited information is known in regards to the mechanism of action by which these products impart this benefit. This study explored the relationship of direct antagonism between the probiotics and a variety of pathogens using an *in vitro* approach. Although adhesion was observed with all bacterial strains, the binding potentials were strain-specific and yeast sample type-specific. Further research is warranted to conclude how various yeast probiotics and paraprobiotics directly interact with pathogenic bacteria. Additionally, future *in vivo* studies are needed to determine how these findings relate to overall impacts in animal health.

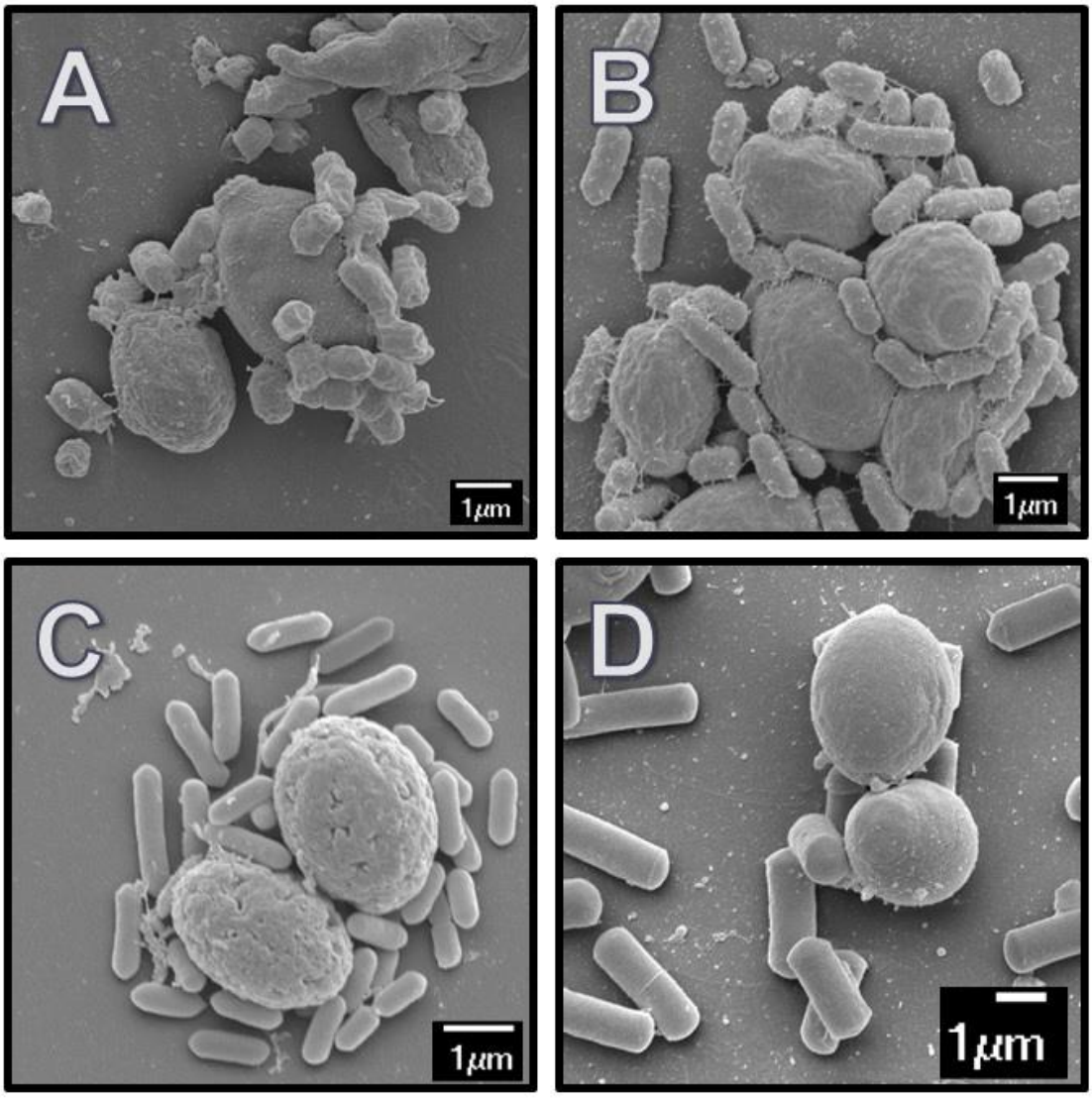


Figure 2.1 SEM images of pathogenic bacteria adhered to yeast probiotics and paraprobiotics via direct antagonism.

(A) *E. coli* O157:H7 bound to yeast paraprobiotic 3711. (B) *S. enterica* serovar Typhimurium bound to yeast paraprobiotic 3711. (C) *L. monocytogenes* F2365 bound to yeast paraprobiotic 2194. (D) *C. perfringens* bound to yeast probiotic Procreatin 7. Samples (A) – (C) were prepared aerobically, and Sample (D) was prepared anaerobically. Yeast probiotics/paraprobiotics were co-incubated with bacteria for 4 hours on Thermonox coverslips and imaged by scanning electron microscopy.

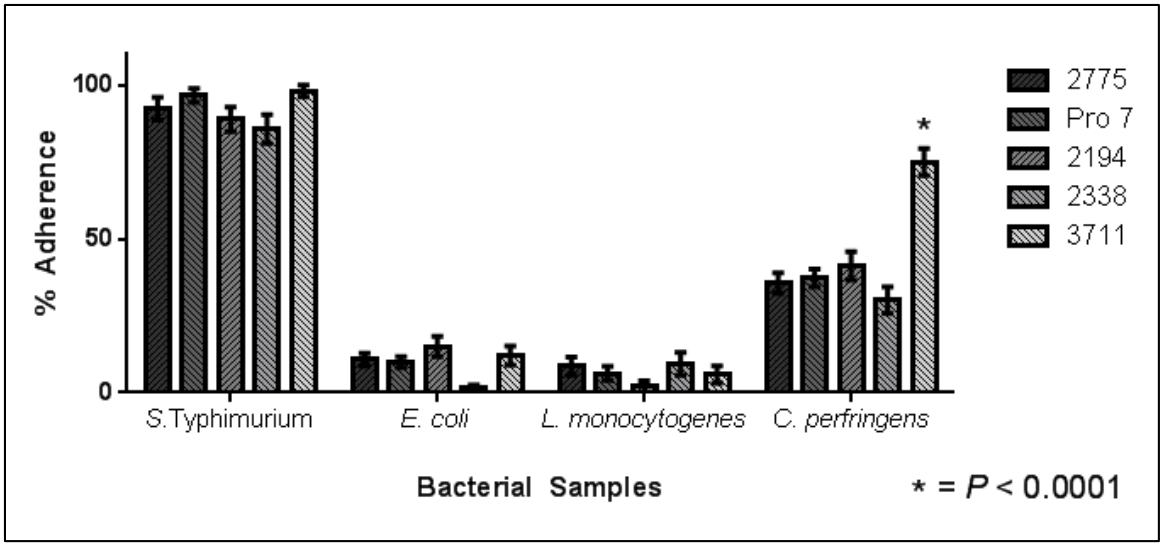


Figure 2.2 Percent adherence of *E. coli* O157:H7, *S.* Typhimurium, and *L monocytogenes* to yeast probiotics and paraprobiotics.

Probiotics 2775 and Pro7 and paraprobiotics 2194, 2338, and 3711 were co-incubated with bacteria in 6-well dishes on Thermonox coverslips and then washed extensively to remove any unadherent bacteria. Coverslips were analyzed using scanning electron microscopy (JEOL 6500F). A sample size, n=50, was used for each sample when counting the amount of yeast binding events (adherence versus non-adherence). Shown are the percent adherences per yeast sample to each bacterium plus and minus standard error. Data analysis was done using SAS using the GLIMMIX Procedure.

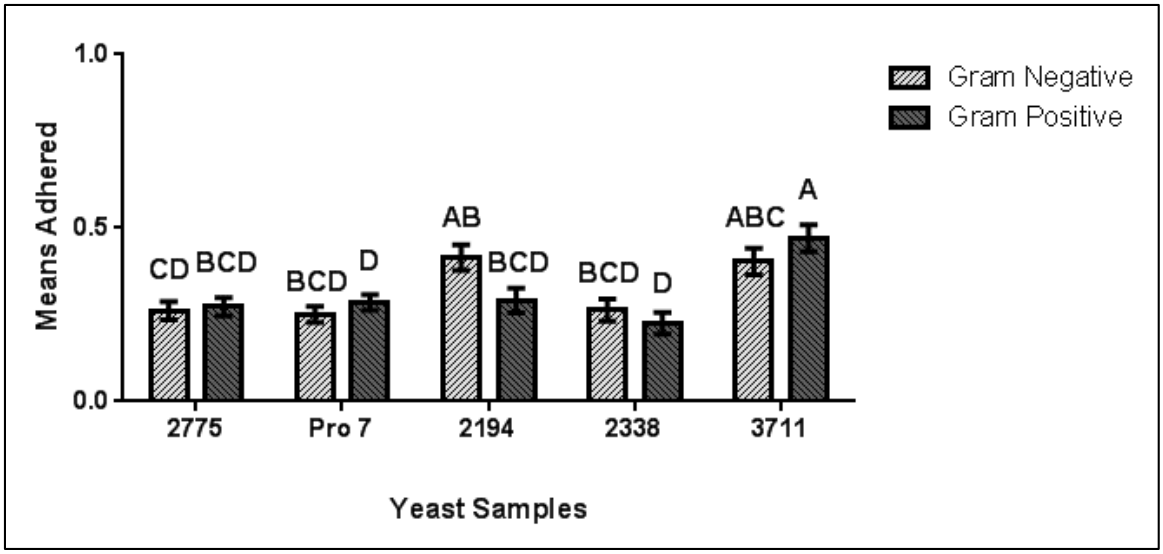


Figure 2.3 Adhesion of Gram-negative and Gram-positive bacteria to yeast probiotics and paraprobiotics.

Probiotics 2775 and Pro7 and paraprobiotics 2194, 2338, and 3711 were co-incubated with bacteria in 6-well dishes on Thermonox coverslip and then washed extensively to remove any unadherent bacteria. Coverslips were analyzed using scanning electron microscopy (JEOL 6500F). A sample size, n=50, was used for each sample when counting the amount of yeast binding events (adherence versus non-adherence). Shown is the mean adherence per yeast sample plus and minus standard error. The letters represent the level of significance between each of the samples with each letter significantly different than the other. Multiple samples share a letter due to not being significantly different. Data analysis was done using SAS using the GLIMMIX Procedure.

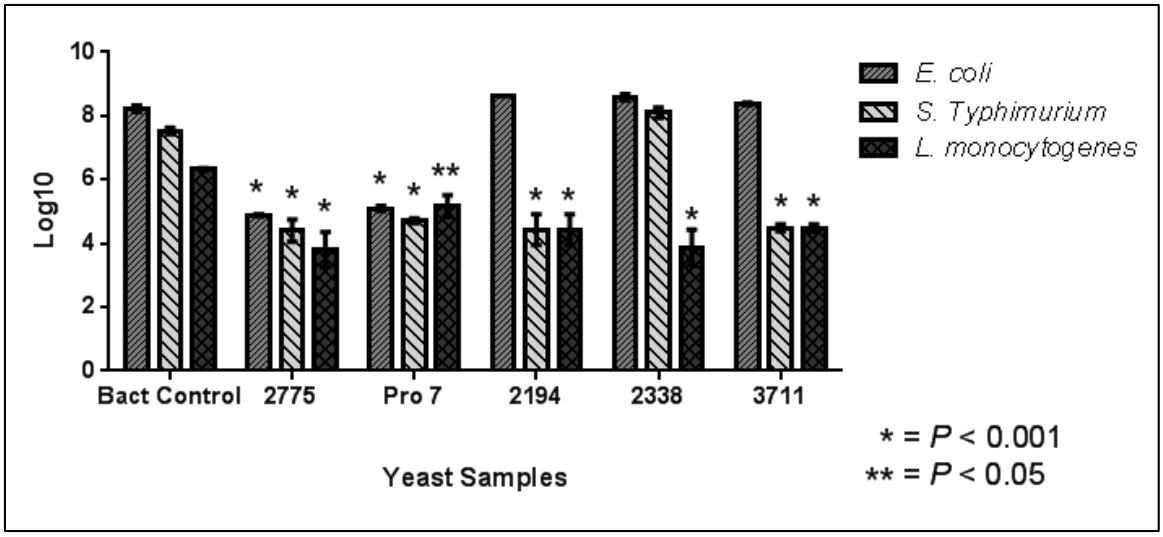


Figure 2.4 Adhesion of *Escherichia coli* O157:H7, *S.* Typhimurium, and *L. monocytogenes* with each yeast probiotic/paraprobiotic.

During the membrane filtration adhesion assay, probiotics 2775 and Pro7 and paraprobiotics 2194, 2338, and 3711 were co-incubated with bacteria and filtered using a 3 µm membrane filter. Binding efficiencies monitored by viable plate counts from filtrate of each yeast sample. Values represent the average $\log_{10}$ CFU/mL values plus and minus standard error. Statistical analysis was performed using SAS using the GLIMMIX Procedure.

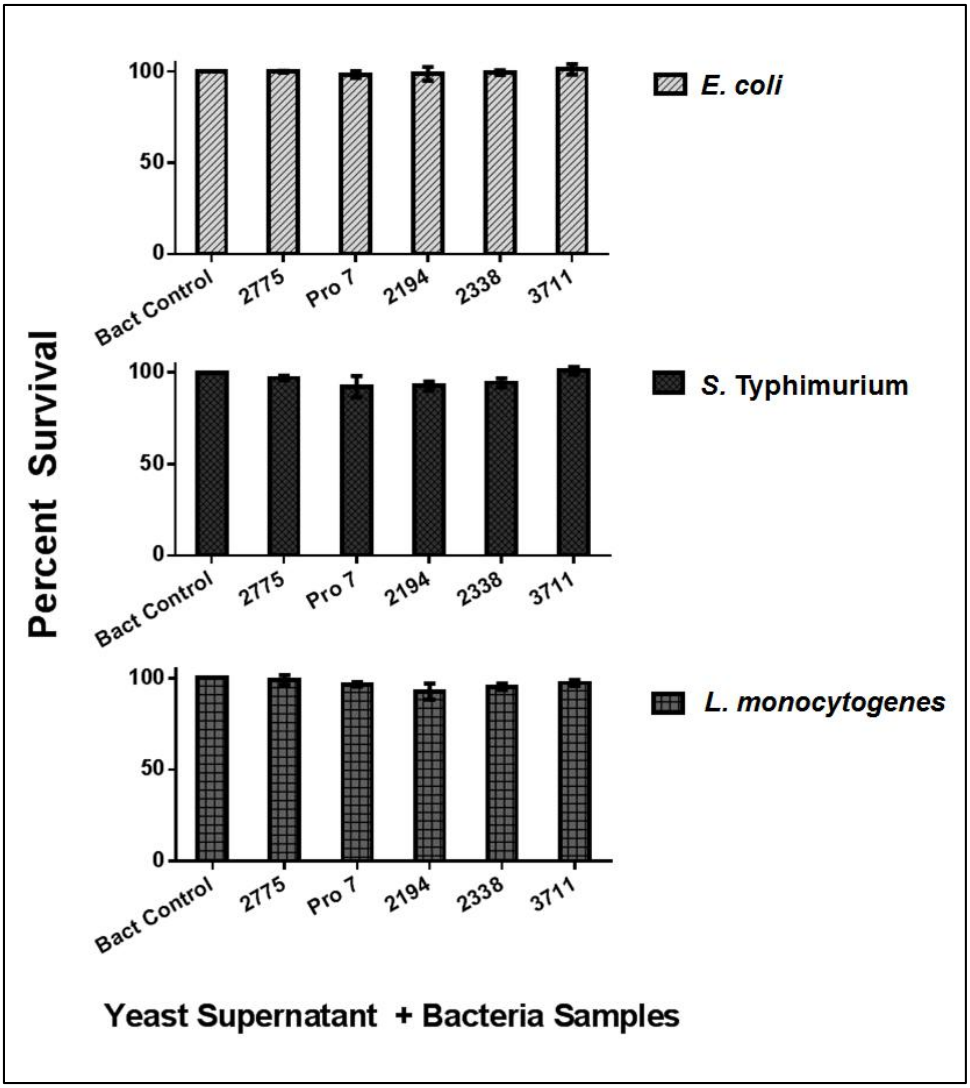
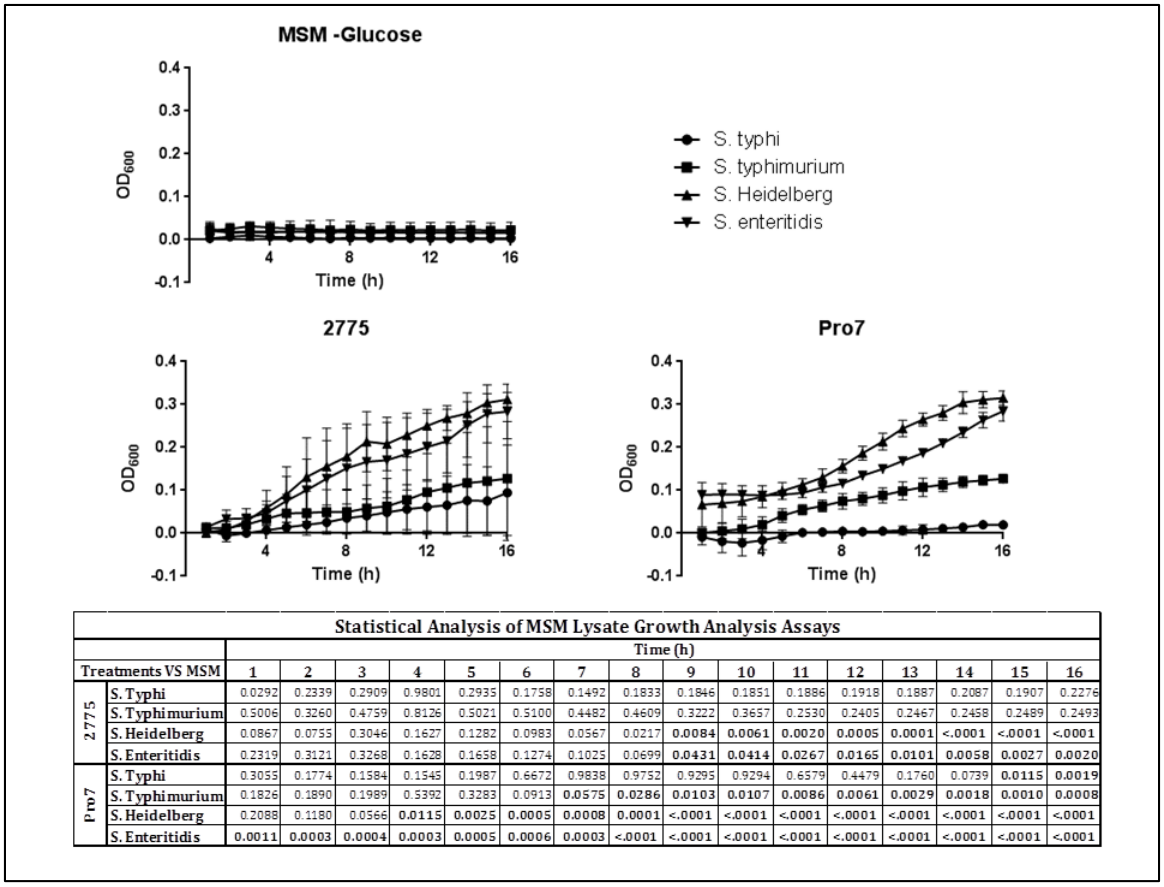


Figure 2.5 Percent survival of *Escherichia coli* O157:H7, *S.* Typhimurium, and *L. monocytogenes* co-incubated with the supernatant of yeast probiotics and paraprobiotics.

During the membrane filtration adhesion assay, the supernatants of probiotics 2775 and Pro7 and paraprobiotics 2194, 2338, and 3711 were collected and co-incubated with bacteria and filtered using a 3 µm membrane filter. Growth monitored by viable plate counts with filtrate of each yeast sample. Values represent the average log10 CFU/mL values plus and minus standard error of each co-incubation set of samples (O157:H7, F2365, *S. typhimurium*) compared to bacterial control sets of each bacterium. There were no significant differences between any co-incubation sets and their corresponding bacterial control set. Data analysis was done using SAS using the GLIMMIX Procedure.

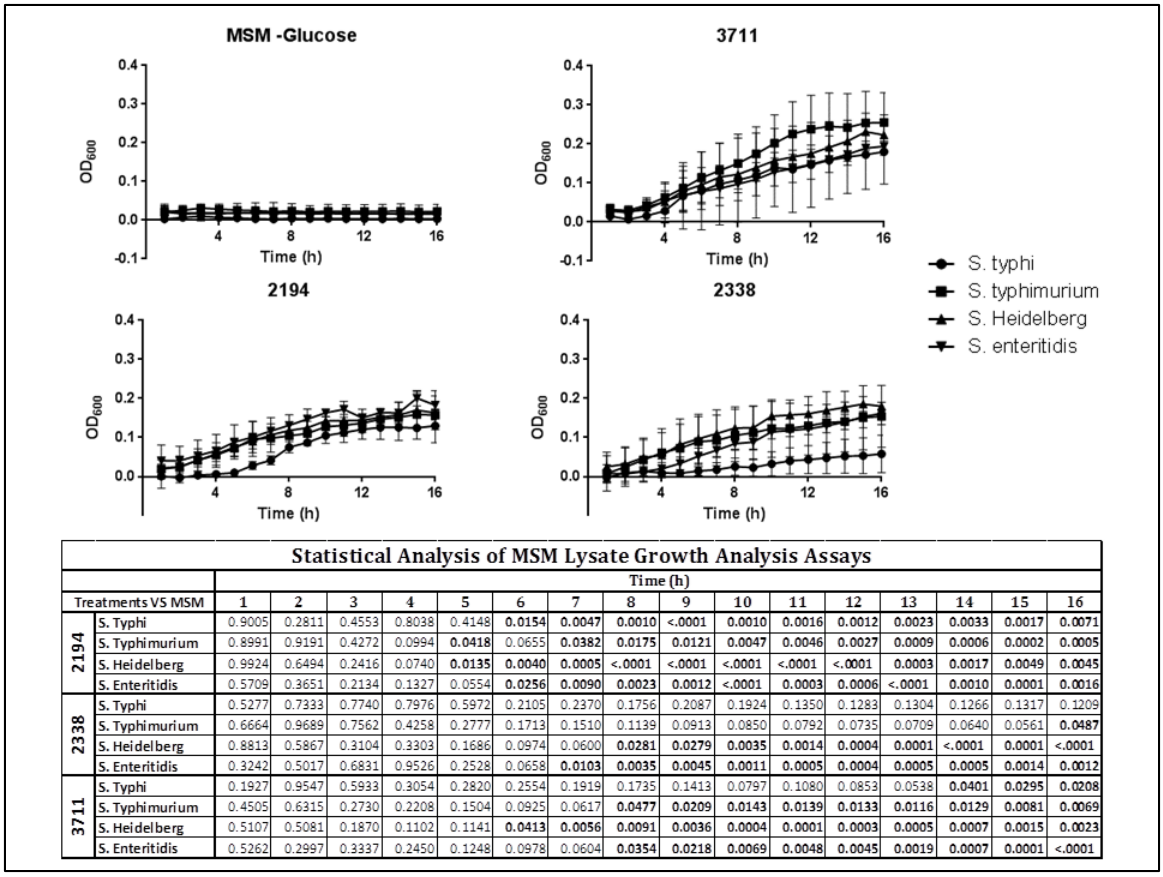


Statistical Analysis of MSM Lysate Growth Analysis Assays

		Time (h)															
Treatments VS MSM		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16
2775	S. Typhi	0.0292	0.2339	0.2909	0.9801	0.2935	0.1758	0.1492	0.1833	0.1846	0.1851	0.1886	0.1918	0.1887	0.2087	0.1907	0.2276
	S. Typhimurium	0.5006	0.3260	0.4759	0.8126	0.5021	0.5100	0.4482	0.4609	0.3222	0.3657	0.2530	0.2405	0.2467	0.2458	0.2489	0.2493
	S. Heidelberg	0.0867	0.0755	0.3046	0.1627	0.1282	0.0983	0.0567	0.0217	**0.0084**	**0.0061**	**0.0020**	**0.0005**	**0.0001**	**<.0001**	**<.0001**	**<.0001**
	S. Enteritidis	0.2319	0.3121	0.3268	0.1628	0.1658	0.1274	0.1025	0.0699	**0.0431**	**0.0414**	**0.0267**	**0.0165**	**0.0101**	**0.0058**	**0.0027**	**0.0020**
Pro7	S. Typhi	0.3055	0.1774	0.1584	0.1545	0.1987	0.6672	0.9838	0.9752	0.9295	0.9294	0.6579	0.4479	0.1760	0.0739	**0.0115**	**0.0019**
	S. Typhimurium	0.1826	0.1890	0.1989	0.5392	0.3283	0.0913	**0.0575**	**0.0286**	**0.0103**	**0.0107**	**0.0086**	**0.0061**	**0.0029**	**0.0018**	**0.0010**	**0.0008**
	S. Heidelberg	0.2088	0.1180	0.0566	**0.0115**	**0.0025**	**0.0005**	**0.0008**	**0.0001**	**<.0001**	**<.0001**	**<.0001**	**<.0001**	**<.0001**	**<.0001**	**<.0001**	**<.0001**
	S. Enteritidis	**0.0011**	**0.0003**	**0.0004**	**0.0003**	**0.0005**	**0.0006**	**0.0003**	**<.0001**	**<.0001**	**<.0001**	**<.0001**	**<.0001**	**<.0001**	**<.0001**	**<.0001**	**<.0001**

Figure 2.6 Growth of various strains of *Salmonella* (*typhi, typhimurium, Heidelberg,* enteritidis) grown with lysates of yeast probiotics in MSM

Each *Salmonella* strain (*typhi, typhimurium, Heidelberg, enteritidis*) was cultured in mineral salts media media (MSM) supplemented with the lysate of each yeast probiotic (2775 and Procreatin 7). A control of each *Salmonella* strain was cultured in MSM (-Glucose). Growth was monitored hourly at 37°C for 16 h by plate reader. Values represent average OD_{600} values plus and minus standard error.

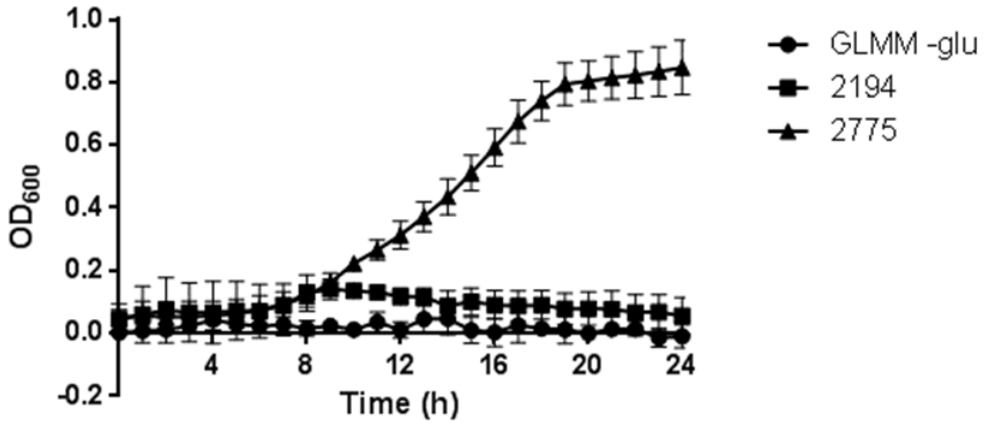


Statistical Analysis of MSM Lysate Growth Analysis Assays

		Time (h)															
Treatments VS MSM		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16
2194	S. Typhi	0.9005	0.2811	0.4553	0.8038	0.4148	**0.0154**	**0.0047**	**0.0010**	**<.0001**	**0.0010**	**0.0016**	**0.0012**	**0.0023**	**0.0033**	**0.0017**	**0.0071**
	S. Typhimurium	0.8991	0.9191	0.4272	0.0994	**0.0418**	0.0655	**0.0382**	**0.0175**	**0.0121**	**0.0047**	**0.0046**	**0.0027**	**0.0009**	**0.0006**	**0.0002**	**0.0005**
	S. Heidelberg	0.9924	0.6494	0.2416	0.0740	**0.0135**	**0.0040**	**0.0005**	**<.0001**	**<.0001**	**<.0001**	**<.0001**	**<.0001**	**0.0003**	**0.0017**	**0.0049**	**0.0045**
	S. Enteritidis	0.5709	0.3651	0.2134	0.1327	0.0554	**0.0256**	**0.0090**	**0.0023**	**0.0012**	**<.0001**	**0.0003**	**0.0006**	**<.0001**	**0.0010**	**0.0001**	**0.0016**
2338	S. Typhi	0.5277	0.7333	0.7740	0.7976	0.5972	0.2105	0.2370	0.1756	0.2087	0.1924	0.1350	0.1283	0.1304	0.1266	0.1317	0.1209
	S. Typhimurium	0.6664	0.9689	0.7562	0.4258	0.2777	0.1713	0.1510	0.1139	0.0913	0.0850	0.0792	0.0735	0.0709	0.0640	0.0561	**0.0487**
	S. Heidelberg	0.8813	0.5867	0.3104	0.3303	0.1686	0.0974	0.0600	**0.0281**	**0.0279**	**0.0035**	**0.0014**	**0.0004**	**0.0001**	**<.0001**	**0.0001**	**<.0001**
	S. Enteritidis	0.3242	0.5017	0.6831	0.9526	0.2528	0.0658	**0.0103**	**0.0035**	**0.0045**	**0.0011**	**0.0005**	**0.0004**	**0.0005**	**0.0005**	**0.0014**	**0.0012**
3711	S. Typhi	0.1927	0.9547	0.5933	0.3054	0.2820	0.2554	0.1919	0.1735	0.1413	0.0797	0.1080	0.0853	0.0538	**0.0401**	**0.0295**	**0.0208**
	S. Typhimurium	0.4505	0.6315	0.2730	0.2208	0.1504	0.0925	0.0617	**0.0477**	**0.0209**	**0.0143**	**0.0139**	**0.0133**	**0.0116**	**0.0129**	**0.0081**	**0.0069**
	S. Heidelberg	0.5107	0.5081	0.1870	0.1102	0.1141	**0.0413**	**0.0056**	**0.0091**	**0.0036**	**0.0004**	**0.0001**	**0.0003**	**0.0005**	**0.0007**	**0.0015**	**0.0023**
	S. Enteritidis	0.5262	0.2997	0.3337	0.2450	0.1248	0.0978	0.0604	**0.0354**	**0.0218**	**0.0069**	**0.0048**	**0.0045**	**0.0019**	**0.0007**	**0.0001**	**<.0001**

Figure 2.7 Growth of various strains of *Salmonella* (*typhi, typhimurium, Heidelberg,* enteritidis) grown with lysates of yeast paraprobiotics in MSM.

Each *Salmonella* strain (*typhi, typhimurium, Heidelberg, enteritidis*) was cultured in mineral salts media media (MSM) media supplemented with the lysate of each yeast paraprobiotic (3711, 2194, and 2338). A control of each *Salmonella* strain was cultured in MSM media (-Glucose). Growth was monitored hourly at 37°C for 16 h by plate reader. Values represent average OD_{600} values plus and minus standard error.



Statistical Analysis of GLMM Lysate Growth Analysis Assays												
Treatment VS GLMM	Time (h)											
	1	2	3	4	5	6	7	8	9	10	11	12
2775	0.1670	0.2218	0.3768	0.7252	0.3301	0.1161	0.1169	**0.0144**	**0.0025**	**0.0003**	**0.0010**	**0.0005**
2194	0.3764	0.3639	0.4886	0.7098	0.4874	0.3940	0.2315	**0.0379**	**0.0058**	**0.0022**	**0.0115**	**0.0074**
Treatment VS GLMM	Time (h)											
	13	14	15	16	17	18	19	20	21	22	23	24
2775	**0.0010**	**0.0009**	**0.0002**	**0.0002**	**0.0002**	**<.0001**	**<.0001**	**<.0001**	**<.0001**	**<.0001**	**<.0001**	**<.0001**
2194	0.0893	0.3709	0.0589	0.0795	0.2306	0.0661	0.1665	0.0670	0.1471	0.2213	0.0898	0.1821

Figure 2.8 Growth of *Listeria* monocytogenes F2365 grown with the lysates of a yeast probiotic and paraprobiotic in GLMM.

Listeria monocytogenes F2365 was cultured in three different glucose limited mineral media (GLMM): GLMM (no glucose), GLMM (with lysate of yeast paraprobiotic 2194), and GLMM (with lysate of yeast probiotic 2775). Growth monitored by plate reader. Growth monitored by plate reader at 37°C for 24 h, OD_{600} reading taken every 1 h. Values represent average OD_{600} values plus and minus standard error.

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CHAPTER III

CONCLUSION

Yeast probiotics and paraprobiotics represent important alternatives to the use of antibiotics for treatment of bacterial infections. Yeast probiotics and paraprobiotics exhibit a wide array of mechanisms of action, ranging from host immune stimulation to host microbial community stabilization to toxin/bacterial cell adhesion (F. Chaucheyras-Durand et al., 2008).

The binding effect of yeast probiotic and paraprobiotics on several different Gram-positive and Gram-negative pathogenic bacteria was investigated through a literature review in Chapter 1 of this thesis. The aim of this review was to explain how yeast probiotics and paraprobiotics interact with bacteria and interfere with bacterial pathogenesis. The review also discusses information regarding the direct adherence of probiotics to bacteria that allow for the removal of pathogens (Gedek, 1999). Unfortunately, variations in the adhesion capabilities between bacteria have complicated research deciphering this mechanism of action (Rajkowska, 2012). Variations in the binding potential demonstrated by Rajkowska and colleagues led to the hypothesis that yeast probiotics and paraprobiotics exhibit specific binding patterns against bacteria.

To further investigate the strain specific interactions of yeast probiotics and paraprobiotics to various pathogenic bacteria, *Escherichia coli* O157:H7, *Listeria monocytogenes, Salmonella* Typhimurium*, and Clostridium perfringens* were co-

incubated with five different strains of *Saccharomyces cerevisiae*-based samples (two probiotics and three paraprobiotics) on Thermonox coverslips under aerobic or anaerobic conditions, depending on the specific bacterial growth requirements. The coverslips were processed for scanning electron microscopy, and the amount of yeast cells with bacteria bound were assessed for percent adherence. While the Gram-positive bacteria overall displayed strain-specific binding, the Gram-negative bacteria overall displayed no significant difference in binding to any of the yeasts.

To quantitate the efficiencies, each co-incubation mixture was filtered to accurately quantitate the binding of each bacterial species to each yeast sample. When the $\log_{10}$ concentrations of each filtrate were compared to their bacterial control, *L. monocytogenes* F2365 exhibited significant binding to all yeast samples, *E. coli* O157:H7 exhibited significant binding to only the yeast probiotics, and *S. typhimurium* exhibited significant binding to all yeast samples except yeast paraprobiotic 2338. This strain specificity led to another question of whether or not the bacteria may be using these paraprobiotics as nutrients. To investigate that question, all yeast samples were lysed and supplemented into minimal media lacking a carbon course. Four strains of *Salmonella* and *L. monocytogenes* strain F2365 were grown in the minimal media supplemented with yeast lysate. Two strains of *Salmonella* and F2365 exhibited growth with the probiotics. All four strains of *Salmonella* exhibited growth with all yeast paraprobiotics, while *Listeria monocytogenes* was able to grow in both probiotic 2775 and paraprobiotic 2194. This result further validated the existence of strain specificity being displayed when bacteria interact with these yeast samples.

Despite these results, there are limitations to this study. *Saccharomyces cerevisiae* is the only type of probiotic/paraprobiotic used in this project. Expanding this sample size to include other strains of *Saccharomyces* as well as include some bacterial probiotics/paraprobiotics would further validate not only the mechanism of direct antagonism with probiotics and paraprobiotics, but also provide a comparison experiment of bacteria to yeast when referring to probiotic mechanisms of action. Expanding the library of pathogenic bacteria would also help to verify the strain specificity.

The importance of this work rests in its ability to provide insight to the interaction of yeast probiotics and paraprobiotics to various pathogenic bacteria. With this knowledge, the idea of treating infections specifically with a particular probiotic is a possibility. Further research is warranted to confirm if this mechanism of direct antagonism can be used as a valid mechanism for probiotic action.

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APPENDIX A

COMPLETE SEM ADHESION ASSAY RESULTS: ALL BACTERIA

Scanning Electron Microscopy Adhesion Assay

Bacterial strains and cultivation methods

The bacterial strains used in this study were *Escherichia coli* O157:H7 (ATCC 43895), *Salmonella enterica* serovar Typhimurium (ATCC 13311), *Listeria monocytogenes* (F2365), and *Clostridium perfringens* (ATCC 13124), *Clostridium difficile* (ATCC NR-32882), *Porphyromonas assacharolyticus* (ATCC 25260), *Bacteroides fragilis* (ATCC 25285), *Arcanobacterium pyogenes* (ATCC 19411), *Fusobacterium necrophorum* (ATCC 25286), *Salmonella enterica* serovar Enteritidis (ATCC 13076), *Salmonella enterica* serovar Heidelberg (ATCC 8326), *Salmonella enterica* serovar Typhi (ATCC 6539), and *Salmonella enterica* serovar Dublin (ATCC NR-28793). *Escherichia coli* O157:H7, *L. monocytogenes* F2365, and all *Salmonella* strains were grown in tryptic soy agar or broth (TSA/TSB) at 37°C. *C. perfringens* and *C. difficile* was grown in clostridial reinforced medium (CRM; BD 218081) anaerobically at 37°C. *B. fragilis and P. assacharolyticus* was grown on Brucella broth with Vitamin K and hemin (BRU-BROTH; Anaerobe Systems AS-105) anaerobically at 37°C. *F. necrophorum* and *A. pyogenes* were grown in Chopped meat glucose broth (CMG; Anaerobe Systems AS-813) anaerobically at 37°C. The *Saccharomyces cerevisiae* yeast samples used in this study were the two live yeast probiotics (Batch 2775 and Procreatin 7), and the three yeast cell wall paraprobiotics (Cell Wall Yeast #2194, Safmannan A #2338, Safmannan #3711). All of the products were reconstituted in yeast peptone dextrose (YPD) media at 37°C at a concentration of 0.1g/mL (~$2x10^8$ CFU/mL). Viability of the live products was verified on YPD agar.

Materials and Methods

Overnight cultures of all bacteria were cultured at 37°C with constant agitation either aerobically or anaerobically in their respective media depending on their growth requirements. Overnight cultures of the yeast probiotic samples Procreatin 7 and Batch 2775 were cultured at 37°C in Yeast Peptone Dextrose broth (YPD). Overnight cultures of the yeast paraprobiotic samples 2194, 2338, and 3711 were cultured at 37°C in YPD broth.

A Thermonox coverslip (Thermonox #174934) was placed in each well of a 6-well culture plate. Overnight cultures of the yeast probiotics and paraprobiotics (2mL, ~$4x10^8$ CFU/ml) were added to coverslip and incubated at 37°C for ~16 h. Overnight bacterial cultures (5 mL) were pelleted for 5 min at 13,000 x g and resuspended in 6 mL of TSB. Bacteria (1 mL) were added to the yeast at a concentration of 2 x 10^{10} CFU/mL, as determined by the optical density (OD_{600}) of 0.2 (Nanodrop ND-1000). The co-culture of yeast and bacteria was incubated for 4 h at 37°C, after which each coverslip was washed with 2-3mL of 1X phosphate buffered saline (PBS) three times. After extensive washings, 2-3mL of 2.5% glutaraldehyde in PO_4 fixative was added to each well. Each coverslip was rinsed with distilled water, post-fixed with 2% osmium tetroxide (OsO_4), rinsed again with distilled water, and then dehydrated in a graded ethanol series (Merritt, 2009). Each coverslip was critical point dried, mounted on aluminum stubs, and coated with 15nm platinum. The coverslips were then viewed under a JEOL JSM-6500F scanning electron microscope (SEM) at 5 kb. Per coverslip, 50 yeast probiotic cells were counted, and of that count, the number of yeast cells found with bacteria bound was used in calculating the percent adherence per sample.

Results and Discussion

The data collected from the complete SEM adhesion assay could not be used. Limiting the initial scope of the assay to only two Gram-positive and two Gram-negative bacteria allowed for the same bacteria to be used throughout all assays. The anaerobic bacteria were difficult to use in the Membrane filtration adhesion assay due to the actual filtration protocol. No growth was exhibited after the filtration and dilution steps with any of the anaerobic bacteria.

The percent adherence of each bacterium to each yeast probiotic and paraprobiotic is displayed in Table A.1, and the statistical analysis of the binding potentials of each bacterium between each yeast sample is displayed in Table A.2.

Table A.1 Percent adherence of pathogenic bacteria to yeast probiotics and paraprobiotics

Bacteria	2775 % Adhere	Pro7 % Adhere	2194 % Adhere	2338 % Adhere	3711 % Adhere
S. typhimurium	92.31%	96.67%	88.89%	85.71%	98.11%
E. coli O157:H7	10.76%	9.93%	15.04%	1.49%	12.04%
L. monocytogenes	8.69%	6.25%	1.96%	9.37%	5.88%
C. perfringens	35.61%	37.32%	41.23%	30.09%	75.00%
C. difficile	17.24%	20.97%	0.00%	5.00%	0.00%
P. assacharolytica	85.63%	31.79%	0.00%	0.00%	0.00%
B. fragilis	55.32%	13.51%	37.50%	15.22%	4.55%
A. pyogenes	39.05%	17.11%	0.00%	0.00%	0.00%
F. necrophorum	13.51%	55.32%	37.50%	15.22%	4.55%
S. enteritidis	49.09%	55.22%	16.67%	0.00%	11.90%
S. Heidelberg	29.30%	30.43%	24.99%	46.43%	22.22%
S. typhi	65.08%	58.83%	21.53%	59.32%	50.00%
S. Dublin	34.00%	9.09%	12.00%	0.00%	6.00%

Table A.2 Statistical analysis of binding potentials of pathogenic bacteria to yeast probiotics and paraprobiotics

Bacteria	2775 vs Pro7 P-value	2775 vs 2194 P-value	2775 vs 2338 P-value	2775 vs 3711 P-value	Pro7 vs 2194 P-value	Pro7 vs 2338 P-value	Pro7 vs 3711 P-value	2194 vs 2338 P-value	2194 vs 3711 P-value	2338 vs 3711 P-value
S. typhimurium	0.8579	0.9723	0.8199	0.6970	0.5217	0.3014	0.9899	0.9853	0.4214	0.2684
E. coli O157:H7	0.9981	0.7937	**0.0435**	0.9970	0.6073	0.0576	0.9744	**0.0111**	0.9668	**0.0361**
L. monocytogenes	0.9649	0.5975	0.9999	0.9647	0.8016	0.9437	1.0000	0.5674	0.8542	0.9452
C. perfringens	0.9952	0.8604	0.8584	**<.0001**	0.9516	0.6568	**<.0001**	0.4093	**<.0001**	**<.0001**
C. difficile	0.9458	**0.0065**	0.1114	**0.0109**	**0.0003**	**0.0125**	**0.0007**	0.8555	1.0000	0.8757
P. assacharolytica	**<.0001**	**<.0001**	**<.0001**	**<.0001**	**<.0001**	**<.0001**	**<.0001**	1.0000	1.0000	1.0000
B. fragilis	**<.0001**	0.2219	**<.0001**	**<.0001**	**0.0095**	0.9970	0.1756	**0.0330**	**<.0001**	0.1057
A. pyogenes	**<.0001**	**<.0001**	**<.0001**	**<.0001**	**0.0361**	**0.0388**	**0.0417**	1.0000	1.0000	1.0000
F. necrophorum	**<.0001**	**0.0095**	0.9970	0.1756	0.2219	**<.0001**	**<.0001**	**0.0330**	**<.0001**	0.1057
S. enteritidis	0.9188	**<.0001**	**<.0001**	**<.0001**	**<.0001**	**<.0001**	**<.0001**	0.1908	0.9735	0.6420
S. Heidelberg	0.9999	0.9892	0.3447	0.9296	0.9720	0.3715	0.8760	0.2002	0.9981	0.1036
S. typhi	0.9495	**<.0001**	0.9666	0.4914	**0.0003**	1.0000	0.8757	**0.0005**	**0.0163**	0.8664
S. Dublin	**0.0005**	**0.0040**	**<.0001**	**<.0001**	0.9890	0.5597	0.9861	0.2980	0.8680	0.8680