DISSERTATION

INVESTIGATION OF DIETARY RICE BRAN FOR PROTECTION AGAINST *SALMONELLA ENTERICA* TYPHIMURIUM INFECTION IN MICE

Submitted by

Ajay Kumar

Department of Clinical Sciences

In partial fulfillment of the requirements

For the Degree of Doctor of Philosophy

Colorado State University

Fort Collins, Colorado

Summer 2014

Doctoral Committee:

Advisor: Elizabeth P. Ryan

Steven Dow
Jan Leach
Alan Schenkel
Tiffany Weir

ABSTRACT

INVESTIGATION OF DIETARY RICE BRAN FOR PROTECTION AGAINST *SALMONELLA ENTERICA* TYPHIMURIUM INFECTION IN MICE

Rice bran is a byproduct of rice milling for white rice. Rice bran is a rich source of nutrients such as vitamins, minerals, soluble and insoluble fibers, fatty acids, polyphenols and proteins. Research has shown the beneficial health effects of rice bran in hyperlipidemia, diabetes, immune modulation, allergies and cancer. This dissertation focuses on evaluation of rice bran for protection against *Salmonella* using a mouse model of oral infection.

Salmonella is a food and water borne pathogen that affects a variety of hosts including plants, animals and humans. *Salmonella* infections are a major public health challenge around the globe. Currently, salmonellosis is treated using high doses of synthetic antimicrobials and the problem of drug resistance has increased. In this scenario, alternative and sustainable interventions are needed to control *Salmonella* infections. Several dietary agents have been studied for protective effects in *Salmonella* infection models. We tested the prophylactic effects of dietary rice bran in a *Salmonella* model of infection using female 129S6/SvEvTac mouse model with infection of *Salmonella enterica* Typhimurium 14028s strain. Feeding of 10% dietary rice bran for one week prior to infection significantly ($p<0.05$) reduced fecal excretion of *Salmonella* in orally infected mice. *Salmonella*-infected, rice bran fed mice also showed a significant decrease in systemic inflammatory cytokines such as TNF-α, IFN-γ and IL-12 as compared to control diet fed animals. The colonization resistance against enteric pathogens is highly influenced by composition of gut microflora. Supplementation of dietary rice bran increased the number of *Lactobacillus spp*. in feces of mice as compared to mice that were fed

control diet. Research has shown that oral administration of some species of *Lactobacillus* reduces the colonization of *Salmonella*. We hypothesized that rice bran components also enhance mucosal protection by preventing *Salmonella* entry into the epithelial cells. Methanolic rice bran extracts were assessed in mouse small intestinal epithelial (MSIE) cells for blocking *Salmonella* entry and intracellular replication. Rice bran extract significantly reduced *Salmonella* entry and intracellular replication into MSIE cells. These results suggest the potential mechanisms for dietary rice bran induced improvement of colonization resistance against *Salmonella*. Given that rice crops have a large variation in genotype and phenotype such as in yield, disease and pest resistance, drought resistance, and nutrient quality, we hypothesized that variation in rice bran across cultivars induces differential protection against *Salmonella* infection due to differences in their phytochemical profile.

A panel of six varieties namely IAC 600, Jasmine 85, IL 121-1-1, Wells, Red Wells and SHU 121 were tested in the in vitro and in vivo model of *Salmonella* infection. We found that rice bran extracts across varieties inhibited *Salmonella* entry into the MSIE and Caco-2 cells to different extents. IAC 600 fed animals significantly ($p<0.05$) reduced *Salmonella* fecal excretion as compared to the control diet fed animals. IAC 600 fed animals also reduced *Salmonella* fecal shedding significantly ($p<0.05$) as compared to SHU 121 diet fed animals at 2 and 6 days post *Salmonella* infection. Histopathological analysis revealed that IAC 600 diet fed animals had better ileal pathological scores as compared to SHU 121 and the control diet fed animals post *Salmonella* infection. SHU 121 and the control diet fed groups showed higher ulceration and inflammatory changes in ileum as compared to IAC 600 fed animals. Next we analyzed the fatty acid profile, mineral profile and total phenolic contents of rice bran. Stearic acid, lignoceric acid, boron and total phenol content were significantly correlated with *Salmonella* fecal shedding in

mice across varieties. However, further studies are required to confirm the role of these nutrients from rice bran in protection against *Salmonella*. These results suggest that the variety of rice plays an important role in bran-induced protection against *Salmonella* infection and this difference in protection across the varieties could be attributed to a combination of bioactive components.

Our studies suggest that dietary rice bran improves colonization resistance against *Salmonella* in mice. Rice bran could have important role in prevention of enteric infections in resource scarce populations and further human clinical studies are required. Rice bran may also be evaluated for supplementing diets of food animals to prevent *Salmonella* infections and therefore could have a potential role in food safety.

ACKNOWLEDGEMENT

My sincere gratitude goes to my graduate committee members Drs. Steven Dow, Alan Schenkel, Jan Leach and Tiffany Weir. Their commendable guidance and invaluable suggestions have been indispensable during entire course of studies. I want to acknowledge my advisor Dr. Elizabeth Ryan for providing the opportunity to work with a diverse team of intellectuals from different specialties. It always has been an honor for me to work with them.

I acknowledge the contributions of Dr. Angela Henderson for helping in flow cytometery and reviewing the manuscript, Dr. Andrew Goodyear for the preparation of *Salmonella* stock, Genevieve M. Forster, Dr. Adam Hueberger and Caleb Schmidt for technical assistance, Dr. Tiffany Weir and Brittney Barnett for fecal microflora determination, Dr. Andres Vazquez-Torres from University of Colorado for providing the strain of *Salmonella* used in these studies, Dr. Anna McClung from the USDA-ARS Dale Bumpers Rice Research Center for providing rice bran.

I acknowledge the contribution of Dr. Michael A Grusak from USDA-ARS, Baylor College of Medicine for rice bran mineral analysis across varieties, Dr. EJ Erhart from Colorado State University for evaluation of histology, and Dr. Ann Hess from Colorado State University for statistical analysis expertise. My sincere gratitude also goes to Barb Rose, Laura Chubb and Lynda Reed from the Animal Cancer Center and Morna Mynard from the Department of Clinical Sciences for their unconditional support.

Finally, I am very thankful to my parents, my brother and my sister in law for their constant emotional support throughout all of my challenges in graduate school. I am grateful to all of my friends who constantly supported me.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

HYPOTHESIS AND SPECIFIC AIMS

Due to antimicrobial resistance, frequent *Salmonella* infections and lack of effective strategies, there is a significant need to develop dietary solutions. The human diet is recognized as an important carrier of bioactive phytochemicals and accumulating literature points to the application of plant-based phytochemicals in the prevention of chronic diseases. Rice (*Oryza sativa*) is a staple food crop around the world and bran is a nutrient rich outer layer of rice grain that is generally removed during rice milling to produce white rice. An estimate of the annual worldwide production of rice bran is 70 million tons. Generally, rice bran is used as an animal feed or discarded as a waste product. Hence, rice bran is accessible and affordable to resource-poor populations. Rice bran is a rich source of vitamins, minerals, fibers, fatty acids and polyphenols such as ferulic acids (Ryan 2011). These components individually possess health benefits. For instance, sulfated glucans isolated from rice bran inhibit entry of cytomegalovirus into the human fibroblasts (Ghosh, Auerochs et al. 2010). Cycloartenyl ferulate is another rice bran component that chelates IgE and attenuates mast cell degranulation in rats (Oka, Fujimoto et al. 2010). Gamma-oryzanol possesses anti-hypercholesterolemiac properties rats (Ghatak and Panchal 2012). Tocotrienol, phytic acid, and tricin decrease the prevalence of cancer progression in animal models (Cai, Hudson et al. 2004, Iqbal, Minhajuddin et al. 2004, Norazalina, Norhaizan et al. 2010). Oil is an important component of rice bran and has been used in many studies. Rice bran oil constitutes a unique profile of fatty acids that have been shown to lower the serum cholesterol in humans (Most, Tulley et al. 2005, Zavoshy, Noroozi et al. 2012). The cholesterol lowering effect is potentially associated with reduced reabsorption of cholesterol in the intestines. (Trautwein, Schulz et al. 2002). Rice bran oil contains a large quantity of unsaponifiable matter such as oryzanol, policosanol, fatty aldehydes and potassium salts of

oryzanol that are presumed to associate with cholesterol lowering effects (Afinisha Deepam and Arumughan 2012). Rice bran oil also possesses immune modulatory properties. Consumption of rice bran oil increased proliferation of B-lymphocytes and Th1 cytokines such as IL-2 and TNF-α in mice (Sierra, Lara-Villoslada et al. 2005). Gamma oryzanol alone is not responsible for this immune-modulatory effect because addition of gamma oryzanol in high oleic sunflower oil did not change the immune response of the mice (Sierra, Lara-Villoslada et al. 2005). Consumption of rice bran oil alters the composition of the gut microflora, especially increased *Lactobacilli* as compared to the non-rice bran oil group of mice (Tamura, Hori et al. 2012).Rice bran fiber concentrate reduces hyperlipidemia and the water-soluble component of the rice bran reduces hyperglycemia in diabetic humans (Qureshi, Sami et al. 2002). Mechanistically, the in-vitro study demonstrated that hemicellulose fraction of rice bran binds to cholesterol and bile acids (Hu and Yu 2013). In addition to components, whole rice bran has also been studied for health benefits. Dietary rice bran may be beneficial in prevention of several types of cancers such as intestinal, lung, breast, liver and pancreatic cancers (Verschoyle, Greaves et al. 2007, Henderson, Ollila et al. 2012). Further, intraperitoneal injection of ethanolic black rice bran extract decreased 2,4-dinitrofluorobenzene (DNFB)-induced allergic dermatitis. Also, feeding of 10% black rice bran as a dietary supplement reduced the DNFB-induced dermatitis on mouse skin (Choi, Kim et al. 2010). The symptoms of atopic dermatitis were also improved in the patients who took rice bran broth bath (Fujiwaki and Furusho 1992). Apart from immunological effects, dietary rice bran improves the digestive system as a supplementation of rice bran in pig diets increased the feed efficiency by improving the ileal mucosa (Herfel, Jacobi et al. 2013). Recently, health effects of rice bran, rice bran oil and rice hull have been reviewed (Friedman 2013). These

studies show the importance of rice bran in prevention of a number of diseases. However, rice bran has never been tested to prevent *Salmonella* infections in mice.

Given the health promoting properties of rice bran in several diseases and rice bran as a rich source of bioactive components, ***we hypothesize that dietary rice bran enhances colonization resistance against Salmonella infection in mice*.** The three specific aims of this dissertation (Chapter 2 and 3) investigate the effects of rice bran using both in vivo and in vitro models of *Salmonella* infection (Figure I.1).

Specific Aim 1: **To investigate the extent to which dietary rice bran (variety Neptune) increases the colonization resistance against *Salmonella* infection in mice**. Following experiments were performed for the specific aim 1.

- Assessment of RBE in the in-vitro *Salmonella* entry assay
- Effect of dietary rice bran on prevention of *Salmonella* fecal shedding in mice
- Measurement of *Salmonella*-induced systemic inflammation
- Effects of rice bran on *Salmonella*-induced shift in gut microbiota

Specific Aim 2: To examine whether dietary rice bran exerts similar effects against *Salmonella* across the six selected diverse varieties. Following experiments were performed for the specific aim 2:

- Assessment of RBE in the in-vitro *Salmonella* entry across the varieties
- Effect of dietary rice bran on prevention of Salmonella fecal shedding in mice across the varieties
- Effect of dietary rice bran on *Salmonella*-induced intestinal inflammation across the varieties

Specific Aim 3: To determine rice bran components across the six diverse varieties and to correlate the rice bran components with *Salmonella* fecal shedding. Following experiments were performed for specific aims 3:

- Determination of rice bran mineral profile across the varieties
- Determination of rice bran fatty acid profile across the varieties
- Determination of rice bran total soluble phenolic compounds across the varieties
- Statistical correlations of components and *Salmonella* fecal shedding across varieties

Chapter 3 will investigate the differences in rice bran across varieties for protection against *Salmonella* using in-vitro and vivo models and analysis of phytochemical profile across varieties and will cover specific aim 2 and 3. Chapter 1 will present a literature of review on the use of dietary supplements in *Salmonella* infections across species relevant to humans.

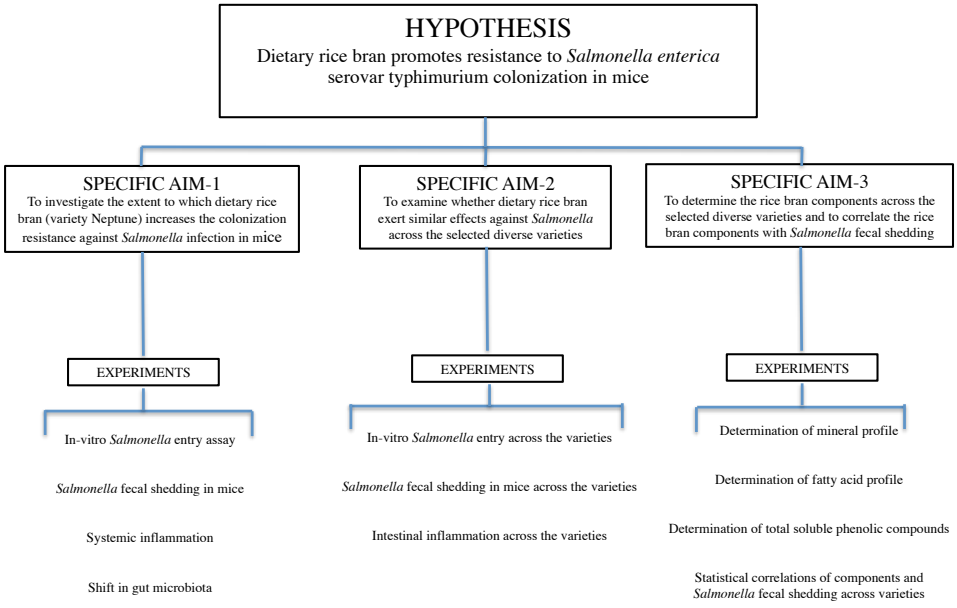


Figure I.1 Shows the schema for study hypothesis, specific aims and experiments conducted

REFERENCES

Afinisha Deepam, L. S. and C. Arumughan (2012). "Effect of saponification on composition of unsaponifiable matter in rice bran oil." J Oleo Sci **61**(5): 241-247.

Cai, H., E. A. Hudson, P. Mann, R. D. Verschoyle, P. Greaves, M. M. Manson, W. P. Steward and A. J. Gescher (2004). "Growth-inhibitory and cell cycle-arresting properties of the rice bran constituent tricin in human-derived breast cancer cells in vitro and in nude mice in vivo." British Journal of Cancer **91**(7): 1364-1371.

Choi, S. P., S. P. Kim, M. Y. Kang, S. H. Nam and M. Friedman (2010). "Protective Effects of Black Rice Bran against Chemically-Induced Inflammation of Mouse Skin." Journal of Agricultural and Food Chemistry **58**(18): 10007-10015.

Friedman, M. (2013). "Rice brans, rice bran oils, and rice hulls: composition, food and industrial uses, and bioactivities in humans, animals, and cells." J Agric Food Chem **61**(45): 10626-10641.

Fujiwaki, T. and K. Furusho (1992). "The effects of rice bran broth bathing in patients with atopic dermatitis." Acta Paediatr Jpn **34**(5): 505-510.

Ghatak, S. B. and S. J. Panchal (2012). "Anti-hyperlipidemic activity of oryzanol, isolated from crude rice bran oil, on Triton WR-1339-induced acute hyperlipidemia in rats." Revista Brasileira De Farmacognosia-Brazilian Journal of Pharmacognosy **22**(3): 642-648.

Ghosh, T., S. Auerochs, S. Saha, B. Ray and M. Marschall (2010). "Anti-cytomegalovirus activity of sulfated glucans generated from a commercial preparation of rice bran." Antiviral chemistry & chemotherapy **21**(2): 85-95.

Henderson, A. J., C. A. Ollila, A. Kumar, E. C. Borresen, K. Raina, R. Agarwal and E. P. Ryan (2012). "Chemopreventive Properties of Dietary Rice Bran: Current Status and Future Prospects." Advances in Nutrition **3**(5): 643-653.

Herfel, T., S. Jacobi, X. Lin, E. van Heugten, V. Fellner and J. Odle (2013). "Stabilized rice bran improves weaning pig performance via a prebiotic mechanism." J Anim Sci **91**(2): 907-913.

Hu, G. and W. Yu (2013). "Binding of cholesterol and bile acid to hemicelluloses from rice bran." Int J Food Sci Nutr **64**(4): 461-466.

Iqbal, J., M. Minhajuddin and Z. H. Beg (2004). "Suppression of diethylnitrosamine and 2-acetylaminofluorene-induced hepatocarcinogenesis in rats by tocotrienol-rich fraction isolated from rice bran oil." European Journal of Cancer Prevention **13**(6): 515-520.

Most, M. M., R. Tulley, S. Morales and M. Lefevre (2005). "Rice bran oil, not fiber, lowers cholesterol in humans." Am J Clin Nutr **81**(1): 64-68.

Norazalina, S., M. E. Norhaizan, I. Hairuszah and M. S. Norashareena (2010). "Anticarcinogenic efficacy of phytic acid extracted from rice bran on azoxymethane-induced colon carcinogenesis in rats." Experimental and Toxicologic Pathology **62**(3): 259-268.

Oka, T., M. Fujimoto, R. Nagasaka, H. Ushio, M. Hori and H. Ozaki (2010). "Cycloartenyl ferulate, a component of rice bran oil-derived gamma-oryzanol, attenuates mast cell degranulation." Phytomedicine **17**(2): 152-156.

Qureshi, A. A., S. A. Sami and F. A. Khan (2002). "Effects of stabilized rice bran, its soluble and fiber fractions on blood glucose levels and serum lipid parameters in humans with diabetes mellitus Types I and II." J Nutr Biochem **13**(3): 175-187.

Ryan, E. P. (2011). "Bioactive food components and health properties of rice bran." J Am Vet Med Assoc **238**(5): 593-600.

Sierra, S., F. Lara-Villoslada, M. Olivares, J. Jimenez, J. Boza and J. Xaus (2005). "Increased immune response in mice consuming rice bran oil." European Journal of Nutrition **44**(8): 509-516.

Tamura, M., S. Hori, C. Hoshi and H. Nakagawa (2012). "Effects of rice bran oil on the intestinal microbiota and metabolism of isoflavones in adult mice." Int J Mol Sci **13**(8): 10336-10349

Trautwein, E. A., C. Schulz, D. Rieckhoff, A. Kunath-Rau, H. F. Erbersdobler, W. A. de Groot and G. W. Meijer (2002). "Effect of esterified 4-desmethylsterols and -stanols or 4,4'-dimethylsterols on cholesterol and bile acid metabolism in hamsters." Br J Nutr **87**(3): 227-237.

Verschoyle, R. D., P. Greaves, H. Cai, R. E. Edwards, W. P. Steward and A. J. Gescher (2007). "Evaluation of the cancer chemopreventive efficacy of rice bran in genetic mouse models of breast, prostate and intestinal carcinogenesis." British Journal of Cancer **96**(2): 248-254.

Zavoshy, R., M. Noroozi and H. Jahanihashemi (2012). "Effect of low calorie diet with rice bran oil on cardiovascular risk factors in hyperlipidemic patients." J Res Med Sci **17**(7): 626-631.

CHAPTER 1

INTRODUCTION TO DIETARY STUDIES IN *SALMONELLA* INFECTIONS ACROSS SPECIES

1.1 Introduction

Despite increased awareness and development of treatments such as antimicrobials and antibiotics, *Salmonella* contributes high economic losses in public health worldwide (Herrick, Buchberger et al. 2012). *Salmonella* are Gram- negative bacteria and the genera is composed of more than 2200 *Salmonella* strains, with about 200 of these being pathogenic. *Salmonella enterica* and *enteritidis* are commonplace and cause non-typhoidal and typhoidal salmonellosis respectively. Food systems and human population's eating habits have changed during the last few decades and may contribute to the incidence of *Salmonella* infections (Cohen 2000, Kearney 2010). *Salmonella* induces acute inflammation in the gut after infection and takes advantage of that inflammation to thrive and colonize in the gut (Stecher, Robbiani et al. 2007, Winter, Thiennimitr et al. 2010, Thiennimitr, Winter et al. 2011). Colonization resistance against *Salmonella* is modulated by gut microflora, intestinal immunity, epithelium and the quality and quantity of digestive fluids. Foods have been shown to modulate these factors and could be a potential intervention to prevent enteric infections.

Over the past 20 years, the role of the dietary agents in shaping the immunity against enteric infections has become increasingly evident (Chandra 1996, Calder and Kew 2002, Harrison, Balan et al. 2013, Hekmatdoost, Wu et al. 2013, Taylor, Cao et al. 2013) and piqued the interest in nutritional interventions for enteric infections. Several types of dietary components ranging from polyphenols, fibers, micronutrients, fatty acids, peptides and carbohydrates of plant

and animal origin are effective against *Salmonella*, *Listeria*, *E. coli* and viruses in various experimental models (Dhaliwal, Shawa et al. 2010, He, Wang et al. 2011, Long, Santos et al. 2011, Daglia 2012, Agerberth, Bergman et al. 2013, Hung, Garner et al. 2013, Roberts, Keita et al. 2013). Dietary fatty acids, amino acids and polyphenols enhance the epithelial barrier, immune response and gut microbiota to up-regulate resistance against infection (Chandra 1996, Ramalingam, Wang et al. 2010, Kau, Ahern et al. 2011, Ulluwishewa, Anderson et al. 2011). Dietary interventions have considerable advantages over synthetic antimicrobials and antibiotics in terms of drug resistance and side effects. However, this is a very complex and dynamic topic as it includes interactions among the diet, gut epithelium, digestive system, immune system and gut microbiota. In this chapter we will review only the *Salmonella* infection studies to understand the effects of dietary agents, the mechanisms of actions and the types of animal models employed.

Dietary interventions can have implications in *Salmonella* infection across species because *Salmonella* is not only a problem in humans but also in food animals. Many of the recent human *Salmonella* infections are a result of shedding by food animals such as chickens, pork and beef (Chai, White et al. 2012, Arguello, Alvarez-Ordonez et al. 2013). Apart from rodents, food animals such as calves and pigs have also been used as a research models for human typhoidal and non-typhoidal *Salmonella* infections (Tsolis, Kingsley et al. 1999, Metcalf, Almond et al. 2000, Santos, Zhang et al. 2001). Despite the use of these animals as a model for human *Salmonella* infection, there are dissimilarities between human and animals that are important to consider. Hence, this chapter will discuss dietary studies and *Salmonella* infections in species that have relevance to humans, and their strengths and weaknesses will be described. The relationships between dietary components and *Salmonella* infections are presented in Figure 1.1.

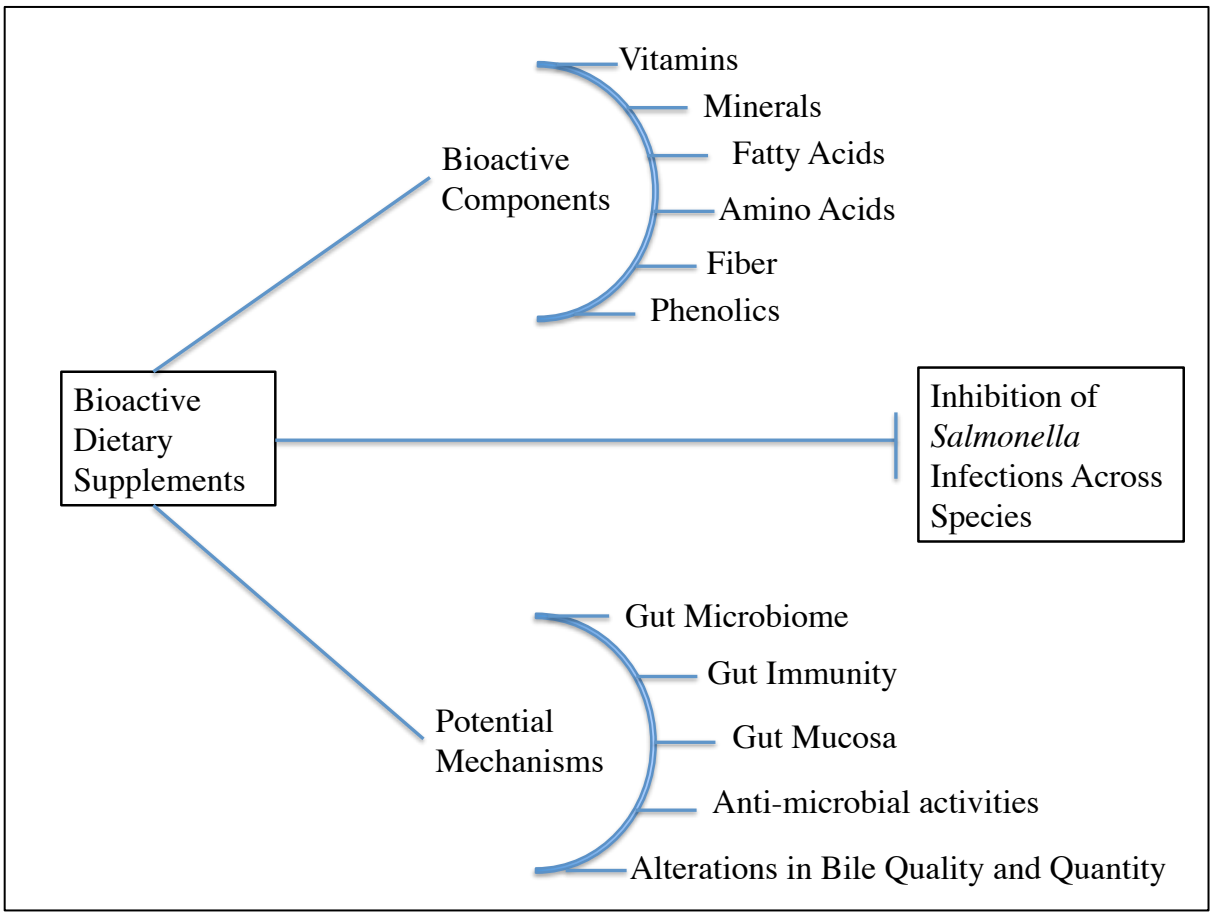


Figure 1.1 Relationships between dietary bioactive components and *Salmonella* infections. Dietary bioactive components such as fiber, amino acids, vitamins and minerals, fatty acids and polyphenols improve the gut epithelium, microbiota and immunity that may eventually lead to increased resistance to *Salmonella* infections.

1.2 Effect of dietary components on *Salmonella* infections across species

Dietary interventions for *Salmonella* infection in humans

A variety of *Salmonella* serotypes can infect humans. Epidemiological studies have shown that typhoidal and non-typhiodal salmonellosis are the predominant types of infections (Sanchez-Vargas, Abu-El-Haija et al. 2011). Typhoidal salmonellosis is caused by *S. typhi* and *paratyphi*.

Table 1.1 Effect of dietary interventions on *Salmonella* infections in humans

***Salmonella* Strains**	**Model**	**Dietary agents**	**Response**	**References**
Salmonella spp.	Children	Gluten free diet	Increased fecal *Salmonella*	(Di Cagno, De Angelis et al. 2011)
S. cholerasusis spp. cholerasuis	Children	Dairy products with special *Lactobacillus* strains	Decreased cytotoxicity of fecal samples, decreased *Salmonella* adhesion to intestinal mucin	(Lara-Villoslada, Sierra et al. 2007)
Non specific persistent diarrhea	Children	Green banana and pectin	Decreased stool frequency and vomiting	(Rabbani, Teka et al. 2001)
Salmonella spp.	Children	Lactic acid fermented cereal gruel	Decreased enteropathogenic colonization	(Kingamkono, Sjogren et al. 1999)

The disease is clinically presented by headache, diarrhea, constipation, abdominal pain, fever, chills, loss of appetite and fever with an incubation time of 7-20 days (Connor and Schwartz 2005). Typhoidal salmonellosis is unlikely to occur in the United States as compared to other developing countries (Sanchez-Vargas, Abu-El-Haija et al. 2011).

However, non- typhoidal salmonellosis presents a challenge for public health authorities. Several outbreaks have caused panic among the food safety authorities, as main causes come from both plant and animal origin. Non-typhoidal salmonellosis is presented by diarrhea, vomiting, dehydration and gastro-enteritis with an incubation period of 6-20 hours in humans (Hohmann 2001). These symptoms disappear in one week without treatment but can lead to the serious complications in immune-compromised subjects if left untreated (Hohmann 2001). Antimicrobial therapy is the first choice of treatment in persistent human salmonellosis. However, the problem of drug resistance emerged due to continuous use and misuse of antimicrobials (Glenn, Lindsey et al. 2013). Hence, dietary prophylactic studies are further required to fight with *Salmonella* infections. A few dietary prophylactic studies have been conducted in children for prevention of *Salmonella* infections (Table 1.1). Stool frequency, vomiting, *Salmonella* fecal shedding are the parameters measured in clinical trials that were mainly conducted in children. Several other disease conditions also affect the incidence of *Salmonella* infections. Di Cagno et al., revealed that administration of gluten free diets in children with celiac disease did not reduce *Salmonella* shedding from stool as compared to healthy children (Di Cagno, De Angelis et al. 2011). Other dietary interventions are effective in reducing *Salmonella* infection. Lara et al., showed that feeding of dairy products containing probiotic mixtures of various strains of *Lactobacillus* to healthy children for six weeks, decreased *Salmonella cholerasusis* adhesion to the intestinal mucin (Lara-Villoslada, Sierra et al.

2007). Dietary interventions can also reduce frequency of stool and vomiting in *Salmonella* infected children. Rabbani et al., revealed that feeding of cooked raw banana for one week in children having persistent diarrhea, reduced frequency of the stool and vomiting as compared to the children fed only the rice diet (Rabbani, Teka et al. 2001). In another clinical trial in children, fermented food (lactic-acid fermented cereal gruel) was fed to healthy children 3 times a day for 2 weeks. After 2 weeks of feeding, stool swabs were taken from the treated and non-treated groups and analyzed for the presence of enteropathogenic bacteria including *Salmonella*. The fermented food reduced the presence of enteropathogenic bacteria as compared to the control diet (Kingamkono, Sjogren et al. 1999). These studies show that dietary interventions can be effective in the management of diarrheal diseases. However, there are several constraints in conducting dietary studies in *Salmonella* infections in humans that are prophylactic in nature. The major issues in conducting human clinical trials are time, cost, availability of appropriate stool and serum biomarkers and overall patient compliance and ethics. Hence, animal models are required to assess the efficacy and mechanisms for dietary interventions to protect against *Salmonella* infections.

Effect of dietary components in rodents for *Salmonella* infections

The effects of dietary interventions have been studied in rodent models of *Salmonella* infection (Table 1.2). *Salmonella* fecal shedding, *Salmonella* tissue colonization, local and systemic inflammatory changes, survival and weight reduction are the major observable changes associated with *Salmonella* infections in rodents. Bovee et al., revealed reduced *Salmonella* fecal shedding when fructooligosaccharides-were fed to the male Wistar rats as compared to the cellulose fed group after 2 weeks of dietary intervention (Bovee-Oudenhoven, ten Bruggencate et al. 2003). Furthermore, dietary fructooligosaccharides

increased fecal *Lactobacilli* count and increased the translocation of *Salmonella* to the liver and spleen with an increase in the fecal mucin as compared to the cellulose fed rats (Harrison, Balan et al. 2013). The author concluded that dietary fructooligosaccharides decreased the *Salmonella* colonization but increased the translocation potentially due to the irritation of the mucosal membrane. Some of the mice strains succumb easily to *Salmonella* infection and hence survival rate is the primary indicator of the dietary efficacy against infection. Hitchins et al., showed that feeding of freeze dried yoghurt to the male weanling Sprague-Dawley rats increased the overall survival rate and weight of the animals after intraperitoneal *Salmonella* challenge as compared to the rats fed on milk diet for one week (Hitchins, Wells et al. 1985). Similarly, dietary feeding of Herba Pogostemonis Extract to Balb/c mice increased the overall survival rate as compared to the control diet fed animals after intraperitoneal *Salmonella* challenge (Kim, Moon et al. 2012). Feeding of Herba Pogostemonis (*Pogostemon cablin* Bantham extract) also reduced *Salmonella* liver damage as compared to the control diet fed animals (Kim, Moon et al. 2012). These studies show that there are measurable markers for *Salmonella* infections in the rodents and they can be used as a model to mimic the *Salmonella* infections in people.

Although several characteristics of the immune system are similar in human and rodents, many striking immune differences also exist. For instance, the human blood is composed of 50-70% neutrophils while mouse blood contains 10-25% neutrophils. Neutrophils are an important source for defensins in humans where as mice neutrophils don't express defensins. In mice, the crypt paneth cells express more than 20 defensins while in humans they express only 2 defensins (Mestas and Hughes 2004, Gibbons and Spencer 2011). Defensins from the paneth cells function in different ways. Neutrophil defensins are effective in killing engulfed bacteria by making the intracellular phagolysozome (Cunliffe 2003).

Both food borne pathogens and dietary components pass through the stomach acid, when ingested. Hence, gastric acidity is one of the important factors in determining stability of enteric pathogens. In a randomized controlled clinical trial, gastric hypochlorhydria (low hydrochloric acid) was found to be associated with increased *Salmonella* infections (Kelly, Shawa et al. 2010). This hypothesis was also confirmed in the rodent model of *Salmonella* infection. Tennant et al., (Tennant, Hartland et al. 2008) showed that treatment of mice with antacids, resulted in the decreased infectious dose of *Salmonella* as compared to the normal mice.

Table 1.2 Effect of dietary intervention on *Salmonella* infections in rodents

***Salmonella* Strains**	**Model**	**Dietary agents**	**Response**	**References**
S. enteritidis	Rat	Freeze dried yoghurt	Decreased mortality and weight gain deceleration	(Hitchins, Wells et al. 1985)
S. enteritidis	Rat	Ovine serum immunoglobulin	Increased goblet cell count, luminal mucin, organ weight, and functional gut morphology	(Balan, Han et al. 2011)

S. enteritidis	Rat	Fructooligosaccharides and inulin	Decreased colonization but increased invasion	(Bovee-Oudenhoven, ten Bruggencate et al. 2003)
S. typhimurium	Mice	Rice hull smoke extract	Decreased mortality and liver pathology	(Kim, Kang et al. 2012)
S. typhimurium	Mice	Camel milk	Improved survival rate	(Cardoso, Ponte et al. 2013)
S. typhimurium	Mice	*Pogostemon cablin* leaf extract	Extended life span of animals	(Kim, Moon et al. 2012)
S. typhimurium	Mice	Zinc	Decreased bacterial load and inflammation in liver	(Rishi, Kaur et al. 2008)
S. typhimurium	Mice	White button mushroom	Higher survival rate	(Wang, Niu et al. 2013)

Similar results were also observed in a constitutively hypochlorhydric mice (proton pump mutation) as compared to the normal mice (Tennant, Hartland et al. 2008). Also, gastric pH not only affects the survival of pathogens but also affects the digestion and absorption of foods. Lucas et al., showed that an increase in pH from 1.5 to 2.5, reduced digestion of the kiwifruit peptides (Lucas, Cochrane et al. 2008). Gastric pH also modulates the absorption of

micronutrients such as zinc. Henderson et al., observed higher plasma zinc levels in the young healthy volunteers at low pH as compared to the plasma level in higher gastric pH volunteers (Henderson, Brewer et al. 1995). The gastric pH is considerably different across the species. For instance, the mean gastric pH in mice is 3.1 - 4.5 and in rats it is 3.2 - 3.9, whereas in human it is 1.5 - 3.5. In addition to the gastric pH, intestinal pH is also different in rodents as compared to humans. Mice and rats have a mean intestinal pH of 5.2 and 6.6 respectively as compared to 7.2 in humans (Kararli 1995). These studies show that gastric and intestinal pH could potentially affect the bioactivity of dietary components and should be considered as one of the important factors in selecting a study model.

In rodents and humans, several disease symptoms can be confounding due to the differences in their anatomy and physiology. For example, in the non-typhoidal salmonellosis, vomiting and diarrhea are the main symptoms in humans. However, anatomically mice cannot vomit and due to this reason, the assessment of diarrhea could be very difficult in mice. In these cases it becomes harder to translate the finding into clinical applications. Hence, these limitations of rodent models should be taken into account while interpreting the results from the dietary intervention studies for *Salmonella* infections in the rodent models for human clinical trials.

Dietary studies in calves for *Salmonella* infection

Calves are considered one of the best models to mimic the human non-typhoidal salmonellosis (Santos, Zhang et al. 2001). After *Salmonella* infection, the calves show similar clinical symptoms as humans such as fever, diarrhea, anorexia and dehydration and the intestinal pathological changes (Santos, Zhang et al. 2001, Costa, Paixao et al. 2012). Hill et al., revealed that feeding of a commercially available blend of butyric acid, coconut oil, and flax oil to the male Holstein calves for 28 days altered the inflammatory response to intraperitoneal *Salmonella*

toxoid as compared to the control group (Hill, Vandehaar et al. 2011). The dietary blend reduced the hyperthermia, hypophagia and serum TNF- α but increased the IL-4 as compared to the control group (Hill, Vandehaar et al. 2011). However, studies in animal models of human *Salmonella* infections with dietary interventions not only should be aligned with the infection pathology but also should be parallel in digestive system. Calves exhibit significant anatomical and physiological differences in the digestive system as compared to humans. A ruminant's stomach is four chambered and contains a large number of microflora that digest fibers, especially cellulose which remain undigested in humans. Sugars are fermented in the ruminant stomach and as a result several volatile fatty acids are produced (Hofmann 1989). Most of the carbohydrates are converted into volatile fatty acids and a very small proportion of carbohydrates are absorbed as glucose. Also, the ruminant microflora differs from the human gut microflora to a great extent (Li, Connor et al. 2012). Hence, the same dietary components may produce different metabolites and physiological effects as compared to humans. Logistically, calves need a large amount of food and it is very expensive to conduct the dietary experimental studies in this model.

Effect of dietary components on *Salmonella* infection in pigs

Pigs have been used in several studies involving dietary interventions (Guilloteau, Zabielski et al. 2010). Pigs have many more similarities to human gastrointestinal tract as compared to rodents. Humans and pigs are similar in the body composition, cardiovascular, renal, nutritional, immunological, metabolic and gastro-intestinal aspects (Guilloteau, Zabielski et al. 2010). Having so many similarities, pigs have been used as a laboratory model for *Salmonella* infections. Several studies have been conducted in the pig model of *Salmonella* infection with dietary interventions (Table 1.3). Michiels et al., demonstrated that

supplementation of a mixture of formic, sorbic and benzoic acid to the piglets for 35 days significantly reduced the *Salmonella* fecal shedding as compared to the control group after oral challenge (Michiels, Missotten et al. 2012). Dietary organic acids increase the fecal cytotoxicity to *Salmonella* but the effect can be dependent upon the environmental temperature. Rajtak et al., revealed that supplementation of a pig diet with organic acid (Potassium- diformate) reduced the survival of *Salmonella* in pig feces when incubated at 22° C but not at 4° C (Rajtak, Boland et al. 2012). Boyen et al., fed the supplemented diet with the coated butyric acid (2 g/kg of diet) to the pigs for 12 days and orally challenged the animals with *Salmonella* (Boyen, Haesebrouck et al. 2008). Fecal shedding of *Salmonella* was decreased in the coated butyric acids fed animals as compared to the un-coated. It was hypothesized that coating prevents the degradation of fatty acids in the intestinal tract (Boyen, Haesebrouck et al. 2008). Dietary supplements also reduce the inflammation after *Salmonella* infection in pigs. Chen et al., supplemented the pig diet with arginine (0.5 %) for one week and infected the pigs intramuscularly with *Salmonella* (Chen, Chen et al. 2012). Fecal *Salmonella* shedding is one of the distinctive biomarker of *Salmonella* infection in pig models. However, *Salmonella* colonization patterns are different in pigs as compared to humans. For instance, *Salmonella* typhimurium has been observed to colonize in the tonsils and respiratory tissues of infected pigs (Boyen, Haesebrouck et al. 2008), where as in humans it does not colonize at these sites. The pig stomach is 2-3 times larger compared to humans (Kararli 1995). This anatomical difference may have impacts on the *Salmonella* survival and digestibility of dietary components. Next the pig cecum is several fold larger than the human cecum and may have implications in the *Salmonella* colonization (Kararli 1995). In humans, stomach pH before eating is around 5 however in pigs it is below 2. Consequently, pigs release much greater extent of bile in the duodenum as compared to humans. Bile is known for its

antimicrobial activities and it impacts colonization of *Salmonella* in the proximal small intestine. Also, it can modulate the digestion and absorption of dietary components. Besides these differences, pigs are different in gastrointestinal thickness of mucins, GI motility and transit as compared to humans. The distal small intestine of pigs contains a larger number of microbes as compared to humans and can degrade some carbohydrates with low digestibility compared to humans (Eberhard, Hennig et al. 2007). Hence, similar dietary interventions in pigs and humans may exhibit different potential. The pig immune system also differs from humans however; implications of this difference have not been studied in regard to enteric infections. For instance, the gut of neonate piglets completely lacks leukocytes where as human infants consist of a few number of leukocytes at birth (Scharek and Tedin 2007). Pig intestine contains a larger number of Peyer's patches as compared to humans throughout the intestine (Scharek and Tedin 2007). This could have implications on *Salmonella* colonization and more studies are needed to establish this relationship.

Table 1.3 Effect of dietary intervention on *Salmonella* infections in pigs

***Salmonella* Strains**	**Model**	**Dietary agents**	**Response**	**References**
S. enterica serovar *Choleraesuis* C500	Weaning piglets	Arginine	Decreased Serum CRP, Myd 88, TLR4,5, NFkB & TNF-alpha	(Chen, Chen et al. 2012)
Salmonella spp.	Weaning	Bamboo charcoal	Decreased fecal *Salmonella*,	(Chu, Jung et

	piglets	and vinegar	Increased fecal IgM and *Lactobacillus*	al. 2013)
S. typhimurium	Piglets	Short chain fatty acids	Decreased *Salmonella* fecal shedding and decreased *Salmonella* in cecal content	(Michiels, Missotten et al. 2012)
S. typhimurium var. *Copenhagen*	Pigs	Barley	Decreased *Salmonella* in intestinal content, lymph nodes	(Pieper, Bindelle et al. 2012)
S. typhimurium	Pigs	Organic acids (effect of environmental temp on effectiveness)	Increased fecal toxicity to *Salmonella* at 22° C but not at 4° C	(Rajtak, Boland et al. 2012)
S. typhimurium	Weaned pigs	Wheat distillers dried grains with soluble or sugar beet pulp	Increased *Lactobacillus* but no effect on *Salmonella*	(Thomson, Pieper et al. 2012)
S. typhimurium	Pigs	Coated short chain fatty acid	Decreased fecal shedding and intestinal colonization	(Boyen, Haesebrouck et al. 2008)

Effect of dietary components against *Salmonella* in in-vitro models

Dietary components may prevent the infection outcome either by suppressing the growth and virulence by direct action on pathogens or by modulating host response to the pathogens. To test the direct effects of dietary components on pathogens, researchers have used food extracts or dietary bioactive components on *Salmonella* cultures. Following the treatment of dietary components, growth, gene expression for virulence and motility are measured (Table 1.4). This model may not reveal the in-vivo mechanisms of action however; at preliminary levels of investigation these models are very useful. It is comparatively less expensive and less cumbersome model to assess the efficacy of dietary components for *Salmonella* infection. For instance, several essential oils were added to *Salmonella* growth media at various doses and *Salmonella* growth was compared with untreated animals (Oussalah, Caillet et al. 2007). Citrus flavonoids were evaluated on *Salmonella* virulence gene expression and motility by using this assay (Vikram, Jesudhasan et al. 2011).

Orally infected *Salmonella* can enter into the circulation through various routes. It can invade several phagocytic and non-phagocytic cells depending upon the serotype. In the murine model, *Salmonella* invades both phagocytic and epithelial non-phagocytic cell types. Hence, in-vitro models of *Salmonella* entry have been developed to assess the effect of test compound on the host. The *Salmonella* entry model could reveal the mechanism of action of the test compound on the organism. However, in-vivo studies are warranted to further verify the claims of in-vitro studies as in-vivo processing of testing compounds can lead to different results. Several human and mouse cell lines such as Caco-2 (Chen, Tsen et al. 2013) and RAW264.7 have been used in the literature to test the efficacy of the compounds against *Salmonella* entry. For example,

secretory immunoglobulin A (SIgA) was tested for inhibition of *Salmonella* entry into polarized monolayers of HeLa cells (Forbes, Eschmann et al. 2008).

Table 1.4 Effect of dietary intervention on *Salmonella* infections in in-vitro

***Salmonella* Strains**	**Model**	**Dietary agents**	**Response**	**References**
S. enterica	Cell free	Essential oil from thyme, oregano, cinnamon, clove bud, allspice, bay leaf, palmarosa, and marjoram oils	50% reduction in *Salmonella* CFU	(Friedman, Henika et al. 2002)
S. enterica sv. *typhimurium* VTT E-981151	Cell free	Phenolic extract from berries	Decreased *Salmonella* growth	(Puupponen-Pimiä, Nohynek et al. 2005)
S. indiana	In-Vitro Disc diffusion	Rosemary and Oregano essential oil	Increased antibacterial activity	(Mathlouthi, Bouzaienne et al. 2012)
S. typhimurium	HeLa Cells	Cranberry anthocynidins	Decreased *Salmonella* entry into the cells	(Harmidy, Tufenkji et al. 2011)
S. typhimurium	U937 Cells & Monocytes	Resveratrol and quercetin	Decreased nitric oxide, apoptosis and entry	(Paolillo, Carratelli et al. 2011)

Salmonella contains pathogenicity islands for the secretion of effector molecules to infect the target cells (Hansen-Wester and Hensel 2001). The molecules released by these secretory systems change the host cell cytoskeleton to facilitate *Salmonella* entry. The in-vitro *Salmonella* entry models are useful in studying the effects of dietary components on *Salmonella* as well as on host cells simultaneously. Dietary components could affect *Salmonella* virulence by affecting secretory systems or by competing with *Salmonella* for the receptors on host cells (Vikram, Jesudhasan et al. 2011). Host cells can also release the cytokines in response to the dietary components that can affect *Salmonella* virulence or motility. Therefore in-vitro models of *Salmonella* infection can have great implications for assessing mechanisms of actions by the dietary components.

1.3 Potential mechanisms by which dietary components protect from *Salmonella* infections

Protection from *Salmonella* involving intestinal epithelium

After crossing the mucin layer in the gut, enteric pathogens need to penetrate the epithelial layer in order to infect the organism. Human gut epithelia consists of a monolayer of epithelial cells. It separates the gut lumen from the lamina- propria. Intestinal epithelial cellular junctions affect the intestinal permeability as well as the transcytosis capacity of individual cells. Strong cellular junctions are necessary to avoid the invasion of pathogens through the epithelium. *Salmonella* can breach the epithelial barrier by employing the para-cellular and trans-cellular mechanisms, including the actin cytoskeleton of epithelial cells and secretion of the effector molecules (Ulluwishewa, Anderson et al. 2011).

Dietary components have been shown to modulate the epithelial barrier (Kosińska and Andlauer 2013). Diet can have both positive and negative impacts on epithelial integrity. Liu et al., showed that when high grain diet was fed to the male goats, it resulted in the disruption of the

ruminal epithelium as measured by the presence of systemic lipopolysacharide (LPS)(Liu, Xu et al. 2013). However, diet can also impact epithelial integrity positively. In a study by Nofrarias et al., pigs were fed resistant starch for 97 days and consequently increased hypertrophy, reduced apoptosis in the crypts, lymphoid nodules in the colon and increased mucin sulfuration were observed. These changes promoted the epithelial protection compared to the control dietary group containing digestible starch (Nofrarias, Martinez-Puig et al. 2007). Dietary components can also modulate the epithelial proteins such as occludins that secure junctions between the adjacent cells in the gut epithelium. Enteric pathogenic bacteria secrete LPS that causes inflammation and escalates the loss of protein occludin that decreases the barrier function of epithelium. Park et al., showed in the rats that dietary administration of gangliosides (a type of lipids) prevents LPS induced degradation of the occludin and reduces the total nitric oxide in the gut mucosa (Park, Thomson et al. 2010). An in vitro study with Caco-2 cells demonstrated that addition of quercetin (a flavonoid) induces expression of zonula occludens-2, occludin, and claudin-1 and the claudin-4 as compared to the control group (Suzuki and Hara 2009). All of these proteins play an important part in maintaining the epithelial integrity. *Salmonella* entry into the epithelial cells can result in the epithelial necrosis and apoptosis. Int 407 cell line (human intestinal cell line) showed significantly lesser extent of necrosis and apoptosis during *Salmonella* infection when treated with sterols and fatty acids found in the root extract of *Hemidusmus indicus* as compared to the untreated cell line (Das and Devaraj 2007). Hence, protection of the epithelium can be considered an important target of the dietary interventions in *Salmonella* infections.

Gut microbiome mediated protections against *Salmonella* infections

The gut contains more than a trillion symbiotic bacteria that play a major role in developing immunity as well as resistance against the enteric infections. Initially it was hypothesized that the genetic factors were responsible for the susceptible and resistant mouse strains against the enteric infections and we now appreciate that the genetic factors are only one of the determinants of the composition and structure of the gut microflora. However, Willing et al., successfully transferred the microbiota from resistant to susceptible mice and observed a delayed colonization of *Citrobacter rodentium* and mortality in the susceptible strain (Willing, Vacharaksa et al. 2011). Again in the same study, the native gut microbiota of resistant mice was depleted by the oral streptomycin (20 mg) 24 hours prior to transplantation and replaced by the microbiota from susceptible mice. As a result, the oral antibiotic treatment reduced the innate defenses and a sever infection pathology was observed as compared to mice in the control group. This experiment demonstrates that the gut microbiota plays an important role in fighting infection (Willing, Vacharaksa et al. 2011). Similarly, mice were given a combination of antibiotics (Streptomycin, Vancomycin, Ampicillin, Neomycin and Metronidazole) for one week in drinking water and later orally challenged with *Salmonella* Typhimurium 14028. The mice on the antibiotics showed a significantly higher number of *Salmonella* DNA in the cecum and the large intestine as compared to the control mice group (Croswell, Amir et al. 2009). The gut microbiota may affect enteric infections by modulating the intestinal immunity or by the direct competition. The symbiotic gut microbiota competes with pathogens for the nutrients such as iron and the carbon sources (Kamada, Chen et al. 2013). Stelter et al., showed that the *Salmonella* induced mucosal lactins, kills the symbiotic gut microflora and then *Salmonella* takes advantage of this process for survival in the GI tract (Stelter, Kappeli et al. 2011). *Salmonella* induces acute inflammation in mice and the neutrophils are recruited at the site of infection. Gill

et al., showed that neutrophil elastases can shift the mice gut microbiota and increase the *Salmonella* colonization, while neutralization of the neutrophil elastases decrease the colonization of *Salmonella* (Gill, Ferreira et al. 2012). These studies show that the gut microbiota play an important role in protection from *Salmonella* infections and the modulation of the gut microflora for prevention of enteric infections warrants further studies.

Given the role of gut microbiota in the protection against *Salmonella*, several studies have been conducted to test the effects of dairy and native gut probiotics on *Salmonella* colonization. Probiotics are the microorganisms that induce the health benefits when consumed in effective doses. *Lactobacillus* and *Streptococcus* are the two widely studied categories of probiotics and their effectiveness against *Salmonella* is well reviewed by Castillo et al., (Castillo, de Moreno de LeBlanc et al. 2012). *Lactobacillus rhamnosus* has been shown to reduce *Salmonella* adhesion to the epithelial cells in the in vitro model of *Salmonella* infection (Burkholder and Bhunia 2009). Probiotics not only compete with *Salmonella* for nutrients but also enhance the protective immunity against it. Castillo et al., showed that oral administration of *Lactobacillus* in mice changes the cytokine production and TLR expression that is protective for the mice against *Salmonella* infection (Castillo, Perdigon et al. 2011). Moreover, probiotics such *Bifidobacterium*, can directly affect the virulence of *Salmonella* by releasing the molecules that down-regulate the expression of pathogenicity islands 1 and 2 (Bayoumi and Griffiths 2010). Hence, *Lactobacillus* and *Biofidobacterium* have emerged as the major players in the protection against enteric infections such as *Salmonella*.

Diet is a major factor in the establishment of gut microbiome. Data supports that a change of diet from low fat, high plant polysaccharide to the high fat, simple sugar diet, changed the structure of the gut microbiota very rapidly (McNulty, Wu et al. 2013). A shift of low fat diet to

the western diet also changed the metabolic pathways and modulates the gene expression in the gut microbiome (Turnbaugh, Ridaura et al. 2009). Humanized mice (mice transplanted with human gut microflora) when fed a western diet, showed an increased adiposity and this trait was transmissible through the transplantation of the gut microbiota in other mice (Turnbaugh, Ridaura et al. 2009). Diet can also modulate the gut microbiota directly by providing the prebiotics and several studies have shown the effect of prebiotics such as dietary fiber, fatty acids and polyphenols on the shift in the gut microflora (Laparra and Sanz 2010, Toward, Montandon et al. 2012). However, data on the protective effects of diet on *Salmonella* infections showing inclusion of native gut microbiota is scarce and need further studies.

Role of intestinal immunity in *Salmonella* infection and dietary modulation of intestinal immunity

The immune system of the GI tract is the largest segment of the mammalian immune system. The gut encounters massive amounts of pathogens and dietary antigens that need to be neutralized. These functions emphasize the importance of the gut immune system. The mucosal immune system is equipped with the innate and adaptive immune defense mechanisms. Innate immunity provides the first line of defense against pathogens. The major players of the innate immune defense are macrophages, monocytes, neutrophils, epithelial cells, Natural Killer (NK) cells and dendritic cells (DCs) (Yuan and Walker 2004). Dendritic cells, macrophages and epithelial cells are also termed as antigen presenting cells (APCs) because of their capacity of processing and presenting the foreign antigens to other cells. APCs have a series of receptors called Pattern Recognition Receptors (PRRs) on their surfaces such as Toll Like Receptors (TLRs) and Nod Like Receptors (NODs) to recognize the pathogens (Kawai and Akira 2010). These receptors recognize the motifs on pathogens known as Pathogen Associated Molecular

Patterns (PAMPs) (Abreu 2010). The innate immune cells release inflammatory cytokines and mediators after sensing the PAMPs (Gordon 2002). However, if the innate immunity fails to resolve the inflammation and eliminate pathogen, adaptive immunity enters this process. In the gut adaptive immune system, predominant response is antibody mediated and is represented by the Immunoglobulin A (IgA) (Macpherson, Mccoy et al. 2008). IgA is chiefly produced by the B cells in the intestinal mucosa triggered by the anti-inflammatory cytokines such as TGF-β and IL-10 (Fagarasan and Honjo 2003). Hence, both the innate and adaptive immune responses are required in protection against infection depends upon the type of pathogen.

The role of the gut immune system in the protection from enteric infections has been studied intensely (Jouanguy, Doffinger et al. 1999, Cross 2002, Nanton, Way et al. 2012). Primary *Salmonella* infection increases interferon gamma (IFN-γ), tumor necrosis factor alpha (TNF-α) and interleukin 12 (IL-12) in the circulation and in the local tissues (Arnold, Niesel et al. 1993, Jouanguy, Doffinger et al. 1999, Bao, Beagley et al. 2000). The major source of IFN-γ and TNF-α are neutrophils and macrophages (Kirby, Yrlid et al. 2002). IL-12 is a cytokine induced in response to several bacteria and mediates onset of Th1 protective response. Natural Killer T (NKT) cells produce IFN-γ in response to IL-12 (Mastroeni, Harrison et al. 1996). Infected macrophages also interact with NK cells in order to produce IFN-γ in humans (Lapaque, Walzer et al. 2009). Even though the initial innate immune response restricts the infection to a certain extent, it fails to inhibit the growth of pathogens in the deeper tissues. Hence, the immune response is switched to the adaptive response after some time and is achieved mainly by the induction of CD4+ T cells, CD8+ T cells and B cells (Mittrucker and Kaufmann 2000). In the experimental models, the depletion of CD4+ T cells had more pronounced effect on the protection from *Salmonella* as compared to the CD8+ T cells. However, the underlying

mechanisms are not clear. The second major adaptive response to *Salmonella* is the induction of B cells to produce antibodies such as IgA. The antibodies bind *Salmonella* and prevent the entry into the deeper tissues. Administration of B cell hybridoma producing *Salmonella* specific IgA has been shown to prevent oral *Salmonella* infection in the mice (Michetti, Mahan et al. 1992). These studies show the role of immunity in *Salmonella* infections and the dietary components could be investigated to modulate the immunity that is involved in *Salmonella* infections.

Dietary components such as the dietary fiber and prebiotics manipulate both the innate and adaptive immunity (Schley and Field 2002). Galdeino et al., demonstrated that the feeding of probiotic fermented milk to the rats increases the number of macrophages and DCs with an increase in IFN-γ, TNF- α and IL-12 after 5 days of nutrition (Galdeano, Nunez et al. 2011). Nutrients such as glutamine, arginine, vitamin A and zinc have protective impacts against enteric infections (Guerrant, Oria et al. 2008). Macrophages play an important role in the clearance of *Salmonella* in primary infections. Modified arabinoxylan rice bran improves the phagocytic function of macrophages in the in vitro models of RAW264.7 cells (Ghoneum and Matsuura 2004). Treatment of macrophages with the modified arabinoxylan rice bran, increased the attachment and phagocytosis of yeast cells with an increase in TNF-α and IL-6 (Ghoneum and Matsuura 2004). Wang et al., showed an enhanced *Salmonella* specific immune response in the orally vaccinated mice with attenuated *Salmonella* and fed with white button mushroom powder as compared to the only vaccinated mice (Wang, Niu et al. 2014). The white button mushroom fed mice had higher number of *Salmonella* specific fecal IgA, IFN- γ and TNF- α in the splenocytes. These mice also showed an increased number of DCs and activation marker CD40 in splenocytes as compared to the control mice (Wang, Niu et al. 2014). These studies show that dietary interventions could modulate the immune efficiency in *Salmonella* infections.

In conclusion, dietary components could have potent effects on prevention of *Salmonella* infections. These effects may involve various mechanisms such as the gut microbiota, immune system and epithelium. Since, the route of dietary components is oral, they are highly sensitive to changes in digestive physiology across species and could exert different clinical outcomes. Similarly, *Salmonella* is also a food and water borne pathogen and travels through the alimentary canal and sensitive to physiological changes across species. Moreover, animal species vary in their dietary requirements that could potentially alter the nutrient-nutrient interactions and eventually the absorption of bioactive components. Therefore selection of an appropriate model is an important aspect in the assessment of dietary components in *Salmonella* infections.

REFERENCES

Abreu, M. T. (2010). "Toll-like receptor signalling in the intestinal epithelium: how bacterial recognition shapes intestinal function." Nature Reviews Immunology **10**(2): 131-143.

Agerberth, B., P. Bergman and G. Gudmundsson (2013). Helping the Host: Induction of Antimicrobial Peptides as a Novel Therapeutic Strategy Against Infections. Antimicrobial Peptides and Innate Immunity. P. S. Hiemstra and S. A. J. Zaat, Springer Basel**:** 359-375.

Arguello, H., A. Alvarez-Ordonez, A. Carvajal, P. Rubio and M. Prieto (2013). "Role of Slaughtering in Salmonella Spreading and Control in Pork Production." Journal of Food Protection **76**(5): 899-911.

Arnold, J. W., D. W. Niesel, C. R. Annable, C. B. Hess, M. Asuncion, Y. J. Cho, J. W. Peterson and G. R. Klimpel (1993). "Tumor necrosis factor-alpha mediates the early pathology in Salmonella infection of the gastrointestinal tract." Microb Pathog **14**(3): 217-227.

Balan, P., K. S. Han, S. M. Rutherfurd, H. Singh and P. J. Moughan (2011). "Dietary supplementation with ovine serum immunoglobulin attenuates acute effects on growth, organ weights, gut morphology and intestinal mucin production in the growing rat challenged with Salmonella enteritidis." Animal **5**(10): 1570-1578.

Bao, S., K. W. Beagley, M. P. France, J. Shen and A. J. Husband (2000). "Interferon-gamma plays a critical role in intestinal immunity against Salmonella typhimurium infection." Immunology **99**(3): 464-472.

Bayoumi, M. A. and M. W. Griffiths (2010). "Probiotics down-regulate genes in Salmonella enterica serovar typhimurium pathogenicity islands 1 and 2." J Food Prot **73**(3): 452-460.

Bovee-Oudenhoven, I. M. J., S. J. M. ten Bruggencate, M. L. G. Lettink-Wissink and R. van der Meer (2003). Dietary fructo-oligosaccharides and lactulose inhibit intestinal colonisation but stimulate translocation of salmonella in rats. **52:** 1572-1578.

Boyen, F., E. Haesebrouck, D. Maes, F. Van Immerseel, R. Ducatelle and F. Pasmans (2008). "Non-typhoidal Salmonella infections in pigs: A closer look at epidemiology, pathogenesis and control." Vet Microbiol **130**(1-2): 1-19.

Boyen, F., F. Haesebrouck, A. Vanparys, J. Volf, M. Mahu, F. Van Immerseel, I. Rychlik, J. Dewulf, R. Ducatelle and F. Pasmans (2008). "Coated fatty acids alter virulence properties of Salmonella Typhimurium and decrease intestinal colonization of pigs." Vet Microbiol **132**(3-4): 319-327.

Burkholder, K. M. and A. K. Bhunia (2009). "Salmonella enterica serovar Typhimurium adhesion and cytotoxicity during epithelial cell stress is reduced by Lactobacillus rhamnosus GG." Gut Pathog **1**(1): 14.

Calder, P. C. and S. Kew (2002). "The immune system: a target for functional foods?" Br J Nutr **88 Suppl 2**: S165-177.

Cardoso, R. R. D., M. Ponte and V. Leite (2013). "Protective Action of Camel Milk in Mice Inoculated with Salmonella enterica." Israel Medical Association Journal **15**(1): 5-8.

Castillo, N. A., A. de Moreno de LeBlanc, C. M. Galdeano and G. Perdigón (2012). "Probiotics: An alternative strategy for combating salmonellosis: Immune mechanisms involved." Food Research International **45**(2): 831-841.

Castillo, N. A., G. Perdigon and A. de Moreno de Leblanc (2011). "Oral administration of a probiotic Lactobacillus modulates cytokine production and TLR expression improving the immune response against Salmonella enterica serovar Typhimurium infection in mice." BMC Microbiol **11**: 177.

Chai, S. J., P. L. White, S. L. Lathrop, S. M. Solghan, C. Medus, B. M. McGlinchey, M. Tobin-D'Angelo, R. Marcus and B. E. Mahon (2012). "Salmonella enterica Serotype Enteritidis: Increasing Incidence of Domestically Acquired Infections." Clinical Infectious Diseases **54**: S488-S497.

Chandra, R. K. (1996). "Nutrition, immunity and infection: from basic knowledge of dietary manipulation of immune responses to practical application of ameliorating suffering and improving survival." Proc Natl Acad Sci U S A **93**(25): 14304-14307.

Chen, C. Y., H. Y. Tsen, C. L. Lin, C. K. Lin, L. T. Chuang, C. S. Chen and Y. C. Chiang (2013). "Enhancement of the immune response against Salmonella infection of mice by heat-killed multispecies combinations of lactic acid bacteria." J Med Microbiol **62**(Pt 11): 1657-1664.

Chen, Y., D. Chen, G. Tian, J. He, X. Mao, Q. Mao and B. Yu (2012). "Dietary arginine supplementation alleviates immune challenge induced by Salmonella enterica serovar

Choleraesuis bacterin potentially through the Toll-like receptor 4-myeloid differentiation factor 88 signalling pathway in weaned piglets." Br J Nutr **108**(6): 1069-1076.

Chu, G. M., C. K. Jung, H. Y. Kim, J. H. Ha, J. H. Kim, M. S. Jung, S. J. Lee, Y. Song, R. I. Ibrahim, J. H. Cho, S. S. Lee and Y. M. Song (2013). "Effects of bamboo charcoal and bamboo vinegar as antibiotic alternatives on growth performance, immune responses and fecal microflora population in fattening pigs." Anim Sci J **84**(2): 113-120.

Cohen, M. L. (2000). "Changing patterns of infectious disease." Nature **406**(6797): 762-767.

Connor, B. A. and E. Schwartz (2005). "Typhoid and paratyphoid fever in travellers." Lancet Infect Dis **5**(10): 623-628.

Costa, L. F., T. A. Paixao, R. M. Tsolis, A. J. Baumler and R. L. Santos (2012). "Salmonellosis in cattle: advantages of being an experimental model." Res Vet Sci **93**(1): 1-6.

Cross, M. L. (2002). "Microbes versus microbes: immune signals generated by probiotic lactobacilli and their role in protection against microbial pathogens." Fems Immunology and Medical Microbiology **34**(4): 245-253.

Croswell, A., E. Amir, P. Teggatz, M. Barman and N. H. Salzman (2009). "Prolonged impact of antibiotics on intestinal microbial ecology and susceptibility to enteric Salmonella infection." Infect Immun **77**(7): 2741-2753.

Cunliffe, R. N. (2003). "Alpha-defensins in the gastrointestinal tract." Mol Immunol **40**(7): 463-467.

Daglia, M. (2012). "Polyphenols as antimicrobial agents." Curr Opin Biotechnol **23**(2): 174-181.

Das, S. and S. N. Devaraj (2007). "Protective role of Hemidesmus indicus R. Br. root extract against Salmonella typhimurium-induced cytotoxicity in Int 407 cell line." Phytotherapy Research **21**(12): 1209-1216.

Dhaliwal, W., T. Shawa, M. Khanam, P. Jagatiya, M. Simuyandi, N. Ndulo, C. L. Bevins, I. R. Sanderson and P. Kelly (2010). "Intestinal antimicrobial gene expression: impact of micronutrients in malnourished adults during a randomized trial." J Infect Dis **202**(6): 971-978.

Di Cagno, R., M. De Angelis, I. De Pasquale, M. Ndagijimana, P. Vernocchi, P. Ricciuti, F. Gagliardi, L. Laghi, C. Crecchio, M. E. Guerzoni, M. Gobbetti and R. Francavilla (2011). "Duodenal and faecal microbiota of celiac children: molecular, phenotype and metabolome characterization." BMC Microbiol **11**: 219.

Eberhard, M., U. Hennig, S. Kuhla, R. M. Brunner, B. Kleessen and C. C. Metges (2007). "Effect of inulin supplementation on selected gastric, duodenal, and caecal microbiota and short chain fatty acid pattern in growing piglets." Arch Anim Nutr **61**(4): 235-246.

Fagarasan, S. and T. Honjo (2003). "Intestinal IgA synthesis: regulation of front-line body defences." Nature Reviews Immunology **3**(1): 63-72.

Forbes, S. J., M. Eschmann and N. J. Mantis (2008). "Inhibition of Salmonella enterica serovar Typhimurium motility and entry into epithelial cells by a protective antilipopolysaccharide monoclonal immunoglobulin a antibody." Infect Immun **76**(9): 4137-4144.

Friedman, M., P. R. Henika and R. E. Mandrell (2002). "Bactericidal Activities of Plant Essential Oils and Some of Their Isolated Constituents against Campylobacter jejuni, Escherichia coli, Listeria monocytogenes, and Salmonella enterica." Journal of Food Protection **65**(10): 1545-1560.

Galdeano, C. M., I. N. Nunez, A. D. de LeBlanc, E. Carmuega, R. Weill and G. Perdigon (2011). "Impact of a probiotic fermented milk in the gut ecosystem and in the systemic immunity using a non-severe protein-energy-malnutrition model in mice." Bmc Gastroenterology **11**.

Ghoneum, M. and M. Matsuura (2004). "Augmentation of macrophage phagocytosis by modified arabinoxylan rice bran (MGN-3/biobran)." Int J Immunopathol Pharmacol **17**(3): 283-292.

Gibbons, D. L. and J. Spencer (2011). "Mouse and human intestinal immunity: same ballpark, different players; different rules, same score." Mucosal Immunol **4**(2): 148-157.

Gill, N., R. B. Ferreira, L. C. Antunes, B. P. Willing, I. Sekirov, F. Al-Zahrani, M. Hartmann and B. B. Finlay (2012). "Neutrophil elastase alters the murine gut microbiota resulting in enhanced Salmonella colonization." PLoS One **7**(11): e49646.

Glenn, L. M., R. L. Lindsey, J. P. Folster, G. Pecic, P. Boerlin, M. W. Gilmour, H. Harbottle, S. Zhao, P. F. McDermott, P. J. Fedorka-Cray and J. G. Frye (2013). "Antimicrobial resistance genes in multidrug-resistant Salmonella enterica isolated from animals, retail meats, and humans in the United States and Canada." Microb Drug Resist **19**(3): 175-184.

Gordon, S. (2002). "Pattern recognition receptors: doubling up for the innate immune response." Cell **111**(7): 927-930.

Guerrant, R. L., R. B. Oria, S. R. Moore, M. O. B. Oria and A. A. M. Lima (2008). "Malnutrition as an enteric infectious disease with long-term effects on child development." Nutrition Reviews **66**(9): 487-505.

Guilloteau, P., R. Zabielski, H. M. Hammon and C. C. Metges (2010). "Nutritional programming of gastrointestinal tract development. Is the pig a good model for man?" Nutr Res Rev **23**(1): 4-22.

Hansen-Wester, I. and M. Hensel (2001). "Salmonella pathogenicity islands encoding type III secretion systems." Microbes and Infection **3**(7): 549-559.

Harmidy, K., N. Tufenkji and S. Gruenheid (2011). "Perturbation of host cell cytoskeleton by cranberry proanthocyanidins and their effect on enteric infections." PLoS One **6**(11): e27267.

Harrison, L. M., K. V. Balan and U. S. Babu (2013). "Dietary fatty acids and immune response to food-born bacterial infections." Nutrients **5**(5): 1801-1822.

He, R. R., M. Wang, C. Z. Wang, B. T. Chen, C. N. Lu, X. S. Yao, J. X. Chen and H. Kurihara (2011). "Protective effect of apple polyphenols against stress-provoked influenza viral infection in restraint mice." J Agric Food Chem **59**(8): 3730-3737.

Hekmatdoost, A., X. Wu, V. Morampudi, S. M. Innis and K. Jacobson (2013). "Dietary oils modify the host immune response and colonic tissue damage following Citrobacter rodentium infection in mice." Am J Physiol Gastrointest Liver Physiol **304**(10): G917-928.

Henderson, L. M., G. J. Brewer, J. B. Dressman, S. Z. Swidan, D. J. DuRoss, C. H. Adair, J. L. Barnett and R. R. Berardi (1995). "Effect of intragastric pH on the absorption of oral zinc acetate and zinc oxide in young healthy volunteers." JPEN J Parenter Enteral Nutr **19**(5): 393-397.

Herrick, R. L., S. G. Buchberger, R. M. Clark, M. Kupferle, R. Murray and P. Succop (2012). "A Markov model to estimate Salmonella morbidity, mortality, illness duration, and cost." Health Econ **21**(10): 1169-1182.

Hill, T. M., M. J. Vandehaar, L. M. Sordillo, D. R. Catherman, H. G. Bateman, 2nd and R. L. Schlotterbeck (2011). "Fatty acid intake alters growth and immunity in milk-fed calves." J Dairy Sci **94**(8): 3936-3948.

Hitchins, A. D., P. Wells, F. E. McDonough and N. P. Wong (1985). "Amelioration of the adverse effect of a gastrointestinal challenge with Salmonella enteritidis on weanling rats by a yogurt diet." Am J Clin Nutr **41**(1): 92-100.

Hofmann, R. R. (1989). "Evolutionary Steps of Ecophysiological Adaptation and Diversification of Ruminants - a. Comparative View of Their Digestive-System." Oecologia **78**(4): 443-457.

Hohmann, E. L. (2001). "Nontyphoidal salmonellosis." Clin Infect Dis **32**(2): 263-269.

Hung, C. C., C. D. Garner, J. M. Slauch, Z. W. Dwyer, S. D. Lawhon, J. G. Frye, M. McClelland, B. M. Ahmer and C. Altier (2013). "The intestinal fatty acid propionate inhibits Salmonella invasion through the post-translational control of HilD." Mol Microbiol **87**(5): 1045-1060.

Jouanguy, E., R. Doffinger, S. Dupuis, A. Pallier, F. Altare and J. L. Casanova (1999). "IL-12 and IFN-gamma in host defense against mycobacteria and salmonella in mice and men." Curr Opin Immunol **11**(3): 346-351.

Kamada, N., G. Y. Chen, N. Inohara and G. Nunez (2013). "Control of pathogens and pathobionts by the gut microbiota." Nat Immunol **14**(7): 685-690.

Kararli, T. T. (1995). "Comparison of the gastrointestinal anatomy, physiology, and biochemistry of humans and commonly used laboratory animals." Biopharm Drug Dispos **16**(5): 351-380.

Kararli, T. T. (1995). "Comparison of the Gastrointestinal Anatomy, Physiology, and Biochemistry of Humans and Commonly Used Laboratory-Animals." Biopharmaceutics & Drug Disposition **16**(5): 351-380.

Kau, A. L., P. P. Ahern, N. W. Griffin, A. L. Goodman and J. I. Gordon (2011). "Human nutrition, the gut microbiome and the immune system." Nature **474**(7351): 327-336.

Kawai, T. and S. Akira (2010). "The role of pattern-recognition receptors in innate immunity: update on Toll-like receptors." Nat Immunol **11**(5): 373-384.

Kearney, J. (2010). "Food consumption trends and drivers." Philosophical Transactions of the Royal Society B-Biological Sciences **365**(1554): 2793-2807.

Kelly, P., T. Shawa, S. Mwanamakondo, R. Soko, G. Smith, G. R. Barclay and I. R. Sanderson (2010). "Gastric and intestinal barrier impairment in tropical enteropathy and HIV: limited impact of micronutrient supplementation during a randomised controlled trial." Bmc Gastroenterology **10**: 72.

Kim, S. P., M. Y. Kang, J. C. Park, S. H. Nam and M. Friedman (2012). "Rice hull smoke extract inactivates Salmonella Typhimurium in laboratory media and protects infected mice against mortality." J Food Sci **77**(1): M80-85.

Kim, S. P., E. Moon, S. H. Nam and M. Friedman (2012). "Composition of Herba Pogostemonis Water Extract and Protection of Infected Mice against Salmonella Typhimurium-Induced Liver Damage and Mortality by Stimulation of Innate Immune Cells." Journal of Agricultural and Food Chemistry **60**(49): 12122-12130.

Kingamkono, R., E. Sjogren and U. Svanberg (1999). "Enteropathogenic bacteria in faecal swabs of young children fed on lactic acid-fermented cereal gruels." Epidemiol Infect **122**(1): 23-32.

Kirby, A. C., U. Yrlid and M. J. Wick (2002). "The innate immune response differs in primary and secondary Salmonella infection." J Immunol **169**(8): 4450-4459.

Lapaque, N., T. Walzer, S. Meresse, E. Vivier and J. Trowsdale (2009). "Interactions between Human NK Cells and Macrophages in Response to Salmonella Infection." Journal of Immunology **182**(7): 4339-4348.

Laparra, J. M. and Y. Sanz (2010). "Interactions of gut microbiota with functional food components and nutraceuticals." Pharmacol Res **61**(3): 219-225.

Lara-Villoslada, F., S. Sierra, J. Boza, J. Xaus and M. Olivares (2007). "[Beneficial effects of consumption of a dairy product containing two probiotic strains, Lactobacillus coryniformis CECT5711 and Lactobacillus gasseri CECT5714 in healthy children]." Nutr Hosp **22**(4): 496-502.

Li, R. W., E. E. Connor, C. J. Li, R. L. Baldwin and M. E. Sparks (2012). "Characterization of the rumen microbiota of pre-ruminant calves using metagenomic tools." Environmental Microbiology **14**(1): 129-139.

Liu, J. H., T. T. Xu, Y. J. Liu, W. Y. Zhu and S. Y. Mao (2013). "A high-grain diet causes massive disruption of ruminal epithelial tight junctions in goats." Am J Physiol Regul Integr Comp Physiol **305**(3): R232-241.

Long, K. Z., J. I. Santos, J. L. Rosado, T. Estrada-Garcia, M. Haas, A. Al Mamun, H. L. DuPont and N. N. Nanthakumar (2011). "Vitamin A supplementation modifies the association between

mucosal innate and adaptive immune responses and resolution of enteric pathogen infections." Am J Clin Nutr **93**(3): 578-585.

Lucas, J. S., S. A. Cochrane, J. O. Warner and J. O. Hourihane (2008). "The effect of digestion and pH on the allergenicity of kiwifruit proteins." Pediatr Allergy Immunol **19**(5): 392-398.

Macpherson, A. J., K. D. Mccoy, F. E. Johansen and P. Brandtzaeg (2008). "The immune geography of IgA induction and function." Mucosal Immunol **1**(1): 11-22.

Mastroeni, P., J. A. Harrison, J. A. Chabalgoity and C. E. Hormaeche (1996). "Effect of interleukin 12 neutralization on host resistance and gamma interferon production in mouse typhoid." Infect Immun **64**(1): 189-196.

Mathlouthi, N., T. Bouzaienne, I. Oueslati, F. Recoquillay, M. Hamdi, M. Urdaci and R. Bergaoui (2012). "Use of rosemary, oregano, and a commercial blend of essential oils in broiler chickens: in vitro antimicrobial activities and effects on growth performance." J Anim Sci **90**(3): 813-823.

McNulty, N. P., M. Wu, A. R. Erickson, C. L. Pan, B. K. Erickson, E. C. Martens, N. A. Pudlo, B. D. Muegge, B. Henrissat, R. L. Hettich and J. I. Gordon (2013). "Effects of Diet on Resource Utilization by a Model Human Gut Microbiota Containing Bacteroides cellulosilyticus WH2, a Symbiont with an Extensive Glycobiome." Plos Biology **11**(8).

Mestas, J. and C. C. Hughes (2004). "Of mice and not men: differences between mouse and human immunology." J Immunol **172**(5): 2731-2738.

Metcalf, E. S., G. W. Almond, P. A. Routh, J. R. Horton, R. C. Dillman and P. E. Orndorff (2000). "Experimental Salmonella typhi infection in the domestic pig, Sus scrofa domestica." Microbial Pathogenesis **29**(2): 121-126.

Michetti, P., M. J. Mahan, J. M. Slauch, J. J. Mekalanos and M. R. Neutra (1992). "Monoclonal Secretory Immunoglobulin-a Protects Mice against Oral Challenge with the Invasive Pathogen Salmonella-Typhimurium." Infect Immun **60**(5): 1786-1792.

Michiels, J., J. Missotten, G. Rasschaert, N. Dierick, M. Heyndrickx and S. De Smet (2012). "Effect of organic acids on Salmonella colonization and shedding in weaned piglets in a seeder model." J Food Prot **75**(11): 1974-1983.

Mittrucker, H. W. and S. H. E. Kaufmann (2000). "Immune response to infection with Salmonella typhimurium in mice." Journal of Leukocyte Biology **67**(4): 457-463.

Nanton, M. R., S. S. Way, M. J. Shlomchik and S. J. McSorley (2012). "Cutting edge: B cells are essential for protective immunity against Salmonella independent of antibody secretion." J Immunol **189**(12): 5503-5507.

Nofrarias, M., D. Martinez-Puig, J. Pujols, N. Majo and J. F. Perez (2007). "Long-term intake of resistant starch improves colonic mucosal integrity and reduces gut apoptosis and blood immune cells." Nutrition **23**(11-12): 861-870.

Oussalah, M., S. Caillet, L. Saucier and M. Lacroix (2007). "Inhibitory effects of selected plant essential oils on the growth of four pathogenic bacteria: E-coli O157 : H7, Salmonella

Typhimurium, Staphylococcus aureus and Listeria monocytogenes." Food Control **18**(5): 414-420.

Paolillo, R., C. R. Carratelli and A. Rizzo (2011). "Effect of resveratrol and quercetin in experimental infection by Salmonella enterica serovar Typhimurium." Int Immunopharmacol **11**(2): 149-156.

Park, E. J., A. B. Thomson and M. T. Clandinin (2010). "Protection of intestinal occludin tight junction protein by dietary gangliosides in lipopolysaccharide-induced acute inflammation." J Pediatr Gastroenterol Nutr **50**(3): 321-328.

Pieper, R., J. Bindelle, G. Malik, J. Marshall, B. G. Rossnagel, P. Leterme and A. G. Van Kessel (2012). "Influence of different carbohydrate composition in barley varieties on Salmonella Typhimurium var. Copenhagen colonisation in a "Trojan" challenge model in pigs." Arch Anim Nutr **66**(3): 163-179.

Puupponen-Pimiä, R., L. Nohynek, S. Hartmann-Schmidlin, M. Kähkönen, M. Heinonen, K. Määttä-Riihinen and K. M. Oksman-Caldentey (2005). "Berry phenolics selectively inhibit the growth of intestinal pathogens." Journal of Applied Microbiology **98**(4): 991-1000.

Rabbani, G. H., T. Teka, B. Zaman, N. Majid, M. Khatun and G. J. Fuchs (2001). "Clinical studies in persistent diarrhea: dietary management with green banana or pectin in Bangladeshi children." Gastroenterology **121**(3): 554-560.

Rajtak, U., F. Boland, N. Leonard, D. Bolton and S. Fanning (2012). "Roles of diet and the acid tolerance response in survival of common Salmonella serotypes in feces of finishing pigs." Appl Environ Microbiol **78**(1): 110-119.

Ramalingam, A., X. Wang, M. Gabello, M. C. Valenzano, A. P. Soler, A. Ko, P. J. Morin and J. M. Mullin (2010). "Dietary methionine restriction improves colon tight junction barrier function and alters claudin expression pattern." Am J Physiol Cell Physiol **299**(5): C1028-1035.

Rishi, P., P. Kaur, J. S. Virdi, G. Shukla and A. Koul (2008). "Amelioratory effects of zinc supplementation on Salmonella-induced hepatic damage in the murine model." Digestive Diseases and Sciences **53**(4): 1063-1070.

Roberts, C. L., A. V. Keita, B. N. Parsons, M. Prorok-Hamon, P. Knight, C. Winstanley, O. K. N, J. D. Soderholm, J. M. Rhodes and B. J. Campbell (2013). "Soluble plantain fibre blocks adhesion and M-cell translocation of intestinal pathogens." J Nutr Biochem **24**(1): 97-103.

Sanchez-Vargas, F. M., M. A. Abu-El-Haija and O. G. Gomez-Duarte (2011). "Salmonella infections: an update on epidemiology, management, and prevention." Travel Med Infect Dis **9**(6): 263-277.

Santos, R. L., S. Zhang, R. M. Tsolis, R. A. Kingsley, L. G. Adams and A. J. Baumler (2001). "Animal models of Salmonella infections: enteritis versus typhoid fever." Microbes Infect **3**(14-15): 1335-1344.

Scharek, L. and K. Tedin (2007). "The porcine immune system--differences compared to man and mouse and possible consequences for infections by Salmonella serovars." Berl Munch Tierarztl Wochenschr **120**(7-8): 347-354.

Schley, P. D. and C. J. Field (2002). "The immune-enhancing effects of dietary fibres and prebiotics." British Journal of Nutrition **87**: S221-S230.

Stecher, B., R. Robbiani, A. W. Walker, A. M. Westendorf, M. Barthel, M. Kremer, S. Chaffron, A. J. Macpherson, J. Buer, J. Parkhill, G. Dougan, C. von Mering and W. D. Hardt (2007). "Salmonella enterica serovar typhimurium exploits inflammation to compete with the intestinal microbiota." Plos Biology **5**(10): 2177-2189.

Stelter, C., R. Kappeli, C. Konig, A. Krah, W. D. Hardt, B. Stecher and D. Bumann (2011). "Salmonella-induced mucosal lectin RegIIIbeta kills competing gut microbiota." PLoS One **6**(6): e20749.

Suzuki, T. and H. Hara (2009). "Quercetin Enhances Intestinal Barrier Function through the Assembly of Zonnula Occludens-2, Occludin, and Claudin-1 and the Expression of Claudin-4 in Caco-2 Cells." Journal of Nutrition **139**(5): 965-974.

Taylor, A. K., W. Cao, K. P. Vora, J. De La Cruz, W. J. Shieh, S. R. Zaki, J. M. Katz, S. Sambhara and S. Gangappa (2013). "Protein energy malnutrition decreases immunity and increases susceptibility to influenza infection in mice." J Infect Dis **207**(3): 501-510.

Tennant, S. M., E. L. Hartland, T. Phumoonna, D. Lyras, J. I. Rood, R. M. Robins-Browne and I. R. van Driel (2008). "Influence of gastric acid on susceptibility to infection with ingested bacterial pathogens." Infect Immun **76**(2): 639-645.

Thiennimitr, P., S. E. Winter, M. G. Winter, M. N. Xavier, V. Tolstikov, D. L. Huseby, T. Sterzenbach, R. M. Tsolis, J. R. Roth and A. J. Baumler (2011). "Intestinal inflammation allows Salmonella to use ethanolamine to compete with the microbiota." Proceedings of the National Academy of Sciences of the United States of America **108**(42): 17480-17485.

Thomson, L. W., R. Pieper, J. K. Marshall and A. G. Van Kessel (2012). "Effect of wheat distillers dried grains with solubles or sugar beet pulp on prevalence of Salmonella enterica Typhimurium in weaned pigs." J Anim Sci **90 Suppl 4**: 13-15.

Toward, R., S. Montandon, G. Walton and G. R. Gibson (2012). "Effect of prebiotics on the human gut microbiota of elderly persons." Gut Microbes **3**(1): 57-60.

Tsolis, R. M., R. A. Kingsley, S. M. Townsend, T. A. Ficht, L. G. Adams and A. J. Baumler (1999). "Of mice, calves, and men - Comparison of the mouse typhoid model with other Salmonella infections." Mechanisms in the Pathogenesis of Enteric Diseases 2 **473**: 261-274.

Turnbaugh, P. J., V. K. Ridaura, J. J. Faith, F. E. Rey, R. Knight and J. I. Gordon (2009). "The Effect of Diet on the Human Gut Microbiome: A Metagenomic Analysis in Humanized Gnotobiotic Mice." Science Translational Medicine **1**(6).

Ulluwishewa, D., R. C. Anderson, W. C. McNabb, P. J. Moughan, J. M. Wells and N. C. Roy (2011). "Regulation of tight junction permeability by intestinal bacteria and dietary components." J Nutr **141**(5): 769-776.

Vikram, A., P. R. Jesudhasan, G. K. Jayaprakasha, S. D. Pillai, A. Jayaraman and B. S. Patil (2011). "Citrus flavonoid represses Salmonella pathogenicity island 1 and motility in S. Typhimurium LT2." International Journal of Food Microbiology **145**(1): 28-36.

Wang, J., X. Niu, X. Du, D. Smith, S. N. Meydani and D. Wu (2014). "Dietary supplementation with white button mushrooms augments the protective immune response to salmonella vaccine in mice." J Nutr **144**(1): 98-105.

Wang, J. P., X. L. Niu, X. G. Du, D. Smith, S. N. Meydani and D. Y. Wu (2013). "Dietary supplementation with white button mushroom augments the protective immune response to Salmonella vaccine in mice." Faseb Journal **27**.

Willing, B. P., A. Vacharaksa, M. Croxen, T. Thanachayanont and B. B. Finlay (2011). "Altering Host Resistance to Infections through Microbial Transplantation." Plos One **6**(10).

Winter, S. E., P. Thiennimitr, M. G. Winter, B. P. Butler, D. L. Huseby, R. W. Crawford, J. M. Russell, C. L. Bevins, L. G. Adams, R. M. Tsolis, J. R. Roth and A. J. Baumler (2010). "Gut inflammation provides a respiratory electron acceptor for Salmonella." Nature **467**(7314): 426-429.

Yuan, Q. and W. A. Walker (2004). "Innate immunity of the gut: mucosal defense in health and disease." J Pediatr Gastroenterol Nutr **38**(5): 463-473.

CHAPTER 2

DIETARY RICE BRAN PROMOTES RESISTANCE TO *SALMONELLA ENTERICA* SEROVAR TYPHIMURIUM COLONIZATON IN MICE[1]

2.1 Research rationale

Salmonella is a food and water borne pathogen and annually affects 1.5 billion people around the world that causes economic loss of $ 0.5 – 2.3 billion (Arshad, Wilkins et al. 2008). Prevention of *Salmonella* infections consumes a large amount of synthetic antimicrobials that are not affordable to the poor population. Literature also suggests that *Salmonella* infections are prevalent in the lower socioeconomic sections of the society because of unavailability of clean water and food (Das, Chisti et al. 2013). Drug resistance is another issue with unjustifiable and continuous use of antimicrobials (Abouzeed, Baucheron et al. 2008, Andersson and Hughes 2010). Therefore, accessible and affordable alternative strategies are required to prevent *Salmonella* infections.

Rice bran is the outer covering of the rice grain and is removed in the process of milling to obtain the white rice. Rice bran is composed of 15-25 % fats and hence requires heat stabilization to prevent rancidity. Annual production of rice bran is 60- 70 million ton and grown in more than 114 countries. Rice bran is generally discarded as a waste product or fed to livestock. Rice bran is a rich source of nutrients and has been studies for several health benefits including diabetes, hyperlipidemia, inflammatory diseases, allergies and cancer. However, rice bran has not been tested for prevention of *Salmonella* infection. We selected rice bran from Neptune variety for our study.

[1] Ajay Kumar, Angela Henderson, Genevieve M Forster, Andrew W Goodyear, Tiffany L Weir, Jan E Leach,

Neptune is a rice variety released in 2007 by Louisiana State University. It is a high yielding, semi dwarf, medium grain variety that is moderately susceptible to sheath blight and moderately resistant to blast and significantly contributes towards total rice production in United States (Sha, Linscombe et al. 2010). We chose mouse model of *Salmonella* infection for this study. Over the years, mouse models have provided insight to human diseases because of genetic and physiological similarities. Mouse models are one of the best models to test the efficacy of dietary components because of the following reasons:

- Rodents are small in size as compared to other animal models of *Salmonella* infections and hence the maintenance is comparatively cheaper in terms of diet and housing.
- Due to their small size, breeding of rodents is easier compared to other large animals.
- Availability of laboratory reagents such as antibodies and different assay kits is easier as compared to other large animals.
- Rodents are good models for mechanistic studies as gene knockouts studies are comparatively easier.

2.2 Summary

Dietary rice bran consists of many bioactive components with disease fighting properties; including the capacity to modulate the gut microbiota. Studies point to the important roles of the gut microbiota and the mucosal epithelium in the establishment of protection against enteric pathogens, such as *Salmonella*. The ability of rice bran to reduce the susceptibility of mice to a *Salmonella* infection has not been previously investigated. Therefore, we hypothesized that the incorporation of rice bran into the diet will inhibit the colonization of *Salmonella* in mice.

Mice were fed diets containing 0%, 10% and 20% rice bran for one week prior to being orally infected with *Salmonella enterica* serovar Typhimurium. Experimentally, appropriate doses of rice bran were determined. We found that mice consuming the 10 and 20% rice bran diets exhibited a reduction in *Salmonella* fecal shedding for up to nine days post-infection as compared to the control diet fed animals ($p<0.05$). In addition, we observed decreased concentrations of the pro-inflammatory cytokines, TNF-alpha, IFN-gamma, and IL-12 ($p<0.05$) as well as increased colonization of native *Lactobacillus* spp. in rice bran fed mice ($p<0.05$). Furthermore, in vitro experiments revealed the ability of rice bran extracts to reduce the *Salmonella* entry into the mouse small intestinal epithelial cells. These results show that administration of dietary rice bran reduces *Salmonella* colonization in mice. Increasing rice bran consumption represents a novel dietary means for reducing susceptibility to enteric infection with *Salmonella,* potentially via induction of native *Lactobacillus* spp.

2.3 Introduction

Salmonella outbreaks are a major health challenge and medical problem around the world. Of the ~2,200 strains, *Salmonella enterica* and *enteridis* cause 75% of total disease incidence (Broide, Shapiro et al. 2005). Disease occurrence has resulted in economic burdens of $0.5 to $2.3 billion due to healthcare costs and productivity loss (Arshad, Wilkins et al. 2008). The emergence of drug resistant *Salmonella* strains is a strong rationale for the development of easily implemented dietary strategies to reduce susceptibility to infection (Abouzeed, Baucheron et al. 2008, Andersson and Hughes 2010). The evidence suggests that presence of some indigestible saccharides and polyphenols in the diet can affect survival and maintenance of gut microflora as well as help prevention of colonization by enteric pathogens (Stecher and Hardt 2008, Selma, Espin et al. 2009, Turnbaugh, Ridaura et al. 2009). For example, non-digestible

carbohydrates can be fermented by native gut *Lactobacilli,* which results in the production of organic acids, such as bacteriocins and hydrogen peroxides. These byproducts are associated with reduced growth of *Salmonella* (Fayol-Messaoudi, Berger et al. 2005, Marianelli, Cifani et al. 2010). Therefore, dietary supplementation represents a novel approach to aid in the induction of protective responses against enteric infections.

Little is known regarding the potential impact of whole foods on the colonization of *Salmonella* in the small intestine because traditional biomedical research methods focus on the effect of single nutrients or isolated dietary small molecules (Mackay 2010). Rice is an important staple food worldwide and the bran portion is typically removed, making rice bran widely available for human and animal consumption. Rice bran contains prebiotic components (Komiyama, Andoh et al. 2011), and is a rich source of bioactive polyphenols, fatty acids and peptides (Hemavathy and Prabhakar 1987, Tsutsumi, Kawauchi et al. 2000, Kong, Lam et al. 2009, Heuberger, Lewis et al. 2010, Ryan 2011). Dietary rice bran intake increased the fecal IgA and native gut *Lactobacillus* spp. in mice (Henderson, Kumar et al.). Also, rice bran controls gastrointestinal cancers, hyperlipidemia and diabetes in rats (Tomita, Kuno et al. 2008, Chou, Ma et al. 2009, Cheng, Ma et al. 2010, Norazalina, Norhaizan et al. 2010) as well as hypercholesterolemia in humans (Gerhardt and Gallo 1998).

The primary goal of this study was to examine the effect of dietary rice bran intake on susceptibility of mice to oral challenge with *Salmonella*. The *Salmonella enterica* serovar Typhimurium strain 14028s was chosen for these studies because it is a translational model of non-lethal, infection in female 129S6/SvEvTac mice (Sebastiani, Blais et al. 2002). The protective effect of rice bran against *Salmonella* infection in mice was measured by decreased fecal shedding following oral challenge. These novel findings of rice bran bioactivity have

practical implications for developing accessible, affordable and effective dietary public health intervention strategies to reduce *Salmonella* infections worldwide.

2.4 Methods

Animals and feeding schedule

Four to six week old female 129S6/SvEvTac (Taconic Farms, Germantown, NY) mice were randomly divided into 3 groups (n=5 in each group) and housed with a 12-hour light/dark cycle at 20-25°C. Animals were provided water and fed a maintenance diet AIN-93M (Harlan Teklad, Madison, WI) *ad libido* for one week. After 1 week, mice were randomized into Group 1- AIN-93M control diet, Group 2- 10% rice bran diet, or Group 3- 20% rice bran diet. The Animal Care and Use Committee at Colorado State University approved all mouse protocols (Protocol number 09-1457A). The study schema is shown in figure 2.1.

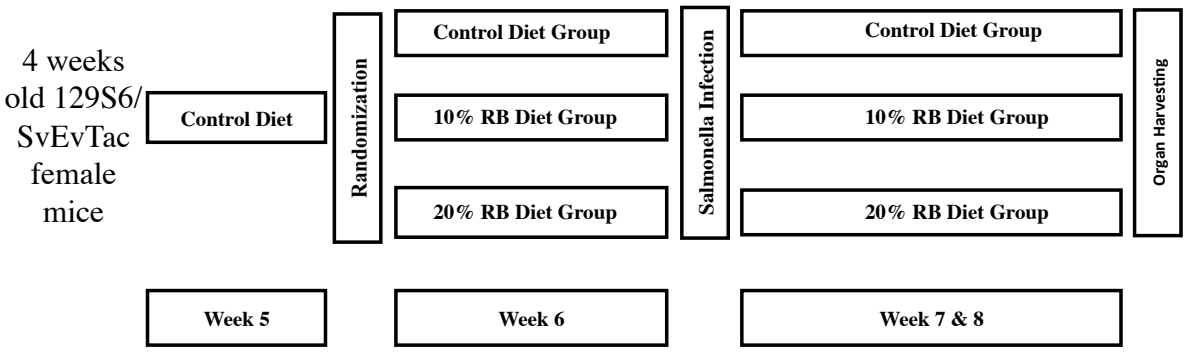


Figure 2.1 Study schema for assessment of dietary rice bran in mice *Salmonella* infection model. Four week old animals were fed AIN-93M control diet. Animals were randomized after one week and divided in two three groups (Group 1- Control Diet, Group 2- 10% Rice Bran (RB) Diet, Group 3- 20% Rice Bran (RB) Diet) and fed respective diets for one week. Mice were orally gavaged with *Salmonella* and feces were collected for two weeks.

Bacterial infection

Salmonella enterica serovar Typhimurium strain 14028s was a generous gift from Dr. Andres Vazquez-Torres (University of Colorado). *Salmonella* was grown in LB broth (Sigma Aldrich) at 37°C overnight to obtain stationary phase cultures, 15% glycerol (Fisher Scientific) was added and stocks were stored at -80°C. Frozen *Salmonella* stock was thawed and diluted with PBS to a final concentration of 2x 10^7 CFU/ml. Mice were infected with ~2×10^7 CFU in a total volume of 200 µl using a 25-gauge gavage needle. Each inoculum used for oral infection was plated on MacConkey agar (BD Biosciences) to confirm bacterial concentration.

Diet composition

Rice bran used in these studies was provided as a gift from Dr. Anna McClung at USDA-ARS Dale Bumpers Rice Research Unit (Stuttgart, AK). Diets were formulated to match macronutrients (e.g. protein, carbohydrates) across groups. Differences in macronutrient composition were balanced using purified diet components. The percent of rice bran incorporated into the diet is expressed as g/100g of diet. Harlan mixed and made pellets of rice bran containing diets using AIN-93M purified components. The composition of rice bran containing diets was calculated based on published reports (Barnes, Clapp et al. 1983, Cara, Dubois et al. 1992, Bird, Hayakawa et al. 2000) that demonstrated chronic disease fighting activity. Diet formulations are shown in table 2.1. The Neptune rice variety was chosen for its availability as U.S. grown rice. The proximate analysis of different diets is shown in table 2.2.

Table 2.1 Composition of control (AIN93-M) and rice bran (RB) supplemented mice pelleted diets

Constituents (g/kg)	Control	10% RB	20% RB	30% RB	50% RB
Casein	140	125	110	95	65
L-Cystine	1.8	1.8	1.8	1.8	1.8
Corn Starch	465.7	422.7	377.7	300.7	155.7
Maltodextrin	155	155	155	155	130
Sucrose	100	100	100	100	100
Corn Oil	40	19	0	0	0
Cellulose	50	29	8	0	0
Mineral Mix	35	35	35	35	35
Vitamin Mix	10	10	10	10	10
Choline Bitartrate	2.5	2.5	2.5	2.5	2.5
TBHQ*	0.008	0.008	0.008	0.008	0.008
Rice Bran (RB)	0	100	200	300	500

*TBHQ- Tertiary butyl-hydroquinone

Table 2.2 Proximate analyses of pelleted and powdered diets

Parameters (%)	Control (Pellet)	10% RB (Pellet)	20% RB (Pellet)	30% RB (Pellet)	50% RB (Pellet)
Moisture	8.33	10.17	9.28	9.48	9.65
Protein	13.13	13.48	12.59	12.37	12.05
Crude Fat	3.86	4.43	3.26	4.79	7.94
Crude Fiber	3.67	2.83	1.51	2.08	2.98
Ash	2.42	3.13	3.69	4.35	5.49

Fecal collection and processing

Fecal pellets were collected and body weights were recorded on day 0 before oral challenge, and on days 2, 5, 7, 9, 12 and 14-post infection. Mice were kept in Tupperware for 30 minutes and pellets from each mouse were weighed and diluted with PBS. After homogenization, fecal matter was serially diluted and plated on MacConkey agar (BD Biosciences) with 50 µg/ml of kanamycin (Fisher Scientific). Agar plates were incubated at 37°C under humid conditions for 24 hours and bacteria were counted as CFU/g of fecal matter. Morphology of *Salmonella* colony in pure culture and infected feces were compared.

Blood and tissue collection

Blood was collected by tail vein (before infection) or cardiac puncture (before necropsy) using 4% Isoflurane (Attane Isoflurane USP, Minard Inc) in anesthesia machine with oxygen at a flow rate of 0.1 L/min. Serum separator tubes (BD Microtainer) were centrifuged at 7500g for 10 minutes and stored at -20°C. Spleen, liver, ileum (distal 10 cm), mesenteric lymph nodes and Peyer's patches were harvested, thoroughly washed with PBS, weighed and transferred to bags (Whirl-Pack, Nasco) and homogenized in stomacher (Seward Stomacher 80, Biomaster Lab Systems). Serial dilutions of homogenized tissues were plated on MacConkey agar with 50 µg/ml of kanamycin.

Serum cytokine analysis

Serum cytokines (TNF-α, IFN-γ and IL-12) were analyzed by cytometeric bead array assay using the mouse inflammation kit (BD Biosciences) and the assay was performed according to the manufacturer's instruction manual. Flow cytometry was performed using a Cyan ADP flow cytometer and Summit software (Beckman Coulter), and FlowJo software (TreeStar Inc) was used for analysis and quantification of serum cytokine data.

Cell culture conditions

Mouse small intestine epithelial cells (MSIE) were a generous gift from Dr. Robert Whitehead at Vanderbilt University and the Ludwig Institute for Cancer Research (Whitehead and Robinson 2009). Briefly, MSIE cells were grown using published methods in RPMI 1640 media supplemented with 2.05 mM L-Glutamine (Hyclone Laboratories). RPMI media was also supplemented with heat inactivated 10% FBS (Atlas Biologicals), 1% antibiotic (Penicillin and Streptomycin) and antimycotic (Amphotericin) solution (Cellgro, Mediatech Inc), 0.1% Thioglycerol Hydrocortisone (Sigma), 0.004% IFN-γ (Peprotech USA), 0.023% Insulin (Regular Human Insulin, Novo Nordik). Cells were grown in 75cm^2 flasks and trypsinized at 80% confluence. Cells were seeded overnight in a 6 well plate at a density of 2×10^5 cell/well. After 12 h (hours) media was aspirated and fresh media was added with rice bran extracts for 24 h at 37°C and 5% CO_2 and 95% humidity.

Rice bran extraction (RBE)

Crude rice bran cannot be reliably tested in cellular assays, and was therefore extracted with 80% methanol to obtain a mixture of rice bran phytochemicals and called a rice bran extract (RBE). Briefly, rice bran (Neptune variety) was removed from the grain and heat stabilized at 110°C for 3 minutes. Ice-cold, 80% methanol was added, vortexed and incubated at -80°C for one hour. Following centrifugation at 1500g for 5 minutes, the supernatant was removed. Methanol was dried by vacuum centrifugation (SpeedVac Concentrator, Thermo Savant Model RT-100). Dried rice bran extract was weighed and then re-suspended with cell culture media to the appropriate doses for treatment of MSIE cells.

Salmonella entry and replication

Salmonella entry assay was done according to the published protocol (Steele-Mortimer 2008). This assay measures the total number of *Salmonella* (the bacteria that is surface attached plus the *Salmonella* internalized in the cell). MSIE cells were grown and treated with RBE for 24 h. Media was aspirated and cells were re-incubated with fresh media containing *Salmonella* and RBE. Frozen stock of *Salmonella* was mixed in RPMI media at a multiplicity of infection (MOI) of 100-120 in the presence (co-incubation with *Salmonella*) or absence of RBE (Figure 2.2). After 30 minutes of incubation, media was aspirated, and MSIE cell monolayer was washed with PBS twice to remove extracellular bacteria. Fresh media was added to cells for additional 1 hour. There are 2 additional cycles of washing with fresh media plus 50 μg/ml of gentamicin (Sigma-Aldrich) following 1-hour incubations under same conditions with 5 μg/ml of gentamicin. Media was aspirated and cell monolayer was washed with PBS twice to remove extracellular gentamicin. The cell monolayer was placed in 1 ml of buffer (PBS containing 1% TritonX-100 and 0.1% SDS) for 5 minutes. The contents were mixed by pipetting and serially diluted on MacConkey agar plates (BD Biosciences) with 50 μg/ml of kanamycin (Fisher Scientific) and incubated at 37°C for 24 hours. Colonies were counted and presented per ml of cell lysate. Intracellular *Salmonella* replication was measured in cells incubated with 5 μg/ml of gentamycin and RBE for 24 hours (Figure 2.3). Cells were lysed and plated on agar media to enumerate the total CFU count (Bowden, Ramachandran et al. 2010).

In-Vitro *Salmonella* Entry Assay Flowchart

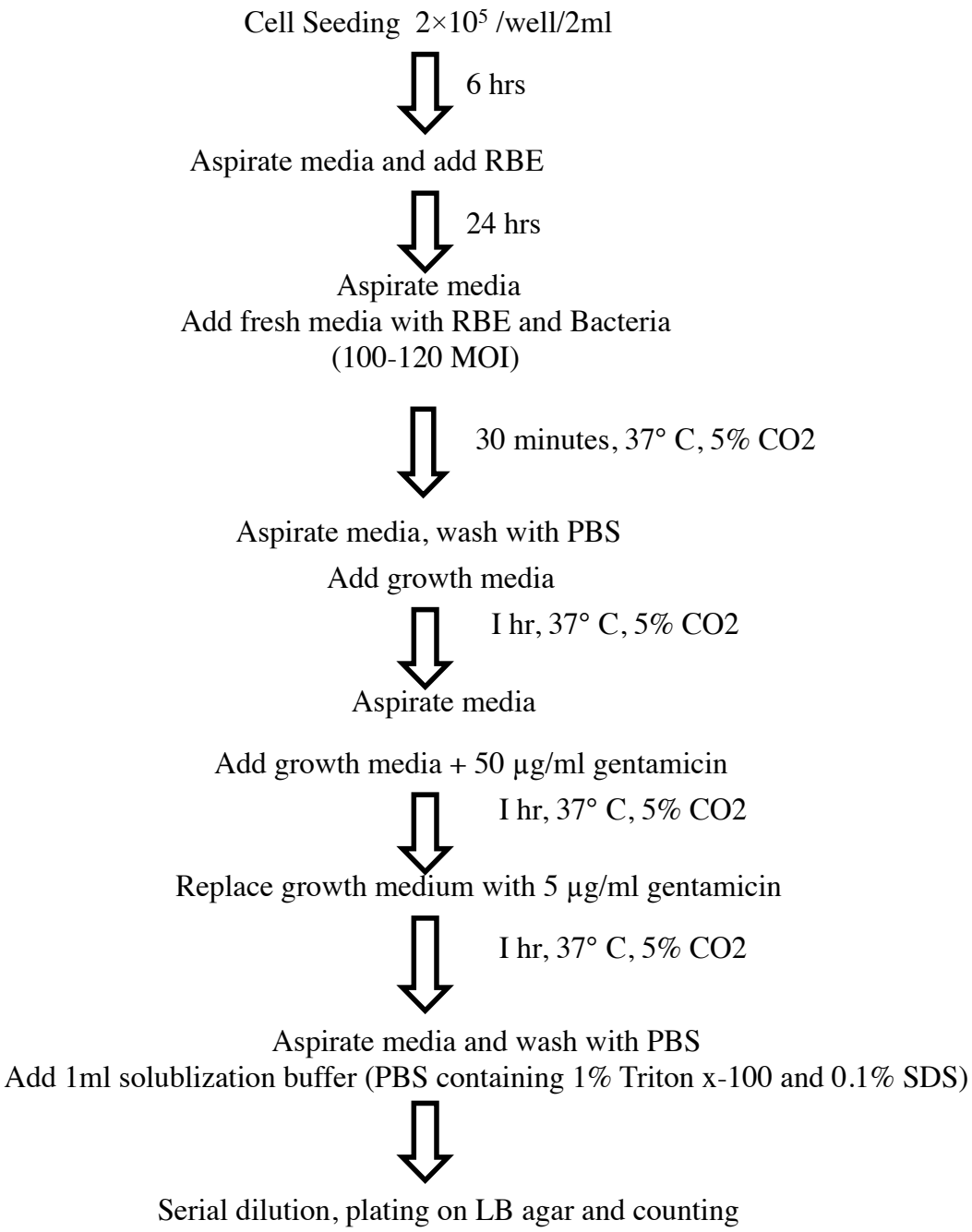


Figure 2.2 Flowchart showing steps in in-vitro *Salmonella* entry assay in epithelial cells

In-Vitro Salmonella Replication Assay Flow Chart

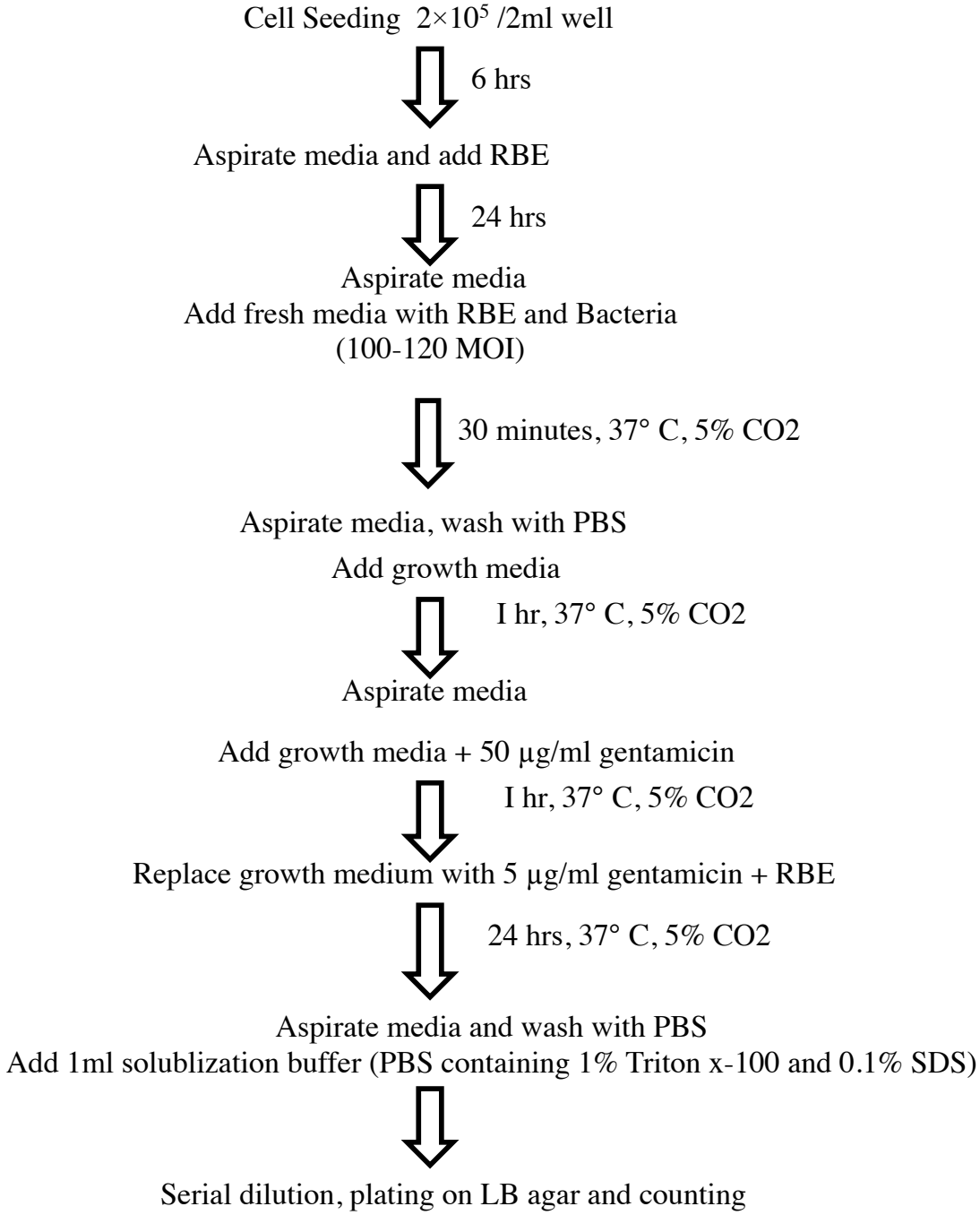


Figure 2.3 Flowchart showing steps in in-vitro *Salmonella* replication assay in epithelial cells

Cell viability

Cell viability was determined using AlamarBlue (Invitrogen). Briefly, cells were seeded in a 96 well plate at $2x10^5$ cells/ml. After 6 hours of cell adherence, cells were treated in the presence and absence of RBE for 24 hours at 37°C, 5% CO_2 in maintenance media. Supernatant was removed and AlamarBlue was added to media (20 μg/ml). Fluorescence was detected at excitation: 530/25; emission: 590/35 in ELISA plate reader (Bio-Tek Synergy HT, Winooski, VT).

Bacterial quantitation

RBE doses of 0, 1, 2, 5 and 10 mg/ml were tested for direct effects on *Salmonella* viability. Bacteria was added to media at a concentration of 2×10^7 CFU/ml and incubated for 6 hours at 37℃. Bacterial suspension was serially diluted, plated on agar plates and counted after 24 hours incubation.

Quantitative PCR for *Lactobacillus* spp.

A dilution of DNA from pure cultures of *Lactobacillus rhamnosus* was used to generate standard curves and DNA from *Pseudomonas aeruginosa* were run as a negative control to ensure primer specificity. DNA was quantified by Nanodrop (Thermo Fisher Scientific) and diluted to 5ng/μl. Real time PCR primers were used from Malinen et al (Malinen, Rinttila et al. 2005) for amplification of *Lactobacillus spp*. Samples were run on an ABI Prism 310 thermocycler (Applied Biosystems) using the following program: 95^0C for 3 min 30 s followed by 30 cycles of 95°C for 15s, 58ºC for 20 s 72°C for 30 s and melt curves were generated by 95°C for 1 min followed by eighty 10 s repeats at set point temperatures incrementally decreasing by 0.5°C.

ELISA for fecal IgA estimation

Fecal IgA was extracted according to the method described by Haneberg et al. (Haneberg, Kendall et al. 1994). Fecal IgA was analyzed by sandwich ELISA methods. Briefly, plates were coated with affinity purified anti-mouse immunoglobin antibody (BD Biosciences) at a concentration of 5µg/ml in 50µl carb-bicarb buffer (ph 9.6) per well in a 96 well plate and incubated overnight. Plate was washed with 3 times with PBS+0.05% Tween-20 (Fisher Scientific, Fair Lawn, NJ and BDH4210, VWR, West Chester, PA) and incubated with 300 µl 3% BSA each well for 1 hour at room temperature and again washed. Samples and diluted standards (50 µl) were incubated for 1 hour at room temperature and washed. Detection Antibody (BD Biosciences) was added to each well (50 µl @ 2 µg/ml in 1% BSA). SA-HRP (BD Biosciences) was added to each well (50 µl @ 1:2000 in 1% BSA). Plate was incubated (30 minutes) and 50 µl of TMB (Fisher) was added to each well and plate was incubated for 20 minutes. Reaction was stopped by 50 µl 1N HCL (Fisher) and absorbance was read at 450 nm.

Statistical analysis

Data was analyzed using Graphpad Prism5 Software. Experiments were repeated a minimum of three times. Raw data were log transformed into a $\log_{10}$ scale for CFU analysis and repeated measures ANOVA and post hoc Tukey's test were used for *Salmonella* fecal shedding and fecal *Lactobacilli* measures. Inflammatory cytokines were analyzed using two-way ANOVA and Bonferroni post hoc test. A nonparametric ANOVA (Kruskal Wallis) was performed, followed by Dunn's test for *in vitro Salmonella* assays. Significance was determined for all studies at $P < 0.05$.

2.5 Results

In order to test the infectivity of the *Salmonella* dose and variation in *Salmonella* fecal shedding, 5 week old mice were taken and gavaged with *Salmonella* at a dose of 2×10^7 in 200 μl Phosphate Buffer Saline (PBS) per mouse. Feces were collected and plated on Mackonkey agar to enumerate Colony Forming Unit (CFU). We found that a dose of 2×10^7 CFU/mouse was sufficient to infect and detect the *Salmonella* in feces (Figure 2.4). The variability was increased in *Salmonella* fecal shedding with time.

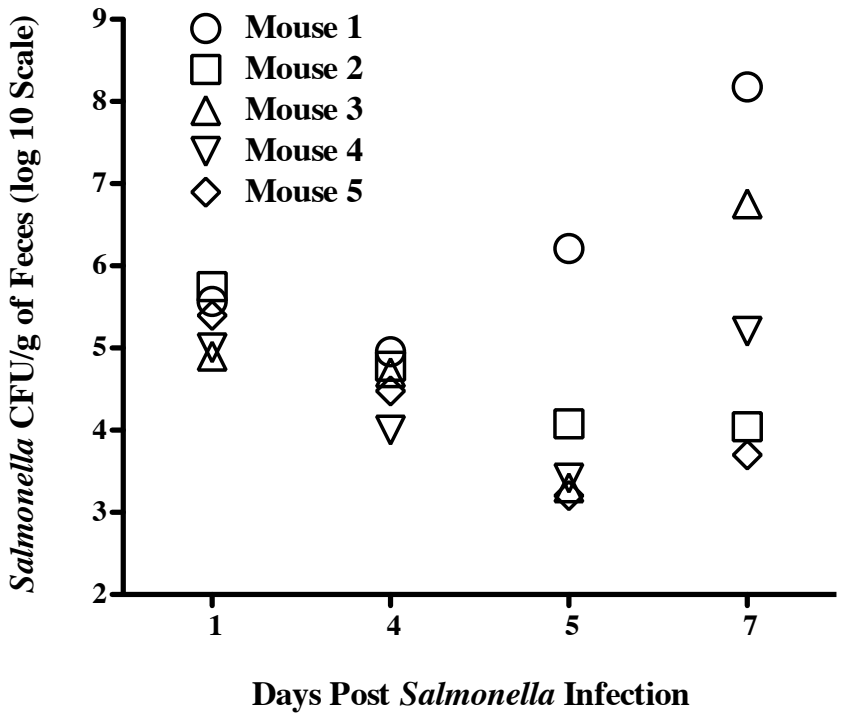


Figure 2.4 Efficacy of *S*. Typhimurium infection in terms of fecal shedding over the time. Fecal shedding was examined in *Salmonella* infected mice fed on chow diet for one week. Data are shown as CFU per gram of feces ($\log_{10}$ Scale) of individual mice (n=5).

Next we evaluated the effect of rice bran diet on *Salmonella* fecal shedding for 4 weeks. Previously, a 10% rice bran diet was found effective in studies across the species (Kahlon, Saunders et al. 1990, Stratton-Phelps, Backus et al. 2002, Gallinger, Suárez et al. 2004). Hence, a 10% rice bran diet was chosen for this experiment. Six to eight week old mice were taken and kept on control diet for one week. Mice were randomized and were fed on powdered rice bran and control diets (Table A.3) for 1 week and then orally infected with *Salmonella*. Fecal shedding of *Salmonella* was observed for 4 weeks. We found that mice fed on 10% rice bran diet showed lower average *Salmonella* fecal shedding up to 18 days post *Salmonella* infection as compared to control diet fed animals (Figure 2.5). However, this difference in *Salmonella* fecal shedding failed to attain statistical significance ($p<0.05$).

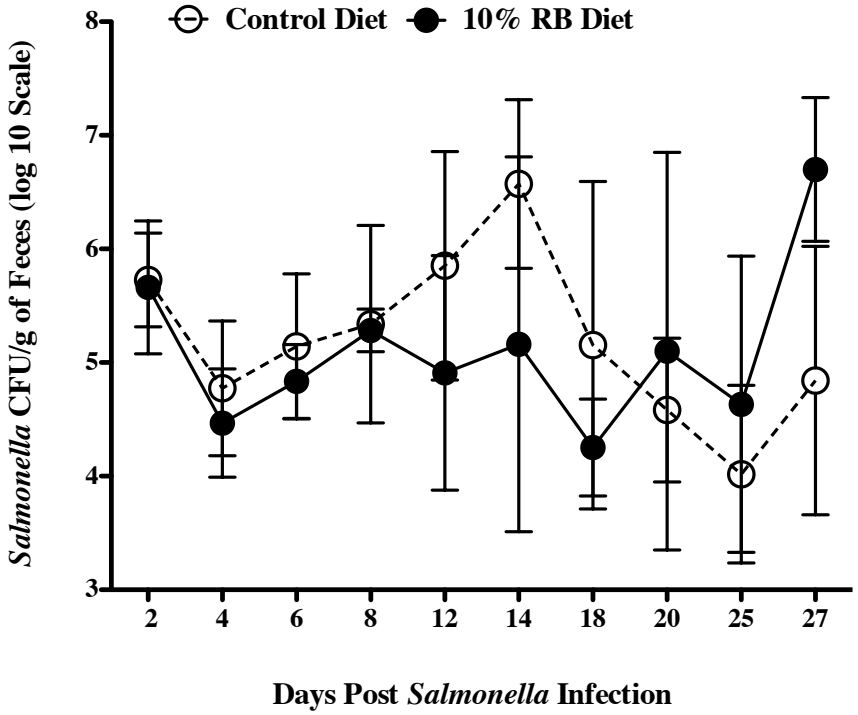


Figure 2.5 Effect of dietary rice bran (powdered) intake on *S.* Typhimurium fecal shedding for 4 weeks. Six to eight week old mice were kept on control diet for one week and randomized and then rice bran diet was fed for one week. Mice were orally infected one week later and fecal shedding was examined in *Salmonella* infected mice for 4 weeks. Data are shown as mean ± standard deviation of mean $\log_{10}$ CFU per gram of feces (n=5 mice/diet group), Repeated measures two-way ANOVA and post hoc Bonferroni test was applied.

Effect of dietary rice bran on *Salmonella* fecal shedding

Further, rice bran was incorporated in the diet at doses of 10, 20, 30 and 50% (w/w) and pellets were made (Table 2.1). Mice were fed rice bran diets and control diet for 1 week and infected with *Salmonella*. Feces were processed and plated on agar for *Salmonella*. Dietary rice bran dose dependently reduced fecal *Salmonella* shedding in mice up to 9 days post infection (Figure 2.6). To determine the effect of pelleted rice bran diet on *Salmonella* fecal shedding, 10 and 20% (w/w) daily dietary rice bran supplementation was examined further using a mouse model of *Salmonella* infection. Control and rice bran diets were fed to mice for one week prior to

oral challenge with *S*. Typhimurium and during infection. Mice consuming the rice bran diet showed a time dependent decrease in the fecal shedding of *S*. Typhimurium as compared to control diet animals (Figure 2.7).

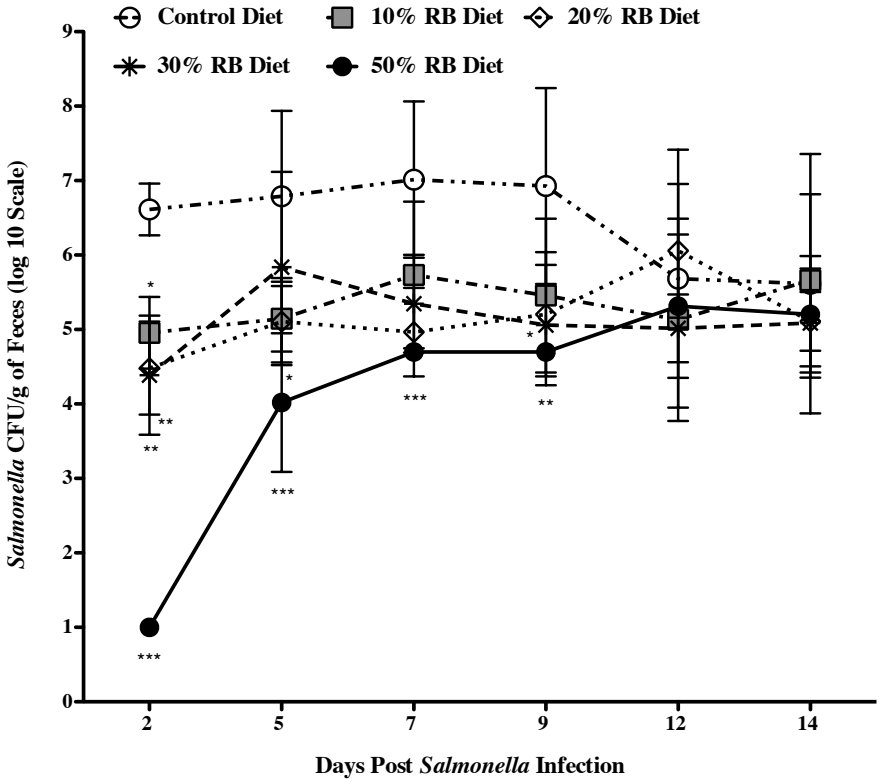


Figure 2.6 Effect of dietary rice bran (pelleted) intake on *S*. Typhimurium fecal shedding for 2 weeks. Four to six week old mice were kept on control diet for one week. Mice were randomized and then rice bran diet was fed for one week. Mice were orally infected one week later and fecal shedding was examined in *Salmonella* infected mice for 2 weeks. Data are shown as mean ± standard deviation of mean $\log_{10}$ CFU per gram of feces (n=5 mice/diet group), Repeated measures two-way ANOVA and post hoc Bonferroni test was applied. Statistical significance was denoted by * ($p<0.05$), ** ($p<0.01$), *** ($p<0.001$).

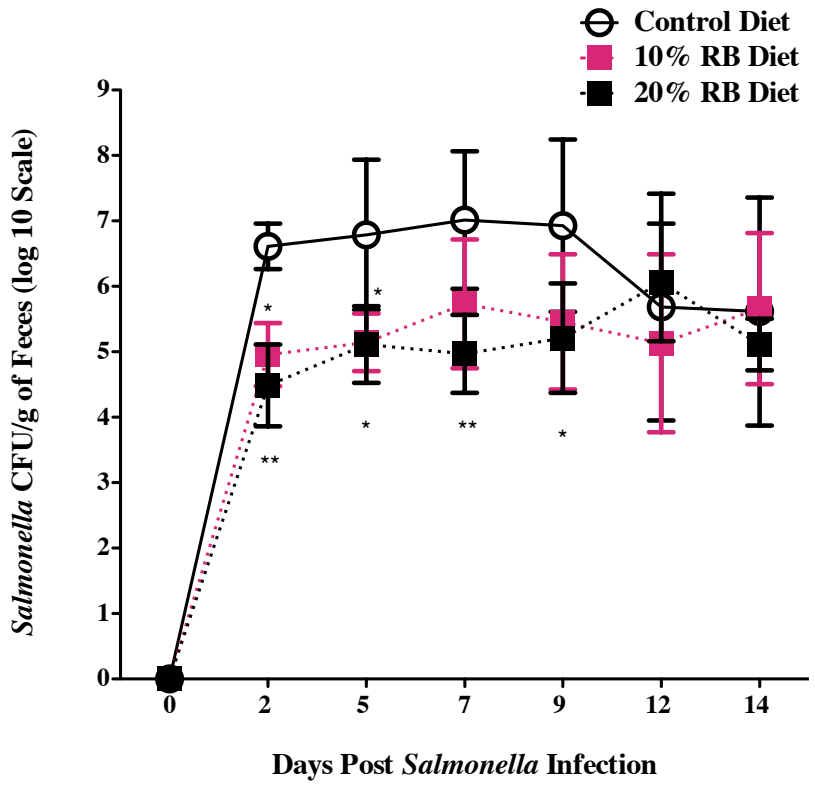


Figure 2.7 Effect of dietary rice bran on *S.* Typhimurium fecal shedding of mice Fecal shedding was examined in *Salmonella* infected animals fed control, 10% and 20% rice bran diet for 3 weeks (one week prior and 2 weeks post challenge). Data are shown as mean ± standard deviation of mean $\log_{10}$ CFU per gram of feces (n=5 mice/diet group), and similar results were obtained in other repetitive experiments. Repeated measures two-way ANOVA and Bonferroni post hoc test were applied. Significance is shown by * (P<0.05) and ** (P<0.01). Similar results were obtained in one additional experiment.

More specifically, animals fed the 10% rice bran diet exhibited decreased *Salmonella* fecal shedding by a $\log_{10}$ value of 1.66, 1.69 and 1.48 in comparison to animals fed the control diet on days 2, 5 and 9 post infection, respectively (p<0.05). Animals fed the 20% rice bran diet showed a reduction in *Salmonella* fecal shedding by a $\log_{10}$ value of 2.13, 1.69, 2.04 and 1.73 in comparison to the animals fed the control diet on days 2, 5, 7 and 9, respectively. No significant difference was observed in *Salmonella* fecal shedding between the 10 and 20 % rice bran diet

groups. These data demonstrate that pre-feeding dietary rice bran for one week reduced the susceptibility of mice to oral infection with the *Salmonella* pathogen as measured by fecal shedding.

Effect of dietary rice bran on weight of animals

Weight of animals was recorded during rice bran diet intervention and post infection to know whether rice bran affect the weight of animals. No significant difference was found amongst the three dietary groups (Figure 2.8).

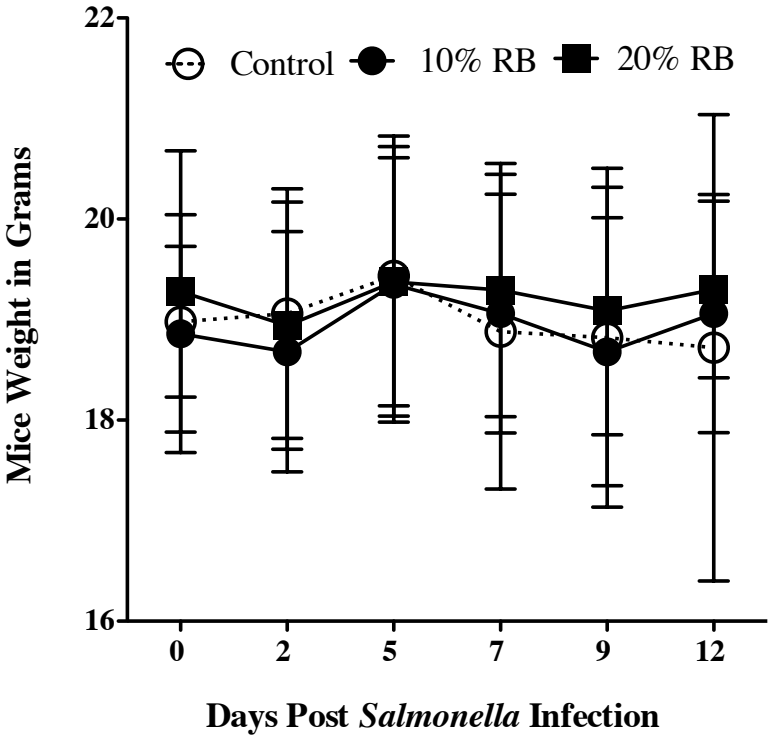


Figure 2.8 Animal weights across dietary groups before and after *S.* Typhimurium challenge. The weight of all animals were taken at regular intervals and presented as average of each dietary group (in grams). No significant difference was detected in weight gain of animals across dietary rice bran and control groups as tested by repeated measures two- way ANOVA, at a p value of 0.05 and Bonferroni post hoc test. Error bars represent mean with standard deviation (n=5). Similar results were observed in one additional experiment.

Effect of dietary rice bran on *Salmonella* tissue invasion in mice

Dietary rice bran decreased *Salmonella* fecal shedding (Figure 2.7) but this decrease in shedding could be due to increased *Salmonella* translocation to tissues. Hence to know the *Salmonella* load in different tissues this experiment was performed. *Salmonella* infected animals were sacrificed at 7 and 14 days after oral challenge with *S*. Typhimurium and selected tissues were homogenized and plated for enumeration of bacteria. Rice bran consumption decreased *Salmonella* translocation into the ileum, Peyer's patches and mesenteric lymph nodes compared to control animals (Figure 2.9). A 10 % rice bran diet reduced *Salmonella* invasion of the ileum (99.95%), Peyer's patches (94.35%) and mesenteric lymph node (90.78%), respectively, at day 7 compared to controls (Figure 2.9 *A-C*). On day 14, a 90.45, 86.51 and 57.78% reduction was detected in the ileum, Peyer's patches and mesenteric lymph node respectively (Figure 2.9 *D-F*). Mice consuming 20% rice bran intake showed 99.68, 88.19 and 99.58% reduced *Salmonella* invasion compared to controls in the ileum, Peyer's patches and mesenteric lymph node, respectively, at day 7. The reduction in *Salmonella* invasion in mice fed the 10 and 20% rice bran diet was not statistically different from control at day 7 and 14. *Salmonella* colonization of the liver and spleen was similar across rice bran and control groups (Figure 10 *A* and *B*). The 10% and 20% rice bran doses displayed differential kinetics of *Salmonella* translocation to the ileum, Peyer's patches and mesenteric lymph nodes. Hence, we did not find increased *Salmonella* translocation in the tissues such as Peyer's patches, mesenteric lymph nodes, liver and spleen. These results show that the reduction in fecal shedding of *Salmonella* in rice bran fed animals was not due to increased translocation to deeper tissues.

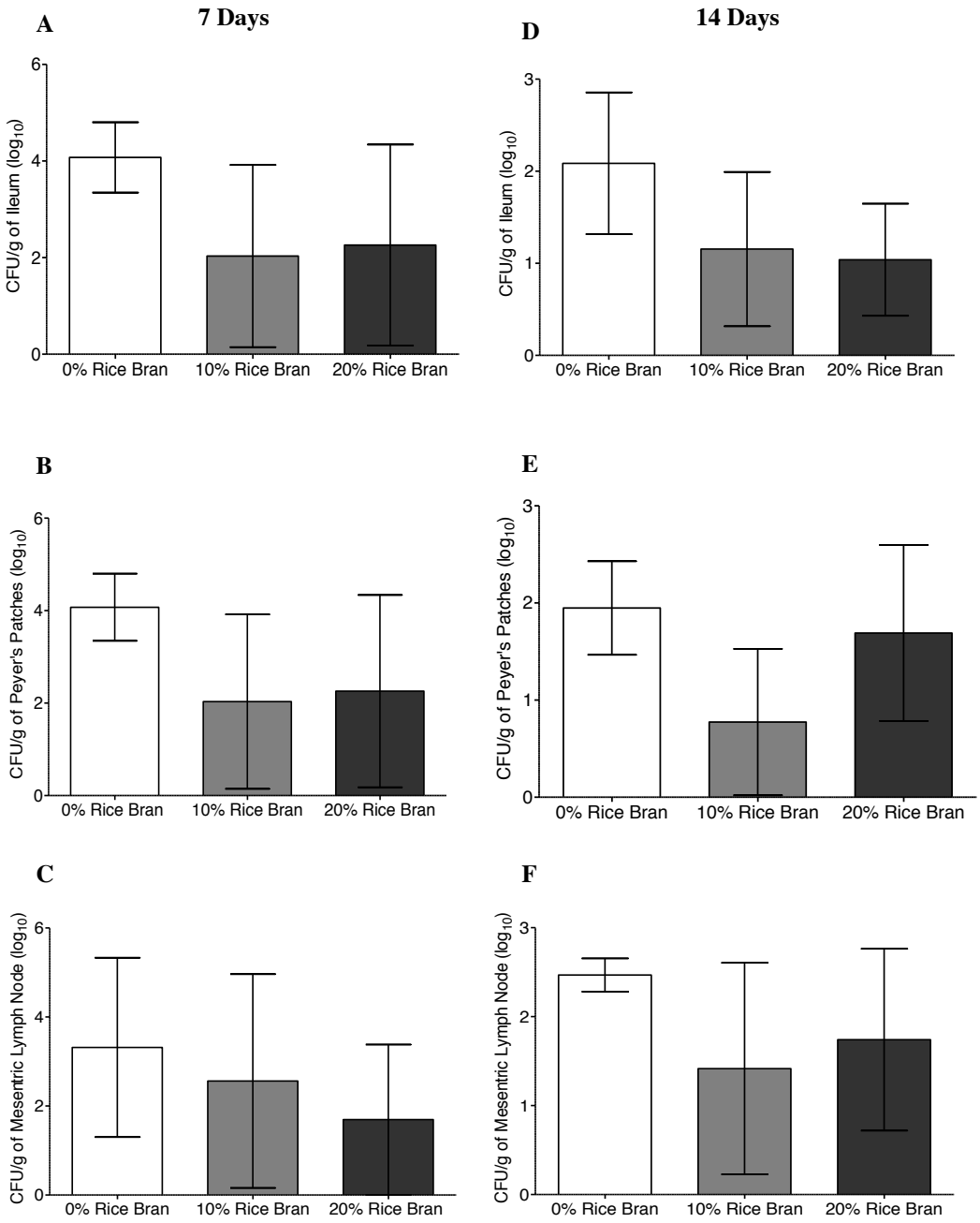


Figure 2.9 Effect of dietary rice bran on *S.* Typhimurium intestinal invasion. *Salmonella* infected animals were sacrificed on days 7 (Figure 2.9 A-C) and 14 (2.9 D-F) following oral challenge and selected tissues were homogenized and plated for enumeration of bacteria. Trends in the data indicate that rice bran supplementation decreased *Salmonella* translocation into the ileum, Peyer's patches and mesenteric lymph nodes. Data were analyzed using repeated measures one-way ANOVA, at a p value of 0.05, n=5 and Dun's multiple post-test. This experiment was not repeated.

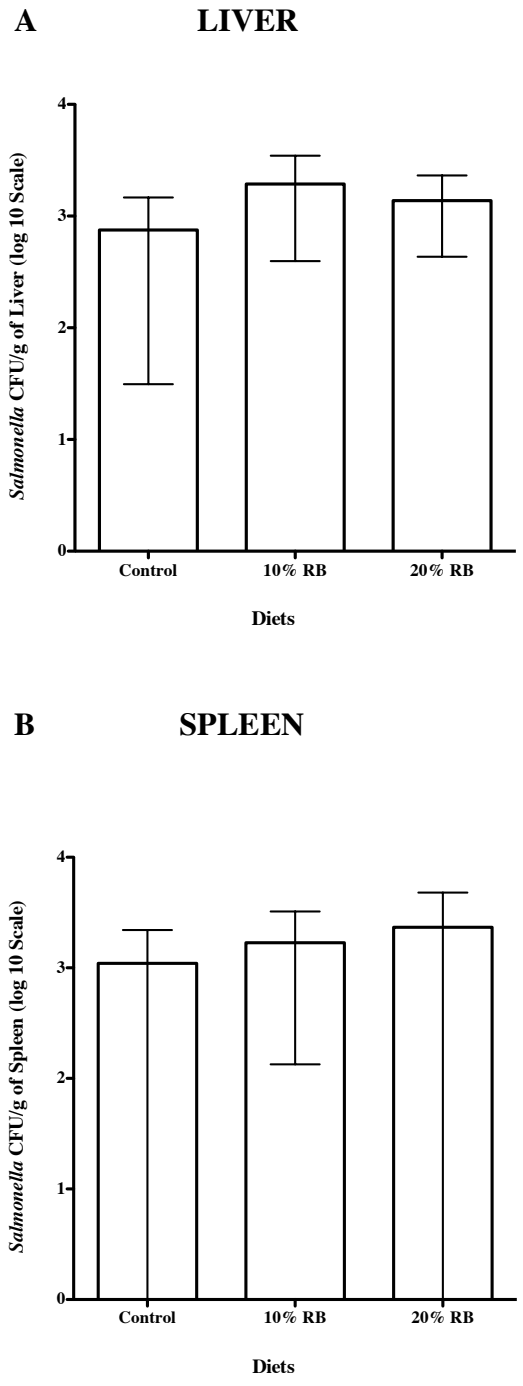


Figure 2.10 Effect of dietary rice bran on *S*. Typhimurium translocation into liver and spleen in mice. No significant differences in the invasion of *S*. Typhimurium in (A) liver and (B) spleen of animals fed 0, 10 and 20 % rice bran were detected at day 14 post-infection. Bacterial counts were enumerated in tissues after 48 hours of incubation and presented as $\log_{10}$ CFU per gram of organ. Data are mean ± standard error of mean with five animals in each group (n=5). Data were compared statistically using non-parametric one-way ANOVA with Dun's multiple means post-test, at a *P*-value of 0.05. This experiment was not repeated.

Effect of dietary rice bran on fecal IgA

IgA protects mice from *Salmonella* infection (Michetti, Mahan et al. 1992). We tested the total fecal IgA in mice to determine the rice bran induced reduction in *Salmonella* fecal shedding is mediated by IgA. Mouse fecal pellets were collected and protein was isolated for IgA determination. IgA was not significantly different amongst all three groups in first one week. However, IgA concentration was 200% higher ($p<0.001$) in control diet fed group as compared to rice bran fed group at 13 day, post *Salmonella* infection. These results indicate that with onset of adaptive immunity, increased burden of *Salmonella* in mice could have induced higher levels of IgA in control mice. Also, these results show that rice bran induced reduction in fecal shedding of *Salmonella* does not involve IgA in the first week of study.

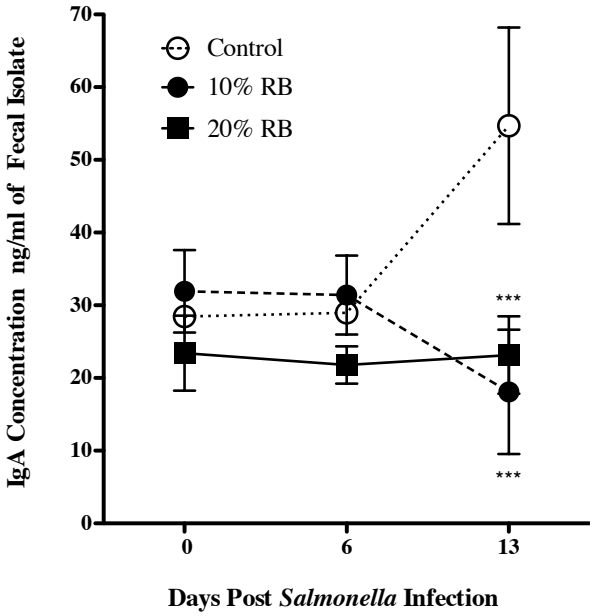


Figure 2.11 Effect of dietary rice bran on fecal IgA level in *S*. Typhimurium infected mice. Fecal IgA was examined in *Salmonella* infected animals fed for 3 weeks (one week prior and 2 weeks post challenge) with 0, 10 and 20 % rice bran. Fecal pellets were tested for IgA at 0, 6 and 13 days post infection. Data are shown as mean ± standard error of mean (n=5 mice/diet group). Repeated measures two-way ANOVA and post hoc Bonferroni test were applied to find significance. This experiment was not repeated.

Effect of dietary rice bran on serum cytokines

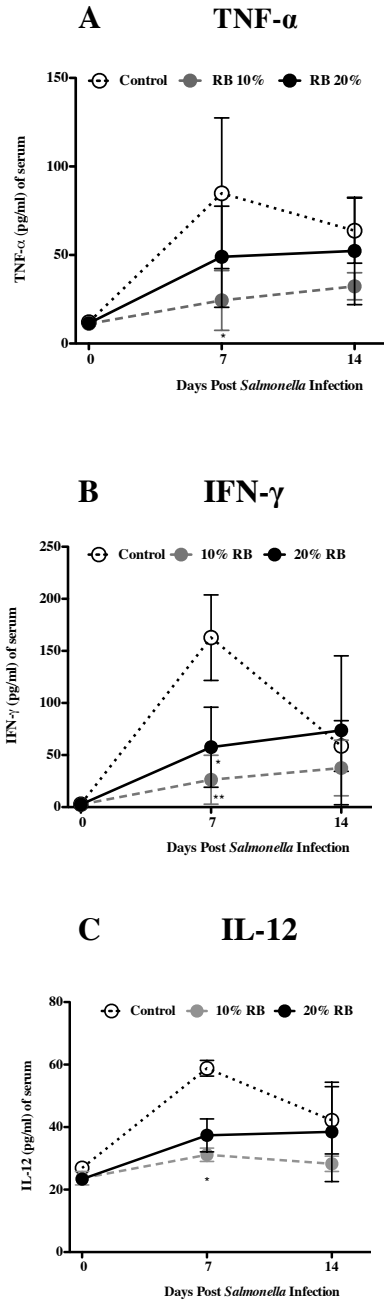


Figure 2.12 Effect of dietary rice bran on serum TNF- α, IFN-γ and IL-12 levels in *S. Typhimurium* infected mice. Blood was drawn at days 0, 7 and 14 following *Salmonella* infection and serum was analyzed for TNF- α (A), IFN-γ (B) and IL-12 (C) levels in control, 10% and 20% rice bran diet groups (RB) fed for 3 weeks. Cytokine levels are expressed in pg/μl. Data are shown as mean ± standard deviation of mean (n=3 mice/diet group). Significance was measured by two-way ANOVA and Bonferroni post hoc test.

Previous research demonstrated that in response to primary *Salmonella* infection, the host immune system releases massive amounts of the cytokines such as TNF-α, IFN-γ and IL-12 locally and systemically (Mittrucker and Kaufmann 2000). The local inflammatory response shifts the microbiota composition allowing *Salmonella* to efficiently colonize the gut (Stecher, Robbiani et al. 2007). Due to the fact that rice bran mediated a decrease in fecal shedding, we next measured the cytokine level in the serum of mice consuming either the 10 or 20% rice bran diets. Mice fed the 10% rice bran diet for 7 days had decreased serum levels of TNF-α, IFN-γ, and IL-12 by 60.4, 136.3 and 27.6 pg/ml respectively in comparison to animals on the control diet ($p<0.05$). Additionally, mice fed the 20% rice bran diet showed decreased levels of serum IFN-γ in comparison to control animals ($p<0.05$). These data (Figure 2.12) suggests that rice bran induced suppression of systemic cytokine production may play a role in reducing the colonization of *Salmonella*.

Effect of dietary rice bran on fecal *Lactobacilli* spp

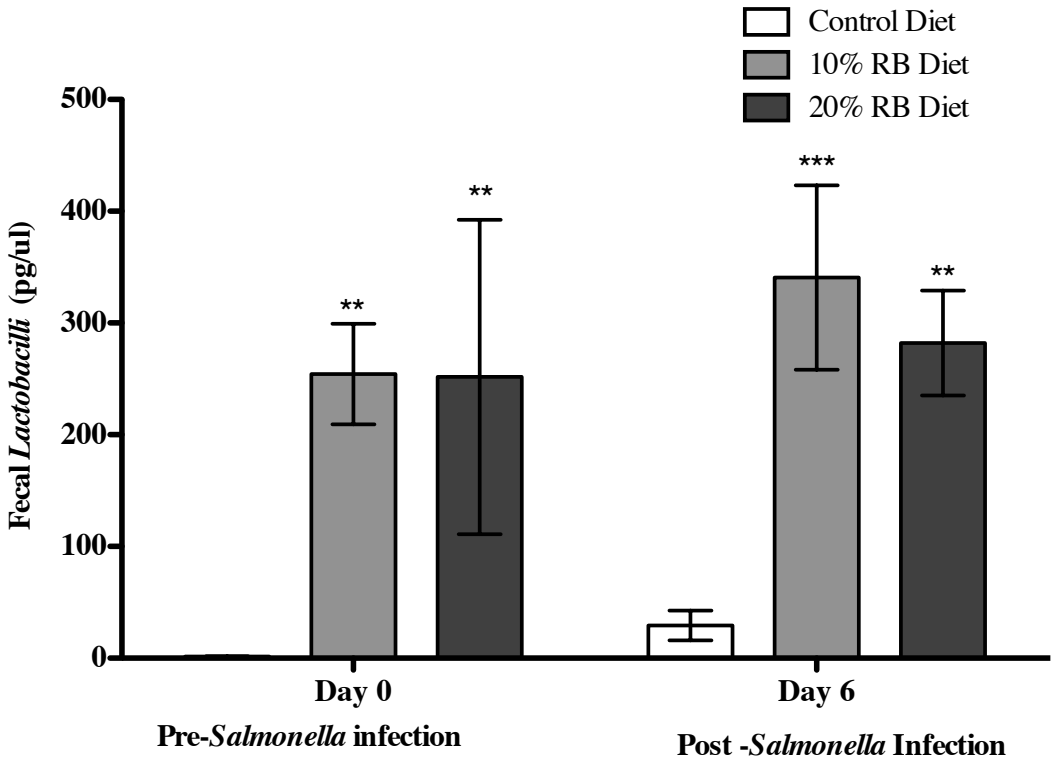


Figure 2.13 Effect of dietary rice bran on fecal *Lactobacilli* spp. Mice were fed 0%, 10% and 20% rice bran diets for 2 weeks. DNA (pg/µl) from fecal pellets of mice before *Salmonella* infection (day 0) and at day 6 (post infection) was determined using qPCR. Error bars indicate standard deviation of mean and * (P<0.05), ** (P<0.01) and *** (P<0.001) denote significant differences in rice bran (RB) fed mice from controls (n=5 mice/diet group). Significance was tested by repeated measured two-way ANOVA and Bonferroni post hoc test. Similar results were observed in additional experiment.

Members of the genus *Lactobacillus* are potent commensal bacteria with potential for eradication of *Salmonella* infection. To know whether dietary rice bran induced reduction in *Salmonella* is mediated by change in intestinal *Lactobacillus*, we measured total number of fecal *Lactobacillus* in rice bran and control diet fed mice. Uninfected mice on the 10 and 20% rice

bran diets had a 170 and 167-fold higher ($p<0.05$) numbers of fecal *Lactobacilli*, respectively, compared to mice on the control diet (Figure 2.13). Following infection, the levels of fecal *Lactobacilli* remained higher (11- and 9-fold) in the mice consuming the rice bran diets than in the control diet fed mice (Figure 2.13). These data suggest that rice bran induced changes in gut microbial ecology may be in part responsible for reduced fecal shedding of *Salmonella*.

Rice bran extract inhibited *Salmonella* entry and replication *in vitro*

The ability of *Salmonella* to invade intestinal epithelial cells is an important step involved in the establishment of infection (Ly and Casanova 2007). The ability of rice bran components to interfere with *Salmonella* entry was tested in the mouse small intestinal epithelial (MSIE) cell model. Concentrations of rice bran extract (RBE) that did not affect MSIE cell viability were used (0-2 mg/ml) in these studies (Figure 2.14 A). RBE (2 mg/ml) reduced the entry of *Salmonella* into MSIE cells by 27% compared to controls ($p<0.05$) (Figure 2.14 C). The RBE in cell culture media did not kill *Salmonella* directly (Figure 2.14 B) and therefore did not confound the results of reduced pathogen entry.

We next assessed the ability of RBE to inhibit the intracellular replication of *Salmonella* in MSIE cells (Figure 2.14 D). After infection and incubation, extracellular bacteria were removed by washing and antibiotic treatment, and kept for 24 h with RBE. The 2 mg/ml dose of RBE reduced intracellular *Salmonella* replication by 30% ($p<0.05$) in comparison to control. No direct effect of RBE on *Salmonella* extracellular growth and replication was detected (Figure 2.14 B). These results suggest that the rice bran extract contains bioactive compounds that block *Salmonella* entry into MSIE cells as well as inhibit intracellular *Salmonella* replication in *in vitro* model.

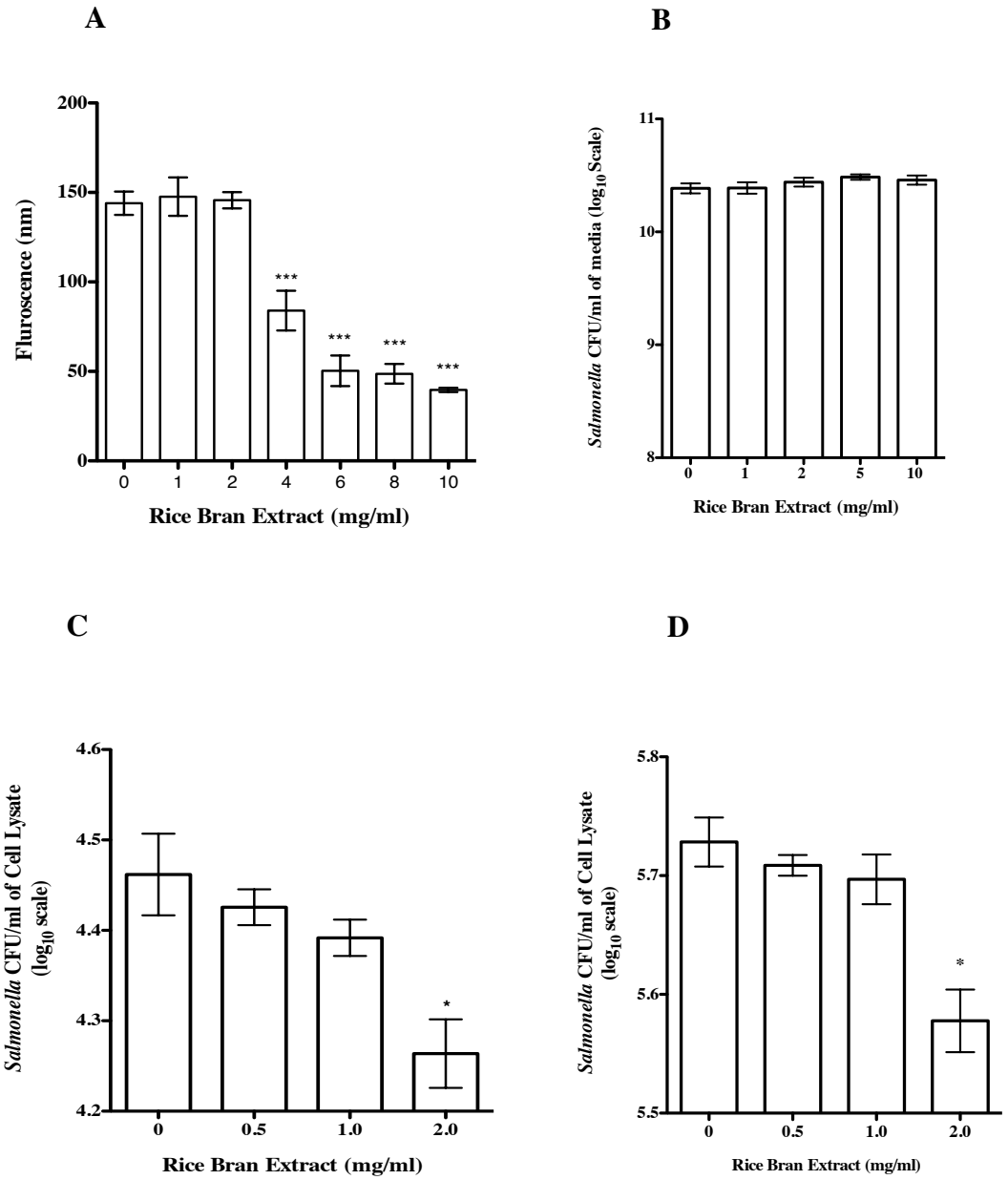


Figure 2.14 Effect of rice bran extract on *S.* Typhimurium entry and intracellular replication in MSIE cells. MSIE cells were pre-incubated with rice bran extract (RBE) at doses of 0, 0.5, 1.0 and 2.0 mg/ml for 24 hours and viability was evaluated (A). RBE was also tested in *Salmonella* culture at doses of 0, 1, 2, 5 and 10 mg/ml for viability (B). MSIE cells were co-incubation with RBE at a dose of 2 mg/ml for 24 hours and infected with *Salmonella*. RBE showed significant inhibition of *Salmonella* entry into MSIE cells (C). RBE was tested for effects on intracellular *Salmonella* replication inside MSIE cells for 24 hours (co-incubated with RBE) (D). Bacteria are shown as mean ± standard deviation of mean $\log_{10}$ CFU per mL of cell lysate (n=3). Significance was determined using a nonparametric (Kruskal Wallis) one-way ANOVA. Statistical differences denoted by * ($P<0.05$) and ** ($P<0.01$). Similar results were observed in additional experiment.

2.6 Discussion

In this study, we examined the ability of dietary rice bran to protect mice against an oral challenge with *Salmonella*. Decreased *Salmonella* fecal shedding is a reliable marker for reduced susceptibility to infection (Monack, Bouley et al. 2004, Wells, Yen et al. 2005, Spiehs, Shurson et al. 2008) and was used herein to determine whether dietary rice bran supplementation reduced susceptibility to *Salmonella* infection. Fecal shedding of *Salmonella* from orally challenged mice fed 10 and 20% rice bran diets was significantly reduced as compared to control (Figure 2.7). Consistent with previous research, the highest number of fecal *Salmonella* in the control diet fed mice was observed on day 7, followed by a reduction in *Salmonella* numbers on days 8-13 (Figure 2.7) (Monack, Bouley et al. 2004). *Salmonella* fecal shedding in rice bran fed mice was consistently lower than control diet fed mice until day 9-post infection. In preliminary studies, no significant difference was found between *Salmonella* fecal shedding of powdered control and rice bran diets (Figure 2.5). This could happen due to slight difference in dietary composition (Table 2.1 and Table A.3) or the age of animals. The age of mice was 6-8 weeks at the initiation of this study (Figure 2.5) where as in next study the mice were 4-6 week old (Figure 2.6). Proximate analysis of diets showed that diet containing 10 and 20% rice bran had lower amount of crude fiber as compared to control diet (Table 2.2). Thus, the total amount of fiber may not be a component for the protection against *Salmonella* infection. However, the quality of fiber may have implication and separate studies are required to assess the efficacy of rice bran fiber in *Salmonella* infections. Similarly protein and crude fat content are also almost similar in control diet and rice bran diets (10 and 20%). These data indicate that control diet and rice bran diets are isocaloric however; the quality of these macronutrients could have healthful effects.

We chose this mouse model of *Salmonella* infection over other models because in contrast to other strains that die 7-14 days after *Salmonella* inoculation, the 129S6/SvEvTac mice do not die from disseminated *Salmonella* infection due to presence of both functional copies of the *nramp1* gene (Monack, Bouley et al. 2004). Although our data suggested that rice bran supplementation showed a decreased trend of *Salmonella* invasion in the ileum, Peyer's patches and mesenteric lymph node of the rice bran fed mice, these values were not statistically significant (Figure 2.9). Thus, the rice bran diet reduced *Salmonella* fecal shedding may be a result of the induction of increased colonization resistance in the intestinal lumen as opposed to the increased horizontal transfer of *Salmonella* into the tissues (Ten Bruggencate, Bovee-Oudenhoven et al. 2004).

Gut inflammation resulting from *Salmonella* presence favors the colonization and growth of the *Salmonella* because of changes in gut ecology and environment (Stecher, Robbiani et al. 2007). Local inflammation in the intestine occurs in conjunction with a massive systemic release of TNF-α, IFN-γ and IL-12 (Lalmanach and Lantier 1999, Mittrucker and Kaufmann 2000, Kirby, Yrlid et al. 2002). The rice bran fed mice showed a significant reduction in serum inflammatory cytokines associated with *Salmonella* infection, namely TNF-α, IFN-γ and IL-12 (Figure 2.12 A-C). The presence of *Salmonella* antigens in the lumen is in part responsible for inducing the inflammatory cytokines in control diet fed animals. Therefore, a reduced *Salmonella* antigen load in the lumen of rice bran fed mice may have diminished this inflammatory response. Determining the mucosal immune cells involved in the development of local and systemic inflammation by *Salmonella* in these mice will be important for understanding the mechanisms by which rice bran modulates the inflammatory response.

Secretory IgA (SIgA) plays a protective role against *Salmonella* (Michetti, Mahan et al. 1992, Mantis and Forbes 2010). SIgA prevent the microbial access to intestinal epithelium by binding the epitopes present on *Salmonella*. The O antigens component of *Salmonella* LPS are targeted by SIgA and affect the expression of *Salmonella* pathogenicity island-1 (SPI-1), a type three secretion system that helps *Salmonella* in epithelial cell invasion (Mantis and Forbes 2010). Another specific set of SIgA could bind the flagellin protein and affect the *Salmonella* motility. However, onset of adaptive immune in response to pathogens takes more than one week in mice depending upon the strain (Cerutti and Rescigno 2008). Our data (Figure 2.11) shows an increase in fecal SIgA in the control group on day 13 but not on day 6 as compared to rice bran fed group. We speculate that control diet fed group mice had a higher burden of *Salmonella* and IgA is one of the responses to contain *Salmonella*. The onset of IgA secretion in feces is also consistent with our *Salmonella* fecal shedding data (Figure 2.7) where animals on control diet showed reduction in *Salmonella* shedding at day 12. Hence, lower fecal IgA in rice bran diet fed animals as compared to control group indicates lower burden of *Salmonella* in intestine. Since we measured non-specific SIgA in feces, future experiments shall determine the specificity of fecal SIgA for *Salmonella*.

Given that *Salmonella* induces changes in the gut microbiome (Stecher, Robbiani et al. 2007, Barman, Unold et al. 2008), we next explored differences in the gut microbial communities between control and rice bran fed mice as a plausible mechanism for the reduced colonization of *Salmonella* (Figure 2.7). Rice bran fed animals demonstrated a ~170 fold increase in fecal *Lactobacilli* content as compared to control before infection (Figure 2.13). Probiotic *Lactobacilli* protect against *Salmonella* infection through production of lactic acid that modulates bacterial virulence gene expression and can help maintain tight junctions of mucosal

epithelial cells (Ibrahim, Yang et al. 2008, de Moreno de LeBlanc, Castillo et al. 2010, Tanaka, Imai et al. 2010). Changes in the gut microbiota by dietary rice bran warrant a separate study to explore this novel mechanism for prevention and reduced susceptibility to *Salmonella* infection.

Rice bran is a collection of numerous bioactive components that may exhibit multiple mechanisms of action for protection against enteric pathogens. Methanol extracts contain bioactive polyphenols and fatty acids from rice bran (Eloff 1998), and were used for the treatment of MSIE cells *in vitro*. RBE reduced the cellular entry of *Salmonella* by 27% in comparison to control (Figure 2.14 C). In addition to reduced *Salmonella* entry, RBE also decreased intracellular *Salmonella* replication by 30% (Figure 2.14 D). These *in vitro* findings merit further investigation of the rice bran effects on the epithelium *in vivo*. Rice bran phytochemicals may inhibit pathogen entry and intracellular replication of *Salmonella* either by modulating the epithelial cytoskeleton, blocking receptors, altering the cellular microenvironment, and/or by influencing virulence gene expression (Santos, Raffatellu et al. 2009, Winter, Keestra et al. 2010). Additional mechanisms may include increased production of bile and gastric acids and increased intestinal motility by dietary rice bran. Future studies are warranted to elucidate these mechanisms and to determine the specific combinations of bioactive rice bran components responsible for protection against infection (Figure 2.15). Our findings provide a rationale for biomedical scientists to work closely with rice crop scientists for advancing our understanding of rice bran-microbe interactions. These findings set the stage for additional work with rice industry, public health and veterinary nutritionists to determine whether the dietary supplementation of rice bran offers greater mucosal protection against enteric infections in people and animals.

Conclusions

Our study reveals dietary rice bran intake mitigates *Salmonella* enterica Typhimurium infection in mice. Increasing consumption of rice bran represents a promising and novel means for reducing susceptibility to enteric infection with *Salmonella*, potentially through the modulation of native gut *Lactobacillus* spp. Further investigation in animal models and human clinical studies will be necessary to elucidate the mechanisms of action.

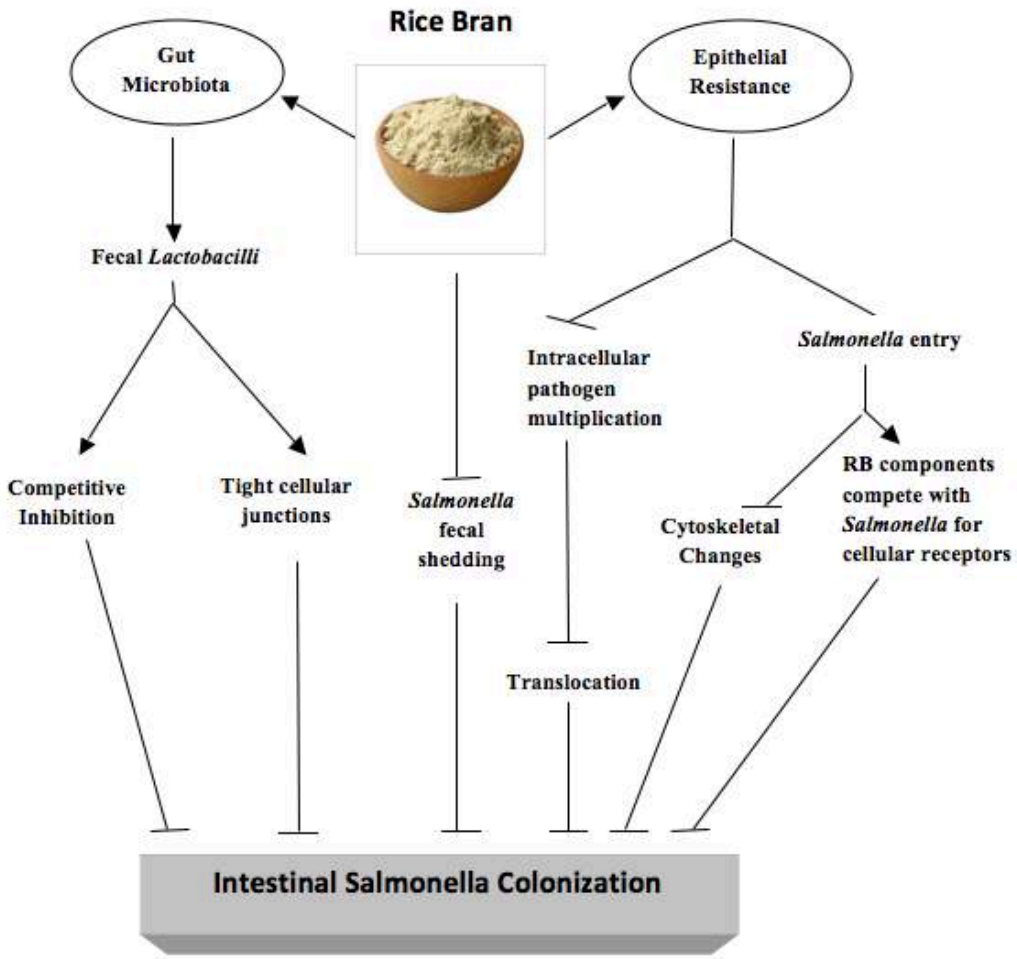


Figure 2.15 Potential mechanisms involved in dietary rice bran induced reduction in susceptibility to *Salmonella* infection. Rice bran may inhibit *Salmonella* colonization via modulation of *Lactobacilli*, preventing cellular entry of *Salmonella*, and inhibiting intracellular replication.

REFERENCES

Abouzeed, Y. M., S. Baucheron and A. Cloeckaert (2008). "ramR mutations involved in efflux-mediated multidrug resistance in Salmonella enterica serovar Typhimurium." Antimicrob Agents Chemother **52**(7): 2428-2434.

Andersson, D. I. and D. Hughes (2010). "Antibiotic resistance and its cost: is it possible to reverse resistance?" Nat Rev Microbiol **8**(4): 260-271.

Arshad, M. M., M. J. Wilkins, F. P. Downes, M. H. Rahbar, R. J. Erskine, M. L. Boulton, M. Younus and A. M. Saeed (2008). "Epidemiologic attributes of invasive non-typhoidal Salmonella infections in Michigan, 1995--2001." Int J Infect Dis **12**(2): 176-182.

Barman, M., D. Unold, K. Shifley, E. Amir, K. Hung, N. Bos and N. Salzman (2008). "Enteric Salmonellosis Disrupts the Microbial Ecology of the Murine Gastrointestinal Tract." Infection and Immunity **76**(3): 907-915.

Barnes, D. S., N. K. Clapp, D. A. Scott, D. L. Oberst and S. G. Berry (1983). "Effects of wheat, rice, corn, and soybean bran on 1,2-dimethylhydrazine-induced large bowel tumorigenesis in F344 rats." Nutr Cancer **5**(1): 1-9.

Bird, A. R., T. Hayakawa, Y. Marsono, J. M. Gooden, I. R. Record, R. L. Correll and D. L. Topping (2000). "Coarse brown rice increases fecal and large bowel short-chain fatty acids and starch but lowers calcium in the large bowel of pigs." J Nutr **130**(7): 1780-1787.

Bowden, S. D., V. K. Ramachandran, G. M. Knudsen, J. C. Hinton and A. Thompson (2010). "An incomplete TCA cycle increases survival of Salmonella Typhimurium during infection of resting and activated murine macrophages." PLoS One **5**(11): e13871.

Broide, E., M. Shapiro, I. Boldur, E. Klinowski, A. N. Kimchi, Y. Gluskin and E. Scapa (2005). "Salmonellosis: an epidemiologic study." Isr Med Assoc J **7**(2): 91-94.

Cara, L., C. Dubois, P. Borel, M. Armand, M. Senft, H. Portugal, A. M. Pauli, P. M. Bernard and D. Lairon (1992). "Effects of oat bran, rice bran, wheat fiber, and wheat germ on postprandial lipemia in healthy adults." Am J Clin Nutr **55**(1): 81-88.

Cerutti, A. and M. Rescigno (2008). "The biology of intestinal immunoglobulin A responses." Immunity **28**(6): 740-750.

Cheng, H. H., C. Y. Ma, T. W. Chou, Y. Y. Chen and M. H. Lai (2010). "Gamma-oryzanol ameliorates insulin resistance and hyperlipidemia in rats with streptozotocin/nicotinamide-induced type 2 diabetes." Int J Vitam Nutr Res **80**(1): 45-53.

Choi, S. P., S. P. Kim, M. Y. Kang, S. H. Nam and M. Friedman (2010). "Protective Effects of Black Rice Bran against Chemically-Induced Inflammation of Mouse Skin." Journal of Agricultural and Food Chemistry **58**(18): 10007-10015.

Chou, T. W., C. Y. Ma, H. H. Cheng, Y. Y. Chen and M. H. Lai (2009). "A rice bran oil diet improves lipid abnormalities and suppress hyperinsulinemic responses in rats with streptozotocin/nicotinamide-induced type 2 diabetes." J Clin Biochem Nutr **45**(1): 29-36.

Das, S. K., M. J. Chisti, M. A. Malek, S. Ahmed, F. D. Farzana, F. Ferdous, K. Shahunja, M. A. Salam, T. Ahmed and A. S. G. Faruque (2013). "Socio-demographic, host and clinical characteristics of people with typhoidal and non-typhiodal Salmonella gastroenteritis in urban Bangladesh." Journal of Biomedical Science & Engineering **6**(8).

de Moreno de LeBlanc, A., N. A. Castillo and G. Perdigon (2010). "Anti-infective mechanisms induced by a probiotic Lactobacillus strain against Salmonella enterica serovar Typhimurium infection." International Journal of Food Microbiology **138**(3): 223-231.

Eloff, J. N. (1998). "Which extractant should be used for the screening and isolation of antimicrobial components from plants?" J Ethnopharmacol **60**(1): 1-8.

Fayol-Messaoudi, D., C. N. Berger, M. H. Coconnier-Polter, V. Lievin-Le Moal and A. L. Servin (2005). "pH-, Lactic acid-, and non-lactic acid-dependent activities of probiotic Lactobacilli against Salmonella enterica Serovar Typhimurium." Appl Environ Microbiol **71**(10): 6008-6013.

Gallinger, C. I., D. M. Suárez and A. Irazusta (2004). "Effects of Rice Bran Inclusion on Performance and Bone Mineralization in Broiler Chicks." The Journal of Applied Poultry Research **13**(2): 183-190.

Gerhardt, A. L. and N. B. Gallo (1998). "Full-fat rice bran and oat bran similarly reduce hypercholesterolemia in humans." J Nutr **128**(5): 865-869.

Haneberg, B., D. Kendall, H. M. Amerongen, F. M. Apter, J. P. Kraehenbuhl and M. R. Neutra (1994). "Induction of specific immunoglobulin A in the small intestine, colon-rectum, and vagina

measured by a new method for collection of secretions from local mucosal surfaces." Infect Immun **62**(1): 15-23.

Hemavathy, J. and J. Prabhakar (1987). "Lipid composition of rice (Oryza sativa L.) bran." Journal of the American Oil Chemists' Society **64**(7): 1016-1019.

Henderson, A. J., A. Kumar, B. Barnett, S. W. Dow and E. P. Ryan "Consumption of Rice Bran Increases Mucosal Immunoglobulin A Concentrations and Numbers of Intestinal Lactobacillus spp." J Med Food.

Heuberger, A. L., M. R. Lewis, M.-H. Chen, M. A. Brick, J. E. Leach and E. P. Ryan (2010). "Metabolomic and Functional Genomic Analyses Reveal Varietal Differences in Bioactive Compounds of Cooked Rice." PLoS One **5**(9): e12915.

Ibrahim, S. A., H. Yang and C. W. Seo (2008). "Antimicrobial activity of lactic acid and copper on growth of Salmonella and Escherichia coli O157:H7 in laboratory medium and carrot juice." Food Chemistry **109**(1): 137-143.

Kahlon, T., R. Saunders, F. Chow, M. Chiu and A. Betschart (1990). "Influence of rice bran, oat bran and wheat bran on cholesterol and triglycerides in hamsters." Cereal chemistry **67**(5): 439-443.

Kirby, A. C., U. Yrlid and M. J. Wick (2002). "The innate immune response differs in primary and secondary Salmonella infection." J Immunol **169**(8): 4450-4459.

Komiyama, Y., A. Andoh, D. Fujiwara, H. Ohmae, Y. Araki, Y. Fujiyama, K. Mitsuyama and O. Kanauchi (2011). "New prebiotics from rice bran ameliorate inflammation in murine colitis models through the modulation of intestinal homeostasis and the mucosal immune system." Scand J Gastroenterol **46**(1): 40-52.

Kong, C. K. L., W. S. Lam, L. C. M. Chiu, V. E. C. Ooi, S. S. M. Sun and Y.-S. Wong (2009). "A rice bran polyphenol, cycloartenyl ferulate, elicits apoptosis in human colorectal adenocarcinoma SW480 and sensitizes metastatic SW620 cells to TRAIL-induced apoptosis." Biochemical Pharmacology **77**(9): 1487-1496.

Lalmanach, A. C. and F. Lantier (1999). "Host cytokine response and resistance to Salmonella infection." Microbes Infect **1**(9): 719-726.

Ly, K. T. and J. E. Casanova (2007). "Mechanisms of Salmonella entry into host cells." Cell Microbiol **9**(9): 2103-2111.

Mackay, A. S. a. D. (2010). "A Commentary on the Nutrient-Chronic Disease Relationship and the New Paradigm of Evidence-Based Nutrition." Natural Medicine Journal **2**(12): 10-18.

Malinen, E., T. Rinttila, K. Kajander, J. Matto, A. Kassinen, L. Krogius, M. Saarela, R. Korpela and A. Palva (2005). "Analysis of the fecal microbiota of irritable bowel syndrome patients and healthy controls with real-time PCR." Am J Gastroenterol **100**(2): 373-382.

Mantis, N. J. and S. J. Forbes (2010). "Secretory IgA: Arresting Microbial Pathogens at Epithelial Borders." Immunological Investigations **39**(4-5): 383-406.

Marianelli, C., N. Cifani and P. Pasquali (2010). "Evaluation of antimicrobial activity of probiotic bacteria against Salmonella enterica subsp. enterica serovar typhimurium 1344 in a common medium under different environmental conditions." Res Microbiol **161**(8): 673-680.

Michetti, P., M. J. Mahan, J. M. Slauch, J. J. Mekalanos and M. R. Neutra (1992). "Monoclonal secretory immunoglobulin A protects mice against oral challenge with the invasive pathogen Salmonella typhimurium." Infect Immun **60**(5): 1786-1792.

Mittrucker, H. W. and S. H. Kaufmann (2000). "Immune response to infection with Salmonella typhimurium in mice." J Leukoc Biol **67**(4): 457-463.

Monack, D. M., D. M. Bouley and S. Falkow (2004). "Salmonella typhimurium persists within macrophages in the mesenteric lymph nodes of chronically infected Nramp1+/+ mice and can be reactivated by IFNgamma neutralization." J Exp Med **199**(2): 231-241.

Norazalina, S., M. E. Norhaizan, I. Hairuszah and M. S. Norashareena (2010). "Anticarcinogenic efficacy of phytic acid extracted from rice bran on azoxymethane-induced colon carcinogenesis in rats." Exp Toxicol Pathol **62**(3): 259-268.

Ryan, E. (2011). "Bioactive food components and health properties of rice bran." Journal of the American Veterinary Medical Association **238**(5): 593-600.

Santos, R. L., M. Raffatellu, C. L. Bevins, L. G. Adams, Ç. Tükel, R. M. Tsolis and A. J. Bäumler (2009). "Life in the inflamed intestine, Salmonella style." Trends in microbiology **17**(11): 498-506.

Sha, X., S. Linscombe, S. Blanche, D. Groth, D. Harrell, L. White and S. Theunissen (2010). "Registration of 'Neptune'southern medium-grain rice." Journal of plant registrations **4**(3): 179-182.

Sebastiani, G., V. Blais, V. Sancho, S. N. Vogel, M. M. Stevenson, P. Gros, J. M. Lapointe, S. Rivest and D. Malo (2002). "Host immune response to Salmonella enterica serovar Typhimurium infection in mice derived from wild strains." Infect Immun **70**(4): 1997-2009.

Selma, M. V., J. C. Espin and F. A. Tomas-Barberan (2009). "Interaction between phenolics and gut microbiota: role in human health." J Agric Food Chem **57**(15): 6485-6501.

Spiehs, M. J., G. C. Shurson and L. J. Johnston (2008). "Effects of two direct-fed microbials on the ability of pigs to resist an infection with Salmonella enterica serovar Typhimurium." Journal of Swine Health and Production **16**(1): 27-36.

Stecher, B. and W. D. Hardt (2008). "The role of microbiota in infectious disease." Trends Microbiol **16**(3): 107-114.

Stecher, B., R. Robbiani, A. W. Walker, A. M. Westendorf, M. Barthel, M. Kremer, S. Chaffron, A. J. Macpherson, J. Buer, J. Parkhill, G. Dougan, C. von Mering and W. D. Hardt (2007). "Salmonella enterica serovar typhimurium exploits inflammation to compete with the intestinal microbiota." PLoS Biol **5**(10): 2177-2189.

Steele-Mortimer, O. (2008). Infection of Epithelial Cells With Salmonella enterica. **431:** 201-211.

Stratton-Phelps, M., R. C. Backus, Q. R. Rogers and A. J. Fascetti (2002). "Dietary rice bran decreases plasma and whole-blood taurine in cats." J Nutr **132**(6 Suppl 2): 1745S-1747S.

Tanaka, T., Y. Imai, N. Kumagae and S. Sato (2010). "The effect of feeding lactic acid to Salmonella typhimurium experimentally infected swine." J Vet Med Sci **72**(7): 827-831.

Ten Bruggencate, S. J., I. M. Bovee-Oudenhoven, M. L. Lettink-Wissink, M. B. Katan and R. Van Der Meer (2004). "Dietary fructo-oligosaccharides and inulin decrease resistance of rats to salmonella: protective role of calcium." Gut **53**(4): 530-535.

Tomita, H., T. Kuno, Y. Yamada, T. Oyama, N. Asano, Y. Miyazaki, S. Baba, A. Taguchi, A. Hara, T. Iwasaki, H. Kobayashi and H. Mori (2008). "Preventive effect of fermented brown rice and rice bran on N-methyl-N'-nitro-N-nitrosoguanidine-induced gastric carcinogenesis in rats." Oncol Rep **19**(1): 11-15.

Tsutsumi, K., Y. Kawauchi, Y. Kondo, Y. Inoue, O. Koshitani and H. Kohri (2000). "Water extract of defatted rice bran suppresses visceral fat accumulation in rats." J Agric Food Chem **48**(5): 1653-1656.

Turnbaugh, P. J., V. K. Ridaura, J. J. Faith, F. E. Rey, R. Knight and J. I. Gordon (2009). "The effect of diet on the human gut microbiome: a metagenomic analysis in humanized gnotobiotic mice." Sci Transl Med **1**(6): 6ra14.

Wells, J. E., J. T. Yen and D. N. Miller (2005). "Impact of dried skim milk in production diets on Lactobacillus and pathogenic bacterial shedding in growing-finishing swine1." Journal of Applied Microbiology **99**(2): 400-407.

Whitehead, R. H. and P. S. Robinson (2009). "Establishment of conditionally immortalized epithelial cell lines from the intestinal tissue of adult normal and transgenic mice." Am J Physiol Gastrointest Liver Physiol **296**(3): G455-460.

Winter, S. E., A. M. Keestra, R. M. Tsolis and A. J. Bäumler (2010). "The Blessings and Curses of Intestinal Inflammation." Cell host & microbe **8**(1): 36-43.

CHAPTER 3

DIFFERENTIAL EFFECTS OF RICE BRAN IN INHIBITION OF *SALMONELLA* FECAL SHEDDING ACROSS RICE VARIETIES[2]

3.1 Research rationale

Despite the constant efforts to curb the *Salmonella* burden, it reappears from time to time and has become a challenge for the healthcare. Recently, dietary prophylactic measures have been tested to combat the burden of *Salmonella*. As we eat food thrice a day, diet can be an effective carrier for the delivery of compounds that are safe and effective for disease prevention. Chapter 2 describes that rice bran inhibits *Salmonella* colonization in mice. Rice is grown all over the world and there are many of diverse varieties that are different in genetic and agronomic traits such as disease and pest resistance, drought resistance and yield (Khush 1997). We and others have shown large differences in phytochemical content across rice varieties (Wu, Yu et al. 2012, Forster, Raina et al. 2013) Rice bran has been evaluated for total metabolite profile variation across the varieties and found to be significantly different amongst varieties (Amissah, Ellis et al. 2003, Ryan 2011). Rice bran from diverse varieties should be tested for efficacy against *Salmonella*.

A panel of six rice varieties containing IAC 600, Jasmine 85, IL 121-1-1, Wells, Red Wells and SHU 121, was chosen for evaluation. Wells and Jasmine 85 have brown, long grains that are commercially grown in United States. SHU 121 has brown bran, long grains and is grown in China. Red Wells and IL 121-1-1 have the red bran while IAC 600 has the purple colored bran and is grown in Brazil (Forster, Raina et al. 2013). The rice variety IAC 600 has

[2] Ajay Kumar, Michael A. Grusak, Jan E. Leach, Steven W. Dow, Ann Hess, Anna McClung, and Elizabeth P. Ryan

been studied for health effects in the past. The grains from IAC 600 had higher amount of total soluble phenolic compounds as compared to the light brown colored grain from other varieties (Walter, Marchesan et al. 2013). Diet containing 20% (w/w) IAC 600 rice grain reduced hypercholestrolemia when fed to rats for 30 days as compared to the control diet (Salgado, de Oliveira et al. 2010). Jasmine 85 is a partially blight resistant rice variety and this resistance to blight is associated with higher content of Salicylic acid (Silverman, Seskar et al. 1995). Salicylic acid has been used as an anti-inflammatory agent in the biopharmaceutical industry for a long time. On the other hand, the variety SHU 121 is susceptible to the blight and contains lower amount of Salicylic acid.

3.2 Summary

Dietary rice bran is effective in mitigation of the *Salmonella* colonization in mice in our studies. Rice is a staple food crop all over the world having hundreds of varieties. We have examined bran across varieties in the colorectal cancer cell lines, however; rice bran has not been examined across varieties for inhibition of *Salmonella* colonization. Therefore we conducted the studies to assess the ability of rice bran to inhibit the salmonellosis across the varieties using the in vitro and in vivo models of *Salmonella* infection. Six rice varieties referred as Wells, Red Wells, IL 121-1-1, Jasmine 85, IAC 600 and SHU 121 with known phytochemical differences were chosen. Mouse small intestinal epithelial (MSIE) and Caco-2 cells were pretreated with methanolic extract of rice bran across the varieties and co-incubated with *Salmonella*. The cells were lysed and plated for *Salmonella* enumeration. We found that the rice bran extract (RBE) across the varieties significantly ($p<0.001$ and $p<0.01$) reduced the *Salmonella* entry into the MSIE to different extents as compared to the control. In Caco-2 cells RBE differentially ($p<0.5$) inhibited *Salmonella* entry across the varieties. We further analyzed the varietal differences in a

mouse model of *Salmonella* infection. We found that the variety IAC 600 significantly ($p<0.05$) reduced the *Salmonella* fecal shedding in the mice at days 2, 4 and 6 post *Salmonella* infection as compared to the control diet fed animals. Moreover, IAC 600 significantly ($p<0.05$) reduced the fecal *Salmonella* content relative to the variety SHU 121.

Further, the fatty acid, total phenolic and mineral contents of rice bran across varieties were assessed for correlation with the *Salmonella* fecal shedding. Spearman correlations with lignoceric acid ($r=-0.94$), stearic acid ($r=-0.94$), boron ($r=-0.89$), total phenols ($r=-0.83$) reveal the potential importance of these components yet further studies are required to confirm how these components influence protection against the *Salmonella* colonization.

3.3 Introduction

Rice is a major staple food crop across the world. It supplies about 21% caloric needs of the world population. Rice bran is the outer nutrient rich layer of the whole rice grain and is generally considered as a byproduct of processing. Rice bran is also used in animal feed, whereas rice bran oil has been included in several cosmetic products and for cooking (Orthoefer 2005). In the last two decades research has shown that there are health benefits for rice bran in hyperlipidemia, diabetes and cancer in the in vivo models and clinical trials (Cara, Dubois et al. 1992, Gerhardt and Gallo 1998, Minhajuddin, Beg et al. 2005, Chen and Cheng 2006). Rice bran oil also modulates gut immunity in the animal models (Sierra, Lara-Villoslada et al. 2005). Chapter 2 shows that intake of 10% dietary rice bran decreases *Salmonella* fecal shedding in mice and a decrease in serum inflammatory biomarkers.

There is vast genomic and agronomic information on rice and the species *Oryza sativa* has thousands of varieties (Khush 1997). Some rice varieties have been a part of traditional diet for centuries and some are new to the human consumption. Newer varieties have high yields,

better disease and pest resistance and are able to grow in the dry conditions (Kennedy and Burlingame 2003). The genomic and agronomic differences amongst rice varieties are constituent with the notion that they have metabolite variation in their grains. Kennedy et al., reviewed the variation in food composition of rice across varieties and found significant differences amongst rice varieties in protein, iron, zinc, thiamine, riboflavin and niacin (Kennedy and Burlingame 2003). Rice varieties with a similar amylose content and chemical composition showed different digestibility of starch and different glycemic index (Panlasigui, Thompson et al. 1991). Not only the rice grain composition is different across varieties but rice bran composition is also different in the components such as total fat and crude fiber contents (Amissah, Ellis et al. 2003). Wu et al., showed that blight resistant cultivars of rice are different in the composition by 154 metabolites as compared to the susceptible rice cultivars (Wu, Yu et al. 2012). In this study, linoleic acid was positively correlated with the increased resistance to blight. Moreover, researchers have reported varietal effects of other cereal grains on disease prevention in humans. Comino et al., demonstrated that dietary oat exhibits different immunogenicity across varieties in the celiac disease patients (Comino, Real et al. 2011). In addition to oat, quinoa is also preferred in celiac diseases. However, all the cultivars of quinoa are not similar in their reactivity. In a study by Zevallos et al., sixteen quinoa cultivars were tested and two of them showed the equal stimulation of cells as of gliadin (an allergen found in wheat) by secretion of IFN-γ and IL-15 in the cultured celiac duodenal biopsies (Zevallos, Ellis et al. 2012). Our laboratory has recently shown that methanolic extract from rice bran differentially inhibits the growth of a human colorectal cancer cell line (Forster, Raina et al. 2013). Hence, there is a need to evaluate the efficacy of rice varieties for bran's ability in health promotion.

Based on findings in chapter 1 that rice bran promotes resistance against *Salmonella* and above-mentioned literature on varietal differences in the health effects of cereals, we hypothesized that there will be differences across varieties in the rice bran induced protection against salmonellosis due to their different phytochemical profiles. To evaluate the efficacy of varieties, we chose the in vitro and in vivo models of infection utilized and described in chapter 2.

The primary goal of this study was to examine the dietary rice bran induced changes in *Salmonella* fecal shedding across a panel of varieties with stoichiometric differences in total lipids, fatty acids, phenolics and minerals. Initially, rice bran extract (RBE) from six varieties (Wells, Red Wells, IL 121-1-1, Jasmine 85, IAC 600 and SHU 121) were evaluated in the in vitro model of *Salmonella* entry using polarized Caco-2 (Holzer and Hensel 2012) and Mouse Small Intestinal Epithelial (MSIE) cell lines (Whitehead and Robinson 2009). Caco-2 is a universal in vitro model for human intestinal epithelium infection as these cells develop similar patterns of microvilli found in human small intestine (Sambuy, Angelis et al. 2005) and MSIE cells are pertinent to the in vivo mouse model.

3.4 Material and methods

Rice varieties

Bran from diverse rice varieties was provided by the USDA-ARS Dale Bumpers National Rice Research Center (Stuttgart, AR). The varieties Wells and Jasmine 85 are commercially produced in the southern USA. IAC 600 has been shown to synthesize anthocyanins (Min, Gu et al. 2012) and first developed and commercialized in Brazil. IAC 600 is a purple colored variety that has been shown to possess health promoting traits (Salgado, de Oliveira et al. 2010). As the

name indicates Red Wells, it exhibits red color bran and is a rich source of pro-anthocyanidins. More information on varieties can be obtained from our publication (Forster, Raina et al. 2013).

Animals and feeding schedule

Four to six week old female 129S6/SvEvTac (Taconic Farms, Germantown, NY) mice were provided water and fed a maintenance diet AIN-93M (Harlan Teklad, Madison, WI) *ad libido* for one week (Figure 3.1). After 1 week, mice were randomly divided into 3 sets. **Set 1**: Group 1: AIN-93M Control Diet, Group 2: 10% rice bran diet (Jasmine 85), Group 3: 10% rice bran diet (SHU 121); **Set 2**: Group 1: AIN-93M Control Diet, Group 2: 10% rice bran diet (Wells), Group 3: 10% rice bran diet (Red Wells). **Set 3**: Group 1: AIN-93M Control Diet, Group 2: 10% rice bran diet (IL 121-1-1), Group 3: 10% rice bran diet (IAC 600) (n=10 in each group) and housed with a 12-hour light/dark cycle at 20-25°C. The Animal Care and Use Committee at Colorado State University approved all mouse protocols (Protocol number 09-1457A).

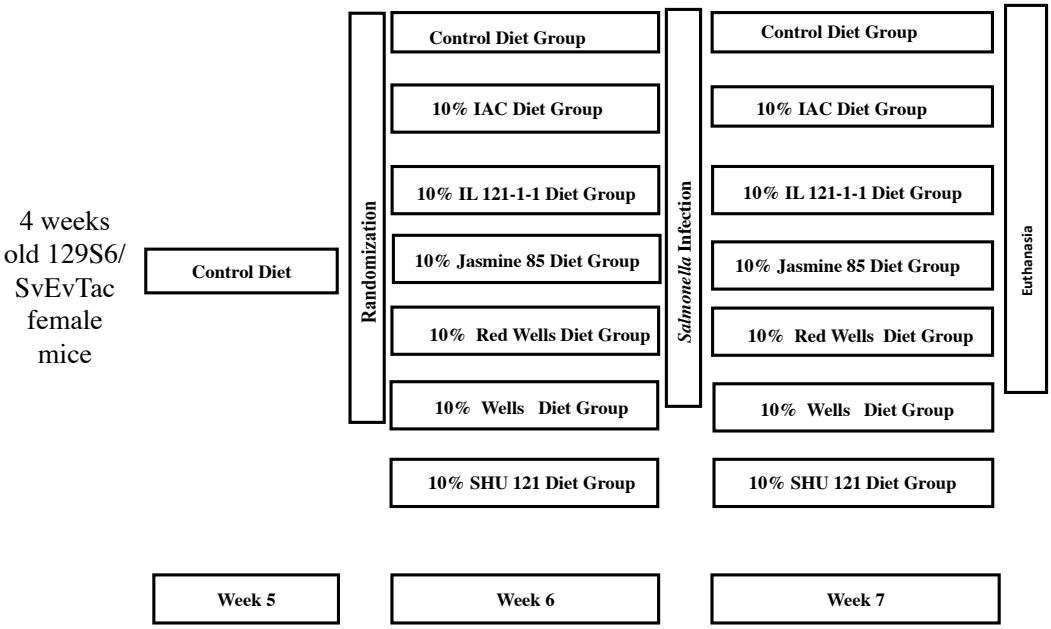


Figure 3.1 Study schema showing the assessment of rice bran for inhibition of *Salmonella* fecal shedding in mice across the rice varieties.

Bacterial infection

Briefly, *Salmonella enterica* serovar Typhimurium strain 14028s stocks were made in 15% glycerol (Fisher Scientific) and stored at -80°C. Frozen *Salmonella* stock was thawed and diluted with PBS and a final concentration of 10^8 CFU/ml was made. Mice were infected with ~2×10^7 CFU in a total volume of 200 µl using a 25-gauge gavage needle.

Diet composition

Diet compositions are shown in the Table 3.1. Purified diet components were used to balance differences in macronutrient. The percent of rice bran incorporated into the diet is expressed as g/100 Kg of diet (Table 3.1). Diet components (Harlan Teklad, Madison, WI) were

homogenized in a mixer. Cornstarch, casein, dextrose and rice bran were mixed for 10 minutes and then premix of cellulose, vitamin mix, mineral mix and choline were added and mixed for 5 minutes. After this, corn oil was added and mixed for another 10 minutes. Powder diets were fed to the mice in the glass dishes (Dytes inc). The composition of rice bran containing the diets was calculated based on the published reports (Barnes, Clapp et al. 1983, Cara, Dubois et al. 1992, Bird, Hayakawa et al. 2000) that demonstrated chronic disease fighting activity.

Fecal collection and processing

Briefly, fecal pellets were collected on the day 0 before *Salmonella* oral challenge, and on the days 2, 4 and 6-post infection. The fecal pellets were diluted with PBS, homogenized and serially diluted and plated on the MacConkey agar (BD Biosciences) with 50 µg/ml of kanamycin (Fisher Scientific). Agar plates were incubated at 37°C under humid conditions for 24 h and bacteria were counted as CFU/g of fecal matter. *Salmonella* was counted and represented as CFU/g of the fecal matter. There was no difference in the morphology of *Salmonella* colonies in the pure culture and infected feces.

Rice bran extract (RBE)

Crude rice bran does not give reliable results in the cellular assays and thereby extracted with 80% methanol called the RBE according to the previously published protocols (Chapter 2). Briefly, heat stabilized rice bran was incubated with 80% methanol at -80°C for 1 h and after centrifugation supernatant was removed and dried. RBE was dried and mixed with the cell culture media to the desired doses for treatment of the MSIE and Caco-2 cell treatment.

Table 3.1 Composition of control (AIN93-M) and rice bran supplemented powdered diets

Constituents (g/kg)	Control	10% RB
Casein	140	125
L-Cystine	1.8	1.8
Corn Starch	465.7	422.7
Maltodextrin	155	155
Sucrose	100	100
Corn Oil	40	19
Cellulose	50	29
Mineral Mix	35	35
Vitamin Mix	10	10
Choline Bitartrate	2.5	2.5
RB	0	100

Cell culture conditions

MSIE Cells: Briefly, MSIE cells were grown in the RPMI 1640 (Mediatech Inc, Manassas, VA) media supplemented with 2.05 mM L-Glutamine (Hyclone Laboratories) supplemented with heat inactivated 10% FBS (Atlas Biologicals), 1% antibiotic (Penicillin and Streptomycin) and antimycotic (Amphotericin) solution (Cellgro, Mediatech Inc), 0.1% Thioglycerol Hydrocortisone (Sigma), 0.004% IFN-γ (Peprotech USA), 0.023% Insulin (Regular Human Insulin, Novo Nordik). The cells were seeded overnight in a 6 well plate at a density of 2×10^5 cell/well. After 12 hours media was aspirated and fresh media was added with rice bran extracts for 24 h at 37°C and 5% CO_2 and 95% humidity.

Caco-2 Cells: Caco-2 cell lines were purchased from American Type Culture Collection (Manassas, VA) and cultured in the RPMI media supplemented with 10% fetal bovine serum (Atlas Biologicals, Fort Collins, CO), 2 mM L-glutamine (Mediatech Inc), 10 mg/mL penicillin, 10,000 IU/mL streptomycin, 25mg/mL amphotericin, 1 mM sodium pyruvate (Mediatech Inc), and 1x MEM nonessential amino acids (Mediatech Inc). RBE was resuspended in the cell culture medium, at a concentration of 2 mg/ml.

Cell viability

The cell viability was determined using AlamarBlue (Invitrogen). Briefly, the cells were seeded in a 96 well plate at $2x10^5$/ml. After 6 hours of adherence, the cells were treated in the presence and absence of RBE for 24 hours at 37°C, 5% CO_2 in maintenance media. Supernatant was removed and alamarBlue was added to the media (20 μg/ml). Fluorescence was detected at excitation: 530/25; emission: 590/35 in ELISA plate reader (Bio-Tek Synergy HT, Winooski, VT).

Salmonella entry and replication

The *Salmonella* entry assay was performed according to the protocol published earlier (Steele-Mortimer 2008). This assay measures the total number of *Salmonella* (the bacteria that is surface attached plus the *Salmonella* internalized in the cell). **The** MSIE cells were grown and treated with RBE for 24 h. Media was aspirated and the cells were re-incubated with fresh media containing *Salmonella* and RBE. Frozen stock of *Salmonella* was mixed in the RPMI media at a MOI (Multiplicity of Infection) of 100-120 in the presence (co-incubation with *Salmonella*) or absence of RBE. After 30 minutes of incubation, media was aspirated, and the MSIE cell monolayer was washed with PBS twice to remove the extracellular bacteria. Fresh media was added to the cells for additional 1 h. There were two additional cycles of washing with fresh media plus 50 μg/ml of gentamicin (Sigma-Aldrich) following 1 h incubations under the same conditions with 5 μg/ml of gentamicin. Media was aspirated and the cell monolayer was washed with PBS twice to remove the extracellular gentamicin. The cell monolayer was placed in 1 ml of buffer (PBS containing 1% TritonX-100 and 0.1% SDS) for 5 minutes. The contents were mixed by pipetting and serially diluted on the MacConkey agar plates (BD Biosciences) with 50 μg/ml of kanamycin (Fisher Scientific) and incubated at 37°C for 24 h. Colonies were counted and presented per ml of cell lysate (Bowden, Ramachandran et al. 2010).

Histological analysis

The terminal ileum from infected mice were harvested and kept in the histological cassettes in a circular loop and stored in 10% neutral buffered formalin. The tissues were then processed, fixed and stained with the hematoxylin and eosin at D-Lab, Colorado State University. Experienced veterinary pathologist at Colorado State University performed the tissue

examination and photography. Tissues were scored on a following scale: (0) no inflammation, (1) mild inflammation (2) moderate inflammation (3) severe inflammation

Rice bran mineral analysis

Dr. Mike Grusak at USDA ARS, Children's Nutrition Research Center Houston, Texas, performed rice bran mineral analysis. Briefly, rice bran was digested in nitric:perchloric acid (4:1) for 1h at 100° C and then samples were dried at 200° C. Samples were resuspended in 15 ml 2% nitric acid and mineral were determined by ICP-OES (CIROS ICP model FCE12; Spectro, Kleve, Germany).

Quantification of total polyphenols and fatty acids

Total soluble polyphenols in rice bran were determined according to the published protocol (Forster, Raina et al. 2013). Briefly 150 μL mixture of Folin– Ciocalteu's reagent/water (1:9) was added to 35 μL of RBE and incubated at 22 °C for 5 min. Later, 115 μL of sodium bicarbonate (7.5% solution) was added and incubated for 30 min at 37 °C. Absorbance was measured at 765 nm (Bio-Tek Synergy HT Multi-Mode microplate reader) after bringing the samples to room temperature. The total soluble polyphenols were estimated using a gallic acid standard curve and expressed as μg of gallic acid equivalents (GAE) per 50 mg of rice bran.

Fatty acids were determined by Folch procedure using chloroform: methanol (2:1, v/v) as solvents and performed by Dr. John E Bauer, Texas A&M University, College Station. Thin layer chromatography was used for fractionation of triacylglycerols. The fatty acid profile was determined using flame ionization and capillary gas chromatography. A full description of these methods can be found in our publication (Forster, Raina et al. 2013).

3.5 Results

Preliminary studies

To evaluate the differences between the varieties for rice bran's effect on *Salmonella*, in vitro experiments were performed first. RBE across the varieties could have different effects on the cell viability. Hence, it was necessary to measure the effect of RBE on cell viability before assessing the *Salmonella* entry assay. The cell viability was determined initially as described in the Section 3.4. RBE was prepared using 80% methanol and cells were pretreated with RBE for 24 h. The cells were treated with RBE at a dose of 2mg/ml as used in the previous experiments in chapter 2. RBE did not decrease the cell viability in the MSIE cells except the extract from Jasmine 85 variety where it reduced 18% viability ($p<0.001$) as compared to the control. Jasmine 85 also reduced the cell viability to a significant extent ($p<0.001$) as compared to other varieties too (Figure 3.2).

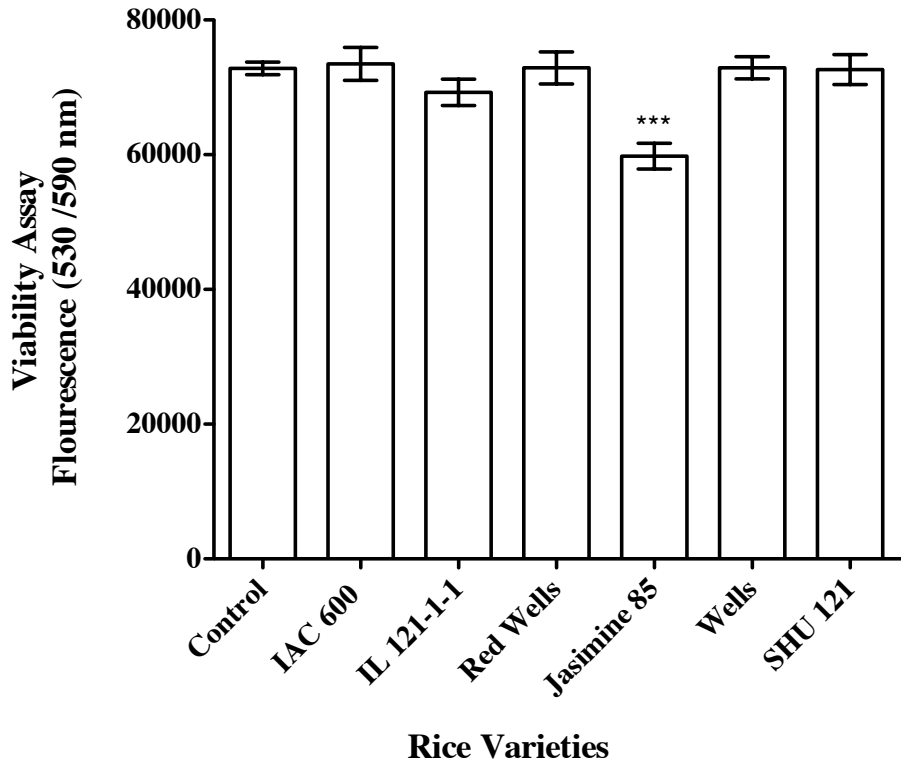


Figure 3.2 Effect of rice bran extract (RBE) across varieties on cell viability of the MSIE cells. Cells were treated with RBE at a dose of 2 mg/ml and incubated for 24 hours. Cell viability was measured using AlamarBlue at 530 nm (excitation)/ 590 nm (emission). Varieties were compared to control (no RBE) treatment. Data was analyzed using one-way ANOVA with Tukey's multiple comparison post hoc tests. Error bars are shown with mean ± standard deviation (SD). Significance is represented by ***$p<0.001$. Similar results were seen in one additional experiment.

Various cell lines can respond differentially to the similar treatments and hence to make sure that differential effect of the varieties against *Salmonella* in the in vitro model is not specific to one cell line, we next evaluated the efficacy of extracts from six varieties in the Caco-2 cells. The Caco-2 cells are essentially human epithelial colorectal adenocarcinoma however; in culture conditions they become polarized and shows similar characteristics to enterocytes of small

intestine by showing microvilli and a number of enzymes and transporters (Pinto, Robineleon et al. 1983, Hidalgo, Raub et al. 1989, Borchardt 2011). The cell viability was evaluated in Caco-2 cells with 24 h treatment with RBE across six varieties at a dose of 2 mg/ml. Both the extracts from varieties Red Wells and SHU 121 reduced the cell viability by 20% ($p<0.05$) as compared to extract free control media. There was no significant difference found in cell viability when all varieties were compared to each other (Figure 3.3).

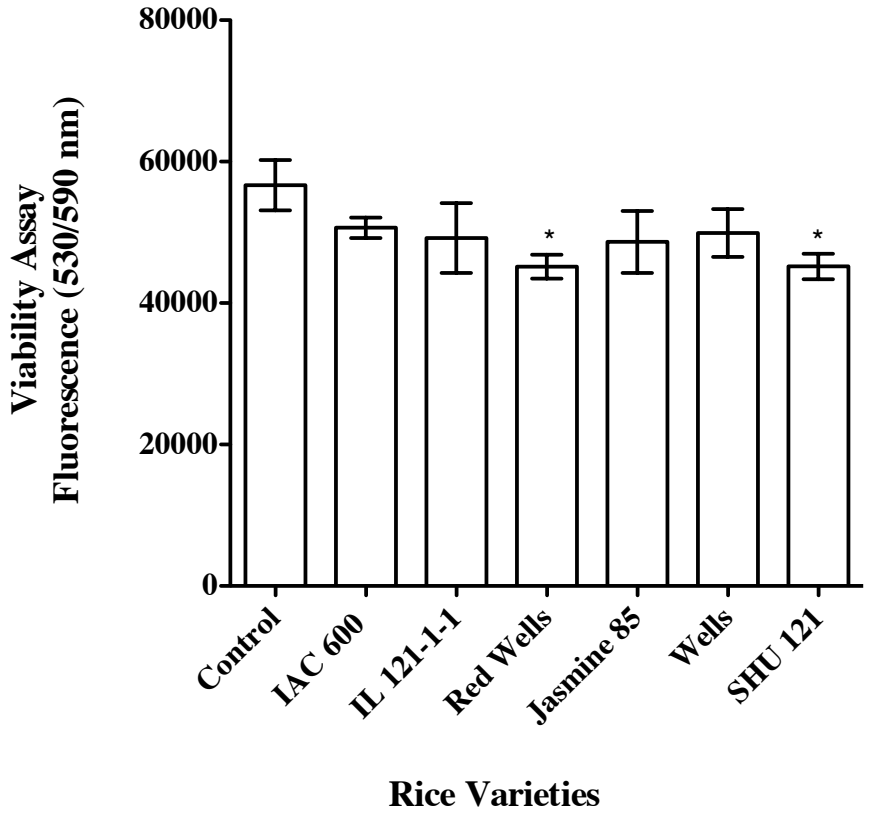


Figure 3.3 Effect of rice bran extract (RBE) across varieties on cell viability of the Caco-2 cells. Cells were treated with RBE at a dose of 2mg/ml and incubated for 24 hours. Cell viability was measured using AlamarBlue at 530 nm (excitation)/ 590 nm (emission). Varieties were compared to control group (no RBE). Data was analyzed using one-way ANOVA with Tukey's multiple comparison post hoc tests (n=3). Error bars are shown with mean ± standard deviation (SD). Significance is represented by *$p<0.05$. Similar results were seen in one additional experiment.

Effect of RBE across varieties on *Salmonella* entry assay in the MSIE cells

Next we evaluated the efficacy of RBE across the varieties against *Salmonella* entry into the MSIE cells to know whether RBE across varieties differentially block *Salmonella* entry into the cells. Briefly, the MSIE cells were incubated with RBE for 24 h. Further, *Salmonella* was added at 100 MOI (Multiplicity of Infection) and the extracellular bacteria were washed with gentamycin. The cells were lysed and bacterial CFU were enumerated as described in the section 2.7. We found that RBE from IAC 600, Red Wells and SHU 121 reduced the *Salmonella* entry into the cell at a dose of 2 mg/ml on an average by 83, 79 and 80% respectively ($p<0.001$) as compared to the extract free control media. The other three varieties IL 121-1-1, Jasmine 85 and Wells reduced the entry of *Salmonella* into the MSIE cell by 52, 59 and 54% respectively ($p<0.001$) as compared to the extract free control media (Figure 3.3). The *Salmonella* entry into the MSIE cells was also significantly ($p<0.5$ to $p<0.001$) different between IAC 600, Wells, SHU 121 and IL 121-1-1, Jasmine 85 and Wells.

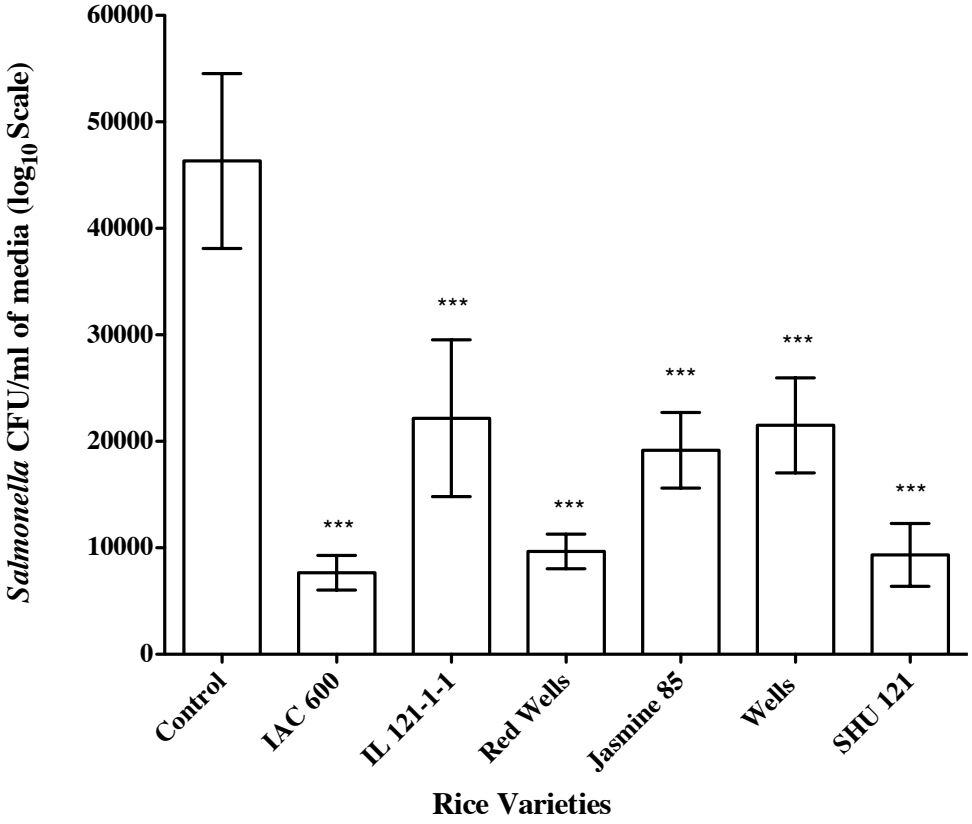


Figure 3.4 Effect of Rice Bran Extract (RBE) on *S.* Typhimurium entry in the MSIE cells. MSIE cells were seeded with RBE from a set of varieties and control at a dose of 2.0 mg/ml for 24 hours before infection. *Salmonella* was co-incubated with RBE and cell were washed, lysed and plated on agar. All varieties were compared with control. Bacteria are shown as mean ± standard deviation CFU per mL of cell lysate (n=6). Significance was determined using a one-way ANOVA, followed by Tukey's multiple comparison tests (n=6). Statistical differences are denoted by ** (P<0.01) and *** (P<0.001). Data is consolidated from two independent experiments.

Effect of RBE across varieties on *Salmonella* entry assay in the Caco-2 cells

Next, we compared the efficacies of RBE from the varieties in the *Salmonella* entry assay in the Caco-2 cells. We found that RBE from varieties IAC 600, Jasmine 85 and SHU 121

reduced *Salmonella* entry into the Caco-2 cells by 60, 71 and 77% respectively ($p<0.001$) as compared to the extract free control group (Figure 3.5).

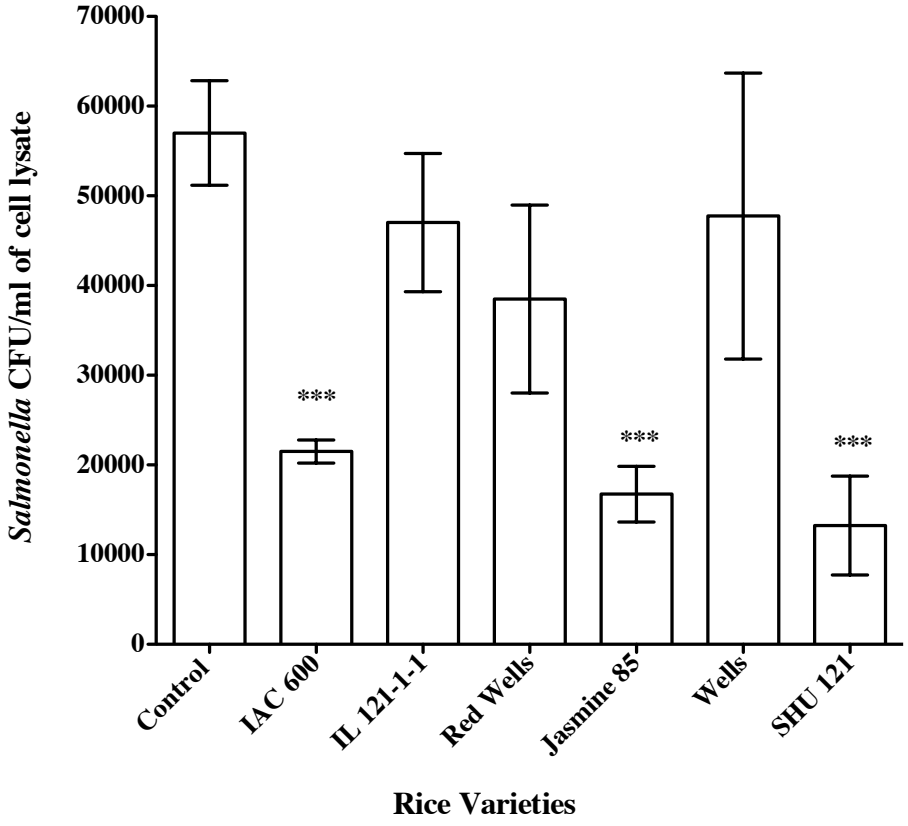


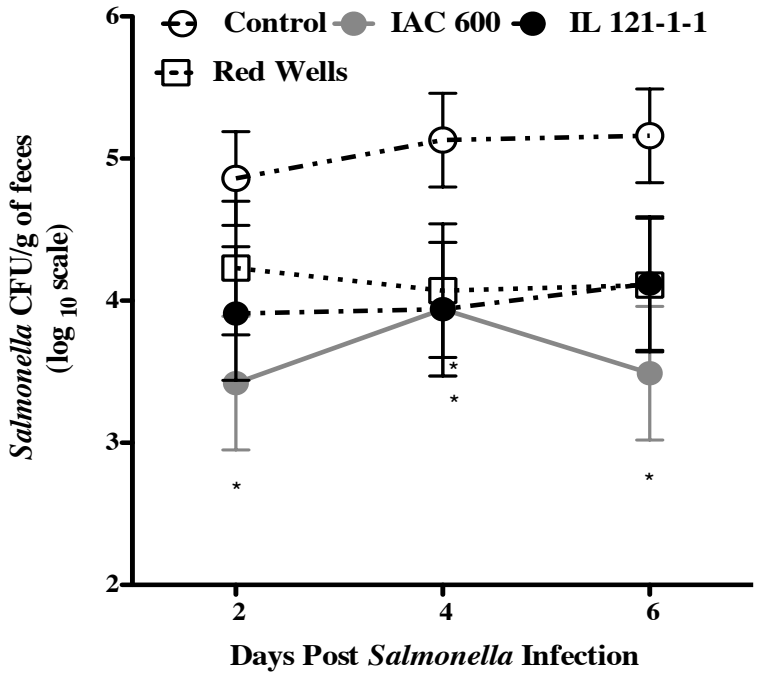
Figure 3.5 Effect of Rice Bran Extract (RBE) on *S.* Typhimurium entry in the Caco-2 cells. Caco-2 cells were seeded with RBE from a set of varieties and control at a dose of 2.0 mg/ml for 24 hours before infection. *Salmonella* was co-incubated with RBE and cell were washed, lysed and plated on agar. All varieties were compared with control. Bacteria are shown as mean ± standard deviation CFU per mL of cell lysate (n=3). Significance was determined using a one-way ANOVA, followed by Tukey's multiple comparison tests (n=3). Statistical differences are denoted by * ($P<0.05$) and *** ($P<0.001$). Similar results were seen in one additional experiment.

RBE from the three varieties, IL 121-1-1, Red Wells and Wells, did not show inhibition of *Salmonella* entry as compared to the control group. Three varieties IAC 600, Jasmine 85 and SHU 121 inhibited *Salmonella* entry into the Caco-2 cells as compared to the control group, but RBE from IL 121-1-1, Red Wells and Wells did not ($p<0.05$). These data show that RBE across the varieties differentially inhibits the *Salmonella* entry into the MSIE and Caco-2 cells.

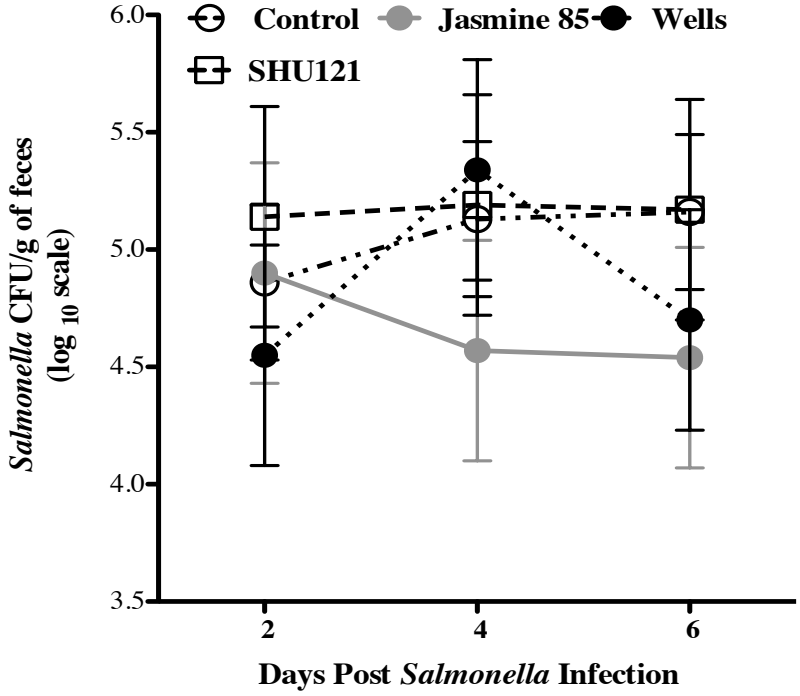
Effect of dietary rice bran on *Salmonella* fecal shedding across varieties

After observing differential effects of varieties against *Salmonella* entry in the in vitro models, we further evaluated the varietal differences in the mouse model for protection against *Salmonella*. To test the varieties in animal model, female 129S6/SvEvTac mice were infected with *Salmonella enterica* Typhimurium14028s using fecal *Salmonella* shedding as a marker of infection. Rice bran (10% w/w) was mixed in the AIN 93-M diet and fed to mice (Table 3.1). The mice were fed control diet for one week and then randomized in to the different dietary groups (n=10). One week later, mice were orally infected with 2×10^7 CFU/animal in 200 µl and *Salmonella* fecal shedding was evaluated by agar plate method at day 2, 4 and 6-post infection (Figure 3.6). Rice bran diet was continued during the post *Salmonella* infection period.

A


Control
IAC 600
IL 121-1-1
Red Wells
Salmonella CFU/g of feces
(log 10 scale)
6
5
4
3
2
*
*
*
2
4
6
Days Post Salmonella Infection

B


Control
Jasmine 85
Wells
SHU121
Salmonella CFU/g of feces
(log 10 scale)
6.0
5.5
5.0
4.5
4.0
3.5
2
4
6
Days Post Salmonella Infection

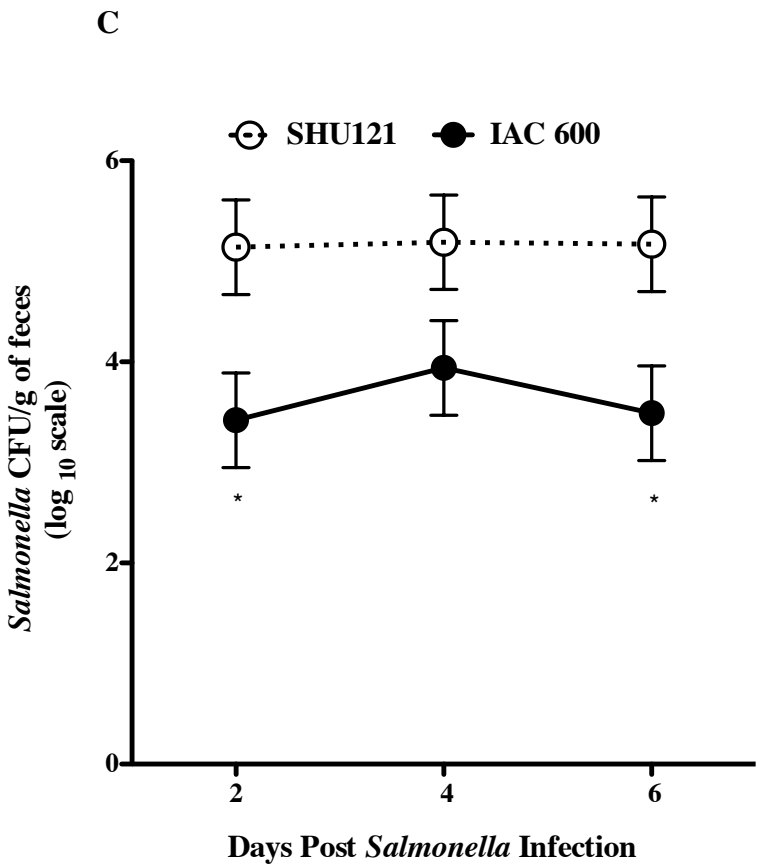


Figure 3.6 Effect of dietary rice bran on *Salmonella* fecal shedding of mice across varieties. Mice (n=10) were fed with rice bran for two weeks (1 week prior to infection and 1 week post infection) from a diverse panel of varieties and fecal shedding (CFU/g of feces) was measured at day 2, 4 and 6 post *Salmonella* infection. Figure 3.6 A shows comparison of control diet with rice bran diets from varieties IAC 600, IL 121-1-1 and Red Wells. The statistical significance is denoted by * ($p<0.05$). Figure 3.6 B shows comparison of control diet with rice bran diets from varieties Jasmine 85, Wells and SHU 121 in terms of *Salmonella* fecal shedding. Figure 3.6 C shows comparison between SHU 121 and IAC 600 rice bran diets and * represents $p<0.05$. Data was analyzed using mixed procedure in SAS system followed by Tukey-Kramer test. Errors bars represent standard error of mean (SEM). Similar results were seen in one additional experiment.

Salmonella fecal shedding is a gold standard measurement of *Salmonella* infection.

Figure 3.6 shows fecal shedding of *Salmonell*a in mice across a set of diverse varieties at day 2, 4

and 6-post infection. We found that IAC 600 rice bran diet significantly inhibited the *Salmonella* fecal shedding as compared to the control diet fed mice on day 2, 4 and 6 ($p<0.05$) (Figure 3.6 A). The IAC 600 significantly reduced the *Salmonella* fecal shedding in comparison to the SHU 121 on day 2 and 6 post infection ($p<0.05$) (Figure 3.6 C). The bran from rice variety IL 121-1-1, significantly ($p<0.05$) reduced the *Salmonella* shedding as compared to the control diet at day 4-post infection however no significant difference was seen at day 2 and 6 (Figure 3.6 A). The Red Wells, Wells, Jasmine 85 and SHU 121, were not significantly different in *Salmonella* fecal shedding as compared to the control diet fed mice (Figure 3.6 B). These results show that dietary rice bran mediated inhibition of the *Salmonella* fecal shedding differs across rice varieties.

Varietal effect of rice bran on terminal ileum of *Salmonella* infected mice

Salmonella induces inflammation in the intestine after colonization and causes inflammatory changes in the epithelium (Stecher, Robbiani et al. 2007, Valdez, Grassl et al. 2009). Higher fecal shedding may associate with escalated inflammation in lower small intestine (Stecher, Paesold et al. 2006). Hence, we evaluated the differences between IAC 600 and SHU 121 using histopathology of *Salmonella* infected terminal ileum. The mice infected with *Salmonella* and fed control diet showed necrosis and ulceration in the ileal mucosa (Figure 3.7). The extent of mucosal necrosis was lesser in the SHU 121 diet fed mice as compared to the control; however, necrosis in the SHU 121 fed mice was more than IAC 600 diet fed animals. The inflammation and epithelial disruption amongst the dietary groups was in the order of a>c>b. The more noticeable differences occurred in the Peyer's patches (Table 3.2). The pathological scores in the Peyer's patches were in the order a>c>.b. However, no significant difference was found across the group. These results suggest that dietary rice bran may protect the mucosa against *Salmonella*-induced inflammation differentially across the varieties.

Control Diet **IAC 600 Diet** **SHU 121 Diet**

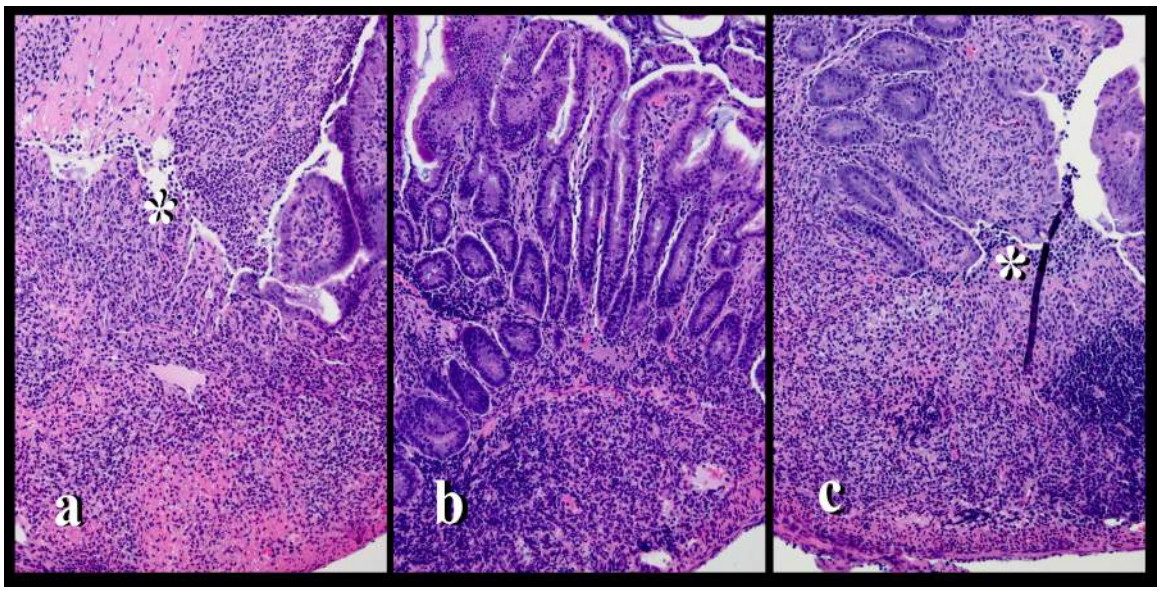


Figure 3.7 Hematoxylin & Eosin staining of *Salmonella* infected mouse terminal ileum

Mice fed with rice bran diets and infected with *Salmonella*, were euthanized at the day 7 post-infection and the ileum was harvested (n=5). Figure 3.7 shows representative terminal ileum from a) control diet fed, b) IAC 600 diet fed, c) SHU 121 diet fed. ∗ Represents mucosal necrosis and ulceration. Pictures are shown at a magnification of 200x in Hematoxylin & Eosin staining. Representative images are shown from similar results obtained from experiments.

Table 3.2 Comparison of inflammatory scores in Peyer's patches among different dietary groups

Parameters	Control Group (n=5)	IAC 600 (n=4)	SHU 121 (n=5)
Inflammation in Peyer's Patches	2.6±0.5	1±1.4	2.2±0.8
Polymorphonuclear Cells in Peyer's Patches	2.6±0.5	1±1.4	2.2±0.8
Mononuclear Cells in Peyer's Patches	1±0	0.75±1.0	1.2±0.8
Epithelial Necrosis	2.2±1.0	0.25±0.5	0.8±1.3
Pseudo Membrane	2.2±1.0	0.5±1	1.6±0.9

(0) no inflammation, (1) mild inflammation (2) moderate inflammation (3) severe inflammation,

± Error represents Standard Deviation

The data was analyzed by using one way ANOVA.

Rice bran components

Rice bran across the varieties exerted differential protection against *Salmonella* in the mice. This differences in *Salmonella* protection across the varieties could be attributed to different phytochemical profile. Hence, we determined the complete profile of fatty acids (Table 3.5), total polyphenols, Vitamin E isoforms, γ Oryzanol (Table 3.4) and complete profile of the mineral contents (Table 3.3) of rice bran across the varieties. The correlation graphs were plotted (Figure 3.8) between components and *Salmonella* fecal shedding across the varieties. The phytochemical profile of rice bran across varieties shows that variety IAC 600 has significantly

($p<0.001$) higher amount of Mn and Zn than SHU 121 (Table 3.3). The variety IAC 600 was also found to have significantly ($P<0.001$) higher content of total phenolics, δ- Tocotrienol and α-Tocopherol than SHU 121 (Table 3.4). IAC 600 had a significantly ($p<0.001$) lower amount of γ-Tocotrienol than in SHU 121 (Table 3.4). Correlation was performed between *Salmonella* fecal shedding and bran components across varieties. The component analyses revealed that the in vivo decrease in *Salmonella* fecal shedding was significantly ($p<0.05$) correlated (spearman) with a higher amount of boron ($r= -0.89$), stearic acid ($r= -0.94$), lignoceric acid ($r= -0.94$) and total soluble phenolic content ($r= -0.84$) (Figure 3.8). However, tocotrienol and tocopherols were not found correlated with *Salmonella* fecal shedding across all the varieties. These results show that rice bran across the varieties differ in their phytochemical contents. Moreover, rice bran across varieties differentially inhibits the *Salmonella* fecal shedding and this difference is potentially due to the rice bran components that act individually or synergistically. However, further studies are required to confirm these statistical correlations.

Table 3.3 Rice bran mineral content* differs across rice varieties

Minerals	IAC 600	IL 121-1-1	Red Wells	Jasmine 85	Wells	SHU 121
B[a]	10.63 ± 0.0	10.28 ± 2.27	10.49 ± 1.28	7.47 ± 0.84	6.09 ± 1.88	6.73 ± 0.72
Co[a]	0.19 ± 0.05	0.2 ± 0.09	0.18 ± 0.00	0.15 ± 0.03	0.25 ± 0.02	0.21 ± 0.02
Cu[a]	7.75 ± 0.41	8.44 ± 0.04	10.1 ± 0.87	8.96 ± 0.15	8.97 ± 0.06	4.54 ± 0.05
Fe[a]	78.26 ±7.09	116.14 ± 19.50	99.31 ± 14.02	83.78 ± 3.86	78.84 ± 0.36	81.68 ± 5.28
Mn[a]	163.47 ± 15.19	163.22 ± 5.10	104.26 ± 9.70	156.08 ± 3.38	275.97 ± 5.83	194.35 ± 0.76***
Mo[a]	1.11 ± 0.10	0.19 ± 0.01	0.84 ± 0.05	0.19 ± 0.05	0.75 ± 0.08	0.4 ± .03
Ni[a]	1.82 ± 0.23	2.4 ± 0.17	1.19 ± 0.18	1.77 ± .09	1.51 ± 0.04	1.11 ± 0.18
Zn[a]	63.84 ± 2.64	78.19 ± 0.63	68.25 ± 4.88	59.21 ± 0.77	64.14 ± 0.70	42.68 ± 1.01***
Ca[b]	0.44 ± 0.04	0.37 ± 0.00	0.28 ± 0.03	0.37 ± 0.01	0.37 ± 0.00	0.29 ± 0.01
K[b]	11.97 ± 0.80	12.88 ± 0.42	13.52 ± 0.98	11.8 ± 0.32	15.66 ± 0.07	12.34 ± 0.07
Mg[b]	5.23 ± 0.53	7.05 ± 0.22	6.37 ± 0.76	6.5 ± 0.23	7.62 ± 0.03	5.46 ± 0.05
P[b]	13.92 ± 1.16	16.55 ± 0.58	15.68 ± 1.83	14.33 ± 0.54	19.1 ± 0.13	12.79 ± 0.05
S[b]	1.77 ± 0.09	1.78 ± 0.02	1.6 ± 0.08	1.48 ± 0.03	1.77 ± 0.02	1.49 ± 0.03

a- μg/g of rice bran, b- mg/g of rice bran

* These experiments were performed by Dr. Mike Grusak's laboratory at USDA-ARS, Baylor College of Medicine, Houston, Texas. Values are presented by mean ± Standard deviation. Duplicate samples were run during the phytochemical estimation. Data was analyzed using two-way ANOVA and Bonferroni post-test for comparison. Comparison is shown between IAC 600 and SHU 121 by *** at $p<0.001$.

Table 3.4 Rice bran components differ across rice varieties revised from Forster et al., (Forster, Raina et al. 2013)

Components	IAC 600	IL 121-1-1	Red Wells	Jasmine 85	Wells	SHU 121
Total Phenolics[a]	369.2 ± 35.8	682.1 ± 4.1	677.9 ± 5.4	224.9 ± 8.4	208.6 ± 6.8	199.5 ± 9.4***
γ-Oryzanol[b]	3.63 ± 0.14	0.93 ± 0.01	4.27 ± 0.01	3.56 ± 0.05	5.13 ± 0.09	3.53 ± 0.01
δ- Tocotrienol[c]	6.03 ± 0.24	6.50 ± 0.04	6.73 ± 0.003	2.07 ± 0.001	5.47 ± 0.01	5.77 ± 0.05***
γ- Tocotrienol[c]	103.53 ± 5.53	126.93 ± 1.41	153.97 ± 0.61	231.60 ± 0.20	184.20 ± 1.79	272.20 ± 3.30***
α- Tocotrienol[c]	98.5 ± 3.8	109.7 ± 2.8	108.3 ±2.1	28.8 ±3.6	98.7 ± 6.4	13.1 ± 0.1
δ- Tocopherol[c]	0.33 ± 0.01	4.33 ± 0.19	0.60 ± 0.09	NA	1.27 ± 0.09	1.37 ± 0.14***
γ- Tocopherol[c]	1.30 ± 0.42	28.82 ± 0.64	14.03 ± 0.61	3.26 ± 0.66	24.37 ± 0.52	41.00 ± 4.15
α- Tocopherol[c]	56.63 ± 1.65	96.55 ± 0.77	56.00 ± 0.47	24.12 ± 3.94	41.63 ± 3.16	5.00 ± 0.28***
Total Fat[d]	9.8	12.05	9.96	5.02	12.6	16.2

a- Total phenolics (GAE) in μg/50 mg of RBE, b- γ-Oryzanol in mg/g of rice bran, c- Tocotrienols and tocopherols in μg/g of rice bran, d- total fat % of rice bran; Values are presented by mean ± Standard deviation. Triplicate samples were run during the phytochemical estimation.

* Vitamin E isoforms and total fat content assays were performed by the laboratory of Dr. John Bauer, Department of Small Animal Clinical Sciences, Texas A&M University, College Station, Texas

Data except total phenolics was analyzed using 2way ANOVA and Bonferroni post-test for comparison. Total phenolics were analyzed by using one-way ANOVA with Tukey's post hoc test. Comparison is shown between IAC 600 and SHU 121 by *** at $p<0.001$.

Table 3.5 Rice bran fatty acid composition differ across rice varieties (Forster, Raina et al. 2013)

Rice Bran Fatty Acid in % of total fats	IAC 600	IL 121-1-1	Red Wells	Jasmine 85	Wells	SHU 121
Myristic Acid 14:0	0.36	0.34	0.31	0.31	0.26	0.35
Palmitic Acid 16:0	16.63	14.21	13.73	18.95	14.76	19.7
Palmitoleic Acid 16:01	0.22	0.14	0.16	0.22	0.17	0.19
Steric Acid 18:0	3.06	2.84	2.32	2.22	1.63	1.81
Oleric Acid 18:1 n9	38.89	43.5	43.32	44.23	42.36	40.78
Vaccenic Acid 18:1 n7	1.44	0.95	0.99	0.98	1.02	1.74
Linoleic Acid 18:2 n6	34.82	33.8	35.22	29.15	36.27	31.08
α- Linolenic Acid 18:3 n3	1.09	0.97	1.09	1.2	1.17	1.27
Arachidic Acid 20:00	1.27	1.37	1	1.08	0.75	1.46
Gadoleic Acid 20:01	0.55	0.67	0.69	0.61	0.7	0.66
Behenic Acid 22:0	0.48	0.44	0.4	0.41	0.32	0.4
Lignoceric Acid 24:00:00	0.81	0.68	0.78	0.64	0.6	0.57

*Fatty acids were determined by the laboratory of Dr. John Bauer, Department of Small Animal Clinical Sciences, Texas A&M University, College Station, Texas.

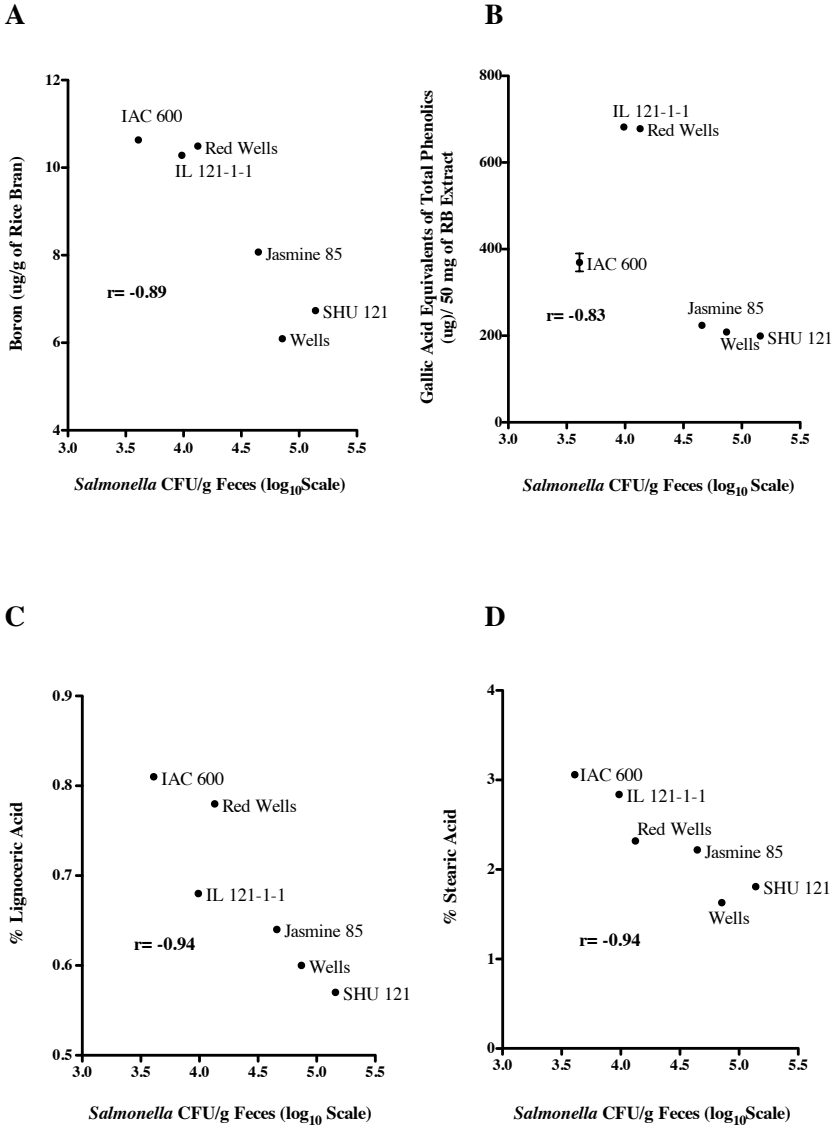


Figure 3.8 Spearman correlation analyses of rice bran components with *S.* Typhimurium fecal shedding in mice. Rice bran components were analyzed and correlated with fecal *Salmonella* shedding by using spearman correlation in Graph-pad Prism. Micro and macro minerals were analyzed in rice bran and a significant correlation (r= -0.89, $p<0.05$) was found between boron and *Salmonella* fecal shedding (Figure 3.8 A). Figure 3.8 B shows correlation between total soluble phenols and fecal *Salmonella* (r=0.83, $p<0.05$). Figure 3.8 C & 3.8 D show correlation for lignoceric (r=-0.94, $p<0.05$) and stearic acid (r=-0.94, $p<0.05$) respectively.

3.6 Discussion

Genetic and agronomic diverse rice varieties differ in their disease and pest resistance and show differences in grain qualities e.g. aroma, size and hardness (Khush 1997, Zhu, Chen et al. 2000, Ni, Colowit et al. 2002, Fitzgerald, McCouch et al. 2009, Huang, Wei et al. 2010). Rice bran from different varieties exhibit a spectrum of anti-oxidant capacity in the in vitro models (Amissah, Ellis et al. 2003, Iqbal, Bhanger et al. 2005, Nam, Choi et al. 2006). Rice bran from the diverse varieties had not yet been examined for differential protection against *Salmonella* infection. Our data shows that RBE differentially inhibited the *Salmonella* entry into the Caco-2 and MSIE cells, respectively (Figures 3.4 and 3.5). The RBE from IAC 600, Jasmine 85 and SHU 121 varieties significantly ($p<0.001$) inhibited the *Salmonella* entry into the Caco-2 cells as compared to the control whereas IL 121-1-1, Wells and Red wells did not inhibit the *Salmonella* entry as compared to the control. In the MSIE cells, RBE from IAC 600, Red Wells and SHU 121 inhibited *Salmonella* entry compared to the control ($p<0.001$), while RBE from Wells, IL 121-1-1 and Jasmine 85 had a lower extent of the *Salmonella* entry inhibition ($p<0.01$). Moreover, IAC 600 significantly reduced the *Salmonella* fecal shedding in comparison to SHU 121 on day 2 and 6 post infection ($p<0.05$) in a mouse infection model (Figure 3.6). These results show that dietary rice bran inhibited *Salmonella* entry and fecal shedding differs across the rice varieties.

After infection, *Salmonella* induces inflammation and necrosis in the intestinal epithelium. The terminal ileums of *Salmonella* infected mice were analyzed for histopathological changes across the varieties. The variety IAC 600 prevented *Salmonella* -induced inflammation and necrosis in mice terminal ileum as compared to the variety SHU 121 (Figure 3.7). The more pronounced effects of varietal difference were observed in the Peyer's patches. IAC 600 fed

mice had a lower histopathological score of PMN's, epithelial necrosis, inflammation and pseudo membrane formation as compared to the mice fed diets containing the SHU 121 variety (Table 3.2). The murine Peyer's patches surrounded with microfold cells, are one of the entry ports for *Salmonella* during the infection (Jepson and Clark 2001), and it is unknown whether rice bran components prevented the *Salmonella* entry into these microfold cells. Furthermore, the differences across the varieties in *Salmonella* fecal shedding could be due to changes in gut microbiota. Sonoyama et al., showed that feeding of rice variety Yukihikari reduced atopic dermatitis related symptoms (Sonoyama, Ogasawara et al. 2010). When the rice variety Yukihikari was compared to other varieties in the BALB/c mouse model, it was found that the gut microbiota pattern in mice was different across the varieties. Also, mice fed with Yukihikari showed lower amounts of *Akkermansia muciniphila*, a mucin degrading bacteria, as compared to brewing rice and waxy rice (Sonoyama, Ogasawara et al. 2010). Additional studies are required to investigate these relationships.

To find the responsible rice bran components in whole bran, we analyzed the mineral content profile, total amount of fat, a series of fatty acids, total soluble phenolic compounds, vitamin E-isoforms and gamma oryzanol in rice bran across the varieties (Table 3.3- 3.5). We found that increased boron in the rice bran is significantly correlated (spearman's correlation coefficient $r = -0.89$) with the decreased amount of *Salmonella* fecal shedding in mice ($p<0.05$). An increase in total soluble phenolic content (measured in Gallic Acid Equivalents) in rice bran was correlated with the decreased amount of *Salmonella* fecal shedding ($p<0.05$, $r= -0.84$). The fatty acids such as the stearic ($p<0.05$, $r= -0.94$) and lignoceric ($p<0.05$, $r= -0.92$) acids were also correlated with the *Salmonella* fecal shedding. These results indicate a possibility for boron,

phenols, stearic acid and lignoceric acid as candidate rice bran bioactive components that may interact to produce the health protective benefits against the *Salmonella* infection.

Boron could play a role in the protection against salmonellosis. *Salmonella* induces inflammation at the infection site and exploits inflammation to colonize itself in the gut environment (Stecher, Robbiani et al. 2007). Dietary boron exerts anti-inflammatory action in a paw swelling model of rat inflammation (Hunt and Idso 1999). Supplementation of dietary boron also attenuated the local inflammatory swelling response in pigs induced by the administration of phytohemagglutinin (Armstrong, Spears et al. 2001, Armstrong and Spears 2003). In rats, dietary boron supplementation also increased the anti-typhoid IgM and IgG concentrations following the intervention of the human typhoid vaccine (Bai, Hunt et al. 1996). Hence further studies are warranted to establish the role of rice bran boron in the prevention of *Salmonella* infection.

The total phenolic content of rice bran was found in the range of approximately 4- 13 GAE mg/g of rice bran across the varieties and was negatively correlated with fecal *Salmonella* shedding. Another study found the total rice bran polyphenols in a range of 2.5- 3.5 GAE mg/g of rice bran (Iqbal, Bhanger et al. 2005). This difference in the polyphenol range could be due to the differences in rice cultivar and their geographical locations. Rice bran phenolic compounds such as ferulic acid and γ- oryzanol possess strong anti-oxidant activities in the in vitro and in vivo models (Srinivasan, Sudheer et al. 2007, Ismail, Al-Naqeeb et al. 2010). It is possible that γ-oryzanol in concert with the other compounds diminishes the oxidative burst of the immune cells during inflammatory phase of *Salmonella* infection. In this study, ferulic acid and γ- oryzanol were not correlated with fecal *Salmonella* shedding across the varieties. Rice bran also contains other polyphenolic compounds such as anisole, *p*-coumaric acid, 4, 7-dihydroxyvanillic acid, protocatechuic acid methyl ester, syringaldehyde, and vanillin (Nam, Choi et al. 2006).

Coumaric acid from *Capsicum annum* extract has been found to inhbit *Salmonella* growth (Dorantes, Colmenero et al. 2000), and a complete polyphenolic profile of rice bran across varieties merits investigation.

We found a significant, negative correlation between stearic acid and the *Salmonella* fecal shedding across the varieties. Stearic acid has been shown to augment the lactic acid production capacity of *Lactobacillus* upon exposure (Kodicek 1945). Increased amounts of lactic acid can reduce *Salmonella* activity (Kim and Marshall 2000). Another study showed that dietary stearic acid reduced cholesterol reabsorption (Schneider, Cowles et al. 2000) and increased the amount of cholesterol in the lumen that may shift the gut microflora (Martinez, Perdicaro et al. 2013). Given that dietary rice bran increases native gut *Lactobacillus spp*., and that there are already published protective effects of *Lactobacillus* in mice against *Salmonella* infection (Asahara, Shimizu et al. 2011), stearic acids from rice bran could directly or indirectly be involved in protection against *Salmonella*. Separate studies are required to evaluate these mechanisms in *Salmonella* infection model of mice. Lignoceric acid is a 24 carbon saturated fatty acid that is found in small amounts in rice bran. Lignoceric acid (tetracosanoic acid) was also negatively correlated with *Salmonella* fecal shedding across varieties. Teponno et al., reported that lignoceric acid extracted from *Dioscora bulbifera*, reduced the motility and growth of *Salmonella* Typh*i* and *paratyphi* when measured using agar diffusion and broth dilution techniques (Teponno, Tapondjou et al. 2006). Taken together, the effects of lignoceric acid on *Salmonella* typhimurium should be determined.

By assessing rice bran across varieties in our *Salmonella* infection model, we have shown that there is a range in the potential for protection against *Salmonella* infections. Our observations also indicate that differences in the fecal *Salmonella* shedding may be attributed to

differences in phytochemical contents. Our approach offers a new pre-clinical tool to assess the functional variation in rice bran bioactivity against *Salmonella* infection.

REFERENCES

Amissah, J. G. N., W. O. Ellis, I. Oduro and J. T. Manful (2003). "Nutrient composition of bran from new rice varieties under study in Ghana." Food Control **14**(1): 21-24.

Armstrong, T. A. and J. W. Spears (2003). "Effect of boron supplementation of pig diets on the production of tumor necrosis factor-alpha and interferon-gamma." J Anim Sci **81**(10): 2552-2561.

Armstrong, T. A., J. W. Spears and K. E. Lloyd (2001). "Inflammatory response, growth, and thyroid hormone concentrations are affected by long-term boron supplementation in gilts." J Anim Sci **79**(6): 1549-1556.

Asahara, T., K. Shimizu, T. Takada, S. Kado, N. Yuki, M. Morotomi, R. Tanaka and K. Nomoto (2011). "Protective effect of Lactobacillus casei strain Shirota against lethal infection with multi-drug resistant Salmonella enterica serovar Typhimurium DT104 in mice." J Appl Microbiol **110**(1): 163-173.

Bai, Y., C. D. Hunt and S. M. Newman Jr (1996). "Dietary boron increases serum antibody (IgG and IgM) concentrations in rats immunized with human typhoid vaccine." North Dakota Academy of Science Proceedings **50**: 181.

Barnes, D. S., N. K. Clapp, D. A. Scott, D. L. Oberst and S. G. Berry (1983). "Effects of wheat, rice, corn, and soybean bran on 1,2-dimethylhydrazine-induced large bowel tumorigenesis in F344 rats." Nutr Cancer **5**(1): 1-9.

Bird, A. R., T. Hayakawa, Y. Marsono, J. M. Gooden, I. R. Record, R. L. Correll and D. L. Topping (2000). "Coarse brown rice increases fecal and large bowel short-chain fatty acids and starch but lowers calcium in the large bowel of pigs." J Nutr **130**(7): 1780-1787.

Borchardt, R. T. (2011). "Hidalgo, I. J., Raub, T. J., and Borchardt, R. T.: Characterization of the Human Colon Carcinoma Cell Line (Caco-2) as a Model System for Intestinal Epithelial Permeability, Gastroenterology, 96, 736-749, 1989-The Backstory." Aaps Journal **13**(3): 323-327.

Bowden, S. D., V. K. Ramachandran, G. M. Knudsen, J. C. Hinton and A. Thompson (2010). "An incomplete TCA cycle increases survival of Salmonella Typhimurium during infection of resting and activated murine macrophages." PLoS One **5**(11): e13871.

Cara, L., C. Dubois, P. Borel, M. Armand, M. Senft, H. Portugal, A. M. Pauli, P. M. Bernard and D. Lairon (1992). "Effects of oat bran, rice bran, wheat fiber, and wheat germ on postprandial lipemia in healthy adults." Am J Clin Nutr **55**(1): 81-88.

Chen, C. W. and H. H. Cheng (2006). "A rice bran oil diet increases LDL-receptor and HMG-CoA reductase mRNA expressions and insulin sensitivity in rats with streptozotocin/nicotinamide-induced type 2 diabetes." J Nutr **136**(6): 1472-1476.

Comino, I., A. Real, L. de Lorenzo, H. Cornell, M. A. Lopez-Casado, F. Barro, P. Lorite, M. I. Torres, A. Cebolla and C. Sousa (2011). "Diversity in oat potential immunogenicity: basis for the selection of oat varieties with no toxicity in coeliac disease." Gut **60**(7): 915-922.

Dorantes, L., R. Colmenero, H. Hernandez, L. Mota, M. E. Jaramillo, E. Fernandez and C. Solano (2000). "Inhibition of growth of some foodborn pathogenic bacteria by Capsicum annum extracts." International Journal of Food Microbiology **57**(1-2): 125-128.

Fitzgerald, M. A., S. R. McCouch and R. D. Hall (2009). "Not just a grain of rice: the quest for quality." Trends Plant Sci **14**(3): 133-139.

Forster, G. M., K. Raina, A. Kumar, S. Kumar, R. Agarwal, M. H. Chen, J. E. Bauer, A. M. McClung and E. P. Ryan (2013). "Rice varietal differences in bioactive bran components for inhibition of colorectal cancer cell growth." Food Chemistry **141**(2): 1545-1552.

Gerhardt, A. L. and N. B. Gallo (1998). "Full-fat rice bran and oat bran similarly reduce hypercholesterolemia in humans." J Nutr **128**(5): 865-869.

Hidalgo, I. J., T. J. Raub and R. T. Borchardt (1989). "Characterization of the Human-Colon Carcinoma Cell-Line (Caco-2) as a Model System for Intestinal Epithelial Permeability." Gastroenterology **96**(3): 736-749.

Holzer, S. U. and M. Hensel (2012). "Divergent roles of Salmonella pathogenicity island 2 and metabolic traits during interaction of S. enterica serovar typhimurium with host cells." PLoS One **7**(3): e33220.

Huang, X. H., X. H. Wei, T. Sang, Q. A. Zhao, Q. Feng, Y. Zhao, C. Y. Li, C. R. Zhu, T. T. Lu, Z. W. Zhang, M. Li, D. L. Fan, Y. L. Guo, A. Wang, L. Wang, L. W. Deng, W. J. Li, Y. Q. Lu, Q. J. Weng, K. Y. Liu, T. Huang, T. Y. Zhou, Y. F. Jing, W. Li, Z. Lin, E. S. Buckler, Q. A.

Qian, Q. F. Zhang, J. Y. Li and B. Han (2010). "Genome-wide association studies of 14 agronomic traits in rice landraces." Nature Genetics **42**(11): 961-976.

Hunt, C. D. and J. P. Idso (1999). "Dietary boron as a physiological regulator of the normal inflammatory response: A review and current research progress." Journal of Trace Elements in Experimental Medicine **12**(3): 221-233.

Iqbal, S., M. I. Bhanger and F. Anwar (2005). "Antioxidant properties and components of some commercially available varieties of rice bran in Pakistan." Food Chemistry **93**(2): 265-272.

Ismail, M., G. Al-Naqeeb, W. A. A. bin Mamat and Z. Ahmad (2010). "Gamma-oryzanol rich fraction regulates the expression of antioxidant and oxidative stress related genes in stressed rat's liver." Nutrition & Metabolism **7**.

Jepson, M. A. and M. A. Clark (2001). "The role of M cells in Salmonella infection." Microbes Infect **3**(14-15): 1183-1190.

Kennedy, G. and B. Burlingame (2003). "Analysis of food composition data on rice from a plant genetic resources perspective." Food Chemistry **80**(4): 589-596.

Khush, G. S. (1997). "Origin, dispersal, cultivation and variation of rice." Plant Molecular Biology **35**(1-2): 25-34.

Kim, J. and D. L. Marshall (2000). "Lactic acid inactivation of Salmonella typhimurium attached to catfish skin." Journal of Food Safety **20**(1): 53-64.

Kodicek, E. (1945). "The effect of unsaturated fatty acids on Lactobacillus helveticus and other Gram-positive micro-organisms." Biochem J **39**(1): 78-85.

Martinez, I., D. J. Perdicaro, A. W. Brown, S. Hammons, T. J. Carden, T. P. Carr, K. M. Eskridge and J. Walter (2013). "Diet-induced alterations of host cholesterol metabolism are likely to affect the gut microbiota composition in hamsters." Appl Environ Microbiol **79**(2): 516-524.

Min, B., L. W. Gu, A. M. McClung, C. J. Bergman and M. H. Chen (2012). "Free and bound total phenolic concentrations, antioxidant capacities, and profiles of proanthocyanidins and anthocyanins in whole grain rice (Oryza sativa L.) of different bran colours." Food Chemistry **133**(3): 715-722.

Minhajuddin, M., Z. H. Beg and J. Iqbal (2005). "Hypolipidemic and antioxidant properties of tocotrienol rich fraction isolated from rice bran oil in experimentally induced hyperlipidemic rats." Food Chem Toxicol **43**(5): 747-753.

Nam, S. H., S. P. Choi, M. Y. Kang, H. J. Koh, N. Kozukue and M. Friedman (2006). "Antioxidative activities of bran extracts from twenty one pigmented rice cultivars." Food Chemistry **94**(4): 613-620.

Ni, J., P. M. Colowit and D. J. Mackill (2002). "Evaluation of genetic diversity in rice subspecies using microsatellite markers." Crop Science **42**(2): 601-607.

Orthoefer, F. T. (2005). Rice Bran Oil. Bailey's Industrial Oil and Fat Products, John Wiley & Sons, Inc.

Panlasigui, L. N., L. U. Thompson, B. O. Juliano, C. M. Perez, S. H. Yiu and G. R. Greenberg (1991). "Rice varieties with similar amylose content differ in starch digestibility and glycemic response in humans." Am J Clin Nutr **54**(5): 871-877.

Pinto, M., S. Robineleon, M. D. Appay, M. Kedinger, N. Triadou, E. Dussaulx, B. Lacroix, P. Simonassmann, K. Haffen, J. Fogh and A. Zweibaum (1983). "Enterocyte-Like Differentiation and Polarization of the Human-Colon Carcinoma Cell-Line Caco-2 in Culture." Biology of the Cell **47**(3): 323-330.

Salgado, J. M., A. G. C. de Oliveira, D. N. Mansi, C. M. Donado-Pestana, C. R. Bastos and F. K. Marcondes (2010). "The Role of Black Rice (Oryza sativa L.) in the Control of Hypercholesterolemia in Rats." J Med Food **13**(6): 1355-1362.

Sambuy, Y., I. Angelis, G. Ranaldi, M. L. Scarino, A. Stammati and F. Zucco (2005). "The Caco-2 cell line as a model of the intestinal barrier: influence of cell and culture-related factors on Caco-2 cell functional characteristics." Cell Biology and Toxicology **21**(1): 1-26.

Schneider, C. L., R. L. Cowles, C. L. Stuefer-Powell and T. P. Carr (2000). "Dietary stearic acid reduces cholesterol absorption and increases endogenous cholesterol excretion in hamsters fed cereal-based diets." J Nutr **130**(5): 1232-1238.

Sierra, S., F. Lara-Villoslada, M. Olivares, J. Jimenez, J. Boza and J. Xaus (2005). "Increased immune response in mice consuming rice bran oil." Eur J Nutr **44**(8): 509-516.

Sonoyama, K., T. Ogasawara, H. Goto, T. Yoshida, N. Takemura, R. Fujiwara, J. Watanabe, H. Ito, T. Morita, Y. Tokunaga and T. Yanagihara (2010). "Comparison of gut microbiota and allergic reactions in BALB/c mice fed different cultivars of rice." Br J Nutr **103**(2): 218-226.

Srinivasan, M., A. R. Sudheer and V. P. Menon (2007). "Ferulic acid: Therapeutic potential through its antioxidant property." Journal of Clinical Biochemistry and Nutrition **40**(2): 92-100.

Stecher, B., G. Paesold, M. Barthel, M. Kremer, J. Jantsch, T. Stallmach, M. Heikenwalder and W. D. Hardt (2006). "Chronic Salmonella enterica serovar Typhimurium-induced colitis and cholangitis in streptomycin-pretreated Nramp1 (+/+) mice." Infection and Immunity **74**(9): 5047-5057.

Stecher, B., R. Robbiani, A. W. Walker, A. M. Westendorf, M. Barthel, M. Kremer, S. Chaffron, A. J. Macpherson, J. Buer, J. Parkhill, G. Dougan, C. von Mering and W. D. Hardt (2007). "Salmonella enterica serovar typhimurium exploits inflammation to compete with the intestinal microbiota." PLoS Biol **5**(10): 2177-2189.

Steele-Mortimer, O. (2008). Infection of Epithelial Cells With Salmonella enterica. **431:** 201-211.

Teponno, R. B., A. L. Tapondjou, D. Gatsing, J. D. Djoukeng, E. Abou-Mansour, R. Tabacchi, P. Tane, H. Stoekli-Evans and D. Lontsi (2006). "Bafoudiosbulbins A, and B, two anti-

salmonellal clerodane diterpenoids from Dioscorea bulbifera L. var sativa." Phytochemistry **67**(17): 1957-1963.

Valdez, Y., G. A. Grassl, J. A. Guttman, B. Coburn, P. Gros, B. A. Vallance and B. B. Finlay (2009). "Nramp1 drives an accelerated inflammatory response during Salmonella-induced colitis in mice." Cell Microbiol **11**(2): 351-362.

Whitehead, R. H. and P. S. Robinson (2009). "Establishment of conditionally immortalized epithelial cell lines from the intestinal tissue of adult normal and transgenic mice." American Journal of Physiology-Gastrointestinal and Liver Physiology **296**(3): G455-G460.

Wu, J., H. C. Yu, H. F. Dai, W. L. Mei, X. Huang, S. F. Zhu and M. Peng (2012). "Metabolite profiles of rice cultivars containing bacterial blight-resistant genes are distinctive from susceptible rice." Acta Biochimica Et Biophysica Sinica **44**(8): 650-659.

Zevallos, V. F., H. J. Ellis, T. Suligoj, L. I. Herencia and P. J. Ciclitira (2012). "Variable activation of immune response by quinoa (Chenopodium quinoa Willd.) prolamins in celiac disease." American Journal of Clinical Nutrition **96**(2): 337-344.

Zhu, Y. Y., H. R. Chen, J. H. Fan, Y. Y. Wang, Y. Li, J. B. Chen, J. X. Fan, S. S. Yang, L. P. Hu, H. Leung, T. W. Mew, P. S. Teng, Z. H. Wang and C. C. Mundt (2000). "Genetic diversity and disease control in rice." Nature **406**(6797): 718-722.

CHAPTER 4

CONCLUSIONS AND FUTURE DIRECTIONS

4.1 Study significance

The research presented in this dissertation provides the important pieces of information needed to understand the potential role for dietary agents in the prevention of *Salmonella* infections. *Salmonella* is a worldwide challenge for healthcare, and every year *Salmonella* outbreaks pose significant financial losses in terms of the direct health care costs and productivity (Scharff 2012). Moreover, these outbreaks may cause government agencies to make stringent rules on food safety that could eventually lead to an increased burden of antibiotics and antimicrobials (Landers, Cohen et al. 2012, Souza-Monteiro and Hooker 2013, Verraes, Van Boxstael et al. 2013). Recently, several cases of antimicrobial resistance have emerged due to the over use of antimicrobials (Glenn, Lindsey et al. 2013).

We show that the dietary rice bran protects mice against *Salmonella*. However, clinical trials in animals and humans are required further to substantiate these preclinical results. If these clinical trials are successful, rice bran can have significant impact on food safety by two different ways. First, *Salmonella* is a major problem in poultry, swine and beef industry. Feeding rice bran to the animals may reduce the *Salmonella* burden in food animals and eventually leads to safer production of meat and poultry foods. Second, rice bran can be fed to humans to enhance the immunity against enteric infection so that if the infectious agents are already present in the food chain, these pathogens can be neutralized without any adverse effects. Although, there are drugs and nutritional supplements in the market to fight with *Salmonella* infections, either they are not accessible or affordable to the resourceless population or have some unwanted side effects. Rice bran can be of great asset in the underdeveloped countries to fight against the enteric infections.

Rice is grown in more than 114 countries and consumed by the 65% population of the world. Hence, supplementation of rice bran in the diet is feasible. However, due to extensive genetic and agronomic variations in rice crop, the rice cultivars have different metabolite profiles in the bran. These variations could have implications in selection of better varieties that efficiently prevent the *Salmonella* infection. We found that bran from diverse rice varieties differentially improves colonization resistance against *Salmonella* in mice.

Table 4.1 Review of major study outcomes

Experiment	Results
Effect of prophylactic feeding of 10% dietary rice bran (Neptune) on the *Salmonella* fecal shedding in mice	Reduced the *Salmonella* fecal shedding at days 2-9 as compared to the control diet fed group
Effect of prophylactic feeding of 10% dietary rice bran (Neptune) on the *Salmonella* invasion in mouse liver, spleen, ileum, Peyer's patches and mesenteric lymph node	Lower the invasion of *Salmonella* in the rice bran fed group as compared to the control diet fed group at day 7
Effect of prophylactic feeding of 10% dietary rice bran (Neptune) on the serum cytokines in *Salmonella* infected mice	Reduced the serum levels of TNFα-, IFN-γ and IL-12 at day 7 post *Salmonella* infection compared to control diet fed group
Effect of prophylactic feeding of 10% dietary	Increased levels of fecal *Lactobacilli*

rice bran (Neptune) on the fecal *Lactobacilli* spp.	spp. at day 0 and 7 post *Salmonella* infection as compared to the control diet fed group
Effect of 10% dietary rice bran (Neptune) on *Salmonella* fecal shedding across six varieties in mice	Rice bran from variety IAC 600 reduced *Salmonella* fecal shedding as compared to the variety SHU 121 at days 2 and 6
Effect of rice bran extract (Neptune) at a dose of 2 mg/ml on *Salmonella* entry and replication into the MSIE cells	Decreased entry and replication in MSIE cells as compared to control (no rice bran extract) treatment
Effect of rice bran extract at a dose of 2 mg/ml on *Salmonella* entry into the MSIE cells across six varieties	Varieties IAC 600, Red Wells and SHU 121 reduced *Salmonella* entry to greater extent than IL-121-1-1, Jasmine 85 and Wells
Effect of rice bran extract at a dose of 2 mg/ml on *Salmonella* entry into the Caco-2 cells across six varieties	Varieties IAC 600, Jasmine 85 and SHU 121 reduced *Salmonella* entry to greater extent than IL-121-1-1, Red Wells and Wells
Spearman correlation between minerals, fatty acids, total soluble polyphenols and *Salmonella* fecal shedding across varieties	Boron, stearic acid, lignoceric acids and total soluble polyphenols were negatively correlated with *Salmonella* fecal shedding

4.2 Conclusions

Effect of dietary rice bran on *Salmonella* fecal shedding

Functional foods have gained popularity for having chronic disease fighting properties. Rice bran is already known for health promoting effects. However, it was never tested for enteric infections. Therefore, chapter 2 investigated the ability of dietary rice bran to promote the colonization resistance of *Salmonella* in mice. Fecal culture has been considered a gold standard in diagnosis of *Salmonella* infections (Kotton, Lankowski et al. 2006). We found that pre-feeding of dietary rice bran to the mice at a dose of 10% reduced the *Salmonella* fecal shedding significantly as compared to the control diet fed animals after days 2, 4 and 6 post *Salmonella* infection. Rice bran doses were tested in a range of 10 to 50% (w/w). Although 50% rice bran dose was the most effective in reducing *Salmonella* fecal shedding, a 10% dose was selected for the future experiments because of the practical applications in clinical trials.

We found some key indicators such as modulation of fecal *Lactobacillus* and systemic inflammation in *Salmonella* infection model that can further the understanding of rice bran effects on enteric infection. Dietary rice bran increased fecal *Lactobacilli* after one week of intervention and that effect was also maintained after one week of infection. *Lactobacilli* have been found to be effective against *Salmonella* infection in animal studies (de LeBlanc Ade, Castillo et al. 2010, Castillo, Perdigon et al. 2011). Administration of *Lactobacilli* showed improved animal survival, reduced intestinal inflammation and reduced horizontal transfer of *Salmonella* with increased secretion of IgA in the mouse model of *Salmonella* infection (Nakazato, Paganelli et al. 2011, Castillo, de LeBlanc et al. 2013). *Lactobacilli* can exert anti *Salmonella* activities by several mechanisms of action. *Lactobacilli* can produce lactic acid and acetic acid that reduces the pH and may inactivate or kill the *Salmonella* (Fayol-Messaoudi,

Berger et al. 2005). *Lactobacilli* may also compete for nutrients with the *Salmonella* and may restrict their growth (van der Wielen, Lipman et al. 2002). However, further investigation is needed to clarify how rice bran increased the levels of fecal *Lactobacilli*.

Onset of inflammation is the next step after *Salmonella* establishment in the gut. Various cytokines such as TNF-α, IFN-γ and IL-12 are released in the local and systemic environment in response to the *Salmonella* infection (Mastroeni, Skepper et al. 1995). *Salmonella* exploits the inflammatory response to grow by several routes such as shift in the gut microbiota by nutrient competition (Stecher, Robbiani et al. 2007, Thiennimitr, Winter et al. 2011). We found a reduction in the serum biomarkers of inflammation (TNF-α, IFN-γ and IL-12) in rice bran fed mice after *Salmonella* infection as compared to the control diet fed group. This reduction in serum levels of TNF-α, IFN-γ and IL-12 could have happened due to several mechanisms. Rice bran could have inhibited *Salmonella* growth and division in the mouse lumen leading to a lesser antigenic and endotoxin load that might have resulted in a reduced inflammatory response. Rice bran increased gut *Lactobacilli* spp. in mice and the increased *Lactobacilli* is known to enhance the epithelial integrity and anti-inflammatory response in the experimental models (Wang, Kirpich et al. 2011, Sudhakaran, Panwar et al. 2013). Improved epithelial integrity and anti-inflammatory response could have prevented horizontal transfer of *Salmonella* and dampened the *Salmonella*-induced inflammatory response in the lumen respectively. The altered nutrient availability also gives chances to grow unwanted microflora such as *Akkermensia muciniphila* that increase the rate of mucin degradation (Winter, Thiennimitr et al. 2010). These pathways could be of interest in the future to elucidate the mechanisms of rice bran induced protection against *Salmonella*.

Rice bran could have multidimensional impacts that mediate the protection from *Salmonella*. Several studies have been conducted using in vitro models to elucidate the mechanisms of *Salmonella* entry into the epithelial cells (Gagnon, Zihler Berner et al. 2013, Yoon, Park et al. 2013). To evaluate the efficacy of rice bran using the in vitro *Salmonella* infection, we prepared a methanolic extract from rice bran and pretreated the cells with the extract before infection. RBE significantly decreased *Salmonella* entry and replication in to the MSIE cells as compared to the control group (with no extract). To confirm that RBE is affecting *Salmonella* entry, we tested the viability of MSIE cells and *Salmonella* with RBE. RBE did not affect the viability of the MSIE cells and *Salmonella* at specific doses. Hence, we can conclude that RBE inhibited the *Salmonella* entry into the MSIE cells. One possible mechanism is for RBE to directly affect *Salmonella* virulence, motility or viability. The viability of *Salmonella* was tested in our study and no effect of RBE on the viability was found. Secondly, components of RBE could bind toll-like receptors (TLRs). TLRs are responsible for sensing the *Salmonella* are TLR-4, TLR-2, TLR-5 and TLR-9 (Gerold, Zychlinsky et al. 2007). Binding of RBE to the TLRs could also stimulate cells to release chemokines or cytokines in the media. These cytokines could have affected the *Salmonella* in virulence, motility or viability. Moreover, components could also have permeated inside the cell and reduced cytolytic activities of *Salmonella*. Future studies are required to understand the mechanisms of *Salmonella* invasion inhibition by RBE.

Effect of dietary rice bran on *Salmonella* fecal shedding across varieties

In chapter 2, we demonstrated that the dietary rice bran promotes colonization resistance against *Salmonella* infection (Figure 4.1). Rice is a universal staple food crop and grown in several countries. Rice cultivars have different agronomic conditions, disease and pest resistance,

yield and genetic constitution. Rice bran across varieties also has different phytochemical composition. However, little is known regarding the functionality of rice bran for protection against *Salmonella* across varieties. We demonstrated that rice bran protects the mice against *Salmonella* to different extents across the varieties. Our in-vitro results also indicate the differential capacity of RBE to inhibit the *Salmonella* entry into the MSIE and Caco-2 cells across the varieties. The lower amount of *Salmonella* shedding in the feces could indicate diminished colonization and inflammation in the intestine. The inflammation in the intestine may cause mucosal ulceration and disruption of epithelial integrity. Consistent to the differences in *Salmonella* fecal shedding, marked differences in histopathological scores in the mouse ileum were also observed across the varieties. We observed that all varieties are not equal in their protection against *Salmonella* infection. To find out the responsible rice bran components, we analyzed fatty acid, polyphenols and mineral profile of the whole rice bran across the varieties. The correlation analyses between components and *Salmonella* fecal shedding across the varieties, indicated that stearic acid, lignoceric acid, boron and total polyphenols could be associated with increased protection against *Salmonella*. These studies revealed that rice bran differentially inhibits *Salmonella* fecal shedding in mice across varieties. Figure 4.1 shows the important outcomes of the study and the potential mechanisms to be studied in the future.

We did not determine the vitamin and carbohydrate profiles of the rice bran. Rice bran is a rich source of oil and may contain fat-soluble vitamins such as A, D and K. We found that total fat content across the six selected varieties range from 5-15%. Hence, there is a strong possibility that quantity of these fat-soluble vitamins also differ amongst varieties. We determined vitamin E content across the varieties but not vitamin A and D. Vitamin E was not correlated with the *Salmonella* fecal shedding in mice across the varieties. Vitamin D modulates immunity and may

have implications in protection against infections. For instance, CD8+ T cells of the vitamin D supplemented healthy volunteers, showed lesser amount of IFN-γ and TNF-α and more IL-5 and TGF-β (Lysandropoulos, Jaquiery et al. 2011). These responses could have implications in reducing the inflammation induced by *Salmonella*. Vitamin D deficiency is associated with the higher incidence of respiratory and enteric infections, pneumonia, clostridium infections, sepsis, influenza and HIV infections (Borella, Nesher et al. 2014). Similarly, supplementation of vitamin A reduced mortality in children suffering from measles and diarrhea (Fawzi, Chalmers et al. 1993, Mayo-Wilson, Imdad et al. 2011). Hence, it is worth to determine the profile of fat-soluble vitamins in rice bran across the varieties.

Another important components of rice bran are quantity and quality of fibers. Rice bran contains dietary fibers such as beta-glucans, pectins and gums. A few studies have shown the effects of these fibers on intestinal infections. For example, prophylactic administration of beta-glucans to the weaned pigs reduced the susceptibility to the *E.coli* infections (Stuyven, Cox et al. 2009). Very recently, it is shown that dietary administration of pectin rich soluble plantain, non - starch polysaccharides, inhibited intestinal invasion of *Salmonella* in chickens (Parsons, Wigley et al. 2014). Rice bran may contain variable amounts of these dietary fibers across the varieties. When total fiber content was compared between the varieties, it was found that varieties of the Japonica origin contain higher amount of fiber than the varieties of Indica origin (Cheng 1993). Based on these facts, it is important to determine the rice bran fiber profile across the varieties.

Suitability of mouse model to assess dietary effects in *Salmonella* colonization

Mouse models are one of the better choices for pre-clinical studies. There are several strains of mice that have been used in *Salmonella* infection models such as C57BL/6, DBA/2, BALB/c, CBA/j and CD1 (Gill, Shu et al. 2001, Stecher, Paesold et al. 2006, Roux, Butler et al.

2010). However, none of the mouse model clearly differentiates between typhoidal and non-typhoidal salmonellosis as it is found in humans. *S*. Typhimurium causes non-typhoidal salmonellosis and *S*. Typhi cause typhoid fever in humans. Unlike humans, the serotype *S*. Typhimurium does not induce the intestinal ulceration and colitis in the murine model. However, *S*. Typhimurium causes systemic infection in the murine model that is similar to *S*. Typhi in humans. The serotype *S*. Typhi does not infect the murine model. Hence it becomes difficult to clearly differentiate the typhoidal and non-typhoidal salmonellosis in murine models. *S*. Typhimurium infection in the streptomycin treated mice induces the similar intestinal pathology as in humans (Barthel, Hapfelmeier et al. 2003). Streptomycin treatment depletes the murine microflora and makes the animal susceptible for intestinal inflammation (Barthel, Hapfelmeier et al. 2003). Very recently researchers have developed the *tlr11* $^{-/-}$ knockout mice where S. Typhi can infect the animals and mimic the typhoid diseases (Mathur, Oh et al. 2012). However, more studies are needed to establish this model because TLR 11 is essential for the mouse physiological functions. The treatment of mice with streptomycin depletes the gut-microbiota and dietary components may protect the organisms via modulation of the gut-microbiota. Hence, streptomycin treated mouse models are not the best to study dietary interventions in rodent models.

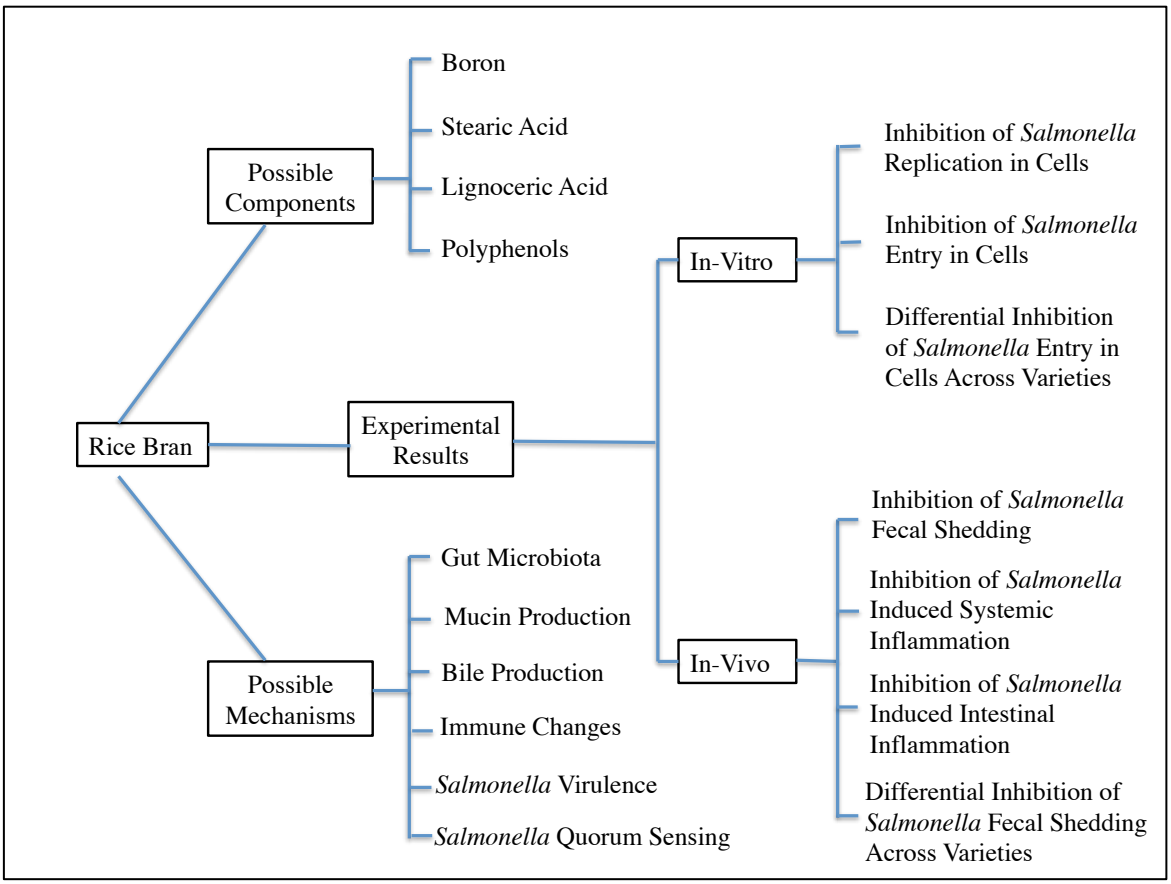


Figure 4.1 Overall representation of dietary rice bran study in prevention of *Salmonella* intestinal colonization

Another model of *Salmonella* infection is 129S6/SvEvTac that represents the chronic non-typhoidal salmonellosis model. These mice contain nramp1 gene and can sustain the chronic *Salmonella* infection up to 300 days. Also, the survival rate is highest in these mice as compared to other mice strain as *Salmonella* cannot reach to the systemic circulation in this strain (Monack, Bouley et al. 2004). Moreover these mice show the intestinal ulceration and inflammation after *Salmonella* infection without streptomycin treatment. Hence, we selected this model of infection for our dietary preclinical studies.

4.3 Future directions

These studies are solid foundations to carry the work to the next level. The mechanisms of dietary rice bran induced protection against *Salmonella* need to be elucidated in future studies. Various studies could be performed in future to elucidate the mechanisms by which rice bran improves colonization resistance. Studies were conducted with pre-feeding of rice bran for one week before *Salmonella* infection. Rice bran can be prophylactically fed to mice for upto one month to see the effects on *Salmonella* fecal shedding. It is possible that rice bran could exert different changes after feeding for a long time. Moreover, rice bran dietary intervention could be evaluated in therapeutic model of *Salmonella* infection. The therapeutic application will make rice bran as a potential candidate for nutritional interventions in *Salmonella* outbreaks.

Effect of dietary rice bran on immunity and gut microbiome in *Salmonella* infection

The effect of dietary rice bran on innate immunity during the *Salmonella* infection needs to be studied in detail. The effect of rice bran on the induction of innate immunity such as macrophages, neutrophils, NK cells, NK T cells and γδ T cells shall be evaluated before and after the *Salmonella* infection. The effect on mucosal immunity may or may not be affected by gut microbiota and hence future mechanistic studies may involve the microbiota depletion to know

the role of gut microbiota in rice bran induced protection. The fecal matter from rice bran fed animals can be gavaged to another set of mice and efficacy of this fecal matter can be tested against the *Salmonella* infection to further elucidate the mechanisms.. Moreover, the effect of rice bran on immunity and gut microbiome should be measured across the varieties using immune phenotyping and pyrosequencing respectively.

Effect of rice bran extract on *Salmonella* virulence and motility

Rice bran could have diminished the virulence of *Salmonella* by alterations in gene expression that are necessary for entry into the cells. *Salmonella* invasion is a multistage process that organizes the spectrum of effector proteins into the host cell using two distinct type three secretion systems (T3SSs) namely *Salmonella* pathogenicity islands 1 and 2 (SPI-1 and 2). Additionally, type VI secretion systems (T6SSs) are known to control virulence in *Salmonella* (McGhie, Brawn et al. 2009). SP1-1 contains several effector proteins such as SipA, SipC, SopB, SopD, SopE and SopE2. These effector proteins are involved in the host cell membrane ruffling, cytoskeleton rearrangement and internalization of *Salmonella* in the vacuole. Moreover, these proteins are also involved in induction of acute inflammation. The effect of RBE could be measured on these proteins using the proteomic approach to elucidate the mechanisms in detail. We observed that RBE not only inhibit *Salmonella* invasion into the cells but also inhibit replication inside the cells. SPI-2 mediates the intracellular survival of *Salmonella*. Hence, it is worth to measure the expression of SPI-2 effectors such as SseB, SseC and SseD (Galan 2001). It is possible that RBE had influenced the components of T6SSs that affect virulence. It encodes between 12-25 proteins and the cluster is located in on SPI-19. T6SSs are responsible for a variety of functions such as inter-bacterial communications, stress sensing and formation of biofilms (Liu, Guo et al. 2013). Hence, expression of T6SSs gene products should be measured

in future. Moreover, RBE components could have reduced the motility of *Salmonella* and it can be measured using agar motility assay (Forbes, Eschmann et al. 2008). These experiments should also be considered to elucidate the varietal differences in rice bran.

Effect of rice bran on bile

Bile is an important digestive fluid synthesized by the liver of many vertebrates and plays a role in the digestion of fats in small intestine by emulsification and micelle formation. As a result, absorption of fat-soluble vitamins such as vitamin A, D, E and K are also increased in the presence of bile. Bile is stored in the gall bladder and released into the duodenum after receiving the stimuli in the form of semi-digested fats and proteins from stomach. After digestion of fats, majority of the bile is reabsorbed in the terminal ileum. Cholycystokinin and secritin hormones in the gut control the process of bile reabsorption (Hofmann and Hagey 2008). Bile is alkaline and composed of phospholipids, bile acids and surfactants and in the duodenum the alkaline pH neutralizes the stomach acids (Hofmann and Hagey 2008).

In addition to the digestive role, bile has been shown to exhibit anti-microbial activity against gut pathogens (Darkoh, Lichtenberger et al. 2010). Both bile quality and quantity may influence the growth of enteric pathogen (Hofmann and Eckmann 2006). Bile salts have been shown to act as an antimicrobial especially on the *Salmonella* and other enteric infections (Prieto, Ramos-Morales et al. 2004, Begley, Gahan et al. 2005, Merritt and Donaldson 2009), while *Salmonella* can also utilize biliary phospholipids for growth if present in excess (Antunes, Andersen et al. 2011). Bile acids induce genes involved in entero-protection by inhibiting pathogenic overgrowth and mucosal injury in the ileum (Inagaki, Moschetta et al. 2006). Both bile quality and quantity have been shown to repress *Salmonella* virulence in the gut environment in the in-vivo models (Prouty and Gunn 2000, Prouty, Brodsky et al. 2004, Ye, Li

et al. 2013). Antunes et al., showed that *Salmonella* could grow in the gall bladder of susceptible mice and causes typhoid (Antunes, Wang et al. 2012). Bile acids exert antimicrobial actions on the pathogens by virtue of their amphipathic properties and damage bacterial DNA (Prieto, Ramos-Morales et al. 2004).

The quality and quantity of bile can be affected by the diet to a great extent. Different dietary fibers affect bile composition and metabolism to different extents (Hillman, Peters et al. 1986) and improve colonization resistance against enteric pathogens. Consumption of dietary medium chain fatty acids increase the fecal bile acids (cholic acid) significantly as compared to the control group in C57BL/6J mice (Xu, Xue et al. 2013). Further, in vitro study in the hepatocytes showed that addition of medium chain fatty acids in the culture media enhance the cell surface expression and transport capacity of bile-salt export pump (BSEP/ABCB11) (Kato, Hayashi et al. 2010). Costarelli et al., compared the diets containing different fatty acids in the healthy premenopausal women and found that the dietary linoleate increases postparandial plasma-bile acid and cholycytokinin as compared to the low fat diet fed subjects (Costarelli and Sanders 2001). Dietary fish oil also increased fecal bile acids in a rat model without increased gene expression for bile synthesis in the liver (Yang, Lan et al. 2012). This study suggests that not necessarily all the fatty acids increase bile acid synthesis in liver, but that some of them reduce bile absorption in ileum.

Dietary factors such as fiber may bind to the bile acids and reduce the reabsorption in colon (Matsumoto, Yokoyama et al. 2010). Oat bran, pectin and guar gum increase bile acids in fecal matter (Andersson, Immerstrand et al. 2010, Chen, Lin et al. 2010, Gunness and Gidley 2010). Reduction in the reabsorption of bile acids in the large intestine modulates gut hormone feedback system and stimulates liver to synthesize more bile acids (Hofmann and Hagey 2008).

This process could reduce the alkalinity of the small intestine, and may increase the gut motility, making the gut environment unfit for *Salmonella* infection (Hofmann and Eckmann 2006). However, there are no studies that directly show the mechanisms of action of dietary intervention in prevention of *Salmonellosis* and more in-depth studies are warranted in the future to reveal the mechanisms involved. To test the effect of dietary rice bran on functionality of bile, bile could be directly withdrawn from the animal's gall bladder and tested for antimicrobial activity. As the rice varieties differ in their quality and quantity of fatty acids, the effect of rice bran on bile quality and quantity should be measured across the varieties using metabolomic techniques.

Effect of dietary rice bran on mucin production

To reach the epithelium, *Salmonella* needs to cross luminal barriers. The **i**ntestinal mucous is the first line of defense to *Salmonella* in the small intestine of rodents and humans (Zarepour, Bhullar et al. 2013). The mucous in the small intestine is single layered and loosely attached to the epithelium as compared to the double-layered mucous of the colon. Mucous is made up of secretory proteins called mucins and the predominant mucin in the small intestine is MUC2 (Hansson 2012) . Abnormalities in mucous layers, underproduction of MUC2 by goblet cells and mutated Muc 2 results in elevated bacterial infection (Kim and Ho 2010). During *Salmonella* infection, mucin layer is disrupted and *Salmonella* gets access to the epithelium (Kim and Khan 2013). Therefore dietary interventions that improve mucin production are likely to improve resistance to enteric pathogens such as *Salmonella*.

Various components of diet have been shown to up-regulate the expression of Muc2 in the intestinal cells. Willemsen et al., showed that treatment of intestinal epithelial and fibroblast co-culture with short chain fatty acids, significantly increased the expression of Muc2 (Willemsen, Koetsier et al. 2003). Ingestion of dietary fibers (soluble and insoluble) has been

shown to increases proliferation of goblet cells and sialylated mucin in the small intestine of rats (Hino, Takemura et al. 2012). In another study, feeding of inulin/fructans to the rats significantly increased the mucous layer thickness in the colon and increased the number of goblet cells in the crypts of distal jejunum as compared to the control fed dietary group (Kleessen and Blaut 2005). Morita et al., showed that intake of dietary resistant starch in rats reduces the endotoxin influx from the intestinal tissue and hypothesized that it could be partially due to the alterations in the mucosal barrier functions (Morita, Tanabe et al. 2004). Given the importance of mucin production in *Salmonella* infection, and dietary studies effectively modulating mucin production, further investigation of mucin stimulating dietary interventions to protect against *Salmonella* is needed. As rice bran contains dietary fiber it presents an attractive dietary intervention for the protection against salmonellosis. Moreover, the effect of rice bran on mucin production should be measured across the varieties.

Rice bran components and *Salmonella* infection

To know the responsible components in rice bran for protection against *Salmonella*, rice bran can be separated into oil and slurry, as it is the most feasible way to separate few of the components. Rice bran oil could be tested in the in vivo *Salmonella* model of infection. On the other hand, the remained slurry after oil extraction should also be tested against *Salmonella* infection model. This information will be of great importance in knowing the responsible rice bran components. These experiments will indicate whether rice bran fats or fibers have stronger effects against *Salmonella*. It is also possible that both groups may not show any protection against *Salmonella*. In that case it will be considered that rice bran components inhibit *Salmonella* colonization in a synergistic effort.

In rice bran component analyses, increased amount of boron was correlated with decreased number of fecal *Salmonella*. Boron is an essential nutrient for plants and plays an important role in maintaining the structure and function of the plasma membrane and cell wall. Boron deficiency in plants leads to malformation of the reproductive tissues (Brown, Bellaloui et al. 2002). Although most of the food have some level of boron, the biggest sources of boron in human diets are avocado, peanut butter, prunes, grapes, pecans and raisin brans (Meacham, Karakas et al. 2010). The maximum safe intake of boron intake is limited to 20 mg/day. In humans, boron affects the metabolism of macrominerals, energy, nitrogen and reactive oxygen. Boron also affects brain function, psychomotor performance and efficacy of estrogen. Boron is also known as a modulator of the immune function (Meacham, Karakas et al. 2010). Traditionally, boron has been used as a food preservative. Boron is also an important component of Boromycin antibiotic that is effective against gram-positive bacteria (Dunitz, Hawley et al. 1971). Boron inhibits the replication of HIV-1 strain in vitro (Kohno, Kawahata et al. 1996). Based on these facts, we hypothesize that boron inhibits *Salmonella* colonization. This hypothesis can be tested in in vitro and in vivo models. Different doses of boron ranging from 0 to 20 mg/ml can be tested in *Salmonella* broth and then further in *Salmonella* invasion model of MSIE or Caco-2 cells. Boron can also be tested in mice using prophylactic model of *Salmonella* infection. We expect that supplementation of boron will decrease the *Salmonella* fecal shedding in mice however it is also possible that boron may not show any protection against *Salmonella*. This could be explained by the fact that the absorption of elemental boron could be different than other associated forms in the plant tissues. In that case, it should be assessed in the similar form, as it is present in the rice bran.

We found that increased levels of stearic acid in rice bran are negatively correlated with *Salmonella* fecal shedding. Stearic acid is a saturated fatty acid with an 18-carbon chain and mainly found in animal and plant fats/oils. It is absorbed in lower amounts in the intestine as compared to other long chain saturated fatty acids (Baer, Judd et al. 2003). Dietary stearic acid decrease plasma and liver cholesterol levels by blocking the intestinal cholesterol absorption (German and Dillard 2004). Hence, some of the unabsorbed stearic acid could be metabolized by the gut microflora. The presence of stearic acid increases survival of *Lactobacilli* in the gastric juice (Corcoran, Stanton et al. 2007) and also increases the growth of some strains of *Lactobacilli* (Endo, Kamisada et al. 2006). We have seen increased levels of fecal *Lactobacilli* after administration of rice bran to the mice. Hence, we hypothesize that supplementation of stearic acid increase the *Lactobacillus* levels in mice and partially responsible for protection against the *Salmonella* infection. This hypothesis can be tested using the in vitro and in vivo models. Various doses of stearic acid can be tested in *Salmonella* broth to check the viability. The maximum tested dose of stearic acid in humans for its bioactivity is 7 g/day and for mice it can be calculated per unit body weight accordingly (Baer, Judd et al. 2003, Terpstra 2004). Stearic acid supplementation in the diet can be optimized for prophylactic effects against *Salmonella* in mice after observing fecal shedding. Further, feces of rice bran fed animals, control diet fed animals and feces inoculated with stearic acid should be incubated and total *Lactobacilli* contents could be evaluated.

Lignoceric acid is another component that was found negatively correlated with *Salmonella* fecal shedding in our study. Lignoceric acid is a 24-carbon chain saturated fatty acid and a byproduct of lignin production in the plants. It is found in small amounts in natural fats. Lignoceric acid has been tested minimally for health promotion. Hence, it needs to be explored

first with different doses in the in vitro and in-vivo experiments. We partially determined the composition of varieties, however, a number of other components can also correlate with the *Salmonella* fecal shedding. Therefore, in future studies, another components such as vitamins and types of fibers, should be determined in rice bran across varieties.

In conclusion, our studies present strong foundation for rice bran induced health effects and warrant the need of rice bran for further testing in different models that are of public interest. Information from component analyses gives important direction for choosing the best varieties for protection against *Salmonella* infection.

REFERENCES

Andersson, K. E., T. Immerstrand, K. Sward, B. Bergenstahl, M. W. Lindholm, R. Oste and P. Hellstrand (2010). "Effects of oats on plasma cholesterol and lipoproteins in C57BL/6 mice are substrain specific." Br J Nutr **103**(4): 513-521.

Antunes, L. C., M. Wang, S. K. Andersen, R. B. Ferreira, R. Kappelhoff, J. Han, C. H. Borchers and B. B. Finlay (2012). "Repression of Salmonella enterica phoP expression by small molecules from physiological bile." J Bacteriol **194**(9): 2286-2296.

Antunes, L. C. M., S. K. Andersen, A. Menendez, E. T. Arena, J. Han, R. B. R. Ferreira, C. H. Borchers and B. B. Finlay (2011). "Metabolomics Reveals Phospholipids as Important Nutrient Sources during Salmonella Growth in Bile In Vitro and In Vivo." J Bacteriol **193**(18): 4719-4725.

Barthel, M., S. Hapfelmeier, L. Quintanilla-Martinez, M. Kremer, M. Rohde, M. Hogardt, K. Pfeffer, H. Russmann and W. D. Hardt (2003). "Pretreatment of mice with streptomycin provides a Salmonella enterica serovar typhimurium colitis model that allows analysis of both pathogen and host." Infect Immun **71**(5): 2839-2858.

Begley, M., C. G. M. Gahan and C. Hill (2005). "The interaction between bacteria and bile." Fems Microbiology Reviews **29**(4): 625-651.

Castillo, N. A., A. D. de LeBlanc, C. M. Galdeano and G. Perdigon (2013). "Comparative study of the protective capacity against Salmonella infection between probiotic and nonprobiotic lactobacilli." Journal of Applied Microbiology **114**(3): 861-876.

Castillo, N. A., G. Perdigon and A. de Moreno de Leblanc (2011). "Oral administration of a probiotic Lactobacillus modulates cytokine production and TLR expression improving the immune response against Salmonella enterica serovar Typhimurium infection in mice." BMC Microbiol **11**: 177.

Chen, H. L., Y. M. Lin and Y. C. Wang (2010). "Comparative effects of cellulose and soluble fibers (pectin, konjac glucomannan, inulin) on fecal water toxicity toward Caco-2 cells, fecal bacteria enzymes, bile acid, and short-chain fatty acids." J Agric Food Chem **58**(18): 10277-10281.

Costarelli, V. and T. A. Sanders (2001). "Acute effects of dietary fat composition on postprandial plasma bile acid and cholecystokinin concentrations in healthy premenopausal women." Br J Nutr **86**(4): 471-477.

Darkoh, C., L. M. Lichtenberger, N. Ajami, E. J. Dial, Z. D. Jiang and H. L. DuPont (2010). "Bile acids improve the antimicrobial effect of rifaximin." Antimicrob Agents Chemother **54**(9): 3618-3624.

de LeBlanc Ade, M., N. A. Castillo and G. Perdigon (2010). "Anti-infective mechanisms induced by a probiotic Lactobacillus strain against Salmonella enterica serovar Typhimurium infection." Int J Food Microbiol **138**(3): 223-231.

Fayol-Messaoudi, D., C. N. Berger, M. H. Coconnier-Polter, V. Lievin-Le Moal and A. L. Servin (2005). "pH-, Lactic acid-, and non-lactic acid-dependent activities of probiotic Lactobacilli against Salmonella enterica Serovar Typhimurium." Appl Environ Microbiol **71**(10): 6008-6013.

Gagnon, M., A. Zihler Berner, N. Chervet, C. Chassard and C. Lacroix (2013). "Comparison of the Caco-2, HT-29 and the mucus-secreting HT29-MTX intestinal cell models to investigate Salmonella adhesion and invasion." J Microbiol Methods **94**(3): 274-279.

Gill, H. S., Q. Shu, H. Lin, K. J. Rutherfurd and M. L. Cross (2001). "Protection against translocating Salmonella typhimurium infection in mice by feeding the immuno-enhancing probiotic Lactobacillus rhamnosus strain HN001." Medical Microbiology and Immunology **190**(3): 97-104.

Glenn, L. M., R. L. Lindsey, J. P. Folster, G. Pecic, P. Boerlin, M. W. Gilmour, H. Harbottle, S. H. Zhao, P. F. McDermott, P. J. Fedorka-Cray and J. G. Frye (2013). "Antimicrobial Resistance Genes in Multidrug-Resistant Salmonella enterica Isolated from Animals, Retail Meats, and Humans in the United States and Canada." Microbial Drug Resistance **19**(3): 175-184.

Gunness, P. and M. J. Gidley (2010). "Mechanisms underlying the cholesterol-lowering properties of soluble dietary fibre polysaccharides." Food Funct **1**(2): 149-155.

Hansson, G. C. (2012). "Role of mucus layers in gut infection and inflammation." Curr Opin Microbiol **15**(1): 57-62.

Hillman, L. C., S. G. Peters, C. A. Fisher and E. W. Pomare (1986). "Effects of the fibre components pectin, cellulose, and lignin on bile salt metabolism and biliary lipid composition in man." Gut **27**(1): 29-36.

Hino, S., N. Takemura, K. Sonoyama, A. Morita, H. Kawagishi, S. Aoe and T. Morita (2012). "Small intestinal goblet cell proliferation induced by ingestion of soluble and insoluble dietary fiber is characterized by an increase in sialylated mucins in rats." J Nutr **142**(8): 1429-1436.

Hofmann, A. F. and L. Eckmann (2006). "How bile acids confer gut mucosal protection against bacteria." Proc Natl Acad Sci U S A **103**(12): 4333-4334.

Hofmann, A. F. and L. R. Hagey (2008). "Bile acids: Chemistry, pathochemistry, biology, pathobiology, and therapeutics." Cellular and Molecular Life Sciences **65**(16): 2461-2483.

Inagaki, T., A. Moschetta, Y. K. Lee, L. Peng, G. X. Zhao, M. Downes, R. T. Yu, J. M. Shelton, J. A. Richardson, J. J. Repa, D. J. Mangelsdorf and S. A. Kliewer (2006). "Regulation of antibacterial defense in the small intestine by the nuclear bile acid receptor." Proc Natl Acad Sci U S A **103**(10): 3920-3925.

Kato, T., H. Hayashi and Y. Sugiyama (2010). "Short- and medium-chain fatty acids enhance the cell surface expression and transport capacity of the bile salt export pump (BSEP/ABCB11)." Biochim Biophys Acta **1801**(9): 1005-1012.

Kim, J. and W. Khan (2013). "Goblet Cells and Mucins: Role in Innate Defense in Enteric Infections." Pathogens **2**(1): 55-70.

Kim, Y. S. and S. B. Ho (2010). "Intestinal goblet cells and mucins in health and disease: recent insights and progress." Curr Gastroenterol Rep **12**(5): 319-330.

Kleessen, B. and M. Blaut (2005). "Modulation of gut mucosal biofilms." Br J Nutr **93 Suppl 1**: S35-40.

Mastroeni, P., J. N. Skepper and C. E. Hormaeche (1995). "Effect of anti-tumor necrosis factor alpha antibodies on histopathology of primary Salmonella infections." Infect Immun **63**(9): 3674-3682.

Mathur, R., H. Oh, D. Zhang, S. G. Park, J. Seo, A. Koblansky, M. S. Hayden and S. Ghosh (2012). "A mouse model of Salmonella typhi infection." Cell **151**(3): 590-602.

Matsumoto, K., S. Yokoyama and N. Gato (2010). "Bile acid-binding activity of young persimmon (Diospyros kaki) fruit and its hypolipidemic effect in mice." Phytother Res **24**(2): 205-210.

Merritt, M. E. and J. R. Donaldson (2009). "Effect of bile salts on the DNA and membrane integrity of enteric bacteria." J Med Microbiol **58**(Pt 12): 1533-1541.

Monack, D. M., D. M. Bouley and S. Falkow (2004). "Salmonella typhimurium persists within macrophages in the mesenteric lymph nodes of chronically infected Nramp1+/+ mice and can be reactivated by IFNgamma neutralization." J Exp Med **199**(2): 231-241.

Morita, T., H. Tanabe, K. Takahashi and K. Sugiyama (2004). "Ingestion of resistant starch protects endotoxin influx from the intestinal tract and reduces D-galactosamine-induced liver injury in rats." J Gastroenterol Hepatol **19**(3): 303-313.

Nakazato, G., F. L. Paganelli, J. C. Lago, F. H. Aoki, C. Mobilon, M. Brocchi, E. G. Stehling and W. D. Silveira (2011). "Lactobacillus Acidophilus Decreases Salmonella Typhimurium Invasion in Vivo." Journal of Food Safety **31**(2): 284-289.

Prieto, A. I., F. Ramos-Morales and J. Casadesus (2004). "Bile-induced DNA damage in Salmonella enterica." Genetics **168**(4): 1787-1794.

Prouty, A. M., I. E. Brodsky, J. Manos, R. Belas, S. Falkow and J. S. Gunn (2004). "Transcriptional regulation of Salmonella enterica serovar Typhimurium genes by bile." FEMS Immunol Med Microbiol **41**(2): 177-185.

Prouty, A. M. and J. S. Gunn (2000). "Salmonella enterica serovar typhimurium invasion is repressed in the presence of bile." Infect Immun **68**(12): 6763-6769.

Roux, C. M., B. P. Butler, J. Y. Chau, T. A. Paixao, K. W. Cheung, R. L. Santos, S. Luckhart and R. M. Tsolis (2010). "Both Hemolytic Anemia and Malaria Parasite-Specific Factors Increase Susceptibility to Nontyphoidal Salmonella enterica Serovar Typhimurium Infection in Mice." Infect Immun **78**(4): 1520-1527.

Scharff, R. L. (2012). "Economic Burden from Health Losses Due to Foodborn Illness in the United States." Journal of Food Protection **75**(1): 123-131.

Stecher, B., G. Paesold, M. Barthel, M. Kremer, J. Jantsch, T. Stallmach, M. Heikenwalder and W. D. Hardt (2006). "Chronic Salmonella enterica serovar Typhimurium-induced colitis and cholangitis in streptomycin-pretreated Nramp1 (+/+) mice." Infect Immun **74**(9): 5047-5057.

Stecher, B., R. Robbiani, A. W. Walker, A. M. Westendorf, M. Barthel, M. Kremer, S. Chaffron, A. J. Macpherson, J. Buer, J. Parkhill, G. Dougan, C. von Mering and W. D. Hardt (2007). "Salmonella enterica serovar typhimurium exploits inflammation to compete with the intestinal microbiota." PLoS Biol **5**(10): 2177-2189.

Thiennimitr, P., S. E. Winter, M. G. Winter, M. N. Xavier, V. Tolstikov, D. L. Huseby, T. Sterzenbach, R. M. Tsolis, J. R. Roth and A. J. Baumler (2011). "Intestinal inflammation allows Salmonella to use ethanolamine to compete with the microbiota." Proceedings of the National Academy of Sciences of the United States of America **108**(42): 17480-17485.

van der Wielen, P. W., L. J. Lipman, F. van Knapen and S. Biesterveld (2002). "Competitive exclusion of Salmonella enterica serovar Enteritidis by Lactobacillus crispatus and Clostridium lactatifermentans in a sequencing fed-batch culture." Appl Environ Microbiol **68**(2): 555-559.

Willemsen, L. E., M. A. Koetsier, S. J. van Deventer and E. A. van Tol (2003). "Short chain fatty acids stimulate epithelial mucin 2 expression through differential effects on prostaglandin E(1) and E(2) production by intestinal myofibroblasts." Gut **52**(10): 1442-1447.

Xu, Q., C. Xue, Y. Zhang, Y. Liu, J. Wang, X. Yu, X. Zhang, R. Zhang, X. Yang and C. Guo (2013). "Medium-Chain Fatty Acids Enhanced the Excretion of Fecal Cholesterol and Cholic

Acid in C57BL/6J Mice Fed a Cholesterol-Rich Diet." Biosci Biotechnol Biochem **77**(7): 1390-1396.

Yang, Q., T. Lan, Y. Chen and P. A. Dawson (2012). "Dietary fish oil increases fat absorption and fecal bile acid content without altering bile acid synthesis in 20-d-old weanling rats following massive ileocecal resection." Pediatr Res **72**(1): 38-42.

Ye, W., Y. Li, Z. Zhou, X. Wang, J. Yao, J. Liu and C. Wang (2013). "Synthesis and antibacterial activity of new long-chain-alkyl bile acid-based amphiphiles." Bioorg Chem **51C**: 1-7.

Yoon, H., B. Y. Park, M. H. Oh, K. H. Choi and Y. Yoon (2013). "Effect of NaCl on Heat Resistance, Antibiotic Susceptibility, and Caco-2 Cell Invasion of Salmonella." Biomed Research International.

Zarepour, M., K. Bhullar, M. Montero, C. Ma, T. Huang, L. Xia and B. A. Vallance (2013). "The mucin Muc2 limits pathogen burdens and epithelial barrier dysfunction during Salmonella Typhimurium colitis." Infect Immun **81**(10):3672-3683.

APPENDIX

Abstracts

1. Andrew Goodyear, **Ajay Kumar**, Anna McClung, Ming-Hsuan Chen, Steven Dow, and Elizabeth P. Ryan. Dietary rice bran effects on gut mucosal immunity and mechanisms for protection against *Salmonella*. **Keystone Symposia on Innate Immune Response in the Pathogenesis of Infectious Disease (E1), Ouro Preto, Brazil, May 10- 15, 2013.**

2. Kumar A, Henderson A, Forster G, Goodyear A, Weir T, Leach J, Dow S, and Ryan E P. Dietary Supplementation with Rice Bran Reduces Susceptibility to *Salmonella* enterica Typhimurium Infection in Mice via Changes in Gut Microbiota. **Keystone Symposia on Malnutrition, Gut-Microbial Interactions and Mucosal Immunity to Vaccines (T1)**, **New Delhi, India, November 7-11, 2011.**

3. Kumar A, Henderson A, Forster G, Goodyear A, Weir T, Leach J, Dow S, and Ryan E P. Rice bran induced changes in gut mucosal immunity. **Grand Challenges in Global Health Symposia**, **New Delhi, India, November 4-7, 2011**.

4. Forster G, Borresen E, **Kumar A**, Keller A, Chen M, Bauer J, Weir T, Wdowik M, Brown R and Ryan E P. Rice Bran Phytochemcials and Dietary Colon Chemoprevention Teamwork. **University of Colorado, Cancer Center Annual Retreat**, **Denver, October 31- November 3, 2011.**

5. Kumar A, Forster G, Leach J, Dow S and Ryan E P. Reduced Susceptibility to Salmonellosis by Dietary Rice Bran. **12TH Annual College of Veterinary Medicine and Biomedical Sciences Research Day**, **Colorado State University, Fort Collins, March 5, 2011.**

6. Kumar A, Forster G, Leach J, Dow S and Ryan E P. Reduced Susceptibility to Salmonellosis by Dietary Rice Bran. **Public Health Symposium on Food Systems, Colorado School of Public Health**, **Colorado State University, Fort Collins, April 4, 2011.**

7. Kumar A, Forster G, Dow S and Ryan E P. Reduced susceptibility to Salmonellosis by Rice Bran. **Global Health Symposium**, **University of Colorado, Denver, October 15, 2010.**

Articles

1. Kumar A, Henderson A, Forster G, Goodyear A, Weir T, Leach J, Dow S, and Ryan E P. Dietary rice bran promotes resistance to *Salmonella enterica* serovar Typhimurium colonization in mice. BMC Microbiology, 4; **12:71**, June 2012.

2. Henderson A, **Kumar A**, Barnett B, Dow S, and Ryan E P. Consumption of Rice Bran Increases Mucosal Immunoglobulin A Concentrations and Numbers of Intestinal *Lactobacillus* spp. J Med Food, 15(5):469-75; May 2012.

3. Borresen E, Henderson A, **Kumar A**, Weir T, and Ryan E P. Fermented foods: Patented Approaches and Formulations for Nutritional supplementation and health promotion. Recent Pat Food Nutr Agric, 1;4(2):134-40, June 2012.

4. Henderson A, Ollila C, **Kumar A**, Borresen E, Raina K, Agarwal R, and Ryan E P. Chemopreventive Properties of Dietary Rice Bran: Current Status and Future Prospects. Advances in Nutrition, 3:643-653 September 2012.

5. Forster G, Raina K, **Kumar A**, Kumar S, Agarwal R, Chen M, Bauer JE, McClung A and Ryan E P. Rice Varietal differences in bioactive bran components for inhibition of colorectal cancer cell growth. Food Chemistry, 141(2) 1545-1552 April 2013.

LIST OF ABBREVIATIONS

AIN- American Institute of American

ANOVA- Analysis of Variance

APCs- Antigen Presenting Cells

BSA- Bovine Serum Albumin

CFU- Colony Forming Unit

CRP- C Reactive Protein

ELISA- Enzyme Linked Immunosorbent Assay

FBS- Fetal Bovine Serum

HCL- Hydrochloric Acid

IFN-γ- Interferon Gamma

IgM- Immunoglobulin M

IL-12- Interleukin 12

IL-6 – Interleukin 6

IU- International Unit

LB- Luria-Bertani

MEM- Minimum Essential Medium

mL- Milliliter

MOI- Multiplicity of Infection

MSIE Cells- Mouse Small Intestinal Epithelial Cells

NFkB- Nuclear Factor kappa-light-chain-enhancer of activated B Cells

NLRs- Nod-Like Receptors

PAMPs- Pathogen Associated Molecular Patterns

PBS- Phosphate Buffer Saline

PCR- Polymerase Chain Reaction

PRRs- Pattern Recognition Receptors

RBE- Rice Bran Extract

RPMI- Roswell Park Memorial Institute medium

SB- *Saccharomyces boulardii*

SDS- Sodium Dodecyl Sulfate

SIgA- Secretory Immunoglobulin A

TBHQ- Tertiary Butyl- Hydroquinone

TLRs- Toll Like Receptors

TMB- 3,3',5,5'-Tetramethylbenzidine

TNF-α- Tumor Necrosis Factor

*μ*g- Microgram

*μ*L- Microliter