POTENTIAL FOR ENHANCING THE HYPOCHOLESTEROLEMIC EFFECT OF *LACTOBACILLUS ACIDOPHILUS* WITH SOLUBLE FIBER AND THE INFLUENCE OF THE *LACTOBACILLUS* ON IMMUNE RESPONSE

By

LACEY MICHELE SMITH

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Texas Tech University

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Thesis Approved:

Stanley E. Gilliland

Scott D. Carter

Barbara J. Stoecker

A. Gordon Emslie

Dean of the Graduate College

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CHAPTER I

INTRODUCTION

In our modern and developed nation, more people die from coronary artery disease (CAD) and stroke than any other cause. Deaths caused by CAD even outnumber deaths from all forms of cancer combined. It is now widely accepted that controlling hypercholesterolemia reduces the morbidity and mortality of CAD (Steinberg and Gotto, 1999). Data from several studies demonstrates the need for both dietary and pharmacologic strategies to prevent both coronary heart disease and cardiovascular disease (Stamler et al., 2000).

Therefore it is important to identify different dietary strategies for people who need to lower their cholesterol. One such strategy is the use of selected strains of *Lactobacillus acidophilus* that have been shown to lower cholesterol by one of two different mechanisms: cholesterol assimilation (Gilliland et al., 1985) and bile salt deconjugation (Gilliland and Speck, 1977; Brashears et al., 1998; Corzo and Gilliland, 1999a.; Pereira et al., 2002). In a study by Danielson et al. (1989), the cholesterol lowering effect of acidophilus yogurt was tested on mature boars that had been fed a high cholesterol diet. The results showed a significant decrease in the serum cholesterol in the boars fed acidophilus yogurt compared to those in the control group.

Another useful substance that can help lower serum cholesterol is the soluble fiber, β-glucan, found in oats. Beta-glucan can bind both free and conjugated bile acids which may result in their being excreted from the body (Cuesta-Alonso and Gilliland, 2003). The body must then synthesize new bile acids using cholesterol in the body which will in turn lower serum cholesterol. Anderson et al. (1984) reported that hypercholesterolemic male subjects consuming an oat bran diet had a 19% decrease in serum cholesterol concentrations. Exocellular polysaccharides produced by some strains of traditional yogurt starter cultures also preferentially bind free bile acids and thus may be helpful in lowering serum cholesterol levels (Pigeon et al., 2002).

There are many positive research results for the hypocholesterolemic effect of both *L. acidophilus* and β-glucan. Results of some studies also have indicated that the combination of the two might have even more positive effects on cholesterol levels. One reason for this is that free bile acids inhibit the bile salt hydrolase activity of *L. acidophilus* (Corzo, 1997). Therefore binding of free bile acids by β-glucan may improve the deconjugation activity of *L. acidophilus* (Cuesta-Alonso and Gilliland, 2003).

The objective of this study was to determine if there was an enhanced hypocholesterolemic effect of a combination of *L. acidophilus* and oat β-glucan administered to pigs fed a high cholesterol diet. Additionally, the immune enhancing capabilities of the probiotic cultures used in the study were tested.

CHAPTER II

REVIEW OF LITERATURE

Health Benefits from the Consumption of Probiotic Cultures

A large body of research has attributed many potential health benefits to the consumption of probiotic bacteria. These benefits include the control of intestinal pathogens, lactose utilization, enhanced immunity, anti-carcinogenic properties, and control of serum cholesterol levels. The first four topics will be covered briefly and the latter in more detail since it is the focus of this study.

Control of intestinal pathogens

Probiotic cultures such as *Lactobacillus acidophilus* have been shown to inhibit intestinal pathogens in both *in vitro* and *in vivo* experiments. In a study done by Gilliland and Speck (1977), *L. acidophilus* NCFM had an inhibitory effect on the growth of *Staphylococcus aureus*, *Salmonella typhimurium*, and *Clostridium perfringens* in MRS broth media. *Staphylococcus aureus*, *S. typhimurium*, and *Escherichia coli* were inhibited by *L. acidophilus* 4962 in a milk base medium.

In an *in vivo* study by Watkins and Miller (1983) baby chicks were used to investigate the effects of *L. acidophilus* on pathogenic *S. typhimurium* and *S. aureus*. The results of the study indicated that a prophylactic treatment was more effective in inhibiting pathogens than a therapeutic treatment. Mortality from the pathogens was significantly ($P<0.01$) reduced. Shedding of both pathogens was also significantly ($P<0.01$) reduced. The shedding of *L. acidophilus* increased as higher numbers of *L. acidophilus* were used for treatment. This decreased pathogen shedding and increased *L. acidophilus* shedding indicates that the lactobacilli were able to competitively inhibit the pathogens.

In recent research, many studies have been conducted to investigate the efficacy of *Lactobacillus* direct-fed microbials for reducing *E. coli* O157:H7 in feedlot cattle. Brashears et al. (2003) found that the feeding of either *L. acidophilus* 747 or *L. crispatus* 750 resulted in a reduction of fecal shedding of *E. coli* O157:H7 and hide contamination at harvest. A similar study by Younts-Dahl et al. (2005) evaluated the effect of three different doses of *L. acidophilus* strain NP51 and a combination of strains NP51 and NP45 on the prevalence of *E. coli* O157:H7 in feedlot steers. The greatest decrease in *E. coli* O157:H7 was found in the treatment group receiving the highest dose of *L. acidophilus* NP51 which was 10^9 CFU per steer/day.

Lactose utilization

People with an inability to digest lactose have an inadequate supply of the enzyme, lactase, to hydrolyze the disaccharide in the small intestine. When lactase deficient people consume dairy products they are plagued with several gastrointestinal symptoms which include cramps, diarrhea, and bloating (Cappello and Marzio, 2005). Many strains of probiotic bacteria are lactic acid bacteria that contain β-galactosidase which enables them to metabolize lactose. The yogurt culture, *S. thermophilus*, produced active β-galactosidase in the digestive tracts of germ-free mice. The enzyme, β-galactosidase, significantly decreased the amount of lactose recovered in the feces of mice who were given *S. thermophilus* orally (Drouault et al., 2002). Other lactic acid bacteria used in fermented products produce β-galactosidase that remains active during passage through the digestive tract. The enzyme aids in lactose digestion in the small intestine after being released from the bacterial cells by bile salts (De Vrese et al., 2001).

A breath hydrogen test can be used to determine lactose malabsorption. More hydrogen will be detected in the breath of people that are unable to utilize lactose (Fernandes et al., 1978). Studies by Kim and Gilliland (1983) showed that lactose malabsorbers who consumed milk containing cells of *L. acidophilus* had reduced hydrogen exhalation. These results indicate that *L. acidophilus* aids lactose malabsorbers in the utilization of lactose. There was, however, some variation in the response of lactase deficient people. This dietary strategy might not be effective for every individual with difficulties metabolizing lactose.

Several research studies have had positive results for improved lactose digestion by administering unfermented acidophilus milk to lactose intolerant test subjects. The permeability of the cells of *L. acidophilus* increased in an environment containing bile so that more lactose can be hydrolyzed within the cell (Noh and Gilliland, 1993; Mustapha et al., 1997). Consumption of the acidophilus milk significantly decreased the common symptoms associated with lactose intolerance.

Enhanced Immunity

There are several proposed mechanisms for enhanced immunity from the consumption of probiotics. Some of these mechanisms include: increased intestinal permeability, altered gut microecology, intestinal immunoglobulin A response, and the balance of pro-inflammatory and anti-inflammatory cytokines (Isolauri et al., 2001).

Research has documented the use of probiotics to treat or prevent certain instances of gastroenteritis and diarrhea. For example infant rotavirus diarrhea has been treated successfully with *Lactobacillus rhamnosus* GG. Treatment with this probiotic reduced the length of the diarrhea episode by almost half (Marteau et al., 2001).

Probiotics also have been used in attempts to lessen allergic reactions. Isolauri et al. (2000) tested a group of 27 infants who exhibited atopic eczema during breast-feeding. The infants were assigned to one of three treatments: hydrolyzed whey formula alone, formula supplemented with *Bifidobacterium*

lactis Bb-12, or formula supplemented with *Lactobacillus* strain GG. The infants receiving the probiotic-supplemented formulas showed a significant improvement in skin condition after two months. Allergic inflammation was also reduced as evidenced by a decrease in shedding of certain cell surface receptor proteins into serum and eosinophilic protein X (EPX) in urine. Chronic inflammation can cause an increase in these molecules. Allergic inflammation of childhood asthma has been shown to be related to urinary EPX.

Enhanced Immunoglobulin Production

Enhanced immunoglobulin production by ingestion of probiotics has been studied in both animals and humans. Link-Amster et al. (1994) studied the effects of milk fermented with *S. thermophilus*, mesophilic streptococci, bifidobacteria Bb12 (a commercial mixed culture from Chris Hansen, Hørshom, Denmark) and containing *L. acidophilus* La1 on the humoral immune response of human volunteers. The volunteers were divided into two groups. Both groups were required to exclude fermented products from their diets two weeks prior to the study. Group A received three 125 g portions of the fermented milk per day for three weeks. Group B (control group) was given no fermented milk and they continued to exclude fermented products from their diet. All volunteers were given the attenuated *Salmonella typhi* Ty21a oral vaccine capsule on days 8, 10, and 12 of the fermented milk intake phase. In Group A serum IgA increased significantly from day 12 of the fermented foods exclusion period to 14 days after the administration of the *S. typhi* Ty21a vaccine capsule. The researchers also

observed a >4-fold rise in specific IgA antibody titers. There were no significant changes in serum IgG. The researchers concluded that the increase in serum IgA response correlated well with previous research that indicated protection from *S. typhi* is an IgA antibody-dependent cytotoxicity. The group receiving the fermented milk showed an increased humoral immune response.

The ingestion of a fermented product was tested for increased immune response in weaned pigs (Lessard and Brisson, 1987). The fermented product was made from rehydrated skim milk powder and fermented by *L. bulgaricus*, *L. casei*, and *S. thermophilus*. The finished product was freeze-dried and used as a powder. The study design was a 2 x 3 factorial arrangement with 2 levels of probiotic treatments: none and 0.1% of the diet and 3 levels of vaccination treatments with transmissible gastroenteritis virus: none, intramuscular, and oral vaccination. There were no differences observed in serum IgG or IgA levels at weaning in pigs fed with or without the fermented product. After three weeks and at the end of the feeding period the vaccinations were administered. At this time the serum IgG levels were significantly higher in pigs fed the fermented product than the pigs on the control diet. The serum IgA levels were not significantly different.

The immune modulating effects of *Lactobacillus rhamnosus* HN001 (DR20^{TM}) was evaluated in mice that had been challenged with *E. coli* O157:H7 (Shu and Gill, 2002). For a week before the experimental period all mice were fed a skim milk powder-based diet (SMP-based diet) ad libitum. After this time period the mice were randomly assigned to either the control group or *L.*

rhamnosus HN001 group. There were 40 mice in the control group and 44 mice in the *L. rhamnosus* HN001 group. The control group was fed the same SMP-based diet as mentioned before. The experimental group was fed the SMP-based diet with 3.0 x 10^8 cfu/g of *L. rhamnosus* HN001. After 7 days *E. coli* O157:H7 (1.0 x 10^7 cfu/mouse) was orally administered to both groups of mice. The mice were given the same diets for 7 days following the *E. coli* O157:H7 challenge. Following the trial 10 mice from each experimental group were used for the analysis of several parameters. The mucosal antibody response was measured as immunoglobulins IgA and IgG from the flushed contents of the small intestine. There was no significant difference in IgG titers for the *L. rhamnosus* HN001 and control group. The *L. rhamnosus* HN001 group had significantly higher mean anti-*E. coli* IgA titers than the control group.

Four commercial yogurt starter cultures were tested for their immune system effects on mice challenged with cholera toxin (Tejada-Simon et al., 1999). Each type of yogurt was made with pasteurized nonfat milk. The four commercial yogurt starter cultures are as follows Ultra-Gro Direct (*S. thermophilus, L. delbrueckii* ssp. *bulgaricus*), Sbifidus Direct (*S. thermophilus, L. delbrueckii ssp. bulgaricus, L. acidophilus, Bifidobacterium* spp.), PY-3 Redi-Set (*S. thermophilus, L. delbrueckii ssp. bulgaricus, L. acidophilus, Bifidobacterium bifidum*), and DPL Yogurt Quick Start ABY-2C (*S. thermophilus*, *L. bulgaricus*, *L. acidophilus*, and *Bifidobacterium infantis*). The control diet consisted of nonfat milk control (no starter cultures) mixed (1:1, wt/wt) with a semipurified diet while the experimental diet had the yogurt mixed (1:1, wt/wt) with the diet. The study

duration was 21 days. The mice were immunized with cholera toxin on day 0 and day 14. Fecal samples were collected on day 1 before immunization and experimental feeding, 1 week after the first immunization (week 1), 2 weeks after the first immunization (week 2), and 1 week after the second immunization (week 3) for IgA anti-cholera toxin (anti-CT) measurement. The IgA anti-CT from fecal samples was a measure of intestinal response. Blood serum was collected 1 week after the second cholera toxin immunization to measure IgA and IgG anti-CT antibodies. This represented a systemic response. IgA anti-CT titers from fecal pellets were very low from day 1 samples. The levels increased slightly for the week 1 samples but there were no significant differences among treatments. There was a decrease in IgA anti-CT for the week 2 samples from the treatment groups and the control group had a slightly higher level. A week after the second dose of cholera toxin (week 3) the IgA anti-CT in the groups that received the yogurts made with Sbifidus Direct and PY-3 Redi-Set were significantly higher than for the controls. The Ultra-Gro Direct yogurt group had an IgA level below that of the control. When IgA anti-CT was measured gravimetrically Sbifidus Direct, PY-3 Redi-Set, and DPL yogurt Quick Start ABY-2C had significantly higher levels than the control. For serum IgA anti-CT the concentrations for all yogurt treatments except Ultra-Gro Direct were higher than the control group. There were no significant differences among groups for IgG levels except Ultra-Gro Direct was significantly lower than the control. The results suggest that yogurt fermented with *L. acidophilus* and *Bifidobacterium* spp. enhanced both mucosal and systemic IgA responses. The Ultra-Gro Direct (*L. bulgaricus and S.*

thermophilus) was considered a conventional yogurt starter culture combination. The Ultra-Gro Direct did not enhance immunoglobulin production on an intestinal or systemic level.

Anticarcinogenic Properties

The anticarcinogenic properties of probiotics have been studied in both human and animal models. One such animal study used male Swiss mice that were implanted with Ehrlich ascites tumor cells (Shahani et al., 1983). The mice were separated into the following treatments: fresh bovine colostrum, colostrum cultured with *L. acidophilus*, colostrum cultured with *L. bulgaricus*, colostrum cultured with *L. bulgaricus* and *S. thermophilus*, milk cultured with *L. acidophilus*, and milk cultured with *L. bulgaricus*. The study results indicated that fresh colostrum did not cause a significant reduction in tumor cells. There was significant tumor inhibition in mice fed colostrum fermented with *L. acidophilus*, *L. bulgaricus*, or *S. thermophilus*. There was a similar level of inhibition for whole milk that was fermented with either *L. acidophilus* or *L. bulgaricus*. The tumor cell inhibition by fermented colostrum or milk did not differ significantly between the different cultures used.

Lactobacillus casei was shown to significantly prevent the recurrence of bladder cancer in some patients (Aso et al., 1995). The product that was administered in this study has been available in Japan for 20 years. It contains approximately 1.0×10^{10} viable organisms per gram. The study was a double blind design with 138 patients. The predominant theorized mechanisms for the

success of the product include: reduction of intestinal production of carcinogens, incorporation of carcinogens, and immune system enhancement.

Control of serum cholesterol levels

Research has shown that some strains of probiotic bacteria have a hypocholesterolemic effect on serum cholesterol levels. Certain probiotics can contribute to cholesterol lowering by two different mechanisms: cholesterol assimilation and bile salt deconjugation (Liong and Shah, 2005). Studies by Gilliland et al. (1985) revealed that some freshly isolated strains of *L. acidophilus* assimilated cholesterol during growth. Studies suggest that under defined conditions, certain strains of *L. acidophilus* are able to integrate steroid structures, such as cholesterol, into the cellular structure (Noh et al., 1997). The cholesterol assimilation was demonstrated both *in vitro* and *in vivo*. In *in vitro* experiments the cholesterol assimilating strain RP32 (ATCC 43121) removed cholesterol from MRS broth supplemented with cholesterol micelles and oxgall (bile source). The other experimental strain, P47, did not remove cholesterol from the media.

Both *L. acidophilus RP32* (ATCC 43121) and P47 were used in a pig feeding trial in which the pigs were being fed a high cholesterol diet. The pigs being fed RP32 (ATCC 43121) had lower levels of serum cholesterol than did either the control group or the group being fed P47. The cholesterol assimilating strain, RP32 (ATCC 43121), was effective in helping to control serum cholesterol levels while the non-cholesterol assimilating strain was not (Gilliland et al., 1985).

Bile salt deconjugation by some strains of probiotics has also been demonstrated experimentally. Gilliland and Speck (1977) found that human isolates of lactobacilli varied in their ability to deconjugate the bile salts, sodium glycocholate and sodium taurocholate. Corzo and Gilliland (1999b.) measured the bile salt hydrolase activity of a strain of *L. acidophilus* using an assay involving HPLC. Bile salt deconjugation can result in a hypocholesterolemic effect because the free bile acids are less likely to be reabsorbed from the intestine and are more likely to be excreted. The body must then synthesize new bile acids from cholesterol in the body which in turn lowers serum cholesterol levels (Brashears et al., 1998).

Danielson et al. (1989) fed 18 mature boars a high cholesterol diet for 56 days. Nine of the boars were then fed a diet that included a yogurt containing *L. acidophilus* LA16. The other nine boars remained on the original diet. This second experimental phase of the study was also 56 days in duration. The *L. acidophilus* strain was originally isolated from the feces of mature boars. The selected strain was chosen over other isolates to use in the study because it exhibited the highest cholesterol reduction in laboratory media. The feeding trial resulted in a 10.5% reduction in serum cholesterol for the 9 boars on the yogurt diet ($P<0.01$). Neither serum high density lipoprotein (HDL) cholesterol nor serum triglycerides were significantly affected. Serum low density lipoprotein (LDL) cholesterol levels were decreased by 9% ($P<0.08$).

A decrease in serum cholesterol was also achieved in a human feeding trial. *Lactobacillus acidophilus* L1 was isolated from humans and selected for its

bile tolerance, bile salt hydrolysis, and cholesterol assimilation. The fermented milk containing *L. acidophilus* L1 reduced serum cholesterol by 2.4% in the first study and 3.2% in the second study. The combined analysis of both studies showed a 2.9% reduction in serum cholesterol. There is a 2 to 3% reduction for the risk for coronary heart disease for every 1% reduction of serum cholesterol. So the findings of these studies translate into a possible 6 to 10% reduced risk for heart disease with the consumption of an active strain of *L. acidophilus* (Anderson and Gilliland, 1999).

Selection of Probiotic Strains

There are several factors to consider when a potential probiotic strain is being selected. In order for a probiotic strain to confer any benefit to the host organism, it is generally accepted that it must be able to grow and/or function in the intestinal tract. A probiotic strain that is most likely to be able to do this must exhibit the following attributes: acid tolerance, bile tolerance, and ability to compete with similar bacteria (Gilliland and Walker, 1990; Pereira and Gibson, 2002).

Bile Tolerance

Bacteria that are selected as probiotics need to have some degree of acid and bile tolerance in order to survive the gastrointestinal tract (Chou and Weimer, 1999; Liong and Shah, 2005). A study conducted by Gilliland et al. (1984) investigated the ability of two strains of *L. acidophilus* with differing levels of bile

tolerance to grow in the intestinal tract of dairy calves. The strain of *L. acidophilus* with the highest degree of bile tolerance had the most growth in the upper small intestines of the calves. An increase in the colonization of probiotics in the upper intestine might be important for controlling the proliferation of intestinal pathogens as they enter the intestinal tract (Gilliland et al., 1984).

The degree of bile tolerance of a potential probiotic also has been found to be important for the improvement of lactose digestion by lactose intolerant individuals. Research has shown that the permeability of *L. acidophilus* cells is increased in the presence of bile. Increased permeability allows more substrate to enter cells so that the β-galactosidase activity of the whole cell is increased (Noh and Gilliland, 1993). Additional studies have supported these findings. Mustapha et al. (1997) found that the *L. acidophilus* strain with the lowest β-galactosidase and lactose transport activity but the highest bile tolerance was the best strain for improving lactose digestion.

Ability to Assimilate Cholesterol

Some strains of probiotics may have a hypocholesterolemic effect upon the host by one or both of their cholesterol lowering properties. Both cholesterol assimilation and deconjugation of bile salts can result in the reduction of serum cholesterol. *Lactobacillus acidophilus* RP32 (ATCC 43121) has been shown to assimilate cholesterol in both *in vitro* and *in vivo* experiments (Gilliland et al., 1985).

Lactobacillus acidophilus RP32 (ATCC 43121) removed cholesterol from laboratory media when grown anaerobically and in the presence of bile. It also has been shown to deconjugate bile acids (Corzo and Gilliland, 1999a.). The ability to assimilate cholesterol was an important factor in enabling *L. acidophilus* RP32 (ATCC 43121) to control serum cholesterol in pigs (Gilliland et al., 1985).

In a study by Noh et al. (1997), most of the cholesterol assimilated by *L. acidophilus* ATCC 43121 (RP32) was recovered. *Lactobacillus acidophilus* ATCC 43121 (RP32) was able to assimilate cholesterol with controlled pH at 6.0 and also with no pH control. Some of the assimilated cholesterol was recovered from the membranes of cells grown under both pH conditions. It also was demonstrated that using unsaturated or saturated fatty acids in the phospholipids did not affect the amount of cholesterol assimilated.

Liong and Shah (2005) reported that cholesterol can be removed from laboratory media by growing, resting, or dead cells of several strains of lactobacilli. There were seven strains of *L. casei* and four strains of *L. acidophilus* used in this study. All of the strains were of human origin. Growing cells of lactobacilli removed cholesterol ranging from 4.53 to 16.03 mg/g of dry weight. The amount of cholesterol removed by heat-killed and resting lactobacilli cells was not significantly different but most strains exhibited higher cholesterol removal in the resting state. The heat-killed and resting cells removed cholesterol ranging from 0.79 to 3.82 mg/g of dry weight.

Bile Salt Deconjugation

Some strains of probiotics have the ability to deconjugate bile salts. This deconjugation coupled with a decrease in pH by the acid production of the bacteria is thought to cause the precipitation of free bile salts and cholesterol. The solubility of bile salts decreases at a pH of 6.0 or less (Brashears et al., 1998).

Gilliland and Speck (1977) investigated different growth conditions that affect bile salt deconjugation. They found that *L. acidophilus* requires a low O/R potential in order to deconjugate bile salts. A greater amount of deconjugation was observed when the culture was grown in MRS broth supplemented with thioglycollate compared to decongugation when grown in MRS broth in a GasPak system. Gilliland and Speck (1977) also tested the deconjugating abilities of lactobacilli isolated from human feces. They observed differences in the deconjugation of taurocholate and glycocholate by the different strains. Some strains deconjugated both bile salts, others deconjugated one of the two bile salts, and some deconjugated neither.

Walker and Gilliland (1993) conducted a study to test the relationships between the bile tolerance, cholesterol assimilation, and bile acid deconjugation of 19 different cultures of *L. acidophilus*. They found that most of the strains of *L. acidophilus* that assimilated cholesterol also deconjugated bile acids. However, the correlations among the amount of cholesterol assimilation, deconjugation, and bile tolerance were not significant.

De Rodas et al. (1996) conducted a feeding trial to determine the cholesterol lowering effects of *L. acidophilus* RP32 (ATCC 43121) and calcium on diet induced hypercholesterolemia. The pigs were fed a diet supplemented with 0.5% crystalline cholesterol and 10% butter for 14 days in order to increase total serum cholesterol. On average the cholesterol was increased from 84.5 to 294.6 mg/dl in 92 kg barrows. The diets of the pigs were then supplemented with 1.4% calcium and/or 2.5 x 10^{11} cfu/g of *L. acidophilus* RP32 (ATCC 43121) per feeding. The results of this study indicate that both the *L. acidophilus* and dietary calcium have hypocholesterolemic properties. The *L. acidophilus* likely helped to lower the serum cholesterol levels by cholesterol assimilation and bile salt deconjugation. The reductions in serum cholesterol caused by the lactobacilli were associated with increased levels of free bile salts in the serum. This suggests the involvement of deconjugation in the cholesterol lowering effect.

Corzo and Gilliand (1999a.) conducted studies to test the bile salt hydrolase activities of three strains of *L. acidophilus*. Two of the strains in the study were of human origin and the third strain was of porcine origin. Bile salt deconjugation of the cultures was tested on both sodium taurocholate and sodium glycocholate with and without pH control. All three of the strains showed deconjugation activity when grown without pH control in MRS broth that was supplemented with both sodium glycocholate and taurocholate. The three strains were then grown in MRS broth with pH maintained at 6.5 and supplementation of a 2.3 molar ratio of sodium taurocholate to sodium glycocholate. This ratio of bile salts is thought to be similar to that of a healthy human. All three strains of *L.*

acidophilus displayed deconjugation activity. ATCC 43121 (RP32) deconjugated more sodium glycocholate than the other strains and all three strains deconjugated more sodium glycocholate than sodium taurocholate. Control of pH at 6.5 was used to simulate the conditions in human intestines.

Klaver and van der Meer (1993) conducted research studies to investigate what they term as the "proposed assimilation of cholesterol". The researchers obtained *L. acidophilus* RP32 (ATCC 43121) from Oklahoma State University. The other bacterial cultures used in the study were *L. casei* MUH117, *L. acidophilus* MUH79, *L. acidophilus* MUH41, *Bifidobacterium bifidum* MUH80, and *L. acidophilus* CH1. The study involved both pH-controlled and acidifying conditions. The researchers concluded that bile salt-deconjugating activity of *L. acidophilus* RP32 (ATCC 43121) was responsible for the disappearance of cholesterol from the growth medium. All of the other strains tested were shown to deconjugate bile acids except for *L. acidophilus* CH1 and *L. casei* MUH117.

When comparing studies of germ-free and normal microflora animals it is evident that the gut microflora has an impact on the metabolism. Germ-free animals have been shown to excrete only conjugated bile acids. Some of the intestinal bacteria of normal microflora animals can deconjugate primary bile acids to form secondary bile acids. Transformation of bile acids causes a decrease in the bile acid pool because some deconjugated bile acids may be excreted more readily. The body must use its stores of cholesterol to synthesize more bile acids (Eyssen, 1973).

Host Specificity

While there may be some exceptions, for the most part probiotics exhibit host specificity. In an *in vitro* study by Barrow et al. (1980), aside from two chicken isolates, only strains from domestic pigs and wild boars were able to adhere to the pig squamous epithelial cells. Danielson et al. (1989) also utilized porcine strains of *L. acidophilus* for a pig feeding trial to avoid host specificity complications. Due to host specificity, attention needs to be given to the selection of strains of lactobacilli for different species of hosts (Gilliland et al., 1975).

Health Benefits of Oat β-glucan

Control of Serum Cholesterol

Soluble fibers such as β-glucan can bind bile salts which may prevent them from being reabsorbed by the body during enterohepatic circulation. The bile salts that have been bound are more likely to be excreted by the body through the feces. The body must then use some of its stores of cholesterol to synthesize bile salts to replace those lost to excretion. This action may ultimately cause a reduction in levels of serum cholesterol (Lia et al., 1995).

There are several proposed mechanisms for the cholesterol lowering action of oat β-glucan. First, studies investigating the consumption of oat bran have shown an increase in fecal bile acid excretion (Kirby et al., 1981; Anderson et al., 1991; Lia et al., 1995; Amundsen et al., 2003). Second, the microflora of

the colon ferment oat bran to produce the short-chain fatty acids acetate, propionate, and butyrate (Anderson et al., 1991). Propionate may cause an inhibition of cholesterol synthesis by the liver (Wright et al., 1990; Chen et al., 1984). Third, diets with more soluble fiber may cause a decrease in insulin secretion. Decreased insulin secretion may result in decreased cholesterol synthesis because insulin is responsible for stimulating HMG-CoA reductase. This enzyme is the rate limiting step for cholesterol production in the liver (Jenkins et al., 1989). A fourth cholesterol lowering property of soluble fibers is the viscosity. Beer et al. (1995) found increased gut viscosity to be a very important factor in lowering serum cholesterol. The increase in viscosity may form a barrier that hinders bile acid reabsorption.

The soluble fiber β-glucan may also help enhance the cholesterol lowering capabilities of some probiotics. Some strains of *L. acidophilus* deconjugate bile salts and in turn aid in lowering serum cholesterol. An accumulation of deconjugated bile salts may cause a feedback inhibition of bile salt hydrolase, which is responsible for deconjugation (Corzo, 1997). Therefore the addition of a soluble fiber to bind the free bile salts may enhance the deconjugation activity of some strains of *L. acidophilus* (Cuesta-Alonso and Gilliland, 2003).

Source of fiber and other benefits

The source of β-glucan for the present study was derived from oats. The product used was actually oat bran called OatWell 22 (Oat Ingredients, Boulder, CO). This particular oat bran contains 22% β-glucan.

Aside from lowering cholesterol, the soluble fiber, β-glucan, has many other beneficial properties. Brennan and Cleary (2005) have summarized the uses and benefits of β-glucan as a functional food ingredient. Dietary fiber with β-glucan can help reduce bowel transit time, prevent constipation, and reduce the risk of colorectal cancer. Beta-glucan fiber can also help regulate blood glucose levels, result in the production of short chain fatty acids, and it can act as a prebiotic to help support the growth of helpful gut microflora.

The Pig as a Human Model

Pigs have been used as a human model for many different research applications. It is possible to use swine because of their key similarities to humans. These similarities include a comparable digestive tract, cardiovascular system, and an omnivorous diet (Cevallos et al., 1979; Miller and Ullrey, 1987).

The pig is an important model for many human nutritional applications. The physiological and anatomical aspects of the pig digestive tract greatly resemble those of man (Miller and Ullrey, 1987). Pigs can be used as a model where it might be unethical to use humans such as research for infant nutrition (Burrin, 2001). Pigs and humans have main bile acids that are hydrophilic as opposed to hydrophobic. Humans and pigs also both form “cholesterol-supersaturated bile” (J.T. Yen, 2001).

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CHAPTER III

POTENTIAL FOR ENHANCING HYPOCHOLESTEROLEMIC EFFECT OF *LACTOBACILLUS ACIDOPHILUS* WITH SOLUBLE FIBER AND THE INFLUENCE OF THE *LACTOBACILLUS* ON IMMUNE RESPONSE

Lacey M. Smith, Scott D. Carter, and Stanley E. Gilliland

Food and Agricultural Products Research and Technology Center

and

Department of Animal Science

Oklahoma State University

Stillwater, Oklahoma 74074

ABSTRACT

The objective of the present study was to determine the effects of *Lactobacillus acidophilus* RP32 (ATCC 43121) and oat β-glucan on the serum cholesterol levels in pigs on a high cholesterol diet. Additionally the immune enhancing capabilities of *L. acidophilus* RP32 (ATCC 43121), *Streptococcus thermophilus* OSU-1, and a combination of the cultures was tested. Twelve crossbred gilts and twelve crossbred barrows were placed in individual pens. Following a one week adjustment period on a corn-based diet four pigs were randomly assigned to each of six treatments (two gilts and two barrows per treatment): 1) control milk, 2) control milk + *L. acidophilus* RP32 (ATCC 43121), 3) milk fermented with *S. thermophilus* OSU-1, 4) milk fermented with *S. thermophilus* OSU-1 + *L. acidophilus* RP32 (ATCC 43121), 5) 5 g oat bran (Oatwell 22) on surface of feed + control milk, and 6) 5 g oat bran (Oatwell 22) on surface of feed and control milk + *L. acidophilus* RP32 (ATCC 43121). Day 0 blood samples were taken for baseline cholesterol levels and the pigs were placed on a high cholesterol diet. The pigs were fed the corn based diet with 1,761 mg cholesterol per kg feed for 14 days. On day 7 and day 14 blood samples were taken and analyzed for total serum cholesterol. Pigs that were given treatments 1, 2, 3, and 4 were inoculated with lipopolysaccharide (LPS) derived from enteropathogenic *Escherichia coli* O111:B4. Temperature and serum IgG, IgA, and IgM were measured at 0, 1.5, 3.0, and 6.0 hours. Enzyme

linked immunosorbent assays (ELISA) were used for the immunoglobulin analyses. Results for the cholesterol feeding trial were highly variable. The control milk containing *L. acidophilus* RP32 (ATCC 43121) caused a significant decrease in cholesterol from day 7 to day 14 in the animals having an initial mean cholesterol level of 85 mg/dl. There were no other significant decreases in cholesterol observed. Immunoglobulins IgG and IgA were both increased by *L. acidophilus* RP32 (ATCC 43121) and *S. thermophilus* OSU-1 when given separately. However, the combination of the cultures did not result in a significant increase in IgG and IgA. The levels of IgM were not significantly different among treatments.

INTRODUCTION

High serum cholesterol levels have been associated to coronary heart disease (CHD) (Cleeman and Lenfant, 1998). Of both males and females, an estimated one in four have some type of cardiovascular disease (CVD). In 2002, 38% of all deaths were attributed to CVD a disease which cost an estimated $393.5 billion in 2005 (American Heart Association, 2005). Hypercholesterolemia and more specifically elevated LDL cholesterol have been identified as major risks for CVD (Levy, 1981; Schaefer, 2002). Because of all of these factors it is imperative to identify different tactics to help lower serum cholesterol levels.

One strategy for lowering serum cholesterol levels that has been identified through research is the ingestion of products containing active cultures of *Lactobacillus acidophilus* (Gilliland et al., 1985; Danielson et al., 1989; Anderson and Gilliland, 1999). *In vitro* laboratory experiments are useful for identifying strains of *L. acidophilus* that possess the characteristics for both survival in the gastrointestinal tract and hypocholesterolemic traits. Potential probiotic strains of *L. acidophilus* should be bile tolerant, acid tolerant, and competitive against intestinal flora (Gilliland and Walker, 1990; Pereira and Gibson, 2002). Probiotic strains intended for cholesterol reduction also demonstrate the ability to remove cholesterol from laboratory media. Cholesterol removal by selected strains of *L. acidophilus* is thought to be achieved by way of one or both of the following

mechanisms: cholesterol assimilation and bile salt deconjugation (Liong and Shah, 2005).

In vitro experiments showed the uptake of cholesterol from laboratory media when the culture was grown in the presence of bile and anaerobically (Gilliland et al., 1985). Certain strains of *L. acidophilus* can also deconjugate bile salts under anaerobic conditions (Gilliland and Speck, 1977). Bile salt hydrolase is the enzyme responsible for bile salt deconjugation (Corzo and Gilliland, 1999). Both of the previously mentioned actions can be involved in reducing serum cholesterol levels.

Another well documented cholesterol-lowering agent is the soluble oat fiber (β-glucan). Anderson et al. (1984) reported that consumption of an oat bran diet by hypercholesterolemic male subjects caused a 19% decrease in serum cholesterol concentrations. Amundsen et al. (2003) reported the cholesterol lowering effect of a diet supplemented with oat bran concentrate (OBC). Subjects on the OBC diet had a significant decrease in serum cholesterol and low-density lipoprotein compared to those in a control group.

Increased bile acid excretion is the mechanism most commonly identified for the cholesterol reducing properties of oat β-glucan (Kirby et al., 1981; Anderson et al., 1991; Lia et al., 1995; Amundsen et al., 2003). The proposed cholesterol-lowering mechanisms of β-glucan other than bile acid excretion are as follows. First, lipoprotein metabolism may be changed due to the consumption of oat bran (Anderson et al., 1991). Second, microorganisms in the colon ferment oat bran to produce short-chain fatty acids such as acetate,

propionate, and butyrate (Anderson et al., 1991). These short-chain fatty acids can decrease the cholesterol synthesis rates in rats (Hara et al., 1999). Similar studies with rats show that propionate significantly reduced cholesterol synthesis in the liver (Chen et al., 1984; Wright et al., 1990). Third, oat bran has been shown to decrease insulin secretion which may reduce cholesterol synthesis (Anderson et al., 1991). A fourth aspect of cholesterol-lowering fibers is viscosity. A study using oat gum by Beer et al. (1995) concluded that increased viscosity in the gut may be the most important quality of β-glucan for lowering serum cholesterol.

A combination of all of the hypocholesterolemic mechanisms of both *L. acidophilus* and oat β-glucan may enhance the cholesterol lowering effects. An accumulation of free bile salts causes feedback inhibition for the bile salt hydrolase enzyme of *L. acidophilus* (Corzo, 1997). Therefore the binding of free bile salts by β-glucan could prevent feedback inhibition and allow the maximum deconjugating ability of *L. acidophilus* (Cuesta-Alonso and Gilliland, 2003).

The objective of this study was to investigate the hypocholesterolemic effect of a combination of both *L. acidophilus* and oat β-glucan fed to pigs on a high cholesterol diet. The pig was used as a human model because of its omnivorous diet and comparable digestive tract and cardiovascular systems (Cevallos et al., 1979; Miller and Ullrey, 1987). A second objective was to determine if *L. acidophilus* and/or fermented milk fed to pigs would influence the levels of IgA, IgG, and IgM formed in response to a lipopolysaccharide challenge.

MATERIALS AND METHODS

Source and Maintenance of Cultures

Lactobacillus acidophilus RP32 (ATCC 43121) and *S. thermophilus* OSU-1 were both acquired from the food microbiology culture collection at Oklahoma State University. Their identities were confirmed by Gram stain, catalase test, growth at 15°C and 45°C plus the API 50 CH system (bioMérieux sa, France) as described by Yap and Gilliland (2000). The fermentation patterns were confirmed using the 9th edition of Bergey's Manual of Determinative Bacteriology (Sneath et al., 1986).

Each week *L. acidophilus* RP32 (ATCC 43121) was subcultured using a 1% inoculum into 10 ml of sterile MRS broth (Difco Laboratories, Detroit, MI). The culture was incubated at 37°C for 18 hrs. The *S. thermophilus* OSU-1 was subcultured weekly using a 1% inoculum into 10 ml of sterile nonfat milk made with 10% non-fat dried skim milk and deionized water. This culture was incubated in a water bath at 45°C for 18 hrs. Both cultures were subcultured three times in their respective media before use in the feeding trial. The cultures were stored at 4°C between subculturing procedures.

Tests for the Enhancement of Cholesterol Assimilation

In vitro tests were conducted to investigate the enhancement of cholesterol assimilation by *L. acidophilus* RP32 (ATCC 43121) with added β-glucan from Oatwell 22 oat bran (Oat Ingredients, Boulder, CO). Cholesterol-

phosphatidylcholine micelles were prepared as described by Razin et al. (1980). A β-glucan solution was prepared by adding 0.5 g Oatwell 22 oat bran to 10 ml deionized water and vortexing for 30 seconds. The mixture was then centrifuged for 10 min. at 15,000 x g and 1-3°C. The supernatant was filter sterilized through a 0.45 µm filter (Fisher Scientific, Waltham, MA) into a sterile container and stored in the refrigerator until use. The prepared β-glucan solution contained approximately 5,000 µg β-glucan/ml (based on solids determination for the filtrate using an oven drying method). The MRS-thio broth used in this experiment was MRS broth supplemented with 0.2% sodium thioglycollate (Sigma Chemical Co., St. Louis, MO) and 0.3% Oxgall (Difco Laboratories, Sparks, MD). Cholesterol assimilation was tested with and without added β-glucan. One ml of cholesterol-phosphatidylcholine micelles and 1 ml β-glucan solution (or 1 ml sterile deionized water) were added to 10 ml tubes of MRS-thio + oxgall broth. A freshly prepared MRS broth culture of *L. acidophilus* RP32 (ATCC 43121) was used to inoculate (1%) both tubes. Uninoculated controls with and without β-glucan were also prepared and used. The tubes were incubated for 16 hours at 37°C. The cells were then removed by centrifugation for 10 min. at 15,000 x g and 1-3°C. The *o*-phthalaldehyde method by Rudel and Morris (1973) was used to determine the amount of cholesterol in the spent broth both with and without β-glucan and in the uninoculated controls. The amount of cholesterol assimilated was calculated as the difference between the amounts in each spent broth and its corresponding uninoculated control.

Tests for the Enhancement of Bile Salt Deconjugation

In vitro tests were conducted to investigate the influence of β-glucan (Oatwell 22 oat bran; Oat Ingredients, Boulder, CO) on bile salt deconjugation by *L. acidophilus* RP32 (ATCC 43121). The methods for testing a culture's ability to deconjugate sodium taurocholate were used as described by Walker and Gilliland (1993). The assay measures the amount of free cholic acid released by the culture. Bile salt deconjugation with and without added β-glucan solution was tested. A volume of 2 ml of β-glucan solution (final concentration of 500 µg β-glucan/ml) and 2 ml of 0.01 M sodium taurocholate (final concentration of 538 µg sodium taurocholate/ml) were added to 16 ml of MRS-thio described in the previous section. A volume of 2 ml of sterile water in place of β-glucan was added to a second tube. A 1% inoculation from a freshly prepared MRS broth culture of *L. acidophilus* RP32 was added to each tube. An uninoculated control for each treatment also was assayed. The amount of cholic acid released was determined by the difference in the inoculated treatment and its corresponding uninoculated control. The tubes were incubated for 16 hours at 37°C followed by collection of spent media and assay for cholic acid (Walker and Gilliland, 1993).

Feeding Trial

Twelve crossbred gilts and twelve crossbred barrows (4 replicates of 6 pigs) were placed into separate pens. For the first week the pigs were fed a corn-based diet twice daily (Table 1). Uneaten feed was cleaned out of the feeders before each feeding. After a one week adjustment period the pigs were

randomly assigned to 1 of 6 treatments (equal numbers of barrows and gilts in each). The treatments are as follows: 1) control milk (25 ml), 2) control milk + cells of *L. acidophilus* (25 ml), 3) control milk fermented with *S. thermophilus* OSU-1 (25 ml), 4) control milk fermented with *S. thermophilus* OSU-1 + cells of *L. acidophilus* RP32 (25 ml), 5) 5 g oat bran (Oatwell 22) on surface of feed + control milk (25 ml), and 6) 5 g oat bran (Oatwell 22) on surface of feed and control milk + cells of *L. acidophilus* RP32 (25 ml). (The treatment assignments for each pig are in Table 2 in the Appendix). The pigs received 25 ml of their respective treatments once daily for 14 days.

Following the adjustment period all of the pigs were placed on a high cholesterol corn-based diet containing 1761 mg cholesterol per kg of diet for 14 days. The diet contained 0.16% crystalline cholesterol (Sigma-Aldrich, Co., St. Louis, MO) as well as 7.5% butter which resulted in a total 1,761 mg cholesterol/kg of diet (Table 1). For the first week the gilts and barrows were fed an average of 0.383 kg and 0.385 kg per meal respectively. The gilts and barrows consumed 0.313 kg and 0.344 kg feed per meal respectively. For the second week the gilts and barrows were fed an average of 0.357 kg and 0.379 kg per meal respectively. Feed intakes for the second week were 0.313 kg per meal for the gilts and 0.344 kg per meal for the barrows. (Feed intakes and feed efficiencies are listed in Table 16 in the Appendix). For both weeks any uneaten feed was removed from the feeders prior to feeding. The uneaten feed was collected and weighed weekly to calculate feed intakes and feed efficiency.

The control milk was dried skim milk (10%) reconstituted with deionized water. After reconstitution it was dispensed in 125 ml volumes into bottles, heated at 100°C for 30 min, and then refrigerated until use. A fresh milk culture of *S. thermophilus* OSU-1 was used to inoculate (2%) milk for treatments 3 and 4. The bottles were incubated for 6 to 8 hours (until firm coagulation) in a 45°C water bath.

Frozen concentrated cultures of *L. acidophilus* RP32 were prepared as described by Gilliland and Rich (1990). The number of viable organisms in the frozen concentrated cultures was confirmed by plating appropriate dilutions on MRS agar (Difco Laboratories, Sparks, MD). Plates were incubated 48 hr at 37°C. The required number of frozen vials (2g) of concentrated culture were thawed and added to milk for treatments 2, 4 (after fermentation), and 6. One vial of culture (7.0×10^{10} cfu) was added to each 125ml treatment bottle just prior to feeding pigs (each pig received 25 ml of treatment containing 1.4×10^{10} cfu).

The β-glucan utilized in the trial was from oat bran with a high percentage of β-glucan. The commercial product called OatWell 22 was obtained from Oat Ingredients (Boulder, CO). It contained 22% β-blucan.

Blood Collection and Cholesterol Analyses

Blood samples for all 24 pigs were collected on days 0, 7, and 14. The day 0 samples represent the baseline cholesterol level before the pigs were consuming the high cholesterol diet. All blood samples were taken following a 12

hr fast. Blood was drawn via the anterior vena cava using sterile vacutainers. The blood was placed on ice after collection and then refrigerated overnight.

The blood samples were centrifuged for 10 min. at 3,000 x g and 1-2°C in order to separate the blood serum. The serum was recovered by using a transfer pipet and dispensed into 3 cryogenic vials per sample. The recovered serum was held in a freezer (-20°C) until all of the samples for days 0, 7, and 14 had been collected.

All blood serum samples were analyzed for total cholesterol, HDL cholesterol, LDL cholesterol, and triglycerides at the Stillwater Medical Center using a Vitros 5,1 FS Chemistry System (Ortho-Clinical Diagnostics, Raritan, NJ).

Escherichia coli Lipopolysaccharide (LPS) Challenge to Test Immune Response

At the end of the 14 day experimental period, all pigs fed treatments 1, 2, 3, and 4 were subjected to a LPS challenge. The source of the LPS was *Escherichia coli* O111:B4 (Sigma-Aldrich, Co., St. Louis, MO). A rectal temperature reading was taken for each of the pigs. The pigs were then injected intraperitoneally with 25 µg LPS (suspended in 9 g/L saline) per kg of body weight (0 hr). In addition to 0 hour sampling, temperature readings and blood collections were taken at hours 1.5, 3.0, and 6.0. All of the blood samples were placed on ice after collection and then in a refrigerator overnight. The blood samples were centrifuged for 10 min. at 3,000 x g and 1-2°C to recover the serum as described previously. Each serum sample was then transferred to

appropriately labeled cryovials. The serum was held in a freezer (-20°C) until immunoglobulin analyses were performed.

Immunoglobulin Analyses

The serum samples were analyzed for the immunoglobulins IgM, IgA, and IgG. An enzyme linked immunosorbent assay (ELISA) quantitation kit for each immunoglobulin was used (Bethyl Laboratories, Inc., Montgomergy, TX). The serum was analyzed according to the manufacturer's directions.

Statistical Analysis

The serum cholesterol data were analyzed using a repeated measures analysis of covariance (ANCOVA) with the SAS software (SAS Institute, 2003). Day 0 cholesterol values were used as a covariate. Subgrouping of treatments allowed for the adequate modeling of the variance. The subgroups were as follows: treatments 1 and 2, treatments 3 and 4, and treatments 5 and 6. Fit statistics were used to pick the best model. The compound symmetry covariance structure was adopted.

The immunoglobulin data for IgG, IgA, and IgM levels were analyzed using a repeated measures analysis with the SAS software (SAS Institute, 2003). For both IgG and IgA an analysis of covariance (ANCOVA) was modeled with time 0 as a covariate. There was no significant covariate effect for time 0 in the IgM data. An analysis of variance (ANOVA) was used for the IgM data. Fit

statistics were analyzed and the Toeplitz covariance structure was adopted for IgG, IgA, and IgM analysis.

Table 1. Composition of diet used in pig feeding trial

Component	Amount (kg)
Corn grain	119.70
Soybean meal (48% CP)	118.89
Whey, dried	68.18
Butter	25.57
Calcium phosphate (dicalcium)	2.86
Limstone, ground	3.16
Sodium chloride	0.85
OSU Trace Mineral mix[a]	0.51
OSU Vitamin mix[a]	0.68
Total	340.40[b]

[a]Vitamin and mineral mix (Oklahoma State University, Stillwater, OK)

[b]After mixing with a Marion mixer (Rapids Machinery Co., Marion, Iowa), 68.18 kg of feed was removed for the adjustment feeding period. 436.3 g of crystalline cholesterol (approx. 95% GC; Sigma Chemical Co., St. Louis, Mo.) was added and mixed into the remaining 272.31 kg of feed for used in the experimental period.

RESULTS

Culture Identification

The identities of *L. acidophilus* RP32 (ATCC 43121) and *S. thermophilus* OSU-1 were confirmed as described in the 9th edition of Bergey's Manual of Determinative Bacteriology (Sneath et al., 1986). *Lactobacillus acidophilus* RP32 (ATCC 43121) is a Gram positive, catalase negative, nonsporeforming rod. *Streptococcus thermophilus* OSU-1 is a Gram positive, catalase negative, cocci. (The complete culure identification characteristics for these cultures are listed in Table 3 and Table 4 respectively in the Appendix.)

Influence of Oatwell 22 β-glucan on Cholesterol Assimilation and Bile Salt Deconjugation

The Oatwell 22 β-glucan solution did not enhance cholesterol assimilation by *L. acidophilus* RP32 as was hoped (Table 5 in the Appendix). The values obtained with and without the β-glucan were virtually the same.

The values obtained for the deconjugation of bile salts with and without the β-glucan solution were also very similar (Table 6 in the Appendix). There was a slight decrease in the amount of cholic acid released from sodium taurocholate with added β-glucan.

Feeding Trial

All of the pigs were healthy throughout the duration of the feeding trial. The day 0 cholesterol values for all groups were highly variable. The starting cholesterol values across groups ranged from 90.5 mg/dl to 118.25 mg/dl. There was also a lot of variability among animals within treatments. All of the variability made the data difficult to analyze. The changes in total serum cholesterol from day 0 of the trial are illustrated in Figure 1. The mean levels of cholesterol decreased in the group receiving the control milk containing added lactobacilli and did not increase for the group receiving the fermented milk plus lactobacilli. However, it increased in all other treatment groups.

The cholesterol data were analyzed using subgrouping in order to adequately model the variance. The subgrouping was organized as follows: treatments 1 and 2, treatments 3 and 4, and treatments 5 and 6. The data were analyzed using a repeated measures analysis of covariance with day 0 cholesterol values as a covariate. There was no overall treatment effect ($p=0.7017$). There also was no overall day effect ($p=0.9846$) or trt*day effect ($p=0.0788$). The only significant effect was an initial cholesterol (day 0 values)*trt*day effect ($p=0.0439$). The initial cholesterol of the pigs had a significant impact on the cholesterol response to the treatments and high cholesterol diet. In order to compare the treatments the baseline cholesterol values were grouped to low, middle, or high serum cholesterol levels. The grouping values are as follows: low 85 mg/ml, middle100 and 115 mg/dl, and a high 130 mg/dl. The least squares means of trt*day at each initial value were

computed. A slice effect for both treatment and day was used to determine significant differences among treatments and within treatments for day 7 and day 14.

The results for the effect of all treatments on total serum cholesterol at adjusted baseline values for 85, 100, 115, and 130 mg/dl are summarized in Table 7 in the Appendix. There were no overall significant treatment effects for day 7 or day 14 cholesterol values when adjusted for a low initial cholesterol level of 85 mg/dl, medium values of 100 or 115 mg/dl, or for an initial high cholesterol level of 130 mg/dl. When adjusted for an initial cholesterol level of 85 mg/dl there was a significant decrease in total serum cholesterol from day 7 to day 14 for treatment 2 (control milk + *L. acidophilus*). For all other treatments there were no significant differences between day 7 and day 14 total cholesterol values. There were no significant differences between day 7 and day 14 cholesterol levels when baseline was adjusted to 100 mg/dl. For the baseline adjustment of 115 mg/dl there were significant increases in cholesterol from day 7 to day 14 for treatments 1 (control) and 2. There were no significant increases or decreases for other treatments at this level. After the adjustment of baseline levels to 130 mg/dl there was a significant increase in cholesterol from day 7 to day 14 for treatments 1 and 2. There were no significant increases or decreases for other treatments at this level.

Lipopolysaccharide Challenge and Immunoglobulin Levels

On day 14 of the feeding trial pigs in treatment groups 1, 2, 3, and 4 were injected with lipopolysaccharide (LPS) derived for enteropathogenic *E. coli* O111:B4. The serum levels for IgG, IgA, and IgM were measured as a means of determining if the treatment influenced the immune response of the pigs. All of the immunoglobulin data were analyzed with a repeated measures analysis. Both IgG and IgA had significant covariate effect for initial immunoglobulin levels so an analysis of covariance (ANCOVA) was conducted. The IgM did not have a significant covariate effect for baseline levels so an analysis of variance (ANOVA) was conducted. (The immunoglobulin results for IgA, IgG, and IgM are summarized in Table 8 in the Appendix.)

While there was a trend toward higher levels of IgA for the group(s) receiving milk and lactobacilli overall there was no significant treatment effect ($p=0.0803$). There were significant overall effects for initial IgA*treatment ($p=0.0002$) and hour of blood sampling ($p=0.0004$). (The hour effects are summarized in Table 9 in the Appendix.) Because of the significant covariate effect of initial IgA values some adjustments were made for comparisons. Baseline IgA levels were adjusted to 8, 10, and 12 mg IgA/ml serum. There were some significant differences when baseline values were adjusted to 10 mg/ml. The treatment 3 group had the highest IgA level of 11.20 mg/ml. The treatment 2 level of 11.00 mg/ml did not differ significantly from treatment 3 ($p=0.6492$) or the control (treatment 1) level of 10.08 mg/ml ($p= 0.0536$). Treatment 4 resulted in the lowest level of IgA at 9.62 mg/ml. When baseline was adjusted to 12 mg/ml

treatment 3 had the highest IgA level of 13.50. Both treatments 1 and 2 did not differ significantly from this level with their values of 12.10 (p=0.1380) and 13.05 (p=0.4341) mg/ml respectively, however treatment 4 had a significantly lower IgA level of 10.79 mg/ml.

The changes from 0 hr for levels of IgA across hours are illustrated in Figure 2. The increase in IgA levels for all treatments peaked at 1.5 hours. Treatment 3 peaked at the highest level while treatment 2 was the next highest. The control treatment had the lowest IgA level at 1.5 hours. The IgA levels decreased for all treatments after 1.5 hours. Treatment 2 had the highest levels at hours 3 and 6 with treatment 3 at the second highest. Treatment 4 dropped off faster than the control.

For IgG there was no overall treatment effect (p=0.0743) or treatment*hour effect (p=0.9004). There were significant effects for initial IgG*treatment (0.0446) and hour of blood sampling (p=0.0022). (The hour effects are summarized in Table 9 in the Appendix.) Baseline IgG values were adjusted because of the significant covariate effect of initial IgG levels. Comparisons were made from baseline adjustments to 15, 30, and 45 mg IgG/ml serum. At the baseline adjustment of 45 mg/ml both treatments 2 and 3 exhibited the highest IgG levels of 43.92 and 37.72 mg/ml respectively. The IgG levels of treatment 1 and 4 were significantly lower than those of treatments 2 and 3 whereas the levels for treatments 1 and 4 were not significantly different from each other (p=0.2185).

The changes from 0 hr for levels of IgG across hours are illustrated in Figure 3. Treatment 3 had the highest IgG level that peaked at 1.5 hours. Treatment 2 had the next highest levels. Treatment 4 decreased from hour 0 and was lower than the control treatment, which also decreased from hour 0.

For IgM there was no overall treatment effects (p=0.2337) or treatment*hour effects (p=0.5430). There was a significant hour effect (p=0.0038). (The hour effects are summarized in Table 9 in the Appendix.) No significant covariate effect for initial IgM levels was observed so no baseline adjustments were needed. Even though the values for treatments including the lactobacilli tended to be higher than their respective controls, there were no significant differences between the treatments for IgM levels.

The IgM level trends across hours are illustrated in Figure 4. All treatments except for the control increase and showed a peak at 1.5 hours, followed by a decrease, and then an increase to hour 6. The treatment 4 group exhibited the highest IgM levels followed by treatments 2 and 3 which had similar responses to one another. The control treatment group had the lowest IgM levels.

Lipopolysaccharide Challenge and Pig Body Temperature

The body temperature of the pigs in the LPS challenge was taken initially and at 1.5, 3.0, and 6.0 hours after the administration of the *E. coli* LPS. (The temperature trends are illustrated in Figure 5 in the Appendix.) Temperatures for all groups (treatments 1-4) appeared to peak at 3.0 hours. From 0 to 3.0 hours

there was a steady increase. After 3.0 hours the temperatures decreased sharply by hour 6.0.

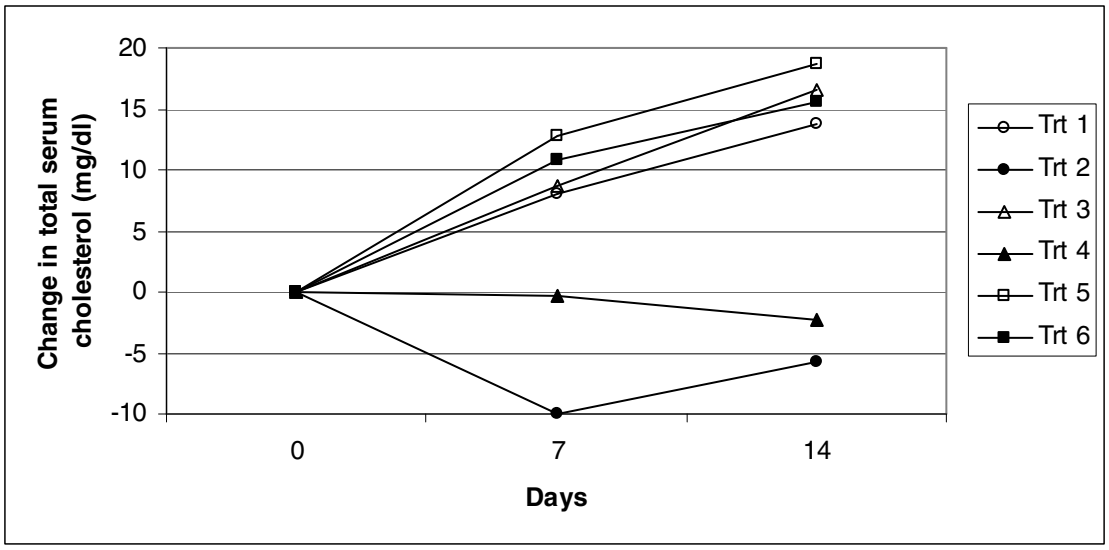

Figure 1. Changes in total serum cholesterol (mg/dl) from day 0 to in response to experimental treatments. Treatment descriptions: 1) control milk, 2) control milk + *L. acidophilus* RP32, 3) control milk fermented with *S. thermophilus* OSU-1, 4) control milk fermented with *S. thermophilus* OSU-1 + *L. acidophilus* RP32, 5) 5 g oatwell 22 + control milk, 6) 5 g oatwell 22 + control milk with *L. acidophilus* RP32.

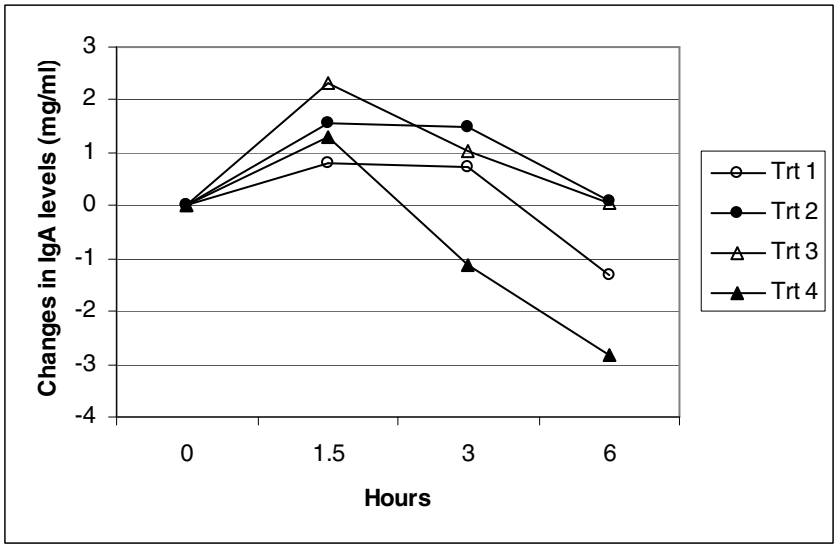

Figure 2. Changes in serum IgA levels (mg/ml) in response to *Escherichia coli* lipopolysaccharide challenge from hour 0 to 1.5, 0 to 3, and 0 to 6. Treatment descriptions: 1) control milk, 2) control milk + *L. acidophilus* RP32, 3) control milk fermented with *S. thermophilus* OSU-1, 4) control milk fermented with *S. thermophilus* OSU-1 + *L. acidophilus* RP32.

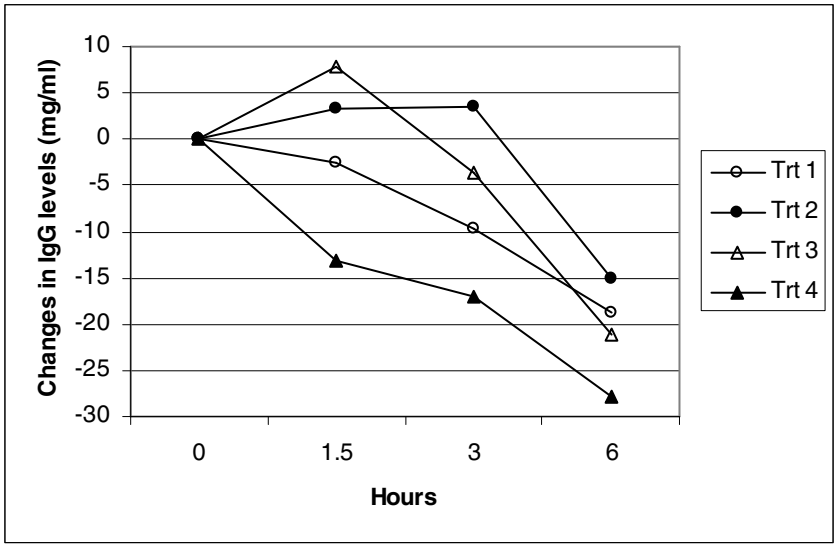

Figure 3. Changes in serum IgG levels (mg/ml) in response to *Escherichia coli* lipopolysaccharide challenge from hour 0 to 1.5, 0 to 3, and 0 to 6. Treatment descriptions: 1) control milk, 2) control milk + *L. acidophilus* RP32, 3) control milk fermented with *S. thermophilus* OSU-1, 4) control milk fermented with *S. thermophilus* OSU-1 + *L. acidophilus* RP32.

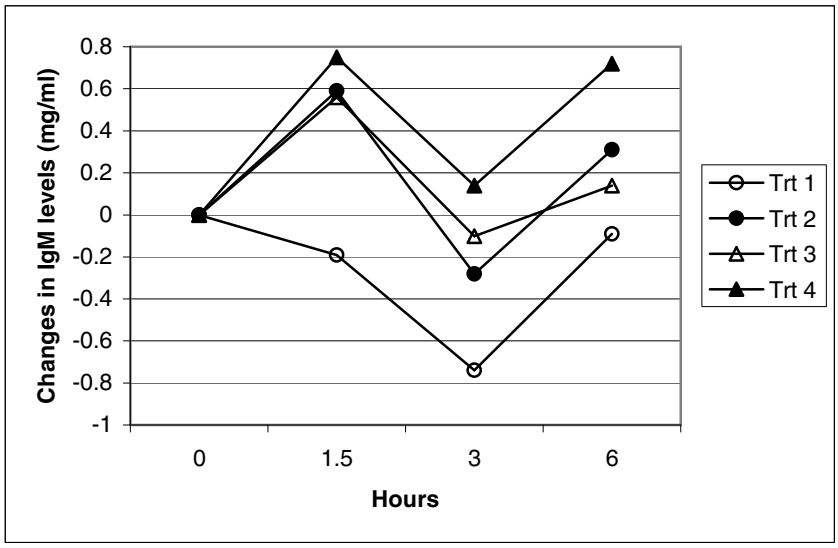

Figure 4. Changes in serum IgM levels (mg/ml) in response to *Escherichia coli* lipopolysaccharide challenge from hour 0 to 1.5, 0 to 3, and 0 to 6. Treatment descriptions: 1) control milk, 2) control milk + *L. acidophilus* RP32, 3) control milk fermented with *S. thermophilus* OSU-1, 4) control milk fermented with *S. thermophilus* OSU-1 + *L. acidophilus* RP32.

DISCUSSION

The strain of *L. acidophilus* used for the present study was one used in previous research in our laboratory because of several of its characteristics. *Lactobacillus acidophilus* RP32 (ATCC 43121) was isolated from the intestine of a pig and is bile tolerant. It can remove cholesterol from laboratory media by assimilation and has also been shown to exert a hypocholesterolemic effect in swine (Gilliland et al., 1985, De Rodas et al., 1996).

This study used young pigs for several reasons. The smaller size of a young pig made for easier handling than a mature animal. It has also been documented that hypercholesterolemia can be induced in young pigs more readily than adult animals (Barnes et al., 1959; Ratcliffe and Luginbühl, 1971). The pig has also been deemed a very acceptable model for both cardiovascular and immunological applications (Ratcliffe and Luginbühl, 1971; Rothkötter et al., 2002).

While there was no overall significant influence on serum cholesterol when all animals in each group were included in the statistical analyses, the trend was for lower levels in two out of three groups fed the lactobacilli. The exeption was those on the feed supplemented with β-glucan.

The results obtained from the cholesterol feeding trial were highly variable among animals. This presented a problem for data analysis because the high variability was masking some of the trends that might be present. In order to minimize the effects of the high variance, the data were divided into subgroups

by treatments for statistical analysis. The subgroups are as follows: milk without and with cells of *L. acidophilus* (treatments 1 and 2), fermented milk without and with cells of *L. acidophilus* (treatments 3 and 4), and β-glucan plus milk without and with cells of the lactobacilli (treatments 5 and 6). Subgrouping allowed for the adequate modeling of the variance. There was a significant initial cholesterol by treatment by day interaction. The data were analyzed with day 0 cholesterol values as a covariate. In order to make comparisons the data was adjusted to baseline cholesterol levels representing a low, two midway, and a high starting cholesterol. The initial cholesterol had a significant impact on the outcome. This revealed a significant decrease in serum cholesterol observed only from day 7 to day 14 for treatment group 2 in the low baseline (85 mg/dl) compared to the control group (treatment 1). This culture has demonstrated cholesterol assimilation (Gilliland et al., 1985; Noh et al. 1997) and bile salt deconjugation in laboratory media (Gilliland et al., 1985; Corzo and Gilliland, 1999a.). The culture of *L. acidophilus* exerted beneficial control of serum cholesterol levels in pigs fed a high cholesterol diet (Gilliland et al., 1985; De Rodas et al., 1996). It is unclear why there were no other significant differences besides the problem of variation on the individual animal level. The young pigs used in the study by Gilliland et al. (1985) had initial serum cholesterol levels ranging from 52 to 56 mg/dl and exhibited less variability in the values. It was also found that the pigs in the present study were slightly younger than those used in the 1985 study. This may have also had some effect on the cholesterol results.

Milk fermented with *S. thermophilus* OSU-1 (treatment 3) compared to the fermented milk with added *L. acidophilus* RP32 (ATCC 43121) (treatment 4) did not indicate any appreciable differences. The inclusion of the fermented milk to the project was done because of the ability of exocellular polysaccharides (EPS) formed by *S. thermophilus* OSU-1 to bind free bile acids. Pigeon et al. (2002) studied several strains of *L. delbrueckii* ssp. *bulgaricus* and *S. thermophilus* that produce EPS. The strains that produced the highest amounts of EPS were tested for the ability to bind free bile acids. *Streptococcus thermophilus* OSU-1 was among those strains tested. It was able to bind the most cholic acid when compared to the other two *S. thermophilus* strains tested.

In theory the bile salt binding ability of *S. thermophilus* OSU-1 may also help to enhance the bile salt deconjugation of *L. acidophilus* RP32 (ATCC 43121) by helping to control feedback inhibition. An accumulation of free bile salts caused feedback inhibition of the bile salt hydrolase enzyme of *L. acidophilus* (Corzo, 1997). This enzyme is responsible for the bile salt deconjugation by *L. acidophilus* RP32 (ATCC 43121). Such activity in the gut may be partially responsible for helping lower serum cholesterol.

Unfortunately neither treatment 3 nor 4 appeared to confer a significant decrease in serum cholesterol. When baseline levels were adjusted to 130 mg/dl there was a decrease in cholesterol from day 7 to day 14 for treatment 4 but it was not significant. For treatment 3 the bile salt binding ability of *S. thermophilus* OSU-1 may not have been high enough to realize a significant difference in an *in vivo* system. The combination of *S. thermophilus* OSU-1 and *L. acidophilus*

RP32 (ATCC 43121) did not exhibit the enhanced combined effect as was hoped.

Treatment 5 was comprised of 5 g of oat bran fiber and control milk while treatment 6 was similar but with the addition of *L. acidophilus* RP32 (ATCC 43121). These treatments were components of the study based on the ability of oat bran to help lower serum cholesterol. Oat β-glucan can bind free bile acids and result in the increased fecal excretion of bile acids (Kirby et al., 1981; Anderson et al. 1991; Lia et al., 1995; Amundsen et al., 2003). There are several other proposed cholesterol-lowering mechanisms for oat β-glucan. Colonic microflora ferment oat bran to short chained fatty acids such as propionate (Anderson et al. 1991) which can inhibit cholesterol synthesis in the liver (Wright et al., 1990; Chen et al., 1984). Increased soluble fiber in the diet causes a decrease in insulin secretion. This may decrease cholesterol synthesis because insulin stimulates HMG-CoA reductase which is involved in the rate limiting step of cholesterol formation in the liver (Jenkins et al., 1989). Increased gut viscosity from oat bran also may form a barrier to deter bile acid reapsorption (Beer et al., 1995).

There were no significant differences for either treatment 5 or 6. However there were some statistically insignificant decreases. When the baseline cholesterol was adjusted to 115 and 130 mg/dl there was a trend toward a slight decrease in cholesterol from day 7 to day 14 for treatment 6. The optimum amount of oat bran to consume to elicit positive health effects has not been determined. We arrived at the amount of oat bran fed in this trial by observing

amounts of oat bran fed in a variety of human studies. The estimated amount of β-glucan fed to test subjects and their average weights were taken into account. This led to an approximate amount to feed on a weight basis which was extrapolated to the approximate weight of the pigs. Because of all of these approximations we may have not incorporated enough oat bran into the diets of treatment groups 5 and 6 to observe more obvious and significant cholesterol lowering effects.

The immunoglobulins measured in this study are the main antibodies present in blood, lymph, and connective tissue (Parham, 2000). The IgG aids phagocytic cells in the engulfment of microorganisms and toxins. A bacterium becomes opsonized by being coated with IgG and this causes a more efficient destruction of the organism. During an immune response IgM is the first antibody secreted. When IgM binds to the pathogen surface the complement system is activated and the pathogen will be engulfed by macrophages. The IgA binds to toxins and pathogens on mucosal surfaces. More IgA is made in the gastrointestinal tract than other isotypes to protect the body from infection that can accompany the uptake of food.

Pigs in treatment group 1, 2, 3, and 4 were subjected to an *E. coli* O111:B4 lipopolysaccharide (LPS) challenge so that possible immune stimulating effects of the probiotics could be tested. This particular LPS was used based on previous research in the nutritional science and animal science departments at Oklahoma State University (Mandali et al., 2000; Mandali et al., 2002). Pigs fed cells of *L. acidophilus* (either in milk or fermented milk) tended to have higher

levels of IgA than did the respective controls. However, overall the differences were not significant. There was considerable variation in the immunoglobulin data which may be the reason for lack of significant differences. Other studies have reported variation in immunoglobulin levels of young pigs (DeBuysscher and Berman, 1980). For analyses a baseline level adjustment into groups of 8, 10, and 12 mg/ml was used for IgA statistical analysis. Using this approach, for the baseline of 10 mg/ml group the treatment 3 group exhibited the highest IgA level of 11.2 mg/ml. Treatment 2 was the second highest at 11.00 mg/ml and this was not significantly different from the treatment 3 result. The control treatment (1) was not significantly different from treatment 2 or the IgA level for treatment 4 which was statistically the lowest at 9.62 mg/ml.

Similar isolated significant differences were noted for IgG using this statistical approach. However, we are not able to say, based on the data from this study, that in general the consumption of cells of *L. acidophilus* exerts significant impact on immunoglobulins. In the case of the fermented milk with and without cells of the lactobacilli there was no trend for apparent differences. This may be due to some sort of interaction between the cultures. Results from a study by Perdigón et al. (1988) revealed a diminished immune enhancement when two cultures were combined in a treatment given to mice compared to the individual cultures. In their study the two cultures were grown together in this study. In the present study the milk was fermented with *S. thermophilus* OSU-1 and a concentrated culture of *L. acidophilus* RP32 (ATCC 43121) was added after fermentation. There may have been unfavorable interactions between the

two cultures *in vivo* that affected the immune enhancing capabilities. For future research it may be necessary to investigate any interactions *in vitro* before a combination is used.

A temperature reading was taken at the same time intervals as the blood sampling. Temperatures for all treatment groups followed the same trend. There was a steady increase and the peak temperatures occurred at 3.0 hours and declined sharply by hour 6.0.

For future research on the enhancement of immunoglobulins it may be beneficial to test other parameters. Webel et al. (1998) tested the effects of vitamin E during an LPS challenge. The immune system markers tested were interleukin-6 (IL-6) and plasma cortisol. The researchers justified this choice of immune parameters because IL-6 is more effective than interleukin-1 (IL-1) or tumor necrosis factor-α (TNF- α) for stimulating specific proteins in response to inflammation. Plasma cortisol was chosen because it is elevated at the same time as IL-6 after an LPS challenge.

It also may be beneficial to measure immunoglobulins specific to the source of the challenge organism. Tejada-Simon et al. (1999) used a cholera toxin to challenge mice being fed a yogurt containing *L. acidophilus* and *Bifidobacterium*. Anti-cholera toxin IgA levels were tested in both fecal pellets and blood serum. Link-Amster et al. (1994) measured both IgG and IgA titers specific to their challenge organism, *Salmonella typhi* Ty21a. Measuring immunoglobulins that are specific to the challenge organism may give a more accurate description of what is occurring due to the experimental procedures.

In the present study LPS from an enteropathogenic *E. coli* O111:B4 was used to challenge the pigs. This organism and other enteric organisms cause intestinal infections. It also may be of more benefit to measure intestinal immunoglobulin levels. Link-Amster et al. (1994) concluded that the consumption of fermented milk may increase the humoral immune response to intestinal pathogens in the intestine. They used fecal immunoglobulins as a measure of intestinal immunoglobulin levels.

In summary the present study shows some positive and some variable results for the use of a probiotic culture and oat bran combinations to help control hypercholesterolemia in pigs fed a high cholesterol diet. More research is needed to adequately test the effects of the treatments without as much animal to animal variability. It may be desirable to prescreen animals to select ones that have less variable levels of serum cholesterol for use in the trials.

The treatments were more promising for the enhancement of immune function. Both IgA and IgG levels were increased. Treatments 2 and 3 seemed to confer the most immunological benefits. Further study is needed to investigate the immune enhancing capabilities of *L. acidophilus* RP32 (ATCC 43121) and *S. thermophilus* OSU-1. This type of research could lead to the development of cultured products to aid in immune enhancement for humans.

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APPENDIX

Table 2. Assignment of pigs to treatments for feeding trial

[a]Treatment	Pig Pen #
1	3
1	5
1	20
1	24
2	1
2	11
2	14
2	18
3	2
3	4
3	13
3	17
4	9
4	10
4	19
4	22
5	6
5	7
5	21
5	23
6	8
6	12
6	15
6	16

[a]Treatment 1 = 25 ml 10% NFMS/day, Treatment 2 = 25 ml 10% NFMS + *L. acidophilus* RP32/day, Treatment 3 = 25 ml 10% NFMS fermented with *S. thermophilus* OSU-1/day, Treatment 4 = 25 ml 10% NFMS fermented with *S. thermophilus* OSU-1 + *L. acidophilus* RP32/day, Treatment 5 = 25 ml 10% NFMS + 5 g Oatwell oat bran (on top of feed)/day, Treatment 6 = 25 ml 10% NFMS + L. acidophilus RP32 + 5 g Oatwell oat bran (on top of feed)/day.

Table 3. Identification of *Lactobacillus acidophilus* RP32 (ATCC 43121) by fermentation of carbohydrates[1]

Carbohydrate	Bergey's[2]	*L. acidophilus* RP32 (ATCC 43121)
D-Arabinose	-	-
Ribose	-	-
D-Xylose	-	-
Galactose	+	+
D-Glucose	+	+
D-Fructose	+	+
D-Mannose	-	-
Rhamnose	-	-
Mannitol	-	-
Sorbitol	-	-
N-acetyl glucosamine		+
Amygdaline	+	+
Arbutine		+
Esculine	+	+
Salicine	+	+
Cellobiose	+	+
Maltose	+	+
Lactose	+	+
Melibiose	+/-	+
Saccharose	+	+
Trehalose	+/-	+
Melezitose	-	-
D-Raffinose	+/-	+
Amidon	+	+
B-Gentibiose		+
D-tagatose		+
Gluconate	-	-
Growth at 15°C	-	-
Growth at 45°C	+	+

[1] Based on the API 50 CH system (bioMérieux sa, France). *L. acidophilus* RP32 (ATCC 43121) is a Gram positive catalase negative rod.
[2] The reactions for *Lactobacillus acidophilus* are from the 9th edition of Bergey's Manual of Determinative Bacteriology.

Table 4. Identification *Streptococcus thermophilus* by carbohydrate fermentation[1]

Carbohydrate	Bergey's[2]	*S. thermophilus* OSU-1
Glycerol	-	-
D-Arabinose	-	-
L-Arabinose	-	-
L-Xylose	-	-
D-Glucose	+	+
D-Fructose	+	-
D-Mannose	+	-
Rhamnose	-	-
Mannitol	-	-
Sorbitol	-	-
Salicine	-	-
Maltose	+/-	-
Lactose	+	+

[1] Based on the API 50 CH system (bioMérieux sa, France). *S. thermophilus* OSU-1 is a Gram positive catalase positive cocci that grows at 45°C and not at 15°C.

[2] The reactions for *Streptococcus thermophilus* are from the 9th edition of Bergey's Manual of Determinative Bacteriology.

Table 5. Influence of oatwell 22 β-glucan solution on cholesterol assimilation of *Lactobacillus acidophilus* RP32 (ATCC 43121)

Treatment	µg cholesterol removed[1,2]
416.67 µg/ml β-glucan	15.33 (1.53)
No β-glucan	14.67(1.53)

[1]Each value is a mean from three replications of the experiment; numbers in parenthesis = standard deviation.

[2]µg of cholesterol removed were determined by subtracting the amount of cholesterol in the spent broths from that in an uninoculated control; the inoculated broth contained approximately 1,000 µg/ml.

Table 6. Influence of β-glucan solution from Oatwell 22 on deconjugation of taurocholate by *Lactobacillus acidophilus* RP32 (ATCC 43121)[1]

Sample	Cholic acid released (μmoles/ml)
No β-glucan	0.73 (0.12)[2]
β-glucan	0.60 (0.03)[2]

[1]Each value is a mean from three trials; during growth for 16 hr in MRS-thio broth supplemented with 538 μg sodium taurocholate/ml.
[2]numbers in parenthesis = standard deviation.

Table 7. Effect of treatments on total serum cholesterol of pigs fed a high cholesterol diet

		Treatments[1]					
Initial cholesterol (mg/dl)	Day	1	2	3	4	5	6
85[2] (79-102)	7	109.19[a]	93.41[a]	101.56[a]	24.70[a]	104.83[a]	100.51[a]
	14	105.85[a]	79.75[b]	107.16[a]	63.94[a]	109.35[a]	107.37[a]
100[2] (86-114)	7	109.01[a]	91.15[a]	101.92[a]	66.79[a]	114.45[a]	102.53[a]
	14	114.19[a]	94.28[a]	111.55[a]	87.42[a]	120.10[a]	103.64[a]
115[2] (86-114)	7	108.83[b]	88.89[b]	102.27[a]	108.88[a]	124.07[a]	104.55[a]
	14	122.54[a]	108.81[a]	115.93[a]	110.91[a]	130.85[a]	99.90[a]
130[2] (102-136)	7	108.65[b]	86.63[b]	102.62[a]	150.97[a]	133.70[a]	106.56[a]
	14	130.88[a]	123.35[a]	120.32[a]	134.40[a]	141.60[a]	96.17[a]

No significant differences were detected between treatments at each baseline cholesterol level.

[ab]Response means within a column with the same letter are not significantly different (p=0.05).

[1]Description of treatments – see Table 2 pg. 67

[2]Baseline value adjustment for treatment comparisons; numbers in parentheses beneath the baseline value represent the range of values included for the initial cholesterol adjustment.

Table 8. Serum immunoglobulin levels for pigs challenged with lipopolysaccharide (LPS) from *E. coli* O111:B4

IgA (mg/ml)						
Trt[1]	$IgA_0 = 8$[2,3]		$IgA_0 = 10$[2,3]		$IgA_0 = 12$[2,3]	
1	8.07[a]	(0.52)	10.08[bc]	(0.55)	12.10[ab]	(0.75)
2	9.02[a]	(0.56)	11.00[ab]	(0.51)	13.05[a]	(0.59)
3	8.89[a]	(0.55)	11.20[a]	(0.52)	13.50[a]	(0.66)
4	8.45[a]	(0.53)	9.62[c]	(0.51)	10.79[b]	(0.51)

IgG (mg/ml)						
Trt	$IgG_0 = 15$[2,3]		$IgG_0 = 30$[2,3]		$IgG_0 = 45$[2,3]	
1	20.14[a]	(5.71)	23.48[ab]	(4.08)	26.83[b]	(4.47)
2	10.66[a]	(5.73)	27.29[a]	(3.96)	43.92[a]	(5.92)
3	12.84[a]	(5.51)	25.28[ab]	(4.07)	37.72[a]	(4.39)
4	19.40[a]	(4.58)	21.19[b]	(4.09)	22.99[b]	(3.96)

IgM (mg/ml) No covariate necessary		
Trt		
1	2.16[a]	(0.25)
2	2.54[a]	(0.25)
3	1.80[a]	(0.25)
4	1.93[a]	(0.25)

[abc] Response means within a column with the same letter are not significantly different (p=0.05).

[1]Treatment descriptions – see Table 2 on pg. 67

[2]Baseline value adjustment for treatment comparisons.

[3]Values are least squares estimates; Numbers in parenthesis = standard error.

Table 9. Hour effects for changes in serum immunoglobulins.

	Immunoglobulins (mg/ml)		
Hour[1]	IgA[2]	IgG[2]	IgM[2]
0[3]	9.75	35.38	1.99[b] (0.16)
1.5	11.41[a] (0.53)	35.79[a] (4.56)	2.42[a] (0.16)
3	10.42[b] (0.53)	30.19[a] (4.56)	1.75[b] (0.16)
6	8.92[c] (0.53)	16.15[b] (4.56)	2.26[a] (0.16)

[1]Hour of blood collection for immunoglobulin analysis.

[2]Least squares means estimate; the numbers in parentheses are standard errors.

[3]Hour 0 averages are included. The least squares means estimates for hour 0 for IgA and IgG are not available because hour 0 was a covariate in the analysis.

[ab]Response means within a column with the same letter are not significantly different (p=0.05).

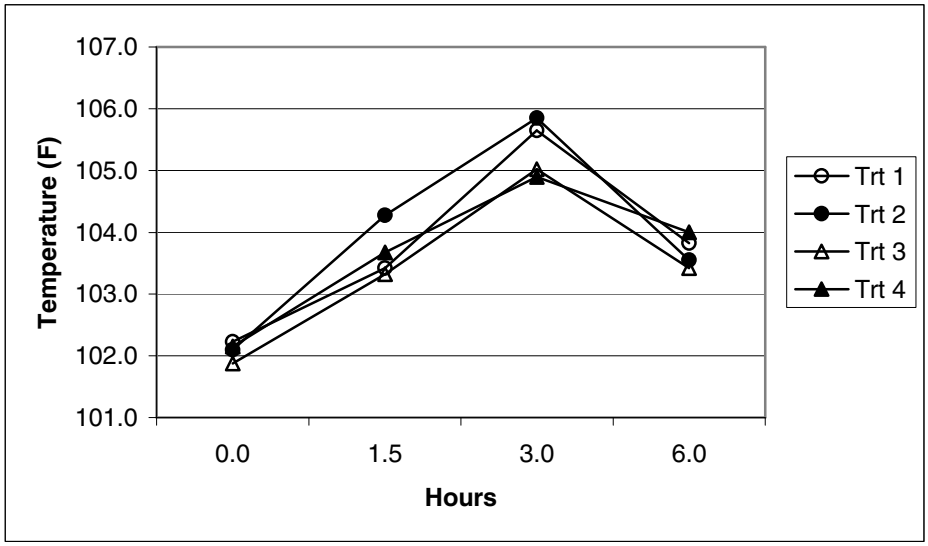

Figure 5. Changes in pig body temperature in response to *Escherichia coli* lipopolysaccharide challenge. Treatment descriptions: 1) control milk, 2) control milk + *L. acidophilus* RP32, 3) control milk fermented with *S. thermophilus* OSU-1, 4) control milk fermented with *S. thermophilus* OSU-1 + *L. acidophilus* RP32.

Table 10. Influence of treatments 1 and 2 on serum cholesterol (mg/dl) in pigs fed a high cholesterol diet (raw data)

	1[a]			2[a]		
	Day 0	Day 7	Day 14	Day 0	Day 7	Day 14
	125	103	125	83	100	81
	82	109	104	98	73	81
	116	116	127	115	85	104
	81	108	103	108	106	115
Means	101	109	114.75	101	91	92.25

[a]See footnote of Table 2 on pg. 67 for treatment descriptions.

Table 11. Influence of treatments 3 and 4 on serum cholesterol (mg/dl) in pigs fed a high cholesterol diet (raw data)

	3[a]			4[a]		
	Day 0	Day 7	Day 14	Day 0	Day 7	Day 14
	84	128	111	111	104	104
	102	138	129	117	119	100
	98	62	89	109	83	112
	88	79	109	136	166	148
Means	93	101.75	109.5	118.25	118	116

[a] See footnote of Table 2 on pg. 67 for treatment descriptions.

Table 12. Influence of treatments 5 and 6 on serum cholesterol (mg/dl) in pigs fed a high cholesterol diet (raw data)

	5[a]			6[a]		
	Day 0	Day 7	Day 14	Day 0	Day 7	Day 14
	113	151	153	79	108	126
	101	127	129	91	103	94
	72	81	88	111	104	106
	133	111	124	81	90	98
Means	104.75	117.5	123.5	90.5	101.25	106

[a] See footnote of Table 2 on pg. 67 for treatment descriptions.

Table 13. Influence of treatments on serum HDL cholesterol (mg/dl) in pigs fed a high cholesterol diet

	1[a]			2[a]		
	Day 0	Day 7	Day 14	Day 0	Day 7	Day 14
	49	36	47	40	36	31
	35	33	33	47	32	36
	43	36	44	42	28	40
	27	30	31	41	32	38
Means	38.5	33.8	38.8	42.5	32.0	36.3
	3[a]			4[a]		
	Day 0	Day 7	Day 14	Day 0	Day 7	Day 14
	33	38	35	51	52	47
	40	46	47	39	55	51
	46	20	31	56	49	62
	34	21	31	72	99	87
Means	38.3	31.3	36.0	48.3	40.8	42.5
	5[a]			6[a]		
	Day 0	Day 7	Day 14	Day 0	Day 7	Day 14
	46	51	56	34	33	40
	39	35	42	36	29	31
	31	31	34	43	35	37
	51	32	41	34	31	33
Means	41.8	37.3	43.3	36.8	32.0	36

[a]Treatment descriptions – see Table 2 pg. 67.

Table 14. Influence of treatments on serum LDL cholesterol (mg/dl) in pigs fed a high cholesterol diet

	1[a]			2[a]		
	Day 0	Day 7	Day 14	Day 0	Day 7	Day 14
	67	59	65	31	53	38
	35	55	61	35	32	31
	60	68	70	64	47	54
	45	66	64	50	66	65
Means	51.8	62.0	65.0	45.0	49.5	47.0
	3[a]			4[a]		
	Day 0	Day 7	Day 14	Day 0	Day 7	Day 14
	43	79	65	46	40	44
	50	76	73	55	42	38
	38	35	51	42	28	40
	42	49	67	50	53	48
Means	43.3	59.8	64.0	54.5	63.8	61.8
	5[a]			6[a]		
	Day 0	Day 7	Day 14	Day 0	Day 7	Day 14
	51	85	76	32	63	74
	42	76	76	43	61	55
	29	44	43	52	64	58
	72	72	74	35	52	58
Means	48.5	69.3	67.3	40.5	60.0	61.3

[a]Treatment descriptions – see Table 2 pg. 67.

Table 15. Influence of treatments on serum triglycerides (mg/dl) in pigs fed a high cholesterol diet

	1[a]			2[a]		
	Day 0	Day 7	Day 14	Day 0	Day 7	Day 14
	45	36	65	58	56	58
	59	104	46	75	46	68
	67	61	64	49	51	52
	42	60	40	87	40	57
Means	53.3	65.3	53.8	67.3	48.3	58.8
	3[a]			4[a]		
	Day 0	Day 7	Day 14	Day 0	Day 7	Day 14
	45	58	56	72	57	62
	60	78	49	116	106	56
	69	34	37	58	28	48
	60	45	57	68	72	63
Means	58.5	53.8	49.8	78.5	65.8	57.3
	5[a]			6[a]		
	Day 0	Day 7	Day 14	Day 0	Day 7	Day 14
	79	69	105	65	61	60
	100	79	59	62	60	44
	61	30	52	81	31	54
	53	36	44	59	36	38
Means	73.3	53.5	65.0	66.8	47.0	49.0

[a]Treatment descriptions – see Table 2 pg. 67.

Table 16. Average daily gain, feed intake, and feed efficiency of pigs recorded during feeding of high cholesterol diet

Treatment[a]	Pen #	Weight gain D0-D14 (kg)	Avg. Daily Feed Intake (kg)	Avg. Daily Gain	Feed Efficiency (F:G)
1	3	7.03	0.66	0.50	1.32
1	5	5.82	0.62	0.42	1.48
1	20	6.71	0.68	0.48	1.42
1	24	5.99	0.64	0.43	1.49
	Avg.	6.39	0.65	0.46	1.41
2	1	6.53	0.66	0.47	1.40
2	11	6.71	0.61	0.48	1.27
2	14	6.98	0.65	0.50	1.30
2	18	6.17	0.66	0.44	1.50
	Avg.	6.60	0.64	0.47	1.36
3	2	6.45	0.67	0.46	1.46
3	4	6.53	0.64	0.47	1.36
3	13	7.31	0.67	0.52	1.29
3	17	6.94	0.70	0.50	1.40
	Avg.	6.81	0.67	0.49	1.37
4	9	6.94	0.64	0.50	1.28
4	10	5.26	0.55	0.38	1.45
4	19	7.78	0.70	0.56	1.25
4	22	6.44	0.66	0.46	1.43
	Avg.	6.61	0.64	0.47	1.36
5	6	5.53	0.64	0.40	1.60
5	7	4.85	0.57	0.35	1.63
5	21	6.94	0.67	0.50	1.34
5	23	7.12	0.70	0.51	1.37
	Avg.	6.11	0.64	0.44	1.45
6	8	5.85	0.59	0.42	1.40
6	12	6.30	0.56	0.45	1.24
6	15	7.62	0.70	0.54	1.30
6	16	7.03	0.62	0.50	1.24
	Avg.	6.70	0.61	0.48	1.27

[a] See footnote of Table 2 on pg. 67 for treatment descriptions.

Table 17. Weights of pigs recorded during feeding of a high cholesterol diet

Treatment[a]	Pen #	Weight (kg)			
		Day 0	Day 7	Day 14	Weight gain
1	3	10.93	14.38	17.96	7.03
1	5	8.60	11.34	14.42	5.82
1	20	10.39	13.79	17.10	6.71
1	24	9.89	12.88	15.88	5.99
	Avg.	9.95	13.10	16.34	6.39
2	1	8.94	11.97	15.47	6.53
2	11	10.07	13.79	16.78	6.71
2	14	10.57	13.65	17.55	6.98
2	18	10.39	13.38	16.56	6.17
	Avg.	9.99	13.20	16.59	6.60
3	2	10.02	13.34	16.47	6.45
3	4	8.94	11.97	15.47	6.53
3	13	10.70	14.24	18.01	7.31
3	17	10.02	13.52	16.96	6.94
	Avg.	9.92	13.27	16.73	6.81
4	9	9.89	13.43	16.83	6.94
4	10	8.98	11.11	14.24	5.26
4	19	10.82	14.97	18.60	7.78
4	22	10.07	13.34	16.51	6.44
	Avg.	9.94	13.21	16.55	6.61
5	6	9.48	11.84	15.01	5.53
5	7	9.66	11.84	14.51	4.85
5	21	9.75	13.15	16.69	6.94
5	23	11.07	14.70	18.19	7.12
	Avg.	9.99	12.88	16.10	6.11
6	8	9.84	12.29	15.69	5.85
6	12	9.53	11.79	15.83	6.30
6	15	11.16	15.06	18.78	7.62
6	16	9.12	12.25	16.15	7.03
	Avg.	9.91	13.03	16.61	6.70

[a] See footnote of Table 2 on pg. 67 for treatment descriptions.

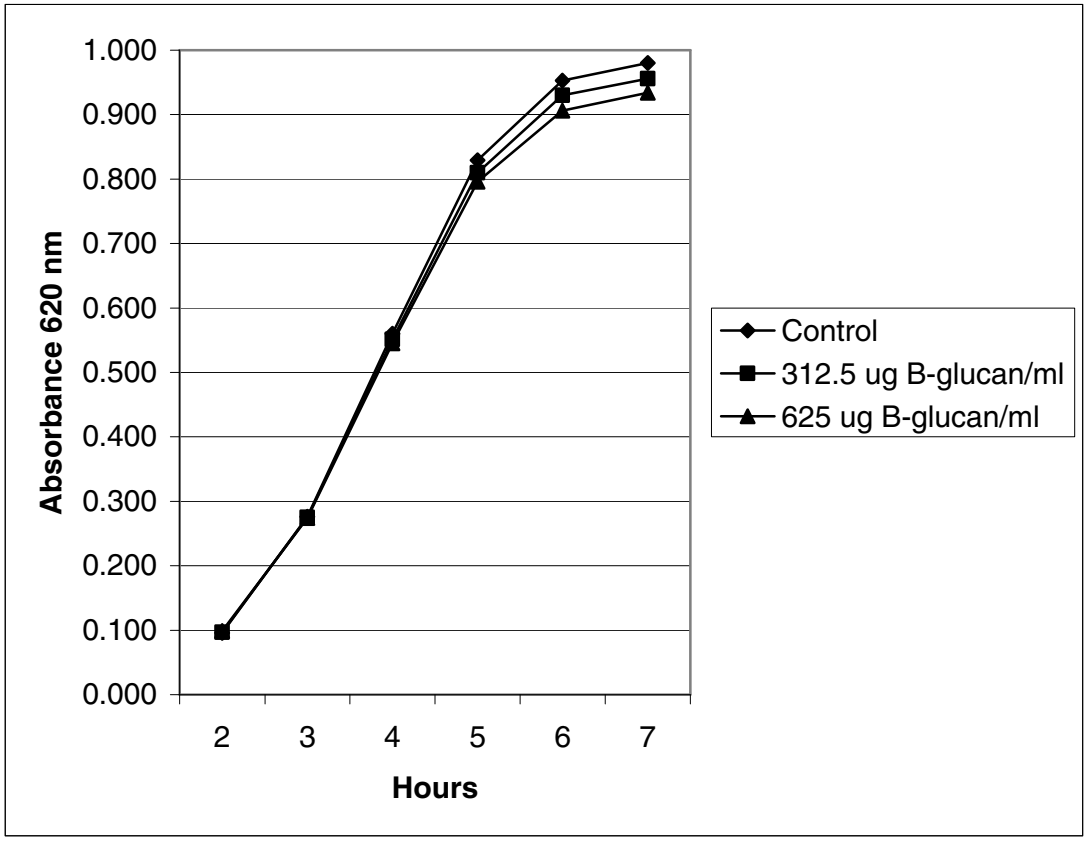

Figure 6. Effect of oatwell 22 oat bran β-glucan on the growth of *Lactobacillus acidophilus* RP32 (ATCC 43121) when grown in MRS-thio (sodium thioglycollate) at 37°

VITA

Lacey Michele Smith

Candidate for the Degree of

Master of Science

Thesis: POTENTIAL FOR ENHANCING HYPOCHOLESTEROLEMIC EFFECT OF *LACTOBACILLUS ACIDOPHILUS* WITH SOLUBLE FIBER AND THE INFLUENCE OF THE LACTOBACILLI ON IMMUNE RESPONSE

Major Field: Food Science

Biographical:

Personal Data: Born in Amarillo, TX, December 4, 1980, the daughter of Mike and Jenny Smith

Education: Graduated from Wheeler High School, Wheeler, TX, in May, 1999; Received the Bachelor of Science degree in Food Technology from Texas Tech University in August, 2003; completed requirements for the Master of Science degree at Oklahoma State University in December, 2006

Experience: Student lab assistant at Texas Tech University, 2001-2003; Research Assistant/Teaching Assistant at Oklahoma State University 2003-present.

Professional Memberships: Institute of Food Technologists, International Association for Food Protection

Name: Lacey Michele Smith Date of Degree: December, 2006

Institution: Oklahoma State University Location: Stillwater, Oklahoma

Title of Study: POTENTIAL FOR ENHANCING THE HYPOCHOLESTEROLEMIC EFFECT OF *LACTOBACILLUS ACIDOPHILUS* WITH SOLUBLE FIBER AND THE INFLUENCE OF THE *LACTOBACILLUS* ON IMMUNE RESPONSE

Pages in Study: 84 Candidate for the Degree of Master of Science

Major Field: Food Science

Scope and Method of Study: The purpose of this study was to determine the hypocholesterolemic effects of *Lactobacillus acidophilus* RP32 (ATCC 43121), *Streptococcus thermophilus* OSU-1, and oat β-glucan in different combinations of six treatments on serum cholesterol in pigs fed a high cholesterol diet for 14 days. Additionally the immune enhancing effects of four of the treatments were tested. Serum IgA, IgG, and IgM were measured.

Findings and Conclusions: The results for both the cholesterol study and immunological study were highly variable. Because of variability among animals with respect to initial levels of serum cholesterol there were no overall significant effects. However, the mean levels of cholesterol tended to decrease in milk and fermented milk containing *L. acidophilus* while the respective controls tended to increase. Statistically, for pigs in a group with low cholesterol initially, the treatment containing cells of *Lactobacillus acidophilus* resulted in a decrease in serum cholesterol from day 7 to day 14. There were no other statistically significant results for controlling serum cholesterol. Both IgA and IgG were increased by treatments containing either *Lactobacillus acidophilus* or *Streptococcus thermophilus*. There were no statistically significant changes in IgM levels.

ADVISER'S APPROVAL: Dr. Stanley Gilliland