INFLUENCE OF pH ON BILE SALT DECONJUGATION AND REMOVAL OF CHOLESTEROL FROM MEDIA BY *LACTOBACILLUS CASEI* AND *LACTOBACILLUS ACIDOPHILUS* DURING GROWTH

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CHAPTER I

INTRODUCTION

High serum cholesterol levels are associated with the development of coronary heart disease, the leading cause of death in the United States (62, 73, 74, 98). However, cholesterol is an essential component of the human body because it is a precursor to vitamin D, steroid hormones, and bile acids. It also helps maintain the fluidity of cellular membranes.

Studies have indicated that some lactobacilli can remove cholesterol from laboratory media and have the potential to reduce serum cholesterol levels in humans (7, 15, 40). While studying the Maasai warriors, Mann and Spoerry (79) found that fermented milk may have a hypocholesterlemic effect in humans. Other studies have revealed that certain strains of lactobacilli have a cholesterol lowering effect in humans (5, 50, 80, 81) and animals (15, 16, 18, 40, 47, 86, 103, 123, 133).

The mechanisms involved in removal of cholesterol from laboratory media and lowering serum cholesterol levels in animals and humans are not well understood. Klayver and Van der Meer (65) said that removal of cholesterol from laboratory media during growth of *L. acidophilus* was due to deconjugation of bile salts resulting in disruption of the cholesterol micelles and "coprecipitation" of the cholesterol with the free bile acids. Deconjugated bile acids are less soluble as the pH drops due to acid production during growth by the cultures (111). However, Noh and Gilliland (92) grew cultures of *L. acidophilus* at a constant pH of 6.0. They found that cholesterol was still removed from the broth and that some of the cholesterol was recovered in the cellular membrane of the organisms thus indicating that the cells actually assimilated cholesterol.

Either mechanism can result in lowering of serum cholesterol levels. Any cholesterol assimilated by the lactobacilli in the small intestine would not be available for absorption into the blood by the human. Also, deconjugation of a large amount of bile salts by the culture could indirectly lead to lower serum cholesterol levels.

The most important route of excretion of cholesterol from the body is through the excretion of bile acids (127). Bile acids are synthesized in the liver from cholesterol and then they are conjugated with glycine or taurine and secreted into the small intestine where some of them may undergo deconjugation. Lactobacilli have been identified as the bacterial species most heavily involved in deconjuation of bile acids in the small intestines (13, 17, 36, 66, 75, 114). When bile acids are deconjugated in the small intestine, they are more readily excreted than conjugated ones (93). Cholesterol from the body is used to replace the bile acids that are excreted thus reducing the amount of cholesterol in the body.

When animals are fed bacterial cultures which have high bile salt hydrolase activity, there are more bile acids excreted in the feces and thus the serum cholesterol levels are reduced (11, 122).

Buck and Gilliland (7) isolated several strains of lactobacilli from human volunteers in an effort to discover a strain(s) having great potential for controlling serum cholesterol in man. One strain of *L. acidophilus* that was isolated removed a very large amount of cholesterol from laboratory media during growth. A study at the University of Kentucky revealed that hypercholesterolemic persons who consumed this strain of *L. acidophilus* had significant reductions in their serum cholesterol levels (unpublished). The strain is currently used to manufacture a yogurt product sold in Holland. The

product is sold making the claim that it can help maintain desirable serum cholesterol levels.

The objective of this study was to determine if *L. acidophilus* and *L. casei* removed cholesterol from laboratory media in a similar manner by 1) determining if there was a difference in the amount of cholesterol removed from the laboratory media when the pH was maintained at a constant level (pH 6.0) compared to cells grown without pH control, 2) determining the amount of sodium taurocholate deconjugated by the cultures during growth with and without pH control, and 3) determining if *L. casei* prefers to deconjugate sodium glycocholate or sodium taurocholate during growth with and without pH control of the medium at pH 6.0.

CHAPTER II

REVIEW OF LITERATURE

Significance of Cholesterol Assimilation by the Lactobacilli

Role of Cholesterol in the Body

Cholesterol is an essential component of the human body. It is a precursor to steroid hormones, Vitamin D and bile acids and is important in maintaining the fluidity of cell membranes (127). However, deposition of excess cholesterol in the arteries can lead to coronary heart disease, the leading cause of death in the United States (62, 73, 98, 124).

The body's cholesterol is synthesized in the liver and/or is absorbed from dietary sources from the intestinal tract (3, 22, 31, 83, 121). The most important route of excretion of cholesterol from the body is through the bile acids. Bile acids are synthesized from cholesterol in the liver and are then conjugated with glycine or taurine before they are excreted into the small intestine. The bile acids act as emulsifiers to aid in the digestion of fats. Most bile acids are recirculated via enterohepatic circulation and are reused. Enterohepatic circulation involves recycling of the bile acids through the liver and intestinal tract by absorbing them and excreting them again and again. Bile acids deconjugated in the small intestines are more likely to be excreted via the feces than are conjugated ones. Conjugated bile acids are more readily absorbed from the small intestine than are deconjugated ones. Less than 1 gram/day of bile acids is excreted. Cholesterol is then converted to more bile acids to replace the excreted ones. According to Voet and Voet (127), this is the only route for excretion of cholesterol from the body.

Relationship between Cholesterol and Coronary Heart Disease

Coronary heart disease is the leading cause of death in the United States (62, 73, 98, 124). Arteriosclerosis is associated with high levels of low density lipoproteins (LDL) in the blood and is the major cause of coronary heart disease (60, 124). Atherosclerosis, the most common form of arteriosclerosis, is characterized by a thickening of the inner wall of the arteries. The arterial thickening is due to the deposition of lipids and cholesterol in the arterial walls. The deposits become calcified and narrow the openings of the arteries and cause the formation of blood clots thus restricting blood flow to the heart. When blood flow is blocked to the heart, myocardial infarctions (heart attacks) occur (127).

The best example of the relationship between cholesterol and coronary heart disease is familial hypercholesterolemia. This is an inherited disease that is characterized by a high level of cholesterol rich, circulating LDL's in the blood which cause cholesterol levels that are 3-5 times higher than normal (127). Persons with this condition develop atherosclerosis at an early age because of the large amounts of cholesterol in the body. Cells in the body are unable to utilize the cholesterol in the LDL's because they lack LDL receptors so the body must make all the cholesterol needed. Death usually occurs within the first 20 years of life due to coronary heart disease directly related to the high plasma LDL levels.

Studies have shown that in persons with primary hypercholesterolemia, lowering the plasma cholesterol levels and LDL cholesterol can reduce the chances of developing coronary heart disease (73, 74). Several methods have been studied to lower serum cholesterol levels.

Methods to Control Serum Cholesterol Levels

About 50% of blood cholesterol is absorbed from dietary and biliary cholesterol in the intestinal tract (11, 20, 21, 94). A study by Conner et. al. (14) reported that there is a positive correlation between dietary cholesterol intake and serum cholesterol levels. This data suggests that the serum cholesterol levels can be controlled somewhat through dietary modifications.

Additionally, there are drugs available to aid in lowering serum cholesterol levels. Most of these drugs inhibit the production of cholesterol by the liver or bind bile acids thus increasing their secretion. There are some undesirable side effects including constipation, heartburn and nausea associated with the drugs (73). Cholestyramine, a drug proven to be effective in lowering serum cholesterol levels (73, 74), is a bile acid sequesterant that causes excess excretion of bile acids. However, this drug and others that function in a similar manner interfere with the absorption of fats and fat-soluble vitamins (97). Dujovne (24) reported that using a combination of Probucol with Colestipol was effective in lowering the serum cholesterol levels from 242 +/- 51 to 171 +/- 41. The combination of the two drugs resulted in fewer physical side effects. However, the undesirable side effects and health risks associated with drugs justify the need for alternative ways to lower serum cholesterol levels.

Van Belle (126) proposed four methods to increase fecal excretion of bile acids and thus lower serum cholesterol levels; 1) alter the oxidation of cholesterol in the liver so that it will be converted to bile acids and thus excreted, 2) use drugs to cause production of bile acids by the liver that are not easily reabsorbed, 3) use drugs that

inhibit the reabsorption of bile acids from the liver, and 4) alter the intestinal microflora to favor the growth of organisms that actively deconjugate bile acids thus increasing their excretion.

A paper by Grundy and Bilheimer (46) also suggested several approaches to reducing serum cholesterol levels. The first approach was a dietary change to reduce the amount of cholesterol in the diet. Secondly, synthesis of cholesterol by the body could be inhibited by various drugs. Last of all, they suggest reducing the absorption of cholesterol from the small intestine. None of the authors suggested specific approaches for their suggestions.

Role of Gut Microflora in Controlling Serum Cholesterol Levels

Excretion of bile acids via the feces is the primary means of elimination of cholesterol from the body (78, 127). Eyssen (28) reported that the intestinal microflora play a major role in interfering with the reabsoprtion of bile acids from the intestine thus promoting their excretion. He studied both conventional and germ free chicks fed a diet high in cholesterol. The germ free chicks had serum cholesterol levels twice as high as the conventional animals. Additionally, the germ free chicks excreted significantly less cholesterol and bile acids than the conventional chicks.

In another study, Chikai et al. (10) inoculated germ free rats individually with either *Bacteroides vulgatus*, *Bifidobacterium longum*, *Clostridium ramosum* or *Escherichia coli*. Following inoculation they were fed a normal diet. Excretion of bile acids increased in all the rats except those inoculated with *E. coli*. *E. coli* does not deconjugate bile acids. Also, more of the bile acids that were excreted in the inoculated

animals were deconjugated compared to the bile acids excreted by the germ free rats. They reported that free bile acids are excreted more readily than conjugated ones. Additionally they speculated that bile acids may bind to microorganisms and/or dietary fiber in the intestinal tract thus leading to excretion.

Effect of Cultured and Culture Containing Dairy Products on Serum Cholesterol Levels

There are many health benefits associated with lactic acid bacteria (29). Many studies have indicated that cultured or culture containing dairy products may lower serum cholesterol levels. Mann and Spoerry (79) were the first to report this phenomenon. While studying the Maasai tribesmen in Africa, they discovered that after the tribesmen consumed large quantities of milk fermented with a "wild" strain of *Lactobacillus* they had reduced serum cholesterol levels. As part of a tribal ritual, the tribesmen consumed the fermented milk for six days and then slaughtered and ate a steer on the seventh day. The ritual was continued for three weeks. The researchers supplemented the milk consumed by one half of the men with Tween 20, a surfactant that was thought to enhance lipid absorption and the other half of the men served as a control by drinking milk supplemented with olive oil. In both groups there was an increase in weight, but a decrease in serum cholesterol levels. The researchers' original hypothesis was that the men who consumed the Tween 20 would have higher serum cholesterol levels than the control group due to the increased absorption of cholesterol. The results were contrary to the hypothesis and they concluded that there was some component in the fermented milk that lowered the serum cholesterol levels of the tribesmen.

Mann (80, 81) continued to study the effect of fermented milk on serum cholesterol levels. He evaluated the serum cholesterol levels of persons who consumed either whole milk fermented with commercial yogurt cultures or nonfermented milk for twelve days. The group that consumed the fermented milk had significant reductions serum cholesterol levels after the twelve day study. Similarly, a study by Thakur and Jha (123) reported a reduction in serum cholesterol levels in rabbits who were fed yogurt.

Bazzarre et al. (5) studied the effect of yogurt on high density lipoproteins (HDL) and serum cholesterol levels of women. They found that there was a significant decline in serum cholesterol levels in the women who consumed yogurt compared to women who did not consume yogurt. Also, the HDL cholesterol:total cholesterol ratio was higher for the women who consumed yogurt compared to those who did not.

Rao et al. (103) Studied the effects of milk fermented with *Streptococcus thermophilus* on the serum cholesterol levels of rats compared to rats fed a diet supplemented with skim milk or water. The rats fed the fermented milk had significantly lower serum cholesterol levels than those who did not receive the fermented milk. They also evaluated the methanol solubles in both fermented and nonfermented milk. They found that feeding rats the methanol solubles from the fermented milk significantly lowered serum cholesterol levels while the solubles from the nonfermented milk did not. They speculated that the metabolites produced during fermentation may be responsible for the hypocholesterolemic effect. Similarly, a study by Thakur and Jha (123) reported that rabbits who were fed a high cholesterol diet supplemented with yogurt had lower serum cholesterol levels than rabbits fed a similar diet supplemented with milk. Over time, the cholesterol levels of rats fed a diet supplemented with the milk did not decline.

The researchers suggested that calcium might be involved. However, Howard and Marks (55) found that a diet supplemented with calcium gluconate daily for 7 days did not affect serum cholesterol levels. This suggests that calcium may not be involved in the hypocholesterolemic effect.

Hepner et al. (51) studied the effect of both pasteurized and nonpasteurized yogurt on serum cholesterol levels. After 12 weeks of the trial, they found that both yogurts significantly reduced serum cholesterol levels in humans compared to those who consumed 2% milk. Because the culture was inactive in the pasteurized yogurt, these authors concluded that the active culture may not be involved in the hypocholesterolemic effect.

Some studies report that there is no effect of cultured dairy products on serum cholesterol levels. Thompson et al (125) evaluated the effect of sweet acidophilus milk, yogurt, skim milk and 2% fat milk on serum cholesterol levels. Sixty eight individuals consumed the dairy products for nine weeks and there was no significant effect on their serum cholesterol levels. It is important to note that these individuals were not hypercholesterolemic and they were not consuming any excess dietary cholesterol during the study.

A study by Jaspers et al. (59) reported that yogurt consumption had no effect on total cholesterol, HDL cholesterol, LDL cholesterol, serum lipoproteins, and serum triglycerides in human males. The total serum cholesterol levels decreased for the first 14 days but then returned to normal after 21 days. Lin et al (71) studied a commercially available product which contains *L. acidophilus* ATCC 4962 *and L. delbrueckii* subsp.

bulgaricus ATCC 33409. They found that it had no effect on the concentrations of serum lipoproteins in humans.

Similarly, Pulsani and Rao (102) reported that there was no effect on serum cholesterol levels in rats fed milk fermented with either *L. acidophilus, S. thermophilus* or *L. bulgaricus*. A study by Modler et al. (85) reported that there was no relationship between consumption of *Bifidobacteria* and reduction of serum cholesterol levels.

Conversely, a study by Kiyosawa et al. (64) found that skim milk was more hypocholesterolemic that yogurt on serum cholesterol levels in male rabbits. Two other studies found similar results in humans (56, 108). Both studies reported that the lower serum cholesterol levels in humans was probably due to the lower lipid content of skim milk compared to yogurt.

Several studies have reported that various naturally occurring substances in bovine milk inhibit cholesterol synthesis in the body. Uric and orotic acid, and 3-hydroxy-3-methylglutarate (HMG) are among the substances identified by many researchers (1, 26, 49, 81, 95). A study by Haggerty et al. (48) reported that the amount of orotic acid in bovine milk declined during yogurt fermentation. Conversely, Ferriera (30) found that there was no decrease in the amount of orotic acid in bovine milk during yogurt fermentation. The differences may be due to strain differences in the starter culture used for making the yogurt. However, these results indicate that some factor other than the orotic acid could be responsible for the hypocholesterolemic effect of yogurt.

In these studies the researchers did not report the strain of the culture or if it was viable or able to assimilate cholesterol. It is possible that the culture used for the

production of the cultured product was not viable when consumed or was not a strain that assimilated cholesterol. Additionally, the researchers did not determine if the microorganisms actually survived and grew in the digestive system. The researchers also did not specifically select host-specific strains for their studies. All of these criteria can play a role in the potential hypocholesterolemic effect of various cultures. The lack of focus on these areas could be the explanation for the inconsistency in the results from one study to another.

A study conducted by Imaizumi et al. (58) illustrates this point. They studied the effect of whey collected from cultured skim milk on the secretion of bile acids by cultured hepatocytes. The differences in the amount of bile acids secreted was more dependent on the strain of bacteria used to culture the milk than on the species. Both *L. casei* 2230 and *Bifidobacterium longum* 2912 increased the activity of 7 a-hydroxylase, the enzyme responsible for the rate limiting step in conversion of cholesterol to bile acids. Stimulation of the enzyme by the two strains resulted in increased secretion of bile acids.

Hypocholesterolemic Activities of *Lactobacillus acidophilus* and *L. casei*

Lactobacilli are part of the normal microflora of the intestinal tract of humans. The lactobacilli help in inhibiting the growth of pathogenic bacteria and aid in the digestion process (2, 27). Many researchers have studied the effect of *L. acidophilus* on assimilation of cholesterol in vitro and on the effect of serum cholesterol levels in humans and animals.

Mott et al. (86) studied germ free rats who were inoculated with *Lactobacillus acidophilus*. Following inoculation, the rats were either allowed to develop a normal intestinal flora or not allowed to develop it. The rats who were allowed to develop a normal intestinal flora had significantly lower serum cholesterol levels than those who, because of being kept in sterile chambers, did not develop the normal flora. The excretion of bile acids by rats in the nonsterile environment was lower than that of the rats in the sterile environment. From these results, the researchers suggested that microbial deconjugation alone was not the only factor effecting the serum cholesterol levels. They suggested that microbial metabolism of cholesterol might play a possible role.

Zacconi et al. (133) reported that mice who had *L. acidophilus* in their intestinal tracts showed a reduction in serum cholesterol levels compared to rats who did not have *L. acidophilus*. Similarly, Danielson and Gustaffson (16) reported that germ-free rats had higher serum cholesterol levels than rats that have normal intestinal flora. In another study, Thakur and Jha (123) reported that normal and cecetomized laying hens had significantly lower serum cholesterol levels after consuming *L. acidophilus* for one month.

Harrison and Peat (50) studied the serum cholesterol levels of newborn infants. They reported that human infants fed milk containing *L. acidophilus* has significantly lower serum cholesterol levels, greater weight gain, and more lactobacilli in their stools than infants fed sterile milk. A study by Robins-Browne et al. (106) also reported that humans excreted more lactobacilli in their stool after consumption of a commercial product containing L. acidophilus than did persons not consuming the product.

Grunewald (47) reported that rats who consumed milk fermented with *L. acidophilus* had significantly lower serum cholesterol levels compared to rats who consumed only milk.

Gilliland et al. (40) reported that *L. acidophilus* assimilated cholesterol in vitro. When the culture was grown under anaerobic conditions in the presence of bile, it removed cholesterol from the growth medium. They evaluated several strains of *L. acidophilus* originally isolated from pigs. They grew the cells in MRS broth and used a pleuropneumonia-like organism (PPLO) serum fraction as the source of cholesterol. The cultures were grown at 37°C and the cells were removed from the broth by centrifugation. There was less cholesterol in the spent broth compared to uninoculated broth. There was variation depending on the strain of *L. acidophilus*. *L. acidophilus* strain RP32 (currently named ATCC 43121) which took up significantly more cholesterol than other strains, was fed to pigs fed a high cholesterol diet. After 10 days, the serum cholesterol levels in the pigs fed a diet supplemented with *L. acidophilus* RP32 were significantly lower than the levels of the pigs fed a high cholesterol diet with no added *L. acidophilus*. *L. acidophilus* strain P47, also isolated from a pig, did not assimilate cholesterol in vitro. It was also fed to pigs fed a high cholesterol diet. The serum cholesterol levels of these pigs were not significantly different from the control.

Danielson et al. (16) also isolated several strains of *L. acidophilus* from adult boars. They found that strain 16 was very active in removal of cholesterol from broth in vitro. They made yogurt with this strain and fed it to boars who were fed a high cholesterol diet for 56 days. The boars who received the *L. acidophilus* strain 16 had significantly lower serum cholesterol levels compared to the controls.

Another study (61, 119) reported that the bacteria responsible for eye formation in swiss cheese, *Propionibacterium freudenreichii*, removed at least 50% of the cholesterol present in the PPLO form in the growth medium. The cholesterol removed from the broth was recovered with the bacterial cells.

In a recent study, Buck and Gilliland (7) isolated *L. acidophilus* (7) and *L. casei* (8) from human volunteers. They found variation in the isolates with regard to the amount of cholesterol assimilated from laboratory media. They suggested using the strain that assimilated the most cholesterol and were the most bile tolerant for dietary adjuncts to reduce serum cholesterol levels in humans.

The strain isolated by Buck and Gilliland was used in a human study at the University of Kentucky. Diets of hypercholesterolemic individuals were supplemented with *L. acidophilus* L1. Individuals who received the *L. acidophilus* L1 had significant reductions in their serum cholesterol levels (not published data). This isolate is currently used in a yogurt drink called Fhysiq in Holland. In Holland the product is sold making the claim that the yogurt drink can help maintain a desirable serum cholesterol level.

De Rodas et al. (18) studied the effect of feeding diets supplemented with *L. acidophilus* ATCC 43121 and calcium on pigs with hypercholesterolemia induced by diet. They reported that pigs fed a diet supplemented with *L. acidophilus* had serum cholesterol levels 11.8% lower than the pigs fed a diet containing no *L. acidophilus*. Similarly, pigs fed diets containing 1.4% calcium had lower serum cholesterol levels than those fed .7% calcium. Additionally, serum bile acids were lower in the pigs fed the *L. acidophilus* and the 1.4% calcium compared to controls. This data suggests that strains

of *L. acidophilus* that actively assimilate cholesterol in vitro may also lower serum cholesterol levels in vivo.

A study by Klaver and Van der Meer (65) reported that the "assumed assimilation of cholesterol" was due to the "coprecipitation" of the cholesterol with the bile salts. Bile salts are deconjugated by the bacteria and the deconjugated bile salts tend to precipitate as the pH of the growth medium drops below pH 6.0. They suggested that the cells do not actually take up the cholesterol. Also, Reynier et al. (105) reported that deconjugated bile salts affected the solubility of cholesterol.

Conversely, Walker and Gilliland (128) evaluated 19 strains of *L. acidophilus* for bile salt deconjugation, cholesterol assimilation, and their ability to grow in the presence of bile. The cultures reached maximum deconjugation activity and cholesterol assimilation in the late exponential phase of growth when they were grown in MRS broth supplemented with sodium taurocholate and cholesterol micelles . However, there were no significant statistical correlations between cholesterol assimilation and the deconjugation of bile salts..

Additionally, a study by Noh and Gilliland (91) determined that cholesterol assimilated by *L. acidophilus* was not metabolically degraded by the cells during growth in MRS broth. They recovered cholesterol removed from the MRS broth in the membranes of the cells of *L. acidophilus*. They also reported that cholesterol was assimilated during growth at pH 6.0. Cells grown in the presence of cholesterol were more resistant to lysis by sonication than those grown without cholesterol further suggesting that growth in the presence of the cholesterol altered the cell wall and/or the cellular membrane making the cells more resistant to lysis.

Bile Salt Deconjugation by the Lactobacilli

Absorption of Lipids from the Small Intestine

Lipids, including cholesterol and other sterols, triglycerides, free fatty acids and fat soluble vitamins, require bile acids for absorption from the small intestine (9, 13, 53, 54, 77). The bile salts act as emulsifiers that help solubulize lipids in the intestinal tract. The bile salts are amphiphatic and form a micellular structure around the lipids so they can cross the intestinal mucosal cells. Studies by Gallo-Torres et al. (33) and Holt (54) reported that conjugated bile acids formed better micellular structures with lipids than deconjugated ones. Therefore, the conjugated bile acids are better at facilitating the absorption of lipids including cholesterol. They also reported that taurocholic acid better facilitated lipid absorption than glycocholic acids. However, there is very little taurine in the diet so glycocholic acid predominates in the intestinal tract of humans (23).

The vast majority of cholesterol uptake occurs in the upper two-thirds of the intestines (25). Holt reported that 2 mM "critical micellular concentration" of bile acids is required in the intestinal tract for proper lipid absorption. When conjugated bile acids are present in the intestinal tract at concentrations less than 2 mM, cholesterol may not be absorbed from the small intestine (33).

Role of Intestinal Microorganisms in Bile Salt Deconjugation

The intestinal microorganisms metabolize bile acids and cholesterol in the intestinal tract. Various metabolites are formed which alter the biological and physiological functions of the bile acids and cholesterol (57). Among the various

metabolites formed are dehydroxylation products of the bile acid nucleus and epimerization of hydroxyl groups of C_3, C_6 and C_7 of the steroid ring in cholesterol.

Jonsson et al. (61) studied microbial bile acid transformation in healthy infants. They reported that 55-63% of the bile acids in the meconium were conjugated with taurine while only 11-32% were conjugated with glycine. However, by one month of age, the majority of bile acids appearing in the feces were deconjugated. The amount of deconjugated bile acids appeared in conjunction with establishment of the intestinal microflora. Among the conjugated bile acids present in the one month olds, glycine conjugated bile acids were present in higher amounts compared to the taurine conjugated ones.

The enzyme bile salt hydrolase is responsible for the deconjugation of bile acids by the intestinal microflora. Several researchers have studied this enzyme and a number of microorganisms have bile salt hydrolase activity. The genera *Lactobacillus*(13, 17, 35, 66, 75, 114), *Bacteroides* (10, 84, 114, 120), *Bifidobacterium* (10, 82, 114) *Fusobacterium* (19, 66) *Clostridium* (44, 82, 84, 88), *Peptostreptococcus* (19, 66), and *Streptococcus* (66, 114) have the ability to deconjugate bile acids through the production of bile salt hydrolase.

Many studies have reported that there is variation in bile salt hydrolase activity among strains of *Lactobacillus*. Kobashi et al. (66) reported that there also was variation among species in preferences of the bile acids. They found that *L. fermentum* deconjugated both glycocholate and taurocholate, while *L. brevis* and *L. plantarum* would deconjugate glycocholate only. They found that *L. xylosus* deconjugated taurocholate

while L. acidophilus and *L. leichmanni* did not deconjugate either glycocholate or taurocholate. They examined only one strain of each organism.

A study by Aries et al. (3) reported that there was no bile salt hydrolase activity detected in 48 strains of lactobacilli tested. Conversely, Hill and Drasar (52) reported that they found only twelve strains of lactobacilli with no bile salt hydrolase activity.

Gilliland and Speck (35) were the first to detect bile salt hydrolase activity in *L. acidophilus*. Several studies since then have reported the same results (13. 17). Dashkevicz and Feighner (17) found that 71% of all the strains of *L. acidophilus* that they tested exhibited some bile salt hydrolase activity.

Tannock et al. (122) studied the effect of *Lactobacillus* on the bile salt hydrolase activity in conventional, lactobacillus free, and reconstituted lactobacillus free mice. The lactobacillus free and reconstituted lactobacillus free mice differed in that the lactobacillus free mice did not contain lactobacilli, enterococci, and filamentous ileal microorganism while the reconstituted mice lacked only lactobacilli compared to the conventional mice. Lactobacilli were detected in the intestinal tracts of conventional mice and in the other two groups after inoculation with lactobacilli. The number of lactobacilli increased distally along the small intestine in the conventional mice and in the reconstituted lactobacillus free mice after inoculation with lactobacilli. The bile salt hydrolase activity in the conventional mice was 86% more than in reconstituted lactobacillus free mice and 98% higher than in the lactobacillus free mice. After inoculation with lactobacilli in the lactobacillus free mice groups, there were no significant differences in bile salt hydrolase activity in the small intestine between them and the conventional mice. Based on these results, lactobacilli play a major role in bile

salt deconjugation in the intestinal tract. The deconjugation of bile acids can lead to excretion of the acids and thus a reduction in the cholesterol stores in the body.

Enterohepatic Circulation of Bile Acids

The enterohepatic circulation of bile acids occurs between the liver and the small intestine. Bile acids are produced in the liver and then concentrated and excreted from the gall bladder into the small intestine. In the small intestine they act as emulsifiers and form micellular structures with lipids thus facilitating their absorption. The bile acids are reabsorbed with the lipids from the small intestine and are transported in the blood stream back to the liver (12). Only very small amounts of the conjugated bile acids are excreted with the feces. The bile acids that are recirculated are usually conjugated with glycine or taurine while the excreted bile acids are usually deconjugated (93).

Absorption of Bile Acids

Bile acids are absorbed by both active transport and passive diffusion depending on the location in the small intestine (20, 21, 23, 70, 76, 111). Active transport occurs primarily in the ileum while passive diffusion occurs in the duodenum and jejunum (22, 23). Active transport requires both ionized molecules and bile acids that have negatively charged side chains (21). A study by Schiff et al. (111) reported that both conjugated and deconjugated bile acids can by absorbed by active diffusion in the ileum but the conjugated bile acids are absorbed more efficiently.

The charge of the side chains of bile acids depends on the pKa. The pKa of cholic acid is around 6 (17, 21), for taurocholic acid is 2 and for glycocholic acid is 5. As

mentioned previously, cholic acid is excreted more readily than its conjugated forms. Also, glycochoic acid predominates over taurocholic acid in the intestinal tract. The pH of the intestinal tract ranges from 4.5 to 6. In this pH range, both the taurocholic acid and glycholic acids would be ionized. Therefore they would be actively absorbed in the ileum. Conversely, cholic acid would be unionized in the small intestine and theoretically would be passively absorbed due to its uncharged side chains. In the ileum, the pH is higher and the cholic acid becomes charged thus facilitating absorption in this portion of the intestinal tract. However, Playhouse et al. (100) and Dietschy et al. (20) reported that cholic acid was not readily reabsorbed when they evaluated absorption using the everted gut sac technique. Conversely, another study (21) reported that cholic acid and conjugated bile acids were absorbed similarly in vivo.

Role of Bile Acids in Establishing Gut Microflora

Deconjugated bile acids have an inhibitory effect on microorganisms (32). However, cholic acid does not have the same effect. It has been reported that deoxycholic acid is the most inhibitory free bile acid and inhibits most gram negative organisms (115). Generally, Gram positive organisms are more sensitive to the inhibitory action of the bile acids than the Gram negative bacteria. However, there is variation among genera and species in the bile salt tolerance.

For example, *L. bulgaricus* can not survive in the intestinal tract because of its sensitivity to bile salts (36). Conversely, *L. acidophilus* is a normal inhabitant of the intestinal tract (87). *L. acidophilus* is very tolerant of the bile salts in the intestinal tract,

but there is variation in the bile salt tolerance among strains (39, 40, 128). The bile salt tolerance may play a role in the hypocholeserolemic effects of the *L. acidophilus*.

A study by Grill et al. (45) evaluated eight strains of bifidobacteria and their relationship with six different conjugated bile acids. The conjugated bile acids were 80% inhibitory toward all eight strains of bifidobacteria. However, all eight strains produced deconjugated bile salts during growth which were identified by the researchers as the growth inhibiting factors.

Other Health and Nutritional Benefits of Lactobacilli

Antagonistic Action Towards Pathogens

Several researchers have studied the antagonistic action of the lactobacilli toward various food-borne pathogens and spoilage organisms. Scientists have hypothesized that the inhibitory action is due to a number of factors including, production of hydrogen peroxide, acid, and/or bacteriocins. *L. acidophilus* appears to inhibit pathogens during refrigerated storage by producing hydrogen peroxide (4, 6, 34). Both *L. casei* and *L. acidophilus* inhibit pathogenic organisms both in vitro (6, 34, 104) and in vivo (9, 96, 99, 109, 116, 117, 129, 130). Among the pathogens inhibited are *Staphylococcus aureus* (34, 104, 116, 117, 129) , *Streptococcus* spp. (116, 117), *Salmonella* spp (34, 129), *Clostridium* spp. (36, 116, 117,), *Vibrio* spp (116, 117), *Listeria* (99, 109), and *E. coli* (6, 34, 104, 130)

Growth Stimulation

Infants who were solely formula fed had significantly less weight gain that those who were only formula fed and supplemented with *L. acidophilus* (107). They also found that breast fed infants gained significantly more weight than either of the two formula fed groups, but the supplement of *L. acidophilus* appeared to help infants gain weight at a rate more comparable to breast fed infants compared to those who were only fed formula.

Benefits to Lactose Maldigestors

Lactose maldigestion occurs because of a deficiency of lactase in the digestive system. After consumption of dairy products, persons with this condition may experience bloating, flatulence, diarrhea, and abdominal pain (68).

Researchers have reported that various species of lactobacilli have the potential to improve lactose digestion in persons who are lactose maldigestors because they produce the enzyme B-galactosidase (38, 67).

However, some have reported that consumption of *L. acidophilus* had no benefit to lactose maldigestors (89, 90, 110). Gilliland (42) reported that these studies did not consider the strains used or how the cells were propagated. The enzyme B-galactosidase is inducible in *L. acidophilus*. Therefore, in order for the *L. acidophilus* to have the greatest potential benefit the lactose maldigestors, the cells should be grown in a medium that has lactose as the sole carbohydrate source to ensure a high B-galactosidase activity. Additionally, Gilliland and Lara (41) reported that some strains of *L. acidophilus* lost their B-galactosidase activity in as little as 7 seven days of storage in nonfermented

acidophilus milk, but some strains remained active up to 21 days. Also, Noh and Gilliland (91) reported a wide variation in the B-galactosidase activity among strains of *L. acidophilus*. This data indicates that selection of the strain of *L. acidophilus* used to benefit lactose maldigestors is a critical step in ensuring that the organism alleviates the symptoms of lactose maldigestion.

Production of Antitumor Compounds

Researchers have reported that lactobacilli may be inhibitory to some types of cancers. A review article by Gilliland (39) reported that *L. acidophilus* could be inhibitory toward some organisms that produced carcinogenic compounds. He also reported that there is evidence that the lactobacilli administered in the diet reduced the proliferation of tumor cells in rats.

Sellars (112) reported similar results. He indicated that *L. acidophilus* may be responsible for reducing the formation of carcinogenic compounds by fecal enzymes. He also mentions epidemiological evidence in countries where the populations consume large amounts of products containing *L. acidophilus* indicating lower rates of colon cancer than in other countries.

Immunomodulating Effects

Many researchers believe that lactic acid bacteria and bifidobacteria have a stimulating effect on the immune system. They have studied the mechanisms responsible for immune stimulation.

Yasui et al. (132) reported that mouse pups who were nursed by dams fed *Bifidobacterium breve* and immunized with rotavirus were more resistant to rotavirus-induced diarrhea than pups nursed by dams who were only immunized. They discovered that there was more antirotavirus IgA in milk of the dams fed the bifidobacteria than in the dams who were only immunized. Similarly, Linc-Amster et al. (72) reported humans who consumed *L. acidophilus* and bifidobacteria were less susceptible to infection when challenged with *S. typhymurium*. Subjects who consumed the cultures had 4 times more IgA than the control subjects. Similar results were found by other researchers (63, 118)

Schriffrin et al. (113) reported that humans who consumed fermented milk products supplemented with *L. acidophilus* or *Bifidobacteriaum bifidum* had no modification of lymphocyte subpopulations. However, in vitro studies indicated that phagocytosis of *E. coli* was enhanced concurrently with the intestinal colonization by the lactic acid bacteria. The increased phagocytosis persisted for six weeks after the products were ingested.

Yasui and Ohwake (131) reported that bifidobacteria enhanced production of anitibodies by Peyer's patches, a gut associated lymphoid tissue. Additionally, researchers have reported that the live cells must be consumed to obtain the immunomodulating effects (78).

Products available that contain *L. acidophilus* and/or *L. casei*

There are a number of products available to consumers that contain various species of lactobacilli. Among these products are fermented and nonfermented dairy products and liquid or dried forms of lactic cultures.

Fermented acidophilus milk is readily available in Scandinavian and Eastern European Countries (112). It has an extremely sour taste and would not be readily accepted in the U.S. (69).

Health food stores and pharmacies sell freeze-dried and liquid forms of products that contain various species of lactic acid bacteria including *L. acidophilus* and *L. casei.* However, no evidence is available to indicate the strains selected for these products were based on possession of specific activities that might provide potential health benefits and the viability is usually low (12, 36, 101).

Nonfermented acidophilus milk is readily available in the U.S. Cells of *L. acidophilus* are added to the pasteurized, lowfat milk. The cells do not have any effect on the flavor of the milk. Gilliland (37, 43) reported that cells of *L. acidophilus* to be added to products as dietary adjuncts should have the following characteristics 1) the organism should normally be found in the intestinal tract, 2) the organisms should produce the desired effects in the intestinal tract and 3) the organisms should remain viable during storage of the product.

Cells of *L. acidophilus* may be added to yogurt after fermentation in a similar way that they are added to nonfermented acidophilus milk.

L. casei is available in several countries in a drink called Yakult. This drink was developed in 1935 by Dr. Minoru Shirota (116). The drink is made by fermenting milk with *L. casei* var. *Shirota*. This organism is believed to be more acid tolerant than *L. acidophilus*. Studies have shown the drink is effective in treating constipation and diarrhea. However, a study indicated that continuous consumption of Yakult may be necessary to maintain high enough numbers of the organism in the intestinal tract (116).

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CHAPER III

INFLUENCE OF pH ON BILE SALT DECONJUGATION AND REMOVAL OF CHOLESTEROL FROM MEDIA DURING GROWTH BY *LACTOBACILLUS CASEI* AND *LACTOBACILLUS ACIDOPHILUS*

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ABSTRACT

Lactobacillus acidophilus L1 and ATCC 43121 and *Lactobacillus casei* N19 and E5 were evaluated for their ability to deconjuate bile acids and remove cholesterol from suspension in MRS broth during growth at a constant pH of 6.0 and during growth without pH control.

Samples grown without pH control dropped to pH levels of 4.2 to 4.5 during 20 h of incubation depending on the culture. The plate counts indicated that populations in all cultures were near their maximum numbers after 16 hours of growth. The amount of cholesterol removed from the broth was similar for both strains of *L. acidophilus* grown with and without pH control. However, the strains of *L. casei* differed significantly in the amount of cholesterol removed from the broth between the two pH treatments. Both cultures of *L. casei* grown at pH 6.0 removed very little cholesterol from the broth while cells grown without pH control removed up to 60 ug of cholesterol/ml. All cultures deconjugated 60 to 90% of the bile salts, depending on the culture. *Lactobacillus acidophilus* L1 was the only culture to have differences between the two pH treatments in the amount of bile salts deconjugated. These results indicate that most of the cholesterol removal from broth by *L. acidophilus* is due to assimilation, perhaps incorporation of cholesterol into the cellular membrane. *Lactobacillus casei* most likely removes cholesterol from broth due to destabilization of cholesterol micelles and coprecipitation of the cholesterol with the deconjugated bile salts at pH levels less than 6.0.

INTRODUCTION

High serum cholesterol levels are associated with the development of coronary heart disease, the leading cause of death in the United States (66, 77, 78, 103). During growth, *Lactobacillus acidophilus* can remove cholesterol from laboratory media supplemented with cholesterol micelles and bile salts and may have the potential to reduce serum cholesterol levels in humans (8,18, 43). *Lactobacillus casei* also can remove cholesterol from a laboratory medium during growth (95).

The mechanism that the organisms use to remove the cholesterol from the laboratory media is not completely clear. Klaver and Van der Meer (69) reported that the removal of cholesterol from laboratory media by *L. acidophilus* was due to the disruption of the cholesterol micelles caused by bile salt deconjugation and precipitation of the cholesterol with the free bile salts as the pH of the media dropped due to acid production during growth.

Both *L. acidophilus* and *L. casei* deconjugate bile acids during growth by producing the enzyme bile salt hydrolase (17, 20, 70). The solubility of cholic acid, a deconjugated bile acid, decreases as the pH of the media decreases (22, 24, 25). The cholic acid is especially insoluble at pH levels less that 5 due to its pK of 5 to 6. As the pH drops due to acid production during bacterial growth, the cholic acid precipitates from the broth and may cause the cholesterol to precipitate also if the cholesterol micelles are disrupted.

However, a study by Noh and Gilliland (96) revealed that *L. acidophilus* incorporates some of the cholesterol removed from laboratory media into the cellular membrane during growth. During growth at pH 6.0, a pH sufficiently high to keep the

deconjugated bile acids in solution, they still observed removal of cholesterol from a growth medium supplemented with cholesterol micelles and conjugated bile salts.

Both deconjugation of bile acids and incorporation of cholesterol into the cellular membrane have the potential to lower serum cholesterol levels in humans. If the cholesterol is incorporated into the cellular membrane of the lactobacilli during growth in the small intestine, it is likely unavailable for absorption into the blood. The release of free bile acids through deconjugation of conjugated bile acids in the small intestine results in excretion of more bile acids in the feces (123). The primary means whereby cholesterol is removed from the body is by excretion in the form of deconjugated bile acids (137). Most conjugated bile acids are recirculated through enterohepatic circulation. The bile acids that are excreted must be replaced by new bile acids which are formed from cholesterol in the body. Thus, the more bile acids that are excreted, the more cholesterol is lost from the body's pool. Futhermore, free bile acids do not support absorption of cholesterol and other lipids from the small intestine as well as do conjugated ones (25, 105).

The objective of this study was to determine if *L. acidophilus* and *L. casei* removed cholesterol from laboratory media in a similar manner by 1) determining if isolates of *L. casei* incorporate cholesterol into the cellular membrane, 2)determining if there was a difference in the amount of cholesterol removed from the laboratory media by both species when it was maintained at pH 6.0 during growth compared to growth without pH control, 3) determining the amount of sodium taurocholate deconjugated by the cultures during growth with and without pH control, and 4) determining if *L. casei*

preferentially to deconjugates sodium glycocholate or sodium taurocholate during growth with and without pH control.

MATERIALS AND METHODS

Source and Maintenance of Cultures

All cultures used in this study were from the stock culture collection of the Dairy Microbiology Laboratory at Oklahoma State University. *Lactobacillus acidophilus* ATCC 43121 was originally isolated from a pig. *L. acidophilus* L1 and *Lactobacillus casei* strains A3, A17, E5, E10, L15, L19, M5, M12, N7, and N19 were all isolated from human intestinal sources by Buck and Gilliland (8). The cultures were maintained by subculturing 1% inocula into MRS broth (Difco Laboratories, Detroit, MI) and incubating them for 18 hours at 37°C. The cultures were stored at 7°C between transfers. Stock cultures were stored in MRS agar stabs. The cultures were subcultured at least three times immediately before they were used experimentally.

Bacterial Growth Media

MRS broth (Difco Laboratories) was used for subculturing of the cultures. It was prepared according to manufacturer's instructions. MRS agar was prepared by adding 1.5% agar to MRS broth.

MRS-THIO broth was prepared by supplementing MRS broth with 0.2% sodium thioglycolate (Sodium Salt, Sigma Chemical Co., St. Louis, MO). This broth was further supplemented with 10% cholesterol micelles (to yield 90-100 ug cholesterol per ml) and 6 mM sodium taurocholate (Sigma chemical Co.) or with 2.8 mM sodium glycocholate (Sigma Chemical co.), and 1.2 mM sodium taurocholate when needed.

All media was sterilized by autoclaving for 15 minutes at 121°C. All media containing sodium thioglycolate was prepared the day they were used experimentally.

Initial Screening of Cultures for Cholesterol Removal and Bile Salt Deconjugation

The cultures of *L. casei* were isolated from human intestinal sources in a previous study (8). They represented some of the lactobacilli other than *L. acidophilus* described in that study. Determination of their identity characteristics, relative abilities to remove cholesterol from the medium and to deconjugate sodium taurocholate were done as described earlier (8). The growth medium used was the same as described above except 0.3% oxgall was used in place of either sodium glycocholate or sodium taurocholate. The medium contained 90-100 ug cholesterol per ml.

Incorporation of Cholesterol into the Cellular Membrane of *L. casei*

Strains E5 and N19 of *L. casei* were examined for their ability to incorporate cholesterol into their cellular membrane. Isolation and evaluation of cholesterol content of the membrane was done as described by Noh and Gilliland (96).

Growth of Cultures with and without pH Control

On the day of the experiment, 800 ml of MRS-THIO broth which contained 6 mM sodium taurocholate was prepared. After cooling, the broth was supplemented with 80 ml (10%) cholesterol-phosphatidylcholine micelles prepared according to Razin et al. (109). The micelles were prepared using egg yolk lecithin (Type III-e, Sigma Chemical Co.). The concentration of cholesterol in the broth was 90-100 ug/ml. The media was mixed thoroughly and 10 ml of the broth was removed and placed into a sterile tube and held at 5°C (Uninoculated control or time 0).

The broth was then inoculated with 8 ml of a freshly prepared MRS broth culture of the appropriate strain to be evaluated. (*L. acidophilus* L1 and ATCC 43121 and *L. casei* E5 and N19 were all examined.) The broth was then aseptically transferred in equal portions into each of two sterile fermentor jars (each approximately 1 l capacity). One of the fermentor jars was equipped with a combination pH electrode and ports for the addition of neutralizer, continuous sparging with nitrogen gas, and sample removal. The automatic pH controller was adjusted to maintain the pH at 6.0 during growth by adding a neutralizer containing 5% sodium carbonate in 5% ammonium hydroxide (46). Nitrogen gas was purged through the broth at a rate of 10 ml/minute to help maintain a low oxidation-reduction potential. The culture in the other fermentor jar was incubated statically, without pH control at 37°C. The temperature for both was maintained at 37°C in a water bath.

Samples were aseptically removed and placed into sterile tubes for evaluation 16, 18, 20, and 22 hours after growth. Samples were stored in a mixture of ice and water until they were examined (not more than 30 min). They were examined for pH, plate counts on MRS agar, bile salt deconjugation and removal of cholesterol from the broth.

Deconjugation of Sodium Glycocholate and Sodium Taurocholate

Experiments were done as described in the previous section. *Lactobacillus casei* E5 and N19 were compared. These experiments differed from the experiments described in the previous section in that there was no cholesterol added to the broth and 2.8×10^{-3} mM/ml of sodium glycocholate and 1.2×10^{-3} mM/ml sodium taurocholate were added to the broth instead of just sodium taurocholate. This concentration was chosen because it

resembles the ratio in the human body (10, 117). All other parameters were similar to the previously described experiment.

Samples were aseptically removed and placed into sterile tubes for evaluation 16, 18, 20, and 22 hours after growth. Samples were stored in a mixture of ice and water until they were examined (not more than 30 min). They were tested for deconjugation of bile acids.

Measurement of Cholesterol Removal

The time 0 sample removed initially from the fermentor served as an uninoculated control for the measurement of cholesterol assimilation. The amount of cholesterol remaining in the MRS broth was determined at 16, 18, 20, and 22 hours after growth for cultures grown with and without pH control. The cells were removed from broth samples by centrifugation at 12,000 x g at 4°C for 10 minutes. The supernatant was recovered and the amount of cholesterol remaining in it was determined.

The o-phthalaldehyde method described by Rudel and Morris (115) was used to determine the amount of cholesterol in the sample. The amount of cholesterol removed from the broth was determined by subtracting the amount in each broth sample (ug/ml) from the amount originally present in the uninoculated control.

Plate Counts of Lactobacilli in Samples

The total numbers of lactobacilli were determined using the pour plate method as described in the *Compendium of Methods for the Microbiological Examination of Foods* (136) on MRS agar. The samples were serial diluted in 99 ml 0.1% peptone (Sigma)

dilution blanks containing 0.01% silicone antifoamer (Sigma chemical Co., St. Louis, MO). Plates were incubated in a 37°C incubator for 48 hours. Colonies were counted with the aid a Quebec Colony Counter and the cfu/ml was determined. The cfu/ml was converted to $\log_{10}$ cfu/ml for statistical examination.

Bile Salt Deconjugation

The amounts of bile salt (sodium taurocholate or sodium glycocholate) deconjugated by the lactobacilli were determined using HPLC analysis as described by Corzo (17). Methanol-acetate buffer pH 5.0 was used as the mobile phase. Two ml of sample were suspended in 8 ml of 0.9% NaCl in 0.1 M NaOH and 6 ml of mobile phase. The bile salts were recovered by passage of the solution through a Sep-Pac cartridge (Waters Assoc.) and the bile salts were eluted from the cartridge with 3 ml of mobile phase. Dexamethasone (0.2 mg/ml) was added to the eluate as an internal standard.

Statistical Analyses

The experimental design of this experiment was repeated measures over time in a split plot with the main units in a completely randomized design. The main unit treatments were cultures, the sub-unit treatments were pH treatments, and the repeated measures were sampling times. PROC MIXED command of the SAS system was used to separate means.

RESULTS

Initial Screening of Cultures for Cholesterol Removal and Bile Salt Deconjugation

The identity of the cultures of *L. casei* compared in this study were confirmed (Table 49 in Appendix C). The identity characteristics of all 10 closely matched those of *L. casei* as presented in *Bergey's Manual of Determinative Bacteriology* (7). There were significant differences ($P<.05$) among the ten strains of *L. casei* with regard to the amounts of cholesterol removed from suspension in the growth medium during 24 h incubation (Table 1). Significant differences ($P<.05$) in deconjugation of sodium taurocholate also were observed among cultures (Table 2). *Lactobacillus casei* E10, E5, L15, and M5 which deconjugated greater amounts of bile salt than the other six strains also were among the most active in removing cholesterol from suspension in the growth medium. Because of the apparent relationship between deconjugation of sodium taurocholate and removal of cholesterol from the growth medium, two strains were chosen for further study. Strains E5 and N19 were among the most active in removing cholesterol from the growth medium. However, they exhibited the greatest difference in deconjugation of sodium taurocholate. Two strains of *L. acidophilus*, L1 and 43121, shown to be very active in removal of cholesterol from growth media in previous studies (8, 43) were selected for comparison with *L. casei* E5 and N19.

Removal of Cholesterol from the Broth

Statistical analysis indicated that there were significant ($P<.05$) culture*time, culture*treatment, and culture*time*treatment interactions. Because of this, we examined the results at each sampling time at each level of the other factors in the interaction. The pattern indicates that each culture behaves differently with regard to removal of cholesterol from the broth at each sampling time and pH treatment.

Since the maximum amounts of cholesterol had been removed after 20 hours of growth for all cultures, the results are summarized for that time in Table 3. Data for all sampling times can be found in Appendix A. *Lactobacillus casei* E5 and N19 removed 56.6 and 36.3 ug of cholesterol/ml, respectively after 20 hours of growth without pH control. These amounts were not significantly different ($P>.05$). However, both differed significantly ($P<.05$) from the amounts initially present in the broth. Both cultures of *L. casei* grown at pH 6.0 removed very little cholesterol from the broth. Strain E5 removed 8.0 ug/ml while N19 removed 10.4 ug/ml. Neither of these amounts was significantly ($P>.05$) different from the amount present initially (Appendix A Table 4). Thus, little or none was removed during growth at pH 6.0.

Both strains of *L. acidophilus* removed significant ($P<.05$) amounts of cholesterol from the broth when grown at pH 6.0 and without pH control. *Lactobacillus acidophilus* L1 grown at pH 6.0 removed 46.9 ug/ml while without pH control 44.7 ug/ml were removed. The amount removed from the broth by strain 43121 grown at pH 6.0 was not significantly different ($P>.05$) from the amounts removed by strain L1. However, strain 43121 grown without pH control removed 60.6 ug of cholesterol/ml, significantly more ($P<.05$) than when grown at pH 6.0.

Deconjugation of Sodium Taurocholate

There was a significant ($P<.05$) interaction between cultures and time. This indicates that the cultures did not deconjugate comparable amount of sodium taurocholate at the various sampling times. However, the cultures behaved similarly between the two pH treatments. We analyzed the results from each sampling time at each level of the other factors in the interaction. The data for 20 h is summarized in Table 3. Data for the other sampling times are in Appendix A Table 5.

Lactobacillus acidophilus 43121 deconjugated the most sodium taurocholate after 20 hours of growth at pH 6.0 and without pH control than did any of the other cultures. There was a total of 4.8 mM of the bile salt deconjugated for cells grown at both pH treatments. *Lactobacillus acidophilus* L1 grown at pH 6.0 deconjugated the least amount of cholesterol removing only 2.1 mM. When grown without pH control, it removed 4.1 mM which was significantly ($P<.05$) more than the amount removed by cells grown at pH 6.0, but not significantly different ($P>.05$) from the amount deconjugated by either strain of *L. casei*.

Lactobacillus casei E5 and N19 deconjugated similar amounts of sodium taurocholate under both growth conditions. Strain E5 deconjugated 4.2 mM and 4.0 mM grown at pH 6.0 and without pH control, respectively, while strain N19 deconjugated 3.6 mM when grown at pH 6.0 and 3.4 when grown without pH control.

Plate Counts of Lactobacilli

There were no significant interactions ($P>.05$) among cultures, time or treatments for the plate count data. Generally, cells grown at pH 6.0 reached higher numbers than

those grown without pH control. There were no significant (P>.05) differences between the two growth conditions for *L. casei* E5 and N19. There also were no significant (P>.05) differences in the numbers reached by the two stains of *L. casei*.

There were significant (P<.05) differences for both strains of *L. acidophilus* grown with and without pH control. Strain 43121 grown at pH 6.0 reached a population of 9.35 $\log_{10}$ cfu/ml after 20 hours of growth while cells grown without pH control were about one log cycle lower, 8.36 $\log_{10}$ cfu/ml. Strain L1 showed a similar pattern. Cells grown at pH 6.0 reached a population of 8.84 $\log_{10}$ cfu/ml while cells grown without pH control were significantly less (P<.05) at a population of 7.99 $\log_{10}$ cfu/ml.

pH of the Broth

The pH of all samples began at 6.5. Cells of all cultures grown with pH control were allowed to produce enough acid to reduce the pH to 6.0 which was then maintained throughout the remainder of the sampling period. *Lactobacillus casei* E5 and N19 and *L. acidophilus* L1 43121 produced enough acid to lower the pH to 4.2, 4.2, 4.3 and, 4.6 respectively after 20 hours of growth.

Deconjugation of Sodium Glycocholate and Sodium Taurocholate

L. casei E5 and N19 varied in the amount of sodium taurocholate and sodium glycocholate deconjugated. Strain E5 appeared to have more deconjugating activity than strain N19. Generally, both strains preferred sodium glycocholate over sodium taurocholate.

Strain E5 deconjugated most of the 2.8 x 10^{-3} mM/ml of sodium glycocholate before it began to deconjugate the sodium taurocholate (Fig 1A and 1B, Appendix A Table 8). The culture grown with pH control appeared to deconjugate the bile acids at a slightly slower rate than the cells grown without pH control. However, by 22 hours of incubation, the cultures grown under both conditions had removed 2.8 mM x 10^{-3}/ml (100%) of the sodium glycocholate from the broth. It did not deconjugate all of the sodium taurocholate under either conditions (57% with pH control and 67% without pH control).

L. casei N19 deconjugated significantly ($P<.05$) less sodium taurocholate than strain E5 at 16, 18, and 20 hours of growth at pH 6.0 and at all sampling times when grown without pH control (Fig 1B and 1C, Appendix A Table 8). Strain N19 also deconjugated significantly less ($P<.05$) sodium glycocholate after 16 and 20 hours of growth at pH 6.0 and at all sampling times for cells grown without pH control.

Examination of Cellular Membrane of *L. casei* for Cholesterol

Examination of the cellular membranes isolated from *L. casei* grown in the broth medium containing cholesterol and sodium taurocholate at pH 6.0 and without pH control revealed no cholesterol. Thus there is no evidence for incorporation of cholesterol in the cellular membrane of *L. casei* under the conditions of growth utilized in this study.

DISCUSSION

Several reports indicate that some lactobacilli can remove cholesterol from suspension in laboratory media during growth (8, 18, 43). Some scientists believe that the cholesterol is removed by lactobacilli because of bile salt deconjugation. They theorize that when bile salts are deconjugated and the pH of the media drops due to natural acid production by the culture, the cholesterol micelles destabilize and cholesterol precipitates with the free bile acids (69). Bile acids are less soluble and are more likely to precipitate at pH levels less than 6.0 (22, 24, 25).

However, a study by Noh and Gilliland (96) revealed that cholesterol was removed from laboratory media by *L. acidophilus* ATCC 43121 and L1 when the pH was maintained at 6.0. At this pH, there should have been very little precipitation of bile acids. They also found that some of the cholesterol was incorporated into the cellular membrane of the organism. In the present study, isolation of the cellular membrane of *L. casei* and examination of cholesterol revealed that *L. casei* under the conditions tested did not incorporate cholesterol into the cellular membrane in measurable amounts.

In our study, the amount of cholesterol removed from the broth was variable depending on the culture and the pH of growth. *L. acidophilus* L1 was the only culture among the ones evaluated that did not differ in the amount of cholesterol removed when grown with or without pH control. Although there were significant differences in the amounts of cholesterol removed during growth with and without pH control, *L. acidophilus* 43121 was similar to *L. acidophilus* L1 in that it removed large amounts of cholesterol even when the broth was maintained at pH 6.0. Since free sodium cholate stays in solution at pH 6.0 (22, 24, 25), these results further suggest that the cholesterol

removed from the broth by *L. acidophilus* was not due just to bile salt deconjugation and coprecipitation of cholesterol with free sodium cholate. Thus it is likely that much of the cholesterol removed from suspension from the broth was not entirely due to destabilization of the cholesterol micelles.

The amount of bile salts deconjugated by *L. acidophilus* L1 was significantly different between the two growth conditions while the amount deconjugated by *L. acidophilus* 43121 was not. The difference in the amounts of bile salts deconjugated during growth with and without pH control and the lack of difference in the amount of cholesterol removed from the broth between the two treatments, and visa versa, further suggests that the cholesterol removal was not entirely related to bile salt deconjugation for *L. acidophilus* L1.

The two strains of *L. casei* exhibited significant differences in the amount of cholesterol removed from the broth during growth with and without pH control. There was very little cholesterol removed from the broth maintained at pH 6.0 while the amounts removed from suspension were comparable to the amount removed by *L. acidophilus* when the cells were grown with no pH control. These results suggest that most of the cholesterol removed from the broth by *L. casei* was due to coprecipitation of the cholesterol with the deconjugated bile salts. There was very little difference in the amount of sodium taurocholate deconjugated for either strain for the two pH treatments. However, the bile salts deconjugated by the cultures grown at pH 6.0 would have remained in solution. Therefore the cholesterol stayed in suspension.

Further experiments indicated that both strains of *L. casei* deconjugated sodium glycocholate better than sodium taurocholate. Sodium glycocholate predominates in the intestinal tract of adults because there is very little taurine in the diet (24). Strains that prefer to deconjugate sodium glycocholate thus may have more potential in lowering serum cholesterol levels if deconjugation of bile salts is important in controlling serum cholesterol.

Either mechanism, bile salt deconjugation or cholesterol assimilation, has potential importance in exerting control of serum cholesterol levels in humans. Cholesterol incorporated into or adhered to the bacterial cells likely would be less available for absorption from the intestine into the blood. Deconjugated bile salts are less well absorbed in the enterohepatic circulation thus are more likely to be excreted in the feces. Excreted bile salts are replaced by synthesis of new ones in the body from cholesterol, thus providing the potential of reducing the body's pool of cholesterol. Furthermore free bile salts do not support absorption of lipids, including cholesterol from the intestines as well as do conjugated bile salts (25, 105). Results from this study suggest that strains of *L. acidophilus* should be selected not only on the basis of bile salt deconjugation, but also should be examined for cholesterol assimilation. When selecting strains of *L. casei* for use as dietary adjuncts, it is important to select strains with the highest bile salt deconjugating activity to maximize the potential for reduction of serum cholesterol levels. Based on our observation it does not appear that cholesterol assimilation of *L. casei* could be a major factor in controlling serum cholesterol.

Table 1. Comparison of amounts of cholesterol removed from suspension in growth medium by isolates of *Lactobacillus casei* from human volunteers.

Culture	Cholesterol Removed[1,2]
E5	73.3^{a}
E10	61.4^{ab}
M5	56.6^{abc}
L15	48.8^{abcd}
N19	45.4^{abcd}
A17	34.5^{bcd}
N7	32.2^{bcd}
L19	30.9^{bcd}
A3	28.6^{cd}
M12	16.9^{d}

[1]ug/ml cholesterol removed by the culture during a 24 hour incubation at 37°C.
[2]Values are the means of three replications. Means with no common superscript letter differ significantly ($P<.05$).

Table 2. Comparison of deconjugation of sodium taurocholate by isolates of *Lactobacillus casei* from human volunteers.

Culture	Bile Salt Deconjugated[1,2]
E10	1.6[a]
E5	1.5[a]
L15	1.4[a]
M5	0.8[b]
N7	0.3[c]
A17	0.2[c]
M12	0.2[c]
A3	0.2[c]
N19	0.1[c]
L19	0.1[c]

[1]uMol/ml bile salt deconjugated after incubation for 15 hours at 37°C
[2]Values are the means of three replications. Means with no common superscript letters differ significantly ($P<.05$)

Table 3. Cholesterol removal, bile salt deconjugation, pH and plate counts on MRS agar of *Lactobacillus casei* and *Lactobacillus acidophilus* grown for 20 hours with and without pH control at 37°C.

Culture	Growth Conditions	Cholesterol Removed[1]	Deconjugation[2]	Plate Counts[3]	pH
E5	pH 6.0	8.0^{a}	4.2^{b}	9.77^{b}	6.0
	Static	56.6^{bc}	4.0^{b}	8.56^{ab}	4.2
N19	pH 6.0	10.4^{a}	3.6^{b}	9.21^{b}	6.0
	Static	36.3^{b}	3.4^{b}	8.62^{b}	4.2
43121	pH 6.0	41.6^{b}	4.8^{c}	9.35^{b}	6.0
	Static	60.6^{c}	4.8^{c}	8.36^{a}	4.3
L1	pH 6.0	46.9^{bc}	2.1^{a}	8.84^{b}	6.0
	Static	44.7^{bc}	4.1^{b}	7.99^{a}	4.6

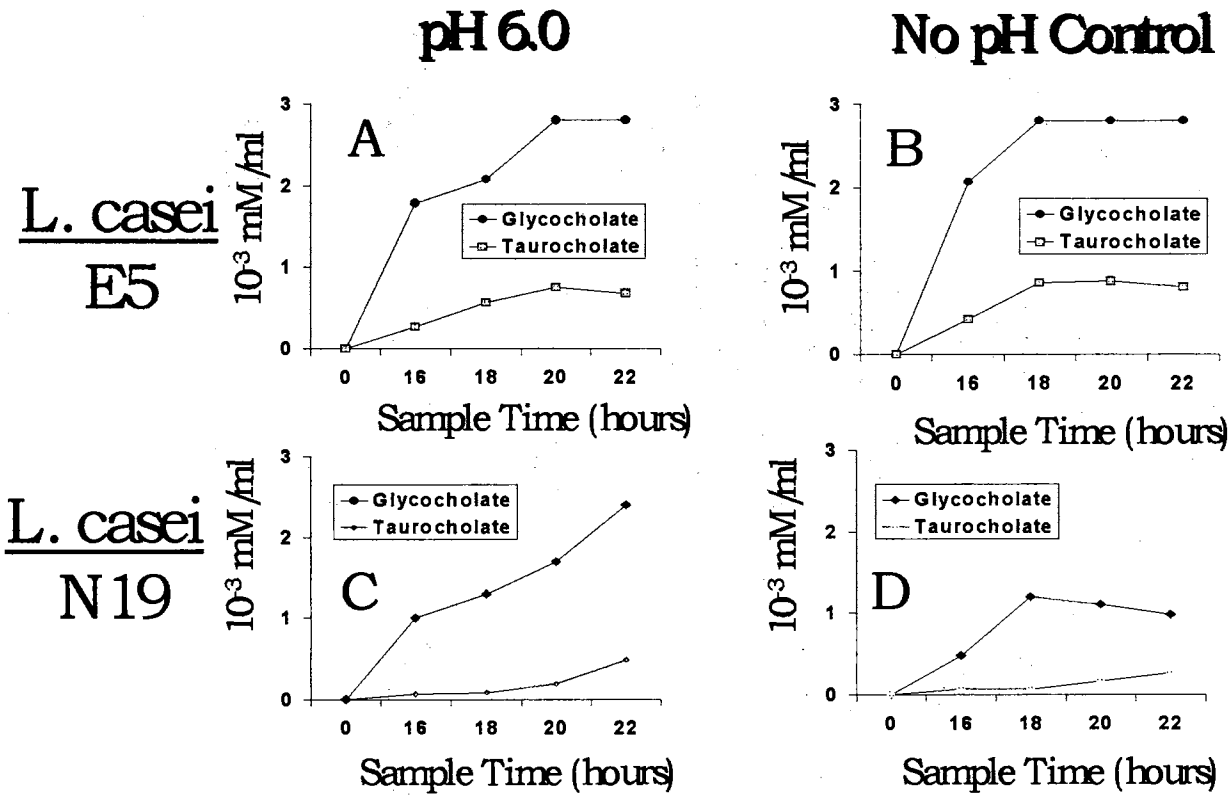
[abc]Values represent the means of three replications. Means with no common superscript letters within each column differ significantly ($P<.05$). Due to the nesting design of this experiment, standard errors have wider ranges when comparing two different cultures compared to standard errors within a single culture.

[1]Cholesterol reported as ug of cholesterol removed/ml of broth.

[2]Deconjugation of sodium taurocholate; initial concentration of 6 mM; reported as mM deonjugated.

[3]Reported as Log_{10} cfu/ml.

Figure 1. Deconjugation of sodium taurocholate (1.2 mM) and sodium glycocholate (2.8 mM) by L. casei grown with and without pH control; Each value represents the mean from three experiments.



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APPENDIX A

COMPREHENSIVE DATA SUMMARY

Table 4. Amount of cholesterol removed from MRS-THIO broth supplemented with 10% cholesterol micelles and 6 mM sodium taurocholate by cultures grown at pH 6.0 and without pH control at 37°C.

Sample	*L. casei* E5[1]		*L. casei* N19		*L. acidophilus* 43121		*L. acidophilus* L1	
Time	pH[2]	No pH[2]	pH	No pH	pH	No pH	pH	No pH
0	0^{a}	0^{a}	0^{a}	0^{a}	0^{a}	0^{a}	0^{a}	0^{a}
16	7.4^{a}	17.6^{abcd}	16.4^{abc}	26.5^{cde}	18.6^{bcd}	46.3^{fghij}	28.5^{cdef}	26.7^{cde}
18	15.5^{abc}	41.1^{efghi}	8.5^{abc}	28.6^{cdefg}	36.9^{defgh}	58.3^{j}	36.3^{defgh}	38.6^{defgh}
20	8.0^{ab}	56.6^{hij}	10.4^{abc}	36.3^{defgh}	41.6^{efghi}	60.6^{j}	46.9^{ghij}	44.7^{efghij}
22	8.9^{ab}	62.2^{j}	13.6^{abc}	$46.8f^{ghij}$	58.5^{j}	67.9^{j}	49.8^{ghij}	57.8^{ij}

[1]All values represent the mean from three trials, reported as ug cholesterol removed from broth; means showing the same superscript letter are not significantly (P>.05) different. Due to the nesting design of this experiment, standard errors have wider ranges when comparing two different cultures compared to standard errors within a single culture.
[2]pH = pH maintained at 6.0; No pH = cultures grown without pH control

Table 5. Amount of sodium taurocholate deconjugated in MRS-THIO broth supplemented with 10% cholesterol micelles and 6 mM sodium taurocholate by cultures grown at pH 6.0 and without pH control at 37°C.

Sample	*L. casei* E5[1]		*L. casei* N19		*L. acidophilus* 43121		*L. acidophilus* L1	
Time	pH[2]	No pH[2]	pH	No pH	pH	No pH	pH	No pH
0	0^{a}	0^{a}	0^{a}	0^{a}	0^{a}	0^{a}	0^{a}	0^{a}
16	2.3^{cd}	2.1^{bc}	1.9^{bc}	3.3^{ef}	4.6^{hi}	4.2^{hi}	1.5^{b}	1.9^{bc}
18	4.6^{hi}	3.3^{ef}	3.4^{fg}	2.9^{ef}	4.6^{hi}	4.1^{gh}	1.7^{b}	2.8^{de}
20	4.2^{gh}	3.9^{gh}	3.6^{fg}	3.4^{fg}	4.8^{i}	4.8^{i}	2.1^{c}	4.1^{gh}
22	5.2^{i}	4.7^{hi}	3.6^{fg}	3.5^{fg}	4.6^{hi}	4.8^{i}	2.5^{de}	4.0^{gh}

[1]All values represent the mean from three trials, reported as mM sodium taurocholate deconjugated from broth; means showing the same superscript letter are not significantly (P>.05) different. Due to the nesting design of this experiment, standard errors have wider ranges when comparing two different cultures compared to standard errors within a single culture.

[2]pH = pH maintained at 6.0; No pH = cultures grown without pH control

Table 6. Plate counts of cultures grown at pH 6.0 and without pH control in MRS-THIO broth supplemented with 10% cholesterol micelles and 6 mM sodium taurocholate

Sample	*L. casei* E5[1]		*L. casei* N19		*L. acidophilus* 43121		*L. acidophilus* L1	
Time	pH[2]	No pH[2]	pH	No pH	pH	No pH	pH	No pH
0	0^{d}	0^{d}	0^{d}	0^{d}	0^{d}	0^{d}	0^{d}	0^{d}
16	9.59^{c}	9.04^{c}	9.03^{c}	8.68^{c}	9.26^{c}	8.72^{c}	8.67^{c}	8.16^{ab}
18	9.58^{c}	8.69^{c}	9.21^{c}	8.63^{c}	9.27^{c}	8.59^{bc}	8.70^{c}	7.86^{a}
20	9.77^{c}	8.56^{bc}	9.21^{c}	8.62^{c}	9.35^{c}	8.36^{bc}	8.84^{c}	7.99^{ab}
22	9.74^{c}	8.33^{abc}	9.26^{c}	8.68^{c}	8.99^{c}	8.18^{abc}	8.63^{c}	8.25^{abc}

[1]All values represent the mean from three trials, reported as Log_{10} cfu/ml; means showing the same superscript letter are not significantly (P>.05) different. Due to the nesting design of this experiment, standard errors have wider ranges when comparing two different cultures compared to standard errors within a single culture.
[2]pH = pH maintained at 6.0; No pH = cultures grown without pH control

Table 7. pH of MRS-THIO broth supplemented with 10% cholesterol micelles and 6 mM sodium taurocholate during growith of cultures at pH 6.0 and without pH control at 37°C.

Sample	*L. casei* E5[1]		*L. casei* N19		*L. acidophilus* 43121		*L. acidophilus* L1	
Time	pH[2]	No pH[2]	pH	No pH	pH	No pH	pH	No pH
0	6.5^{a}	6.5^{a}	6.5^{a}	6.5^{a}	6.5^{a}	6.5^{a}	6.5^{a}	6.5^{a}
16	6^{b}	4.2^{gh}	6^{b}	4.3^{fg}	6^{b}	4.4^{ef}	6^{b}	4.7^{c}
18	6^{b}	4.2^{gh}	6^{b}	4.2^{gh}	6^{b}	4.4^{ef}	6^{b}	4.6^{cd}
20	6^{b}	4.2^{gh}	6^{b}	4.2^{gh}	6^{b}	4.3^{fg}	6^{b}	4.6^{cd}
22	6^{b}	4.1^{h}	6^{b}	4.2^{gh}	6^{b}	4.2^{gh}	6^{b}	4.5^{d}

[1]All values represent the mean from three trials, reported as pH of broth; means showing the same superscript letter are not significantly (P>.05) different. Due to the nesting design of this experiment, standard errors have wider ranges when comparing two different cultures compared to standard errors within a single culture.
[2]pH = pH maintained at 6.0; No pH = cultures grown without pH control

Table 8. Amount of sodium glychocholate and sodium taurocolate deconjugated by *L. casei* during growth with and without pH control at 37°C in MRS-THIO broth supplemented with 1.2 mM sodium taurocholate and 3.8 mM glychocolic acid

	Sodium Taurocholate				Sodium Glychocolate			
Sample	*L. casei* E5		*L. casei* N19		*L. casei* E5		*L. casei* N19	
Time	pH	No pH	pH	No pH	pH	No pH	pH	No pH
16	$.27^{bc}$	$.42^{c}$	$.07^{a}$	$.07^{a}$	1.8^{cd}	2.1^{cde}	1.0^{ab}	$.48^{a}$
18	$.56^{cd}$	$.86^{d}$	$.09^{a}$	$.07^{a}$	2.1^{cde}	2.8^{e}	1.3^{bc}	1.2^{ab}
20	$.75^{d}$	$.88^{d}$	$.19^{ab}$	$.16^{ab}$	2.8^{e}	2.8^{e}	1.7^{cd}	1.1^{ab}
22	$.68^{cd}$	$.80^{d}$	$.49^{cd}$	$.27^{bc}$	2.8^{e}	2.8^{e}	2.4^{de}	$.98^{ab}$

[1]All values represent the mean from three trials, reported as mM deconjugated/ml ; means showing the same superscript letter are not significantly (P>.05) different. Due to the nesting design of this experiment, standard errors have wider ranges when comparing two different cultures compared to standard errors within a single culture.
[2]pH = pH maintained at 6.0; No pH = cultures grown without pH control

APPENDIX B

RAW DATA FROM TREATMENTS

TABLE 9

Plate Counts on MRS agar of cells of *Lactobacillus casei* E5 Grown with no pH Control at 37°C in MRS-THIO Broth Supplemented with 3% Sodium Taurocholate and 10% Cholesterol Micelles

		Log $_{10}$ cfu/ml		
Harvest Time	Rep 1	Rep 2	Rep 3	Average
16	9.04	9.08	8.99	9.04
18	8.75	8.57	8.75	8.69
20	8.82	8.41	8.46	8.56
22	8.85	8.32	7.83	8.33

TABLE 10

Plate Counts on MRS agar of cells of *Lactobacillus casei* E5 Grown at pH 6.0 at 37°C in MRS-THIO Broth Supplemented with 3% Sodium Taurocholate and 10% Cholesterol Micelles

		Log $_{10}$ cfu/ml		
Harvest Time	Rep 1	Rep 2	Rep 3	Average
16	9.91	9.88	8.18	9.59
18	8.86	9.72	8.76	9.58
20	9.93	9.86	8.88	9.77
22	9.86	9.88	8.79	9.74

TABLE 11

Plate Counts on MRS agar of cells of *Lactobacillus casei* N19 Grown with no pH Control at 37°C in MRS-THIO Broth Supplemented with 3% Sodium Taurocholate and 10% Cholesterol Micelles

		Log $_{10}$ cfu/ml		
Harvest Time	Rep 1	Rep 2	Rep 3	Average
16	8.64	8.41	9.00	8.68
18	8.56	8.41	8.93	8.63
20	8.59	8.41	8.85	8.62
22	8.76	8.38	8.90	8.68

TABLE 12

Plate Counts on MRS agar of cells of *Lactobacillus casei* N19 Grown at pH 6.0 at 37°C in MRS-THIO Broth Supplemented with 3% Sodium Taurocholate and 10% Cholesterol Micelles

		Log $_{10}$ cfu/ml		
Harvest Time	Rep 1	Rep 2	Rep 3	Average
16	9.59	9.32	9.00	9.03
18	9.54	9.32	9.15	9.21
20	9.41	9.34	9.52	9.21
22	9.65	9.34	9.48	9.26

TABLE 13

Plate Counts on MRS agar of cells of *Lactobacillus acidophilus* 43121 Grown with no pH Control at 37°C in MRS-THIO Broth Supplemented with 3% Sodium Taurocholate and 10% Cholesterol Micelles

		Log $_{10}$ cfu/ml		
Harvest Time	Rep 1	Rep 2	Rep 3	Average
16	8.67	8.77	8.72	8.72
18	8.59	8.71	8.48	8.59
20	8.20	8.59	8.28	8.36
22	7.08	9.15	8.32	8.18

TABLE 14

Plate Counts on MRS agar of cells of *Lactobacillus acidophilus* 43121 Grown at pH 6.0 at 37°C in MRS-THIO Broth Supplemented with 3% Sodium Taurocholate and 10% Cholesterol Micelles

		Log $_{10}$ cfu/ml		
Harvest Time	Rep 1	Rep 2	Rep 3	Average
16	9.11	9.28	9.38	9.26
18	9.08	9.04	9.67	9.27
20	9.08	9.38	9.58	9.35
22	9.20	8.43	9.34	8.99

TABLE 15

Plate Counts on MRS agar of cells of *Lactobacillus acidophilus* L1 Grown without pH control at 37°C in MRS-THIO Broth Supplemented with 3% Sodium Taurocholate and 10% Cholesterol Micelles

		Log $_{10}$ cfu/ml		
Harvest Time	Rep 1	Rep 2	Rep 3	Average
16	9.11	9.00	7.89	8.67
18	9.17	9.04	7.98	8.70
20	9.15	9.18	8.15	8.84
22	9.20	9.23	7.46	8.63

TABLE 16

Amount of cholesterol removed from MRS-THIO Broth Supplemented with 3% Sodium Taurocholate and 10% Cholesterol Micelles by *Lactobacillus casei* E5 grown with no pH control at 37°C.

	ug cholesterol removed from broth			
Harvest Time	Rep 1	Rep 2	Rep 3	Average
16	28.98	7.73	15.93	
18	46.44	28.21	48.56	
20	76.0	41.65	52.01	
22	83.29	44.14	49.30	

TABLE 17

Amount of cholesterol removed from MRS-THIO Broth Supplemented with 3% Sodium Taurocholate and 10% Cholesterol Micelles by *Lactobacillus casei* E5 grown at pH 6.0 at 37°C.

	ug cholesterol removed from broth			
Harvest Time	Rep 1	Rep 2	Rep 3	Average
16	2.88	5.57	17.08	7.36
18	2.88	9.47	14.00	15.5
20	5.88	8.64	6.52	7.97
22	11.33	10.94	13.82	8.36

TABLE 18

Amount of cholesterol removed from MRS-THIO Broth Supplemented with 3% Sodium Taurocholate and 10% Cholesterol Micelles by *Lactobacillus casei* N19 grown with no pH control at 37°C.

	ug cholesterol removed from broth			
Harvest Time	Rep 1	Rep 2	Rep 3	Average
16	16.89	49.33	13.24	26.49
18	21.12	32.63	32.05	28.6
20	22.65	54.51	31.67	36.28
22	39.35	57.39	43.57	46.76

TABLE 19

Amount of cholesterol removed from MRS-THIO Broth Supplemented with 3% Sodium Taurocholate and 10% Cholesterol Micelles by *Lactobacillus casei* N19 grown at pH 6.0 at 37°C.

	ug cholesterol removed from broth			
Harvest Time	Rep 1	Rep 2	Rep 3	Average
16	9.22	23.03	13.63	16.44
18	1.54	9.98	34.16	8.51
20	1.34	23.42	9.40	10.43
22	8.64	18.43	4.61	13.63

TABLE 20

Amount of cholesterol removed from MRS-THIO Broth Supplemented with 3% Sodium Taurocholate and 10% Cholesterol Micelles by *Lactobacillus acidophilus* 43121 grown with no pH control at 37°C.

	ug cholesterol removed from broth			
Harvest Time	Rep 1	Rep 2	Rep 3	Average
16	28.4	43.95	66.59	46.31
18	55.66	45.87	73.51	58.35
20	58.34	51.43	71.97	60.58
22	75.62	55.28	73.13	67.98

TABLE 21

Amount of cholesterol removed from MRS-THIO Broth Supplemented with 3% Sodium Taurocholate and 10% Cholesterol Micelles by *Lactobacillus acidophilus* 43121 grown at pH 6.0 at 37°C.

	ug cholesterol removed from broth			
Harvest Time	Rep 1	Rep 2	Rep 3	Average
16	27.25	13.82	14.78	18.62
18	36.08	27.83	46.25	36.89
20	50.28	29.44	44.53	41.58
22	62.95	54.89	57.58	58.47

TABLE 22

Amount of cholesterol removed from MRS-THIO Broth Supplemented with 3% Sodium Taurocholate and 10% Cholesterol Micelles by *Lactobacillus acidophilus* L1 grown with no pH control at 37°C.

	ug cholesterol removed from broth			
Harvest Time	Rep 1	Rep 2	Rep 3	Average
16	21.88	26.3	31.88	26.69
18	26.87	50.09	38.77	38.58
20	34.35	69.1	30.71	44.72
22	48.79	75.43	49.14	57.79

TABLE 23

Amount of cholesterol removed from MRS-THIO Broth Supplemented with 3% Sodium Taurocholate and 10% Cholesterol Micelles by *Lactobacillus acidophilus* 43121 grown at pH 6.0 at 37°C.

	ug cholesterol removed from broth			
Harvest Time	Rep 1	Rep 2	Rep 3	Average
16	4.19	61.23	20.15	28.52
18	17.47	62.19	29.18	36.28
20	35.69	63.92	41.08	46.89
22	32.63	65.65	51.06	49.78

TABLE 24

Amount of sodium taurocholate removed from MRS-THIO Broth Supplemented with 3% Sodium Taurocholate and 10% Cholesterol Micelles by *Lactobacillus casei* E5 grown with no pH control at 37°C.

	mM Sodium Taurocholate removed from broth			
Harvest Time	Rep 1	Rep 2	Rep 3	Average
16	3.06	2.06	1.05	
18	4.97	2.65	2.29	
20	5.26	3.08	3.56	
22	5.40	4.24	4.34	

TABLE 25

Amount of sodium taurocholate removed from MRS-THIO Broth Supplemented with 3% Sodium Taurocholate and 10% Cholesterol Micelles by *Lactobacillus casei* E5 grown at pH 6.0 at 37°C.

	mM Sodium Taurocholate removed from broth			
Harvest Time	Rep 1	Rep 2	Rep 3	Average
16	1.95	1.65	1.25	2.29
18	4.22	4.47	3.16	4.59
20	4.22	3.71	4.75	4.17
22	4.33	4.76	5.00	5.16

TABLE 26

Amount of sodium taurocholate removed from MRS-THIO Broth Supplemented with 3% Sodium Taurocholate and 10% Cholesterol Micelles by *Lactobacillus casei* N19 grown with no pH control at 37°C.

	mM Sodium Taurocholate removed from broth			
Harvest Time	Rep 1	Rep 2	Rep 3	Average
16	2.98	3.69	3.16	3.28
18	3.08	2.55	3.18	2.94
20	2.80	3.39	3.92	3.37
22	2.95	3.83	4.09	3.53

TABLE 27

Amount of sodium taurocholate removed from MRS-THIO Broth Supplemented with 3% Sodium Taurocholate and 10% Cholesterol Micelles by *Lactobacillus casei* N19 grown at pH 6.0 at 37°C.

	mM Sodium Taurocholate removed from broth			
Harvest Time	Rep 1	Rep 2	Rep 3	Average
16	3.06	1.36	3.29	1.89
18	2.99	4.41	2.90	3.44
20	3.04	4.26	3.37	3.56
22	3.17	4.13	3.44	3.58

TABLE 28

Amount of sodium taurocholate removed from MRS-THIO Broth Supplemented with 3% Sodium Taurocholate and 10% Cholesterol Micelles by *Lactobacillus acidophilus* 43121 grown with no pH control at 37°C.

	mM Sodium Taurocholate removed from broth			
Harvest Time	Rep 1	Rep 2	Rep 3	Average
16	4.00	4.79	3.91	4.24
18	4.31	3.93	4.05	4.09
20	4.98	5.29	4.05	4.78
22	4.53	4.84	5.17	4.84

TABLE 29

Amount of sodium taurocholate removed from MRS-THIO Broth Supplemented with 3% Sodium Taurocholate and 10% Cholesterol Micelles by *Lactobacillus acidophilusi* 43121 grown at pH 6.0 at 37°C.

	mM Sodium Taurocholate removed from broth			
Harvest Time	Rep 1	Rep 2	Rep 3	Average
16	4.16	5.39	4.35	4.63
18	3.86	5.34	4.49	4.57
20	4.91	5.16	4.36	4.81
22	5.00	4.29	4.36	4.55

TABLE 30

Amount of sodium taurocholate removed from MRS-THIO Broth Supplemented with 3% Sodium Taurocholate and 10% Cholesterol Micelles by *Lactobacillus acidophilus* L1 grown with no pH control at 37°C.

	mM Sodium Taurocholate removed from broth			
Harvest Time	Rep 1	Rep 2	Rep 3	Average
16	2.43	2.15	1.14	1.91
18	3.58	2.39	2.43	2.79
20	3.76	4.61	3.78	4.05
22	3.74	4.74	3.63	4.03

TABLE 31

Amount of sodium taurocholate removed from MRS-THIO Broth Supplemented with 3% Sodium Taurocholate and 10% Cholesterol Micelles by *Lactobacillus acidophilus* L1 grown at pH 6.0 at 37°C.

	mM Sodium Taurocholate removed from broth			
Harvest Time	Rep 1	Rep 2	Rep 3	Average
16	1.46	1.05	2.21	1.14
18	1.71	1.15	1.54	2.43
20	2.11	1.05	1.49	3.78
22	2.46	1.78	1.98	

TABLE 32

pH of MRS-THIO Broth Supplemented with 3% Sodium Taurocholate and 10% Cholesterol Micelles after growth of *Lactobacillus casei* E5 at 37°C.

	pH			
Harvest Time	Rep 1	Rep 2	Rep 3	Average
0	6.5	6.5	6.5	6.5
16	4.2	4.2	4.2	4.2
18	4.2	4.2	4.2	4.2
20	4.2	4.2	4.2	4.2
22	4.2	4.0	4.2	4.1

TABLE 33

pH of MRS-THIO Broth Supplemented with 3% Sodium Taurocholate and 10% Cholesterol Micelles after growth of *Lactobacillus casei* N19 at 37°C.

	pH			
Harvest Time	Rep 1	Rep 2	Rep 3	Average
0	6.5	6.5	6.5	6.5
16	4.3	4.5	4.2	4.3
18	4.3	4.4	4.0	4.2
20	4.2	4.4	4.0	4.2
22	4.2	4.4	4.0	4.2

TABLE 34

pH of MRS-THIO Broth Supplemented with 3% Sodium Taurocholate and 10% Cholesterol Micelles after growth of *Lactobacillus acidophilus* 43121 at 37°C.

	pH			
Harvest Time	Rep 1	Rep 2	Rep 3	Average
0	6.5	6.5	6.5	6.5
16	4.3	4.4	4.4	4.4
18	4.3	4.4	4.3	4.4
20	4.3	4.3	4.3	4.3
22	4.2	4.2	4.2	4.2

TABLE 35

pH of MRS-THIO Broth Supplemented with 3% Sodium Taurocholate and 10% Cholesterol Micelles after growth of *Lactobacillus acidophilus* 43121 at 37°C.

	pH			
Harvest Time	Rep 1	Rep 2	Rep 3	Average
0	6.5	6.5	6.5	6.5
16	4.8	4.6	4.6	4.7
18	4.8	4.5	4.5	4.6
20	4.8	4.5	4.5	4.6
22	4.8	4.3	4.3	4.5

TABLE 36

Amount of sodium taurocholate from MRS-THIO Broth Containing 3.8 mM sodium taurocholate and 2.2 mM glycocholic acid by *Lactobacillus casei* E5 grown with no pH control at 37°C.

	mM bile salt removed from broth			
Harvest Time	Rep 1	Rep 2	Rep 3	Average
16	.45	1.74	1.8	1.33
18	3.15	1.77	3.3	2.74
20	3.08	2.02	3.3	2.8
22	3.09	2.38	2.99	2.82

TABLE 37

Amount of glychocolic acid removed from MRS-THIO Broth Containing 3.8 mM sodium taurocholate and 2.2 mM glycocholic acid by *Lactobacillus casei* E5 grown with no pH control at 37°C.

	mM bile salt removed from broth			
Harvest Time	Rep 1	Rep 2	Rep 3	Average
16	2.2	2.2	1.81	2.07
18	2.2	2.2	2.2	2.2
20	2.2	2.2	2.2	2.2
22	2.2	2.2	2.2	2.2

TABLE 38

Total bile salts removed from MRS-THIO Broth Containing 3.8 mM sodium taurocholate and 2.2 mM glycocholic acid by *Lactobacillus casei* E5 grown with no pH control at 37°C.

	mM bile salt removed from broth			
Harvest Time	Rep 1	Rep 2	Rep 3	Average
16	2.65	3.94	3.61	3.40
18	5.35	3.97	5.50	4.94
20	5.28	4.22	5.50	5.00
22	5.29	4.58	5.17	5.01

TABLE 39

Amount of sodium taurocholate removed from MRS-THIO Broth Containing 3.8 mM sodium taurocholate and 2.2 mM glycocholic acid by *Lactobacillus casei* E5 grown at pH 6.0 at 37°C.

	mM bile salt removed from broth			
Harvest Time	Rep 1	Rep 2	Rep 3	Average
16	.77	1.05	.84	0.87
18	2.18	1.29	1.82	1.76
20	1.75	1.24	2.07	1.69
22	1.89	2.04	2.6	2.18

TABLE 40

Amount of glychocolic acid removed from MRS-THIO Broth Containing 3.8 mM sodium taurocholate and 2.2 mM glycocholic acid by *Lactobacillus casei* E5 grown at pH 6.0 at 37°C.

	mM bile salt removed from broth			
Harvest Time	Rep 1	Rep 2	Rep 3	Average
16	2.2	1.65	1.49	1.78
18	2.2	1.85	2.2	2.08
20	2.2	2.2	2.2	2.2
22	2.2	2.2	2.2	2.2

TABLE 41

Total bile salts removed from MRS-THIO Broth Containing 3.8 mM sodium taurocholate and 2.2 mM glycocholic acid by *Lactobacillus casei* E5 grown at pH 6.0 at 37°C.

	mM bile salt removed from broth			
Harvest Time	Rep 1	Rep 2	Rep 3	Average
16	2.97	2.7	2.33	2.67
18	4.38	3.14	4.02	3.85
20	3.95	3.44	4.27	3.85
22	4.09	4.24	4.8	4.38

TABLE 42

Amount of sodium taurocholate from MRS-THIO Broth Containing 3.8 mM sodium taurocholate and 2.2 mM glycocholic acid by *Lactobacillus casei* N19 grown with no pH control at 37°C.

	mM bile salt removed from broth			
Harvest Time	Rep 1	Rep 2	Rep 3	Average
16	.27	.18	0	0.23
18	.26	.44	0	0.23
20	.25	1.27	0	0.51
22	.13	1.6	.85	0.86

TABLE 43

Amount of glychocolic acid removed from MRS-THIO Broth Containing 3.8 mM sodium taurocholate and 2.2 mM glycocholic acid by *Lactobacillus casei* N19 grown with no pH control at 37°C.

	mM bile salt removed from broth			
Harvest Time	Rep 1	Rep 2	Rep 3	Average
16	.53	.62	0	.38
18	.79	1.05	0	0.92
20	.57	1.24	.72	0.84
22	.77	1.33	.23	0.78

TABLE 44

Total bile salts removed from MRS-THIO Broth Containing 3.8 mM sodium taurocholate and 2.2 mM glycocholic acid by *Lactobacillus casei* N19 grown with no pH control at 37°C.

	mM bile salt removed from broth			
Harvest Time	Rep 1	Rep 2	Rep 3	Average
16	.8	.8	0	0.53
18	1.05	1.49	0	0.85
20	.82	2.6	1.55	2.49
22	.9	2.84	.4	1.38

TABLE 45

Amount of sodium taurocholate removed from MRS-THIO Broth Containing 3.8 mM sodium taurocholate and 2.2 mM glycocholic acid by *Lactobacillus casei* N19 grown at pH 6.0 at 37°C.

	mM bile salt removed from broth			
Harvest Time	Rep 1	Rep 2	Rep 3	Average
16	.33	0	0	0.11
18	.38	.05	0	0.14
20	.28	.62	0	0.31
22	1.24	.24	.85	0.78

TABLE 46

Amount of glychocolic acid removed from MRS-THIO Broth Containing 3.8 mM sodium taurocholate and 2.2 mM glycocholic acid by *Lactobacillus casei* N19 grown at pH 6.0 at 37°C.

	mM bile salt removed from broth			
Harvest Time	Rep 1	Rep 2	Rep 3	Average
16	.61	.61	0	0.41
18	.71	.82	0	0.51
20	.88	1.04	0	0.96
22	.89	.70	.44	0.68

TABLE 47

Total bile salts removed from MRS-THIO Broth Containing 3.8 mM sodium taurocholate and 2.2 mM glycocholic acid by *Lactobacillus casei* E5 grown at pH 6.0 at 37°C.

	mM bile salt removed from broth			
Harvest Time	Rep 1	Rep 2	Rep 3	Average
16	.94	.87	0	0.60
18	1.09	1.66	0	0.92
20	1.16	.94	0	0.70
22	2.13	.61	1.29	1.34

APPENDIX C

IDENTITY OF ISOLATES OF *L. CASEI*

TABLE 49

IDENTITY OF ISOLATES OF *LACTOBACILLUS CASEI*

Test[1]	Lc[2]	A3	A17	E5	E10	L15	L19	M5	M12	N7	N19
Amygdalin	+	+	+	+	+	+	+	-	-	+	+/-
Arabinose	-	+/-	+	+	-	-	-	-	-	-	-
Arginine	-	-	-	-	-	+	-	-	-	-	-
Cellobiose	+	+	+	+	+	+	+	+	+	+	+
Esculin	+	+	+	+	+	+	+	+	+	+	+
Galactose	+	+	+	+	+	+	+	+	+	+	+
Glucose	+	+	+	+	+	+	+	+	+	+	+
Lactose	+			+	+	+	+	+	+	+	+/-
Maltose	+/-	+	+	+	+	+	+	+	+/-	+/-	+
Mannitol	+	+	+	+	+	+	+	+	+	+	+
Mannose	+	+	+	+	+	+	+	+	+	+	+
Melezitose	+	+	+	+	+	+/-	+	+	+	+	+
Melibiose	-	+	+	-	+	-	-	-	-	-	-
Raffinose	-	+/-	+/-	-	+/-	-	-	-	-	-	-
Rhamnose	-	-	-	-	-	-	-	+	+	+	-
Salicin	+	+	+	+	+	+	+	+	+	+	+
Sorbitol	+	+	+			+	+	+	+	+	+
Sucrose	+/-	+	+	+	+	+	+	+	+/-	-	-
Trehalose	+	+	+	+	+	+	+	+	+	+	+
Xylose	-	+/-	-	-	-	-	-	-	-	-	-
Growth at 45°C		+	-	+	+	-	-	+	+	+	+

[1]All isolates were Gram positive, catalase negative rods which grew at 15 °C.

[2]Lc = *Lactobacillus casei*; reactions as listed in the 8th Edition of *Bergey's Manual of Determinative Bacteriology*.

APPENDIX D

EXPERIMENTAL DESIGN

EXPERIMENTAL DESIGN

	DF
Total	119
Among Broth Mix	11
Culture	5
Error a	8
Within Broth Mix	12
Treatment	1
T x C	3
Error b	8
Among Time	96
Time	4
Ti x Tr	4
Tr x Culture	12
Ti x Tr Culture	12
Error c	64

VITA

Mindy Brashears

Candidate for the Degree of

Doctor of Philosophy

Thesis: INFLUENCE OF pH ON BILE SALT DECONJUGATION AND REMOVAL OF CHOLESTEROL FROM MEDIA BY *LACTOBACILLUS CASEI* AND *LACTOBACILLUS ACIDOPHILUS* DURING GROWTH

Major Field: Food Science

Biographical:

Personal Data: Born in Amarillo, Texas on May 13, 1970, the daughter of Gary and Becky Hardcastle. Married August 12, 1989 to Todd Brashears. Daughter, Bailey, born December 12, 1994.

Education: Graduated from Wheeler High School, Wheeler, Texas in May 1988 with an advanced diploma with honors; graduated Magna Cum Laude with Bachelor of Science degree in Food Technology from Texas Tech University, Lubbock, Texas in 1992. Completed the requirements for the Master of Science degree with a major in Food Science at Oklahoma State University, Stillwater, Oklahoma in May, 1994. Completed the requirements for the Doctor of Philosophy degree with a major in Food Science at Oklahoma State University in July, 1997.

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