THE IMPORTANCE OF BILE SALT HYDROLASE ACTIVITY BY *LACTOBACILLUS ACIDOPHILUS* AND ITS GENETIC LOCATION

By

DEREK KEITH WALKER

Bachelor of Science in Agriculture
Oklahoma State University
Stillwater, Oklahoma
1984

Master of Science
Oklahoma State University
Stillwater, Oklahoma
1986

Submitted to the Faculty of the
Graduate College of the
Oklahoma State University
in partial fulfillment of
the requirements for
the Degree of
DOCTOR OF PHILOSOPHY
May, 1990

THE IMPORTANCE OF BILE SALT HYDROLASE ACTIVITY BY LACTOBACILLUS ACIDOPHILUS AND ITS GENETIC LOCATION

Thesis Approved:

Stanley E. Gilliland
Thesis Adviser

H Glen Dobrogosz

Charles V. Maxwell

Alan Harker

Norman N. Durham
Dean of the Graduate College

PREFACE

The relationship among bile tolerance deconjugation by bile acids and assimilation of cholesterol by Lactobacillus acidophilus was determined. In addition, the genetic location of bile salt hydrolase activity was determined.

I wish to express my sincere thanks to Dr. S.E. Gilliland for his patience and guidance as major advisor during my time at Oklahoma State University. I am also thankful to Dr. H. G. Dolezal, Dr. Alan Harker, and Dr Charles Maxwell for serving on the Advisory Committee.

Appreciation is also extended to Dr. Thomas Rehberger and Dr. Lawrence Yates for their assistance, guidance, and friendship during this study.

Finally, the author thanks his wife Brenita for her love, patience and understanding during this course of study, and especially for the preparation of this dissertation. Also, the author thanks his daughter Ashley for her love and encouragement.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

CHAPTER I

INTRODUCTION

Individuals that have elevated serum cholesterol levels have a higher risk of having coronary heart disease than individuals with normal levels (15,39,40). By reducing serum cholesterol (low-density lipoprotein) levels, the incidence of coronary heart disease can be reduced in hypercholesterolemic individuals (39,40). People that have primary hypercholesterolemia synthesize excess cholesterol because there is no control over endogenous synthesis and therefore a means for reducing the serum cholesterol level is necessary.

There are several potential approaches to lowering serum cholesterol levels (28,70). The most direct appoach is to control the diet and subsequent intake of fat. The other approaches are (1) inhibit cholesterol synthesis, (2) inhibit absorption of cholesterol from the small intestines and (3) prevent reabsorption of bile acids during normal enterohepatic circulation.

The intestinal tract is the major route for the removal of cholesterol from the body. Gnotobiotic animals fed a diet containing cholesterol attained levels of cholesterol in the blood twice as high as did conventional animals fed

the same diet (17). Chikai et al(6) and Eyssen(17) reported that more bile salts are excreted in the feces of conventional animals than gnotobiotic animals. Most of the bile acids excreted in the conventional animals are in the deconjugated form.

Some bacteria in the small intestine may influence the absorption of cholesterol into the bloodstream. For example, Gilliland et al (25) using young pigs as an experimental model showed that dietary supplementation with skim milk containing a selected strain of Lactobacillus acidophilus could reduce serum cholesterol levels in pigs fed a high cholesterol diet. Recently, it was confirmed that L. acidophilus exhibited hypocholesterolemic activity (8). They fed mature boars acidophilus yogurt and found that the animals receiving the acidophilus yogurt had lower serum cholesterol levels than those animals in the control group.

Lipid absorption from the small intestines requires bile salts (33). Bile acids are synthesized in the liver and conjugated with glycine or taurine. Conjugated bile acids better facilitate lipid absorption than deconjugated bile acids. Gullo-Torres et al (20) reported that taurine conjugates better facilitate cholesterol absorption than bile acids conjugated with glycine.

Bile salts undergo extensive metabolism in the intestines by gut microflora (35). The most common transformation is the hydrolysis of the peptide bond of

taurine or glycine conjugates of bile acids (26,62). Bile salt hydrolase (BSH) is the enzyme that catalyzes this reaction,

Several genera of bacteria indigenous to the intestinal tract have been reported to have BSH activity (1,6,7,9,22) Gilliland and Speck (22) reported that L. acidophilus preferentially hydrolyzes taurocholic acid over glycocholic acid. Tannock et al. (64) reported that lactobacilli are the predominant organisms that produce BSH in the proximal murine gastrointestinal tract. Gilliland and Speck (22) and Dashkevicz and Feighner (9) reported that several species of lactobacilli could deconjugate bile salts. The latter researchers showed that one of eighteen strains of L. acidophilus could not deconjugate taurocholic or taurodeoxycholic acid. They suggested that the variation in BSH activity among different species of lactobacilli might be plasmid mediated.

This BSH activity may be important in controlling serum cholesterol levels because Eyssen (17) stated that deconjugated bile acids function poorer in the absorption cholesterol from the intestines than conjugated bile acids Also because L. acidophilus has the ability to reduce serum cholesterol levels in some animals (25,27,29).

The purposes of this study were to (1) determine the relationship between bile tolerance, BSH activity and assimilation of cholesterol by L. acidophilus and (2)

determine if there was a common plasmid among L. acidophilus encoding BSH activity.

CHAPTER II

REVIEW OF LITERATURE

Synthesis and Role of Cholesterol in Vivo

Cholesterol is a natural and required component of the body (1). It is used as a precursor for synthesis of steroid hormones, vitamin D and bile acids. Cholesterol also regulates cell fluidity when it is incorporated into cell membranes. Synthesis of cholesterol occurs primarily in the liver; also, it can be acquired from absorbing undigested cholesterol from dietary sources (1). Cholesterol synthesis is controlled by a feedback mechanism by which dietary cholesterol inhibits the initial step that catalyzes cholesterol biosynthesis (13).

In vivo, cholesterol is transported by plasma lipoproteins (1). The lipoproteins primarily involved are low-density lipoproteins (LDL) and high-density liproproteins (HDL). LDL transports free and esterified cholesterol to nonhepatic tissues where receptors on these tissues bind the LDL-cholesterol complex and absorb the cholesterol. HDL transports unesterified cholesterol from nonhepatic tissues to the liver for excretion (58).

Relationship Between Cholesterol, Atherosclerosis, and Coronary Heart Disease

It has been well established that accelerated atherosclerosis is associated with elevated LDL cholesterol levels in the blood (68). The best evidence is familial hypercholesterolemia, in which massive LDL concentrations are evident and result in death within twenty years after birth. Jones (41) and the National Heart and Lung Institute (64) reported that atherosclerosis is the major cause of most cardiovascular disease. In addition, cardiovascular disease accounts for one half the deaths in the U.S. each year (82) and is the major cause of death in Europe (83)

Lowering total plasma cholesterol and LDL cholesterol can decrease the incidence of coronary heart disease and myocardial infarction in persons with primary hypercholesterolemia (47,48); thus, for hypercholesterolemic individuals, a method of reducing serum cholesterol levels is essential.

Approaches to Lowering Serum Cholesterol Levels

Use of Drugs to Lower Serum Cholesterol

Some drugs are effective in reducing serum cholesterol levels. Cholestyramine was used successfully to lower serum cholesterol levels in men with primary hypercholesterolemia (47,48). The study involved over 3000 middle aged men Two

groups, one receiving cholestyramine and the other a placebo were monitored for seven years. The group receiving cholestyramine had significantly lower serum cholesterol levels than the placebo group. Cholestyramine is a bile acid sequesterant that cannot be metabolized or absorbed and functions by binding the bile salt cholesterol mixed micelle and facilitates its excretion from the gastrointestinal (GI) tract. However, cholestyramine and other drugs (clofirbrate, cholestipol, probucol and nicotinic acid) that function similarly have all been shown to produce side effects such as constipation, heartburn, nausea (47) and loosening of the stool (18). These side effects warrant the need for alternative approaches to lowering serum cholesterol levels.

Alternative Approaches to Lowering Serum Cholesterol

There are several alternative approaches to lowering serum cholesteol levels (31,83). The primary approach is to control the diet. Van Belle (83) suggested enhancing the catabolism of cholesterol to bile acids and increasing fecal excretion of bile acids. He proposed four methods to achieve increased fecal bile acid excretion: (1) affect the oxidation of cholesterol in the liver thus facilitating its conversion to bile acids which may result in increased fecal elimination, (2) affect formation of bile acids in the liver with drugs that produce bile acids that are less well

reabsorbed but retain their digestive properties, (3) affect the reabsorption of bile acids from the ileum using pharmaceutical agents to block enterohepatic circulation (EHC) and (4) alter the intestinal microflora to favor the growth of organisms involved in bile acid transformations that render the bile acids less well reabsorbed. Grundy and Bilheimer (31) proposed (1) inhibiting the absorption of cholesterol from the GI tract (2) preventing reabsorption of bile acids in normal EHC and (3) inhibiting cholesterol synthesis. Neither of the aforementioned authors proposed specific methods for implementing these approaches.

Role of Gut Microflora in Controlling Serum Cholesterol Levels

The GI tract is the primary route for removal of cholesterol from the body (54). Eyssen (20) reported that when fed a diet high in cholesterol, gnotobiotic (germ free) rats and chicks accumulated twice as much cholesterol in the blood as their conventional counterparts. These germ free animals also excreted less cholesterol in the feces than the conventional animals. Furthermore, more bile acids were excreted in the feces of conventional animals than germ free animals. He concluded that intestinal microorganisms interfere with the absorption of cholesterol from the small intestine. Chikai et al (8) inoculated germ free rats with Bacteroides vulgatus, Bifidobacterium longum, Clostridium ramosum and Escherichia coli and fed them a normal diet

Monitoring of fecal bile acid excretion revealed that fecal bile acid excretion increased in all the inoculated rats except those inoculated with E. coli, which does not deconjugate. They also reported that more of the excreted bile acids in conventional animals were deconjugated than germ free animals. They speculated that free bile acids are excreted more rapidly than conjugated ones and that adhesion of these free bile acids to microorganisms or dietary fiber may be the route of passage. This increased fecal bile acid excretion may be another mechanism by which lowering of serum cholesterol levels may be acheived.

Mott et al (60) reported that when germ free piglets were monocontaminated with Lactobacillus acidophilus and allowed to develop a normal flora there was a decrease in serum cholesterol when compared to those animals that were not allowed to develop a natural flora. There was an increase in fecal microorganisms in the nonsterile environment but no change in fecal steroids; also, bile acid excretion was lower in animals in the nonsterile environment than in the sterile environment. This suggests that L. acidophilus alone may not be invloved in lowering serum cholesterol levels. In fact, they speculated that factors besides microbial metabolism of steroids might be involved and hypothesized that interference with absorption of cholesterol might be a possibility.

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

Mann and Spoerry (52) were suprised to discover that when Maasai tribesmen consumed large quantities of milk fermented with a "wild" strain of Lactobacillus, there were a significant reductions in serum cholesterol levels. Twenty four volunteers consumed fermented milk for six days and slaughtered and consumed a steer on the seventh day. This regime was followed for three weeks. The fermented milk for one halfof the men was supplemented with a surfactant (Tween 20) which was thought to enhance lipid absorption and that for the other half was supplemented with a placebo (olive oil). As consumption of the fermented milk increased weight gain increased but serum cholesterol levels decreased for both groups. The greater the weight gain the lower the serum cholesterol levels. The decreased serum cholesterol levels were attributed to the consumption of the fermented milk. These findings were contrary to their hypothesis because they expected the surfactant to enhance the absorption of cholesterol resulting in higher serum levels in the group receiving the Tween 20 than in the control group. They concluded that some component in the fermented milk was responsible for inhibiting cholesterol synthesis.

Mann (53) substantiated his previous hypothesis when he reported that consumption of yogurt resulted in significant ($P < .05$) reductions in serum cholesterol levels in humans when compared to fresh whole milk over a twelve day trial. A similar study reported that both pasteurized and nonpasteurized yogurt significantly ($P< .01$) reduced serum cholesterol levels in fifty four individuals during a twelve week feeding trial when compared to 2% fat milk (33). These authors concluded that the culture may not be involved in the hypocholesterolemic effect.

Thompson et al (79) found that sweet acidophilus milk, yogurt, skim milk, and 2% fat milk had no effect on serum cholesterol during a nine week study on sixty eight healthy individuals. However these individuals were not hypercholesterolemic and nothing was fed to create a hypercholesterolemic condition thus no reductions should be expected.

Rao et al (67) reported that rats fed milk fermented with Streptococcus theromophilus had significantly lower serum cholesterol levels when compared to rats fed diets supplemented with skim milk or water. Also diets supplemented with methanol solubles from the Thermophilus milk significantly lowered plasma cholesterol levels when compared to methanol solubles from nonfermented milk. They concluded that metabolites produced during fermentation exerted a hypocholesterolemic effect. Thakur and Jha (78) found that when rabbits on a high cholesterol diet were fed

yogurt, there was a significant reduction in plasma cholesterol levels compared to rabbits on the same diet and fed milk. However serum cholesterol levels in the latter group did decrease over time. They suggested that in addition to some unknown factor in the milk that calcium was involved; however, Howard and Marks (38) reported that 2.4 g of calcium gluconate daily administered to seven individuals for twelve days did not effect serum cholesterol levels This suggests that calcium is not a hypocholesterolemic agent in milk or fermented milk.

Other than the report of Mott et al(60) and perhaps Mann and Spoerry(52), the cultured products mentioned do not contain bacteria that can survive and grow in the intestinal tract. Therefore, the hypocholesterolemic effect they exert may be due to metabolites produced by the culture or alterations in the milk during fermentation.

Hypocholesterolemic Effect of *Lactobacillus acidophilus*

Several reports have shown that consumption of *L. acidophilus* can result in decreased serum cholesterol levels. After consuming *L. acidophilus* for one month cecetomized and normal laying hens had significantly lower serum cholesterol levels (80). Harrison and Peat (32) reported that human infants fed milk containing *L. acidophilus* had significantly ($P<.001$) lower serum cholesterol levels, geater weight gains ($P<.05$) and

increased numbers of lactobacilli in their stools ($P<.001$) when compared to infants receiving sterile milk formula Grunewald (30) found that rats consuming skim milk fermented with L. acidophilus and methanol solubles from this fermented milk had significantly ($P < .05$) lower plasma cholesterol levels than rats fed water or control milk . She concluded that the culture produced a factor during fermentation that reduced cholesterol synthesis following its ingestion.

Gilliland et al (28) were the first to report that L. acidophilus could act directly on cholesterol. They found that L. acidophilus could remove cholesterol from a growth medium when grown under anaerobic conditions in the presence of bile. They found that supplementing a high cholesterol diet in growing pigs with a strain L. acidophilus capable of assimilating cholesterol in vitro significantly reduced serum cholesterol levels when compared to pigs receiving a diet supplemented with a strain that did not assimilate cholesterol in vitro or to pigs on the same diet without supplementation with lactobacilli.

Recently, Danielson et al (10) confirmed this hypocholesterolemic effect of L acidophilus. Mature boars consumed a high cholesterol diet for fifty six days prior to the experiment. After pretreatment, the boars were divided into two groups. One received acidophilus yogurt and the high cholesterol diet while the other received the high cholesterol diet only. After fifty six additional days the

animals receiving the acidophilus yogurt had significantly ($p < .01$) lower serum cholesterol levels than those in the control group. This research also showed the importance of creating hypercholesterolemic conditions to observe the hypocholesterolemic effect of L. acidophilus because there was considerable variation in serum levels within animals during the experiment.

Absorption of Lipids from the Small Intestine

Lipid absorption from the small intestine requires bile salts (5,9,36,37,51). The micellar form of cholesterol is necessary for mucosal uptake. Most of the cholesterol is absorbed in the proximal two-thirds of the small intestine (19). Gallo-Torres et al (23) and Holt (37) stated that conjugated bile acids better facilitate lipid absorption than deconjugated (free) bile acids. In fact, taurine conjugates better facilitate cholesterol absorption than glycine conjugates and of these taurochenodeoxycholic acid is the best. Although the liver preferentially conjugates with taurine, there is so little in the diet that glycocholate is the predominant bile acid found in man (17) Holt (37) reported that a critical micellar concentration (CMC) of 2mM is required to facilitate lipid absorption from the small intestine. Failure to reach this CMC is probably why conjugated bile acids at low levels fail to promote cholesterol absorption (23).

Deconjugation of Bile Acids by Intestinal Microorganisms

The intestinal microflora of man and animals can transform bile acids and cholesterol into various metabolites. These biotransformations are deconjugation of bile acids,
7 - and 7 ß dehydroxylation of the bile acid nucleus and epimerization of hydroxyl groups of C_3, C_6 and C_7 of the steroid ring. These transformations alter the physical characteristics and physiological function of bile acids (39)

Deconjugation of bile acids is due to the action of certain microorganisms in the intestinal tract (39). The enzyme responsible for this action is bile salt hydrolase (BSH). Members of the genera Bacteroides (8,57,72,75), Bifidobacterium (8,21,54,72), Fusobacterium (14,72), Clostridium (29,54,57,63), Peptostreptococcus (14,44), Streptococcus (44,72) and Lactobacillus (9,11,25,44,49,72) exhibit deconjugation activity.

There is considerable variation in BSH activity among Lactobacillus species. Aries et al (1) and Hill and Drasar (34) did not detect BSH activity in forty eight and twelve, respectively, strains of lactobacilli. Kobashi et al (44) found that L. brevis, L. plantarum would deconjugate glycocholate (GC), L. fermentum would deconjugate GC and taurocholate (TC), L. xylosus would deconjugate TC and that

L. leichmannii and L. acidophilus were inactive on either TC or GC. However only one species of each organism was tested. Gilliland and Speck (25) were the first to report BSH activity by L. acidophilus. Other studies have since shown that L. acidophilus exhibits BSH activity on both TC and GC (9,11). In fact, Dashkevicz and Feigher (11) reported that 71% of all L. acidophilus tested exhibited some BSH activity in vitro.

Tannock et al (77) used conventional (CV), lactobacillus free (LF), and reconstituted lactobacillus free (RLF) mice to study the influence of lactobacilli on BSH activity in the murine intestinal tract. CV mice had a normal flora, LF mice lacked lactobacilli, enterococci, and, filamentous ileal microbes. RLF mice have a flora similar to CV mice but lack lactobacilli. They showed that lactobacilli could be detected along the entire GI tract in CV mice, and in LF and RLF that had been inoculated with selected cultures of lactobacilli but not in LF and RLF mice that were not inoculated with lactobacilli. Populations of lactobacilli down the intestinal tract of CV and RLF mice following inoculation with lactobacilli were not considerably different. Log_{10} counts increased distally along the small intestine in both CV, and RLF mice inoculated with lactobacilli. BSH activity in the ileum was 86% less in RLF mice and 98% less in LF mice than in CV mice. There were no significant ($p < .05$) differences in BSH activity between CV and RLF inoculated with lactobacilli

within the three segments of the small intestine. These results suggests that lactobacilli play the major role in the deconjugation activity in the murine intestinal tract and that this activity is found along the entire intestinal tract with maximum activity in the small intestine occurring in the ileum.

Enterohepatic Circulation of Bile Acids, Fecal Bile Acid Excretion, and Absorption of Bile Acids

Enterohepatic Circulation of Bile Acids

Bile acids are produced in the liver, conjugated with glycine or taurine, concentrated in the gall bladder, and released into the small intestine where they solubilize dietary lipids and thus enhance lipid absorption (76). These bile acids are then reabsorbed from the small intestine and transported back to the liver for reuse. This process is known as enterohepatic circulation (EHC) of bile acids. Norman and Sjovall (65) reported that bile acids are found conjugated in bile but that in the feces they are primarily deconjugated.

Fecal Bile Acid Excretion

Microbial modification of bile acids can influence their EHC (24). This action can result in high levels of bile acids being excreted in the feces (3). Chikai et al

(8) reported that germ free rats inoculated with bacteria able to deconjugate bile acids in vivo excreted more bile acids in their feces than uninoculated germ free animals or germ free animals inoculated with E. coli, which cannot deconjugate bile acids. The excreted bile acids were in the deconjugated form; thus, they concluded that the presence of deconjugating microorganisms increased fecal bile acid excretion and consequently resulted in decreased reabsorption of bile acids. This increased fecal excretion could result in serum cholesterol levels being reduced, because the body would use cholesterol to synthesize needed replacement bile acids (2,76).

Absorption of Bile Acids

Bile acids can be reabsorbed by an active transport or a passive diffusion mechanism (15,16,17,45,50,71). Passive diffusion occurs along the proximal small intestine (duodenum and jejunum) while active transport occurs primarily in the ileum (16,17). Passive diffusion is less efficient than active transport (3). Passive diffusion requires nonionized molecules while active transport prefers ionized molecules. In addition active transport requires bile acids that have negatively charged side chain (16). Schiff using the everted gut sac technique (71) reported that both free (deconjugated) and conjugated bile acids can be absorbed actively from the ileum; however the process is more effective for conjugated bile acids. However Dietschy

(16) reported that the everted gut sac technique does not necessarily indicate the in vivo absorption rates of bile acids.

The pKa of bile acids plays a significant role in determining the type of absorption possible. The pKa of cholic acid (CA) is 5-6 (11,16) compared to 2 and 5 for TC and GC respectively . The pH along the small intestine ranges from 4.5 to 6.0. In this pH range TC and GC would both be ionized and not passively absorbed while CA would be unionized and thus could be passively absorbed in the upper small intestine. All three bile acids are actively absorbed in the ileum because the pH is high enough to ionize CA; however cholic acid is reportedly not as well reabsored via the everted gut sac technique (15,66). Dietschy (16) reported that in vivo there is no difference in absorption between CA and conjugated bile acids. However if free bile acids are less well reabsorbed from the ileum than conjugated bile acids it is possible that higher levels of bile acids may be excreted in the feces.

Role of Bile Acids in Gut Microecology

Bile acids play an important role in establishing gut microecology (70). Deconjugated bile acids are more inhibitory to microorganisms than conjugated bile acids (22). Deoxycholic acid is the most inhibitory of all free bile acids (73) and can inhibit most gram negative organisms. Gram positive organisms such as the lactobacilli

are more sensitive to bile acids than gram negative bacteria of enteric origin. L. bulgaricus which is commonly used in yogurt manufacture cannot survive in the intestinal tract (26). In contrast, L. acidophilus which is a normal inhabitant of the intestinal tract of humans and other mammals can grow and help control the intestinal microflora (62); thus bile resistance (tolerance) may be an important characteristic that enables L. acidophilus to survive and function in the intestinal tract. Gilliland et al (27,28) reported that there was considerable variation in bile resistance among various strains of L. acidophilus; however the required degree of bile resistance is unknown. This characteristic is probably very important in enabling L. acidophilus to survive and exert its hypocholesteremic effect(s) in the small intestine.

Plasmids

Many bacteria contain extrachromosomal, autonomously replicating DNA molecules called plasmids. Most plasmids from procaryotes are covalently closed circular DNA molecules. In general, plasmids are considered nonessential because their presence is not usually required for the survival of the cell. Plasmids range in size from less than one megadalton to greater than 300 megadaltons and can be maintained in the cell as a single copy or in multiple copies.

Importance of Plasmids in Dairy Starter Cultures

In recent years, genetic studies of the lactic acid bacteria have established that numerous metabolic functions are encoded by plasmid DNA. Plasmids have found to to be associated with carbohydrate fermentation, proteolytic activity, bacteriocin production, citrate utilization, restriction and modification systems, phage adsorption, antibiotic resistance, cell aggregation, and other metabolic properties. McKay (56), Davies and Gasson (12) and Sandine (69) have reviewed the genetics of the lactic acid bacteria.

The instability of metabolic properties such as lactose metabolism and proteinase activity in the Lactococcus group has been well established (56). Lactococci are dependent on these functions for growth and acid production. Plasmid association of these functions explains their instability and estblishes the important role of plasmids in strain performance for dairy starter cultures.

Chassy et al. (6) were the first to detect plasmids in the lactobacilli and Klaenhammer and Sutherland (42) were the first to report the presence of plasmids in Lactobacillus acidophilus. A general method for the isolation of plasmid DNA from lactobacilli has been established (43). In the lactobacilli, plasmids have been shown to encode for lactose fermentation (7,35,46), drug resistance (40,59,84,86), glucosamine fermentation (74) and

bacteriocin production and immunity (61). Dashkevicz and Feighner (11) suggested that the variation in BSH activity among lactobacilli may be because this trait is encoded by plasmid DNA.

REFERENCES

1. Aries, V., J.S. Crawther, B.S.Drasar, and M.J. Hill 1969. Degredation of bile salts by human intestinal bacteria. Gut. 10:575.

2. Bloch, K., B.N. Berg, and D. Rittenberg. 1943. The biological conversion of cholesterol to cholic acid. J. Biol. Chem. 149:511.

3. Brandt, L.J. and L.H. Bernstein. 1976. Bile salts: Their role in cholesterol synthesis, secretion and lithogenesis. Am. J. Gastroenterol. 65:1513

4. Carey, M.C. and D.M. Small. 1972. Micelle formation by bile salts. Arch. Intern. Med. 130:506

5. Carey, M.C. 1982. The enterohepatic circulation In Arias, I.M., H. Popper, D.Schachter, and D.A. Shafritz (Eds.), The Liver pp.429-465. Raven Press, New York.

6. Chassy, B.M., E. Gibson, and A. Giuffrida. 1976. Evidence for extrachromosomal elements in lactobacilli. J. Bacteriol. 127:1576-1578.

7. Chassy, B.M., E. Gibson, and A. Giuffrida. 1978. Evidence for plasmid associated lactose metabolism in Lactobacillus casei. Curr. Microbiol. 1:141-144.

8. Chikai, T., H. Nakao, and K, Uchida. 1987. Deconjugation of bile acids by human intestinal bacteria implanted in germ free rats. Lipids. 22:669.

9. Cole, C.B. and R. Fuller. 1984. Bile acid deconjugation and attachment of chicken gut bacteria: Their possible role in growth depression. Brit. Poultry Sci. 25:227.

10. Danielson, A.D., E.R. Peo, K.M. Shahani, A.J.Lewis, P.J. Whalen, and M.A. Amer. 1989. Anticholesteremic property of Lactobacillus acidophilus yogurt fed to mature boars. J. Animal Sci. 67:966--974.

11. Dashkevicz, M.P. and S.D. Feighner. 1989. Development of a diferential medium for bile salt hydrolase-active Lactobacillus spp. Appl. Environ. Microbiol 55:11.

12. Davies, F. L. and M. J. Gasson. 1981. Reviews of the progress of dairy science: genetics of lactic acid bacteria. J. Dairy Res. 48:363-376.

13. Dempsey, M.E. 1974. Regulation of steroid biosynthesis. An. Rev. Biochem. 43:967.

14. Dickenson, A.B., B.E. Gustafasson, and A. Norman. 1972. Determination of bile acid conversion potencies of intestinal bacteria by screening in vitro and subsequent establishment in germ free rats. Acta. Path. Microbiol. Scand. B79:691.

15. Dietschy, J.M., H.S. Salomon, and M.D. Siperstein 1966. Bile acid metabolism. I. Studies on the mechanisms of intestinal transport. J. Clin. Inves 45:832.

16. Dietschy, J.M. 1968. Mechanisms for the intestinal absorption of bile acids. J. Lipid Res. 9 297

17. Dowling, R.H. 1972. Progress In Gastroenterology: The enterohepatic circulation. Gastroenterology 62:122.

18. Dujovne, C.A., P. Krehbiel, S. Decoursey, B. Jackson, S.B. Chernoff, A. Pitterman, and M. Garty. 1984 Probucol with colestipol in the treatment of hypercholesterol-emia. Ann. Itern. Med. 100 477.

19. Dulery, C., and D. Reisser. 1982. Intestinal absorption of biliary and exogenous cholesterol in the rat. Biochem. Biophys. Acta 710:164-171.

20. Eyssen, H. 1973. Role of gut microflora in metabolism of lipids and sterols. Proc. Nutr. Sci. 32:59

21. Ferrari, A., N. Pacini, and E. Canzi. 1980. A note on bile acids transformations by strains of bifidobacterium. J. Appl. Bacteriol. 49:193

22. Floch, M.M., H.J. Binder, B. Filburn, and W. Tershengoren. 1972. The effect of bile acids on intestinal microflora. J. Clin. Nutr. 25:1418

23. Gallo-Torres, M.E., O.N. Miller, and J. G. Hamilton 1969. A comparison of the effects of bile salts on the absorption of cholesterol from the intestine of the rat. Biochimica et Biophysica Acta. 176:605

24. Garbutt, J.T., R.M. Wilkins, L. Lack, and M P. Tyor 1970. Bacterial modification of taurocholate during enterheptic recirculation in normal man and patients

with small intestinal disease. Gastroenterology. 59:553.

25. Gilliland, S.E. and M.L. Speck. 1977. Deconjugation of bile acids by intestinal lactobacilli. Appl. Environ. Microbiol. 33:15.

26. Gilliland, S.E. and M.L. Speck. 1977. Instability of Lactobacillus acidophilus in yogurt. J. Dairy Sci 60:1394.

27. Gilliland, S.E., T.E. Staley, and L.J. Bush. 1984. Importance of bile tolerance of Lactobacillus acidophilus used as a dietary adjunct. J. Dairy Sci. 67:3045.

28. Gilliland, S.E., C.R. Nelson, and C. Maxwell. 1985 Assimilation of Cholesterol by Lactobacillus acidophilus. Appl. and Environ. Microbiol 49 377

29. Gopal-Srivastava, R. and P.B. Hylemon. 1988 Purification and characterization of bile salt hydrolase from Clostridium perfringes. J. Lip. Res 29:1079.

30. Grunewald, K.K. 1982. Serum cholesterol levels in rats fed skim milk fermented by L. acidophilus J. Food Sci. 47:2078.

31. Grundy, S.M. and D.W. Bilheimer. 1984. Low density lipoprotein receptors and enterohepatic circulation In Paumgartner, G., A. Steihl, and W. Gerok (Eds), Enterohepatic Circulation of Bile Acids and Sterol Metabolism. MIT Press LTD, Boston.

32. Harrison, V.C. and G. Peat. 1975. Serum cholesterol and bowel flora in the newborn. Am. J. Clin. Nutr. 28:1351.

33. Hepner, G., R. Fried, S. St.Jeor, L. Fusetti, and R. Morin. 1979. Hypocholesterolemic effect of yogurt and milk. Am. J. Clin. Nutr. 32:19.

34. Hill, M.J. and B.S. Drasar. 1968. Degradation of bile salts by human intestinal bacteria. Gut. 9:22.

35. Hofer, F. 1977. Involvement of plasmids in lactose metabolism in Lactobacillus casei suggested by genetic experiments. FEMS Microbiol. Lett. 1:167-170.

36. Hoffman, A.F. 1977. The enterohepatic circulation of bile acids in man. Clin. Gastroenterol. 6 3.

37. Holt, R. 1972. The roles of bile acids during the process of normal fat and cholesterol absorption. Arch. Intern. Med. 130:574.

38. Howard, A.N. and J. Marks. 1977. Hypocholestrolemic effect of milk. The Lancet. 255.

39. Hylemon, P.B. and T.L. Glass. 1983. Biotransformation of bile acids and cholesteol by the intestinal microflora. pp. 189-213. In D.J. Hentages (Ed.), Human Intestinal Microflora in Health and Disease. Academic Press, Inc., N.Y., New York.

40. Ishiwa, H. and S. Iwata. 1980. Drug resistance plasmids in Lactobacillus fermentum. J. Gen. Microbiol. 26:71-74.

41. Jones, R.J. (ed). 1970. Atherosclerosis - Proceedings of the Second Annual International Symposium. Springer-Verlag. N.Y., New York.

42. Klaenhammer, T. R. and S. M. Sutherland. 1980. Detection of DNA in an isolate of Lactobacillus acidophilus. Appl. Environ. Microbiol. 35:592-600.

43. Klaenhammer, T.R. 1984. A general method for plasmid isolation in lactobacilli. Curr. Microbiol. 10:23-28.

44. Kobashi, K., I. Nishizawa, and T. Yamada. 1978. A new hydrolase specific for taurine-conjugates of bile acids. J. Biochem. 84:495.

45. Lack, L. and I.M. Weiner. 1963. Intestinal absorption of bile salts and some biological implications. Fed. Proc. 22:1334.

46. Lee, L-J., J.B. Hansen, E.K. Jagusztyn-Krynicka, and B.M. Chassy. 1982. Cloning and expression of the ß-D-phosphogalactoside galactohydrolase gene of Lactobacillus casei in Escherichia coli K12. J. Bacteriol. 152:1138-1146.

47. Lipids Research Clinics Program. 1984. The lipid research clinics primary prevention trial results I. Reduction in incidence of coronary heart disease J. Am. Med. Assoc. 251:351.

48. Lipids Research Clinics Program. 1984. The lipid research clinics primary prevention trial results II.The relationship between reduction in incidence of coronary heart disease to cholesterol lowering J. Am. Med. Assoc. 251:365-374.

49. Lundeen, S.G. and D.C. Savage. 1989. Purification of the Bile Salt Hydrolase from Lactobacillus sp strain 100-100. Abstract of the annual meeting of the American Society for Microbiology.

50. MacDonald, I.A., V.D. Bokkenheuser, J. Winter, A.M. McLernon, and E.H.Musbach. 1983. Degradation of steroids in the human gut. J. Lipid Res. 24:675.

51. Mallory, A., F. Kern, J. Smith, and D. Savage. 1973. Patterns of bile acids and microflora in the human small intestine. Gastroenterology. 64:26.

52. Mann, C.V. and A. Spoerry. 1974. Studies of surfactant and cholesterol in the Maasai. Am. J. Clin. Nutr. 27:464.

53. Mann, C.V. 1977. A factor in yogurt which lowers cholesterol in man. Atherosclerosos. 26:335

54. Masuda, N. 1981. Deconjugation of bile salts by bacteroides and clostridium. Microbiol. Immunol. 25:1.

55. McIntyre, N. and K.J. Isselbacher. 1973. Role of the small intestine in cholesterol metabolism. Amer J. Clin. Nutr. 26:647.

56. McKay, L.L. 1983. Functional properties of plasmids in lactic streptococci. Antonie van Leeuwenhoek 49:259-274.

57. Midvedt, T. and A. Norman. 1967. Bile acid transformations by microbial strains belonging to the genera found in intestinal contents. Acta. Pathol. Microbiol. Scand. 71:1341.

58. Miller, N.E. 1979. Plasma lipoproteins, lipid transport, and atherosclerosis: Recent developments. J. Clin. Path. 42:639.

59. Morelli, L., M. Vescovo, and V. Bottazzi. 1983. Plasmids and antibiotic resistance in Lactobacillus helveticus and Lactobacillus bulgaricus isolated from natural whey culture. Microbiologia. 6:145-154.

60. Mott, G.E., R.W. Moore, H. Redmond, and R. Reiser. 1973. Lowering of serum cholesterol by intestinal bacteria in cholesterol-fed piglets. Lipids. 8:428.

61 Muriana, P.M. and T.R. Klaenhammer. 1987. Conjugal transfer of plasmid- encoded determinants for

bacteriocin production and immunity in Lactobacillus acidophilus 88. Appl. Environ Microbiol. 53:553-560.

62. Murotomi, M., T. Watanabe, N. Suegara, Y. Kawai, and M. Mutai. 1975. Distribution of indigenous bacteria in the digestive tract of conventional and gnotobiotic rats. Infect. Immun. 11:962.

63. Nair, P.P., M. Gordon, and J. Reback. 1967. The enzymatic cleavage of the carbon-nitrogen bond in 3-, 7-, 12- Trihydroxy-5-ß-cholan-24-oylglycine. J. Biol. Chem. 242:7.

64. National Heart and Lung Institute Task Force on Atherosclerosis. 1971. Vol 2. DHEW Publication no. (NIH) 72:219.

65. Norman, A. and J. Sjovall. 1953. On the transformation and enterohepatic circulation of cholic acid in the rat. J. Physiol. 233:872.

66. Playhoust, M.R. and K.J. Isselbacher. 1964. Studies on the transport and metabolism of conjugated bile salts by intestinal mucosa.

67. Rao, D.R., B. Chawan, and S.R. Pulusani. 1981. Influence of milk and Thermophilus milk on plasma cholesteol levels and hepatic cholesterogenesis in rats. J. Food. Sci. 46:1339.

68. Rudel, L.L., J.S. Parks, F.L. Johnson, and J. Babiak 1986. Low density lipoproteins in atherosclerosis J. Lipid. Res. 27:465.

69. Sandine, W.E. 1987. Looking backward and forward at the practical applications of genetic researches on the lactic acid bacteria. FEMS Microbiol. Rev. 46:205-220.

70. Sakai, K., T. Makino, Y. Kawai, and M. Mutai. 1980. Intestinal microflora and bile acids. Effect of Bile Acids on the Distribution of Microflora and Bile Acid in the Digestive Tract of the Rat. Microbiol. Immunol. 24:187.

71. Schiff, E.R., N.C. Small, and J.H. Dietschy. 1972. Characterization of the kinetics of the passive and active transport mechanisms for bile acid absorption in the small intestine and colon of the rat. J. Clin. Inves. 51:1351.

72. Shimuda, K., K.S. Bricknell, and S.M. Finegold. 1969 Deconjugation of bile acids by intestinal bacteria

Review of literature and additional studies. J. Infect. Dis. 119:273.

73. Shimada, K. V.L. Sutter, and S.M. Finegold. 1970. Effect of bile and deoxycholate on Gram negative anaerobic bacteria. Appl. Microbiol. 20:737.

74. Smiley, M.B. and V. Fryder. 1978. Plasmids, lactic acid production and N-acetyl-D-glucosamine fermentation in Lactobacillus helveticus subsp. jugurti. Appl. Environ. Microbiol. 35:777-781.

75. Stellwag, E.J. and P.B. Hylemon. 1976. Purification and characterization of bile salt hydrolase from Bacteroides fragilis subsp. fragilis. Biochim Biophys Acta. 452:165.

76. Stryer, L. "Biosynthesis of Macromolecular Precursors". Biochemistry 2nd ed. Wilt Freeman and Co. San Francisco.

77. Tannock, G.W., M.P. Dashkevicz, and S.D. Feighner 1989. Lactobacilli and Bile Salt Hydrolase in the Murine Intestinal Tract. Appl. Environ. Microbiol 55:1848.

78. Thakur, C.P. and A.N. Jha. 1981. Influence of milk yogurt and calcium on cholesterol and induced atheroslcerosis in rabbits. Atherosclerosis. 89:211.

79. Thompson, L.U., D. Jenkins, M.A. VicAmer, R. Reichert, A. Jenkins, and J. Kamulsky. 1982. The effect of fermented and unfermented milks on serum cholesterol.

80. Totuero, F., A. Brenes, and J. Rioperez. 1975. Influence of intestinal (cecal) flora on serum and egg yolk cholesterol levels in laying hens. Poultry Sci. 54:1935.

81. Troll, W. and R.K. Cannon. 1952. A modified photo metric ninhydrin method for the analysis of amino and imino acids. J. Biol. Chem. 200:803.

82. United States Bureaof the Census. 1986. Statistical Abstracts of the U.S.

83. Van Belle, H. 1965. Cholesterol, Bile acids, and Atherosclerosis. North Hollan Publishing Company. Amsterdam.

84. Vescovo, M., L. Morelli, and V. Bottazzı. 1981. Evidence of plasmid DNA in Lactobacillus. Microbiologia 4:413-419.

85. Weiner, I.M. and L. Lack. 1968. Bile salt absorption· enterohepatic circulation . In Code, D.F. (Ed.), Handbook of Physiology: Ailimentary Canal. p. 1439. Washington, D.C.: American Physiology Society.

86. West, C.A. and P.J. Warner. 1985. Plasmid profiles and transfer of plasmid-encoded antibiotic resistance in Lactobacillus plantarum. Appl. Environ. Microbiol. 50:1319-1321.

CHAPTER III

RELATIONSHIPS AMONG BILE TOLERANCE, BILE SALT HYDROLASE ACTIVITY, AND ASSIMILATION OF CHOLESTEROL BY *LACTOBACILLUS ACIDOPHILUS*

Derek K. Walker and Stanley E. Gilliland

Department of Animal Science, Oklahoma State University, Stillwater, Oklahoma 74078

ABSTRACT

The relationships among growth in the presence of bile, bile salt hydrolase activity on sodium taurocholoate (NaTC), and assimilation of cholesterol by Lactobacillus acidophilus were examined. Cultures of L. acidophilus were grown at 37°C in broth containing 0.2% sodium thioglycollate, 004 M NaTc and .0012 M cholesterol (cholesterol phosphatidyl choline micelles). Maximum deconjugation activity was achieved in the late exponential phase of growth. This also coincided with maximum cholesterol assimilation. Nineteen cultures of L. acidophilus were compared for bile tolerance, deconjugation activity, and the ability to assimilate cholesterol. There was significant variation among cultures in their ability to grow in the presence of bile, deconjugate NaTC, and assimilate cholesterol. However, statistical analyses revealed that there were no significant correlations among the three.

INTRODUCTION

Heart disease is a major cause of death in the United States (22). Lowering total plasma cholesterol levels can reduce the incidence of heart disease in individuals suffering from primary hypercholesterolemia (17). The association between hypercholesterolemia and heart disease has led to considerable research geared towards lowering plasma cholesterol levels.

Consumption of some cultured or culture containing dairy products supplemented with Lactobacillus acidophilus can result in a reduction of the level of serum cholesterol (4,9,11,12,13,21). The presence of bile and anaerobic conditions during growth are two factors that enable L. acidophilus to assimilate cholesterol (9). Such action in the small intestine may be important in reducing the absorption of cholesterol from the digestive system

Deconjugation of bile acids occurs during enterohaptic circulation in healthy humans (23). The primary bile acids found in man are cholic and chenodeoxycholic acids conjugated with glycine or taurine. Several genera of bacteria indigenous to the intestinal tract can deconjugate bile acids (16). The enzyme responsible for this activity is bile salt hydrolase (BSH). Aries et al (1) reported that lactobacilli do not deconjugate bile acids. However, Floch

et al (6) reported that an unidentified species of Lactobacillus of human origin deconjugated glycocholic, glycodeoxycholic, and glycochenodeoxycholic acids. Gilliland and Speck (7) showed that L. acidophilus could deconjugate both taurocholic and glycocholic acids under anaerobic conditions.

Deconjugation of bile acids in the small intestine may be important in controlling serum cholesterol levels because deconjugated bile acids function poorer in the solubilization and absorption of lipids than do conjugated ones (5). Also Chikai et al.(3) reported that fecal bile acid excretion was significantly higher in rats inoculated with intestinal microorganisms than in gnotobiotic rats. Most of these bile acids were in the deconjugated form. They suggested that free bile acids adhere to bacteria or dietary fiber ,thus resulting in enhanced excretion of bile acids. This action may trigger the feed-back mechanism that regulates hepatic cholesterol synthesis and subsequent transformation into bile acids which may reduce serum cholesterol levels.

Bile tolerance is considered to be an important characteristic of L. acidophilus enabling it to survive, grow and exert its action in the small intestine. Gilliland et al. (8) compared a strain L. acidophilus having relatively low bile resistance and one having higher bile resistance. The one having the higher bile resistance was able to increase the number of facultative lactobacilli in

the upper part of the small intestines of dairy calves significantly better than did the strain having low bile tolerance. Using plueropnuemonia like organism (PPLO) serum as a source of cholesterol, Gilliland et al. (9) reported that L. acidophilus could remove cholesterol from a growth medium when grown anaerobically and in the presence of bile (oxgall). As the concentration of bile increased from 0 to 0.5%, the amount of cholesterol removed from the growth medium also increased. The PPLO serum and oxgall can be replaced with cholesterol phophatidyl cholıne micelles and purified bile acids respectively and still permıt L. acidophilus to assimilate cholesterol during growth (10) Use of these two constituents allowed for better manipulation of the growth medium to assess the factors that enable L. acidphilus to assimilate cholesterol.

The purpose of this study was to determine the relationship among bile tolerance, deconjugatıon, and assimilation of cholesterol by L. acidophilus.

MATERIALS AND METHODS

Source and Maintenance of Cultures

The nineteen cultures of L. acıdophılus used in thıs study were from our laboratory stock culture collectıon. All strains were maintained by subculturıng in lactobacılli MRS broth (Difo Laboratories, Detroit,MI) using a 1% ınocula and 18 to 20 h of incubation at 37°C. The cultures were

stored at 1- 2°C between transfers. Each culture was subcultured at least two times in MRS broth prior to experimental use.

Comparison of Cultures for Bile Tolerance

Cultures of L. acidophilus were compared for their ability to grow in the presence of bile by inoculating them individually (1%) into sterile MRS-THIO (lactobacilli MRS broth supplemented with 0.2% sodium thioglycollate) broth with and without 0.3% oxgall. They were incubated in a 37°C waterbath. The A_{620nm} was measured initially and after 3 hr incubation. Increases in absorbance during the 3 h incubation were used to compare growth of the cultures.

Determination of Incubation Time Required for Maximum Bile Salt Hydrolase Activity

Thirty ml volumes of MRS THIO broth containing .001M sodium taurocholate (NaTC) were inoculated (1%) with L. acidophilus ATCC 43121, ATCC 4356, or NCFM-L and incubated at 37°C. One tube of each culture was removed at 3 h intervals through 18 h incubation. A 1:10 dilution was made from each using sterile peptone diluent (1%) and the A_{620nm} was measured to determine the relative amounts of growth. A modification of the method of Irvin et al (18) was used to measure the amount of free cholic acid liberated by each culture. Twenty ml volumes of each culture were adjusted to pH 7.0 with 1 N NaOH, adjusted to 25 ml with distilled water

and the cells removed by centrifugation for 10 min at 12,000 x g and 1°C. Fifteen ml of the resulting supernatant fluid was adjusted to pH 1.0 using 10 N HCl and brought up to 24 ml with distilled water. Three ml portions of each sample were transferred to glass stoppered test tubes to which were added 9 ml volumes of ethyl acetate. The contents of each tube were vortexed and the phases allowed to separate. Three ml of the ethyl acetate layer from each tube was transferred to a clean test tube and evaporated to dryness at 60°C under a flow of nitrogen gas. One ml of 0.01 N NaOH was added to each tube to dissolve the residue Six ml of 16 N H_2SO_4 was then added to each followed by the addition of 1 ml of 1% furfuraldehyde. The tubes were mixed, heated for 13 min.in a 65°C water bath and cooled to room temperature. Five ml of glacial acetic acid was added, the tube contents mixed , and the A_{660nm} was read against a reagent blank. The A_{660nm} was compared with a standard curve to determine the concentration of free cholic acid. Results were expressed as micromoles of cholic acid per milliliter.

Screening of Cultures for Simultaneous Bile Salt Hydrolase Activity and Cholesterol Assimilation

Freshly prepared MRS broth cultures of L. acidophilus were inoculated (1%) individually into tubes containing 20 ml of MRS-THIO broth supplemented with 0.004 M NaTC and

0.0012 M cholesterol (cholesterol phosphatidyl choline micelles). The cholesterol micelles were prepared according to the method of Razin et al (18). Cultures were incubated at 37°C for 10 h. Cells were removed from 10 ml of the broth culture by centrifugation for 10 min at 12,000 x g and 1°C. The spent broth was analyzed for cholesterol content (19). Relative amounts of deconjugation activity were determined by making appropriate dilutions of the remaining broth cultures and analyzing for free cholic acid as described in the previous section.

Statistical Analyses

Bile tolerance, BSH activity, and cholesterol assimilation data were analyzed using the general linear models (GLM) procedure from SAS (20). The GLM least significant difference mean procedure was used to determine if statistically significance differences occurred among means. The GLM correlation procedure was used to determine if significant relationship(s) occurred among the three traits.

RESULTS

Comparison of Cultures for Bile Tolerance

Considerable variation in the ability to grow in the presence of bile occurred among strains (Table 1). Strains ATCC 43121 and 251 grew significantly ($P < .0001$) better

than the other strains. Cultures could be subjectively categorized based on bile tolerance. Strains ATCC 43121, 251, HM_2, 14, 12, P16, GP4A, GP1B, RP43 and RP42 grew well in the presence of bile. Strains RP34, 15, 5, NCFM-F, NCFM-L, 14F1, and 2 grew moderately well in the presence of bile Strains ATCC 4356 and DKW-9 were very sensitive to bile exhibiting significantly ($p < .0001$) less growth than all other strains.

Determination of Incubation Time Required for Maximum Bile Salt Hydrolase Activity

Most rapid deconjugation by strains ATCC 4356, ATCC 43121, and NCFM-L was attained from 3 to 6 h incubation at 37°C which coincided with most rapid growth for each strain (Fig. 1). Strain ATCC 4356 exhibited approximately twice as much BSH activity at 3 h and reached maximum amount of BSH activity sooner than did strains ATCC 43121 and NCFM-L. However, ATCC 4356 did not exhibit as rapid growth as did ATCC 43121 (Fig.1). Maximum amount of BSH activity had been reached at approximately 10 h incubation for all three cultures. This corresponded to the late stationary phase of growth in each case (Fig 1). Although ATCC 43121 grew faster and reached higher cell density than the other two strains, there was little difference among the three with regard to the final amount of free cholic acid liberated.

Simultaneous Screening of Cultures for Bile Salt Hydrolase Activity and Assimilation of Cholesterol

Results from screening cultures for simultaneous BSH and assimilation of cholesterol are shown in Table 2. There were significant ($P < .005$) variations among strains in the ability to deconjugate NaTC. Strains ATCC 43121, DKW-9, 251, RP42, GP4A, P16, GP1B, RP43, NCFM-L, ATCC 4356, and RP34 liberated significantly ($P < .005$) more free cholic acid than the other strains. Cultures HM_2, NCFM-F, 14, 15, 2, 12, and 5 deconjugated significantly ($p< .005$) more NaTC than strain 14F1 which exhibited little activity.

There were also a significant ($P < .001$) variations among strains in the ability to assimilate cholesterol. The amount of cholesterol taken up ranged from 0 ug/ml for strains ATCC 4356 and 14F1 to 50 ug/ml for strain ATCC 43121. Strains ATCC 43121 , RP34, 251, and RP42 took up significantly ($P < .005$) more cholesterol than any of the other strains. While strain RP43 took up significantly less cholesterol than the afore mentioned strains, it took up significantly ($p<.005$) more than did strains 2, 5, 12, 15, 14, 14F1, P16, HM_2, DKW-9, ATCC 4356, NCFM-F, NCFM-L, GP4A and GP1B. Interestingly, strains ATCC 4356, DKW-9, NCFM-L, P16, and GP1B which did not take up as much cholesterol as the other cultures were among the most active in deconjugating NaTC.

Statistical analyses revealed that there was no significant correlation between bile tolerance and cholesterol assimilation (r = 0.51), bile tolerance and BSH activity (r = 0.27) and cholesterol assimilation and BSH activity (r = 0.55).

DISCUSSION AND CONCLUSIONS

All strains of L. acidophilus tested exhibited some degree of bile tolerance. Interestingly, strains DKW-9 and ATCC 4356, which were the least bile resistant showed considerable bile salt hydrolase activity. Perhaps some of the difference in bile resistance is due to the natural difference in growth of the individual strains. Gilliland et al (7) reported that strains of L. acidophilus that are less bile resistant tended to grow slower in the broth without bile. If this is the case, then strains that tend to have a longer lag time coupled with expression of very active BSH would be more subject to inhibition due to the release of more free cholic acid prior to initiation of growth. Floch et al.(6) reported that deconjugated bile acids are more inhibitory to microorganisms than conjugated ones. They suggested that this inhibitory effect may play a role in inhibiting microbial growth in the intestinal tract L. acidophilus ATCC 4356, which was the least bile resistant strain tested, deconjugated approximately twice as much NaTC as did strains ATCC 43121 or NCFM-L during 3 h incubation. This higher level of BSH activity may explain

why this strain is less bile resistant than the other two strains.

The cessation of BSH activity following 18 h incubation probably corresponds to the cultures entering the stationary phase of growth. The low pH resulting from acid produced by the culture likely inhibited enzyme activity. The optimum pH for activity of BSH of the lactobacilli has been reported to be approximately 6.0 (2, 7, 14).

Some species of lactobacilli can deconjugate both NaTC and sodium glycocholate(NaGC) (7). Aries et al (1) and Hill and Drasar (14) reported that lactobacilli were incapable of deconjugation of bile acids. The difference in findings by these researchers may have been due to differences in growth conditions, types of conjugated bile acids tested, and assay conditions. Aries et al (1) grew lactobacilli under anaerobic conditions in reinforced clostridium medium containing 0.1% of various bile acids. This concentration of bile acids may have been insufficient to detect BSH activity in lactobacilli via the thin layer chromatography method used in their study. Gilliland and Speck (7) found that deconjugation of bile acids by L. acidophilus required a low oxidation- reduction (O-R) potential and that these organisms exhibited more BSH activity when grown in MRS broth supplemented with sodium thioglycollate than MRS broth incubated in an anaerobic Gas Pak system. They concluded that the time required for the

O-R potential to equilibrate in the latter was responsible for the lower amount of BSH activity.

Comparison of cultures of L. acidophilus for BSH activity after 10 h incubation assured that the cultures had grown sufficiently and expressed considerable activity. Gilliland and Speck (8) found the deconjugating system of L. acidophilus was constitutively expressed. The ability of strain ATCC 4356 to liberate twice the amount of free cholic acid after only 3 h incubation when compared to ATCC 43121 or NCFM-L suggests that the deconjugating system is active early during growth. The inocula had not been grown in a medium containing bile salts. This observation supports the constitutive nature of the enzyme.

Measurements of cholesterol assimilation with simultaneous measurements of deconjugation revealed variation among cultures. In general, most strains that assimilated cholesterol expressed BSH activity and bile resistance. However, there was no significant correlation among the three traits.

REFERENCES

1. Aries, V. J. S., S. Crowther, B. S. Drasar, and M. J. Hill. 1969. Degradation of bile salts by human intestinal bacteria Gut 10:575-576.

2. Aries, V., and M. J. Hill. 1970. Degradation of steroids by intestinal bacteria.I. Deconjugation of bile salts. Biochim. Biophys. Acta 202:526-534.

3. Chikai, T. , H. Naka, and K. Ushida. 1987. Deconjugation of bile acids by human intestinal bacteria implanted in germ free rats. Lipids 22: 669-671.

4. Danielson, A. B., E. R. Peo, K. M. Shahani, A. J. Lewis, P. J. Whalen and M. A. Amer. 1989 Anticholesteremic property of Lactobacillus acidophilus yogurt fed to mature boars. J. Anim. Sci. 67:966-974.

5. Eyssen, H. 1973. Role of gut microflora in metabolism of lipids and sterols. Proc. Nutr. Soc. 32:59.

6. Floch, M. M., H. J. Binder, B. Filburn, and W. Tershengoren. 1972. The effect of bile acids on intestinal microflora. J. Clin. Nutr. 25:1418-1426

7. Gilliland, S. E., and M. L. Speck. 1977. Deconjugation of bile acids by intestinal lactobacilli. Appl. Environ. Microbiol. 33:15-18.

8. Gilliland, S. E., T. E. Staley, and L. J. Bush. 1984 Importance of bile tolerance of Lactobacillus acidophilus used as a dietary adjunct. J. Dairy Sci. 67:3045-3051.

9. Gilliland, S. E., C. R. Nelson, and C. Maxwell. 1985. Assimilation of Cholesterol by Lactobacillus acidophilus. Appl. Environ. Microbiol. 49:377-381.

10. Gilliland, S. E., J. W. Nielsen, and H. A. Wyckoff. 1986. Comparison of alternate sources of cholesterol and purity of bile salts on cholesterol uptake by Lactobacillus acidophilus. J. Dairy Sci (Abstract).

11. Grunewald, K. K. 1982. Serum cholesterol levels in rats fed skim milk fermented by L. acidophilus. J. Food Sci. 47:2078-2079.

12. Harrison, V. C. and G. Peat. 1975. Serum cholesterol and bowel flora in the newborn. Am. J. Clin. Nutr. 28:1351-1355.

13. Hepner, G., R. Fried, S. St.Jeor, L. Fusetti, and R. Morin. 1979. Hypercholesterolemic effect of yogurt and milk. Am. J. Clın. Nutr. 32:19-24.

14. Hill, M. J., and B. S. Drasar. 1968. Degradation of bile salts by human intestinal bacteria gut. 9:22-27.

15. Irvin, J. L., C. G. Johnson, and J. Kopalo. 1944. A photometric method of the determination of cholates in bile and blood. J. Biol. Chem. 153:439-457.

16. Lewis, R. and S. Gorbach. 1972. Modication of bile acids by intestinal bacteria. Arch. Intern. Med. 130:545-549.

17. Lipid Research Clinics Program. 1984. The lipid research clinics primary prevention trial results. I. Reduction in incidence of coronary heart disease. J. Am. Med. Assoc. 251:351-363.

18. Razin, S., S. Kutner, H. Efrati, and S. Rottem. 1980. Phospholipid and cholesterol uptake by mycoplasma cells and membranes. Biochim. Biophys. Acta. 598:628-640.

19. Rudel, L. L., and M. D. Morris. 1973. Determınatıon of cholesterol using 0-phthalaldehyde. J. Lipid Res 14: 364-366.

20. SAS Institute Inc. 1987. SAS Guide for personal computers, Version 6. SAS Instıtute Inc. Cary, NY.

21. Totuero, F., A. Brenes, and J. Rioperez. 1975. Influence of intestinal (cecal) flora on serum and egg yolk cholesterol levels in laying hens. Poultry Sci. 54:1935-1938.

22. United States Bureau of the Census. 1983. Statistical abstract of the United States: 1984. 104th ed. Washington, D. C.

23. Van Belle, H. 1965. Cholesterol, Bile acids, and Atherosclerosis. North Holland Publishing Co. Amsterdam.

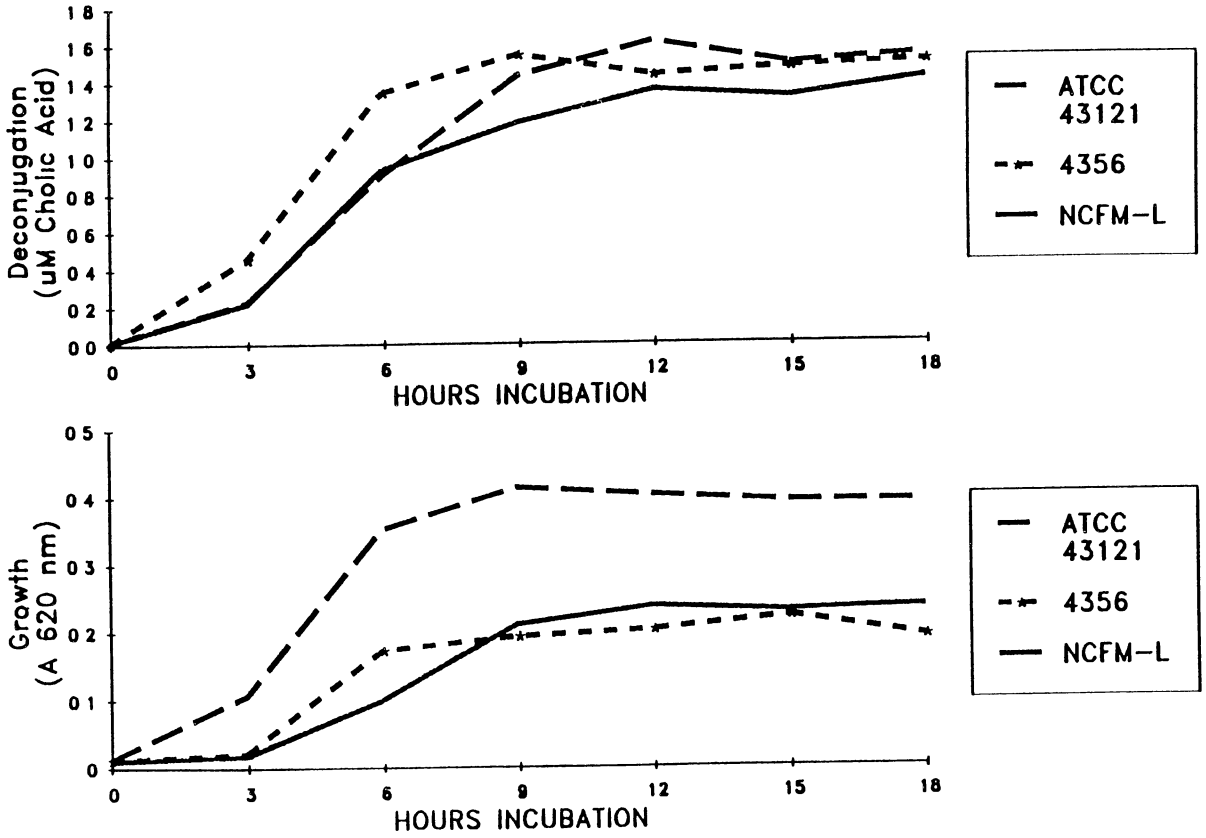


Figure 1. Comparison of growth and bile salt hydrolase activity on sodium taurocholate by Lactobacillus acidophilus.

Table 1. COMPARISON OF BILE TOLERANCE OF CULTURES OF *LACTOBACILLUS ACIDOPHILUS*[1]

Culture	Change in A_{620nm} @ 3 hr incubation
251	0.494^{a}
ATCC 43121	0.443^{a}
RP43	0.339^{b}
GP1B	0.314^{b}
HM_2	0.284^{bc}
P16	0.269^{bc}
14	0.257^{bc}
RP42	0.209^{c}
12	0.205^{c}
GP4A	0.202^{c}
NCFM-F	0.181^{c}
5	0.174^{c}
15	0.166^{c}
2	0.163^{c}
NCFM-L	0.160^{c}
14F1	0.131^{c}
RP34	0.130^{c}
ATCC 4356	0.072^{cd}
DKW-9	0.028^{d}

[1]Based on growth in MRS-THIO broth containing 0.3% oxgall Each value is an average of 2 trials; Means with different superscript letters differ significantly ($p < .0001$)

TABLE 2. COMPARISON OF CHOLESTEROL ASSIMILATION AND BILE SALT HYDROLASE ACTIVITY BY CULTURES OF *LACTOBACILLUS ACIDOPHILUS*[1]

Culture	ug Cholesterol removed	Cholic Acid (uM/ml)
ATCC 43121	50.00[a]	4.30[a]
DKW-9	11.67[c]	4.30[a]
251	47.33[a]	4.15[a]
RP42	48.00[a]	4.06[a]
GP4A	10.67[c]	3.93[a]
P16	5.33[c]	3.89[a]
GP1B	8.00[c]	3.86[a]
RP43	39.33[b]	3.77[a]
NCFM-L	6.67[c]	3.69[a]
ATCC 4356	0.00[c]	3.59[a]
RP34	48.67[a]	3.38[a]
HM_2	4.00[c]	2.97[b]
NCFM-F	5.33[c]	2.85[b]
14	9.33[c]	2.19[b]
15	14.67[c]	2.17[b]
2	3.33[c]	1.78[b]
12	9.33[c]	1.60[b]
5	7.33[c]	1.40[b]
14F1	0.00[c]	0.17[c]

[1]Each value is an average from 3 trials; Means in the same column with different superscript letters differ significantly ($p < .005$). MRS-THIO broth contained .004M sodium taurocholate and .0012M cholesterol.

CHAPTER IV

DETERMINATION OF THE RELATIONSHIP BETWEEN PLASMID DNA AND BILE SALT HYDROLASE ACTIVITY OF *LACTOBACILLUS ACIDOPHILUS*

Derek K. Walker, Thomas G. Rehberger and
Stanley E. Gilliland

Department of Animal Science, Oklahoma State University,

Stillwater, Oklahoma 74078

ABSTRACT

A simple method using MRS broth containing 0.5% individual conjugated bile salts revealed that twenty strains of L. acidophilus (of human and porcine origin) deconjugated both taurine derivatives and showed variable activity on glycine derivatives. To determine if plasmids were involved in the bile salt hydrolase activity of these strains, plasmids were isolated using a simple and rapid method. Extraction of plasmid DNA and agarose gel electrophoresis revealed that eleven of twenty strains contained plasmid DNA which ranged from 2.3 to greater than 33 Mdal. Strains contained from one to as many as four plasmids. Several strains were found to contain plasmids of the same molecular weight. Strains ATCC 43121 and 251 contained a 2.3 and 8.0 Mdal plasmid while strains GP1B and GP4A contained a 3.5 Mdal plasmid. These results suggest that there is not a common plasmid among these strains of L acidophilus responsible for bile salt hydrolase activity

INTRODUCTION

Bile salts undergo extensive metabolism in the intestines by gut microflora (11). The most common transformation is the hydrolysis of the peptide bond of taurine or glycine conjugates of bile acids (11,21) by bile salt hydrolase (BSH) (Figure 2). An agar medium permitting detection of bile salt hydrolase positive and negative bacteria has been developed (8).

Several genera of bacteria indigenous to the intestinal tract exhibit BSH activity (1,5,8,11,21). Lactobacilli are the predominant organisms that produce BSH in the proximal murine gastrointestinal tract (22). Several species of lactobacilli could deconjugate bile salts (8,10). Gilliland and Speck (10) reported that L. acidophilus preferentially hydrolyzes taurocholic acid over glycocholic acid. Dashkevicz and Feighner (8) also showed that only one of eighteen strains of L. acidophilus could not deconjugate taurocholic or taurodeoxycholic acid and that eighteen of forty six Lactobacillus species tested did not exhibit BSH activity. They speculated that this variation in BSH activity may be because the trait is encoded by plasmid DNA.

Genetic studies of lactic acid bacteria have established that many functions are encoded by plasmid DNA (19). Carbohydrate metabolism, proteolytic activity,

bacteriocin production, citrate utilization restriction modification systems, phage adsorption, antibiotic resistance and cell aggregation have been shown to be associated with plasmid DNA. Association of these functions with plasmids shows the important role plasmids play in the strain performance of dairy starter culture bacteria.

The purpose of this study was to determine if there was a common plasmid among L. acidophilus encoding bile salt hydrolase activity.

MATERIALS AND METHODS

Organisms and Growth Conditions

The twenty cultures of Lactobacillus acidophilus used in this study were from our laboratory stock culture collection. All strains were maintained by subculturing in MRS broth (Difco Laboratories, Detroit, MI) using 1% inocula and 18 to 20 h of incubation at 37°C. The cultures were stored at 1 to 2°C between transfers. Each culture was subcultured at least two times in MRS broth prior to experimental use.

Preparation of Agar containing Bile Salts and Detection of Bile Salt Hydrolase Activity

Tests plates were prepared by adding 0.5% (w/v) of sodium taurocholate (NaTC), sodium taurodeoxycholate (NaTDC), sodium glycocholate (NaGC) and sodium glyco-

deoxycholate (NaGDC) and 1.7% agar to reconstituted MRS broth as described by Dashkevicz and Feighner (8), except plates were allowed to solidify aerobically.

For the detection of BSH activity, 10 ul of each broth culture was inoculated on to the surface of MRS agar containing the desired bile salt. The plates were incubated (upright) anaerobically in a GasPak System for 18h at 37°C. After incubation, the plates were removed and observed for zones of white precipitate surrounding the colonies of lactobacilli or opaque white colonies.

Preparation of Cells for Plasmid DNA Isolation

Lactobacillus strains grown for 20 h at 37°C in MRS broth were used as the inocula(1%) for cultures from which plasmids were to be extracted. Cultures for plasmid isolation were incubated at 37°C until they reached late log phase. Cells were harvested by centrifugation and washed once with one tenth volume of 15% sucrose in 50 mM Tris-HCl, 10 mM EDTA, pH 8.0, and transferred to a 1.5 ml microcentrifuge tube. Cells were used immediately or stored at - 20°C for later use.

Rapid microscale screening procedure

The washed cell pellet from 10 ml of culture was resuspended to a final volume of 250 ul with a buffer that contained 15% sucrose in 50 mM Tris-HCl, 10 mM EDTA, pH 8.0

Lysozyme (30 mg/ml in 50 mM Tris-HCl, 10 mM Na_2EDTA, pH 8.0) was added to a final concentration of 3 mg/ml. The sample was thoroughly mixed with a vortexer and incubated on ice for 1 h. Pronase E (10 mg/ml in 50 mM Tris-HCl, 5 mM Na_2EDTA, 50 mM NaCl, pH 8.0) which had been preincubated for 1 h at 37°C, was added to a final concentration of 1 mg/ml. The mixture was gently agitated by inverting the tube several times, and incubated for 30 min at 37°C. Then, 111 ul of 0.25 M Na_2EDTA at pH 8.0 were added and mixed by gentle inversion. The tube was incubated for 10 min at room temperature (RT) and returned to ice for and additional 10 min. Next, 111 ul of 20% SDS was added; the tubes were mixed and incubated on ice for 10 min. Then, 65 ul of 3 M sodium acetate was added. After thorough mixing the tube was incubated on ice for 30 min. Cell debris was pelleted by centrifugation at 14,000 x g for 15 min. The supernatant was transferred to a clean 1.5 ml microcentrifuge tube to which 1 ml of cold (-20°C) 95% ethanol was added followed by several inverions to mix. The sample was incubated at -20°C for 1 h, after which the precipitated plasmid DNA was collected by centrifugation at 14,000 x g for 15 min. The resulting crude DNA pellet was thoroughly dried, dissolved in 50 ul of 10 mM Tris-HCl, 1 mM Na_2EDTA, pH 7.5, and analyzed by agarose gel electrophoresis.

Preparative Plasmid DNA Isolation

The washed cell pellet from a 750 ml culture was resuspended in approximately 25 ml of 15% sucrose in 50 mM Tris-HCL, 10 mM Na_2EDTA, pH 8.0. The optical density (OD) at 600 nm of the cell suspension was adjusted to 20 by adding additional buffer as needed. Cells from 50 ml of this suspension were harvested by centrifugation at 10,000 x g for 10 min. at 1°C and resuspended to a final volume of 25 ml. Cell lysis was achieved as previously described; the volumes of all regents were increased 10-fold. Following the addition of 20% SDS and incubation at room temperature and in ice for 10 min each, lysates were purified by the alkaline denaturation method of Currier and Nester (7). A 3 M solution of NaOH was added until a pH of 12.25 was reached. Next 10 ml of 2 M Tris-HCl, pH 7.0 was added followed by the addition of 7.5 ml of 5 M NaCl. Fifty ml of phenol saturated with 3% NaCl was added followed by vigorous shaking; the sample was held at room temperature for 5 min Twenty ml of chloroform was then added. Next, phases were separated by centrifugation at 10,000 x g for 10 min at 1°C. The aqueous phase was removed and transferred to a clean centrifuge bottle and extracted with 80 ml of chloroform: isoamyl alcohol (24:1) and held for 5 min at RT; the phases were separated by centrifugation again as described above. The aqueous phase containing plasmid DNA was removed and transferred to a clean centrifuge bottle to which two

volumes of cold (-20°C) 95% ethanol were added to precipitate plasmid DNA overnight at -20°C. The plasmid DNA was collected by centrifugation at 10,000 x g for 15 min. The DNA pellet was dried, dissolved in 10 mM Tris-HCl, 1 mM Na_2EDTA, pH 7.5, and further purified by cesium chloride ethidium bromide density gradient centrifugation.

Agarose Gel Electrophoresis

Plasmid DNA samples were subjected to electrophoresis in horizontal gels of 0.7% agarose dissolved in Tris-borate buffer at pH 8.1. Electrophoresis was carried out at 55 - 70 volts for 20 - 24 h. Size determinations were based on mobility reference plasmids isolated from E. coli V517. by the method of Birnboim and Doly (2).

Gels were stained in ethidium bromide (0.5 ug/ml in distilled water), observed on a UV transilluminator (Foto 300, Fotodyne Inc., New Berlin, WI) and photographed through 23A and 2B Wratten filters with a Polaroid MP4 camera (film type 55).

RESULTS

Presence of BSH Activity and Substrate Specificity

Table 3 shows the reliability of the plate assay for detecting BSH activity when compared to the colorimetric assay used in the previous study (24). All strains of L.

acidophilus were bile salt hydrolase positive when tested on NaTC and NaTDC by the plate assay (Table 4). Most strains hydrolyzed NaGC and NaGDC, however strains 4962, ATCC 4356, RP34, GP4A, and GP1B did not hydrolyze NaGC. Strain ATCC 4356 also was unable to hydrolyze NaGDC.

Plasmid Screening

Eleven of twenty strains of L. acidophilus screened for plasmid DNA using a rapid microscale procedure contained plasmid DNA. Preparative plasmid DNA isolation followed by agarose gel electrophoresis revealed that these plasmids ranged in size from 2.3 to greater than 33 Mdal (Table 5). Three strains contained one plasmid, six strains contained two plasmids, one strain contained three plasmids and another strain contained four plasmids. Plasmid profiles of the 11 strains of L. acidophilus are shown in Figure 3. Strain ATCC 43121 and 251 each contained a 2.8 and 6.0 Mdal plasmid. Strains GP1B and GP4A each contained a 3.5 Mdal plasmid and a unique 7.5 and 8.8 Mdal plasmid respectively. Strains ATCC 4356 and 4962 each contained single plasmids greater than 33 Mdal. Strain HM_2 contained two large plasmids, 28.5 and 33 Mdal. Strain RP43 contained two large plasmids, 15 and greater than 33 Mdal. Strain RP42 contained four plasmids of 2.3, 7, 8, and 19.5 Mdal. Strain RP34 contained three plasmids of 11.5, 14, and 26 Mdal. Strain P16 contained two large plasmids greater than 19.5 Mdal (not visible on the gel)

DISCUSSION AND CONCLUSIONS

The instability of metabolic properties such as lactose utilization in Lactococcus species is due to these properties being encoded by plasmid DNA (17). This substantiates the important role plasmids play in strain performance of dairy starter cultures. In lactobacilli plasmids have been found that code for lactose fermentation (4), drug resistance (15,18,20,23), glucosamine fermentation (25) and bacteriocin production and immunity. Dashkevicz and Feighner (8) have suggested that the variation in bile salt hydrolase activity among the lactobacilli may be due to involvement of plasmids.

They incorporated NaTDC into MRS agar and developed a differential medium for detecting bile salt hydrolase positive and negative bacteria. They also reported that the use of glycine derivatives could result in partial precipitation of NaGC or NaGDC without the concominant hydrolysis of the amide bond.

In this study NaTC, NaTDC, NaGC and NaGDC were used to detect bile salt hydrolase activity in L. acidophilus. This method does not allow for quantitative determinations of substrate specificity. Some cultures tended to produce the smallest zones on NaGC; this was also associated with minimal growth (colony formation) when compared to growth and activity on other substrates. Perhaps NaGC exhibits more of an inhibitory effect on some strains of L.

acidophilus. It has been shown that there is approximately 80% recovery of Lactobacillus species when grown on MRS agar containing 0.5% NaTDC (8).

Agarose gel electrophoresis revealed that eleven out of twenty (55%) strains of bile salt hydrolase positive L. acidophilus contained plasmid DNA. Vescovo et al. (23) reported that 19% of the strains of L. acidophilus screened contained plasmid DNA. This difference may be due to lysis protocols or to variations among strains. The distribution of these plasmids is interesting and this diversity may be host organism related. Strains ATCC 4356 and 4962 (human origin) contained large plasmid(s) only. Strains GP1B, GP4A, ATCC 43121, HM_2, P16, RP34, RP42, RP43, and 251 contained a greater diversity of plasmid profiles. However, these data reveal that there is not a common plasmid among these strains of L. acidophilus, suggesting that a plasmid is not responsible for encoding bile salt hydrolase activity.

Future studies will encompass, testing cured derivatives of GP1B, ATCC 43121, RP42, and HM_2 for bile salt hydrolase activity to confirm whether or not this activity is encoded by plasmid DNA. Also, restriction digestion analysis of these plasmids and construction of genetic maps will help determine their similarities and give better estimation of their size. In addition, southern hybridization analysis of plasmids of similar size should be conducted to determine if they are related.

REFERENCES

1. Aries, V., J.S. Crawther, B.S. Drasar, and M.J. Hill. 1969. Degredation of bile salts by human intestinal bacteria. Gut. 10:575.

2. Birnboim, H.C. and J. Doly. 1979. A rapid alkaline extraction procedure for screening recombinant plasmid DNA. Nucl. Acids Res. 7:1513-1523.

3. Chassy, B.M., E. Gibson, and A. Giuffrida. 1976. Evidence for extrachromosomal elements in lactobacilli. J. Bacteriol. 127:1576-1578.

4. Chassy, B.M., E. Gibson, and A. Giuffrida. 1978. Evidence for plasmid associated lactose metabolism in Lactobacillus casei. Curr. Microbiol. 1:141-144.

5. Chikai, T., H. Nakao, and K. Uchida. 1987. Deconjugation of bile acids by human intestinal bacteria implanted in germ free rats. Lipids. 22:669.

6. Cole, C.B. and R. Fuller. 1984. Bile acid deconjugation and attachment of chicken gut bacteria: Their possible role in growth depression. Brit. Pultry Sci. 25:227.

7. Currier, T.C. and E.W. Nester. 1976. Isolation of covalently closed circular DNA of high molecular weight from bacteria. Anal. Biochem. 76:431-441

8. Dashkevicz, M.P. and S.D. Feighner. 1989. Development of differential medium for bile salt hydrolase-active Lactobacillus spp. Appl. Environ. Microbiol 55:11.

9. Davies, F.L. and M.J. Gasson. 1981. Reviews of the progress of dairy science: genetics of lactic acid bacteria. J. Dairy Res. 48:363-376.

10. Gilliland, S.E. and M.L. Speck. 1977. Deconjugation of bile acids by intestinal lactobacilli. Appl. Environ. Microbiol. 33:15.

11. Gopal-Srivastava, R. and P.B. Hylemon. 1988. Purification and characterization of bile saly hydrolase from Clostridium perfringes. J. Lip. Res. 29:1079.

12. Hofer, F. 1977. Involvement of plasmids in lactose metabolism in Lactobacillus casei suggested by

genetic experiments. FEMS Microbiol. Lett. 1:167-170.

13. Hoffman, A.F. 1977. The enterohepatic circulation of bile acids in man. Clin. Gastroenterol. 6:3.

14. Hylemon, P.B. and T.L. Glass. 1983. Biotransformation of bile acids and cholesterol by the intestinal microflora. pp. 189-213. In D.J. Hentages (Ed.), Human Intestinal Microflora in Health and Disease. Academic Press, Inc., N.Y., New York.

15. Ishiwa, H. and S. Iwata. 1980. Drug resistance plasmids in Lactobacillus fermentum. J. Gen. Microbiol. 26:71-74

16. Klaenhammer, T.R. and S.M. Sutherland. 1980. Detection of DNA in an isolate of Lactobacillus acidophilus. Appl. Environ. Microbiol. 35:592-600.

17. McKay, L.L. 1893. Functional properties of plasmids in lactic streptococci. Antonie van Leeuwenhoek 49:259-274.

18. Morelli, L., M. Vescovo, and V. Bottazzi. 1983. Plasmids and antibiotic resistance in Lactobacillus helveticus and Lactobacillus bulgaricus isolated from natural whey culture. Microbiologia. 6:145-154.

19 Sandine, W.E. 1987. Looking backward and forward at the practical applications of genetic researches on the lactic acid bacteria. FEMS Microbiol. Rev. 46:205-220.

20. Smiley, M.B. and V. Fryder. 1978. Plasmids, lactic acid production and N-acetyl-D-glucosamine fermentation in Lactobacillus helveticus subsp. jugurti. Appl. Environ. Microbiol. 35:777-781.

21. Stellwag, E.J. and P.B. Hylemon. 1976. Purification and characterization of bile salt hydrolase from Bacteroides fragilis subsp. fragilis. Biochim Biophys Acta. 452:165.

22. Tannock, G.W., M.P. Dashkevicz, and S.D. Feighner. 1989. Lactobacilli and Bile Salt Hydrolase in the Murine Intestinal Tract. Appl. Environ. Microbiol. 55:1848.

23. Vescovo, M., L. Morelli, and V. Bottazzi. 1981. Evidence of plasmid DNA in Lactobacillus. Microbiologia 4:413-419.

24. Walker, .K. and S.E. Gilliland. 1990. Relationships among ile tolerance, bile salt hydrolase activity, and a imilation of cholesterol by Lactobacillus acido ilus. (In press).

25. West, C. . and P.J. Warner. 1985. Plasmid profiles and t nsfer of plasmid-encoded antibiotic resist nce in Lactobacillus plantarum. Appl. Envir . Microbiol. 50:1319-1321.

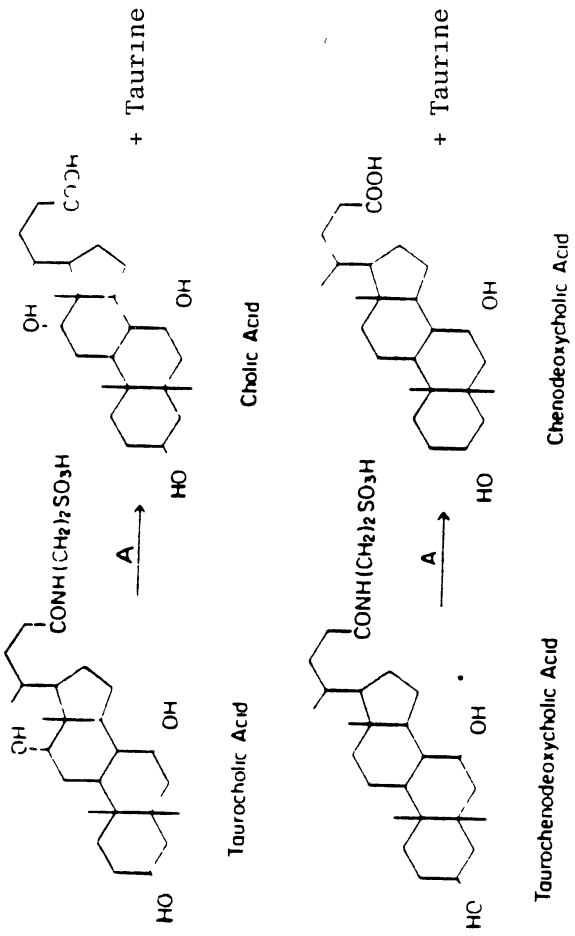


Figure 2. Bacterial bile salt hydrolase transformation.
A = Bile salt hydrolase.

Table 3. COMPARISON OF BILE SALT HYDROLASE ACTIVITY BY LACTOBACILLUS ACIDOPHILUS ON AN AGAR AND LIQUID MEDIUM[a]

Culture	Colorimetric Assay	Plate Assay[b]
GP4A	+	+
GP1B	+	+
RP43	+	+
ATCC 4356	+	+
RP42	+	+
RP34	+	+
14F1	+	+
ATCC 43121	+	+
2	+	+
12	+	+
15	+	+
NCFM-F	+	+
251	+	+
5	+	+
HM_2	+	+
14	+	+
DKW-9	+	+
NCFM-L	+	+
P16	+	+

[a]Each method was performed three times TDC
[b]MRS agar contained 0.5% sodium taurocholate (NaTC); plates were incubated anaerobically for 48 hours at 37°C

Table 4. SUBSTRATE SPECIFICITY OF BILE SALT HYDROLASE BY CELLS OF LACTOBACILLUS ACIDOPHILUS[1]

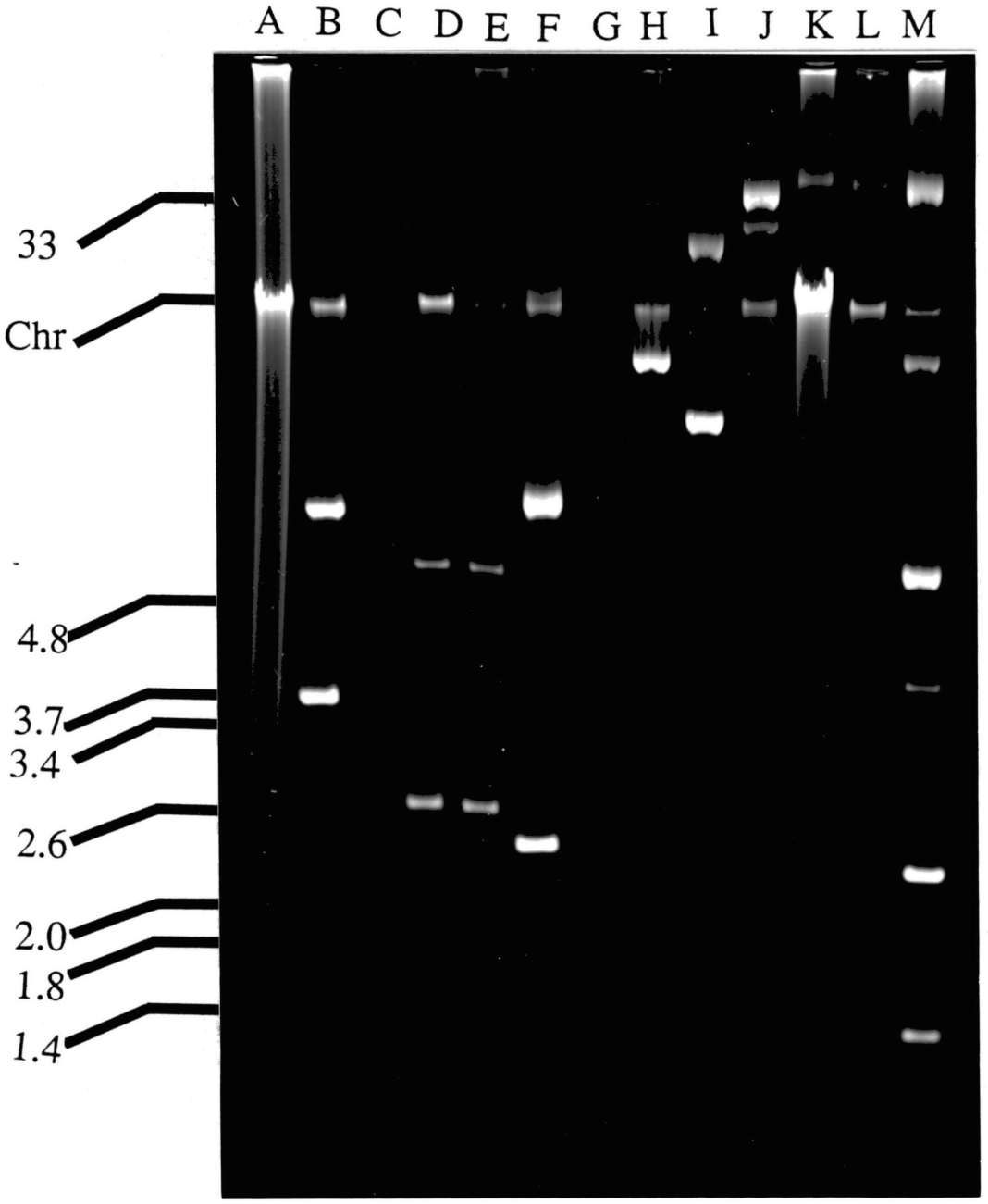
	Substrate (BSH activity)			
Culture	NaTC	NaTDC	NaGC	NaGDC
4962	+	+	-	+
ATCC 4356	+	+	-	-
HM_2	+	+	+	+
RP34	+	+	-	+
RP43	+	+	+	+
RP42	+	+	+	+
ATCC 43121	+	+	+	+
251	+	+	+	+
GP4A	+	+	-	+
GP1B	+	+	-	+
P16	+	+	+	+

[1]Each method was performed three times; MRS agar contained 0.5% of desired bile salt; plates were incubated anaerobically for 48 hours at 37°C

Table 5. PLASMID CONTENT OF LACTOBACILLUS ACIDOPHILUS STRAINS USED IN THIS STUDY

Culture	Host Organism	Plasmid content Number	Size (Mdal)
ATCC 43121	porcine	2	2.8,6
251	porcine	2	2.8,6
RP42	porcine	4	2.3,7,8 19.5
GP4A	porcine	2	3.5,8 8
P16	porcine	2	>19.5
GP1B	porcine	2	3.5,7 5
RP43	porcine	2	15.0,>33
RP34	porcine	3	11.5,14,26
DKW-9	human	none	
NCFM-L	human	none	
ATCC 4356	human	1	>33
HM_2	human	2	28.5,33
4962	human	1	>33
NCFM-F	human	none	
14	human	none	
15	human	none	
2	human	none	
12	human	none	
5	human	none	
14F1	human	none	

Figure 3. Agarose gel electrophoresis showing plasmids isolated from *Lactobacillus acidophilus*. Lane A,P16 ; lane B,GP1B ; lane C,GP4A ; lane D,251 ; lane E,ATCC 43121 ; lane F,RP42 ; lane G, mobility reference standard from *E. coli* V517; lane H,RP43 ; lane I,RP34 ; lane J,HM_2 ; lane K,ATCC 4356 ; lane L,4962 ; lane M,mobility reference standard from *S. lactis* ML3 .


A B C D E F G H I J K L M
33
Chr
4.8
3.7
3.4
2.6
2.0
1.8
1.4

CHAPTER V

SUMMARY AND CONCLUSIONS

The relationships among bile tolerance, bile salt hydrolase (BSH) activity and assimilation of cholesterol were ascertained. Moreover the relationship between plasmid DNA content and bile salt hydrolase activity was determined

Studies comparing the bile resistance of nineteen strains of L. acidophilus indicated that there were significant variation among strains. Three strains of varying degrees of bile resistance were selected and compared for growth and BSH activity.

L. acidophilus ATCC 43121, NCFM-L, and ATCC 4356 were inoculated into MRS broth containing 0.2% sodium thioglycollate and .001M sodium taurocholate (NaTC) and monitored for growth (OD 620nm) and bile salt hydrolase activity at 37^{o}C. At three hours incubation L. acidophilus ATCC 4356 exhibited about twice the BSH activity as ATCC 43121 and NCFM-L. Maximum BSH activity was attained by 12 h incubation.

Studies comparing simultaneous BSH activity and assimilation of cholesterol by nineteen strains of L acidophilus revealed that there was significant variation among cultures in BSH activity and assimilation of cholesterol. However,

cultures that exhibited considerable BSH activity did not necessarily exhibit considerable ability to assimilate cholesterol.

All strains were screened for plasmid DNA. Eleven of twenty strains of L. acidophilus screened were found to contain plasmids, which range in size from 2.3 to greater than 33 Mdal. Two strains were found to contain one plasmid, seven strains contained two plasmids one strain contained three plasmids and another strain contained four plasmids. Fifteen plasmids of different molecular mass were detected on agarose gels. Two strains contained identical 2.8 and 6.0 Mdal plasmids. Two other strains contained an identical 3.5 Mdal plasmid. Four strains contained plasmids equal to or greater than 33 Mdal.

All strains (those with and without plasmids) exhibited BSH activity when inoculated on MRS agar containing 0.5% sodium taurodeoxycholic acid. Substrate spectificity data revealed that NaTC and NaTDC were preferentially hydrolyzed when compared to sodium glycocholate and sodium glycodeoxycholate.

These studies reveal that BSH activity among L. acidophilus varies and that there does not appear to be a significant correlation among BSH activity, bile tolerance, and assimilation of cholesterol. Also, results suggested that there is not a common plasmid among these strains of L acidophilus that encodes BSH activity.

Future studies should entail generating cured derivatives of L. acidophilus and testing them for BSH activity. In addition, restriction digestion analysis and southern hybridizations should be performed to characterize these plasmids of similar size.

COMPREHENSIVE BIBLIOGRAPHY

1. Aries, V., J.S. Crawther, B.S.Drasar, and M.J. Hill 1969. Degredation of bile salts by human intestinal bacteria. Gut. 10:575.

2. Bloch, K., B.N. Berg, and D. Rittenberg. 1943. The biological conversion of cholesterol to cholic acid J. Biol. Chem. 149:511.

3. Brandt, L.J and L.H. Bernstein 1976 Bile salts Their role in cholesterol synthesis, secretion and lithogenesis. Am. J. Gastroenterol. 65 1513

4. Carey, M.C. and D.M. Small. 1972. Micelle formation by bile salts. Arch. Intern. Med. 130:506

5. Carey, M.C. 1982. The enterohepatic circulation In Arias, I.M., H. Popper, D.Schachter, and D A Shafritz (Eds.), The Liver pp.429-465. Raven Press, New York.

6. Chassy, B.M., E. Gibson, and A. Giuffrida. 1976 Evidence for extrachromosomal elements in lactobacilli. J. Bacteriol. 127:1576-1578

7 Chassy, B.M., E. Gibson, and A. Giuffrida 1978 Evidence for plasmid associated lactose metabolism in Lactobacillus casei. Curr. Microbiol 1 141-144.

8. Chikai, T., H. Nakao, and K, Uchida. 1987 Deconjugation of bile acids by human intestinal bacteria implanted in germ free rats. Lipids 22:669.

9. Cole, C.B. and R. Fuller. 1984. Bile acid deconjugation and attachment of chicken gut bacteria: Their possible role in growth depression Brit. Poultry Sci. 25:227.

10. Danielson, A.D., E.R. Peo, K.M. Shahani, A J Lewis, P.J. Whalen, and M.A. Amer. 1989. Anticholesteremic property of Lactobacillus acidophilus yogurt fed to mature boars J Animal Sci 67:966--974.

11. Dashkevicz, M.P. and S.D. Feighner. 1989. Development of a diferential medium for bile salt hydrolase-active Lactobacillus spp. Appl. Environ Microbiol 55:11.

12. Davies, F. L. and M. J. Gasson. 1981. Reviews of the progress of dairy science: genetics of lactic acid bacteria. J. Dairy Res. 48:363-376.

13. Dempsey, M.E. 1974. Regulation of steroid biosynthesis. An. Rev. Biochem. 43:967.

14. Dickenson, A.B., B.E. Gustafasson, and A. Norman. 1972. Determination of bile acid conversion potencies of intestinal bacteria by screening in vitro and subsequent establishment in germ free rats. Acta. Path. Microbiol. Scand. B79·691

15. Dietschy, J.M., H.S. Salomon, and M.D Siperstein 1966. Bile acid metabolism. I. Studies on the mechanisms of intestinal transport. J. Clin Inves 45:832.

16. Dietschy, J.M. 1968. Mechanisms for the intestinal absorption of bile acids. J. Lipid Res. 9:297.

17. Dowling, R.H. 1972. Progress In Gastroenterology The enterohepatic circulation. Gastroenterology 62:122.

18 Dujovne, C.A., P. Krehbiel, S. Decoursey, B. Jackson, S.B. Chernoff, A. Pitterman, and M. Garty. 1984 Probucol with colestipol in the treatment of hypercholesterol-emia. Ann. Itern. Med. 100 477

19. Dulery, C., and D. Reisser. 1982. Intestinal absorption of biliary and exogenous cholesterol in the rat. Biochem. Biophys. Acta 710:164-171

20. Eyssen, H. 1973. Role of gut microflora in metabolism of lipids and sterols. Proc. Nutr. Sci. 32·59

21. Ferrari, A., N. Pacini, and E. Canzi. 1980. A note on bile acids transformations by strains of bifidobacterium. J. Appl. Bacteriol. 49·193.

22. Floch, M.M., H.J. Binder, B. Filburn, and W Tershengoren. 1972. The effect of bile acids on intestinal microflora. J. Clin. Nutr. 25:1418

23. Gallo-Torres, M.E., O.N. Miller, and J. G. Hamilton 1969. A comparison of the effects of bile salts on the absorption of cholesterol from the intestine of the rat. Biochimica et Biophysica Acta 176 605

24. Garbutt, J.T., R.M. Wilkins, L. Lack, and M.P. Tyor 1970. Bacterial modification of taurocholate during enterheptic recirculation in normal man and patients with small intestinal disease. Gastroenterology. 59:553.

25. Gilliland, S.E. and M.L. Speck. 1977. Deconjugation of bile acids by intestinal lactobacilli. Appl Environ. Microbiol. 33:15.

26. Gilliland, S.E. and M.L. Speck. 1977. Instability of Lactobacillus acidophilus in yogurt. J. Dairy Sci 60:1394.

27. Gilliland, S.E., T.E. Staley, and L.J. Bush. 1984. Importance of bile tolerance of Lactobacillus acidophilus used as a dietary adjunct. J. Dairy Sci. 67:3045.

28. Gilliland, S.E., C.R. Nelson, and C. Maxwell. 1985 Assimilation of Cholesterol by Lactobacillus acidophilus. Appl. and Environ. Microbiol. 49·377

29. Gopal-Srivastava, R. and P.B. Hylemon. 1988. Purification and characterization of bile salt hydrolase from Clostridium perfringes. J. Lip. Res 29:1079.

30. Grunewald, K.K. 1982. Serum cholesterol levels in rats fed skim milk fermented by L. acidophilus J Food Sci. 47:2078.

31. Grundy, S.M. and D.W. Bilheimer. 1984. Low density lipoprotein receptors and enterohepatic circulation In Paumgartner, G., A. Steihl, and W. Gerok (Eds), Enterohepatic Circulation of Bile Acids and Sterol Metabolism. MIT Press LTD, Boston.

32. Harrison, V.C. and G. Peat. 1975. Serum cholesterol and bowel flora in the newborn. Am. J. Clin. Nutr 28:1351.

33. Hepner, G., R. Fried, S. St.Jeor, L. Fusetti, and R. Morin. 1979. Hypocholesterolemic effect of yogurt and milk. Am. J. Clin. Nutr. 32:19.

34. Hill, M.J. and B.S. Drasar. 1968. Degradation of bile salts by human intestinal bacteria. Gut 9 22

35. Hofer, F. 1977. Involvement of plasmids in lactose metabolism in Lactobacillus casei suggested by genetic experiments. FEMS Microbiol. Lett 1 167-170.

36. Hoffman, A.F. 1977. The enterohepatic circulation of bile acids in man. Clin. Gastroenterol. 6:3.

37. Holt, R. 1972. The roles of bile acids during the process of normal fat and cholesterol absorption. Arch. Intern. Med. 130:574.

38. Howard, A.N. and J. Marks. 1977. Hypocholestrolemic effect of milk. The Lancet. 255.

39. Hylemon, P.B. and T.L. Glass. 1983. Biotransformation of bile acids and cholesteol by the intestinal microflora. pp. 189-213. In D.J. Hentages (Ed.), Human Intestinal Microflora in Health and Disease. Academic Press, Inc., N.Y., New York.

40. Ishiwa, H. and S. Iwata. 1980. Drug resistance plasmids in Lactobacillus fermentum. J. Gen. Microbiol. 26:71-74.

41. Jones, R.J. (ed). 1970. Atherosclerosis - Proceedings of the Second Annual International Symposium Springer-Verlag. N.Y., New York.

42. Klaenhammer, T. R. and S. M. Sutherland. 1980. Detection of DNA in an isolate of Lactobacillus acidophilus. Appl. Environ. Microbiol. 35:592-600

43. Klaenhammer, T.R. 1984. A general method for plasmid isolation in lactobacilli. Curr. Microbiol 10 23-28.

44. Kobashi, K., I. Nishizawa, and T. Yamada. 1978. A new hydrolase specific for taurine-conjugates of bile acids. J. Biochem. 84:495.

45. Lack, L. and I.M. Weiner. 1963. Intestinal absorption of bile salts and some biological implications Fed. Proc. 22:1334.

46. Lee, L-J., J.B. Hansen, E.K. Jagusztyn-Krynicka, and B.M. Chassy. 1982. Cloning and expression of the ß-D-phosphogalactoside galactohydrolase gene of Lactobacillus casei in Escherichia coli K12. J. Bacteriol. 152:1138-1146.

47. Lipids Research Clinics Program. 1984. The lipid research clinics primary prevention trial results I. Reduction in incidence of coronary heart disease J. Am. Med. Assoc. 251:351.

48. Lipids Research Clinics Program. 1984. The lipid research clinics primary prevention trial results

II.The relationship between reduction in incidence of coronary heart disease to cholesterol lowering J. Am. Med. Assoc. 251:365-374.

49. Lundeen, S.G. and D.C. Savage. 1989. Purification of the Bile Salt Hydrolase from Lactobacillus sp strain 100-100. Abstract of the annual meeting of the American Society for Microbiology.

50. MacDonald, I.A., V.D. Bokkenheuser, J. Winter, A.M. McLernon, and E.H.Musbach. 1983. Degradation of steroids in the human gut. J. Lipid Res. 24·675.

51. Mallory, A., F. Kern, J. Smith, and D. Savage. 1973. Patterns of bile acids and microflora in the human small intestine. Gastroenterology. 64:26.

52. Mann, C.V. and A. Spoerry. 1974 Studies of surfactant and cholesterol in the Maasai. Am. J Clin. Nutr. 27:464.

53. Mann, C.V. 1977. A factor in yogurt which lowers cholesterol in man. Atherosclerosos. 26:335

54. Masuda, N. 1981. Deconjugation of bile salts by bacteroides and clostridium. Microbiol. Immunol 25:1.

55. McIntyre, N. and K.J. Isselbacher. 1973. Role of the small intestine in cholesterol metabolism. Amer. J. Clin. Nutr. 26:647.

56. McKay, L.L. 1983. Functional properties of plasmids in lactic streptococci. Antonie van Leeuwenhoek 49:259-274.

57. Midvedt, T. and A. Norman. 1967. Bile acid transformations by microbial strains belonging to the genera found in intestinal contents. Acta Pathol. Microbiol. Scand. 71:1341.

58. Miller, N.E. 1979. Plasma lipoproteins, lipid transport, and atherosclerosis: Recent developments. J. Clin. Path. 42:639.

59. Morelli, L., M. Vescovo, and V. Bottazzi. 1983. Plasmids and antibiotic resistance in Lactobacillus helveticus and Lactobacillus bulgaricus isolated from natural whey culture. Microbiologia. 6 145-154.

60. Mott, G.E., R.W. Moore, H. Redmond, and R Reiser 1973. Lowering of serum cholesterol by intestinal

bacteria in cholesterol-fed piglets. Lipids. 8:428.

61 Muriana, P.M. and T.R. Klaenhammer. 1987. Conjugal transfer of plasmid- encoded determinants for bacteriocin production and immunity in Lactobacillus acidophilus 88. Appl. Environ Microbiol. 53:553-560.

62. Murotomi, M., T. Watanabe, N. Suegara, Y. Kawai, and M Mutai. 1975. Distribution of indigenous bacteria in the digestive tract of conventional and gnotobiotic rats. Infect. Immun. 11:962.

63. Nair, P.P., M. Gordon, and J. Reback. 1967. The enzymatic cleavage of the carbon-nitrogen bond in 3-, 7-, 12- Trihydroxy-5-ß-cholan-24-oylglycine J Biol. Chem. 242:7.

64. National Heart and Lung Institute Task Force on Atherosclerosis. 1971. Vol 2. DHEW Publication no. (NIH) 72:219.

65. Norman, A. and J. Sjovall. 1953. On the transformation and enterohepatic circulation of cholic acid in the rat. J. Physiol. 233:872.

66. Playhoust, M.R. and K.J. Isselbacher. 1964. Studies on the transport and metabolism of conjugated bile salts by intestinal mucosa.

67. Rao, D.R., B. Chawan, and S.R. Pulusani. 1981 Influence of milk and Thermophilus milk on plasma cholesteol levels and hepatic cholesterogenesis in rats. J. Food. Sci. 46:1339.

68. Rudel, L.L., J.S. Parks, F.L. Johnson, and J. Babiak. 1986. Low density lipoproteins in atherosclerosis J. Lipid. Res. 27:465.

69. Sandine, W.E. 1987. Looking backward and forward at the practical applications of genetic researches on the lactic acid bacteria. FEMS Microbiol. Rev. 46:205-220.

70. Sakai, K., T. Makino, Y. Kawai, and M. Mutai. 1980 Intestinal microflora and bile acids. Effect of Bile Acids on the Distribution of Microflora and Bile Acid in the Digestive Tract of the Rat. Microbiol. Immunol. 24:187.

71. Schiff, E.R., N.C. Small, and J.M. Dietschy. 1972 Characterization of the kinetics of the passive and active transport mechanisms for bile acid absorption

in the small intestine and colon of the rat. J. Clin. Inves. 51:1351.

72. Shimuda, K., K.S. Bricknell, and S.M. Finegold. 1969. Deconjugation of bile acids by intestinal bacteria: Review of literature and additional studies. J. Infect. Dis. 119:273.

73. Shimada, K. V.L. Sutter, and S.M. Finegold. 1970. Effect of bile and deoxycholate on Gram negative anaerobic bacteria. Appl. Microbiol. 20:737.

74. Smiley, M.B. and V. Fryder. 1978. Plasmids, lactic acid production and N-acetyl-D-glucosamine fermentation in Lactobacillus helveticus subsp. jugurti. Appl. Environ. Microbiol. 35:777-781.

75. Stellwag, E.J. and P.B. Hylemon. 1976. Purification and characterization of bile salt hydrolase from Bacteroides fragilis subsp. fragilis. Biochim Biophys Acta. 452:165.

76. Stryer, L. "Biosynthesis of Macromolecular Precursors". Biochemistry 2nd ed. Wilt Freeman and Co. San Francisco.

77. Tannock, G.W., M.P. Dashkevicz, and S.D. Feighner 1989. Lactobacilli and Bile Salt Hydrolase in the Murine Intestinal Tract. Appl. Environ. Microbiol. 55:1848.

78. Thakur, C.P. and A.N. Jha. 1981. Influence of milk yogurt and calcium on cholesterol and induced atheroslcerosis in rabbits. Atherosclerosis 89:211.

79. Thompson, L.U., D. Jenkins, M.A. VicAmer, R. Reichert, A. Jenkins, and J. Kamulsky. 1982. The effect of fermented and unfermented milks on serum cholesterol.

80. Totuero, F., A. Brenes, and J. Rioperez. 1975. Influence of intestinal (cecal) flora on serum and egg yolk cholesterol levels in laying hens. Poultry Sci. 54:1935.

81. Troll, W. and R.K. Cannon. 1952. A modified photo metric ninhydrin method for the analysis of amino and imino acids. J. Biol. Chem. 200:803.

82. United States Bureaof the Census. 1986. Statistical Abstracts of the U.S.

83. Van Belle, H. 1965. Cholesterol, Bile acids, and Atherosclerosis. North Hollan Publishing Company Amsterdam.

84. Vescovo, M., L. Morelli, and V. Bottazzi. 1981. Evidence of plasmid DNA in Lactobacillus. Microbiologia 4:413-419.

85. Weiner, I.M. and L. Lack. 1968. Bile salt absorption enterohepatic circulation . In Code, D.F. (Ed.), Handbook of Physiology: Ailimentary Canal. p. 1439 Washington, D.C.: American Physiology Society.

86. West, C.A. and P.J. Warner. 1985. Plasmid profiles and transfer of plasmid-encoded antibiotic resistance in Lactobacillus plantarum. Appl. Environ. Microbiol. 50:1319-1321.

APPENDIX A

DATA FROM TREATMENTS

TABLE 6

COMPARISON OF GROWTH AND BILE SALT HYDROLASE ACTIVITY OF *LACTOBACILLUS ACIDOPHILUS* ATCC 4356

Run	Hours Incubation	uM Cholic acid	A_{620nm}
1	0	-	008
	3	0.30	.020
	6	1.30	.184
	9	1.48	201
	12	1.30	.210
	15	1.38	.205
	18	1.42	192
2	0	-	008
	3	0.60	020
	6	1.38	.160
	9	1.60	.199
	12	1.56	.190
	15	1.56	.240
	18	1.58	.184

TABLE 7

COMPARISON OF GROWTH AND BILE SALT HYDROLASE ACTIVITY OF LACTOBACILLUS ACIDOPHILUS ATCC 43121

Run	Hours Incubation	uM Cholic Acid	A_{620nm}
1	0	-	.009
	3	0.18	.107
	6	0.80	.345
	9	1.30	.378
	12	1.34	.399
	15	1.42	389
	18	1 50	400
2	0	-	013
	3	0.26	.101
	6	0.99	355
	9	1.56	.444
	12	1.88	.410
	15	1.55	396
	18	1.58	.373

TABLE 8

COMPARISON OF GROWTH AND BILE SALT HYDROLASE ACTIVITY OF LACTOBACILLUS ACIDOPHILUS NCFM-L

Run	Hours Incubation	uM Cholic Acid	A_{620nm}
1	0	-	.008
	3	0.20	.018
	6	1.08	.106
	9	1.24	.221
	12	1.40	.250
	15	1.32	215
	18	1.38	237
2	0	-	008
	3	0.22	.013
	6	0.27	.088
	9	1.12	.195
	12	1.30	.220
	15	1.30	.241
	18	1.44	.231

TABLE 9

COMPARISON OF BILE TOLERANCE OF CULTURES
OF LACTOBACILLUS ACIDOPHILUS

Culture	Change in A620nm @ 3 hour incubation Trial I	Trial II
ATCC 43121	.416	.443
RP34	.149	.130
251	.558	.494
ATCC 4356	.096	.072
DKW-9	.036	.028
HM_2	.300	.284
2	.139	163
14F1	.141	131
NCFM-F	.253	.181
NCFM-L	.188	.160
15	.134	.166
14	.212	.257
12	.204	.205
5	.171	.174
P16	.226	.269
GP4A	.215	.202
RP43	.278	.339
RP42	.195	.207
GP113	.292	.314

TABLE 10

COMPARISON OF ASSIMILATION OF CHOLESTEROL
BY LACTOBACILLUS ACIDOPHILUS

Culture	ug Cholesterol removed		
	Trial I	Trial II	Trial III
ATCC 43121	50	50	50
RP34	40	58	48
251	46	50	46
ATCC 4356	0	0	0
DKW-9	10	10	15
HM_2	2	8	2
2	0	10	0
14F1	0	0	0
NCFM-F	0	8	8
NCFM-L	0	10	10
151	12	18	14
14	14	4	10
12	9	10	9
5	4	10	8
P16	4	6	6
GP4A	6	16	10
RP43	38	40	40
RP42	50	46	48
GP1B	12	8	4

TABLE 11

COMPARISON OF BILE SALT HYDROLASE ACTIVITY OF CULTURES OF LACTOBACILLUS ACIDOPHILUS

Cultures	uM Cholic Acid Trial I	Trial II	Trial III
ATCC 43121	4.00	3.90	4 2
RP34	2.51	4.41	3.2
251	4.20	4.30	4.0
ATCC 4356	4.0	3.32	3.5
DKW-9	3.9	4.70	4.3
HM_2	2.5	3.40	3.0
2	1.6	1.80	1.9
14F1	0.2	0.11	0 2
NCFM-F	1.6	4.00	3.0
NCFM-L	3.9	3.50	3.6
15	2.2	1.9	2.4
14	2.3	2.1	2.2
12	1.8	1.6	1.4
5	1.6	1.2	1 4
P16	4.1	3.7	3.9
GP4A	4.3	3.6	3.9
RP43	3.5	4.2	3.6
RP42	4.0	4.3	3.9
GP1B	3.8	3.9	3.9

APPENDIX B

STATISTICAL ANALYSES

TABLE 12

ANALYSIS OF VARIANCE TABLE - COMPARISON OF BILE TOLERANCE OF CULTURES OF LACTOBACILLUS ACIDOPHILUS

Source	Degrees of Freedom	Sum of Squares	Mean Square	F Ratio
Culture	19	.49200	.02590	10.38
Rep	1	.00008	.00008	0.03
Total	20	.49200	.02460	

OSL < .0001

TABLE 13

ANALYSIS OF VARIANCE TABLE - COMPARISON OF CULTURES OF LACTOBACILLUS ACIDOPHILUS FOR THEIR ABILITY TO ASSIMILATE CHOLESTEROL

Source	Degrees of Freedom	Sum of Squares	Mean Square	F Ratio
Culture	19	19427.3	1022.5	78.0
Rep	2	87.0	43.5	3.3
Total	21	19514.3	929.3	

OSL < .0001

TABLE 14

ANALYSIS OF VARIANCE TABLE - COMPARISON OF CULTURES OF *LACTOBACILLUS ACIDOPHILUS* FOR BILE SALT HYDROLASE ACTIVITY ON SODIUM TAUROCHOLATE

Source	Degrees of Freedom	Sum of Squares	Mean Square	F Ratio
Culture	19	78.59	4.17	22.35
Rep	2	0.20	0.10	0.55
Total	21	78.80	3.75	

OSL < .0001

APPENDIX C

CULTURE IDENTIFICATION

TABLE 15

BIOLOGICAL CHARACTERISTICS OF CULTURES OF LACTOBACILLUS ACIDOPHILUS

Test	CULTURE				
	Bergey's	ATCC 43121	RP34	251	ATCC 4356
Gram Stain	+	+	+	+	+
Cellular Morphology	r[a]	r	r	r	r
Catalase	-	-	-	-	-
Growth @ 15°C	-	-	-	-	-
Growth @ 45°C		+	+	+	+
Amygdalin	+	+	+	+	+
Arabinose	-	-	-	-	-
Arginininine	-	-	-	-	-
Cellobiose	+	+	+	+	+
Esculin	+	+	+	+	+
Galactose	-	+	+	+	+
Glucose	+	+	+	+	+
Lactose	+	+	+	+	+
Maltose	+	+	+	+	+
Mannitol	-	-	-	-	-
Mannose	+	+	-	+	+
Melezitose	-	-	-	-	-
Melibiose		+	-	+	+
Raffinose		+	+	+	+
Rhamnose	-	-	-	-	-
Salacin	+	+	+	+	+
Sorbitol	-	+	-	-	-
Sucrose	+	+	+	+	+
Trehalose		+	-	+	+
Xylose	-	-	-	-	-

[a]Rod shaped

TABLE 16

BIOLOGICAL CHARACTERISTICS OF CULTURES OF LACTOBACILLUS ACIDOPHILUS

Test	CULTURE				
	Bergey's	RP43	RP42	GP1B	NCFM-F
Gram Stain	+	+	+	+	+
Cellular Morphology	r[a]	r	r	r	r
Catalase	-	-	-	-	-
Growth @ 15°C	-	-	-	-	-
Growth @ 45oC		+	+	+	+
Amygdalin	+	-	-	-	+
Arabinose	-	-	-	-	-
Argininine		-	-	-	-
Cellobiose	+	+	+	+	+
Esculin	+	+	+	+	+
Galactose	-	+	+	+	+
Glucose	+	+	+	+	+
Lactose	+	+	+	+	+
Maltose	+	+	+	+	+
Mannitol	-	-	-	-	-
Mannose	+	-	-	-	-
Melezitose	-	-	-	-	+
Melibiose		+	+	+	+
Raffinose		+	+	+	+
Rhamnose	-	-	-	-	-
Salacin	+	+	+	+	+
Sorbitol	-	-	-	-	-
Sucrose	+	+	+	+	+
Trehalose		-	-	-	+
Xylose	-	-	-	-	-

[a]Rod shaped

TABLE 17

BIOLOGICAL CHARACTERISTICS OF CULTURES OF LACTOBACILLUS ACIDOPHILUS

Test	CULTURE				
	Bergey's	15	14	12	5
Gram Stain	+	+	+	+	+
Cellular Morphology	r[a]	r	r	r	r
Catalase	-	-	-	-	-
Growth @ 15°C	-	-	-	-	-
Growth @ 45°C	+	+	+	+	+
Amygdalin	+	+	+	+	+
Arabinose	-	-	-	-	-
Argininine	-	-	-	-	-
Cellobiose	+	+	+	+	+
Esculin	+	+	+	+	+
Galactose	-	+	+	+	+
Glucose	+	+	+	+	+
Lactose	+	+	+	+	+
Maltose	+	+	+	+	+
Mannitol	-	-	-	-	-
Mannose	+	+	+	+	+
Melezitose	-	-	-	-	-
Melibiose		+	+	+	+
Raffinose		+	+	+	+
Rhamnose	-	-	-	-	-
Salacin	+	+	+	+	+
Sorbitol	-	-	-	-	-
Sucrose	+	+	+	+	+
Trehalose		+	+	+	+
Xylose	-	-	-	-	-

[a]Rod shaped

TABLE 18

BIOLOGICAL CHARACTERISTICS OF CULTURES OF LACTOBACILLUS ACIDOPHILUS

Test	CULTURE				
	Bergey's	2	GP4A	NCFM-L	14F1
Gram Stain	+	+	+	+	+
Cellular Morphology	r[a]	r	r	r	r
Catalase	-	-	-	-	-
Growth @ 15°C	-	-	-	-	-
Growth @ 45°C	+	+	+	+	+
Amygdalin	+	+	-	+	+
Arabinose	-	-	-	-	-
Argininine	-	-	-	-	-
Cellobiose	+	+	+	+	+
Esculin	+	+	+	+	+
Galactose	+	+	+	+	+
Glucose	+	+	+	+	+
Lactose	+	+	+	+	+
Maltose	+	+	+	+	+
Mannitol	-	-	-	-	-
Mannose	+	+	-	-	+
Melezitose	-	-	-	+	-
Melibiose		+	+	+	+
Raffinose		+	+	+	+
Rhamnose	-	-	-	-	-
Salacin	+	+	-	+	+
Sorbitol	-	-	-	-	-
Sucrose	+	+	+	+	+
Trehalose		+	-	+	+
Xylose	-	-	-	-	-

[a]Rod shaped

TABLE 19

BIOLOGICAL CHARACTERISTICS OF CULTURES
OF LACTOBACILLUS ACIDOPHILUS

Test	CULTURE			
	Bergey's	DKW-9	HM_2	P16
Gram Stain	+	+	+	+
Cellular Morpholoy	r[a]	r	r	r
Catalase	-	-	-	-
Growth @ 15°C	-	-	-	-
Growth @ 45°C	+	+	+	+
Amygdalin	+	+	+	+
Arabinose	-	-	-	-
Argininine				
Cellobiose	+	+	+	+
Esculin	+	+	+	+
Galactose	-	-	-	-
Lactose	+	+	+	+
Maltose	+	+	+	+
Mannitol	-	-	-	-
Mannose	+	+	+	+
Melezitose	-	-	-	-
Melibiose		+	+	+
Raffinose		+	+	+
Rhamnose	-	-	-	-
Salacin	+	+	+	+
Sorbitol	-	-	-	-
Sucrose	+	+	+	+
Trehalose		-	-	-
Xylose	-	-	-	-

[a]Rod shaped

VITA

Derek Keith Walker

Candidate for the Degree of

Doctor of Philosophy

Thesis: IMPORTANCE OF BILE SALT HYDROLASE ACTIVITY BY LACTOBACILLUS ACIDOPHILUS AND ITS GENETIC LOCATION

Major Field: Food Science

Biographical:

Personal Data: Born in Philadelphia, Pennsylvania, July 31, 1961, the son of Wilmar and Mary Walker. Married to Brenita Randle on August 13, 1982. Daughter, Ashley Chanelle, born March 21, 1984.

Education: Graduated from Franklin Delano Roosevelt High School, Dallas, Texas, in May, 1979; received Bachelor of Science degree in Animal Science -- Food Industry option from Oklahoma State University in May 1984; received Master of Science Degree in Food Science from Oklahoma State University in July 1986; completed requirements for the Doctor of Philosophy Degree at Oklahoma State University in May, 1990.

Professional Experience: Graduate Research Assistant, Animal Science Department, Oklahoma State University, August, 1984, to May, 1990.

Organizations: American Dairy Science Association, American Society of Microbiology, International Association of Milk, Food, and Environmental Sanitarians, Oklahoma Chapter of the Institute of Food Technologists.