BILE SALT DECONJUGATION BY THREE STRAINS OF *LACTOBACILLUS ACIDOPHILUS* AND CHARACTERIZATION OF THEIR BILE SALT HYDROLASE

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CHAPTER I

INTRODUCTION

Humans and animals are in constant interaction with the microbiology of the environment. Perhaps the most interesting part concerns the interaction between microorganisms and their digestive systems. Food is the major source of microorganisms that can improve health or cause illnesses to the human or animal host. Digestive secretions such as saliva, gastric juice, immunoglobulins, bile and proteolytic enzymes are factors that determine which single species of microorganisms or a group of them can inhabit the gastrointestinal tract (GIT) of humans and animals. The intestinal tract is of great importance since most of the nutrients are absorbed through it. Bile and pancreatic secretions as well as bowel movement are perhaps the most important factors for determining the selection of microorganisms which can remain in the GIT. Of these, bile secretion may be the most important since it is linked to mechanisms that can improve health or create health problems in humans. Among those microorganisms, which can survive conditions of the gastrointestinal tract, is Lactobacillus acidophilus.

Lactobacillus acidophilus is a gram positive microorganism that is strictly fermentative and has complex nutritional requirements. It grows in a variety of habitats where large levels of soluble carbohydrates, protein breakdown products, vitamins, and low oxygen tension occur. The gastrointestinal tracts of humans and animals such as pigs, calves and chickens are suitable habitats for it. Lactobacillus acidophilus can colonize the neonatal infant such as the human, piglet, and calf whose intestinal tracts are sterile at birth. Such colonization is achieved probably with the mother's vaginal and perianal population (Knoke and Bernhardt, 1986). The balance of the microbial population is then controlled

by acid secretion in the stomach that may be buffered by the milk imbibed by the infant; so that, lactobacilli may become the predominant microorganism. Bile resistant lactobacilli such L. acidophilus prevail in the upper part of the small intestine (Gilliland et al, 1984), but in the large intestine strict anaerobes such bacteroides predominate (Mitsuoka, 1978). This desirable predominance of lactobacilli in the upper intestine helps to prevent diarrhea or scouring that occurs in young animals when enterophatogenic bacteria proliferate in the upper gastrointestinal tract. One of the most interesting characteristic of L. acidophilus is its bile salt deconjugation activity that may provide health benefits to humans. Moreover, L. acidophilus is a microorganism generally recognized as safe (GRAS) which gives it more advantages over others as a dietary component for human and animal consumption. To obtain strains of L. acidophilus that may improve the health of humans or animals, it is necessary to isolate microorganisms that can survive the acidic conditions of the stomach, the bile acids and digestive enzymes of the upper digestive tract. Also, L. acidophilus should resist the human or animal body's immune response and should be able to compete with other microorganisms of the intestinal microflora.

The objectives of this research were to study the bile salt deconjugation ability of three bile resistant strains of L. acidophilus under *in vitro* conditions and to characterize the bile salt hydrolase from these microorganisms.

CHAPTER II

REVIEW OF LITERATURE

Enterohepatic circulation

Eutherian mammals usually synthesize cholic and chenodeoxycholic acids as primary bile acids, which are conjugated in the liver with glycine or taurine; in the remainder of the animal kingdom only taurine conjugates are found (Elliot, 1985). Bile acids are C_{24} steroids with a carboxyl group at the end of the side chain. Bile acids are structurally related to cholesterol from which they are formed. After birth most of the bile acids are conjugated with taurine (Jönsson et al, 1995). However, for the adult man the glycine to taurine ratio in conjugated bile salts is 2.2 to 3.0, and the cholic to chenodeoxycholic ratio (trihydroxy to dihidroxy ratio) is 1.0 to 1.2 (Burnett, 1965; Haslewood, 1967, 1978; Mallory et al, 1973; Sandine, 1979). Bile salts represent from 40 to 50% of the bile composition (Haslewood, 1967, 1978). The bile acids are secreted by the liver as constituents of bile which is carried through the biliary duct system to the gallbladder. Bile helps to emulsify the dietary lipids increasing the contact between lipases and lipid substrates. The dispersion of lipids in the gut also facilitates their absorption by the intestinal mucous (Margalith, 1986). Bile is concentrated in the gallbladder for ultimate discharge into the duodenum where the bile salts are intimately associated with dietary lipids and various digestive products. Most of the latter constituents are absorbed in the upper small intestine. While conjugated bile salts are absorbed from all sites of the small gastrointestinal tract, most are absorbed in the ileum by active transport mechanisms. Bacteria in the intestines can biotransform bile salts by three principal types of reactions:

hydrolysis of the amide linkage of the conjugate to liberate free bile acids (deconjugation), removal of hydroxyl groups principally the 7 carbon hydroxyl group of the cholic acid moiety, and oxidative and reductive reactions of the existing hydroxyl groups (Drasar and Hill, 1974). After absorption, this mixture of modified bile acids is in part returned to the liver by the hepatic portal circulation in the process known as enterohepatic circulation. Some bile acids are lost from the body as fecal bile acids. Bile acids chemically modified by the intestinal microflora can be returned to the liver. The modified products which survive the enterohepatic cycle several times are known as secondary bile acids such as deoxycholic and lithocholic acids.

Gastrointestinal flora and bile acids

Bile acids are excreted into the human intestine as conjugates with glycine or taurine, and less frequently with sulfate or ornithine via bile. The bile acid conjugation is performed in the liver while in the intestines microbial activity can split the conjugates into free acids (Midtvedt, 1974). The peptide-like bond between the bile acid and taurine or glycine is not cleaved by most proteolytic enzymes, but it is by bile salt hydrolases. Deconjugation has been demonstrated *in vitro* with intestinal genera of Clostridium (Aries and Hill, 1970a), Lactobacillus (Gilliland and Speck, 1977), Streptococcus (Aries and Hill, 1970a), Bacteroides (Stellwag and Hylemon, 1976), and Bifidobacterium (Aries and Hill, 1970a). The fecal microflora is a very complex ecosystem and consists of a multitude of bacteria, predominantly obligatory anaerobes. The composition of the fecal microflora of a normal adult is comprised of less than one percent of aerobic microorganisms. Bile salt hydrolases produced by intestinal microorganisms are involved in the first steps of bile acid transformations. They catalyze the hydrolysis of conjugated bile acids (Hylemon, 1985). In fact, feces do not contain any conjugated bile acids. That intestinal bacteria are responsible for this deconjugation can be demonstrated with gnotobiological (germ-free) animals

where the conjugates appear intact in the feces.

The formation of the typical secondary bile acids (deoxy- and litho-cholic acids) from their primary counterparts (cholic and chenodeoxycholic acids) depends mainly on the 7□-dehydroxylation reaction. Hydrolysis of the conjugated bile acids is a prerequisite for dehydroxylation. Dehydroxylating bacteria seem to inhabit the cecum. Most intestinal bacteria capable of such reaction have been identified as members of the genera Clostridium (Hayakawa, 1973; Stellwag and Hylemon, 1979) and Eubacterium (Hirano et al, 1981; Gustafsson et al, 1966)

Deconjugated bile acids also are subject to oxidation and reduction processes by intestinal microflora. Oxidation at C-3, C-7, and C-12 may be caused by various microorganisms yielding the corresponding oxo or ketonic compounds. These oxidoreductases known as bile acid dehydrogenases (HSDH) are nicotinamide adenine dinucleotide (NAD^+) or nicotinamide adenine dinucleotide phosphate ($NADP^+$) dependent. The major transformation of bile acids resulting form HSDH activities is the epimerization of various hydroxyl groups through oxo intermediates which may represent a significant fraction of human fecal bile acids (Margalith, 1986). The epimerization of bile acids can be carried out by a single organism containing both □- and □-HSDH or by a collaboration of two organisms one species containing the □-HSDH and a second containing the □-HSDH (Hylemon, 1985). HSDH activities are found in members of the genera: Bacteroides, Eubacterium, Clostridium, Bifidobacterium and Escherichia (Aries and Hill, 1970b; Mcdonald et al, 1979; Hirano and Masuda, 1981; Hylemon and Sherrod, 1975).

Potential health benefits from Lactobacillus acidophilus

The growth and/or action of L. acidophilus in the gastrointestinal system of humans has some potential health benefits, such as prevention of bacterial infection, anticarcinogenic

action, prevention of gallstone formation, and control of serum cholesterol among others.

First, the production of antimicrobial substances by lactobacilli may contribute to protecting the young and adult animal against intestinal infections. Antibacterial substances produced by L. acidophilus include bacteriocins such as acidolin (Hamdan and Mikolajcik, 1974), lactacin B (Barefoot and Klaenhammer, 1983) and acidophilin (Shahani et al, 1977).

Second, secondary bile acids formed in the large intestine after enzymatic deconjugation and 7□-dehydroxylation of primary bile acids can have tumor-promoting capacities in animal experiments (Aries et al, 1995). The activity of intestinal bacterial enzymes, implicated in colon carcinogenesis, may be elevated by high meat consumption. In serum and bile of patients with colonic adenomas, more deoxycholic acid was detected than in healthy controls (Bayerdorffer et al, 1995). Secondary bile acids are toxic to several cell systems at physiological concentrations. The exact mechanism by which these acids exert their action is not well understood, but they might act through membrane damage or genotoxic effects (Nagengast et al, 1995). Intestinal bacteria capable of carrying 7□-dehydroxylation of primary bile acids represent a small fraction of the human intestinal flora and do not include lactic acid bacteria such as bifidobacteria and lactobacilli which are considered to improve and maintain health (Takahashi and Morotomi, 1994). Fermentation of starch by bifidobacteria and lactobacilli in the large intestine can enhance an acidic pH in colonic lumen by producing short chain fatty acids and lactic acid which inhibit the bacterial degradation of primary to secondary bile acids (Bartram et al, 1994; Van Munster et al, 1994). Some strains of L. acidophilus have been shown to have anticarcinogenic activities. Colon cancer patients given fermented milk with L. acidophilus showed a decrease in levels of soluble fecal bile acids and fecal bacterial enzymes, two risk markers for colon cancer (Lidbeck, et al, 1992). Moreover, Some strains of L. acidophilus have the ability to reduce the activity of fecal bacterial enzymes such a □-glucoronidase, azoreductase and nitroreductase that catalyze the conversion of procarcinogens to

carcinogens in the large bowel (Goldin and Gorbach, 1984; McConnell and Tannock, 1993).

Third, intestinal bacteria effect the turnover and secretion of bile acids (Eyseen, 1973). Some strains of L. acidophilus have been shown to stimulate the secretion of bile acid and the activity of cholesterol 7□-hydroxylase an enzyme which controls bile acid synthesis from cholesterol (Imaizumi et al, 1992). By increasing synthesis of bile acids from cholesterol the level of serum cholesterol can be decreased or maintained in homeostatic levels thus helping avoid cholesterol related health problems in humans. Other ways for decreasing serum cholesterol by L. acidophilus are cholesterol uptake or cholesterol adsorption by the lactobacilli cells during growth of the organism in the intestines. Cholesterol might be lowered as free bile acids or cholesterol itself if excreted from the body via the intestinal route. Some strains of L. acidophilus as well as other inhabitants of the gastrointestinal tract produce an enzyme named bile salt hydrolase (BSH). Such an enzyme hydrolyzes conjugated bile acids forming free bile acids in the GIT under *in vivo* conditions (Tannock et al, 1989; Tannock et al, 1994). For example, the presence of bile salt hydrolase activity and free bile salts in intestinal contents of mice is due mainly to the presence of lactobacilli (Tannock, 1995). Deconjugation of bile acids by BSH can decrease serum cholesterol (De Rodas et al, 1996). Since conjugated bile acids are necessary to emulsify cholesterol and other hydrophobic materials during food digestion, deconjugation of bile acids by BSH can decrease intestinal absorption of cholesterol. Free bile acids such as cholic and chenodeoxycholic acids as well as cholesterol are less soluble than the conjugated bile acids. Therefore, few free bile acids and cholesterol are absorbed through the enterohepatic circulation and most of them are easily excreted via feces. Serum cholesterol can then be reduced from the body's pool by synthesizing new conjugated bile acids to replace the excreted ones in the form of free bile acids (De Smet et al, 1994). Moreover, dietary cholesterol is not totally absorbed due to bile salt deconjugation. Thus, bile salt hydrolase from L. acidophilus may be an important factor in

reducing serum cholesterol and gallstone formation. Gallstone formation is positively correlated with high serum cholesterol and negatively correlated with the excretion of bile acids (Danzinger et al, 1972; Hosomi et al, 1982). Since gallstones are caused mainly by cholesterol accumulation into the gallbladder (Haslewood, 1967), increasing excretion of free bile acids and cholesterol also may help prevent gallstone formation.

Bile salt hydrolase

Bile salt hydrolase is an enzyme which has been observed in several bacterial species of the gastrointestinal tract. Some researchers have purified BSH from Lactobacillus sp. strain 100-100 (Lundeen and Savage, 1990), Bifidobacterium longum BB536 (Grill et al, 1995), Clostridium perfringens MCV815 (Gopal-Srivastava and Hylemon, 1988), Bacteroides fragilis ssp. fragilis (Stellwag and Hylemon, 1976). These all were active on both glycine and taurine conjugated bile salts. Such enzymes have more activity on sodium glycocholate than on sodium taurocholate conjugates. Other researchers have shown that specific enzymes for sodium taurocholate and sodium glycocholate exist in other microorganisms; for example, Kobashi et al (1978) reported an enzyme which only hydrolyzed taurine conjugates of bile acids. Such an enzyme was obtained from Peptostreptococcus intermedius. The enzyme was active on taurocholate, taurodeoxycholate and taurochenodeoxycholate bile salts, but not on glycine conjugated bile salts. However, the enzyme in Streptococcus faecalis and Lactobacillus brevis preferentially hydrolyzed the glycine conjugates. Also, Kawamato et al (1989) purified an enzyme from Bacteroides vulgatus which was active only on taurine conjugates. Thus BSH can be specific for taurine or glycine conjugated bile salts or for both taurine and glycine conjugated bile salts.

Physical and chemical characteristics of bile salt hydrolases

Nair et al (1967) partially purified the BSH from Clostridium perfringens. The enzyme was intracellular and had a pH optimum between 5.6 and 5.8. It was inhibited by iodoacetate and p-chloromercuribenzoate suggesting the presence of sulfhydryl groups on the enzyme.

Aries and Hill (1970a) studied the conditions for production and optimal activity of the BSH in two species each of Enterococcus, Bacteroides, Bifidobacterium, and Clostridium. They observed that BSH suffered irreversible loss of activity in the absence of a reducing agent or in the presence of O_2. Sodium thioglycolate was a more effective reducing agent than was cystine, glutathione, or □-mercaptoethanol. The BSH from Streptococcus faecalis was stored at -10°C for six months with an 18 % reduction in activity, but the enzymes from other microorganisms lost all their activity. Most of the enzymes had a pH optimum between 5 and 6. The BSH produced by these microorganisms, except for Bifidobacterium, was intracellular.

Stellwag and Hylemon (1976) studied the BSH from Bacteroides fragilis. It was able to hydrolyze taurine and glycine conjugates of cholic acid, chenodeoxycholic acid and deoxycholic acid. Lithocholic acid conjugates were not hydrolyzed. The enzyme had a molecular weight of 250 KDa.

Gilliland and Speck (1977) grew cultures of Lactobacillus species in MRS broth containing 1×10^{-3} M sodium taurocholate under low oxidation/reduction conditions using the GasPak system or sodium thioglycolate. The bile salt deconjugation of sodium taurocholate by L. acidophilus strain NCFM required low oxidation/reduction conditions and such deconjugation by resting cells had an optimum pH of 6.0. Also, Gilliland and Speck (1977) pointed out that cholic acid was not metabolized and the enzyme system was constitutive in L. acidophilus NCFM.

Lundeen and Savage (1990, 1992a, 1992b) reported that bile salt hydrolase activity in

Lactobacillus sp. strain 100-100 was strictly intracellular and was regulated by a factor induced by conjugated bile salts. The authors proposed that the factor facilitated the entry of conjugated bile salts into the cell where they were hydrolyzed by the enzyme. They found four isozymes of bile salt hydrolase with a pH optima between 4.2 and 4.5. The molecular weights were 115, 105, 95 and 80 KDa. They suggested that the isoenzymes were trimers of two peptide units of 42 and 38 KDa respectively.

Walker and Gilliland (1993) studied 19 cultures of L. acidophilus in Mann-Rogosa-Sharp (MRS) broth supplemented with sodium taurocholate. The cultures ATCC 4356, ATCC 43121 and NCFM-L deconjugated the sodium taurocholate within 18 hours. They suggested that the deconjugation activity was correlated with the cell growth and was inhibited by an acidic pH.

Grill et al (1995) purified the BSH from Bifidobacterium longum BB536. This BSH was capable of hydrolyzing taurine and glycine conjugates of cholate, deoxycholate, and chenodeoxycholate. The optimum pH was from 5.5 to 6.5 and the optimum temperature from 35 to 42°C. It had a molecular weight of 250 KDa in non-denaturing gels. Moreover, the enzyme was probably a hexamer as its relative molecular weight in denaturing gels was about 40 KDa. They also indicated that BSH was constitutive in this microorganism.

Genetics of bile salt hydrolase

Although many biological functions in lactobacilli are encoded by plasmid DNA (i.e., carbohydrate metabolism, proteolytic activity, citrate utilization, bacteriocins) (Sandine, 1987), there is no evidence of BSH being plasmid encoded. Studies on the BSH gene have been done by isolating the gene from the chromosomal DNA of Lactobacillus plantarum and cloning it into an Escherichia coli plasmid (Christiaens, et al, 1992). After cloning the plasmid into a Lactobacillus vector and introducing it into the parental strain, a homologous double cross-over recombination occurred suggesting that the BSH gene was

more likely chromosomal than plasmid encoded (Leer et al, 1993). The BSH gene also has been cloned from the chromosomal DNA of Clostridium perfringens and expressed in E. coli (Coleman and Hudson, 1995). Walker (1990) reported no evidence of plasmid encoded BSH in nine strains of L. acidophilus.

The importance of bile salt hydrolases

Gilliland and Speck (1977) have suggested that deconjugation of bile salts by bile salt hydrolase may enhance antagonist action of autochthonous microorganisms of the intestinal flora such as lactobacilli against pathogens in the intestines. Deconjugation of bile salts results in the formation of cytotoxic secondary bile salts such as cholic acid (Floch et al, 1972; Van der Meer et al, 1991). Therefore, deconjugated bile acids may have a higher effect on pathogenic bacteria than on indigenous microorganisms in the intestinal tract.

BSH also may have potential benefits in lowering both serum cholesterol and gallstone formation by increasing biliary secretion. Excretion of bile acids can cause the catabolism of cholesterol to form replacement bile acids (Eyseen, 1973; Gilliland et al, 1985, De Smet et al, 1994).

Feighner and Dashkevicz (1988) have demonstrated a correlation between growth depression of poultry and elevated levels of bile salt hydrolase activity in the intestines. High concentrations of BSH may influence lipid absorption since free bile salts do not have the emulsifying activity of conjugated bile salts. Such an effect could affect growth of animal species because conjugated bile acids are required for efficient lipid absorption. However, De Smet (1996) in swine and Bateup et al (1995) in mice did not find any significant differences in body weight between aminals treated with an active BSH lactobacilli and control group animals. In some species, phospholipids play a larger role in emulsifying lipids than do conjugated bile acids. Therefore, growth depression by high

levels of bile salt hydrolase might not be as important in swine and mice as in other species.

Methods of bile salt hydrolase quantification

Bile salt hydrolase activity has been quantified by radiochemical assays using tauro (carbonyl-^{14}C) cholic acid as a substrate and measuring (carbonyl-^{14}C) cholic acid as the product of such enzymatic activity after an ethyl acetate extraction under acidic conditions (Feighner and Dashkevicz, 1988; Lundeen and Savage, 1990). Also, BSH activity has been measured by quantification of bile conjugates and free acids using spectrophotometry at 385 nm (Aries and Hill, 1970a) or at 660 nm (Walker and Gilliland, 1993). BSH activity can be quantified by measuring taurine and glycine as products of bile conjugates using spectrophotometry at 570 nm (Stellwag and Hylemon, 1976). Qualitative assays of bile acid deconjugation have also been performed by thin-layer chromatography (Aries and Hill, 1970a; Stellwag and Hylemon, 1976). Recently, a continuous spectrophotometric method involving the chromophore, 5-amino-2-nitro-benzoic acid has been developed (Jupille, 1979; Kirby et al, 1995). Because of bile salt hydrolases have activity on bile salt conjugates enzymatic activity can be measured by the enzymatic deconjugation of sodium taurocholate or sodium glycocholate to sodium cholate. These three bile salts as well as other bile acids can be detected by HPLC using a C_{18} column or cartridge with a suitable mobile phase (Scalia, 1987; Dekker et al, 1991; Klaver and Van der Meer, 1993; De Smet et al, 1994). Therefore, bile salt hydrolase activity can be quantified by measuring the formation of cholic acid or by the disappearance of sodium glycocholate or sodium taurocholate using HPLC methods of analysis. An internal standard can be used to obtain a response without too much variation (Muraca and Ghoos, 1985). Since radiochemical assays are quite expensive and since spectrophotometric assays may be not very accurate because of the possible interference of other substances that absorb light at the wavelength

of the assay, HPLC assays offer the best approach.

CHAPTER III

MATERIALS AND METHODS

Sources and maintenance of cultures

The three strains of Lactobacillus acidophilus used in this study were obtained from the stock culture collection of the Food Microbiology Lab in the Department of Animal Science at Oklahoma State University (see Table A1 in Appendix A). All cultures were maintained by subculturing in MRS broth (Difco laboratories, Detroit, MI) using 1% inocula and 18 to 24 hours incubation at 37°C. They were stored at 5-7°C between subcultures in MRS agar stabs.

Bile salt deconjugation during growth without pH control

MRS broth (100 ml) supplemented with 1 mM sodium taurocholate and 1 mM sodium glycocholate (Sigma Chemicals Co., St. Louis, MO) was prepared and placed in 100 ml volumes into dilution bottles of about 180 ml capacity. The bottles containing the broth were autoclaved at 121 °C for 15 min. After cooling, freshly prepared Mann-Rogosa-Sharp broth cultures of L. acidophilus were inoculated (1%) into the medium. After mixing for 1 min, a series of 10 ml aliquots were withdrawn aseptically from the bottle and placed into sterile screw cap test tubes (12 x 1.8 cm). Cultures were incubated at 37 °C for 24 hours. A tube was taken every two hours for 14 hours and at the end of the 24 hours incubation. Growth was monitored by plate count and absorbance at 620 nm. Samples also were analyzed for pH and bile salt deconjugation.

Bile salt deconjugation during growth with pH control

MRS broth (300 ml) supplemented with 1 mM sodium taurocholate and 1 mM sodium glycocholate was prepared and placed into a fermentor of about 1 liter capacity. The fermentor was equipped with an autoclavable combination pH electrode (Ingold Electrodes, Inc., Wilmington, MA). It also was equipped with a port for the addition of neutralizer and a line to permit continuous sparging with nitrogen gas. The fermentor containing the broth was autoclaved at 121 °C for 15 min. After cooling, it was placed in a 37 °C water bath. The pH was controlled by using a mixed solution of 10 % sodium carbonate and 10 % ammonium hydroxide (v/v) as described by Gilliland and Rich (1990). The flask, containing the neutralizer, was connected to the fermentor. The neutralizer was delivered to the fermentor by a peristaltic pump (Masterflex, Cole-Parmer Inst. Co., Chicago, IL) connected to an automatic pH controller (Model 5997, Horizon Ecology Co., Chicago, IL) that was adjusted to maintain the desired pH of the broth. Nitrogen gas was continuously sparged through the broth from bottom to top at about 5 ml per min. After mixing for 5 min, a freshly prepared MRS broth culture of L. acidophilus was inoculated (1%) into the fermentor. After mixing for 1 min, 10 ml was withdrawn aseptically from the fermentor and placed into a sterile test tube to serve as the initial sample (i.e. 0 hour). The fermentor containing the cultures was incubated at 37 °C for 24 hours. Samples were taken aseptically every three hours for 12 hours incubation and at the end of the 24 hours. Growth was monitored by plate count and absorbance at 620 nm. Samples also were analyzed for bile salt deconjugation.

Measuring of culture growth

A 1:10 dilution was made from each sample using sterile peptone diluent (1 %) and the absorbance at 620 nm (Spectronic 21D, Milton Roy, Rochester, NY) was measured to

determine the relative cell growth. The absorbance readings were plotted against incubation time. Growth also was measured by plate count using MRS agar. Appropriate dilutions, prepared using 1 % sterile peptone, were plated by the pour plate method with MRS agar. The plates were overlayed with the same medium and incubated 48 hours at 37 °C after which colonies were counted with the aid of a Quebec colony counter. Colony forming units (CFU) per ml expressed as Log10 were plotted against incubation time.

Bile acids analyses

Instruments

An ISCO HPLC model 2350 equipped with a variable wavelength ultraviolet (UV) detector model V^4 (ISCO Inc. Lincoln, NE) and Valco injector, model C6W (Valco Instruments Co. Inc., Houston, TX), was used. A radial compression module, RCM-100 (Waters Ass., Milford, MA), was used with a flexible-walled reverse-phase column of 100 mm x 8 mm (I.D.) packed with Nova-Pak C_{18} (4 µm) from Waters Associates. The flow rate was adjusted at 1.0 ml/min for detection of conjugated bile salts and at 2.0 ml/min for detection of free bile salts. Ultraviolet detection was performed at 205 nanometers and 0.2 AUF. The injection quantity was 20 µl. Peak areas were calculated by the ChemResearch software version 2.4 (ISCO Inc.).

Chemicals

All organic solvents were high purity, methanol was HPLC grade (EM Industries, Inc., Gibbstown, NJ) and water had a resistivity of 18.2 megaohm-cm (Milli Q plus, Millipore Co., Bedford, MA). Conjugated and free bile salts used as standards had a purity of 98 % or more. Sodium taurocholate, sodium taurodeoxycholate, sodium

taurochenodeoxycholate, sodium glycocholate, sodium glycochenodeoxycholate and sodium cholate were purchased from Sigma Chemicals Co. Sodium glycodeoxycholate acid was purchased from Steraloids Inc., Wilton, NH. Dexamethasone and testosterone were purchased from Sigma Chemicals Co. Sep-pak C_{18} cartridges were obtained from Waters Associates.

Solvents

As mobile phase, 700 ml methanol and 300 ml 0.02 M acetic acid was used. The pH of this mixture was increased to exactly 5.4 by adding 5 M NaOH. Further purification through a 0.45 µm nylon filter (Gelman Sciences, Ann Arbor, MI) was performed. The mixture used for dilution of supernatants from cell cultures was the mobile phase, as described above and 0.2 M NaOH in 0.9 % NaCl (mixture A).

Recovery of bile salts from MRS broth and quantification of conjugated and free bile salts.

The method of Ruben and Berge-Henegouwen (1982) was modified. A five milliliter sample of culture was centrifuged (10 min, 10,000 x g). Supernatants were filtered through a 0.45 µm polysulfone filter (Whatman Inc., Clifton, NJ). Mixture A (14 ml) was added two milliliters of filtrates using a Vortex mixer. The solution was passed through a Sep-Pak C_{18} cartridge (Waters Ass.) after preparation of the cartridge as indicated in the manufacturer's instructions. Subsequently, the Sep-Pak cartridge was washed once with 10 ml water, once with 5 ml 10 % acetone and again with 10 ml water (Zhang et al, 1988). The conjugated and free bile acids were extracted from the cartridge by 5 ml methanol. The methanolic filtrate was evaporated to dryness under a stream of nitrogen gas at 60 °C. The residue was dissolved in 2 ml mobile phase and filtered through a 0.45 µm polysulfone filter to clarify the sample.

Quantification of conjugated bile salts. Two hundred microliters of sample or bile salt standards were mixed with 50 µl of an internal standard (dexamethasone, 0.2 mg/ml), then 20 µl of the mixture were injected into the HPLC. Sodium glycocholate and sodium taurocholate were recovered at 100.2 % ± 8.38 %. Detection of sodium glycocholate and sodium taurocholate was linear from 0 to 25 nmoles. Their determination coefficients were 0.996 and 0.998 respectively (see Figures A1 and A2 in Appendix A).

Quantification of free bile salts. Five hundred microliters were taken for cholic acid derivatization according to "method C" of Iida et al (1985). Two hundred microliters of the final free bile salt derivative was filtered through a 0.45 µm polysulfone filter and mixed with 50 µl of an internal standard (testosterone, 2 mg/ml). Then, 20 µl of this mixture was injected into the HPLC. Detection of sodium cholate was linear from 0 to 37 nmoles and its determination coefficient was 0.998 (see Figures A1 and A3 in Appendix A).

Assay for bile salt hydrolase activity

A methanolic solution of 0.01 M sodium taurocholate and 0.01 M sodium glycocholate was prepared. Twenty five microliters of the methanolic solution was pippeted into small screw cap test tubes (10 x 1.2 cm) that had been previously washed with 50 % nitric acid. The methanol was evaporated under a stream of nitrogen gas at room temperature. The tubes with 0.25 µmoles of each conjugated bile salt were stored at room temperature for no more than four weeks.

The enzyme activity was measured placing 100 µl of sample containing BSH into the assay tubes. After vortexing, the samples were incubated at 37 °C for 5 to 30 min. One hundred microliters of HPLC mobile phase was added to the assay tubes to stop the enzymatic reaction. Then, the mixture was clarified by filtering through a 0.45 µm polysulfone filter (Whatman Inc.). The filtrates were placed on ice at 0 °C and the contents

analyzed by HPLC.

One BSH enzymatic unit was defined as the nanomoles of either sodium taurocholate or sodium glycocholate deconjugated per minute based on disappearance of each from the assay mixture. The validation of the assay for bile salt hydrolase activity is shown in Appendix B.

Purification of bile salt hydrolase

Bile salt hydrolase was purified from cell free extracts from cultures grown in MRS broth without conjugated bile salts, and from spent broth from cultures grown in MRS broth supplemented with 2 mM each sodium taurocholate and sodium glycocholate.

After 24 hours growth in MRS broth without conjugated bile salts, the broth culture (200 ml) was centrifuged at 10,000 x g for 10 min at 5 °C. The spent broth was discarded. The cell pellet was resuspended and washed twice with acetate buffer (50 mM, pH 4.0, 20 ml) and centrifuged at 10,000 x g for 10 min at 5 °C. The cells were resuspended in 50 mM (pH 5.4) to 0.1 of the volume of the original culture. The cells were disrupted by sonication (5μm peak to peak of amplitude, and 85 watts delivered to the cell suspension) five times for 2 min each at 5 °C with a Sonic Dismembrator model 550 (Fisher Scientific, Pittsburgh, PA). The lysed cell suspension (cell free extract) was used for enzyme isolation.

After 24 hours growth in MRS broth containing 2 mM sodium glycocholate and 2 mM sodium taurocholate, the broth culture (200 ml) was centrifuged at 10,000 x g for 10 min at 5 °C. The cell pellet was discarded and the spent broth was saved for enzyme isolation.

Spent broth or cell free extract was mixed with methanol in a ratio 2 to 1 to obtain a final concentration of 33% methanol (v/v). After one hour, the spent broth-methanol or cell free extract-methanol mixture was centrifuged at 10,000 x g for 10 min at 5 °C to pellet the precipitate. The precipitate was dissolved in 50 mM acetate and 1 mM EDTA

buffer pH 5.4 (buffer A).

The dissolved methanol precipitate was fractionated by ammonium sulfate precipitation (40-80% saturation). The precipitated fraction was harvested by centrifugation at 5,000 x g for 15 min at 5 °C and resuspended in buffer A. The ammonium sulfate fractions (4 ml) were vortexed for complete dissolution and dialyzed for 18 hours in buffer A (2 L) through dialysis membranes (Spectra/Por, Spectrum Medical Instruments, Inc. Los Angeles, CA) with molecular cut off from 12 to 14 KDa. The dialyzed ammonium sulfate fraction was stored at -20°C for no more than four weeks.

Three grams of Sephadex G-200 powder with a particle size from 40 to 120 μm (Pharmacia, Sweden) was added to 100 ml working buffer B (50 mM acetate pH 5.4 with 1 mM EDTA and 0.02% sodium azide) with slow stirring. The suspension was held 24 hours at 40°C and then, stored at 5°C for one day. The slurry was poured into a 2.5 x 50 cm glass column (Bio-Rad, Richmond, CA). After the gel was settled completely, excess buffer was removed and the column was washed with two to four times its volume of 250 ml. A 1 % blue dextran (Sigma Chemicals) was used as a marker to determine the void volume of the column (Stellwagen, 1990). The flow rate of the mobile phase was controlled at 0.16 ml/min by using a pump (Cole-Parmer, Chicago, IL) attached to the column inlet. The sample with a protein concentration of no more than 2 mg/ml and a volume of no more than 2 ml was loaded onto the column and samples of 0.8 ml each were collected by an automatic fraction collector (Retriever II, ISCO Co.), and monitored manually for absorbance at 280 nm (Spectronic 21D, Milton Roy). The fractions also were analyzed for BSH activity.

Fractions with BSH activity from spent broth were pooled and concentrated with Centricell 60 membranes (Polysciences, Warrington, PA) with molecular cut off of 30 KDa. The pooled fractions were centrifuged at 1,500 x g for 20 to 60 min at 4 °C or until desired concentration were reached. Volumes of pooled fractions and filtrates were recorded.

Protein determination

The protein content of the fraction(s) at each step of purification as well as fractions from the gel chromatography was determined by the method of Bradford (1976). Bovine serum albumin (Sigma Chemicals Co.) was used as the standard.

Polyacrylamide gel electrophoresis

Non-denaturing protein separations were carried out in 7.5 % slab gels. Peptide separation by sodium dodecyl sulfate-polyacryl-amide gel electrophoresis (SDS-PAGE) also was carried out in 10 % slab gels (Laemmli, 1970) using Mini-Protein slab cell (Bio-Rad Laboratories, Inc., Hercules, CA). Gels were developed using coomassie brilliant blue G-250 and silver stain (Bio-Rad Laboratories, Inc.).

Statistical analysis

Bile salt deconjugation, growth and pH were analyzed using the general linear model correlation procedure from SAS to determine if significant relationships occurred among these variables. Specific bile salt deconjugation and growth rates of L. acidophilus were analyzed by the modified logistic model (Zwietering et al, 1990) using the non-linear model procedure from SAS (see Table F3 in Appendix F). The least significant difference method (LSD) was used to determine if statistically significance differences occurred among means. Physical and chemical properties affecting enzymatic activity such as temperature, EDTA, pH, and □-mercaptoethanol, also were analyzed as dependent variables using the GLM procedure of SAS (see Table F4 in Appendix F).

CHAPTER IV

RESULTS

Bile salt deconjugation by Lactobacillus acidophilus

Influence of sodium thioglycolate and heat sterilization on bile salt deconjugation

Three different treatments were applied to MRS broth media containing the conjugated bile salts (1 mM sodium glycocholate and 1 mM sodium taurocholate). For the first one the broth was sterilized by heating at 121°C for 15 min. The second one included supplementation of the broth with 0.2% sodium thioglycolate to provide low oxidation-reduction (O/R) potential prior to being heat sterilized (121°C for 15 min). The third one involved sterilization of the broth (without sodium thioglycolate) by passage through a sterile membrane filter (0.45 μm pores). The three media were inoculated (1 %) using a freshly prepared MRS broth culture of L. acidophilus ATCC 43121. Sample controls were taken immediately after inoculation. All three inoculated media were incubated at 37 °C for 24 hours. The amounts of conjugated bile salts remaining in the three media at the end of the incubation compared to the amounts at the beginning showed that deconjugation was not affected by heat sterilization and that low O/R potential was not an important factor for deconjugation by L. acidophilus ATCC 43121 (Table 1). The sodium glycocholate and sodium taurocholate in the media were almost completely deconjugated during the 24 hours incubation. The disappearance of the conjugated bile salts in each case was associated with increases in free cholic acid (data not shown).

TABLE 1

DECONJUGATION OF SODIUM GLYCOCHOLATE AND SODIUM TAUROCHOLATE BY L. ACIDOPHILUS ATCC 43121 AS INFLUENCED BY DIFFERENT TREATMENTS OF THE CULTURE MEDIUM

Treatment	Glycocholate[1] (mM)		Taurocholate[1] (mM)	
	0 hours	24 hours	0 hours	24 hours
Heat sterilization plus 0.2 % thioglycolate	0.98(0.03)	0.002(0.002)	0.98(0.04)	0.021(0.020)
Heat sterilization	1.01(0.02)	0.012(0.009)	0.99(0.01)	0.008(0.002)
Sterilization by filtration	0.99(0.04)	0.010(0.010)	0.97(0.04)	0.010(0.006)

[1]Each value is the average of two trials; numbers in parentheses represent the standard deviations. There were no significant differences among treatments for either time period for either bile salt ($P>0.05$).

Bile salt deconjugation and cell growth in static cultures

Three strains of L. acidophilus were grown in MRS broth supplemented with 1 mM each sodium glycocholate and sodium taurocholate without pH control. The media were heat sterilized and did not contain 0.2 % thioglycolate to provide low O/R potential.

Figure 1A shows the deconjugation of sodium taurocholate and sodium glycocholate by L. acidophilus O16 during incubation at 37 °C for 24 hours. The pH dropped as growth of the culture occurred (Figure 1A).

Growth of the culture monitored by plate counts and absorbance increased during the first 8-12 hours of incubation (Fig. 1B). The plate count decreased after 12 hours showing less viable cells towards the end of the incubation time. The reduction in plate count beyond 12 hours was probably because of bile salt deconjugation. Sodium cholate, the

product of the hydrolysis of sodium glycocholate and sodium taurocholate, is toxic for L. acidophilus and other intestinal microorganisms (Aries and Hill, 1970). However, the absorbance, an indirect method for measuring cell growth, was constant indicating cell lysis was not involved.

Figure 2A shows the bile salt deconjugation of L. acidophilus L1. Sodium taurocholate was deconjugated slower than sodium glycocholate. The pH was also reduced as deconjugation occurred and was related to growth of the culture (Fig. 2A and 2B). Figure 2B shows the growth of L. acidophilus L1 as measured by plate count and absorbance. After 10 hours of incubation a decline in plate count was observed, but the absorbance remained constant.

Figure 3 shows the bile salt deconjugation and the growth of L. acidophilus ATCC 43121. Both conjugated bile salts were hydrolyzed completely during the 24 hours of incubation. The viable count and absorbance during the growth of L. acidophilus ATCC 43121 had quite similar patterns to those of the other strains of L. acidophilus analyzed. The plate count of strain ATCC 43121 was reduced earlier than those of strains of O16 and L1, but the absorbance remained constant after reaching its maximum value.

Bile salt deconjugation and growth of all three strains of L. acidophilus were analyzed by the modified logistic equation according with Zwietering et al (1990). The purpose of such analysis was to determine if there were any significant differences in the specific deconjugation rate between sodium glycocholate and sodium taurocholate.

Since deconjugation of sodium taurocholate and sodium glycocholate releases free sodium cholate which could affect the survival of the culture, these data were analyzed to determine if significant differences in the kinetic parameters of growth of the microorganisms occurred when using absorbance and plate count as measurements of cell growth.

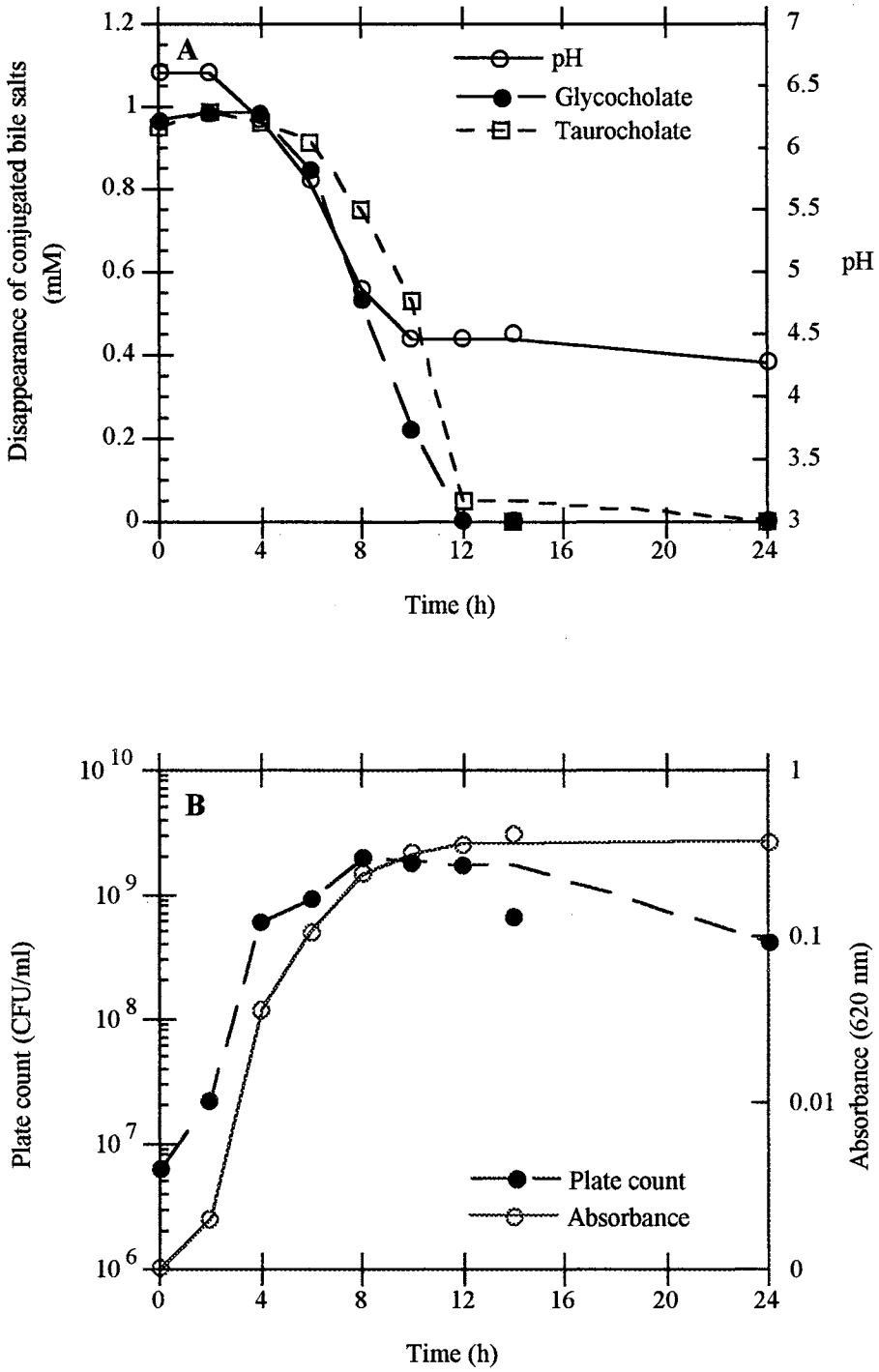

Figure 1. Bile salt deconjugation (A), acid production (A), and growth (B) of Lactobacillus acidophilus O16 in MRS broth supplemented with 1 mM sodium glycocholate and 1 mM sodium taurocholate. Each point represents a mean of three trials (S.E. sodium glycocholate = 0.043; S.E. = sodium taurocholate = 0.016; S.E. pH = 0.078; S.E. Log10 of count plate = 0.13; S.E. absorbance = 0.004; 40 df).

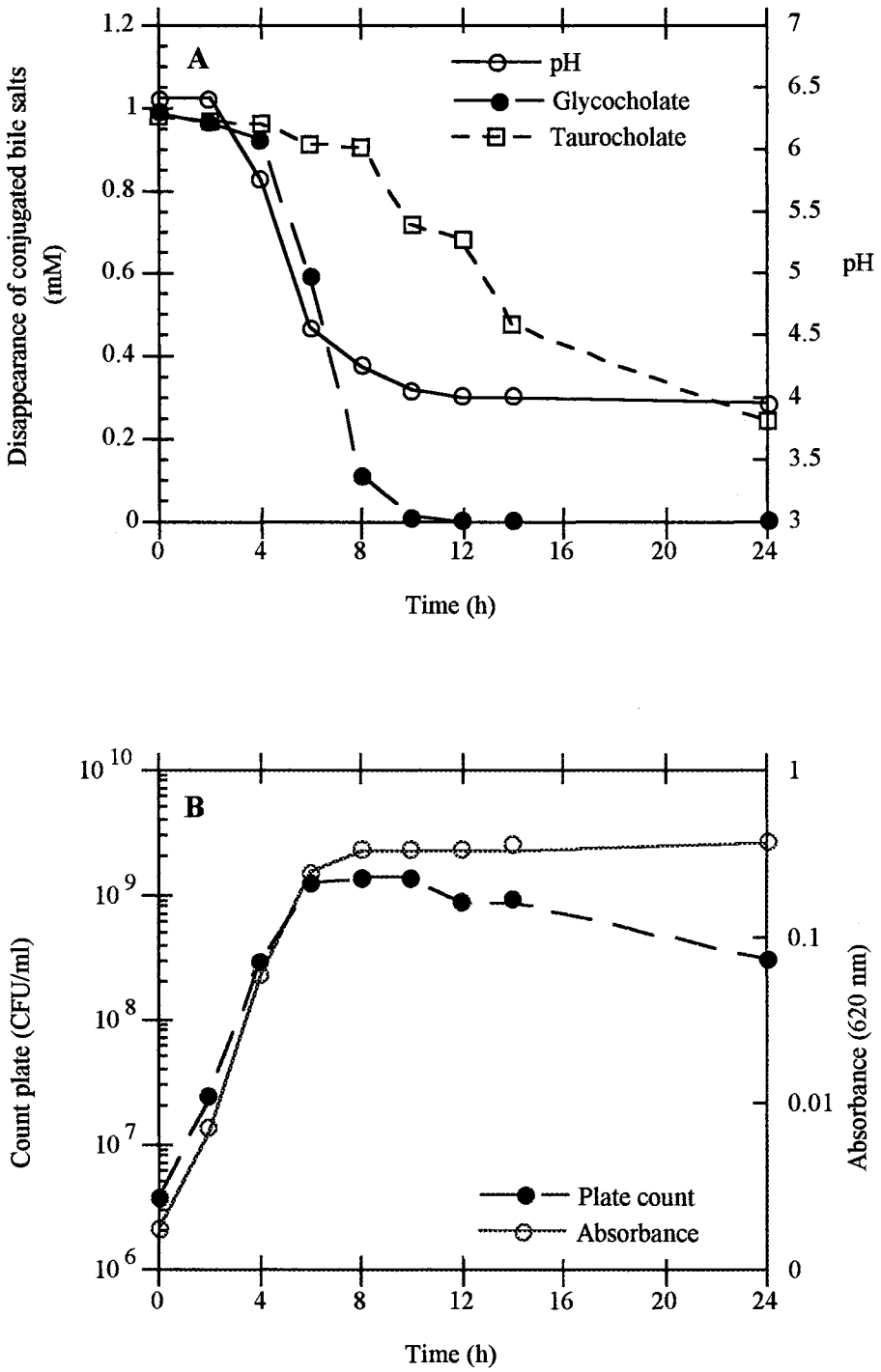

Figure 2. Bile salt deconjugation (A), acid production (A), and growth (B) of Lactobacillus acidophilus L1 in MRS broth supplemented with 1 mM sodium glycocholate and 1 mM sodium taurocholate. Each point represents a mean of three trials (S.E. sodium glycocholate = 0.005; S.E. = sodium taurocholate = 0.004; S.E. pH = 0.031; S.E. Log10 of count plate = 0.045; S.E. absorbance = 0.0006; 40 df).

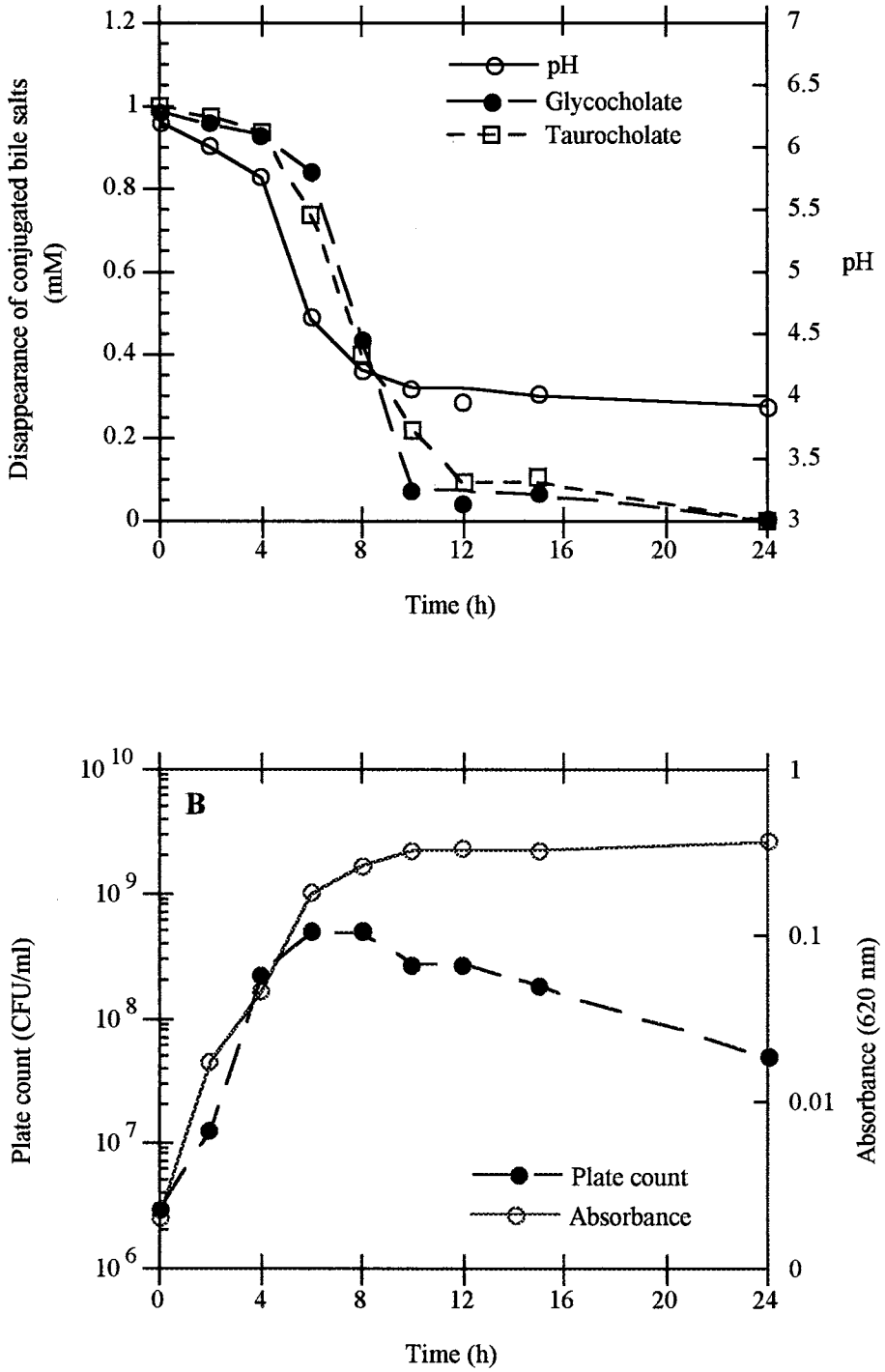

Figure 3. Bile salt deconjugation (A), acid production (A), and growth (B) of Lactobacillus acidophilus ATCC 43121 in MRS broth supplemented with 1 mM sodium glycocholate and 1 mM sodium taurocholate. Each point represents a mean of three trials (S.E. sodium glycocholate=0.013; S.E. sodium taurocholate= 0.014; S.E. pH=0.06; S.E. Log10 of count plate=0.08; S.E. absorbance=0.0002; 40 df).

Table 2 shows the deconjugation rates of both sodium taurocholate and sodium glycocholate in static cultures. The deconjugation rates of sodium glycocholate did not differ significantly among strains of L. acidophilus. The deconjugation rates of sodium taurocholate were not significantly different for strains ATCC 43121 and O16. However, L. acidophilus L1 exhibited a significantly lower deconjugation rate ($P<0.05$) for taurocholate than did the other two strains. Glycocholate was totally deconjugated by all strains during the 24 hours of incubation. However, taurocholate was totally deconjugated only by strains O16 and ATCC 43121. L. acidophilus L1 had deconjugated only 76% of taurocholate after 24 hours incubation. No significant differences in the lag phases were observed among the strains of L. acidophilus on either bile salt. The lag phases, however, for the deconjugation of sodium glycocholate for all three strains appeared shorter than for taurocholate. These results suggest that L. acidophilus ATCC 43121, O16 and L1 might prefer deconjugating sodium glycocholate better than sodium taurocholate.

The logistic model was not efficient in modeling the growth of L. acidophilus when using the colony forming units per milliliter because of the death (loss of CFU/ml) that occurred after 10 hours of incubation. The plate count was strongly correlated to the absorbance at 620 nm during the first 10 hours of growth ($P<0.0001$, Table F2 in Appendix F). However, when correlating the same variables for 24 hours of incubation the correlation factor dropped from 0.770 to 0.446. Therefore, the kinetic parameters of the cell growth for all three strains of L. acidophilus were analyzed by the plate count and by absorbance only from the lag phase to the stationary phase. The death phase was not considered. Table 3 shows the cell growth parameters of all three strains of L. acidophilus in static cultures. The lag time and specific growth rate of all strains of L. acidophilus were not significantly different ($P>0.05$) when measuring the growth by either. However, the maximum growth was significantly higher ($P<0.05$) for the two strains of human origin than that of pig origin when measuring the cell growth by plate count, but significant differences were not detected by the absorbance.

TABLE 2

KINETIC PARAMETERS OF BILE SALT DECONJUGATION OF LACTOBACILLUS ACIDOPHILUS 016, L1, AND ATCC 43121 IN STATIC CULTURES[1]

Deconjugation	Lag phase (hours)	Deconjugation rate (mM/h)	Deconjugation (%)
Glycocholate			
O16	5.51(0.65)[a]	0.18(0.041)[a]	102(6.9)[a]
L1	4.23(0.19)[a]	0.26(0.024)[a]	100(1.7)[a]
ATCC 43121	4.82(0.36)[a]	0.20(0.026)[a]	99(3.4)[a]
Taurocholate			
O16	6.60(0.48)[a]	0.17(0.024)[a]	108(5.3)[a]
L1	6.53(0.56)[a]	0.07(0.010)[b]	76(3.3)[b]
ATCC 43121	5.64(0.50)[a]	0.18(0.031)[a]	98(5.2)[a]

[1]Based on growth in MRS broth supplemented with 1 mM glycocholate and 1 mM taurocholate bile salts. Each value is the average of three trials; numbers in parentheses represent the standard errors. Values in the same column within the same substrate followed by different superscript letters differ significantly ($P<0.05$).

TABLE 3

KINETIC PARAMETERS OF CELLULAR GROWTH OF LACTOBACILLUS ACIDOPHILUS 016, L1, AND ATCC 43121 IN STATIC CULTURES[1]

Cell growth	Lag phase (hours)	Growth rate (1/h)	Maximum growth[2]
Plate count			
O16	1.62(0.44)[a]	1.49(0.31)[a]	5.54(0.23)[a]
L1	1.18(0.37)[a]	1.54(0.26)[a]	5.92(0.22)[a]
ATCC 43121	1.25(0.07)[a]	1.74(0.06)[a]	5.14(0.04)[b]
Absorbance			
O16	1.63(0.47)[a]	1.07(0.17)[a]	5.71(0.31)[a]
L1	0.58(0.27)[a]	1.19(0.12)[a]	5.86(0.15)[a]
ATCC 43121	0.59(0.47)[a]	0.89(0.12)[a]	5.12(0.12)[a]

[1]Based on growth in MRS broth supplemented with 1 mM glycocholate and 1 mM taurocholate bile salts. Each value is the average of three trials; numbers in parentheses represent the standard errors. Values in the same column and within same method of cell growth measurements followed by different superscript letters differ significantly ($P<0.05$).

[2]Maximum growth is represented as Ln (x/xo).

Bile salt deconjugation and culture growth in pH controlled cultures

Some conjugated bile salts such the sodium glycocholate have a pKa between 3 and 4. Disappearance of sodium glycocholate from the medium during growth thus, could be the result of precipitation because of its low solubility at acidic pH created by acid produced by the lactobacilli during growth. Therefore, experiments were conducted by growing the cultures in MRS broth supplemented with conjugated bile salts maintained at pH higher than the pKa of sodium glycocholate to prevent any precipitation due to low pH.

Figure 4A shows the bile salt deconjugation of both sodium glycocholate and sodium taurocholate by L. acidophilus O16 during growth at pH 5.0. Sodium glycocholate was deconjugated faster than sodium taurocholate. L. acidophilus O16 did not grow well at pH 5.0 as observed by small increases in plate count and absorbance (Figure 4B). The plate count of L. acidophilus decreased 3 Log10 cycles during the 24 hours incubation. The deconjugation, thus, was apparently due to the enzymatic activity of the inoculum.

Figures 5A and 5B show the bile salt deconjugation and growth, respectively, of L. acidophilus O16 at pH 5.4. Bile salt deconjugation appeared faster at pH 5.4 than observed at pH 5.0 in Figure 4A. Moreover, cell growth was also greater than that shown in Figure 4B at pH 5.0. The plate count declined slightly beyond 14 hours. However, the absorbance remained constant.

Figure 6A shows the bile salt deconjugation of L. acidophilus O16 during growth at pH 6.0. Deconjugation of sodium taurocholate seemed slightly faster than that of sodium glycocholate. Figure 6B shows that the plate count decreased 1 Log10 cycle during the period beyond 8 hours. There also was a slight decrease in the absorbance toward the end of the incubation.

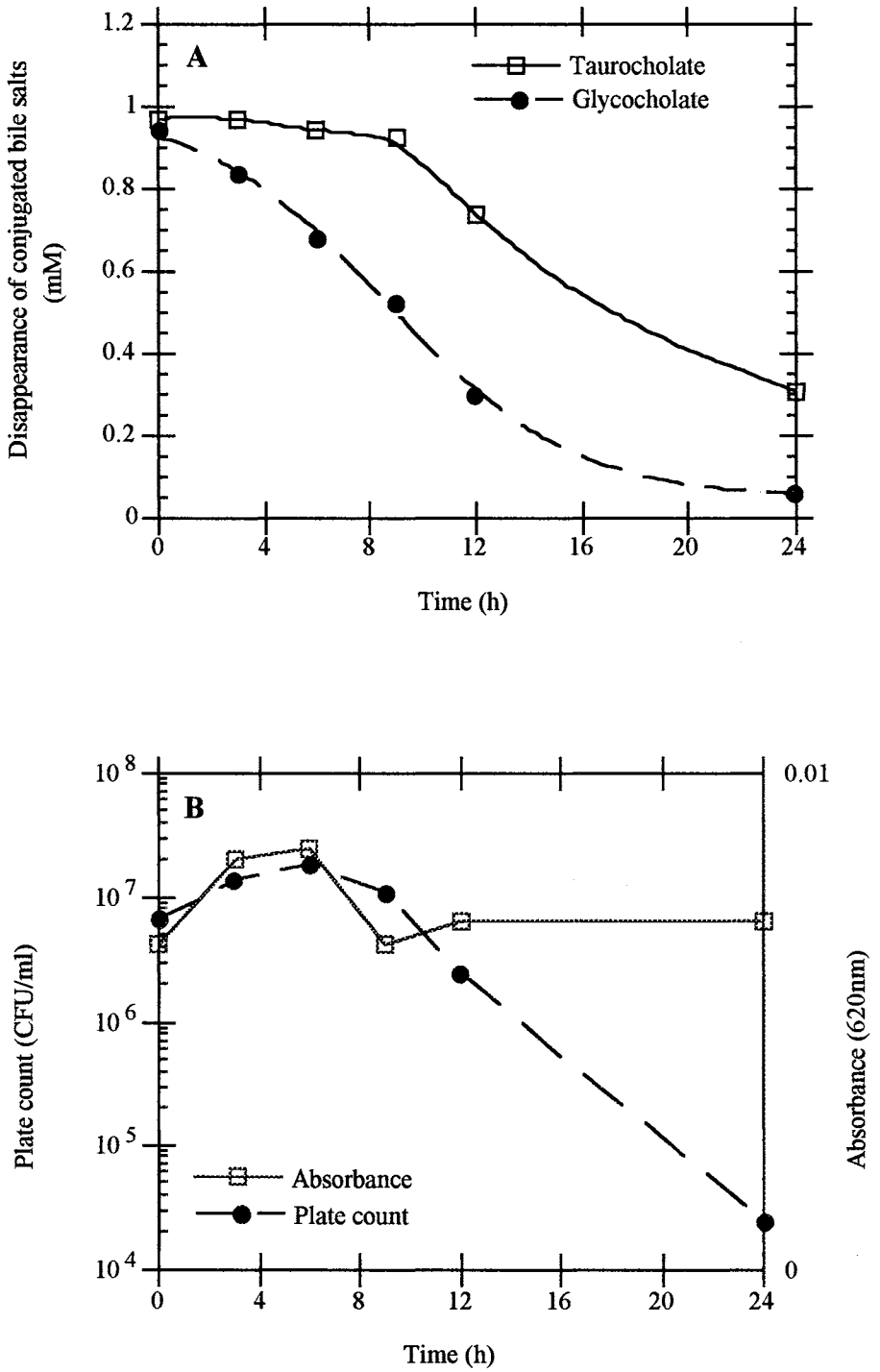

Figure 4. Disappearance of conjugated bile salts (A), and growth (B) of Lactobacillus acidophilus O16 in MRS broth supplemented with 1 mM sodium glycocholate and 1 mM sodium taurocholate at pH 5.0. Each point represents a mean of two trials (S.E. sodium glycocholate = 0.008; S.E. = sodium taurocholate = 0.037; S.E. Log10 of count plate = 0.28; S.E. absorbance = 7.6e-7; 18 df).

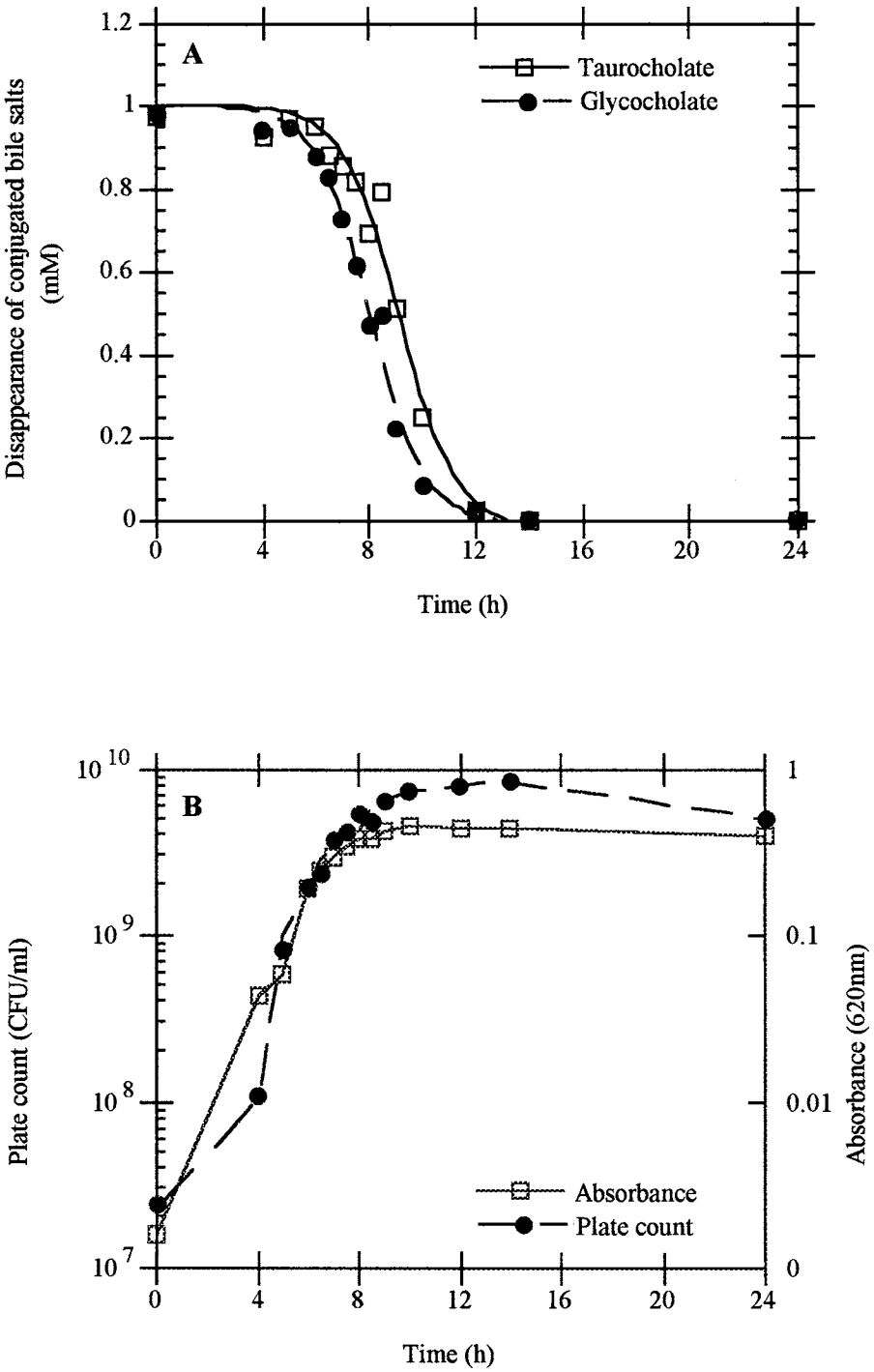

Figure 5. Disappearance of conjugated bile salts (A), and growth (B) of Lactobacillus acidophilus O16 in MRS broth supplemented with 1 mM sodium glycocholate and 1 mM sodium taurocholate at pH 5.4. Each point represents a mean of three trials (S.E. sodium glycocholate = 0.009; S.E. = sodium taurocholate = 0.011; S.E. Log10 of count plate = 0.10; S.E. absorbance = 0.002; 55 df).

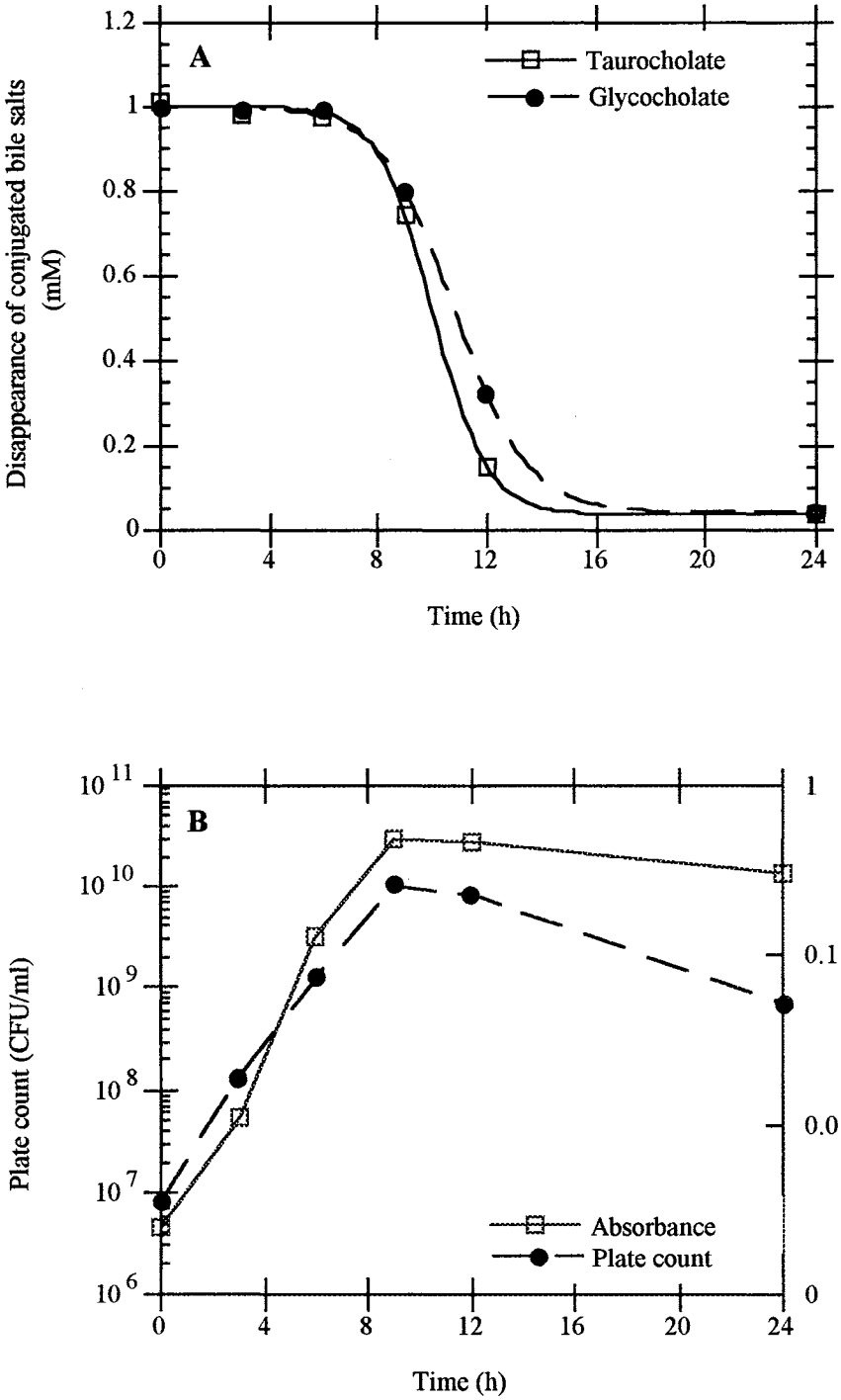

Figure 6. Disappearance of conjugated bile salts (A), and growth (B) of Lactobacillus acidophilus O16 in MRS broth supplemented with 1 mM sodium glycocholate and 1 mM sodium taurocholate at pH 6.0. Each point represents a mean of two trials (S.E. sodium glycocholate = 0.006; S.E. = sodium taurocholate = 0.010; S.E. Log10 of count plate = 0.010; S.E. absorbance = 0.0004; 18 df).

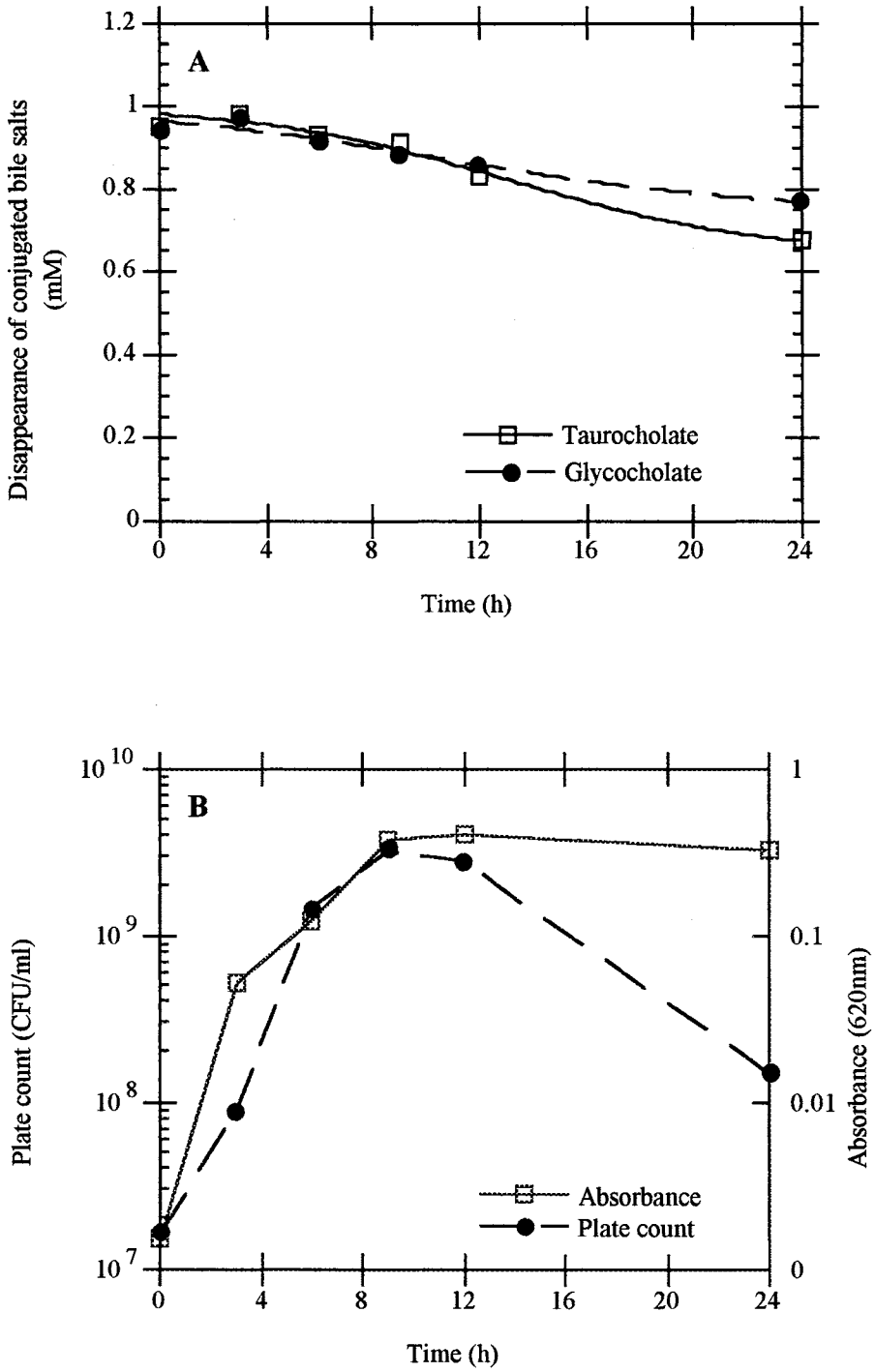

Figure 7. Disappearance of conjugated bile salts (A), and growth (B) of Lactobacillus acidophilus O16 in MRS broth supplemented with 1 mM sodium glycocholate and 1 mM sodium taurocholate at pH 7.0. Each point represents a mean of two trials (S.E. sodium glycocholate = 0.006; S.E. = sodium taurocholate = 0.002; S.E. Log10 of count plate = 0.04; S.E. absorbance = 0.0005; 18 df).

Figure 7 shows the bile salt deconjugation and growth of L. acidophilus O16 at pH 7.0. Bile salt deconjugation of both sodium glycocholate and sodium taurocholate was much slower at pH 7.0 than at pH 5.0, 5.4 and 6.0 shown in the previous figures. The plate count decreased dramatically toward the end of the incubation. However, no decrease in absorbance was noted.

Deconjugation and growth at the different pH levels also were analyzed by the logistic equation to determine the lag phase, maximum rates, and percentages of total deconjugation by L. acidophilus O16.

Table 4 shows that the lag phase of deconjugation of sodium glycocholate was significantly longer ($P<0.05$) at pH 5.4 than at pH 5.0, 6.0 and 7.0. It also shows that the lag phase of deconjugation of taurocholate was significantly longer ($p<0.05$) at pH 5.0, 5.4, and 6.0 than that at pH 7.0. The deconjugation rate of sodium glycocholate by L. acidophilus O16 was significantly higher ($P<0.05$) at pH 5.4 than any other pH tested. The deconjugation rate of sodium taurocholate was significantly higher ($P<0.05$) at pH 5.4 and 6.0 than at either pH 5.0 or 7.0. The significant differences in deconjugation of both glycocholate and taurocholate bile salts at different pHs indicate there was an optimum pH for deconjugating sodium glycocholate and another optimum pH for sodium taurocholate by the culture. Low bile salt deconjugation for both sodium taurocholate and sodium glycocholate was observed at pH 7.0 where significant cell growth was observed. However, high bile salt deconjugation was detected at pH 5.0 where cellular growth was not observed. An explanation for this result could be that some cells of lactobacilli were disrupted at low pH and the bile salt hydrolase (BSH), which is responsible for bile salt deconjugation, was released to the medium. Then, the enzyme hydrolyzed the conjugated bile salts. Most intestinal microorganisms contain an intracellular BSH, and their optimum pH for BSH is in a range from 3.8 to 5.5 rather than at neutral pH (Grill et al, 1995; Stellwag and Hylemon, 1976; Lundeen and Savage, 1990; Gopal-Srivastava and Hylemon, 1988).

TABLE 4

KINETIC PARAMETERS OF BILE SALT DECONJUGATION OF LACTOBACILLUS ACIDOPHILUS 016 IN DYNAMIC CULTURES[1]

Deconjugation	Lag phase (hours)	Deconjugation rate[1] (mM/h)	Deconjugation (%)
Glycocholate			
5.0	0.04(0.85)^{b}	0.06(0.006)^{c}	96(4.5)^{a}
5.4	6.05(0.15)^{a}	0.26(0.020)^{a}	100(2.6)^{a}
6.0	2.25(1.14)^{b}	0.18(0.022)^{b}	101(6.8)^{a}
7.0	2.35(1.70)^{b}	0.01(0.004)^{d}	29(3.1)^{b}
Taurocholate			
5.0	8.35(1.98)^{a}	0.07(0.042)^{b}	70(9.2)^{b}
5.4	6.90(0.18)^{a}	0.23(0.021)^{a}	102(3.6)^{a}
6.0	8.00(0.36)^{a}	0.24(0.044)^{a}	96(4.8)^{a}
7.0	3.60(1.32)^{b}	0.01(0.003)^{b}	36(4.9)^{c}

[1]Based on growth in MRS broth supplemented with 1 mM glycocholate and 1 mM taurocholate bile salts. Each value is the average of three trials; numbers in parentheses represent the standard errors. Values in the same column within the same substrate followed by different superscript letters differ significantly ($P<0.05$).

TABLE 5

KINETIC PARAMETERS OF CELL GROWTH OF LACTOBACILLUS ACIDOPHILUS 016 IN DYNAMIC CULTURES[1]

Cell growth	Lag phase (hours)	Growth rate (1/h)	Maximum growth[2]
Plate count			
5.0	-	-	-
5.4	1.38(0.74)^{a}	0.84(0.13)^{a}	6.05(0.32)^{b}
6.0	0.88(0.43)^{a}	1.04(0.11)^{a}	7.60(0.55)^{a}
7.0	1.92(0.41)^{a}	1.16(0.19)^{a}	5.34(0.31)^{b}
Absorbance			
5.0	-[3]	-	-
5.4	1.29(0.25)^{a}	1.11(0.07)^{a}	5.68(0.06)^{a}
6.0	1.84(0.40)^{a}	0.97(0.12)^{a}	5.45(0.33)^{ab}
7.0	0.55(0.65)^{a}	1.23(0.32)^{a}	5.29(0.16)^{b}

[1]Based on growth in MRS broth supplemented with 1 mM glycocholate and 1 mM taurocholate bile salts. Each value is the average of three trials; numbers in parentheses represent the standard errors. Values in the same column and within same method of cell growth measurements followed by different superscript letters differ significantly ($P<0.05$).

[2]Maximum growth is represented as Ln (x/xo).

[3]It was not determined.

The deconjugation capacity for L. acidophilus O16 for sodium glycocholate was not significantly different ($P<0.05$) at pH 5.0, 5.4 and 6.0. It was, however, lower ($P<0.05$) at pH 7.0 than at the lower pH levels. Deconjugation of sodium taurocholate was not significantly different ($P<0.05$) at pH 5.4 and 6.0, but significantly less ($P<0.05$) was deconjugated at pH 5 and 7.

Table 5 shows the analysis of data from the plate count and the absorbance values by the modified logistic equation. The lag time and specific growth rate at all pH conditions of L. acidophilus O16 cultures were not significantly different ($P>0.05$) when measuring the cell growth by either plate count or absorbance (Data for pH 5 was not included since the culture failed to grow at this pH). There were significant differences in the values of maximum growth. Significantly higher ($P<0.05$) growth was obtained at pH 6.0 when measuring the cell growth by plate count. However, the maximum growth was significantly higher ($P<0.05$) at pH 5.4 when measuring the growth by the absorbance than it was at pH 7.

Deconjugation of sodium glycocholate, based on its disappearance from the growth medium, was observed 1.6 pH units above its pKa; consequently, most of the glycocholate was ionized. Thus the apparent deconjugation was not due to precipitation of the bile salt. Moreover, the uninoculated controls at pH 5.0, 5.4, 6.0, and 7.0 showed no precipitation.

Bile salt deconjugation at pH 6.5. Additional experiments were conducted for all three strains of L. acidophilus in which the growth medium was maintained at pH 6.5. This pH level is similar to that of the small intestine in healthy humans (Mitsuoka, 1978). For these experiments, the concentration of sodium glycocholate and sodium taurocholate were modified to more closely resemble the ratio encountered in healthy humans. This involved a sodium glycocholate to sodium taurocholate molar ratio of 2.3 (Burnett, 1965; Haslewood, 1967; Mallory et al, 1973; Sandine, 1979) so that the growth medium was supplemented with 2.8 mM sodium glycocholate and 1.2 mM sodium taurocholate. Such

culture conditions were an approach to observe the behavior of L. acidophilus at higher concentrations of conjugated bile salts and a nearly neutral pH that may resemble the intestinal environment. Figure 8 shows the bile salt deconjugation and plate count data for L. acidophilus O16 during growth at pH 6.5. After 24 hours incubation 46 % of the sodium glycocholate and 79 % of the sodium taurocholate had been deconjugated. The plate count decreased 1 Log10 cycle beyond 8 hours of incubation.

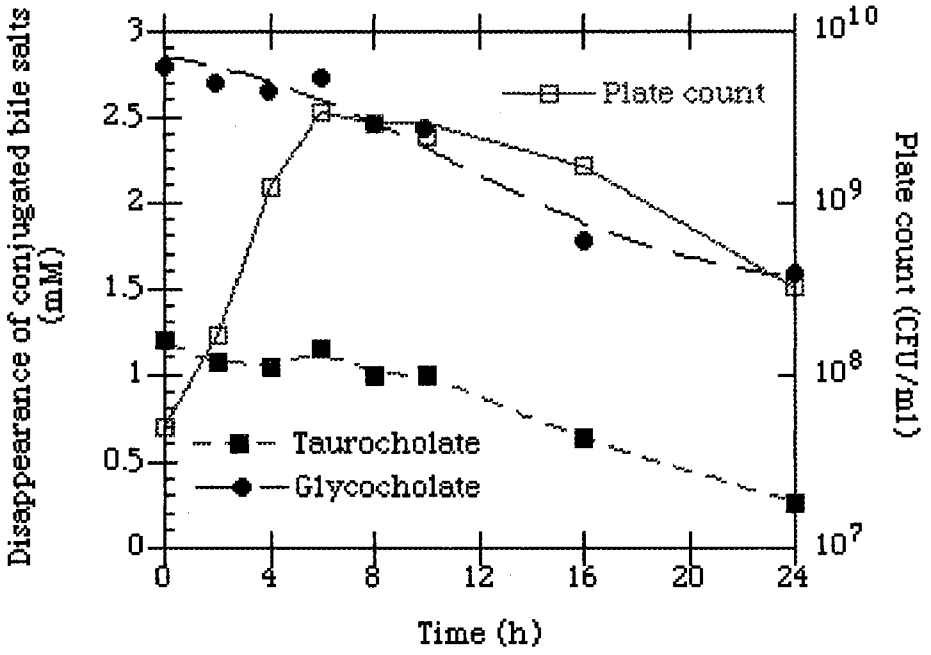

Figure 8. Disappearance of conjugated bile salts and growth of Lactobacillus acidophilus O16 in MRS broth supplemented with 2.8 mM sodium glycocholate and 1.2 mM sodium taurocholate at pH 6.5. Each point represents a mean of two trials (S.E. sodium glycocholate = 0.011; S.E. = sodium taurocholate = 0.007; S.E. Log10 of count plate = 0.013; 26 df).

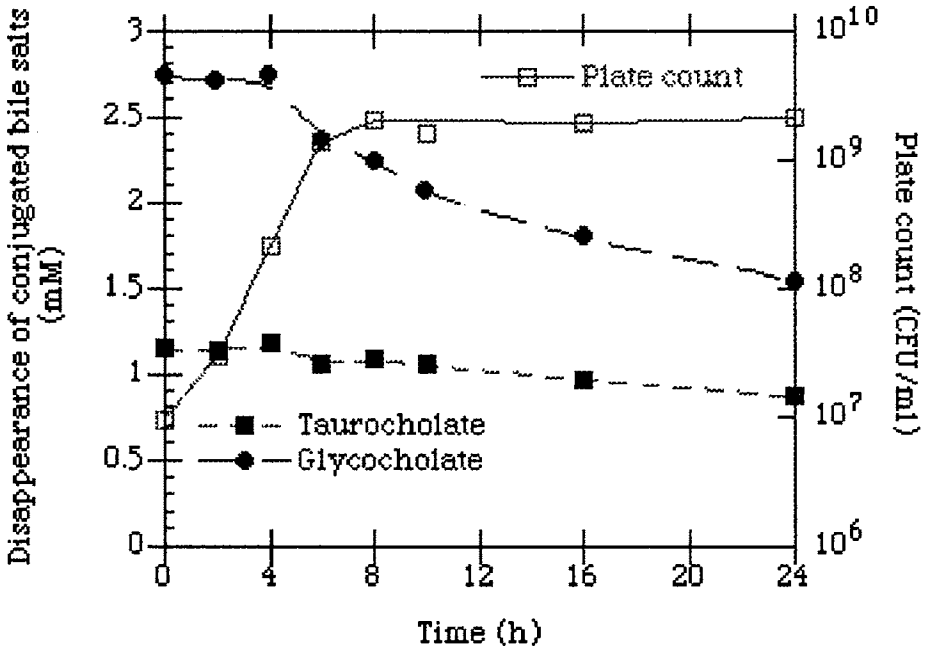

Figure 9. Disappearance of conjugated bile salts and growth of Lactobacillus acidophilus L1 in MRS broth supplemented with 2.8 mM sodium glycocholate and 1.2 mM sodium taurocholate at pH 6.5. Each point represents a mean of two trials (S.E. sodium glycocholate = 0.005; S.E. = sodium taurocholate = 0.002; S.E. Log10 of count plate = 0.009; 26 df).

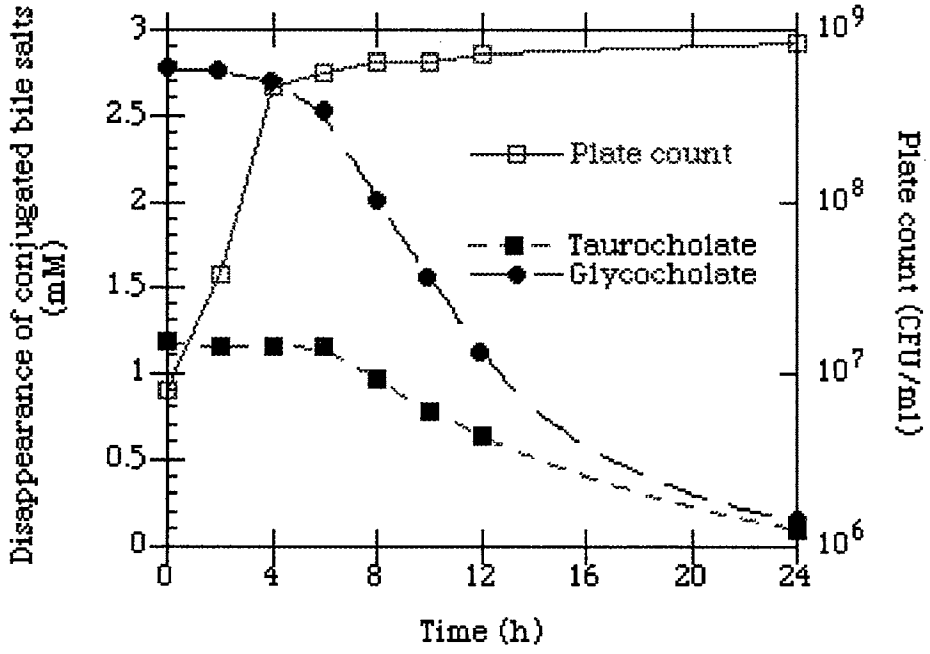

Figure 10. Disappearance of conjugated bile salts and growth of Lactobacillus acidophilus ATCC 43121 in MRS broth supplemented with 2.8 mM sodium glycocholate and 1.2 mM sodium taurocholate at pH 6.5. Each point represents a mean of two trials (S.E. sodium glycocholate = 0.006; S.E. = sodium taurocholate = 0.002; S.E. Log10 of count plate = 0.003; 25 df).

Figure 9 shows that L. acidophilus L1 deconjugated less sodium taurocholate than sodium glycocholate. The plate count did not decrease during incubation beyond 8 hours. Figure 10 shows that both sodium taurocholate and sodium glycocholate were almost totally deconjugated by L. acidophilus ATCC 43121. The plate count for this culture did not decline toward the end of incubation.

Table 6 shows that the deconjugation rates for both sodium glycocholate and sodium taurocholate by L. acidophilus ATCC 43121 were significantly higher ($P<0.05$) than those for strains L1 and O16. The deconjugation rate of sodium glycocholate was higher for all three strains of L. acidophilus than that of sodium taurocholate ($P<0.05$).

TABLE 6

SPECIFIC DECONJUGATION RATES OF ALL THREE STRAINS OF LACTOBACILLUS ACIDOPHILUS AT pH 6.5[1]

Strain	Deconjugation rate (mM/h)	
	Glycocholate	Taurocholate
O16	0.10(0.018)$^{\mathbf{bA}}$	0.058(0.005)$^{\mathbf{bB}}$
L1	0.09(0.012)$^{\mathbf{bA}}$	0.011(0.009)$^{\mathbf{cB}}$
ATCC 43121	0.28(0.015)$^{\mathbf{aA}}$	0.107(0.010)$^{\mathbf{aB}}$

[1]Based on growth in MRS broth supplemented with 2.8 mM glycocholate and 1.2 mM taurocholate bile salts. Each value is the average of two trials; numbers in parentheses represent the standard errors. Means in the same column and without common superscript letters differ significantly ($P<0.05$). Means in the same row and without common upper case superscript letters differ significantly ($P<0.05$).

Effect of conjugated bile salt and cholic acid on culture growth

A death phase was observed for the cultures in some experiments after reaching their maximum growth. Such a death phase was speculated to be the result of the release of free cholic acid by deconjugation. This death was more intense in the cultures grown without pH control. The combined effect of the cholic acid and low pH could be toxic to the cultures. Therefore, L. acidophilus O16 was grown in different bile salt concentrations to determine if apparent cell death was related to the amount of deconjugation. Table 7 shows the effect of various concentrations of conjugated bile salts on the plate counts. Plate counts and pH were determined following 8 and 20 hours of incubation.

TABLE 7

EFFECT OF THE CONCENTRATION OF CONJUGATED BILE SALTS ON THE GROWTH OF LACTOBACILLUS ACIDOPHILUS O16 IN STATIC CULTURES.

Conjugated bile salts[1] (mM)	pH[2]		Log10 Plate count[2] (Log10 CFU/ml)	
	8 hours	20 hours	8 hours	20 hours
0	4.35±0.07**b**	3.95±0.07**c**	9.2±0.03**a**	9.4±0.04**a**
1	4.35±0.07**b**	4.10±0.00**c**	9.2±0.03**a**	9.2±0.12**b**
2	4.40±0.00**b**	4.20±0.00**b**	9.2±0.02**a**	7.7±0.63**c**
4	4.55±0.07**a**	4.65±0.07**a**	8.9±0.02**b**	4.4±0.77**d**

[1]Conjugated bile salts represent the total of equal amounts of sodium glycocholate and sodium taurocholate.

[2]Each value is the average of two trials; numbers in parentheses represent the standard deviations. Means without common superscript letters differ significantly ($P<0.05$).

During the first 8 hours of incubation, there were no significant differences ($P>0.05$) among the control and the media containing 1 and 2 mM conjugated bile salts, but the medium containing 4 mM bile salts had significantly lower ($P<0.05$) numbers compared to the other three media. At 8 hours the populations (plate count) were significantly higher ($P<0.05$) for the culture grown in the media containing 0, 1, and 2 mM bile salts than in the one containing 4 mM bile salts. After 20 hours of incubation, the plate count decreased significantly ($P<0.05$) with each increase in concentration of bile salts. The control maintained a higher viable population than any of the media supplemented with bile salts. Analysis of the media for conjugated bile salts following 20 hours indicated complete deconjugation of the bile salts (data not shown).

Bile salt hydrolase from Lactobacillus acidophilus

Location of bile salt hydrolase

Lactobacillus acidophilus O16 was grown in MRS broth with and without sodium taurocholate, sodium glycocholate, a mixture of both sodium taurocholate and sodium glycocholate. After 18 hours incubation, the spent broth of all media which had contained conjugated bile salts exhibited more BSH activity than did the spent broth of the media which had been prepared without conjugated bile salts. These results suggested that the enzyme was inducible rather than constitutive. It further suggests that the BSH is an extracellular enzyme. However, BSH could have been released into the medium as a consequence of cell lysis caused by cholic acid formation. Experiments with resting cells that were collected from cultures grown in MRS broth without conjugated bile salts were conducted to observe the BSH activity in all three strains of L. acidophilus. Cells of the lactobacilli were harvested after 18 hours of growth and washed twice with 50 mM (pH 5.4) acetate buffer. The cells were resuspended in the buffer (0.1 the volume of the

original culture) and were used for measuring BSH activity within 30 min. Plate counts were performed on the suspensions. After sonication, plate counts were again determined and BSH activity was measured.

Bile salt hydrolase activity of whole cells of L. acidophilus L1 was significantly greater ($P<0.05$) than for L. acidophilus O16 but not significantly greater ($P>0.05$) for strain ATCC 43121 (Table 8). After disrupting the cells by sonication, the BSH activity of the strain 43121 was increased significantly ($p<0.05$) to 32-fold compared to the whole cells. The BSH of strains 016 and L1 for the lysed cells also was somewhat greater 1.08 and 1.15 times respectively than the intact cells ($P>0.05$). For the lysed cells the BSH for strain 43121 was significantly ($P<0.05$) greater than for either of the other two strains. These data suggest that most BSH was intracellular or membrane bound, and that the enzyme was released by sonication.

TABLE 8

BILE SALT HYDROLASE ACTIVITY ON SODIUM GLYCOCHOLATE BY RESTING CELLS AND BY LYSED CELLS OF ALL THREE STRAINS OF LACTOBACILLUS ACIDOPHILUS

	BSH activity[1] ($\times 10^{-9}$ nmol/min/CFU)	
Strain	Whole cells	Lysed cells
ATCC 43121	2.4(0.24)**ab**	79(6.8)**a**
O16	0.86(0.30)**b**	0.93(0.4)**b**
L1	2.6(0.58)**a**	3.0(0.36)**b**

[1]Each value is the average of two trials; numbers in parentheses represent the standard deviation. Means in the same column followed by different superscript letters differ significantly ($P<0.05$).

L. acidophilus O16 was grown in MRS broth free of conjugated bile salts and the cells were collected for locating the BSH. Cells were harvested after 18 hours of growth and washed twice with 50 mM (pH 5.4) acetate buffer. The cells were resuspended in buffer to the original volume of the culture and they were used for measuring BSH activity within 30 min. Such cells were fractionated according with the procedure of Otto et al (1982). The membranes and the intracellular material were tested for BSH activity. The enzyme was present in the cytosol and the cell membrane fractions (Table 9). Whole cells and cell membranes had significantly higher ($P<0.05$) total BSH activity than did the cytosol. Protein content was also higher in whole cells and membranes than in cytosol.

TABLE 9

LOCATION OF BSH ACTIVITY IN LACTOBACILLUS ACIDOPHILUS O16

Fraction	BSH activity[1] (nmol/min/ml)	Protein[1] (mg/ml)	Specific activity[1] (nmol/min/mg)
Whole cells	13.9(2.3)^{a}	0.21(0.01)^{a}	66.8(15.6)^{a}
Cell membranes	17.9(2.7)^{a}	0.24(0.01)^{a}	74.6(13.9)^{a}
Cytosol	3.8(0.76)^{b}	0.08(0.02)^{b}	48.1(21.1)^{a}

[1]Each value is the average of two trials; numbers in parentheses represent the standard deviation. Means in the same column followed by different superscript letters differ significantly ($P<0.05$).

The specific activity was similar in all three cell fractions. The results suggested that the enzyme might be bound to the cell membrane of L. acidophilus. Cell membranes of L. acidophilus were treated with Tween 80 and Tween 20 to try to remove the enzyme from the cell membrane, but non-significant increases in activity were obtained when compared to the control without Tween (data not shown).

Effect of conjugated bile salts on bile salt hydrolase

Because bile salt hydrolase was always found in the spent broth when strains of L. acidophilus were incubated in the presence of conjugated bile salts, L. acidophilus O16 was tested for its BSH content in spent broth and cells at different concentration of conjugated bile salts. Cells of the lactobacilli were collected from MRS broth with and without conjugated bile salts after 18 hours of growth and washed twice with 50 mM (pH 5.4) acetate buffer. The cells were resuspended in buffer to the original volume of the culture and disrupted by sonication. Five milliliters of spent broth from media with conjugated bile salts was dialyzed through 12, 000 to 14, 000 cut off dialysis membranes against 1 liter 50 mM (pH 5.4) acetate buffer for 18 hours. BSH activity of both cell extracts and spent broth was measured within 30 min.

Table 10 shows the effect of the concentration of sodium glycocholate and sodium taurocholate on the BSH activity in the spent broth and cells. The BSH activity in spent broth from cultures with concentrations of 2 and 4 mM of conjugated bile salts was significantly higher ($P<0.05$) than the BSH activity in cultures without and with 1 mM conjugated bile salts. Contrary to the results of BSH activity in the spent broth, BSH activity in cells grown from cultures with concentrations of 2 and 4 mM conjugated bile salts was significantly lower ($p<0.05$) than the BSH activity in cultures grown without and with 1 mM conjugated bile salts.

To demonstrate that bile salt hydrolase activity in the spent broth was a consequence of cell lysis at high concentrations of conjugated bile salts, L. acidophilus ATCC 43121 was grown in the presence of 4 mM conjugated bile salts (2.8 mM sodium glycocholate and 1.2 mM sodium taurocholate), and without conjugated bile salts (control). The BSH activity was quantified at 4 hours intervals during 24 hours of incubation.

TABLE 10

EFFECT OF THE CONCENTRATION OF CONJUGATED BILE SALTS IN GROWTH MEDIUM ON THE BILE SALT HYDROLASE OF LACTOBACILLUS ACIDOPHILUS O16.

Conjugated bile salts[1] (mM)	Bile salt hydrolase activity (nmol/min/ml)	
	Spent broth[2]	Cells[2]
0	4.8(1.7)^{c}	14.2(2.5)^{a}
1	12.4(3.3)^{c}	13.1(1.8)^{a}
2	31.3(6.8)^{b}	4.6(1.2)^{b}
4	43.9(5.7)^{a}	2.1(0.2)^{b}

[1]Conjugated bile salts represents 50:50 mixture of sodium taurocholate and sodium glycocholate.

[2]Each value is the average of two trials; numbers in parentheses represent the standard deviation. Means in the same column followed by different superscript letters differ significantly ($P<0.05$).

Figure 11 shows the growth and BSH activity of L. acidophilus ATCC 43121 when growing in the presence of conjugated bile salts (after 24 hours incubation, all sodium taurocholate and sodium glycocholate were deconjugated). The number of CFU decreased seven log cycles after 4 hours in the broth containing the conjugated bile salts. The spent broth from the culture contained very low BSH activity, and after reaching its maximum growth, the plate count remained constant through the end of the incubation for this medium. The increases in BSH activity of the spent broth from the bile salt culture medium coincided with decreases in the number of CFU/ml in the same culture medium.

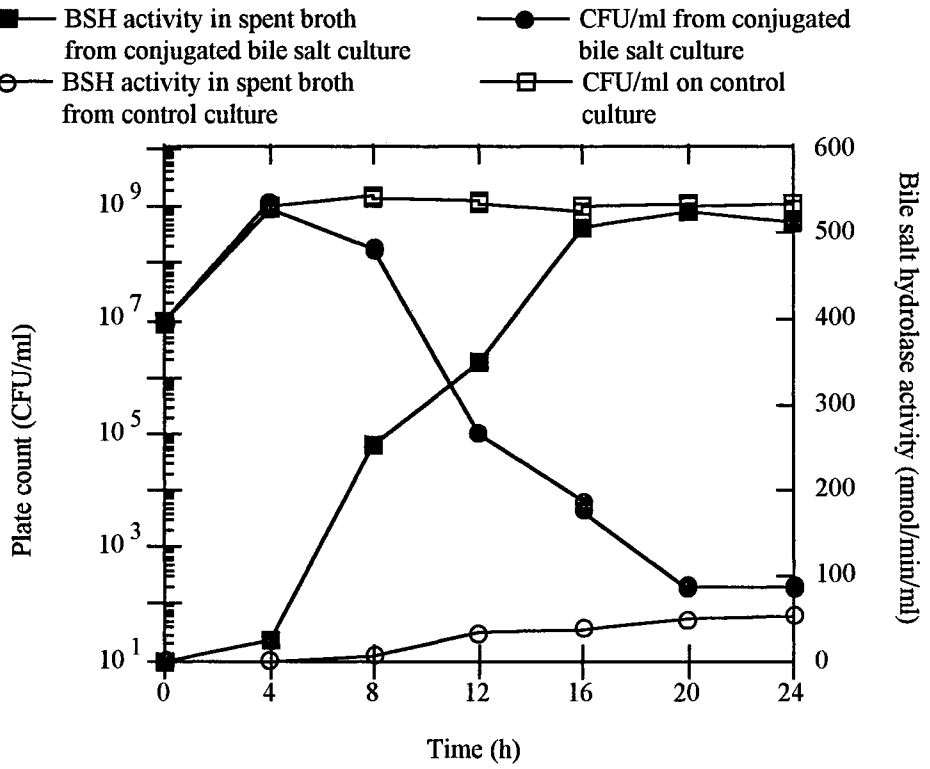

Figure 11. Plate count and bile salt hydrolase activity (spent broth) in cultures of Lactobacillus acidophilus ATCC 43121 supplemented with 2.8 mM sodium glycocholate and 1.2 mM sodium taurocholate. Bile salt hydrolase activity was measured using sodium glycocholate as a substrate.

The spent broths of from L. acidophilus L1 and O16 also were observed to contain high amounts of BSH when the microorganisms were grown in MRS broth supplemented with 2.8 mM sodium taurocholate and 1.2 sodium taurocholate. Table 11 shows the content of BSH activity in spent broth of all three strains of L. acidophilus grown in static cultures. The spent broth from L. acidophilus ATCC 43121 had much more BSH activity on both sodium taurocholate and sodium glycocholate than did the spent broth from strains L1 and O16 ($P<0.05$).

TABLE 11

BILE SALT HYDROLASE ACTIVITY IN SPENT BROTH AFTER CELL GROWTH IN MRS BROTH SUPPLEMENTED WITH 2.8 mM SODIUM GLYCOCHOLATE AND 1.2 mM SODIUM TAUROCHOLATE

Strain	Bile salt hydrolase activity[1] (nmol/min/ml)	
	Sodium taurocholate	Sodium glycocholate
O16	37.2(4.1)^{c}	152.9(6.8)^{b}
L1	63.8(10.4)^{b}	189.0(1.6)^{b}
ATCC 43121	477.1(7.9)^{a}	577.7(13.5)^{a}

[1]Each value is the average of two trials; numbers in parentheses represent the standard deviation. Means in the same column followed by different superscript letters differ significantly ($P<0.05$).

Purification of bile salt hydrolase

Bile salt hydrolase was partially purified from the spent broth of cultures of L. acidophilus which had been grown in MRS broth supplemented with 2 mM sodium glycocholate and 2 mM sodium taurocholate and from cell free extracts of cultures of L. acidophilus grown in the absence of conjugated bile salts. All strains of lactobacilli were grown in static cultures.

Methanol precipitation. Table 12 shows that fractions precipitated by 0-33.3 % of the enzyme solution with methanol contained the highest total activity and the highest specific activity. The specific activity in fraction 33.3-50 % was significantly higher ($P<0.05$) than

that in fraction 50-66.6 %, but significantly lower ($P<0.05$) than in the 0-33.3 %.

TABLE 12

EFFECT OF METHANOL IN PRECIPITATING BILE SALT HYDROLASE FROM SPENT BROTH FROM LACTOBACILLUS ACIDOPHILUS O16 AT pH 4.0

Methanol fraction (% v/v)	Bile salt hydrolase activity[1] (nmol/min/ml)	Specific activity[1] (nmol/min/mg)
0-33.3	23.9(0.70)^{a}	183.5(4.94)^{a}
33.3-50.0	5.56(0.65)^{b}	148.0(8.97)^{b}
50.0-66.6	0.25(0.20)^{c}	10.62(8.54)^{c}

[1]Each value is the average of three trials; numbers in parentheses represent the standard deviation. Means in the same column followed by different superscript letters differ significantly ($P<0.05$).

Ammonium sulfate precipitation. As a second step in BSH purification, the BSH, in the methanol fraction, was dissolved in 10 ml buffer A (see Materials and Methods) and precipitated by ammonium sulfate. Four concentrations of ammonium sulfate were tested for recovering BSH. Table 13 shows that fractions precipitated by 40-60 % and 60-80 % saturation of the enzyme solution with ammonium sulfate contained the highest total activity and the highest specific activity. The specific activity in fraction 0-20 was significantly higher ($P<0.05$) than that in fraction 20-40, but significantly lower ($P<0.05$) than in the 40-60% and 60-80% fractions. Similar recoveries of BSH were obtained from metanolic fractions of spent broth and cell free extracts from strains L1 and ATCC 43121 (data not shown).

TABLE 13

AMMONIUM SULFATE PRECIPITATION OF BILE SALT HYDROLASE OF LACTOBACILLUS ACIDOPHILUS O16

Ammonium sulfate fraction (% of saturation)	Activity[1] (nmol/min/ml)	Specific activity[1] (nmol/min/mg)
0-20	1.67(0.38)^{c}	23.97(5.01)^{b}
20-40	4.90(1.97)^{b}	7.29(3.62)^{c}
40-60	24.17(3.09)^{a}	51.22(8.11)^{a}
60-80	22.35(1.97)^{a}	43.42(1.69)^{a}

[1]Each value is the average of three trials; numbers in parentheses represent the standard deviation. Means in the same column followed by different superscript letters differ significantly ($P<0.05$).

Gel filtration. The bile salt hydrolase obtained by the methanol and ammonium sulfate fractionation from spent broth and from intracellular material was further purified by gel chromatography. Figure 12 shows the elution profile from gel chromatography of BSH purified from the spent broth of L. acidophilus ATCC 43121. Three absorbance (280nm) peaks were observed in this chromatogram. The first one was eluted with the void volume and contained no enzyme activity. The enzyme activity was eluted between the second and third absorbance peaks. The largest portion of material absorbing light at 280 nm was in the third peak in which only small amounts of enzyme activity were found in comparison with the second peak.

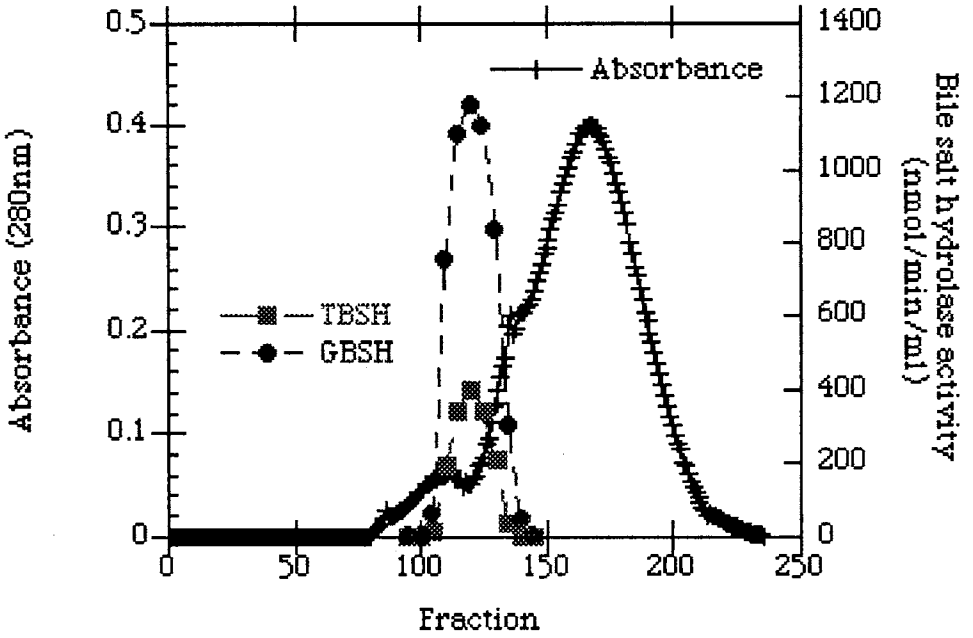

Figure 12. Elution profile of ammonium sulfate precipitate from spent broth of bile salt hydrolase activity produced by Lactobacillus acidophilus ATCC 43121 on Sephadex G-200. Each 0.8 ml fraction was monitored at 280 nm and fractions containing protein were assayed for bile salt hydrolase activity.

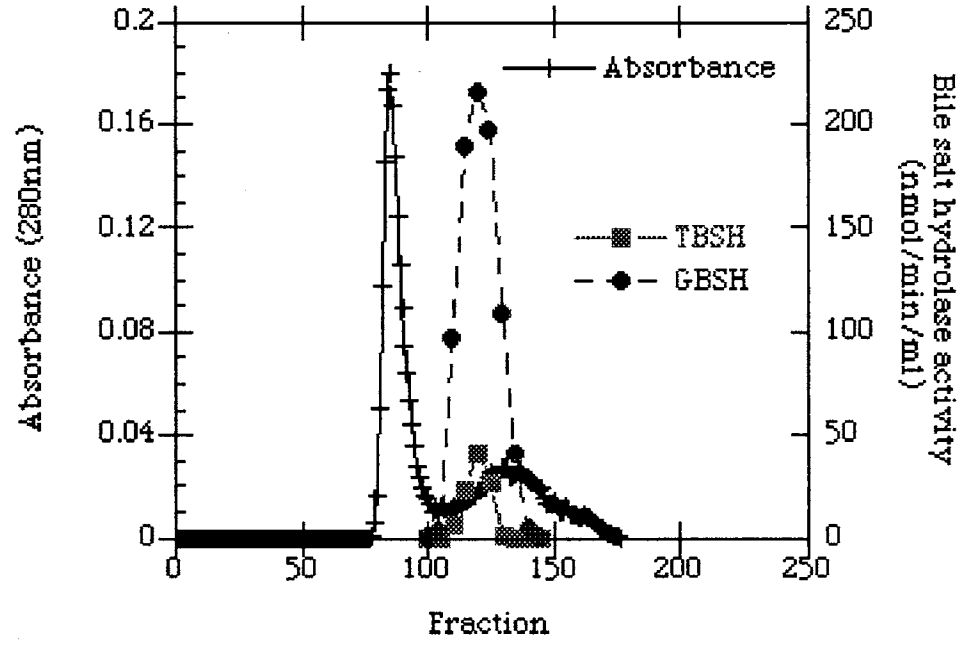

Figure 13. Elution profile of ammonium sulfate precipitate from cell extracts of bile salt hydrolase activity produced by Lactobacillus acidophilus ATCC 43121 on Sephadex G-200. Each 0.8 ml fraction was monitored at 280 nm and fractions containing protein were assayed for bile salt hydrolase activity.

Figure 13 shows the gel chromatogram of BSH from the intracellular material of a L. acidophilus ATCC 43121. Two absorbance peaks were eluted in that chromatogram. An absorbance peak was observed in the fraction corresponding to the void volume. The first peak also had a much larger portion of material absorbing light at 280 nm than that of the second peak. In addition, the first peak did not contain BSH activity. The BSH activity was associated with the second peak, but the maximum absorbance value did not correspond to the maximum enzymatic activity. Enzyme purification from strain ATCC 43121 was higher from the spent broth than that from crude cell extracts. Similar gel chromatograms were observed for strains 016 and L1 (Figures C1 through C4 show both BSH from spent broth and BSH from intracellular material chromatograms from those strains in Appendix C). The active fractions from gel chromatography of all spent broth material were pooled and concentrated with Centricell membranes. The concentrated solutions increased the purification of BSH from 59.7 in L. acidophilus L1 to 115 in L. acidophilus ATCC 43121 (Tables C1 through C6 in Appendix C show the BSH purification from spent broth and intracellular material using both glycocholate and taurocholate bile salts). The BSH from all three strains of L. acidophilus from different sources (spent broth and intracellular) were quite similar in their elution times in gel chromatography suggesting that the BSH molecular weight was similar in all three strains of L. acidophilus.

Molecular weight of BSH from L. acidophilus

A protein cocktail with three different proteins of different molecular weights were chromatographed on Sephadex G-200. The conditions of elution were similar to those

used on the BSH gel chromatography. Blue Dextran was included to show the void volume of the column. The protein cocktail and Blue Dextran 2000 chromatogram were compared to the chromatogram of BSH from L. acidophilus ATCC 43121. Bile salt hydrolase activity was eluted between the peaks corresponding to Immunoglobulin G and Human Serum Albumin with a molecular weight of 158 KDa and 69 KDa respectively (see Figure D1 in Appendix D). The retention factor of all three proteins and BSH were obtained and plotted against their molecular weight (see Figure D2 in Appendix D). A molecular weight of about 126 KDa for BSH from strain ATCC 43121 was calculated by gel filtration chromatography. Bile salt hydrolase from L. acidophilus strains O16 and L1 had similar elution times; so that, their molecular weights were calculated to be similar to that of the BSH of ATCC 43121.

Polyacrylamide gels

The active fractions from gel chromatography from L. acidophilus ATCC 43121 were electrophoresed in non-denaturing gels. As demonstrated in Figure 14, two major bands showed increasing levels of Coomassie blue dye. Those two bands as well as the protein bands observed between those two were removed from a gel without Coomassie blue stain treatment. The small pieces of polyacrylamide gel containing the proteins were submerged in buffer A supplemented with 2.5 mM glycocholate.

After 30 min reaction, the amount of BSH activity was measured. Most of the BSH activity was found in the second major band that presented increases in protein specificity. Little bile salt hydrolase activity was observed in other protein bands.

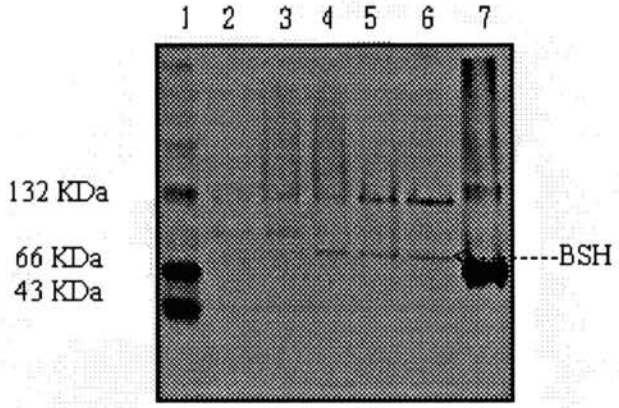

Figure 14. Non-denaturing gel chromatograms of several fractions of Lactobacillus acidophilus 43121. Lane 1 and 7, protein markers; Lane 2, spent-broth; Lane 3, methanol fraction; Lane 4, Ammonium sulfate fraction; Lane 5, Sephadex G-200 fraction (1); Lane 6, Sephadex G-200 fraction (2).

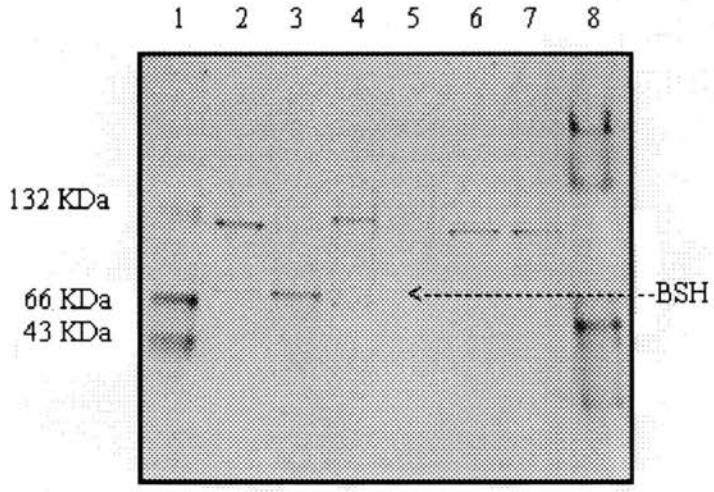

Figure 15. Non-denaturing gel chromatograms of Sephadex G-200 fractions of all three strains of Lactobacillus acidophilus. Lane 1 and 8, protein markers; Lane 2, ATCC 43121 cells; Lane 3, ATCC 43121 spent-broth; Lane 4, L1 cells; Lane 5, L1 spent-broth; Lane 6, O16 cells; Lane 7, O16 spent-broth.

Gel chromatography fractions of the other strains of L. acidophilus from both spent broth and intracellular content also were electrophoresed on non-denaturing gels, but no BSH activity was detected in those fractions (Figure 15). L. acidophilus ATCC 43121 produced more BSH than that of the other two strains. The lower levels of BSH activity in the other two strains may have made it impossible to detect activity in the gel fractions.

Physical and chemical characteristics of BSH

Effect of pH on BSH activity. Table 14 shows the pH ranges supporting optimum BSH activity from all three strains of L. acidophilus on both sodium glycocholate and sodium taurocholate. In general, all three strains of L. acidophilus had optima BSH activity at relatively low pH (see figures E1, E2 and E3 in Appendix E). The optimum pH range was not the same for each conjugated bile salt. Bile salt hydrolase from L. acidophilus O16 was most active on sodium taurocholate at pH 5.0-6.0 and on sodium glycocholate at pH 3.5-5.0. However, strains L1 and ATCC 43121 were most active on taurocholate at pH 3.5-4.5 and on glycocholate at pH 4.0-6.0.

TABLE 14

OPTIMUM pH OF BILE SALT HYDROLASE FROM LACTOBACILLUS ACIDOPHILUS

Strain	Taurocholate[1]	Glycocholate[1]
O16	5.0-6.0	3.5-5.0
L1	3.5-4.0	4.0-6.0
ATCC 43121	3.5-4.5	4.0-5.5

[1]Based on activity on acetate-phosphate buffer 50 mM, 1 mM EDTA at different pH. Each value is the average of two trials.

Enzymatic efficiency of BSH. Enzyme efficiency, defined as Vmax/Ks[=]μM/min/mM, was higher for strain ATCC 43121 than for either strains L1 (150-fold) or O16 (500-fold)(Figure 16).

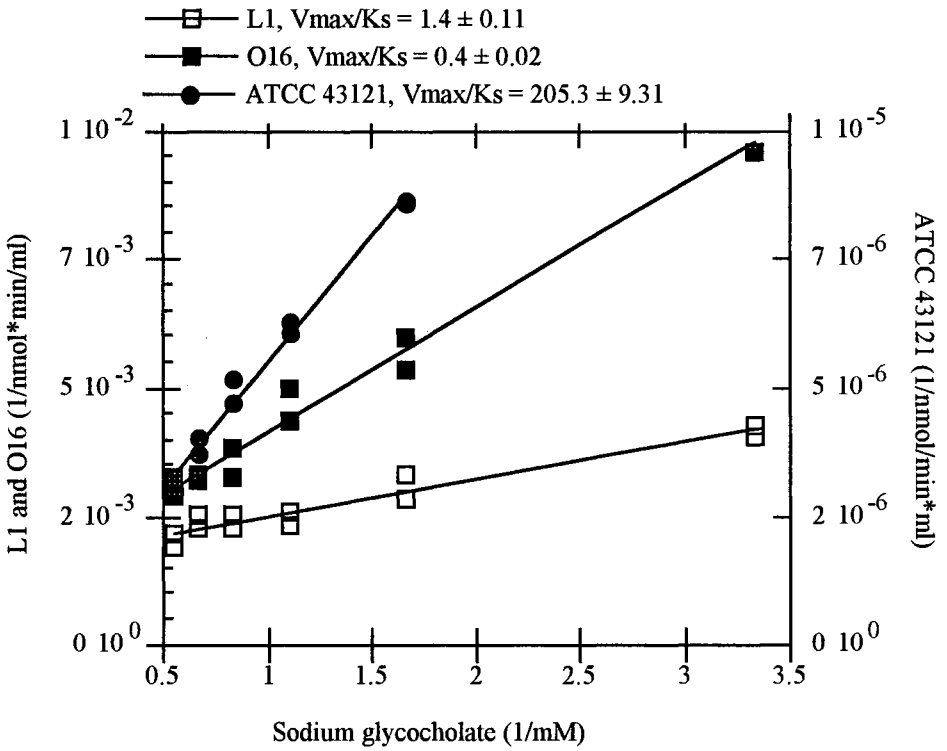

Figure 16. Lineweaver-Burk plots based on sodium glycocholate as the substrate.

Substrate competition and product inhibition in BSH. Table 15 and 16 shows that bile salt hydrolase activity on sodium taurocholate was inhibited 31 % by the presence of 2 mM sodium glycocholate and on sodium glycocholate was inhibited 5 % by the presence of 2 mM sodium taurocholate for strain ATCC 43121. Bile salt hydrolase activity on sodium taurocholate was inhibited 58 % in the presence of 2 mM sodium glycocholate and 16 % on sodium glycocholate in the presence of 2 mM sodium taurocholate for strain L1. Bile salt hydrolase activity on sodium taurocholate was more inhibited by the presence of sodium glycocholate. This may be explained because of a higher affinity of BSH for

sodium glycocholate than that for sodium taurocholate. Besides substrate inhibition of BSH, it was found that sodium cholate was a strong inhibitor of BSH activity on both sodium taurocholate and sodium glycocholate (Figures E4 and E5 in Appendix E).

TABLE 15

BILE SALT HYDROLASE ACTIVITY IN THE PRESENCE OF TAUROCHOLATE AND A MIXTURE OF BOTH GLYCOCHOLATE AND TAUROCHOLATE

Strain	Bile salt hydrolase activity (nmol/min/ml)	
	TBSH[1]	TBSH + Glycocholate[2]
O16	nd[3]	nd
L1	17.1(0.6)**a**	7.1(1.1)**b**
ATCC 43121	89.4(3.5)**a**	61.0(0.1)**b**

[1]Based on BSH activity on 2 mM taurocholate; [2]Based on BSH activity on 2 mM taurocholate in the presence of 2 mM glycocholate; Each value is the average of two trials; numbers in parentheses represent the standard deviation. Treatments in the same row followed by different superscript letters differ significantly ($P<0.05$).[3]It was not determined.

TABLE 16

BILE SALT HYDROLASE ACTIVITY IN THE PRESENCE OF TAUROCHOLATE AND A MIXTURE OF BOTH GLYCOCHOLATE AND TAUROCHOLATE

Strain	Bile salt hydrolase activity (nmol/min/ml)	
	GBSH[1]	GBSH + Taurocholate[2]
O16	69.3(0.5)**A**	68.8(0.63)**A**
L1	102.8(1.7)**A**	86.5(5.7)**B**
ATCC 43121	2337.0(9.1)**A**	2212.9(18)**B**

[1]Based on BSH activity on glycocholate; [2]Based on BSH activity on glycocholate on the presence of 2 mM taurocholate. Each value is the average of two trials; numbers in parentheses represent the standard deviation. Treatments in the same row followed by different superscript letters differ significantly ($P<0.05$).

Activity of BSH on different conjugated bile salts. Bile salt hydrolase from L. acidophilus ATCC 43121 was tested for its activity towards dihydroxy and trihydroxy conjugated bile salts. Table 17 shows that BSH from strain ATCC 43121 had significantly more activity towards dihydroxy-glycoconjugated bile salts than towards trihydroxy-glycoconjugated bile salts. There were no significant differences ($P>0.05$) among the bile salt conjugates of taurine.

TABLE 17

BILE SALT HYDROLASE ACTIVITY IN THE PRESENCE OF TRIHYDROXY AND DIHYDROXY BILE SALT CONJUGATES OF GLYCINE AND TAURINE

Substrate	Bile salt hydrolase activity [1] (nmol/min/ml)
Glycocholate (3□,7□,12□-trihydroxy-)	1,405(70.7)**b**
Taurocholate	122(38.1)**c**
Glycochenodeoxycholate (3□,7□-dihydroxy-)	4,685(77.0)**a**
Taurochenodeoxycholate	174(10.6)**c**
Glycodeoxycholate (3□,12□-dihydroxy-)	4,755(70.7)**a**
Taurodeoxycholate	72(4.24)**c**

[1]Based on BSH activity on 5 mM glycoconjugated or 5 mM tauroconjugated bile salts; Each value is the average of two trials; numbers in parentheses represent the standard deviation. Means followed by different superscript letters differ significantly ($P<0.05$).

CHAPTER V

DISCUSSION

The HPLC method for quantifying BSH permitted the detection of both sodium taurocholate and sodium glycocholate that have different retention times. Bile salt hydrolase from all three strains of L. acidophilus presented a kinetic of zero order at pH 5.4 and at 37 °C. Substrate limitation was observed after 90 % of consumption. Therefore, special care was taken when higher enzymatic activities were observed and more than 90 % of the substrate was consumed. In such a case lesser amounts of enzyme were used and/or shorter time of reaction was employed.

Bile salt hydrolase stability was improved in the presence of EDTA. This salt complexes with heavy metal ions that may inhibit some proteases. Proteases might adversely affect BSH activity as suggested by Lundeen and Savage (1990). The enzyme was also stable in various buffer salts such a citrate, phosphate and acetate. These buffer salts have been used during purification of BSH from different intestinal microorganisms (Lundeen and Savage, 1990; Grill et al, 1995; Gopal-Srivastava and Hylemon, 1988; Stellwag and Hylemon, 1976). Reducing agents such a □-mercaptoethanol did not improve bile salt hydrolase activity. This suggests the BSH is not susceptible to oxidative damage. Storage at -20 °C was more efficient for maintaining BSH activity than was 5 °C. Proteases may also cause failure of BSH activity at 5 °C.

Because bile salt deconjugation was observed in media without thioglycolate, low oxidation/reduction (O/R) potential was not necessary for the deconjugation activity. Masuda (1981) found that BSH activity of four strains of Bacteroides and four strains of

Clostridium, which were strict anaerobic intestinal microorganisms, was insensitive to oxygen. However, Aries and Hill (1970a) reported that anaerobiosis was necessary for optimal production of bacterial BSH in the intestinal tract. Moreover, Gilliland and Speck (1977) also reported that deconjugation of bile salts by L. acidophilus required low O/R potential. Contrary to the results of Aries and Hill (1970a), and Gilliland and Speck (1977), but in agreement with the results of Masuda (1981), the results described here show that deconjugation does not require low O/R potential and there were no harmful effects on BSH activity in the absence of a reducing agent such a thioglycollic acid or □-mercaptoethanol.

Grill et al (1995b) reported that heat sterilization of conjugated bile salts such a sodium taurocholate in broth caused partial hydrolysis of the bile salt. However, Gilliland and Speck (1977) observed that heat sterilization of MRS broth supplemented with sodium taurocholate did not affect the bile salt or deconjugation. In agreement with the results of Gilliland and Speck (1977), it was observed that heat sterilization of both sodium taurocholate and sodium glycocholate did not affect the concentration of conjugated bile salts nor bile salt deconjugation.

Bile salt deconjugation, as a response of BSH activity, increased only when the cultures reach their maximum cell growth in static culture conditions. High BSH activity was observed at the stationary phase of growth of B. fragilis by Hylemon and Stellwag (1976), and of Lactobacillus sp. strain 100-100 by Lundeen and Savage (1990). The latter researchers suggested that the BSH activity was regulated by the growth phase. Since the lowest pH of all strains of L. acidophilus in static cultures was always found in the stationary phase and the BSH activity occurred preferentially at low pH, BSH deconjugation likely was not the result of the growth phase, but the result of the pH. The pH and the cell growth were positively and negatively correlated, respectively, with both sodium taurocholate and sodium glycocholate bile salt deconjugation ($P<0.0001$, see

Table G1 in Appendix F). Acid hydrolysis of conjugated bile acids is not common (Haslewood, 1967), but precipitation at acidic pH can occur. The pKa of sodium glycocholate is 3.9 ± 0.1 (Fini and Roda, 1987) and that of sodium taurocholate is 0.0 ± 1.0 (Irving et al, 1982). Therefore, conjugated bile acids are less soluble at low pH than at neutral or alkaline pH. Apparent bile salt deconjugation could be due to precipitation or hydrolysis at acidic pH (Zhu and Brown, 1990). Moreover, bile salt precipitation by salt formation with calcium ions that may be present in biological fluids and culture media has been observed (Hofmann and Mysels, 1992).

In some experiments cholic acid released during deconjugation was measured. Theoretically, 2 mM cholic acid should have been detected at the end of a total bile salt deconjugation of a mixture of 1 mM sodium glycocholate and 1 mM sodium taurocholate. However, the solubility of cholic acid at acidic pH is lower than that of conjugated bile acids. Because of this, measurements of cholic acid had more variability than that of conjugated bile salts. The low solubility of cholic acid at low pH was probably responsible for the crystals observed at the bottom of the sample tubes after 24 hours of sample storage. Such crystals were difficult to dissolve and thus probably affected the concentration of cholic acid detected. However, cholic acid measurements were valuable for verifying that deconjugation was truly occurring due to enzymatic hydrolysis. Stability of conjugated bile salts at different pH levels also was verified by incubating MRS broth containing conjugated bile salts at pH 5.0, 5.4, 6.0 and 7.0 for 24 hours at 37 °C without microorganisms. There was no chemical hydrolysis or bile salt precipitation at these pH levels. Moreover, sodium glycocholate in 5 mM concentration was tested in a range of pH 1 to 7. There was no significant ($P>0.05$) precipitation at pH 4.0 which was the lowest pH observed after 24 hours incubation of cultures of L. acidophilus in MRS broth (Figure B2 in Appendix B). Thus, disappearance of sodium glycocholate in the cultures was not due to precipitation by low pH but due to deconjugation.

All three strains of L. acidophilus used were previously compared for their cholesterol

assimilation capacity and their bile tolerance. All them showed non-significant differences in their ability to assimilate cholesterol (Buck and Gilliland, 1994). Also, L. acidophilus ATCC 43121 and O16 did not show significant differences in their ability to deconjugate taurocholate. However, L. acidophilus L1 had significantly less ability to deconjugate sodium taurocholate compared to the strains ATCC 43121 and O16. The results by Buck and Gilliland (1994) are in agreement with those results shown in this study. That is, L. acidophilus L1 had a lower deconjugation rate on sodium taurocholate than did the two other strains.

In this study, sodium glycocholate also was used as a substrate for testing bile deconjugation activity. L. acidophilus L1 had a higher deconjugation rate on sodium glycocholate than on sodium taurocholate as did strain O16. Therefore, these two strains might be important for potential hypocholesterolemic activity based on their capacity to hydrolyze sodium glycocholate since it is the major conjugated bile salt in human beings. The cell growth was measured by absorbance and count plate. The lag time of all three strains of L. acidophilus was not significantly different when measuring the cell growth in static cultures by absorbance or by plate count. However, absorbance measurements could not detect the reduction of viable cells in later phases of growth in static cultures after bile salt deconjugation began. Moreover, the absorbance measurements could not discriminate between the maximum cell growth among all three strains of L. acidophilus in static and pH controlled cultures. The formation of free cholic acid at acidic pH may have affected absorbance measurements. Therefore, culture growth should be measured by plate count when free bile salts or any other precipitable components are present in the culture media.

Deconjugation of both sodium glycocholate and sodium taurocholate was pH dependent. L. acidophilus O16 had its maximum cell growth at pH 6.0, but its maximum deconjugation rate for sodium glycocholate was observed at pH 5.4 and its maximum deconjugation rate for sodium taurocholate was observed at pH 5.4 and at pH 6.0. There is no data available in the literature about bile salt deconjugation in culture conditions by

any intestinal microorganisms. However, Stellwag and Hylemon (1976) observed that whole cell suspensions of Bacteroides fragilis subsp. fragilis deconjugated sodium glycocholate optimum at pH 4.2. Also Gilliland and Speck (1977) observed that resting cells of L. acidophilus NCFM deconjugated sodium taurocholate optimum by at a pH near 6.0. By growing L. acidophilus O16 in pH controlled cultures, it was observed that pH is more important than cell growth for bile salt deconjugation. That is, L. acidophilus O16 deconjugated both sodium glycocholate and sodium taurocholate at pH 5.0 where little cell growth was observed. However, L. acidophilus O16 exhibited poor deconjugation of both sodium glycocholate and sodium taurocholate at pH 7.0 where much more growth ocurred than that at pH 5.0.

Conjugated bile salts are found in a 2.2-3.0 molar ratio of glycocholate per taurocholate in the gallbladder of the human adult, and the amount of conjugated bile salts secreted to the gastrointestinal tract (GIT) is about 10 to 15 mmol (Hofmann, 1977). Considering that the volume of the gallbladder is smaller than that of the GIT, the conjugated bile salts are diluted when entering to the upper part of the small intestine. The GIT from the duodenum to the ileum has an approximate volume between 3.5 and 4 liters (Bodansky et al, 1957). Therefore, the highest bile salt molarity that might occur throughout the small intestine is between 2.8 and 4 mM as a consequence of such a dilution. However, a higher concentration might be expected in the duodenum where the bile is secreted, and a lower concentration might be found at the end of the ileum because of diffusion mechanisms, microbial transformations, and absorption of bile salts to the portal vein throughout the intestinal wall. The GIT may also undergo pH changes during food digestion. Since the stomach has a pH between 1 and 3 and its content is mixed with an alkaline pH between 7 and 9 of pancreatic and bile secretions, a variable pH is expected in the upper part of the GIT. However, a pH of 6.5 to 7.1 might be expected because of the digestive buffer system in the intestinal lumen (Mitsuoka, 1978).

All three strains of L. acidophilus deconjugated both sodium taurocholate and sodium glycocholate at pH 6.5. These two strains of human origin had lower deconjugation rates than strain ATCC 43121 of pig origin. Studies by De Rodas et al (1996) and Gilliland et al (1985) have shown that strain ATCC 43121 of pig origin is able to reduce the serum cholesterol in pigs. This strain has been shown to assimilate cholesterol (Gilliland et al, 1985) as have the two strains of human origin (Buck and Gilliland, 1994). While cholesterol assimilation is not correlated to sodium taurocholate deconjugation (Walker and Gilliland, 1993), high bile salt deconjugation at neutral pH is another important characteristic to consider when selecting strains for their potential use to help to reduce serum cholesterol. Strains L1 and O16 had three times less deconjugation activity than did strain ATCC 43121 when grown in culture conditions at pH 6.5.

The effect of conjugated bile salts and cholic acid on bacterial growth was pointed out by Floch et al (1970, 1972). They found that conjugated bile salts were less inhibitory than free bile salts to intestinal microorganisms at pH 7.2. The results in the present study revealed that L. acidophilus ATCC 43121 grew much more in the presence of 4 mM conjugated bile salts than in the presence of 4 mM free sodium cholate. The acidity of the media enhanced the inhibitory effect of unconjugated bile salts on L. acidophilus ATCC 43121. The mechanisms by which conjugated and unconjugated bile salts inhibit bacterial growth are still unclear.

Sodium glycocholate was hydrolyzed by all resting cells of L. acidophilus. However, after sonication (i.e. lysed cells), much more BSH activity was detected than when intact resting cells were tested. This difference was most pronounced for strain ATCC 43121. Thus, bile salt deconjugation activity may be affected by the permeability of cells to substrates. For example, Kobashi et al (1978) demonstrated that resting cells of Streptococcus faecalis and L. brevis split glycine conjugates only, but that their cell-free

extracts hydrolyzed both glycine and taurine conjugates. De Smet et al (1995) demonstrated by ^{14}C-labeled glycocholic acid that conjugated bile salt hydrolysis by resting cells of L. plantarum occurred intracellularly and the product cholic acid was released to the medium. De Smet (1994) also found that resting cells of L. plantarum had higher activity towards sodium glycochenodeoxycholate than towards taurochenodeoxycholate at pH 6.5. No one has suggested in the literature that cell permeability or lysis may be responsible for differences in an organism's ability to deconjugate different conjugated bile acids. The only comparisons that have been reported are from comparing actions of purified or partially purified BSH enzymes. It has been suggested that BSH from intestinal microorganisms shows different substrate specificity depending on the glycine, taurine or hydroxyl groups in the bile salt moiety (Christiaens et al, 1992, Gopal-Srivastava and Hylemon, 1988; Kawamoto et al, 1989; Lundeen and Savage, 1990; Stellwag and Hylemon, 1976; Nair et al, 1967).

All three strains of L. acidophilus contained BSH activity, irrespective of having been grown in the presence or absence of a conjugated bile, indicating that BSH was synthesized constitutively. The enzyme was found in cell membranes and in the cytosol. These results agreed with those found by Aries and Hill (1970a). They found BSH activity in both intracellular and cell membranes of gram positive intestinal microorganisms such Clostridia, Bacteroides and Streptococci. They also found extracellular activity in Bifidobacterium species. Most of the BSH that has been purified from intestinal microorganisms was intracellular (Lundeen and Savage, 1990; Grill et al, 1995; Gopal-Srivastava and Hylemon, 1988; Stellwag and Hylemon, 1976).

In the present study, BSH was released into the growth medium during bile salt deconjugation. Bile salt deconjugation may occur in the cytosol and alter cell permeability causing an increase in cellular permeability and/or some cell lysis. This is probably the reason that many intestinal microorganisms exhibit extracellular BSH activity. Masuda

(1981) found more BSH in culture supernatants after 24 hours of incubation than at earlier times for different strains of Bacteroides and of Clostridium. Kishinaka et al (1994) reported that high concentrations of conjugated bile acids induced extracellular BSH in C. perfringens. The specific BSH activity per cell in C. perfringens increased as the concentration of conjugated bile acids was increased. Moreover, Taranto et al (1996) showed that the most bile sensitive strain demonstrated the better deconjugation. It is likely the permeability of the most bile sensitive strain would be increased in the presence of conjugated and unconjugated bile salts thus permitting bile salt hydrolase to be released into the growth medium.

According to Scopes (1982), methanol is a good organic precipitant for proteins. He studied the effect of various alcohols on yeast glyceraldehyde phosphate deshydrogenase activity. He found that the longer the aliphatic chain the more the denaturating the alcohol was. Thus, it was suggested that the methanol was organic solvent of choice for precipitation of enzymes. Moreover, methanol is a suitable solvent for extracting conjugated and unconjugated bile acids, the latter of which has been shown to be an strong inhibitor of BSH (Nair et al, 1967). Therefore, the use of methanol to precipitate the enzyme would prevent any precipitation of residual bile acids from the spent broth which could interfere during enzyme activity assays.

After extracting the BSH from the spent broth, the total BSH activity in the methanol fraction was higher than that in spent broth. Bile salt hydrolase might have been inhibited by intracellular factors because both the BSH from spent broth and the BSH from cellular extracts resulted in low total BSH compared to the total BSH in the methanol fraction (see Tabless C1 to C6 in Appendix C). Similar observations were reported by Nair et al (1967) when purifying the BSH from cellular extracts of C. perfringens. They attributed such differences in BSH yield to the presence of an inhibitor within the crude extracts of C. perfringens. However, BSH from cellular extracts was recovered in lower percentages

from their methanol fractions. These results suggested that the stability of BSH from spent broth might be better than that from cell free extracts. Instability of BSH might be linked to proteases, since Lundeen and Savage (1990) reported a decline in BSH purity during gel filtration. They suggested that proteases that attacked BSH were involved in such a reduction. Bile salt hydrolase was better purified from the ammonium sulfate fraction resulting from 40 to 80 % saturation than from the fraction resulting from 20 to 40 % saturation. Several studies on BSH purification have shown that 40 to 75 % with ammonium sulfate resulted in best recovery of BSH (Lundeen and Savage, 1990; Gopal-Srivastava and Hylemon, 1988). Gel chromatograms of BSH from all three strains of L. acidophilus showed that ammonium sulfate fractions from spent broth were more contaminated than ammonium sulfate fractions from cell free extracts. Gel chromatograms of the ammonium sulfate protein fraction of L. acidophilus O16 showed that it may contain two types of bile salt hydrolase. One of them for deconjugating sodium taurocholate and another for deconjugating sodium glycocholate. Gel fractions showed that the peak of highest BSH on sodium glycocholate (GBSH) did not correspond to the peak of highest BSH on sodium taurocholate (TBSH). To our knowledge, this is the first report of two BSH from a single microorganism having different specificity for sodium taurocholate and sodium glycocholate. Lundeen and Savage (1992) found four isozymes of BSH from Lactobacillus sp. strain 100-100 which had different values in their kinetic parameters towards the same substrate (sodium taurocholate). Bile salt hydrolase active on sodium taurocholate (TBSH) may have higher molecular weight than that active on sodium glycocholate (GBSH) as observed by the elution profile on G-200 Sephadex column gel. The fact that the optimum pH of TBSH is 5.4 and that of GBSH is about 4.0 to 5.0 also indicates the possibility of two different BSH in L. acidophilus O16. The molecular weight of the BSH from all three strains of L. acidophilus was approximately 126 KDa, which is smaller than the molecular weight reported for other BSH (i.e. 250 000 in B. fragilis subsp. fragilis, Stellwag and Hylemon, 1976; B. longum BB536, Grill et al,

1995; 250 000 C. perfringens, Gopal-Srivastava and Hylemon, 1988), but it is in the range of the BSH from 115 000 in Lactobacillus sp. strain 100-100 (Lundeen and Savage, 1992) to 140 000 in B. vulgatus (Kawamato et al, 1989). The similar molecular weights of BSH suggests that the BSH of strains of human origin (O16 and L1) and of the strain ATCC 43121 of pig origin are closely related.

There are no reports in the literature on the optimum pH for deconjugation of sodium taurocholate or sodium glycocholate by the same enzyme from the same microbial origin. In this study, the optimum pH for BSH of both L. acidophilus strains ATCC 43121 and L1 was in the range of 4.0 to 5.0 . The optimum pH for BSH of L. acidophilus strain O16 was between 5.5 and 6.5. The values are in good agreement with most of the optimal values of BSH previously reported. Lundeen and Savage (1990), who studied BSH from Lactobacillus sp., found an optimum pH between 3.8 and 4.5 when using taurocholate as a substrate. Kawamoto et al (1989) and Gilliland and Speck (1977) showed that the optimum pH for taurocholate deconjugation by Bacillus vulgatus was in the range of 5.6 to 6.4 and by L. acidophilus NCFM was 6.0, respectively. The pH optimum in B. longum (Grill et al, 1995) and C. perfringens (Gopal-Srivastava and Hylemon, 1988) was in the range of 5.5 to 6.5. Stellwag and Hylemon (1976) reported an optimum pH of 4.2 for BSH of B. fragilis subsp. fragilis.

Enzyme efficiency in L. acidophilus ATCC 43121 was approximately similar to those in B. longum BB536 from human origin (Grill et al, 1995) and in B. fragilis subsp. fragilis (Stellwag and Hylemon, 1976). Moreover, it was higher than that found in Lactobacillus sp. strain 100-100 from rat stomach (10-fold)(Lundeen and Savage, 1992) and in C. perfringens (1,000-fold)(Gopal-Srivastava and Hylemon, 1988).

Bile salt hydrolase has been purified from different intestinal microorganisms in which BSH activities range from micromoles per minute to millimoles per minute. For example,

Gopal-Srivastra and Hylemon (1988) reported 1.34 μmol/min/mg in C. perfringens. Lundeen and Savage (1990) reported 30.3 μmol/min/mg in Lactobacillus sp., and Stellwag and Hylemon (1976) reported a maximum of 160.2 μmol/min/mg in B. fragilis subsp. fragilis ATCC 25285. Moreover, Grill et al (1995) have reported the highest BSH activity of 178.2 mmol/min/mg in B. longum which is a microorganism widely used in fermented milks as probiotic. The BSH activities found in the strains O16, L1 and ATCC 43121 were 2.3, 3.8, and 25.8 μmol/min/mg respectively.

During the isolation of BSH, sodium glycocholate was used as the principal substrate for measuring enzyme activity because of the high affinity of BSH towards this substrate. Lundeen and Savage (1990) and Grill et al (1995) did not find any significant differences in BSH deconjugating sodium taurocholate or sodium glycocholate in Lactobacillus sp. 100-100 and B. longum BB536 respectively. There are reports of some stains capable of splitting glycine conjugates only (Aries and Hill, 1970a; Dickinson et al, 1971; Norman and Grubb, 1955) and some strains hydrolyzing taurine conjugates only (Dickinson et al, 1971; Kobayashi et al, 1978). In this study, the BSH from all three strains of L. acidophilus were more active against glycine conjugates than taurine conjugates, and they hydrolyzed both trihydroxi- and dihydroxi-bile salt conjugates. In using L. acidophilus for treatment of hypocholesterolemia by bile salt deconjugation, a wide substrate specificity towards di- and tri-hydroxy conjugates of taurine and glycine is recommended. Since glycine conjugates are more prevalent in human bile (Burnett, 1965; Haslewood, 1967, 1978; Mallory et al, 1973; Sandine, 1979), the preference of the human strains toward these conjugates should be advantageous.

Lactobacilli are used commonly as a probiotics that are characterized as having potential for improving health conditions of animals and humans. A microbial strain may be a good candidate for a probiotic if it is genetically stable, survives the environmental conditions of the host until reaching the location where it is expected to exert maximum

activity and is safe for human or animal consumption. It also must produce the desired effect.

Since the half-life of C^{14}-cholic acid in germ-free rats is five times larger than in normal animals (Gustafsson et al, 1957), intestinal microorganisms are intimately related to the bile and steroid metabolism in those animals. It also has been observed that supplementation of the diet of pigs with L. acidophilus increased the output of free bile salts (Mott et al, 1973). Such results may be extrapolated to other species including man. Lin et al (1989) reported that ingestion of commercially available Lactobacillus tablets, which contain about 2 x 10^6 CFU/tablet of L. acidophilus and L. bulgaricus cells in a dose of four tablets per day, did not affect serum cholesterol concentrations. The strains used did assimilate some cholesterol only when cells were alive at numbers above 10^8 CFU/ml, but BSH activity was not tested. Tannock and McConnell (1995) reported that lactobacilli inhabiting the digestive tract of mice did not influence serum cholesterol concentrations, but they failed to report the number of viable cells within the intestinal tract nor were the animals reported to be hypocholesterolemic. High BSH activity in intestinal microorganism has not been entirely correlated to health benefits in animals, since high BSH activity in chicks resulted in poor weight gain (Feighner and Dashkevicz, 1988). However, high BSH activity, among other characteristics, may be an important factor in selecting microorganisms to induce health benefits in humans and animals. De Smet (1996), De Rodas et al (1996) and Gilliland et al (1985) have reported that strains of lactobacilli with high BSH activity decrease significantly serum cholesterol and serum bile acids in pigs. De Smet (1996) in swine and Bateup et al (1995) in mice did not find any significant differences in body weight between aminals treated with an active BSH lactobacilli and control group animals. Since cholesterol is not well absorbed in the presence of unconjugated bile acids as it is in the presence of conjugated ones (Holt, 1972), bile salt hydrolase activity may also reduce the absorption of cholesterol.

In this study lactobacilli from human origin (L1 and O16) showed less deconjugation

activity than a strain from pig (ATCC 43121). Strain ATCC 43121 has been associated with cholesterol uptake in vitro and also has been shown to reduce blood serum cholesterol in pigs (Gilliland et al, 1985; De Rodas et al, 1996). Isolates of L. acidophilus from pigs seem to have the higher and more frequent BSH activity than several other animal species. De Smet (1996) in a screening of BSH positive lactobacilli from different species found that the lactobacilli from pigs had much more BSH activity than did those isolates from man, rat, dog and rabbit. All isolates also had more activity towards glycocholate than towards taurocholate. Dashkevics and Feighner (1989) also found a high frequency of Lactobacillus spp. with BSH activity from swine origin when developing a differential medium for identifying bile salt hydrolase-active Lactobacillus spp.

The results of the present study suggest that cells of L. acidophilus should be present at numbers above 10^8 CFU/ml to obtain a significant deconjugation rate at pH 6.5. If a strain of L. acidophilus is used for reducing blood serum cholesterol by increasing the cholic acid output through feces, the lactobacilli should reach the upper part of the GIT in a desirable cell concentration from 10^8 to 10^9 CFU per gram. Moreover, the microorganisms may have to remain in the GIT from 6 to 8 hours to deconjugate significant amounts of bile salts. Normal concentration of lactobacilli from the duodenum to the ileum is from 10^2 to 10^7 CFU per gram in a human adult (Mitsuoka, 1978), and the transit time is from 3 to 10 hours. The conditions for reducing serum blood cholesterol by bile salt deconjugating bacteria are not far from real since a dairy diet including 10^9 CFU/g of viable microorganisms is accessible for many consumers. For example, L. acidophilus O16 and L1 deconjugated sodium glycocholate in a rate of 0.1 mM/h and 0.09 mM/h at pH 6.5, respectively. Such rates were obtained with $2x10^9$ CFU/ml. A 200 ml diet of any of those microorganisms can deconjugate 120 micromoles of sodium glycocholate in an average of 6 hours of transit time throughout the small intestine. If 40 % of the deconjugated bile salts are excreted in the feces (De Smet, 1994) then 48 micromoles of free bile salts would be excreted, the same amount needs to be synthesized from cholesterol to replenish the

lost free bile salts. An heterozygous familial hypercholesterolemic patient with 2.5 times more LDL-cholesterol (8.0 mM) than a normal patient (3.2 mM) would have 44, 000 micromoles (8.0 mM) of LDL-cholesterol in his body (i.e. the average of blood in a human adult is 5.5 L). Such an amount would have to be reduced to 17, 600 micromoles (3.2 mM) of LDL-cholesterol to be in normal conditions. Therefore 26, 400 micromoles of serum cholesterol could be reduced in 275 days with a consumption of 400 ml diet of L. acidophilus strain O16 or L1. Using a strain such ATCC 43121 the same amount of cholesterol could be reduced in 99 days. It is important to obtain human strains of L. acidophilus with higher BSH activity to reduce the time of treatment. Moreover, some dietary and bile cholesterol also would be excreted because of less efficient absorption in absence of adequate conjugated bile salts.

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APPENDIX A

API 50 CH CHARACTERIZATION OF LACTOBACILLUS ACIDOPHILUS O16, L1 AND ATCC 43121

TABLE A1

IDENTIFICATION OF THE THREE STRAINS OF LACTOBACILLUS ACIDOPHILUS BY THEIR FERMENTATION OF VARIOUS CARBOHYDRATES[1]

Carbohydrate	Bergey's[2]	O16	ATCC 43121	L1
Galactose	+	+	+	+
D-Glucose	+	+	+	+
D-Fructuose		+	+	+
D-Mannose	+	+	+	+
L-Sorbose		+	+	+
Mannitol	-	-	-	+
N-acetyl glucosamine		+	+	+
Amygdalin	+	+	+	+
Arbutin		+	+	+
Esculin	+	+	+	+
Salicin	+	+	+	+
Cellobiose	+	+	+	+
Maltose	+	+	+	+
Lactose	+	+	+	+
Melibiose	+/-	-	-	+
Sucrose	+	+	+	+
Trehalose	+	+	+	+
D-Raffinose	+/-	-	+	+
Starch		+	+	+
Glycogen		-	-	+
b-Gentibiose		+	+	+
D-Tagatose		+	+	-
Ribose	-	-	-	-
Rhamnose	-	-	-	-
Arabinose	-	-	-	-
Melezitose	-	-	-	-
Sorbitol	-	-	-	-
Xylose	-	-	-	-

[1] Based in the API 50 CH system (bioMérieux sa, France). All three strains were Gram positive catalase negative rods.

[2] Reactions that are listed for Lactobacillus acidophilus in the 8th edition of Bergey's Manual of Determinative Bacteriology.

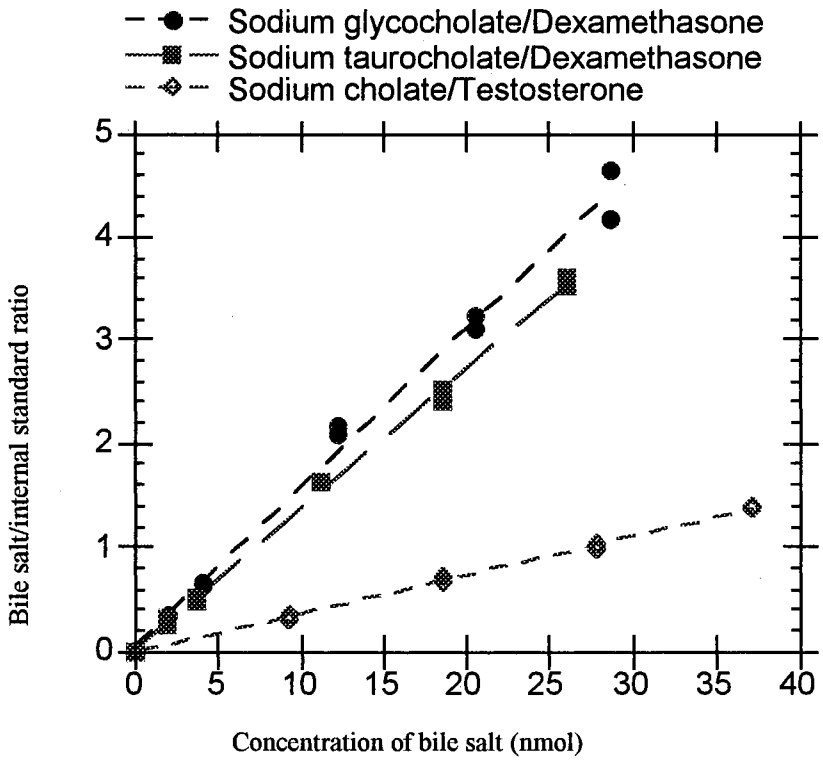

Figure A1. Standard curves of the ratios of sodium glycocholate, sodium taurocholate and sodium cholate with their respective internal standard. The concentration of internal standard was kept constant at different concentrations of bile salts.

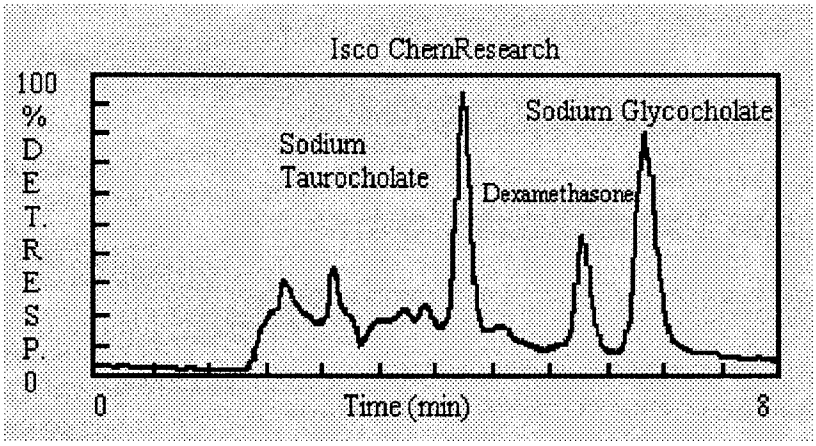

Figure A2. Chromatograms of sodium glycocholate and sodium taurocholate from an MRS broth sample.

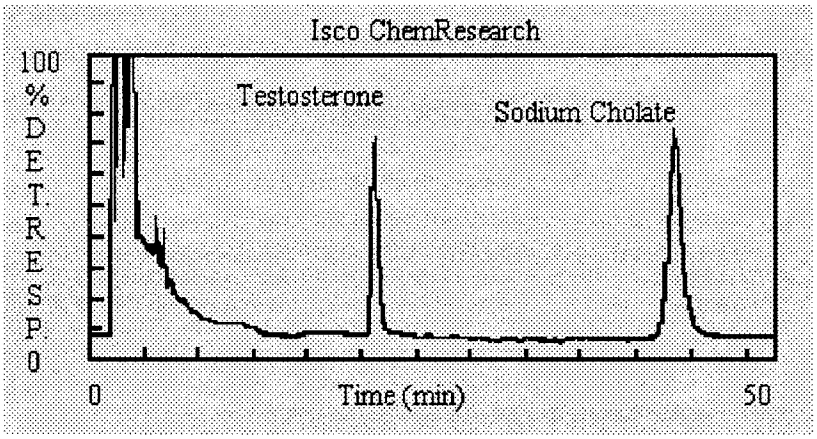

Figure A3. Chromatogram of sodium cholate from an MRS broth sample.

APPENDIX B

DETERMINATION RATE OF THE BILE SALT HYDROLASE AND STABILITY OF THE BILE SALT HYDROLASE IN THE PRESENCE OF EDTA AND AT DIFFERENT STORAGE TEMPERATURES

Determination of rate of bile salt hydrolase

As mentioned in Material and Methods, Bile salt hydrolase activity (BSH) activity was measured by quantification of sodium glycocholate or sodium taurocholate deconjugation at pH 5.4 and at 37 °C. The substrates were in 2.0 mM concentration in 50 mM acetate buffer, pH 5.4. Bile salt hydrolase was measured using fixed time assay. To assure that the measurements would provide true reaction rates, BSH activity was measured at different times to determine the range of time where reaction rates were constant.

Table B1 shows that BSH activity based on 30 to 120 min incubation did not differ significantly ($P<0.05$). However, other analyses involving samples with higher BSH activity than that of samples used in Table B1 resulted in reaction rates based on substrate limiting conditions which can be a typical error when using fixed-time enzymatic assays. This problem was improved by carefully selecting a shorter incubation time for the measurement. Another way used to improve the BSH fixed-time assay for samples having different amounts of activity was by diluting samples containing the enzyme and correcting the activity using the dilution factor.

Table B2 the BSH sample which was diluted 1:1 contained 0.60 mg/ml protein. and consumed all substrate during the 120 min of incubation. After diluting the sample, less substrate was consumed and the rate of activity based on 120 min incubation increased. The increase in rate of enzymatic activity was because the enzyme in the dilute samples were not under substrate limiting conditions by the end of the 120 min incubation.

The bile salt hydrolase enzymatic assay was stopped by adding one volume of methanol to the enzymatic reaction mixture and placing it on ice. Table B3 shows that there were no significant differences ($P>0.05$) in BSH activity when the amount of remaining conjugated bile acid was quantitated from 0 to 24 hours after stopping the reaction with such a solvent. Methanol might function in one of two ways to stop the BSH activity. First,

methanol could denature the enzyme; second, methanol could sequester the conjugated bile salts and make them inaccessible for the enzyme. Bile salt hydrolase activity was measured by deconjugation of the substrates sodium glycocholate or taurocholate. The product of deconjugation, cholic acid, was formed in stoichiometric amounts as shown in Figure B1. Since the measurement of sodium taurocholate and sodium glycocholate was faster and simpler than the measurement of cholic acid, the BSH activity assay was based on the disappearance of sodium glycocholate or sodium taurocholate. To demonstrate that bile salts did not merely precipitate at pH 5.4, sodium glycocholate (5 mM) was dissolved in 25 mM acetate-phosphate buffer and adjusted at different pH levels and held for 12 hours at 37 C. Fifty percent of sodium glycocholate was precipitated at pH 1, such a precipitation was dependent of pH from pH 1 to 4, but no significant differences were observed from pH 5 to 7 (Figure B2). Most of the research on purification and activity of BSH has involved the use of buffers containing □-mercaptoethanol to maintain reduced condition in the proteic solution (Gopal-Srivastava and Hylemon, 1988; Grill et al, 1995). Also, those buffers always contained phosphates and EDTA that were important in maintaining stability of BSH against proteases. In the present study citrate, acetate and phosphate salts were tested for buffering the BSH enzymatic assay at pH 5.4. The effect of □-mercaptoethanol in each of the buffers on BSH activity also was analyzed. As suggested by the results in Table B4, any of the buffers analyzed at pH 5.4 was appropriate for measuring BSH activity. Moreover, low R/O potential created by the presence of □-mercaptoethanol did not increase bile salt hydrolase activity.

TABLE B1

EFFECT OF INCUBATION TIME ON DETERMINATION OF RATE OF FIXED-TIME BILE SALT HYDROLASE ACTIVITY

Time (min)	Bile salt hydrolase activity[1] (nmoles/min/ml)
30	6.14(0.91)^{ab}
60	6.87(0.57)^{a}
90	6.31(0.99)^{a}
120	5.98(0.57)^{ab}
180	4.66(0.57)^{bc}
240	3.92(0.05)^{c}
420	2.34(0.16)^{d}

[1]Based on the deconjugation of 2 mM sodium glycocholate at 37°C and pH 5.4 by an enzymatic sample from methanol precipitate from spent broth produced by Lactobacillus acidophilus O16. Each value is the average of two trials; numbers in parentheses represent the standard deviation. Means in the same column without common superscript letters differ significantly ($P<0.05$).

TABLE B2

EFFECT OF ENZYME DILUTION ON FIXED-TIME BILE SALT HYDROLASE ACTIVITY

Protein (mg/ml)	Dilution factor	Sodium glycocholate consumed (nmol)	Final bile salt hydrolase activity[1] (nmol/min/ml)
0.60	1:1	197.0(4.24)[a]	8.2(0.14)[d]
0.45	3:4	195.5(2.12)[a]	10.8(0.11)[c]
0.30	1:2	187.0(1.41)[b]	15.6(0.12)[b]
0.15	1:4	109.5(2.12)[c]	18.2(0.28)[a]

[1]Based on 200 nmol total sodium glycocholate deconjugation in 120 min at 37°C and pH 5.4 by an enzymatic sample from methanol precipitate from spent broth produced by Lactobacillus acidophilus O16. Each value is the average of two trials; numbers in parentheses represent the standard deviation. Means in the same column followed by different superscript letters differ significantly ($P<0.05$).

TABLE B3

BILE SALT HYDROLASE ACTIVITY INACTIVATION BY METHANOL

Time after enzyme inactivation (hours)	Bile salt hydrolase activity[1] (nmol/min/ml)
0	25.5(0.65)[a]
1	25.2(0.46)[a]
2	25.3(0.45)[a]
24	25.6(0.36)[a]

[1]Based on the deconjugation of 2 mM sodium glycocholate at 37°C and pH 5.4 by an enzymatic sample from methanol precipitate from spent broth produced by Lactobacillus acidophilus O16. Each value is the average of two trials; numbers in parentheses represent the standard deviation. Means in the same column followed by different superscript letters differ significantly ($P<0.05$).

TABLE B4

EFFECT OF BUFFER AND □-MERCAPTOETHANOL IN THE ACTIVITY OF BILE SALT HYDROLASE.

Buffer	Bile salt hydrolase activity[1] (nmol/min/ml)	
	with Mercaptoethanol[A]	without Mercaptoethanol[A]
Acetate	14.15(0.21)[a]	13.40(0.70)[a]
Phosphate	13.10(0.42)[a]	12.85(0.35)[a]
Citrate	12.15(1.34)[a]	12.40(0.14)[b]

[1]Based on the deconjugation of 2 mM sodium glycocholate at 37 °C and pH 5.4 by an enzymatic sample from methanol precipitate from spent broth produced by Lactobacillus acidophilus O16. Concentration of buffers were 50 mM each and 10 mM □-mercaptoethanol when needed. Each value is the average of two trials; numbers in parentheses represent the standard deviation. Values in the same column followed by different lower case superscript letters differ significantly ($P<0.05$); values in the same row followed by different upper case superscript letters differ significantly ($P<0.05$).

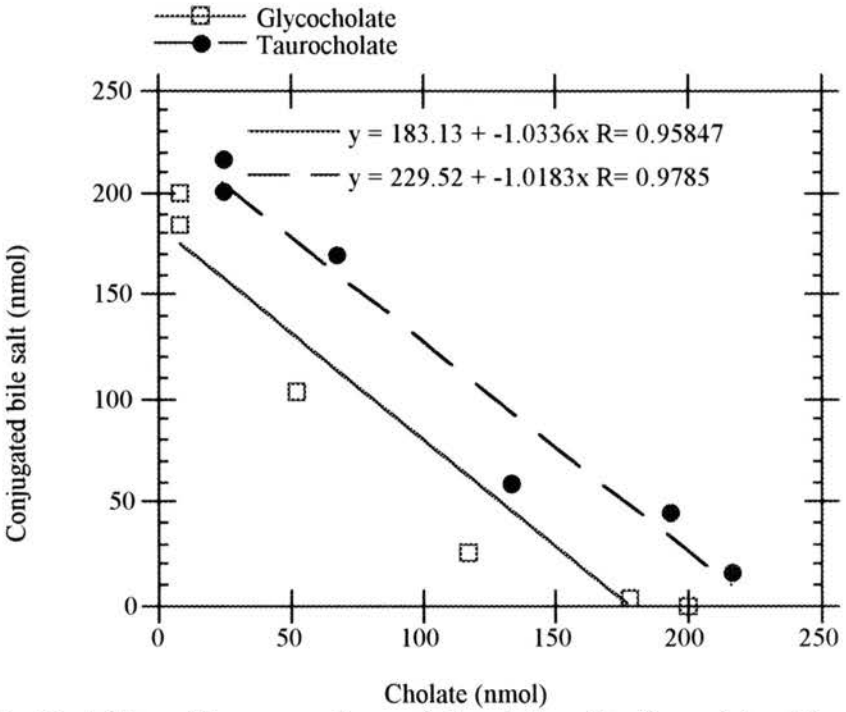

Figure B1. Stoichiometric comparison of free bile salts formed by bile salt hydrolase activity from Lactobacillus acidophilus O16 on sodium glycocholate and sodium taurocholate.

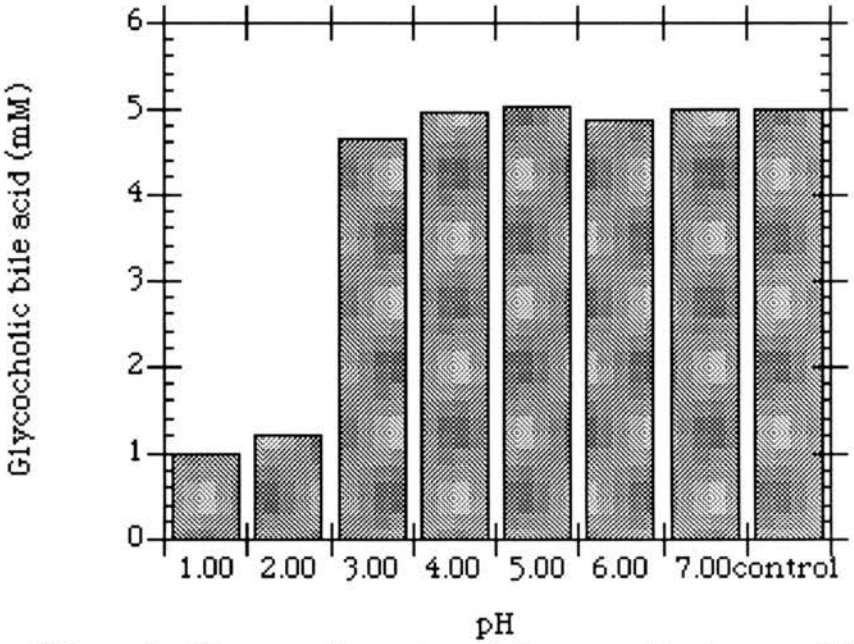

Figure B2. Effect of pH on sodium glycocholate precipitation at 37°C. Sodium glycocholate (5 mM) was dissolved in 25 mM acetate-phosphate buffer and adjusted at different pH levels and held for 12 hours at 37 C.

Stability of bile salt hydrolase

Effect of EDTA and storage temperature on BSH activity.

Bile salt hydrolase had a relative short half life time at 5°C. Bile salt hydrolase activity on sodium glycocholate was reduced significantly after 2 or 3 days of storage. L. acidophilus is a microorganism which uses carbohydrates as its mainly carbon source while its nitrogen is mainly obtained by the breakdown of proteins. Species of lactobacilli contain high levels of acidic proteases and peptidases that are generally bound to the cell wall. These proteases also may act on cell material after lysis (Hammes et al, 1992). EDTA has been used mainly for its chelative properties in controlling proteases which need minerals for presenting enzyme activity. Different concentrations of EDTA were used to observe BSH stability.

The results in Table B5 showed that BSH activity can be stored for short periods at 5 °C in the presence of EDTA. BSH activity probably is lost during storage at 5 °C due to the action of proteases. Buffer solutions with concentrations of EDTA above 1 mM maintained significantly more activity than those without EDTA. Moreover, buffer solutions with concentrations of 2 and 4 mM EDTA can maintain initial activities of BSH up to 2 days at 5 °C.

Bile salt hydrolase stability was also observed at temperatures below 0°C. BSH from intestinal microorganisms has been reported to maintain significant levels of activity after storage at -20°C for up to 8 weeks.

The results in Table B6 showed that BSH can be stored at -20°C up to one month in the presence of at least 1 mM EDTA with no significant ($P>0.05$) decreases in its activity for up to 3 weeks.

TABLE B5

EFFECT OF EDTA ON BILE SALT HYDROLASE ACTIVITY OF LACTOBACILLUS ACIDOPHILUS O16 AT 5°C

	EDTA[1] (mM)			
Time (days)	$_0$**B**	$_1$**A**	$_2$**A**	$_4$**A**
0.0	14.96(0.23)^{a}	15.15(0.63)^{a}	15.03(0.28)^{a}	15.10(0.28)^{a}
1.0	12.26(0.20)^{b}	13.55(0.07)^{b}	14.45(0.35)^{ab}	14.75(0.49)^{ab}
2.0	9.95(0.21)^{c}	13.50(0.42)^{b}	14.40(0.28)^{ab}	14.83(0.10)^{ab}
3.0	8.28(0.31)^{d}	13.85(0.21)^{b}	13.55(0.77)^{b}	14.59(0.34)^{b}
4.0	5.50(0.30)^{e}	12.56(0.15)^{c}	12.80(0.28)^{c}	12.95(0.21)^{c}
5.0	3.82(0.17)^{f}	10.50(0.28)^{d}	11.24(0.48)^{d}	11.20(0.14)^{d}
6.0	1.15(0.49)^{g}	9.60(0.14)^{e}	9.80(0.14)^{e}	10.85(0.91)^{e}

[1]Based on the deconjugation of 2 mM sodium glycocholate at 37 °C and pH 5.4 by an enzymatic sample from methanol precipitate from spent broth produced by Lactobacillus acidophilus O16. Each value is the average of two trials; numbers in parentheses represent the standard deviation. Values in the same column followed by different lower case superscript letters differ significantly ($P<0.05$); values in the same row followed by different upper case superscript letters differ significantly ($P<0.05$).

TABLE B6

EFFECT OF EDTA ON BILE SALT HYDROLASE ACTIVITY OF LACTOBACILLUS ACIDOPHILUS O16 AT -20°C.

	EDTA[1] (mM)			
Time (weeks)	$_0$**A**	$_1$**A**	$_2$**A**	$_4$**A**
0.0	14.96(0.23)^{a}	14.95(0.35)^{ab}	15.03(0.28)^{a}	15.10(0.28)^{a}
1.0	15.20(0.84)^{a}	15.65(0.63)^{a}	15.55(0.63)^{a}	14.64(0.36)^{a}
2.0	14.30(0.42)^{ab}	14.40(0.42)^{bc}	14.45(0.63)^{ab}	14.17(0.89)^{ab}
3.0	13.44(0.36)^{bc}	14.70(0.14)^{bc}	15.29(0.13)^{a}	14.75(0.21)^{a}
4.0	12.80(0.14)^{c}	13.75(0.21)^{c}	13.80(0.42)^{b}	13.25(0.49)^{a}

[1]Based on the deconjugation of 2 mM sodium glycocholate at 37 °C and pH 5.4 by an enzymatic sample from methanol precipitate from spent broth produced by Lactobacillus acidophilus O16. Each value is the average of two trials; numbers in parentheses represent the standard deviation. Values in the same column followed by different lower case superscript letters differ significantly ($P<0.05$); values in the same row followed by different upper case superscript letters differ significantly ($P<0.05$).

APPENDIX C

ENZYME PURIFICATION

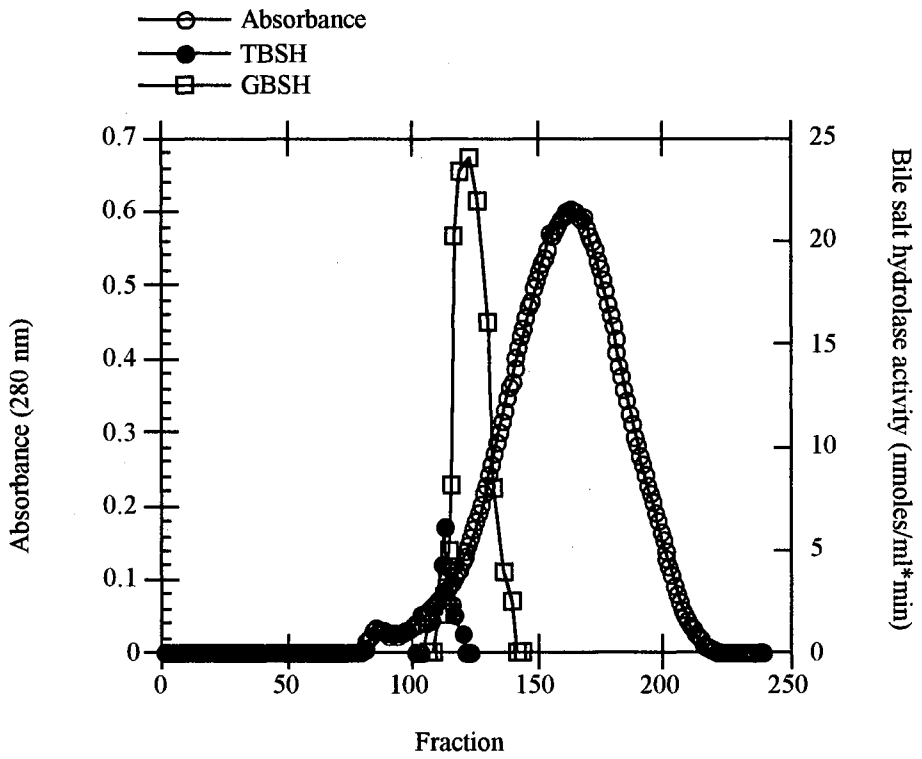

Figure C1. Elution profile of ammonium sulfate precipitate from spent broth of bile salt hydrolase activity produced by Lactobacillus acidophilus O16 on Sephadex G-200. Each 0.8 ml fraction was monitored at 280 nm and fractions containing protein were assayed for bile salt hydrolase activity.

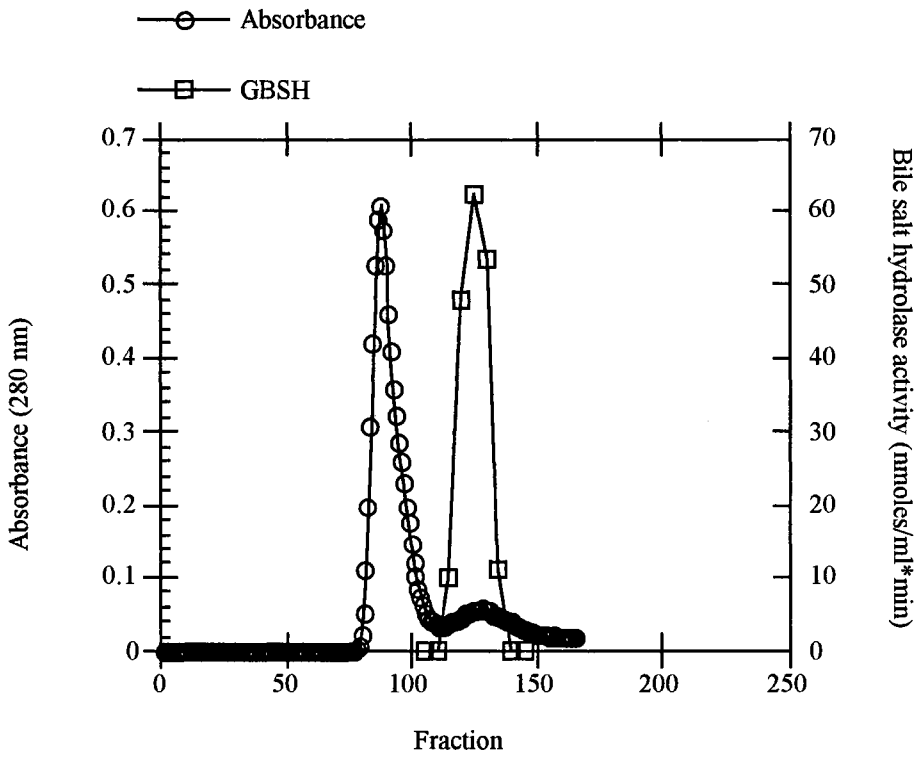

Figure C2. Elution profile of ammonium sulfate precipitate from cell extracts of bile salt hydrolase activity produced by Lactobacillus acidophilus O16 on Sephadex G-200. Each 0.8 ml fraction was monitored at 280 nm and fractions containing protein were assayed for bile salt hydrolase activity.

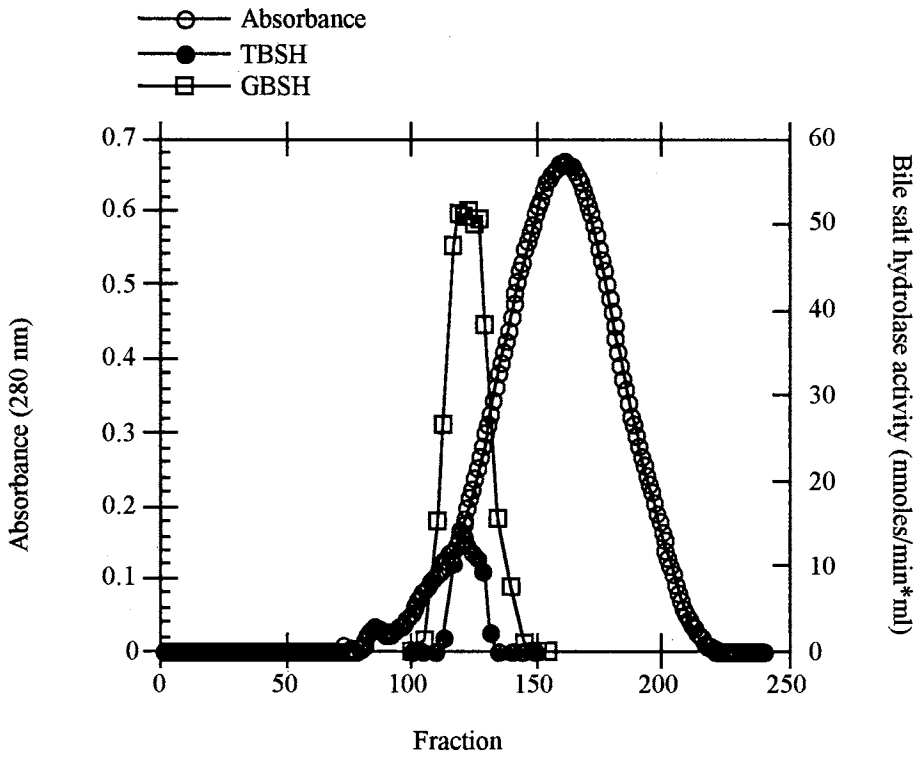

Figure C3. Elution profile of ammonium sulfate precipitate from spent broth of bile salt hydrolase activity produced by Lactobacillus acidophilus L1 on Sephadex G-200. Each 0.8 ml fraction was monitored at 280 nm and fractions containing protein were assayed for bile salt hydrolase activity.

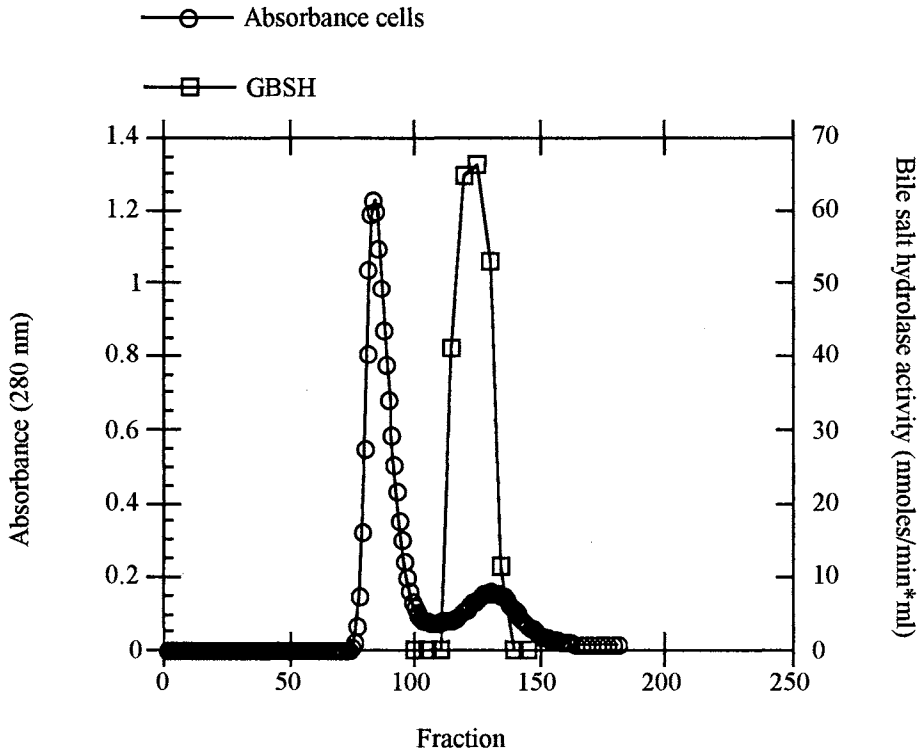

Figure C4. Elution profile of ammonium sulfate precipitate from cell extracts of bile salt hydrolase activity produced by Lactobacillus acidophilus L1 on Sephadex G-200. Each 0.8 ml fraction was monitored at 280 nm and fractions containing protein were assayed for bile salt hydrolase activity.

TABLE C1

PURIFICATION OF BILE SALT HYDROLASE FROM SPENT BROTH OF CULTURE OF L. ACIDOPHILUS 016 USING SODIUM GLYCOCHOLATE AS A SUBSTRATE

Step	Volume (ml)	Total protein (mg)	Total activity (U)	Sp act (U per mg of protein)	Recovery (%)	Purification (fold)
Spent broth	200.0	17.2	3376	196	100.0	1.0
Methanol	20.0	4.8	3058	637	90.6	3.25
$(NH_4)_2SO_4$	4.0	2.4	3453	1463	102.3	7.45
Gel Sp-200	20.0	0.182	384	2136	11.4	10.8
Micro-concentrate	1.5	0.15	342	2280	10.1	11.6

TABLE C2

PURIFICATION OF BILE SALT HYDROLASE FROM SPENT BROTH OF CULTURE OF L. ACIDOPHILUS L1 USING SODIUM GLYCOCHOLATE AS A SUBSTRATE

Step	Volume (ml)	Total protein (mg)	Total activity (U)	Sp act (U per mg of protein)	Recovery (%)	Purification (fold)
Spent broth	200.0	20.0	1378	68.9	100.0	1.0
Methanol	20.0	5.52	3758	681	272.7	9.88
$(NH_4)_2SO_4$	4.0	2.4	3450	1437	250.3	20.9
Gel Sp-200	12.0	0.156	580	3717	42.1	53.9
Micro-concentrate	1.0	0.150	568	3786	41.2	59.7

TABLE C3

PURIFICATION OF BILE SALT HYDROLASE FROM SPENT BROTH OF CULTURE OF L. ACIDOPHILUS ATCC 43121 USING SODIUM GLYCOCHOLATE AS A SUBSTRATE

Step	Volume (ml)	Total protein (mg)	Total activity (U)	Sp act (U per mg of protein)	Recovery (%)	Purification (fold)
Spent broth	200.0	39.2	8150	208	100.0	1.0
Methanol	20.0	10.4	11554	1110	141.7	5.33
$(NH_4)_2SO_4$	4.0	4.8	11657	2429	143.0	11.6
Gel Sp-200	10.0	0.64	10701	16720	131.3	80.4
Micro-concentrate	0.41	0.32	7686	24018	94.31	115

TABLE C4

PURIFICATION OF BILE SALT HYDROLASE FROM CELLS OF L. ACIDOPHILUS 016 USING SODIUM GLYCOCHOLATE AS A SUBSTRATE

Step	Volume (ml)	Total protein (mg)	Total activity (U)	Sp act (U per mg of protein)	Recovery (%)	Purification (fold)
Cell free extracts	10.0	53.6	3731	69.6	100.0	1.0
Centrifug.	10.0	7.80	315	40.3	8.44	0.57
Methanol	7.5	4.87	1427	293	38.2	4.20
$(NH_4)_2SO_4$	2.0	3.1	977	315	26.1	4.52
Gel Sp-200	1.2	0.04	62.8	1570	1.68	22.5

TABLE C5

PURIFICATION OF BILE SALT HYDROLASE FROM CELLS OF L. ACIDOPHILUS L1 USING SODIUM GLYCOCHOLATE AS A SUBSTRATE

Step	Volume (ml)	Total protein (mg)	Total activity (U)	Sp act (U per mg of protein)	Recovery (%)	Purification (fold)
Cell free extracts	10	44.8	1696	37.8	100.0	1.0
Centrifug.	10	12.9	334	25.9	19.7	0.68
Methanol	9	9.09	1571	173	92.6	4.57
$(NH_4)_2SO_4$	3	6.0	1172	195	69.1	5.15
Gel Sp-200	1.2	0.04	79.3	1983	4.67	52.4

TABLE C6

PURIFICATION OF BILE SALT HYDROLASE FROM CELLS OF L. ACIDOPHILUS ATCC 43121 USING SODIUM GLYCOCHOLATE AS A SUBSTRATE

Step	Volume (ml)	Total protein (mg)	Total activity (U)	Sp act (U per mg of protein)	Recovery (%)	Purification (fold)
Cells free extracts	10	66.3	53726	810	100.0	1.0
Centrifug.	10	14.8	38918	2630	72.4	3.24
Methanol	8	9.0	35588	3954	66.2	4.88
$(NH_4)_2SO_4$	3	3.1	22324	7201	41.5	8.89
Gel Sp-200	1.2	0.01	258	25800	0.5	31.8

APPENDIX D

MOLECULAR WEIGHT DETERMINATION

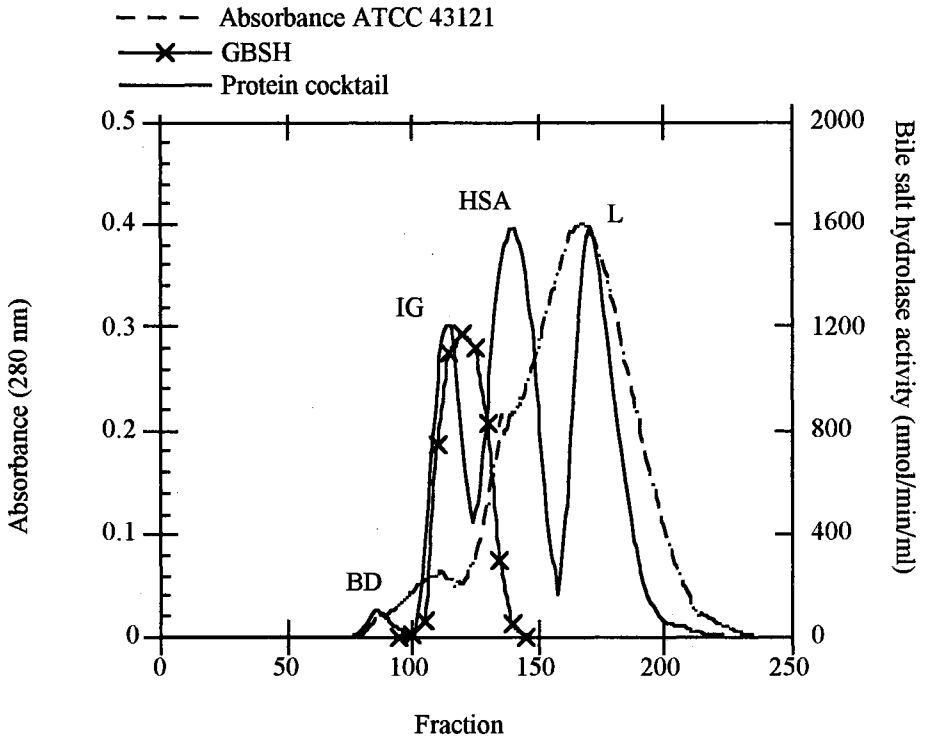

Figure D1. Gel chromatography of a protein cocktail to determine the relative molecular weight of bile salt hydrolase on Sephadex G-200. BD (Blue dextran 2000 KDa); IG (Immunoglobulin G, M.W. 158 KDa); HSA (Human Serum Albumin, M.W. 69 KDa); L (Lyzozyme, M.W. 14.3 KDa).

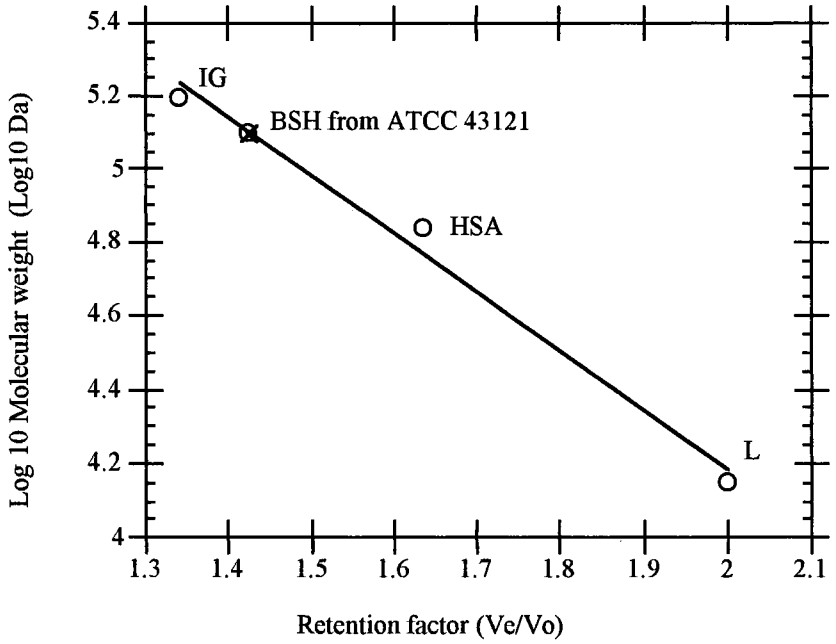

Figure D2. Estimation of the molecular weight of the bile salt hydrolase by Sephadex G-200 gel filtration. BD (Blue dextran 2000 KDa); IG (Immunoglobulin G, M.W. 158 KDa); HSA (Human Serum Albumin, M.W. 69 KDa); L (Lyzozyme, M.W. 14.3 KDa). Ve, volume of elution of protein; Vo, volume of elution of blue dextran.

APPENDIX E

EFFECT OF pH ON THE PURIFIED ENZYME

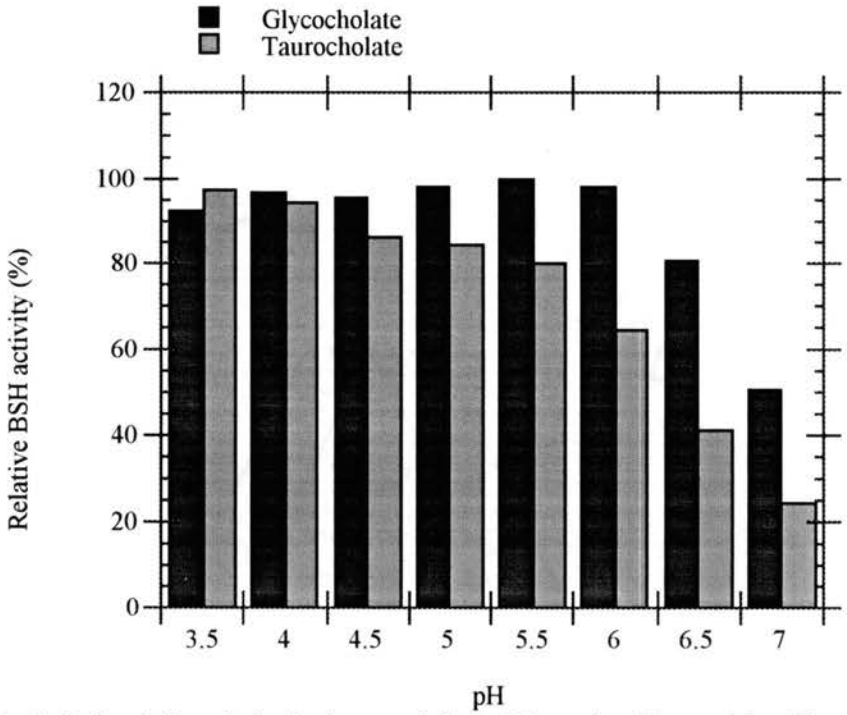

Figure E1. Relative bile salt hydrolase activity of Lactobacillus acidophilus L1 on sodium glycocholate and sodium taurocholate at different pH.

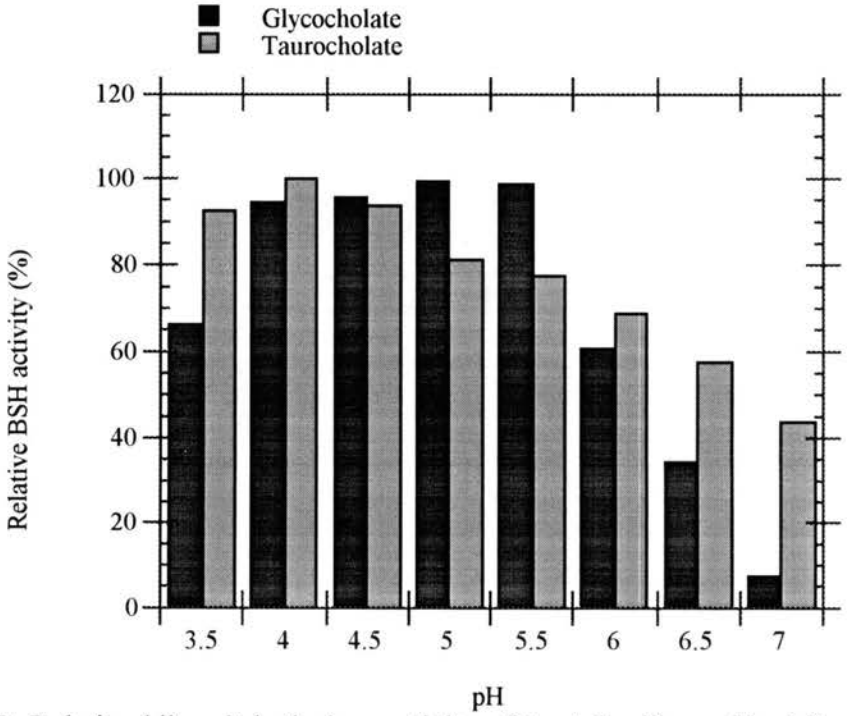

Figure E2. Relative bile salt hydrolase activity of Lactobacillus acidophilus ATCC 43121 on sodium glycocholate and sodium taurocholate at different pH.

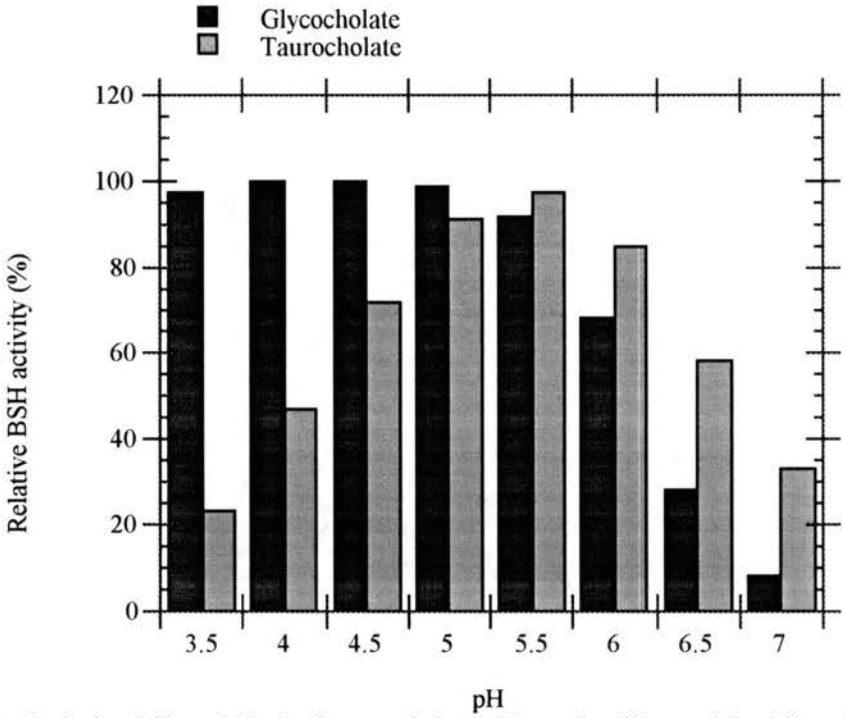

Figure E3. Relative bile salt hydrolase activity of Lactobacillus acidophilus O16 on sodium glycocholate and sodium taurocholate at different pH.

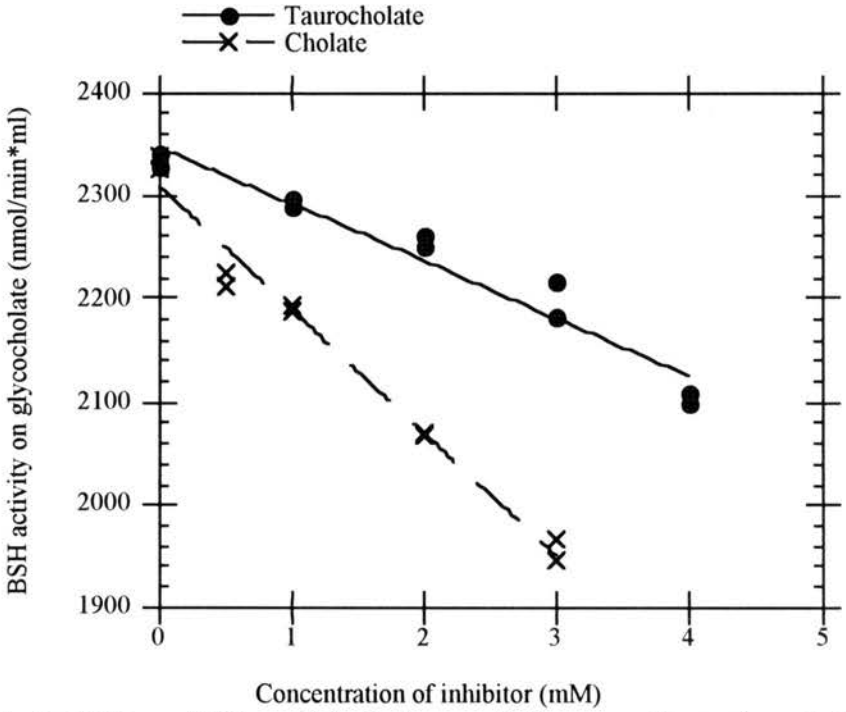

Figure E4. Inhibition of bile salt hydrolase activity on sodium glycocholate by sodium taurocholate and sodium cholate.

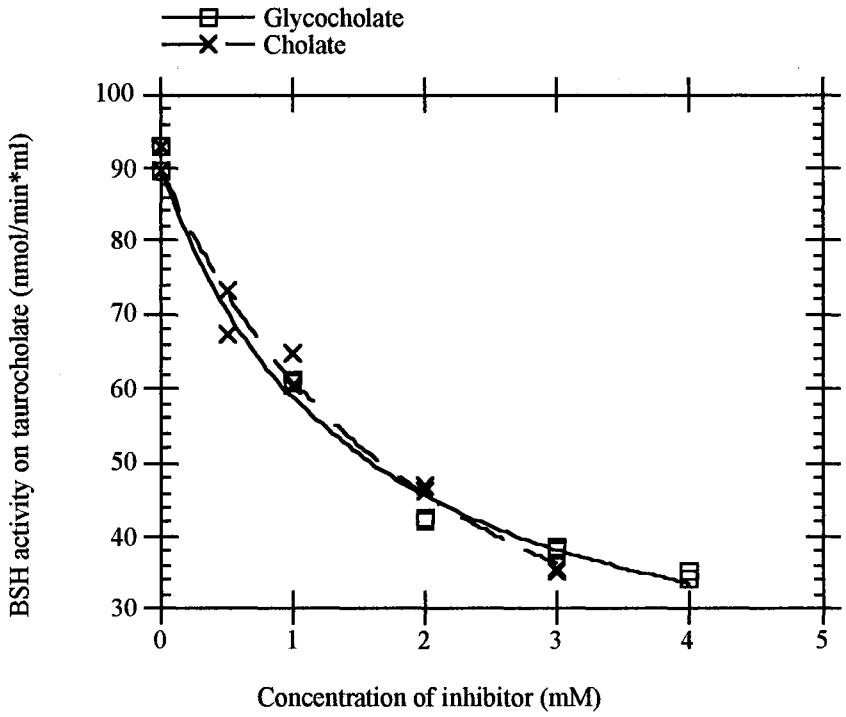

Figure E5. Inhibition of bile salt hydrolase activity on sodium taurocholate by sodium glycocholate and sodium cholate.

APPENDIX F

STATISTICAL ANALYSES

TABLE F1

CORRELATION ANALYSIS OF ABSORBANCE AND pH WITH BOTH SODIUM TAUROCHOLATE AND SODIUM GLYCOCHOLATE DECONJUGATION

Variable	n	Mean	Std Dev	Sum	Minimum	Maximum
Absorbance	112	0.2008	0.1588	22.4910	0.0010	0.401
pH	112	5.1089	1.0242	572.200	3.8000	6.700
Glycocholate	112	0.5082	0.4436	56.9210	0	1.000
Taurocholate	112	0.6525	0.3711	73.0800	0	1.000

Pearson Correlation Coefficients / Prob > | R | under Ho: Rho=0 / N= 112

	Sodium glycocholate deconjugation	Sodium taurocholate deconjugation
Absorbance	-0.94362 (0.0001)	-0.79235 (0.0001)
pH	0.90171 (0.0001)	0.71915 (0.0001)

TABLE F2

CORRELATION ANALYSIS BETWEEN ABSORBANCE AND COUNT PLATE NUMBER FOR 0 TO 10 HOURS OF INCUBATION

Variable	n	Mean	Std Dev	Sum	Minimum	Maximum
Absorbance	78	0.2152	0.1757	54.2550	0.001	0.4870
Count plate	78	$2.1x10^9$	$2.8x10^9$	$5.3x10^{11}$	1300	$1.3x10^{10}$

Pearson Correlation Coefficients / Prob > | R | under Ho: Rho=0 / N= 78

	Absorbance	Count plate
Absorbance	1.00000 (0.000)	0.77090 (0.0001)
Count plate	0.77090 (0.0001)	1.00000 (0.000)

TABLE F3

ANALYSIS OF VARIANCE OF TABLE 1 - KINETIC PARAMETERS OF BILE SALT DECONJUGATION OF LACTOBACILLUS ACIDOPHILUS 016, L1, AND ATCC 43121 IN STATIC CULTURES

Source	DF	Sum of squares	Mean square	F Value
Strain O16				
Model	3	22.747360184	7.582453395	192.2363
Error	45	1.774953816	0.039443418	
Total	48	24.522314000		
Parameter		Estimate	Std. error	
Lag phase		5.511003758	0.657557612	
Deconjugation rate		0.188825526	0.041425140	
Total deconjugation		1.020579823	0.069432252	
Source	DF	Sum of squares	Mean square	F Value
Strain L1				
Model	3	17.901329917	5.967109972	1270.7875
Error	45	0.211306083	0.004695691	
Total	48	18.112636000		
Parameter		Estimate	Std. error	
Lag phase		4.234572266	0.1993361722	
Deconjugation rate		0.260897302	0.0247702211	
Total deconjugation		1.003042328	0.0172469605	
Source	DF	Sum of squares	Mean square	F Value
Strain ATCC 43121				
Model	3	20.667543911	6.889181304	550.24531
Error	45	0.563410089	0.012520224	
Total	48	21.23095400		
Parameter		Estimate	Std. error	
Lag phase		4.823595700	0.3610185483	
Deconjugation rate		0.202554183	0.0266844630	
Total deconjugation		0.991708041	0.0340422357	

Pooled standard error (pse)= $\sqrt{se^2_1 + se^2_2}$

$t = \mu_1 - \mu_2/pse$

degrees of fredom = n_1-3 + n_2 -3

t = Student's t, μ = means, n = observations

TABLE F4

ANALYSIS OF VARIANCE OF TABLE B5 - EFFECT OF EDTA ON BILE SALT HYDROLASE ACTIVITY OF LACTOBACILLUS ACIDOPHILUS O16 AT 5°C

Source	DF	Sum of squares	Mean square	F Value
Model	3	262.46900536	87.48966845	11.23
Error	52	405.21929286	7.79267871	
Total	55	667.68829821		

OSL < 0.05

$LSD_{0.05} = 2.1172$

VITA

Gerardo A. Corzo Burguete

Candidate for the degree of

Doctor of Philosophy

Thesis: BILE SALT DECONJUGATION BY THREE STRAINS OF LACTOBACILLUS ACIDOPHILUS AND CHARACTERIZATION OF THEIR BILE SALT HYDROLASE

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