ASSIMILATION OF CHOLESTEROL BY *BIFIDOBACTERIUM LONGUM* AND ITS INCORPORATION INTO THE CELLULAR MEMBRANE

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CHAPTER I

INTRODUCTION

Rigidity of the cell membrane is due to the presence of cholesterol, which makes it an vital component of the membrane. It also serves as precursor for steroids and bile acids. Absorption of biliary and dietary cholesterol in the gut is an important source of cholesterol in humans. Despite the fact that cholesterol is an essential constituent in the system, excess blood cholesterol has been implicated as a primary cause of atherosclerosis, which in turn leads to cardiovascular disease and coronary heart disease (CHD). In a healthy man, an intricate balance is maintained between the biosynthesis, utilization and transportation of cholesterol, keeping its harmful deposition to a minimum. A possible method for ameliorating CHD is by reducing total serum cholesterol. Diet plays an important role in regulating this blood cholesterol level.

Prophylactic and/or therapeutic value of fermented milk products has been reviewed extensively, since Metchnikoff mentioned the possible beneficial effects by the consumption of the same. Of the lactic acid bacteria used as a dietary adjunct maximum interest has been centered on *L. acidophilus*, a normal inhabitant of the intestinal tract. In North America, *Bifidobacteria* sp., which is also a natural inhabitant of intestine, has received recent attention. It prevails in the large intestine whereas lactobacillus is present in the small intestine.

Bifidobacteria were first identified in breast-fed infants by Tissier in 1899. Decreased infant mortality in breast-fed infants compared to bottle-fed infants was ascribed to the resistance offered by bifidobacteria. In addition, bifidobacteria can be isolated at any age from humans, however, difference in species exists.

Bifidobacteria are unique; they lack both aldolase and glucose-6-phosphate dehydrogenase enzyme, therefore they do not metabolize carbohydrate by either glycolysis or the hexose monophosphate pathway. An alternate to these enzymes is fructose-6-phosphate phosphoketolase (F6PPK) which cleaves fructose-6-phosphate into acetlyphosphate and erythrose-4-phosphate. This helps to distinguish this genera from the rest. Accordingly, the end product of carbohydrate metabolism is acetic acid and lactic acid (3:2 ratio) and small amounts of formic acid and alcohol.

Certain factors are to be considered for a culture to serve as a dietary adjunct. It should be a normal inhabitant of the intestinal tract, function by surviving and growing in the intestinal tract (bile tolerance), compete and grow well in the presence of similar bacteria and be viable in the carrier food. Some of the beneficial effects of bifidobacteria are due to their potential to produce antimicrobial activity, anticarcinogenic and antitumorgenic activity, alleviate lactose intolerance, synthesize vitamins and reduce levels of serum cholesterol.

Considering the feasibility of using bifidobacteria in fermented milk products, the purpose of this project was to: (1) study the bile tolerance of *B. longum*; (2) study the factors affecting the amount of cholesterol taken up by *B. longum*; (3) measure the bile

salt dehydrogenase activity of *B. longum*; and (4) measure the cholesterol content in the cell membrane fraction of *B. longum* I.

CHAPTER II

REVIEW OF LITERATURE

In early 1900's Metchnikoff thought that aging and premature death caused by intestinal putrefaction could be prevented by ingestion of soy milk (45). Later in 1910, he suggested that the routine consumption of fermented milk is beneficial to the health of the consumer (62). Since then extensive research has focused on the possible therapeutic and prophylactic properties of certain cultured milk products. *Lactobacillus acidophilus* used in the manufacture of some cultured milk products has received more attention in this regard than any other organism used in the manufacture of such products. However, over the past decade *Bifidobacteria* sp. has attracted a great deal of attention, since they also frequently are associated with potential health-promoting effects in human and animal intestinal tract, similar to those attributed to *L. acidophilus.*

Occurrence and growth of bifidobacteria in human intestines

Bifidobacteria were first isolated from the feces of infants and characterized in 1899 by Henry Tissier (33, 45). They occur principally in the large intestine, especially in the area of the caecum (31, 37, 47, 62). The majority of them occupy the lumen of the

colon and the walls of the colon. While they occur in largest numbers in the large intestine, they have been detected throughout the intestinal tract (4, 45, 61).

In infants bifidobacteria comprise a larger portion of the intestinal flora than in adults (47). In healthy breast-fed infants, one-fourth of the intestinal flora is bifidobacteria, whereas lactococci, enterococci and coliforms constitute less than 1% of the total microflora with the absence of harmful bacteria like clostridia, proteus etc. (31, 53). The numbers of bifidobacteria found in the feces of breast-fed infants were approximately 10^{11}/g; the numbers decreased in adults to about 10^{9}/g (50).

Changes in diet that occur as people age result in the decrease in numbers of bifidobacteria in the intestinal tract (31, 47). Even though the numbers decrease with age, bifidobacteria can be isolated from the feces of humans at any stage of life. Some of the frequently isolated species in infants are *B. infantis, B. breve, B. bifidum* and *B. longum* (45, 46, 47, 62). In adults, *B. longum, B. adolescentis* and *B. bifidum* are the most often encountered species (45, 46, 62).

Pochart et al. (49) examined the ability of bifidobacteria to survive and/or grow in the upper gastro-intestinal tract of healthy human adults after ingestion of fermented milk containing *Bifidobacteria* sp. strain BB (2.5 x 10^{7}/g). The average number of viable bifidobacteria reaching the terminal ileum after 8 hr was 1 x 10^{6}/ml of ileal fluid. In a similar study, after the consumption by healthy volunteers of fermented milk containing *Bifidobacteria* sp. strain BOSR (1.6 x 10^{9}/g), a mean of 6.3 x 10^{8}/g was recovered from the stool which represented a 20-fold increase of mean daily fecal excretion of bifidobacteria (6).

Clark et al. (10) analyzed the tolerance of *B. longum, B. adolescentis, B. infantis* and *B. bifidum* in the simulated pH of human stomach. They found *B. longum* to have an excellent resistance at pH 1.0, 2.0 and 3.0 followed by *B. infantis* and *B. adolescentis* at pH 2.0 and 3.0. However, survival of *B. bifidum* was poor at pH 1.0 and 2.0. Additionally, Rao et al. (51) observed that microencapsulated *B. pseudolongum* survived in the simulated gastric environment in larger numbers than cells of non-encapsulated strains.

Survival and growth in the presence of physiological concentrations of bile salt is an important characteristic to be considered for the microorganisms to be used as dietary adjuncts (7, 24, 25). Ibrahim and Bezkorovainy (35) studied the growth of four *Bifidobacterium* sp. in the presence of 0, 0.06, 0.15 and 0.3% sodium glycocholate. A general trend of decrease in rate of growth was observed with increasing concentration of bile salt for all four cultures.

Potential health benefits from bifidobacteria

Bifidobacteria potentially can have a hypocholesterolemic effect (33, 52), inhibit the growth of other microorganisms (2, 8, 33, 34, 50, 54, 59), directly and indirectly exert anticarcinogenic actions (21, 30, 33, 36, 41, 54) and improve lactose utilization in lactose malabsorbers (1, 28). Thus there is considerable interest in their use as dietary adjuncts for humans.

Control of serum cholesterol

Cholesterol is an important constituent in maintaining the rigidity and fluidity of the membrane in eukaryotes (66). Sources of cholesterol in the body include that synthesized in the liver from acetyl co-A and, to a lesser extent, that absorbed from the intestine from food. On contrary to its benefits, high levels of blood cholesterol are related to cardiovascular disease and coronary heart disease (39). Thus there is interest in ways to control levels of serum cholesterol in hypercholesterolemic persons. Consumption of certain cultured or culture containing milk products has resulted in reductions in serum cholesterol in humans (27, 40, 52) as well as in animals (14, 17, 26, 33).

Mann and Spoerry (40) found that consumption of fermented milk prepared with wild type *Lactobacillus*, by the Maasai people, led to lower levels of cholesterol in the blood. They suggested that the lactic acid bacteria produced hydroxymethylglutarate (HMG) during fermentation of the milk, which inhibited HMG-Co A reductase involved in cholesterol synthesis in the body. Hughes and Hoover (33) suggested that lowered serum cholesterol brought about by feeding bifidobacteria might have involved HMG co-A reductase.

A significant reduction in levels of serum cholesterol and triglycerides was observed in humans suffering from elevated serum cholesterol levels following consumption of bifidobacteria (52). Rasic et al. (52) reported that cholesterol was assimilated by *B. bifidum* in laboratory media. The degree of uptake varied from strain to strain. Homma (28), in an *in vitro* study of the effect of bifidobacteria on cholesterol

metabolism of human lymphocytes suggested that bifidobacteria retarded the formation of LDL receptors and thus reduced cholesterol synthesis.

Harrison et al. (27) observed lower levels of serum cholesterol in conjunction with increased numbers of lactobacilli in the stools of infants fed milk based formula containing *L. acidophilus* compared to ones receiving control formula. The researchers speculated that the lactobacilli caused the effect on levels of serum cholesterol.

Grunewald (26) observed a reduction in serum cholesterol levels in rats after feeding milk fermented with *L. acidophilus* compared to rats fed non-fermented milk. Gilliland et al. (25) reported that pigs on a high cholesterol diet fed cells of a strain of *L. acidophilus* that assimilated cholesterol in laboratory media had significantly lower levels of serum cholesterol than did pigs on the same diet without *L. acidophilus*. In another study, decrease in the concentrations of total serum cholesterol and low-density lipoprotein cholesterol were observed in boars on high cholesterol diet when fed acidophilus yogurt (14). De Rodas (17) reported a significantly greater decrease in serum cholesterol when pigs with diet induced hypercholesterolemia were fed *L. acidophilus* than in those not receiving *L. acidophilus*.

Buck and Gilliland (7) observed removal of cholesterol from the laboratory media by strains of *L. acidophilus* isolated from the fecal flora of human volunteers. Significant variation occurred among the strains in the ability to assimilate cholesterol. Gilliland et al. (25) demonstrated that isolates of *L. acidophilus* from pigs grown under anaerobic condition in the presence of cholesterol and bile salts assimilated a portion of the cholesterol from the laboratory media. A strain which did not assimilate cholesterol

from laboratory media was not effective in helping control serum cholesterol in pigs. While these cultures varied in tolerance to bile acid, there was no direct relationship between the bile tolerance and the ability to assimilate cholesterol (24, 25). In a similar study, no significant correlations were observed among bile tolerance, ability to deconjugate bile acid and assimilation of cholesterol (67).

The ability of lactobacilli and related intestinal bacteria to deconjugate bile acids in the small intestine is another activity of these bacteria that may be important in controlling serum cholesterol levels (22). Gilliland and Speck (23) reported laboratory strains of *L. acidophilus* deconjugated both glycocholic and taurocholic acids. Buck and Gilliland (7) and Walker and Gilliland (67) reported variations among strains of *L. acidophilus* with respect to relative abilities to deconjugate bile acids and to assimilate cholesterol. However, the two activities were not related. De Rodas (17) on the other hand, reported a significant relationship between deconjugation of bile acids in the intestine and reductions in serum cholesterol level caused by consumption of *L. acidophilus*. Hence suggested the importance of deconjugation in lowering concentrations of serum cholesterol.

Bifidobacteria also can deconjugate bile acids. *B. adolescentis* hydrolyzed only glycine conjugates whereas *B. bifidum* and *B. longum* hydrolyzed both glycocholic and taurocholic acids. Chikai et al. (9) reported that *B. longum* and *B. vulgatus* preferentially deconjugated taurcholic acid and tauro-β-muricholic acids respectively, whereas *Clostridium ramosum* deconjugated both glycine and taurine conjugated bile acids.

Antimicrobial effects

Bifidobacteria exert antagonistic action towards growth of enteric pathogenic bacteria and other bacteria. Those inhibited by bifidobacteria include *Escherichia coli, Bacillus cereus, Salmonella typhosa, Staphylococcus aureus, Shigella dysenteriae, Psuedomonas fluorescens* and *Micrococcus flavus* (2, 8, 33, 50, 54).

The antimicrobial activity of bifidobacteria has been attributed to their production of acetate and lactate along with small amounts of formate from fermentation of carbohydrates (47, 50). The intestinal pH lowered by the production of acetic acid along with lactic acid can have an inhibitory effect on species of *Escherichia, Clostridium, Shigella* and *Salmonella* (28).

Anand et al. (2) reported that *Bifidobacterium bifidum* was inhibitory towards *E. coli, B. cereus, S. typhosa, S. aureus, S. dysenteriae, Ps. fluorescens* and *M. flavus* in laboratory media. The authors inferred that inhibition was due to the presence of some antibacterial substance(s) other than the acid produced from fermentation of sugars.

Children with various gastrointestinal problems to whom milk containing bifidobacteria was administered, exhibited increased weight gain and resistance to infection (36). Colombel et al. (12) examined the antidiarrheal effect of bifidus yogurt in erythromycin-induced gastroenteritis in adults. Abdominal discomfort was noticed only in 10% of the treated group after consuming bifidus yogurt containing *B. longum* compared to all of the control group. The ingestion of *B. bifidum* in combination with lactulose by children suffering from enteric infections led to eradication of *E. coli* in more than 80% of the cases studied (54).

Mice fed *B. longum* lived longer after intragastric or intravenous administration of a lethal dose of pathogenic *E. coli* or an intravenous injection of the endotoxin than did mice not fed the bifidobacteria (59). No clinical signs of pathogenic effect were observed when *B. longum* or mixtures of *B. longum* and *E. coli* were fed to gnotobiotic mice compared to the control (47).

Bifidobacteria can stimulate the host's immune system which can aid in the control of intestinal pathogens. An immune response was produced through increased secretion of immunoglobin A in monoassociated mice fed *B. longum* (28, 36). This immunoglobin A prevents the absorption of antigens by the mucosal epithelium by acting as a barrier to the foreign matter.

Strains of bifidobacteria that attach to epithelial cells significantly interfere with the attachment of enteropathogenic microbes and/or their toxins onto human epithelial cells (5, 62, 68). This represents another possible mechanism where by the bifidobacteria can control intestinal infections.

Some have speculated that bifidobacteria produce an antimicrobial like substance having properties of bacteriocin (2, 34, 43). This substance was found to be heat stable, active at pH 2-10 and active against other gram-positive bacteria such as other bifidobacteria, lactobacilli, clostridia and some streptococci (43). Anand et al. (3) reported that *B. bifidum* produces such a substance which can be stored at 4-5°C for more than three months without affecting its inhibitory activity.

Anticarcinogenic activity

Bifidobacteria may be able to exert some control on certain types of cancer. Feeding cells of *B. bifidum* to mice having tumor cells was followed by reduction in the number of tumor cells (54). In another study, when mice were administered cells *of B. breve* and fructooligosaccahrides there was a reduction in excreted carcinogens (33). The presence of *E. coli, Enterococcus fecalis* and *Clostridium paraputrificum* in the intestine of the mice was associated with the development of liver tumors (33). However, in the presence of *B. longum* proliferation of the tumors decreased.

Whole cells of *B. infantis* as well as the cell walls contain an antitumor constituent (21). Hence, injecting cell wall fractions of *B. infantis* into the growing tumors of mice increased the immune response (36, 54). In addition, ingestion of dead or live cells of bifidobacteria reduced tumor formation in mice and also the lethal effects (30).

Improved lactose utilization

Another potential health benefit of consuming bifidobacteria is their ability to breakdown lactose which is beneficial for lactose malabsorbing persons. Lactose maldigestion is due to insufficient amounts of β-galactosidase in the small intestine to hydrolyze lactose. Since bifidobacteria are resistant to bile and can hydrolyze lactose they can grow in the intestine to provide β-galactosidase to facilitate digestion of lactose (28, 33).

Cholesterol in bacterial membranes

Cholesterol is an integral part of the membrane in the eukaryotic cells. It is believed to maintain the structural rigidity and fluidity by its plasticizer effect (32).

Mycoplasma are unique organisms among prokaryotes because they do not have a cell wall. Cholesterol in their membranes is said to increase the tensile strength of the membrane (55). This permits their growth and survival without cell walls. Since they lack the ability to synthesize cholesterol, they require an external source of cholesterol for growth (29, 55, 58). Mycoplasmas when grown in the presence of cholesterol incorporate it into their cell membranes without any alteration (29, 63, 65). Incorporation is largely dependent upon the composition of other lipids and phospholipids of the membrane (56).

Mycoplasma capricolum when grown in the presence of horse serum (4%) incorporated 127 μg cholesterol/mg of membrane protein (44). Dahl et al. (13) observed an increase in cholesterol content of *M. capricolum* membranes from 14 to 28 mol % of the total lipid with corresponding increase in viscosity. Clejan et al. (11) reported an increase from 28 to 127 μg cholesterol/mg of membrane protein in 5 hr in the presence of 10% fetal calf serum in *M. capricolum*.

Mycoplasma gallisepticum when grown in the presence of cholesterol incorporated 52% of unoxidized cholesterol into their membrane (29). On the other hand, assimilation of oxidized cholesterol into the membrane had inhibitory effects in the growth of mycoplasma.

Osmotic stability of liposomes prepared with membrane lipids of *M. orale* was greater because of cholesterol and in *S. aureus* due to the presence of cardiolipin (48). The presence of cholesterol was found to decrease the transport of branched-chain amino acid, lysine, in membrane vesicles of *S. cremoris* (69).

Razin (57) reported incorporation of cholesterol by gram-positive bacteria, including *Micrococcus lysodeikticus, Bacillus megaterium* and *Proteus mirabilis*, when grown in the presence of cholesterol. However, the quantity incorporated was much less than observed for the mycoplasmas. In another study, membrane fluidity was reduced in *Bacillus megaterium* by incorporated cholesterol (63). Similarly, *E. coli* TB4 incorporated 0.147 mg of cholesterol/mg of protein in the membrane when grown in the presence of cholesterol at 37°C which caused a large decrease in the membrane fluidity (19).

Relationship between cholesterol and bile acids

Cholesterol is a vital constituent of cell membranes and the precursor of steroid hormones and bile acids (60, 66). Even though it is quite essential to life, its deposition in arteries has been associated with heart disease and atherosclerosis, two leading causes of death in humans.

Enterohepatic Circulation of

Cholesterol and Bile acid

Most of the endogenous cholesterol is synthesized in the liver and to a lesser extent in the intestine (60, 66). The liver is the only organ which has the capability to synthesize bile acids from cholesterol. The conversion of cholesterol to bile acids in the liver involves (1) saturation of the 5,6-double bond, (2) epimerization of the 3β-OH group, (3) introduction of OH groups into 7α and 12α positions; and (4) oxidation of C number 24 to carboxylate (60, 66). Conjugation of C number 24 with glycine or taurine results in respective conjugated bile acids. This accounts for 30-60% of the cholesterol utilized in the human body (60).

A substance is said to undergo an enterohepatic circulation when it is metabolized in the liver, excreted into the bile, passed into the lumen of the intestine, reabsorbed through the intestinal wall and then returned to the liver in the portal circulation (60). Cholesterol in the liver is converted into primary bile acids namely cholic acid and chenodeoxycholic acid (15, 18, 20). These are conjugated with the amino acid glycine or taurine to form respective conjugated bile acids. These bile acids are then stored in the gallbladder and released into the duodenum in response to a meal (42, 53, 66). The principle function of conjugated bile acids is to emulsify lipids and aid in efficient dispersion and absorption in the upper part of the small intestine. These bile acids are returned to the liver following reabsorption from the small intestine into the blood and back to the liver for reuse (45, 53, 60, 66).

In the small intestine, approximately 80-95% of the bile salts undergo the enterohepatic circulation by absorption through the wall of the small intestine (42, 45, 53). The remaining bile acids that escaped enterohepatic circulation are excreted through the feces which is the only route for cholesterol elimination from the body (15, 16, 20, 66). The fraction of the bile acid pool lost from the enterohepatic circulation is replaced by *de nova* synthesis of bile acids each day (38).

Bile salt hydrolase is the key bacterial enzyme for deconjugation of conjugated bile acids and it is synthesized constitutively (23, 42, 64). It is an extracellular enzyme in bifidobacteria which deconjugates bile acids to release cholic and chenodeoxycholic acids. These unconjugated secondary and tertiary bile acids are excreted more rapidly than conjugated ones (9). Thus deconjugation of bile acids can increase their excretion. This in turn increases the catabolism of cholesterol into bile acids and thus can reduce the pool of cholesterol in the body.

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CHAPTER III

CHOLESTEROL ASSIMILATION BY *BIFIDOBACTERIUM LONGUM* AND ITS INCORPORATION INTO THE CELLULAR MEMBRANE

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ABSTRACT

Four strains of *B. longum* isolated from feces of human volunteers were tested along with four commercially available strains of *B. longum* for bile tolerance, the ability to deconjugate sodium taurocholate and the ability to assimilate cholesterol. The cultures varied with respect to each test. There appeared to be no relationship between bile tolerance and ability to assimilate cholesterol. Relative amounts of deconjugation of sodium taurocholate also were not related directly to the amount of cholesterol assimilated by the cultures. However, inclusion of sodium taurocholate in the growth medium enhanced the ability of *B. longum* to remove cholesterol from the medium during growth. A portion of the cholesterol removed from the growth medium by *B. longum* was recovered in the cellular membrane fraction suggesting some assimilation of cholesterol into the membrane. Cells grown in the presence of cholesterol and taurocholate were more resistant to sonication than were those grown in the control broth and in broth containing the bile salt without cholesterol. This is further evidence that cell membrane was affected by the cholesterol.

INTRODUCTION

Coronary heart disease is one of the leading causes of death (42%) in the United States (38). Elevated levels of serum cholesterol have been associated with the occurrence of coronary heart disease (22). Thus, there has been much interest in reducing this risk by lowering serum cholesterol levels.

The consumption of milk products containing some lactobacilli or bifidobacteria can have a beneficial effect in lowering concentrations of serum cholesterol (6, 7, 11, 12, 13, 16, 18, 23, 26). Assimilation of cholesterol during growth by *L. acidophilus* has been observed by some researchers (2, 6, 10, 11, 40), an activity providing potential for helping control serum cholesterol if it occurs in the intestine. On the contrary, only a few reports have shown the potential hypocholesterolemic effect of bifidobacteria (16, 26, 35). Consumption of *B. bifidum*, shown to assimilate cholesterol in laboratory tests, for 6 weeks resulted in reduction of levels of serum cholesterol in humans having elevated levels of serum cholesterol (26).

Deconjugation of bile acids and excretion of the resulting free bile acids can result in reduction of serum cholesterol (8, 33). Chikai et al. (3) and Eyssen (8) observed greater excretion of bile acids, mostly deconjugated, in conventional animals than in germ-free animals. When fed to germ free rats, *B. longum* deconjugated taurine-conjugated bile acids and caused increased excretion of free bile acid (3, 17, 20). The increased excretion of bile acids has the potential to lower serum cholesterol concentrations, since their replacement would require utilization of some cholesterol in the body.

Mycoplasma sp. are unique prokaryotes which do not posses a cell wall. Cholesterol in their membranes increases the tensile strength of the membrane enabling

them to survive without cell walls (27, 41). They lack the ability to synthesize cholesterol, therefore require cholesterol in the growth media which is incorporated into their cell membranes (4, 5, 14, 24, 29). *Micrococcus lysodeikticus* and *Bacillus megaterium* also incorporate cholesterol into their cellular membrane during growth in the presence of cholesterol (28, 36). *Lactobacillus acidophilus* also incorporates it into the membrane during growth (25).

The purpose of this study was to determine whether or not cholesterol assimilation by *B. longum* was related to bile salt deconjugation and if it was incorporated into the membrane.

MATERIALS AND METHODS

Source and Maintenance of Cultures

An agar medium containing a mixture of chemicals selective for bifidobacteria (21) was utilized to isolate cultures of *B. longum*. The base medium was MRS agar prepared by dissolving agar (1.5%) in lactobacilli MRS broth (Difco Laboratories, Detroit, MI) prior to sterilization (15 min at 121°C). Just prior to use, an aqueous solution (rendered free of bacterial cells by passage through a sterile 0.45μ membrane filter) containing sufficient amounts of neomycin sulfate, paramycin sulfate, nalidixic acid and lithium chloride was added to 100 ml of melted agar to yield final concentrations of 6 mg/100 ml, 12 mg/100 ml, 0.9 mg/100 ml and 180 mg/100 ml respectively.

Human fecal samples were provided by three volunteers on sterile dacron swabs (Fisher Scientific Co., Pittsburgh, PA) in 10 ml volumes of lactobacilli MRS broth containing 0.2% sodium thioglycolate (Sigma Chemical Co., St. Louis, MO). This broth was referred to as MRS-THIO broth. The tubes containing the fecal samples were held in a refrigerator in an ice-water mixture for not more than 48 hours before microbiological analysis was done. The tubes containing the samples were vortexed (Fisher Scientific Co., Model 232) for approximately 15 seconds then appropriate dilutions were made using 1% peptone dilution blanks (39) and plated using the spread plate method onto plates of the selective agar medium. The plates were incubated for 48 hours at 37°C in a Gas Pak (Baltimore Biological Laboratories, Dickinson and Company, Maryland) anaerobic system. Following incubation, ten isolated colonies were picked using sterile inoculating needles and transferred into sterile tubes of MRS-THIO broth. The tubes were incubated

at 37°C until growth was indicated by turbidity. The isolated cultures were identified as described below. Those identified as *B. longum* were used for further study.

Three other strains of *B. longum* (I, II, III) were obtained from commercial suppliers and one (ATCC 15707) from the American Type Culture Collection. All cultures were maintained by subculturing (1% inoculum) into lactobacilli MRS-THIO broth incubating 16 to 18 hours at 37°C. The cultures were stored at 5 to 7°C between transfers. Prior to experimental use each culture was subcultured at least two times.

Identification of Cultures

Preliminary identification of the cultures was based on catalase test, gram-stain reaction and the presence or absence of fructose-6-phosphate phosphoketolase (15). Cultures composed of catalase negative, gram-positive rod shaped cells and positive for fructose-6-phosphate phosphoketolase were considered to be bifidobacteria. Additional characteristics were determined using API-50 CH kits (bioMérieux Vitek Inc., Hazelwood, MO). For this, cells were harvested from 10 ml MRS-THIO broth cultures by centrifugation (10 min at 12,000 x g at 4°C). The cells were washed once with 10 ml of sterile 0.75% sodium chloride solution (Fisher Scientific Co.) and resuspended in 5 ml of the same solution. The API-50 CH kits were inoculated using the cell suspensions following the manufacturer's direction except mineral oil was not added to each test well. The inoculated kits were incubated in a Gas Pak anaerobic system for 48 hours at 37°C. Determination of the species was accomplished by comparing observed reactions with those given in the ninth edition of *Bergey's Manual of Systemic Bacteriology* (15).

Comparison for Bile Tolerance and Bile Salt Deconjugation

The bile tolerance of each culture was measured by the procedure described by Gilliland and Walker (10), except MRS-THIO broth was used in place of MRS broth.

Results were expressed as hours required for the optical density (O.D.) at 620 nm to increase by 0.3 units. The bile salt deconjugation ability of the cultures was based on deconjugation of sodium taurocholate (40). Results were expressed as cholic acid released (mmoles/ml) during 18 or 24 hour incubation at 37°C.

Measurement of Cholesterol Assimilation

Sterile MRS-THIO broth was supplemented with the desired amount of sodium taurocholate (Sigma Chemical Co.). The sodium taurocholate solution was filtered through a sterile 0.45µ membrane filter prior to use. The broth was freshly prepared on the day of the experiment.

For each culture to be tested, one ml of cholesterol-phosphatidylcholine micelles prepared according to Razin et al. (30) was added to 9 ml of the MRS-THIO broth containing sodium taurocholate. Following mixing, 2 ml of the broth was removed into a sterile test tube and held as an uninoculated control. The remaining broth was inoculated (1%) with a freshly grown culture and incubated for the desired time in a water bath maintained at 37°C. After incubation cells were removed by centrifugation for 10 min at 12,000 x g and 4°C.

The o-phthaladehyde method (32) was used to measure cholesterol in the spent broth and uninoculated control broth. The amount of cholesterol assimilated (expressed in µg/ml) was determined by subtracting the amount remaining in the spent broth from that in the uninoculated control. In order to measure the cholesterol associated with the cells, the pellet from the culture was resuspended in a volume of distilled water equal to that of the original culture and assayed by the o-phthaladehyde method.

Cholesterol Assimilation During Growth at pH 6.5

MRS-THIO broth (450ml) supplemented with 0.006 M sodium taurocholate and cholesterol-phosphatidylcholine micelles (100µg cholesterol/ml) was placed in a

fermentor of about one liter capacity equipped with a pH electrode and ports for the addition of neutralizer and sparging with nitrogen gas (from bottom to top). The entire assembly containing the broth was autoclaved 15 min at 121°C. The fermentor was then placed in 37°C water bath and connected to an automatic pH controller (Model 5997, Horizon Ecology Co., Chicago, IL) which was adjusted to maintain the broth at pH 6.5. The neutralizer for the pH control unit was prepared by adding 5% ammonium hydroxide to a sterile solution of 5% sodium carbonate (9). After mixing the broth for 2-3 min, 10 ml of the broth was withdrawn aseptically using syringe and placed in a sterile screw cap test tube to serve as an uninoculated control. The remaining broth was inoculated with 5 ml of a freshly grown MRS-THIO broth culture of *B. longum* I. Nitrogen gas was sparged through the broth continuously at the flow rate of 5 cc/min. Following 18 hour incubation, a 10 ml sample was withdrawn and centrifuged (10 min at 12,000 x g at 4°C) to remove the cells. The spent and uninoculated control broths were assayed for cholesterol (32). A static culture (without pH control) in the same medium but without being sparged with nitrogen was incubated along side the culture being grown at pH 6.5 for comparison.

Isolation of Cellular Membrane

B. longum I cells were grown in 200 ml of MRS-THIO broth supplemented with 0.006 M sodium taurocholate and cholesterol-phosphatidylcholine micelles (100 μg cholesterol/ml). After 18 hour of incubation, the cells were harvested by centrifuging at 12,000 x g and 4°C for 10 min. The cell pellets were washed with distilled water and membrane isolation was carried out according to the method described by Thorne and Barker (37). The washed cells and membrane fractions were assayed for cholesterol (32), ATPase activity and protein content.

ATPase Activity and Protein Measurement

Adenosine triphosphatase (ATPase) activity was assayed by the method described by Rottem and Razin (31). The specific ATPase activity was expressed as μmoles of inorganic phosphate released per mg protein/min. The protein content was measured by the method of Bradford (1) using human albumin (Sigma Chemical Co.) as a standard.

Resistance of Cells to Sonic Disruption

B. longum I was grown in MRS-THIO (Medium A); MRS-THIO supplemented with 0.006 M sodium taurocholate (Medium B) and MRS-THIO supplemented with 0.006 M sodium taurocholate and cholesterol-phosphatidylcholine micelles (100μg cholesterol/ml) (Medium C) for 18 hours in a 37°C water bath. Cells were recovered by centrifugation at 12,000 x g and 4°C for 10 min. Each cell pellet was resuspended in distilled water to yield a density of approximately 2.5 x 10^9 cells/ml. Twenty ml volumes of cell suspensions were transferred to 50 ml beakers placed in an ice-water bath and sonicated for 10 min in a Sonic Dismembrator (Fisher Scientific, Pittsburgh, PA) adjusted to a maximum output. The number of intact cells were counted at 0 and 10 min by direct microscopic count (39). Results were expressed as percentage of cells lysed.

Statistical Analysis

All experiments were replicated three times. Analysis of variance for randomized complete block was used to determine if significant variation occurred among culture means in each experiment. Least significant difference test was used to determine which means differed significantly for the different strains of bifidobacteria within the experiment (34).

RESULTS

Identification of Isolates

The identification characteristics of each culture used in this study are listed in Tables A.1 and A.2 of the appendices. Each of the commercial strains (I, II and III) and ACT 15707 were confirmed to be *B. longum* based on identity characteristics published in *Bergey's Manual of Systemic Bacteriology* (15). Four cultures from intestinal samples from 3 different persons were identified as *B. longum*. These were included in the study.

Bile Tolerance

Variations were observed among strains with respect to growth in MRS-THIO supplemented with 0.3% oxgall (Table I). The times for the O.D. to increase by 0.3 units in the presence of 0.3% oxgall varied from 4.56 hr (strain I) to greater than 10 hr (strain III). Strain I was significantly ($P < 0.05$) more bile tolerant than strains K5, L5, II and III but not from strains S9, ATCC 15707 and S12. Strain III was significantly ($P < 0.05$) less bile tolerant than all other strains tested.

Influence of Sodium Taurocholate on Assimilation of Cholesterol

The amount of cholesterol removed (assimilated) from the growth medium by *B. longum* S9 was influenced by the concentration of sodium taurocholate in the medium (Table II). The amount of cholesterol removed increased with increasing concentrations of taurocholate from 0.0 M to 0.006 M then declined as the concentration of taurocholate increased beyond 0.006 M. Of the concentrations tested, significantly ($P < 0.05$) more

cholesterol was assimilated from the media containing 0.006 M taurocholate than from media containing any other concentration.

Simultaneous Screening for Bile Salt Hydrolase Activity
and Assimilation of Cholesterol

Initially the eight strains were compared in two groups of four simultaneously for bile salt hydrolase activity and ability to assimilate cholesterol from the growth media, during 24 hours of incubation at 37°C (Table B.3 and B.4 of the appendices). Minimal differences in bile salt deconjugation were observed except strain III was significantly ($P < 0.05$) less active than I, II and ATCC 15707. Strains S9, S12, K5 and L5 did not differ ($P < 0.05$) in amounts of deconjugation. The four strains that assimilated the most cholesterol (I, II, S9, ATCC 15707) were reassayed as a group using 18 hour incubation. Though the strains did not differ significantly ($P > 0.05$) in the ability to deconjugate sodium taurocholate (Table III), they did vary significantly ($P < 0.05$) in the amount of cholesterol assimilated. There was no significant difference between the amounts assimilated by strains I and II. However, both assimilated more ($P < 0.05$) cholesterol than did either strains S9 or ATCC 15707. The latter two strains did not differ ($P > 0.05$) in the amount assimilated.

There was no apparent relationship between the amounts of cholesterol assimilated and the amounts of bile salt deconjugated.

Cholesterol Uptake by *B. longum* I During Growth at pH 6.5

Some researchers (19) have suggested that removal of cholesterol from media containing cholesterol and conjugated bile salts by bifidobacteria results from it being coprecipitated with free bile salts released from deconjugation of the bile acid during growth of the organism. Since cholic acid released through deconjugation of taurocholate precipitates at pH below 6.0 (19), experiments were conducted in which the growth

medium was maintained at pH 6.5 throughout the growth period. During 18 hr of incubation more cholesterol ($P < 0.05$) was assimilated by *B. longum* I when grown at pH 6.5 than when grown as a static culture without pH control (Table IV).

Cholesterol in the Membrane Fraction of *B. longum* I

Cholesterol was measured in the membrane fraction of *B. longum* I after growing for 18 hour at pH 6.5 (Table V). The amount of cholesterol associated with the membrane was expressed as µg of cholesterol/mg of protein. The specific activity of the ATPase was significantly ($P < 0.05$) higher in the membrane fraction than in the washed cells showing that much of the non-membrane components of the cells had been removed during the isolation procedure. Significantly ($P < 0.05$) more cholesterol per mg protein was detected in the washed whole cells than in the membrane fraction. Thus, even though cholesterol was detected in the membrane, much of that associated with the cells apparently was not in the membrane.

Resistance of *B. longum* I to Sonic Disruption

Cells of *B. longum I* grown in medium C which contained both cholesterol and sodium taurocholate were significantly ($P < 0.05$) more resistant to lysis by sonication than were cells grown in either of the other media without cholesterol (Table VI). Cells grown in MRS-THIO (Medium A) and in MRS-THIO containing sodium taurocholate (Medium B) did not differ ($P > 0.05$) in their resistance to lysis by sonication; 91.1% and 91.6% lysis was observed for these media respectively. However, only 85% ($P < 0.05$) of the cells grown in MRS-THIO containing cholesterol and sodium taurocholate (Medium C) were lysed.

DISCUSSION

Previous studies have shown the need for bile salts in order for lactobacilli to assimilate cholesterol (2, 10, 11, 19). Data from the present study shows the importance of bile salts in enhancing the removal of cholesterol from growth media by *B. longum*. Growth is required for this phenomenon (35), thus the organism must be able to grow in the presence of bile. The degree of bile tolerance (i.e. measured as growth in media containing 0.3% oxgall) varied among strains of *B. longum* included in this study. The ability to assimilate cholesterol also varied among strains. However, the most bile tolerant strains did not necessarily assimilate the most cholesterol. For example, strain II was significantly less bile tolerant than strain S9, however it assimilated significantly more cholesterol than did S9. Thus a culture with high bile tolerance does not necessarily assimilate more cholesterol than one with lower bile tolerance.

Gilliland et al. (11) indicated that cholesterol removed from laboratory media during growth of *L. acidophilus* was assimilated by the culture. Klaver and van der Meer (19) on the other hand suggested that the cholesterol merely co-precipitated along with free bile acids released through deconjugation of conjugated bile acids in the growth medium. This was based largely on their observation that no cholesterol was removed when the growth medium was maintained at pH 6.0 which would prevent the precipitation of free bile acids. However, in the present study cholesterol was removed from the growth medium by *B. longum* during growth at pH 6.5. Furthermore, there appeared to be no relationship between the amount of cholesterol assimilated and the degree of bile salt deconjugation. Tahri et al. (35) reported that the removal of cholesterol from the growth medium by bifidobacteria was not entirely due to its precipitation.

Less cholesterol uptake was observed when *B. longum* was grown statically without pH control compared to the amount taken up in a culture maintained at pH 6.5. This could be due to greater growth of the culture in the medium at pH 6.5 than in the static culture or perhaps the agitation required for pH control facilitated better continuous contact between the cells and cholesterol micelles. Assay of membrane fractions isolated from cells of *B. longum* grown in the presence of cholesterol at pH 6.5 revealed cholesterol to be associated with the membrane. The fact that less cholesterol per mg protein was detected in the membrane fraction than in the whole cells suggests much of the cholesterol was not closely associated with the membrane. It may have been intracellular or merely adsorbed onto the cell surface.

The greater resistance to lysis by sonication of cells grown in the presence of cholesterol compared to those grown in its absence suggests differences in the cell membrane. *Mycoplasma* sp. require cholesterol for their growth which is incorporated into their membranes (4, 5, 14, 24, 28, 29). This increases the tensile strength of the membranes (27, 41). It may have a similar effect on the membrane of the bifidobacteria.

In summary, growth of *B. longum* in media containing cholesterol and sodium taurocholate resulted in part of the cholesterol being removed along with the bacterial cells. This removal was not related directly to the ability of the culture to deconjugate free bile acids that would cause the cholesterol to co-precipitate as the growth medium reached pH of less than 6.0. A part of the cholesterol removed from the growth media by *B. longum* was assimilated into the membrane. This incorporation appeared to increase resistance of the cells to sonic disruption. The strains of *B. longum* included in this study did not appear to be as active in removing cholesterol from a laboratory growth medium as has been reported for selected strains of *L. acidophilus* (2, 40). Thus, if this activity is important in having beneficial effect on serum cholesterol the bifidobacteria may not be the best choice of a culture for such use.

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TABLE I

COMPARISON OF GROWTH OF STRAINS OF *B. longum* IN BROTH SUPPLEMENTED WITH 0.3% OXGALL

Strains of *B. longum*	Bile Tolerance[1,2]
I[3]	4.56
S9	5.26[a,b]
ATCC 15707	5.40[a,b]
S12	6.44[a,b,c]
K5	7.20[b,c]
L5	7.43[b,c]
II[3]	8.35[c]
III[3]	>10.00[d]

1 Time in hours for the culture to increase the O.D. by 0.3 at 620nm at 37°C in MRS-THIO broth containing 0.3% oxgall.

2 Values are the means of three replications. Means with no common superscript letters differ significantly ($P < 0.05$). S.E. = 1.29; 12 df.

3 Commercial cultures.

TABLE II

INFLUENCE OF SODIUM TAUROCHOLATE ON ASSIMILATION OF CHOLESTEROL BY *B. longum* S9

Sod. Taurocholate[1]	Cholesterol Assimilated[2,3]
(Molar conc.)	(μg/ml)
0.000	6.11[a]
0.002	17.26[b]
0.004	23.46[c]
0.006	30.68[d]
0.008	22.62[c]
0.010	20.68[b,c]

[1] Molar concentration of sodium taurocholate in MRS-THIO broth containing cholesterol (83.14μg/ml) as cholesterol-phosphatidylcholine micelles.

[2] μg/ml of cholesterol assimilated by the culture during 24 hr growth at 37°C.

[3] Values are the means of three replications. Means with no common superscript letters differ significantly ($P < 0.05$). S. E. = 7.40; 7 df.

TABLE III

ASSIMILATION OF CHOLESTEROL AND BILE SALT DECONJUGATION BY SELECTED STRAINS OF *B. longum*[1]

Culture	Cholesterol Assimilated[2, 4]	Bile salt Deconjugated[3, 4]
II	34.14[a]	2.89[a]
I	31.05[a]	2.93[a]
S9	22.47[b]	2.65[a]
ATCC 15707	21.36[b]	2.96[a]

[1] During 18 hr growth at 37°C in MRS-THIO containing cholesterol (87.38µg/ml) as cholesterol-phosphatidylcholine micelles and 0.006 M sodium taurocholate.

[2] µg/ml of cholesterol assimilated. S.E. = 10.33; 6 df.

[3] mmoles/ml of cholic acid liberated. S.E. = 0.11; 6 df.

[4] Values are the means of three replications. Means with no common superscript letters differ significantly ($P < 0.05$).

TABLE IV

ASSIMILATION OF CHOLESTEROL BY *B. longum* I
DURING GROWTH[1] WITH AND WITHOUT
CONTROL OF GROWTH MEDIUM
AT pH 6.5

Growth conditions	Cholesterol assimilated[2]
	(μg/ml)
Static	30.62[a]
Controlled pH	43.34[b]

[1] During 18 hr growth at 37°C in MRS-THIO containing cholesterol (97.66μg/ml) as cholesterol-phosphatidylcholine micelles and 0.006 M sodium taurocholate.

[2] Values are the means of three replications. Means with no common superscript letters differ significantly ($P < 0.05$). S.E. = 11.28; 4df.

TABLE V

CHOLESTEROL CONTENT AND ATPase ACTIVITY
OF CELLS AND MEMBRANE OF *B. longum* I[1]

Fraction	Cholesterol Assmilated [2,4]	ATPase [3,4] (Specific activity)
Washed whole cells	78.71[a]	0.14[a]
Membrane	14.42[b]	1.63[b]

1 Cells were grown with control of growth medium (pH 6.5) in MRS-THIO containing cholesterol (81.48µg/ml) as cholesterol-phosphatidylcholine micelles and 0.006 M sodium taurocholate for 18 hr at 37°C.

2 µg of cholesterol/mg of protein. S.E. = 702.62; 2df.

3 The specific ATPase activity is expressed as µmoles/min/mg protein. S.E. = 0.00; 2 df.

4 Values are the means of three replications. Means in the same column with no common superscript letters differ significantly ($P < 0.05$).

TABLE VI

COMPARISON OF CELL LYSIS BY SONICATION OF CELLS OF *B. longum* I[1]

Growth Medium[2]	Additive	% Lysis[3]
A	None	91.2[a]
B	Sodium Taurocholate[4]	91.7[a]
C	Sodium Taurocholate[4]+ Cholesterol[5]	85.0[b]

[1] Cells harvested after 18 hr growth in the indicated medium at 37°C and sonicated with Sonic Dismembrator for 10 min after resuspending the cells in water to have an initial count of 2.5 X 10^9 cfu/ml (0% lysis). Number of nonlysed cells were counted by Direct Microscopic Count method and results are expressed in terms of percentage of lysis.

[2] MRS-THIO broth with indicated additives.

[3] Values are the means of three replications. Means with no common superscript letters differ significantly ($P < 0.05$). S.E. = 2.77; 6df.

[4] 0.006 M sodium taurocholate.

[5] 100μg/ml of cholesterol as cholesterol-phosphatidylcholine micelles.

APPENDICES

APPENDIX A : IDENTIFICATION CHARACTERS

TABLE A.1 IDENTIFICATION CHARACTERISTICS OF FRESHLY ISOLATED STRAINS OF *Bifidobacterium longum*[1]

API Tests[2]	Bergey[3]	S9	S12	K5	L5
L-Arabinose	+	+	+	+	+
Ribose	+	+	+	+	+
D-Xylose	+/-	+	+	+	+
Galactose	+	+	+	+	+
D-Fructose	+	+	+	+	+
D-Mannose	+/-	-	-	-	-
Mannitol	-	-	-	-	-
Sorbitol	-	-	-	-	-
Salicine	-	-	-	-	-
Cellobiose	-	-	-	-	-
Maltose	+	+	+	+	+
Lactose	+	+	+	+	+
Mellibiose	+	+	+	+	+
Saccharose	+	+	+	+	+
Trehalose	-	-	-	-	-
Inuline	-	-	-	-	-
Melezitose	+	+	+	+	+
D-Raffinose	+	+	+	+	+
Amidon	-	-	-	-	-
Gluconate	-	-	-	-	-

1 All isolates were Catalase negative, Gram-positive rods and positive for fructose-6-phosphate phosphoketolase.

2 API-50 CH kit

3 Reactions for *Bifidobacterium longum* as listed in the ninth edition of *Bergey's Manual of Systemic Bacteriology.*

TABLE A.2 IDENTIFICATION CHARACTERISTICS OF COMMERCIALLY AVAILABLE STRAINS OF *Bifidobacterium longum*[1]

API Tests[2]	Bergey[3]	I	II	III	ATCC 15707
L-Arabinose	+	+	+	+	+
Ribose	+	+	+	+	+
D-Xylose	+/-	+	+	+	+
Galactose	+	+	+	+	+
D-Fructose	+	+	+	+	+
D-Mannose	+/-	-	-	-	-
Mannitol	-	-	-	-	-
Sorbitol	-	-	-	-	-
Salicine	-	-	-	-	-
Cellobiose	-	-	-	-	-
Maltose	+	+	+	+	+
Lactose	+	+	+	+	+
Mellibiose	+	+	+	+	+
Saccharose	+	+	+	+	+
Trehalose	-	-	-	-	-
Inuline	-	-	-	-	-
Melezitose	+	+	+	+	+
D-Raffinose	+	+	+	+	+
Amidon	-	-	-	-	-
Gluconate	-	-	-	-	-

1 All isolates were Catalase negative, Gram-positive rods and positive for fructose-6-phosphate phosphoketolase.

2 API-50 CH kit

3 Reactions for *Bifidobacterium longum* as listed in the ninth edition of *Bergey's Manual of Systemic Bacteriology.*

APPENDIX B : DATA FROM TREATMENTS

Table B.1 INFLUENCE OF 0.3% OXGALL ON GROWTH OF *Bifidobacterium longum*

Culture	Time In Hours[1]			
	Rep. 1	Rep. 2	Rep.3	Mean[2]
S9	6.59	5.47	4.26	5.44[a,b]
S12	7.52	8.25	4.45	6.74[a,b,c]
K5	9.48	8.48	4.45	7.34[b,c]
L5	8.16	10.00	5.00	7.72[b,c]
I	4.48	6.02	4.32	4.94[a]
II	8.32	9.21	8.23	8.59[c]
III[3]	>10.00	>10.00	>10.00	>10.00[d]
ATCC 15707	5.43	6.46	5.13	5.67[a,b]

[1] Time for O.D. to increase by 0.3 units at 620nm at 37°C in MRS-THIO broth supplemented with 0.3% oxgall
[2] Means with no common superscript differ significantly
[3] Strain III was excluded from Analysis of Variance since all three values were greater than 10.00

Analysis of Variance Table

Source	DF	Sum of squares	Mean square	F Value
Model[1]	8	57.0883048	7.1360381	5.53
Error	12	15.4985905	1.2915492	
Total	20	72.5868952		

[1] Model includes culture and replication.
OSL < 0.05; $LSD^{.05} = 2.02$

Table B.2 INFLUENCE OF SODIUM TAUROCHOLATE ON ASSIMILATION OF CHOLESTEROL BY *B. longum* S9

Molar Conc. of Sod. Taurocholate	µg/ml of Cholesterol Assimilated			
	Rep. 1	Rep. 2	Rep.3	Mean[1]
0.000	8.88	7.96	1.49	6.11[a]
0.002	14.08	18.89	18.82	17.26[b]
0.004	22.78	22.78	24.81	23.46[c]
0.006	27.23	32.22	32.60	30.68[d]
0.008	19.82	24.71	23.34	22.62[e]
0.01	16.67	21.66	23.70	20.68[b,c]

[1] Means with no common superscript differ significantly

Analysis of Variance Table

Source	DF	Sum of squares	Mean square	F Value
Model[1]	7	1034.421489	147.774498	19.97
Error	10	74.015556	7.401556	
Total	17	1108.437044		

[1] Model includes culture and replication
OSL < 0.05; LSD $^{.05}$ = 4.95

Table B.3 CHOLESTEROL ASSIMILATED AND BILE SALT DECONJUGATED BY NEWLY ISOLATED STRAINS OF *B. longum* DURING 24 H OF INCUBATION AT 37°C

Culture	μg/ml of Cholesterol Assimilated				mM/ml of Bile Salt Deconjugated			
	Rep.1	Rep.2	Rep.3	Mean[1]	Rep.1	Rep.2	Rep.3	Mean[1]
S9	34.36	34.44	32.08	33.86[a]	3.09	3.35	2.26	2.90[a]
S12	26.11	22.99	23.70	24.27[c]	3.52	3.52	3.18	3.41[a]
K5	30.92	26.48	23.56	27.65[b]	3.34	2.06	2.45	2.62[a]
L5	26.67	27.78	24.07	26.17[b,c]	2.69	2.82	2.99	2.83[a]

[1] Means with no common superscript differ significantly

Analysis of Variance Table - For Cholesterol Assimilation

Source	DF	Sum of squares	Mean square	F Value
Rep	2	17.8763167	8.9381583	4.15
Culture	3	155.2011667	51.7337222	24.05
Error	6	12.9072833	2.1512139	
Total	11	185.9847667		

OSL < 0.05; LSD $^{.05}$ = 2.93

Analysis of Variance Table - For Bile Salt Deconjugation

Source	DF	Sum of squares	Mean square	F Value
Rep	2	0.38721667	0.19360833	0.93
Culture	3	1.00589167	0.33529722	1.62
Error	6	1.24418333	0.2736389	
Total	11	2.63729167		

OSL < 0.05; LSD $^{.05}$ = 0.99

Table B.4 CHOLESTEROL ASSIMILATED AND BILE SALT DECONJUGATION BY COMMERCIAL STRAINS OF *B. longum* DURING 24 H OF INCUBATION AT 37°C

Culture	µg/ml of Cholesterol Assimilated				mM/ml of Bile Salt Deconjugated			
	Rep.1	Rep.2	Rep.3	Mean[1]	Rep.1	Rep.2	Rep.3	Mean[1]
I	38.33	40.00	39.27	39.20^{b}	3.30	3.40	2.20	2.97^{a}
II	37.04	43.33	43.34	$41.24^{a,b}$	3.06	2.89	2.60	2.85^{a}
III	8.14	9.08	7.59	8.27^{c}	1.75	0.95	1.77	1.49^{b}
ATCC 15707	42.04	46.48	41.66	43.39^{a}	3.40	2.84	2.70	2.98^{a}

[1] Means with no common superscript differ significantly

Analysis of Variance Table - For Cholesterol Assimilation

Source	DF	Sum of squares	Mean square	F Value
Rep	2	22.266050	11.133025	3.17
Culture	3	2477.623367	825.874456	235.37
Error	6	21.052683	3.508781	
Total	11	2520.942100		

OSL < 0.05; LSD $^{.05}$ = 3.74

Analysis of Variance Table - For Bile Salt Deconjugated

Source	DF	Sum of squares	Mean square	F Value
Rep	2	0.64321667	0.32160833	1.81
Culture	3	4.7107000	1.57023333	8.86
Error	6	1.06365000	0.17727500	
Total	11	6.4175667		

OSL < 0.05; LSD $^{.05}$ = 0.84

Table B.5 ASSIMILATION OF CHOLESTEROL AND BILE SALT DECONJUGATION BY SELECTED STRAINS OF *B. longum*[1] DURING 18 H OF INCUBATION AT 37°C

Culture	µg/ml of Cholesterol Assimilated				mM/ml of Bile Salt Deconjugated			
	Rep.1	Rep.2	Rep.3	Mean[1]	Rep.1	Rep.2	Rep.3	Mean[1]
I	35.37	34.44	23.33	31.05[a]	2.69	2.75	3.35	3.00[a]
II	41.86	30.92	29.63	34.14[a]	2.06	3.18	3.42	2.89[a]
ATCC 15707	28.52	17.41	18.15	21.36[b]	2.72	2.85	3.32	2.96[a]
S9	25.74	22.03	19.63	22.47[b]	2.55	2.85	2.55	2.65[a]

[1] The best four of the eight cultures were selected based on the amount of cholesterol assimilated in previous experiments (Table B.3 and B.4).

[2] Means with no common superscripts differ significantly

Analysis of Variance Table - For Cholesterol Assimilation

Source	DF	Sum of squares	Mean square	F Value
Rep	2	214.2168500	107.1084250	10.37
Culture	3	358.2396250	119.4132083	11.56
Error	6	61.9671500	10.3278593	
Total	11	634.4236250		

OSL < 0.05; LSD $^{.05}$ = 6.42

Analysis of Variance Table - For Bile Salt Deconjugated

Source	DF	Sum of squares	Mean square	F Value
Rep	2	0.89971667	0.44985833	4.25
Culture	3	0.22009167	0.073363889	0.69
Error	6	0.63448333	0.10574722	
Total	11	1.75429167		

OSL < 0.05; LSD $^{.05}$ = 0.65

TABLE B.6 ASSIMILATION OF CHOLESTEROL BY *B. longum* I DURING 10, 14 AND 18 H (STATIC) INCUBATION AT 37°C

Time (Hr)	µg/ml of Cholesterol Assimilated			
	Rep.1	Rep.2	Rep.3	Mean[1]
10	16.86	12.96	19.81	16.53[b]
14	26.86	26.85	28.70	26.67[a]
18	34.82	28.70	28.33	30.62[a]

[1] Means with no common superscripts differ significantly

Analysis of Variance Table

Source	DF	Sum of squares	Mean square	F Value
Rep	2	16.9488222	8.4744111	1.01
Culture	2	316.1430889	158.0715444	18.90
Error	4	33.4461778	8.3615444	
Total	8	366.5380889		

OSL < 0.05; $LSD^{.05}$ = 6.56

Table B.7 ASSIMILATION OF CHOLESTEROL BY *B. longum* I AT CONTROLLED pH (6.5) AFTER 10, 14 AND 18 H INCUBATION AT 37°C

Time (Hr)	μg/ml of Cholesterol Assimilated			
	Rep.1	Rep.2	Rep.3	Mean[1]
10	20.75	30.37	20.56	23.89[c]
14	29.08	36.66	34.44	33.93[b]
18	39.82	45.19	45.00	43.34[a]

[1] Means with no common superscripts differ significantly

Analysis of Variance Table

Source	DF	Sum of squares	Mean square	F Value
Rep	2	85.0950889	42.5475444	6.35
Culture	2	567.1630889	283.5815444	42.35
Error	4	26.7837111	6.6959278	
Total	8	679.0418889		

OSL < 0.05; $LSD^{.05} = 5.87$

Table B.8 CHOLESTEROL CONTENT OF *B. longum* I CELLS AND MEMBRANE DURING 18 H (pH-6.5) INCUBATION AT 37°C

Fraction	µg of Cholesterol /mg of Protein			
	Rep.1	Rep.2	Rep.3	Mean[1]
Washed whole cells	90.84	60.24	85.06	78.17[a]
Membrane	15.31	12.50	15.45	14.42[b]

[1] Means with no common superscripts differ significantly

Analysis of Variance Table

Source	DF	Sum of squares	Mean square	F Value
Rep	2	319.868433	159.934217	1.49
Fraction	1	6200.449067	6200.449067	57.87
Error	2	214.271233	107.135617	
Total	5	6734.588733		

OSL < 0.05; $LSD^{.05}$ = 36.36

Table B.9 ATPase ACTIVITY OF *B. longum* I CELL AND MEMBRANE DURING 18 H (pH-6.5) INCUBATION AT 37°C

Fraction	Sp. Activity of ATPase			
	Rep.1	Rep.2	Rep.3	Mean[1]
Washed whole cells	0.15	0.14	0.12	0.14[b]
Membrane	1.64	1.64	1.62	1.63[a]

[1] Means with no common superscripts differ significantly

Analysis of Variance Table

Source	DF	Sum of squares	Mean square	F Value
Rep	2	0.00143333	0.0071667	14.33
Fraction	1	3.33015000	3.33015000	66603.00
Error	2	0.0001000	0.0005000	
Total	5	3.33168333		

OSL < 0.05; $LSD^{.05}$ = 0.65

Table B.10 LYSIS BY SONICATION OF *B. longum* I GROWN IN THE PRESENCE OF BILE AND CHOLESTEROL

		Rep.1		Rep.2		Rep.3		
Media[2]	Time (min)	DMC[1]/ml	% Lysed	DMC[1]/ml	% Lysed	DMC[1]/ml	% Lysed	Mean % Lysed[2]
A	0	3.6×10^9	-	1.7×10^9	-	2.1×10^9	-	0.0
	10	3.6×10^8	90.0	1.4×10^8	91.8	1.8×10^8	91.4	91.1[a]
B	0	2.8×10^9	-	1.1×10^9	-	1.6×10^9	-	0.0
	10	2.3×10^8	91.8	1.1×10^8	90.0	1.1×10^8	93.0	91.6[a]
C	0	4.0×10^9	-	2.2×10^9	-	2.1×10^9	-	0.0
	10	6.6×10^8	83.5	3.5×10^8	84.1	2.6×10^8	87.6	85.1[b]

[1] Direct Microscopic Count

[2] A = MRS. THIO broth (0.2%); B = MRS. THIO broth (0.2%) + sodium taurocholate (0.006 M); C = MRS. THIO broth (0.2%) + sodium taurocholate (0.006 M) cholesterol-phosphatidylcholine micelles (1% - final conc.)

[3] Means with no common superscripts differ significantly

Analysis of Variance Table - 10 min

Source	DF	Sum of squares	Mean square	F Value
Media	2	80.28222222	40.14111111	14.47
Error	6	16.64000000	2.77333333	
Total	8	96.92222222		

OSL < 0.05; $LSD^{.05} = 3.3272$

2

VITA

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