Binding of Free Bile Acids by Cells of Yogurt Starter Culture Bacteria¹

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ABSTRACT

Several strains of *Lactobacillus delbrueckii* ssp. *bulgaricus* and *Streptococcus thermophilus*, which produced exocellular polysaccharides (EPS), varied in the amount produced. The streptococci tended to produce the most EPS per milliliter of culture; however, when compared on the basis of amounts per 10⁷ cfu, the lactobacilli produced the most. *Lactobacillus delbrueckii* ssp. *bulgaricus* strains Lb-18 and Lb-10442 and *S. thermophilus* St-143 produced significantly larger amounts per 10⁷ cfu than did other strains tested. These three cultures plus two strains of the streptococci that produced the greatest amounts of EPS per ml of culture were tested for the ability to bind bile acids from laboratory media. The two cultures of *L. delbrueckii* ssp. *bulgaricus* (Lb-18 and Lb-10442) bound significantly higher amounts of cholic acid than did the three strains of streptococci. These two cultures of lactobacilli bound up to 15.3% of the cholic acid present in laboratory media, up to 452 µg/mg of EPS and 2.9 µg/10⁷ cfu. None of the cultures tested in this study were able to bind the conjugated bile acid, glycocholic acid.

(Key words: bile salt, exocellular polysaccharide, *Lactobacillus*, *Streptococcus*)

Abbreviation key: EPS = exocellular polysaccharide, NDM = nonfat dry milk.

INTRODUCTION

Elevated levels of serum cholesterol can lead to complications such as stroke and coronary heart disease (Pekkanen et al., 1990). Certain lactic acid bacteria such as lactobacilli have the potential to aid in the control of serum cholesterol levels. Mann and Sperry (1974) first demonstrated that milk fermented with lactobacilli might exhibit hypocholesterolemic effects in

humans. Other researchers have since found that some lactobacilli exhibit a cholesterol-lowering ability in man (Anderson and Gilliland, 1999) and animals (Grunewald, 1982; Gilliland et al., 1985).

Several researchers have attempted to elucidate the mechanism involved in the hypocholesterolemic action of these strains of lactic acid bacteria. One proposed mechanism is assimilation of cholesterol by the cells of *Lactobacillus acidophilus* during growth (Gilliland et al., 1985; Buck and Gilliland, 1994). Noh et al. (1997) demonstrated the ability of a strain of *L. acidophilus* to incorporate cholesterol from growth medium into cellular membranes during growth. Removal or assimilation of cholesterol by intestinal organisms in the small intestine could reduce the amount of cholesterol available for absorption from the intestine, thus exerting some control on serum cholesterol levels. Another plausible mechanism is deconjugation of bile acids by lactobacilli that produce the enzyme bile salt hydrolase. Deconjugated or free bile acids are more likely than are conjugated ones to be excreted from the body (Dietschy et al., 1966; Schiff et al., 1972). Thus, an increase in bile acid deconjugation in the intestines would continuously drain the cholesterol pool as more bile acids are synthesized. Many researchers have found bile salt hydrolase activity in strains of lactic acid bacteria, including *L. acidophilus* (Gilliland and Speck, 1977; Kobashi et al., 1978; Tannock, et al, 1989).

The exocellular polysaccharides produced by lactic acid bacteria are either excreted into the growth medium as free exocellular polysaccharides (EPS) or they adhere to the cell as capsular EPS (Sutherland, 1972). Due to the potential benefits of these bacteria to the quality of fermented dairy food products, several thermophilic and mesophilic lactic acid bacteria, and the polymers they produce, have been studied. *Streptococcus thermophilus* (Doco et al., 1990; Cerning et al., 1992), *Lactobacillus delbrueckii* ssp. *bulgaricus* (Bouzar et al., 1992; Grobbsen et al., 1995; Uemura et al., 1998.), other lactobacilli (Dunican and Seeley, 1965; Mozzi et al., 1994) and *Bifidobacterium longum* (Andaloussi et al., 1995) have been included in these studies. Most of these focused on the structure and characterization of the EPS. Very few have studied the potential health-promoting effects of EPS. Among these, Nakaj-

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ima et al. (1992) focused on the cholesterol-lowering activity of milk fermented with an EPS producing lactic acid bacteria. The consumption of this fermented milk significantly decreased serum cholesterol levels in rats, where as the consumption of milk fermented with a non-EPS producing strain did not. There is potential for these polymers to interfere with the absorption of cholesterol or of bile acids from the intestines by binding them and removing them from the body in a manner similar to that reported for plant-based polysaccharides or dietary fiber (Kritchevsky and Story, 1975).

The objective of this study was to determine whether different strains, *S. thermophilus* and *L. delbrueckii* ssp. *bulgaricus*, that produce capsular EPS have the ability to bind bile acids in vitro.

MATERIALS AND METHODS

Source and Maintenance of Cultures

The cultures used in this study were obtained from two sources. *L. delbrueckii* ssp. *bulgaricus* Lb-18 and Lb-10442, as well as *S. thermophilus* St-143 were obtained from Chris Hansen Inc. (Milwaukee, WI). *Streptococcus thermophilus* St-OSU1, St-OSU2, St-OSU3, *L. delbrueckii* ssp. *bulgaricus* Lb-OSU4 and Lb-OSU5 were all from the stock culture collection of the Food Microbiology Laboratory of the Food and Agricultural Products Center at Oklahoma State University. The cultures were maintained by subculturing weekly using 1% inocula in lactobacilli MRS broth made from individual ingredients according to the manufacturer's directions (Difco Laboratories, Detroit, MI), except that 4% lactose was used in the place of glucose (MRSL). The inoculated broth was incubated for 18 h at 37°C. The cultures were stored at 7°C between transfers. Stock cultures were stored in MRSL agar stabs (MRSL broth plus 1.5% agar). The cultures were subcultured at least three times immediately before experimentation. In addition, the cultures were subcultured using 1% inocula in sterile 10% reconstituted nonfat dry milk (NDM) at least three times before experimentation involving milk as the growth medium. The milk subcultures were incubated at 37°C for 18 h and stored at 7°C between transfers.

Preparation of Milk for EPS Production

The milk was prepared by reconstituting a commercial instantized NDM in deionized water at 10% (wt/vol). For use in experiments involving the measurement of EPS production, the 10% NDM was pasteurized at 80°C for 30 min and then refrigerated at 7°C until inoculation. It was prepared and used the same day.

Measurement of Capsular EPS

The measurement of capsular EPS material is complicated by its close association with the bacterial cells (Sutherland, 1972). The concentrations were measured based on the amount of carbohydrate expressed as glucose present in washed cell suspensions (Gilliland and Speck, 1974). The carbohydrate concentrations were determined by either a variation of the anthrone method (Gilliland and Speck, 1974) or the phenol-sulfuric method (Dubois et al., 1956), with glucose as the standard and the amount of EPS expressed as μg of glucose/ml of culture.

Enumeration of Lactobacilli and Streptococci

The numbers of lactobacilli and streptococci in the experimental samples were determined using the pour plate with overlay method (Vanderzant and Splittstoesser, 1992) on MRSL agar. Dilutions were prepared using 99-ml volumes of a sterilized solution of 0.1% peptone (Difco Laboratories) and 0.01% silicone antifoam (Sigma Chemical Co.). Plates were incubated at 37°C for 48 h, and colonies were counted with the aid of a Quebec colony counter (American Optical Co. Buffalo, NY).

Initial Screening of Cultures for Capsular EPS Production

The strains of *S. thermophilus* and *L. delbrueckii* ssp. *bulgaricus* were initially tested to determine the level of capsular EPS produced in 10% reconstituted NDM. Volumes of 10 ml of freshly pasteurized 10% NDM were inoculated with each of the eight cultures using 1% inocula and incubated at 45°C for 8 h. The capsular EPS content was based on sugar content of the cells and was determined in a manner similar to that of Gilliland and Speck (1974). The milk proteins were solubilized by adding 1.1 ml of 20% (wt/vol) sodium citrate to 5.5 ml volumes of each culture, and then 4.4 ml of deionized water was added, bringing the final concentration to 2% sodium citrate. The cells were collected by centrifugation at $12,000 \times g$ for 15 min at 0°C. Each pellet was washed once with 11 ml of cold deionized water and resuspended in 5.5 ml of cold deionized water. The total sugar content of the cell suspension was determined by the anthrone method. The amount of capsular EPS was expressed as μg of glucose/ml of original culture. In addition, plate counts were determined by the pour plate method as described above. This allowed the amount of EPS per 10^7 cfu to be calculated and compared among strains.

Measurement of Bile Acids

The quantification of free bile acid (cholic acid) was accomplished by the method described by Walker and Gilliland (1993). A standard curve was prepared using the sodium salt of cholic acid (Sigma Chemical Co.).

Measurement of Bile Acid Binding

To determine whether the strains were able to bind bile acids, presumably to the capsular EPS, an experiment was designed with cells grown in the MRSL broth. Bottles containing 75 ml of MRSL were inoculated (1%) with selected strains and incubated at 45°C for 8 h. Following incubation, two 22.5-ml volumes of each culture were transferred into sterile 50-ml Erlenmeyer flasks. To one, 2.5 ml of a 15 mM solution of cholic acid (sodium salt) was added, bringing the final concentration of bile acid to 1.5 mM (646 µg/ml). The second received similar mM concentration of the sodium salt of glycocholic acid. These samples were held in a 37°C shaker water bath, with gentle agitation for 2 h to allow contact between the cells and the bile acids.

Following the shaking step, the pH of each mixture was adjusted to 7.0 and the cells removed by centrifugation (12,000 × *g* at 0°C for 10 min). Any bound bile acid would be removed when the cells were removed. The supernatant fluids were assayed for bile salt using the colorimetric assay (Walker and Gilliland, 1993). An uninoculated control was included to permit a calculation of the amounts of bile salts removed (i.e., bound to the cells).

A portion of the initial broth culture was also analyzed for capsular EPS content using the same procedure as for the milk cultures, except that sodium citrate was not necessary to recover the cells from the broth culture. The phenol-sulfuric method was used to quantify the capsular EPS content of the cells. This information allowed calculations of the amount of bile acid bound per milligram of EPS present.

Effect of Initial pH on the Bile Binding Assay

Since the solubility of cholic acid decreases as the pH drops below 6.0, we evaluated the potential influence of acidity of broth on its recovery in our assay system. To test whether or not the pH of the broth cultures might have affected the removal of the bile acid, MRSL broth containing 1.5 mM cholic acid (sodium salt) was prepared and portions adjusted to the pH 6.5, 6.0, 5.0, 4.0, 3.0, 2.0, and 1.0. The broth was held in a shaker water bath for 2 h, adjusted to pH 7.0, centrifuged (12,000 × *g* at 0°C for 10 min), and the supernatant was analyzed for cholic acid following the same procedure described in the previous section.

Table 1. Comparison of amounts of capsular exocellular polysaccharide (EPS) produced by strains of *Lactobacillus delbrueckii* ssp. *bulgaricus* and *Streptococcus thermophilus* grown in 10% nonfat dry milk.

Culture ¹	µg/ml ^{2,4}	cfu/ml	µg/10 ⁷ cfu ^{3,4}
St-OSU3	857 ^a	2.1×10 ⁹	4.1 ^c
St-OSU2	505 ^b	9.3×10 ⁸	5.5 ^c
St-OSU1	478 ^b	1.3×10 ⁹	4.4 ^c
Lb-OSU4	471 ^b	3.9×10 ⁸	12.3 ^c
Lb-OSU5	451 ^b	2.8×10 ⁸	16.6 ^c
Lb-10442	448 ^b	1.3×10 ⁸	47.4 ^b
St-143	439 ^b	9.6×10 ⁷	47.0 ^b
Lb-18	398 ^b	5.8×10 ⁷	70.6 ^a

¹Lb = *Lactobacillus delbrueckii* ssp. *bulgaricus*; St = *Streptococcus thermophilus*.

² EPS is expressed as glucose µg/ml of cell suspension isolated from 8-hour cultures grown at 45°C.

³ EPS is expressed as glucose µg/10⁷ cfu in 8-hour cultures grown at 45°C.

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

Statistical Analyses

Experiments were conducted in triplicate. Each value was the mean of three independent trials. The EPS concentration and the degree of binding of the bile acids were analyzed as independent variables in each respective experiment. The analysis involved the analysis of variance procedure of JMPIN statistical software (Sall and Lehman, 1996). The means with significant differences were separated using the least significant difference method within the statistical software.

RESULTS

Initial Screening of Cultures for Capsular Exopolysaccharide Production

Significant differences (*P* < 0.05) were observed among the strains in the amounts of EPS produced per 10⁷ cfu in milk (Table 1). When considering the amount of EPS in µg/ml, *S. thermophilus* St-OSU3 produced significantly (*P* < 0.05) more than all other strains of both species in the study. The others, which were not significantly different, produced from 398 to 505 µg/ml. The degree of difference among cultures was greater when compared on the basis of µg/10⁷ cfu. *Lactobacillus delbrueckii* ssp. *bulgaricus* Lb-18 produced 70.6 µg/10⁷ cfu, which was significantly greater (*P* < 0.05) than other cultures tested. Strains St-143 and Lb-10442 followed, with 47.0 and 47.4 µg/10⁷ cfu, respectively. These amounts were significantly higher (*P* < 0.05) than the remaining five strains that produced 4.1 to 16.6 µg/10⁷ cfu. Based on the amount of EPS produced per 10⁷ cfu, three cultures (Lb-18, Lb-10442, and St-143) were selected for further study. In addition, St-OSU3 and St-OSU1 were included to compare strains with low

Table 2. Binding of cholic acid (sodium salt) by capsular exocellular polysaccharide produced by strains of *Lactobacillus delbrueckii* ssp. *bulgaricus* and *Streptococcus thermophilus* grown in MRS Broth containing 4% lactose as the source of carbohydrate.

Culture ¹	% Bound ^{2,5}	μg cholic acid/ mg EPS ^{3,5}	μg EPS/ ml	μg cholic acid/ 10^7 cfu ^{4,5}
Lb-10442	15.3 ^a	422 ^a	152	2.9 ^a
Lb-18	8.2 ^b	452 ^a	76	1.1 ^{ab}
St-OSU1	4.2 ^c	224 ^b	75	0.6 ^b
St-143	1.5 ^d	86 ^c	75	0.4 ^b
St-OSU3	0.6 ^d	56 ^c	54	0.2 ^b

¹Lb = *Lactobacillus delbrueckii* ssp. *bulgaricus*; St = *Streptococcus thermophilus*.

²Values are expressed as a binding %, percentage of cholic acid removed by contact with cells from 8 hour cultures grown at 45°C.

³Values are expressed as μg of cholic acid bound per mg of glucose (EPS).

⁴Values are μg of cholic acid bound per 10^7 colony forming units (cfu).

⁵Values are the means of three replications. Means within columns with no common superscript letters differ significantly ($P < 0.05$).

production activity per colony-forming units to the more active strains.

Measurement of Bile Acid Binding

There were significant differences ($P < 0.05$) among the five strains with respect to the binding of cholic acid (Table 2). The degree of binding was measured as percentage of cholic bound and micrograms of cholic acid bound per milligram of EPS. Strain Lb-10442, which produced the most EPS, bound the greatest amount ($P < 0.05$) of bile acid measured at 15.3%, which was nearly twice that of strain Lb-18, which bound 8.2%. Culture St-OSU1 was able to remove 4.2%, while the two remaining strains, St-143 and St-OSU3, were able to bind only minimal amounts. The latter two produced the least amount of EPS. When the results of the microgram of cholic acid bound per milligram of EPS were analyzed, very similar differences were observed. However, there was no difference ($P > 0.05$) between strain Lb-18 (452 $\mu\text{g}/\text{mg}$ of EPS) and Lb-10442 (422 $\mu\text{g}/\text{mg}$ of EPS). The strains of streptococci St-OSU1, St-143, and St-OSU3 bound significantly less ($P < 0.05$) than the two strains of lactobacilli with 224, 86, and 56 $\mu\text{g}/\text{mg}$ of EPS, respectively. In addition, strain Lb-10442 was able to bind 2.9 $\mu\text{g}/10^7$ cfu, which was significantly higher than all the streptococci. Results from experiments to test for the binding of glycocholic acid indicated that none of the strains in this study would bind this conjugated bile acid (data not shown).

Effect of Initial pH on the Bile Binding Assay

To validate that sodium cholate was being associated with the bacterial cells and not just precipitating due to low pH of the cultures, an experiment was conducted to determine the effect of the pH on the removal of cholic

acid from MRSL broth. The series of broth samples at various pH levels were treated and analyzed exactly as mentioned in the procedure to test the cultures for the ability to bind bile acids. After incubation for 2 h at 37°C in the broth at pH 6.5, 6.0, 5.0, 4.0, 3.0, 2.0, and 1.0, then adjusting each to pH 7.0 and centrifuging all the bile acid was recovered in the supernatant fluid. Thus, none was removed by exposure to low pH levels.

DISCUSSION

Some species of lactic acid bacteria, especially lactobacilli, have the potential to assist in the reduction of elevated serum cholesterol levels (Mann and Sperry, 1974; Anderson and Gilliland, 1999). This beneficial action could involve their ability to assimilate cholesterol into cellular membranes (Noh et al., 1997) or to deconjugate bile acids (Gilliland and Speck, 1977; Kobashi et al., 1978; Tannock et al., 1989). Nakajima et al. (1992) found significant differences between serum cholesterol levels among rats consuming fermented milk made with cultures that do or do not produce exocellular polysaccharides. The authors theorized that the observed differences were due to reduced absorption in the intestines, of cholesterol or bile acids due to the exocellular polysaccharides. Due to the resistance of the polysaccharide to digestive enzymes and its water-holding capacity, they hypothesized that the bacterial polymer acted in a manner similar to a dietary fiber.

Other researchers have recognized the possible effects of intestinal organisms on the binding of bile acids or interference in their absorption from the intestine. Fecal sterol composition can be highly dependent upon the presence of bacteria in the intestines (Mott et al., 1973). Several possible factors were cited, including binding of bile acids by intestinal bacteria as being responsible for sterol balance in the body. Chikai et al.

(1987) studied the bile acid deconjugation ability of several species of bacteria. They found that an increase in bile acid deconjugation resulted in increased bile acid excretion. They speculated that adhesion of free bile acids, resulting from the deconjugation, to bacteria as well as dietary fibers might also contribute to the large amounts of bile acid excretion.

The results of this study indicate variation among cultures with respect to binding of cholic acid. The culture, *L. delbrueckii* ssp. *bulgaricus*, which produced the greatest amount of EPS, bound the most cholic acid per mg of EPS or $2.9 \mu\text{g}/10^7$ cfu from laboratory media. Since the cultures did not bind a conjugated bile acid (glycocholic acid), this indicates a possible action in enhancing increased excretion of free bile acids from the body after consumption of a fermented product made with such a culture. This could result in reduction of the cholesterol pool as cholesterol is used to synthesize more bile acid in the enterohepatic circulation to replace those excreted with the EPS via the feces.

In the screening portion of this study there were variable amounts of EPS produced in the milk by the cultures tested. The greatest amounts of EPS in $\mu\text{g}/\text{ml}$ were produced by the streptococci. However, the growth of the streptococci in milk resulted in much higher cell numbers in cfu/ml than the lactobacilli. As a measure of how much EPS per cell was produced, μg of EPS per 10^7 cfu was calculated. This allowed comparisons of strains on total amount produced, as well as the amount surrounding each cell. The lactobacilli produced the highest amounts per 10^7 cfu. The five strains selected for further study exhibited variable production capabilities in both the total production and amount produced per cell. The amount of cholic acid bound was greatest for the cultures that produced the greatest amounts of EPS per 10^7 cfu. However, glycocholic acid, a conjugated bile acid, was not bound by the cells of any of the five cultures.

Theoretically, consumption of yogurt manufactured with the cultures that produce EPS could bind free bile acids in the intestine, thus enhancing excretion of the bile acids via the feces. Figure 1 shows a comparison among the cultures in this study with regard to the amount of cholic acid that could theoretically be removed or bound when an 8-oz serving of the fermented milk or yogurt is consumed. These values are based on the populations and amounts of EPS that would be expected during growth of the cultures in milk. As with the other results, the most bile acid bound would be from the consumption of milk fermented with the *L. delbrueckii* ssp. *bulgaricus* strains Lb-10442 and Lb-18. Up to 45 mg of cholic acid could be removed by a serving yogurt made with Lb-10442. The theoretical amounts that could be bound by strains of streptococci

Figure 1. Comparison of the theoretical amounts of cholic acid that could be bound and removed from the body by consuming an 8 oz serving of cultured product containing each strain.

ranged from 9 to 26 mg of cholic acid/8-oz serving. In addition, other experiments in our laboratory indicate that some of these cultures produce free EPS that are not associated with the cell (data not shown). This also may contribute to the total amount of bile acids that could be bound due consumption of the yogurt. In the present study, the strains of both species of cultures were examined individually. In the actual manufacture of traditional yogurt in which one or more strains of both species might be used, the amounts of EPS might vary which could influence the amounts of bile acids bound.

The binding of bile acids to capsular EPS may contribute to the development of a product containing a probiotic that uses a variety of mechanisms to control serum cholesterol levels. Strain or strains of starter cultures that have the ability to bind free bile acid could be used to produce a yogurt product in conjunction with a probiotic organism known to deconjugate bile acids. The strains in this study did not bind conjugated bile salts (sodium glycocholate). Increasing deconjugation of glycocholic due to the action of a deconjugating probiotic organism to release cholic acid would make more cholic available for binding and removal from the body. Even though the free bile acids are more likely to be excreted via the feces, the presence of an EPS that preferentially binds them over conjugated bile acids could enhance their excretion. Thus the combination of a probiotic culture such as *L. acidophilus* that deconjugates bile acids with selected strains of *L. delbrueckii* ssp. *bulgaricus* and *S. thermophilus* could be used to manufacture cultured yogurt that would have enhanced hypocholesterolemic activity. Such a product would be useful for hypercholesterolemic individuals.

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