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## ANTICHOLESTEREMIC PROPERTY OF *LACTOBACILLUS ACIDOPHILUS* YOGURT FED TO MATURE BOARS<sup>1,2,3</sup>

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### ABSTRACT

Three strains of *Lactobacillus acidophilus* (LA) were isolated from the feces of mature boars that were not being fed antibiotics from the Nebraska Gene Pool (NGP). All three LA isolates were screened in vitro for anticholesteremic and antimicrobial activities. One strain, LA16, caused the greatest reduction in cholesterol and inhibited both *Bacillus subtilis* and *Escherichia coli* the most. LA16 was used to produce 16, 18.9-liter quantities of *acidophilus* yogurt (AY), over a period of 8 wk, for use as a feed ingredient in diets for the NGP boars. Colony forming units (cfu), pH, protein, energy, Ca and P were consistent across all 16 batches of yogurt. All of the 18 boars were fed a high-cholesterol diet for a period of 56 d at a rate of 2.268 kg/(hd-d) to furnish 6.661 g/(hd-d) of cholesterol. Nine of the boars then were fed 1.81 kg/(hd-d) of a second diet that was supplemented with .454 kg/(hd-d) of AY. The other nine boars were fed the original diet. Cholesterol intake was the same for the two dietary treatments. Blood samples were collected weekly from the brachial-jugular region and the sera were analyzed for lipids. *Acidophilus* yogurt reduced serum cholesterol ( $P < .01$ ) and low density lipoproteins ( $P < .08$ ), but it had no effect on serum triglycerides ( $P > .23$ ) or on high density lipoproteins ( $P > .11$ ).

(Key Words: *Lactobacillus acidophilus*, Yogurt, Cholesterol, Triglycerides, Lipoproteins, Boar.)

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### Introduction

*Lactobacillus acidophilus* (LA) is a gram positive, fastidious organism that has adapted to complex organic substrates. It has been isolated from the intestinal tract of man and animals (Kandler and Weiss, 1986). Metchnikoff (1907) proposed that the longevity of

Rumanian and Bulgarian farmers was due to their regular consumption of milk and buttermilk cultured with LA and *Lactobacillus bulgaricus*. The therapeutic value of LA has since been established for many diseases and disorders of the digestive tract (Shahani and Chandan, 1979; Deeth and Tamime, 1981).

High blood cholesterol, high blood pressure, smoking, obesity and intake of saturated fats have been implicated in heart disease of humans (Pelkonen et al., 1977). Recently, it has been shown that yogurt and other fermented milk products contain some substance(s) that lower serum cholesterol in humans (Mann, 1977), swine (Stahelin et al., 1981), rats (Grunewald, 1982) and rabbits (Thakur and Jha, 1981).

Because the pig is considered to be a good animal model for studies of human atherosclerosis (Pond et al., 1986; Miller and Ullrey, 1987), the objective of our research was to determine the effect of feeding LA yogurt on serum cholesterol, triglycerides and lipopro-

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teins of adult intact male pigs fed diets high in cholesterol.

### Experimental Procedure

*Experiment 1.* This experiment involved culturing of bacteria isolated from the feces of mature Nebraska Gene Pool (NGP) boars that were not being fed antibiotics. The objective was to find a strain of porcine LA that was capable of assimilating cholesterol (Gilliland et al., 1985) and competing with other organisms within the intestinal tract (Shahani et al., 1976). It generally is accepted that LA is capable of establishment within the gastrointestinal tract (Alm and Pettersson, 1980). By culturing these bacteria from the porcine intestinal tract, the controversy over the adhesion capabilities of LA when transferred from one species of animal to another is eliminated.

Eighteen sexually mature NGP boars were randomly selected. Fecal samples were obtained from the rectum via manual stimulation of the internal and external anal sphincters. The feces were collected directly into sterile polyethylene bags and placed on ice. Subsamples of each fecal sample then were diluted with a phosphate buffer (Butterfield's in Leininger, 1976), and plated on acidified *Lactobacillus* selection (LBS) agar<sup>7</sup>. The plates were incubated at 37°C for 36 to 48 h in a 99% CO<sub>2</sub> environment at 37°C. After the incubation period, colonies were selected for gram stain. Regular, non-spore forming gram positive rods were the only bacteria that were retained for the determination of species. These were transferred to Lactobacilli MRS broth<sup>8</sup>, where they were grown for 24 h. All strains were tested for the iron-porphyrin enzyme catalase. A sample of each bacteria was placed on a sterile petri plate and a drop of H<sub>2</sub>O<sub>2</sub> was added. All strains that were positive for catalase were discarded.

Thirty-six *Lactobacillus* strains were speciated using a procedure developed by S. K. Frank (personal communication). This proce-

dures employs MRS broth for general propagation, performance of catalase tests, determination of growth temperatures, and obtaining bacterial cell suspensions for use in other tests. Initial separation of the different strains of *Lactobacillus* was accomplished by fermenting glucose with a Durham insert (Frobisher et al., 1974). Biochemical and physiological tests were performed to differentiate the strains of lactobacilli. After this was completed, additional non-essential confirmation tests were conducted (Rogosa, 1974).

Each strain of LA that was differentiated was screened for bile tolerance, cholesterol reduction and antimicrobial activities. Bile tolerance was determined by growing the different strains in MRS broth supplemented with two levels (.2 and .4%) of ox gall<sup>8</sup> (dried, unfractionated bovine bile salts). The organisms were determined to be bile tolerant if appreciable growth occurred after incubation for 24 h at 37°C. Cholesterol assimilation was determined by adding a water soluble form of cholesterol (polyoxyethanyl-cholesteryl sebacate<sup>9</sup>) to MRS broth at a rate of 10 mg cholesterol/dl. This was done in duplicate at .2 and .4% added ox gall to mimic the physiological conditions of the intestinal tract. The bacterial strains were added and incubated in a 99% CO<sub>2</sub> environment for 24 h at 37°C. Bacterial cells then were removed by centrifugation and the supernatant fluid was analyzed at the University of Nebraska-Lincoln Health Center Laboratory<sup>10</sup> for cholesterol<sup>11</sup>.

Antimicrobial activity was tested by a disc-assay procedure. The medium employed was antibiotic medium 2<sup>8</sup> (Penassay base agar). The agar was placed in sterile petri plates and lawns of both *Bacillus subtilis* and *Escherichia coli* were made on separate plates. Sterile discs were inoculated with each strain of LA and placed on each set of plates. The zone of inhibition (lack of microbial growth) was measured after incubation at 37°C for 6, 12 and 24 h. The 6-h measurement was used to evaluate inhibition because no additional growth appeared after 12 or 24 h.

Each LA strain was used in a preliminary study to produce *acidophilus* yogurt (AY) (Danielson, 1987). This was done to determine fermentation time, pH of the final product, degree of whey production, and quality of curd formation. The procedure used to produce the yogurt is outlined in Figure 1. This procedure was scaled down to produce 500 ml of yogurt.

<sup>7</sup>BBL Microbiological Systems, Becton Dickinson Co., Cockeysville, MD.

<sup>8</sup>Difco Laboratories, Detroit, MI.

<sup>9</sup>Sigma Chemical Co., St. Louis, MO.

<sup>10</sup>15th and U, Lincoln, NE.

<sup>11</sup>Central Technical Services, Beckman Instruments Inc., Fullerton, CA.

TABLE 1. ANALYSIS OF PORCINE *ACIDOPHILUS* YOGURT<sup>a</sup>

Item	pH	Plate count (log CFU/g <sup>d</sup> )	GE <sup>b,c</sup> , cal/g	CP <sup>b,d</sup> , %	Ca <sup>b</sup> , %	P <sup>b</sup> , %
Range	4.47-4.55	7.08-7.77	4,574-4,844	29.3-32.1	.84-.98	.79-.91
Mean	4.51	7.56	4,708	30.9	.91	.86

<sup>a</sup>Analysis of 16, 18.9-liter batches of yogurt.

<sup>b</sup>100% dry matter.

<sup>c</sup>GE = gross energy.

<sup>d</sup>Log of colony forming units/g.

*Experiment 2.* The objective of the research conducted was to determine the effect of feeding AY on serum cholesterol, triglycerides, high density lipoproteins (HDL) and low density lipoproteins (LDL) of mature NGP boars fed a high-cholesterol diet.

The AY was produced at the University of Nebraska Food Processing Center<sup>5</sup>. The nonfat dry milk (NFDM) used to produce AY was of food grade quality. Pasteurization (85°C for 30 min) of the milk and NFDM mix was implemented to destroy the thermoduric bacteria normally present in milk. Denaturation of the whey proteins is necessary to obtain a firm curd (Chandan, 1982). The inoculation rate was 2% LA and 2% *Streptococcus thermophilus* for the first five batches of yogurt processed (16 total batches processed), whereas the inoculation rate was 4% LA and 2% *Streptococcus thermophilus* for the remaining 11 batches, *S. thermophilus* was added to the yogurt for a symbiotic effect and to provide a shorter coagulation time. *S. thermophilus* was maintained in reconstituted NFDM (10% solids) at 43°C; LA was maintained in the same medium with the addition of yeast extract<sup>8</sup> at 37°C. Only 20- to 24-h-old vigorously growing bacteria cells were used to inoculate the milk base.

Sixteen 18.9-liter batches of yogurt were produced, and pH, plate count, gross energy, crude protein, Ca and P were determined to assess consistency among batches (Table 1). The pH was measured by a standard pH meter; the probe was placed directly into the yogurt. The pH was checked every hour. Plate count was determined by plating a sample of yogurt on acidified LBS agar. These plates were

counted after 24 h of incubation in a CO<sub>2</sub> atmosphere at 37°C. Yogurt samples were freeze-dried prior to analysis for gross energy, crude protein, Ca and P. Gross energy was determined using an adiabatic calorimeter<sup>12</sup>. Crude protein was determined by the Kjeldahl method (AOAC, 1984). A method described by AOAC (1984) was used to determine Ca; phosphorus was determined by the molybdenum blue method (Fiske and Subbarow, 1925).

Eighteen NGP boars (177 kg, 18 mo.) were used in this phase. The boars were tethered in individual stalls (.7 × 2.3 m) and given access to automatic waterers. A pre-test diet high in cholesterol (HC) (Table 2) was fed to all animals for 56 d prior to the initiation of the experiment. This diet was fed at the rate of

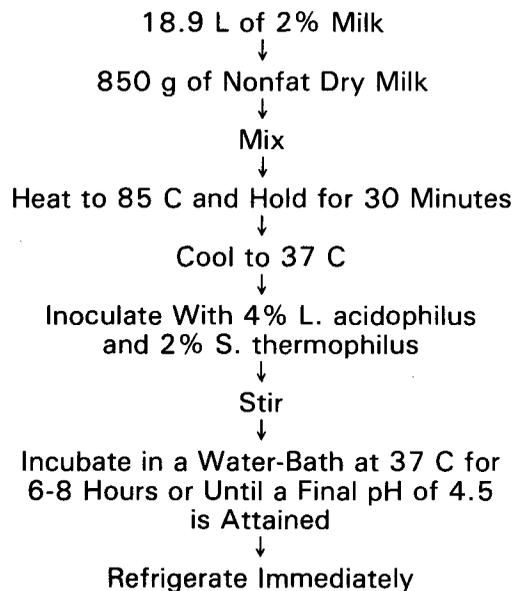


Figure 1. Flow chart for the production of porcine *acidophilus* yogurt.

<sup>12</sup>Procedure described in Manual No. 153, Parr Instrument Co., Moline, IL.

1.134 kg/animal every 12 h. The diet contained dried egg yolk, which was added to provide 6.61 g/(animal-d) of cholesterol (3.305 g/cholesterol per animal per feeding). Blood samples were obtained at the end of the 56-d pre-test period.

Immediately after the pre-test period, nine of the boars were removed from the HC diet and fed the yogurt diet shown in Table 2. This diet was fed at the rate of .907 kg/animal every 12 h plus an additional supplementation of .227 kg/animal AY (as fed). The yogurt was blended into the diet with a small mixer for 5 min prior to feeding. The other nine boars remained on the HC diet (1.134 kg/animal every 12 h). The two respective diets were formulated to contain equal amounts of cholesterol, energy, Ca, P and crude protein, but not dry matter. Dry matter was not equalized because humans consuming yogurt would simply substitute yogurt for other feedstuffs. The experimental period was an additional 56 d.

After a 12-h fast, blood samples were obtained from the brachial-jugular region on d 0, 7, 14, 21, 28, 35, 42, 49 and 56. The blood was collected in sterile Corvac<sup>13</sup> integrated serum separator tubes (transport style, 15 ml) and was centrifuged at 2,000 × g. Serum samples were analyzed for cholesterol, triglycerides and lipoproteins using a Technicon SMAC system<sup>14</sup> (sequential modular analysis with computer, model 397).

Fecal samples were collected at d 28, 35, 42, 49 and 56 using the same procedure described for Exp. 1. Total lactobacilli were determined by plating a sample of the feces on acidified LBS agar; the number of fecal coliforms was determined on violet red bile agar<sup>8</sup>.

**Statistical Analysis.** Serum cholesterol, triglycerides, HDL and calculated LDL were analyzed by least-squares analyses of variance using the General Linear Models procedure as outlined by SAS (1982). Because initial sera values were different among pigs, they were used as covariates. Fecal counts were com-

pared being multivariate analysis with a polynomial transformation.

## Results

**Experiment 1.** Three out of 36 strains of *Lactobacillus* were determined to be of the species *acidophilus*. The key employed in the identification of species was based partially on Bergey's manual of determinative bacteriology (Rogosa, 1974)<sup>15</sup>. Glucose fermentation without carbon dioxide production indicates that a strain belongs to Group I, homofermentative, which can be further divided by ribose fermentation into either *Thermobacterium* IA (ribose negative) or *Streptobacterium* IB (ribose positive). The species *acidophilus* belongs to Group IA, homofermentative *Thermobacterium*. The results of the essential biochemical and physiological tests were:

TABLE 2. COMPOSITION OF HIGH-CHOLESTEROL DIETS FOR MATURE BOARS

Ingredient	Diet	
	High cholesterol (HC)	Yogurt <sup>a</sup>
	% composition	
Ground corn	62.70	54.70
Soybean meal (44% protein)	13.60	18.70
Dried egg yolk <sup>b</sup>	10.60 <sup>c</sup>	13.30 <sup>c</sup>
Beet pulp	5.00	5.00
Tallow	3.00	3.00
Dicalcium phosphate	2.75	2.75
Vitamin premix <sup>d</sup>	1.00	1.00
Limestone	.60	.80
Iodized salt	.50	.50
DL-Methionine	.15	.15
Trace mineral premix <sup>e</sup>	.05	.05
Selenium premix <sup>f</sup>	.05	.05

<sup>a</sup>Fed in combination with .454 kg/(animal-d) *acidophilus* yogurt.

<sup>b</sup>Henningsen Foods Inc., David City, NE.

<sup>c</sup>Provided 2,914 and 3,644 mg of cholesterol/kg of HC and Yogurt diet, respectively.

<sup>d</sup>Provided the following per kg of complete diet: vitamin A, 5,512 USP; vitamin D<sub>3</sub>, 551 ICU; vitamin E, 22 IU; riboflavin, 5.5 mg; d-pantothenic acid, 19.8 mg; niacin, 33.1 mg; choline chloride, 551.2 mg; vitamin B<sub>12</sub>, .017 mg; vitamin K, 2.2 mg; ethoxyquin, 4.4 mg.

<sup>e</sup>Provided the following per kg of complete diet: Fe, 87.5 mg; Zn, 75 mg; Mn, 30 mg; Cu, 8.75 mg; I, 1 mg.

<sup>f</sup>Provided .1 mg Se/kg diet.

<sup>13</sup>Sherwood Medical, St. Louis, MO.

<sup>14</sup>Roche Biomedical Laboratories, Wichita, KA.

<sup>15</sup>A procedure partially based on Bergey's manual of systematic bacteriology (Kandler and Weiss, 1986) keyed these same three strains to belong to one of two complexes: *Lactobacillus acidophilus/gasseri* complex or *Lactobacillus acidophilus/amylovorus/crispatius/gasseri* complex.

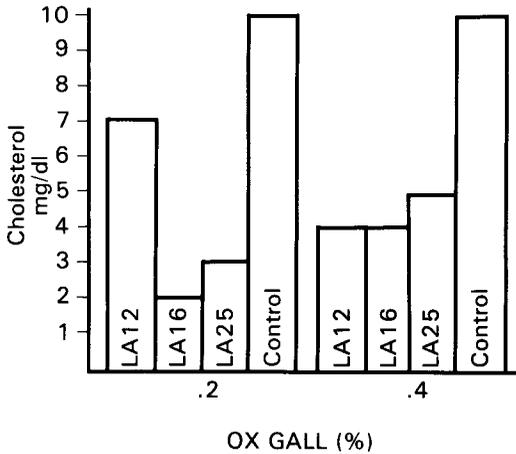


Figure 2. Residual cholesterol following 24 h in vitro incubation with porcine *Lactobacillus acidophilus* at two levels of ox gall (bile salts) (SE = 0).

esculin hydrolysis (+), mannitol (-), melibiose (-), lactose (+), galactose (+) and amygdalin (+); additional non-essential confirmation tests results were: cellobiose (+), maltose (+), salicin (+), sorbitol (-), sucrose (+), trehalose (-),  $\text{NH}_3$  from arginine (-) and motility (-). The three strains that fit this systematic design were then named LA12, LA16 and LA25. All three strains were considered to be highly bile tolerant because all produced heavy growth in MRS broth supplemented with either .2 or .4% ox gall. In vitro cholesterol assimilation (Figure 2) indicated that strain LA16 lowered cholesterol by 80 and 60% at .2 and .4% ox gall, respectively. Strain LA12 showed a 60% lowering of cholesterol at the .4% level of ox gall but only 30% of the .2% level. Strain LA25 lowered cholesterol by 70 and 50% at the two respective levels of ox gall. In vitro antimicrobial activity against both *Bacillus subtilis* and *Escherichia coli* (Figure 3) was the greatest for LA16, followed by LA12 and LA25.

LA12, LA16 and LA25 exhibited similar fermentation durations (curd formation starting 5 to 6 h and final pH attained by 8 h), degree of whey production and quality of curd formation. Because LA16 showed bile tolerance, the greatest cholesterol reduction (70% average), the greatest antimicrobial activity, and similar quality when made into yogurt, it was selected for yogurt production.

**Experiment 2.** During the 56-d test period, 16, 18.9-liter batches of yogurt were produced

(one batch every 3.5 d). This was done to ensure highly viable bacteria within the *acidophilus* yogurt. The 16 batches were consistent for pH, plate count, gross energy, crude protein, calcium and phosphorus (Table 1). The plate count varied because the inoculation rate was changed from 2% to 4% LA after the fifth batch of yogurt was produced. This change was implemented to increase the number of viable bacteria consumed by the boars.

The effect of yogurt on serum lipids is shown in Table 3 and Figure 4. Serum cholesterol was reduced by 10.5% in boars fed the yogurt diet ( $P < .01$ ). Serum triglycerides and HDL were not affected ( $P > .23$  and  $P > .11$ , respectively), but serum LDL were reduced (9%,  $P < .08$ ) by feeding the yogurt diet. Because the LDL were calculated from serum cholesterol, triglycerides and HDL values, this response would be expected. The response of

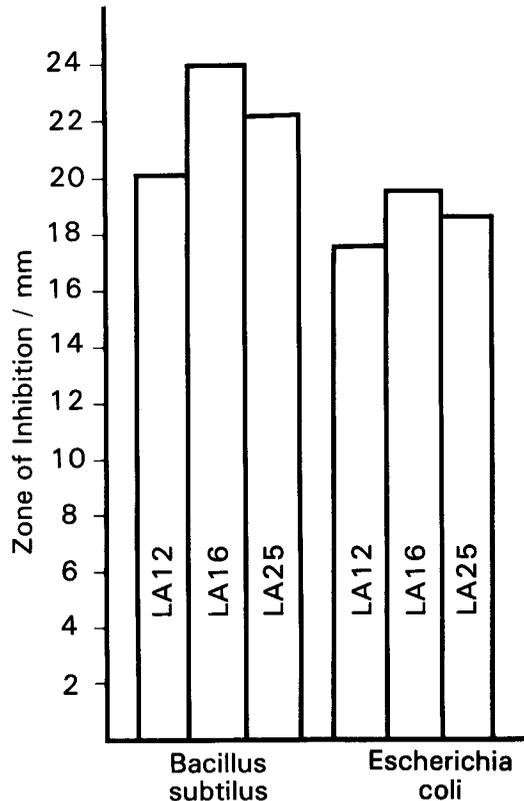


Figure 3. In vitro antimicrobial activity of porcine *Lactobacillus acidophilus* tested against *Bacillus subtilis* and *Escherichia coli* (SE = 0).

TABLE 3. EFFECT OF PORCINE *ACIDOPHILUS* YOGURT ON SERUM LIPIDS (d 56)<sup>a</sup>

Liquid component	Diet			CV, %
	High cholesterol	Yogurt	Difference, %	
Cholesterol, mg/dl <sup>b</sup>	91.1	81.5	-10.5	17.8
Triglycerides, mg/dl <sup>c</sup>	52.2	48.8	-6.5	33.3
HDL, mg/dl <sup>d</sup>	44.7	43.2	-3.4	13.0
LDL, mg/dl <sup>e</sup>	33.7	30.8	-8.6	29.6

<sup>a</sup>Least square means adjusted for initial sera values using covariate analysis.

<sup>b</sup> $P < .01$ .

<sup>c</sup> $P > .23$ .

<sup>d</sup>HDL = high density lipoproteins ( $P > .11$ ).

<sup>e</sup>LDL = calculated low density lipoproteins ( $P < .08$ ); by Friedewalds' equation: LDL = serum cholesterol - HDL - (serum triglycerides/5).

serum cholesterol, triglycerides, HDL and LDL for the 56-d experiment is shown in Figure 4. Serum cholesterol and LDL exhibited the same patterns with the widening of the response traits for the two treatments occurring

after d 14 and d 21, respectively. No distinguishable patterns were demonstrated for serum triglycerides and HDL.

The effect of dietary treatments on fecal lactobacilli and coliforms are shown in Figure

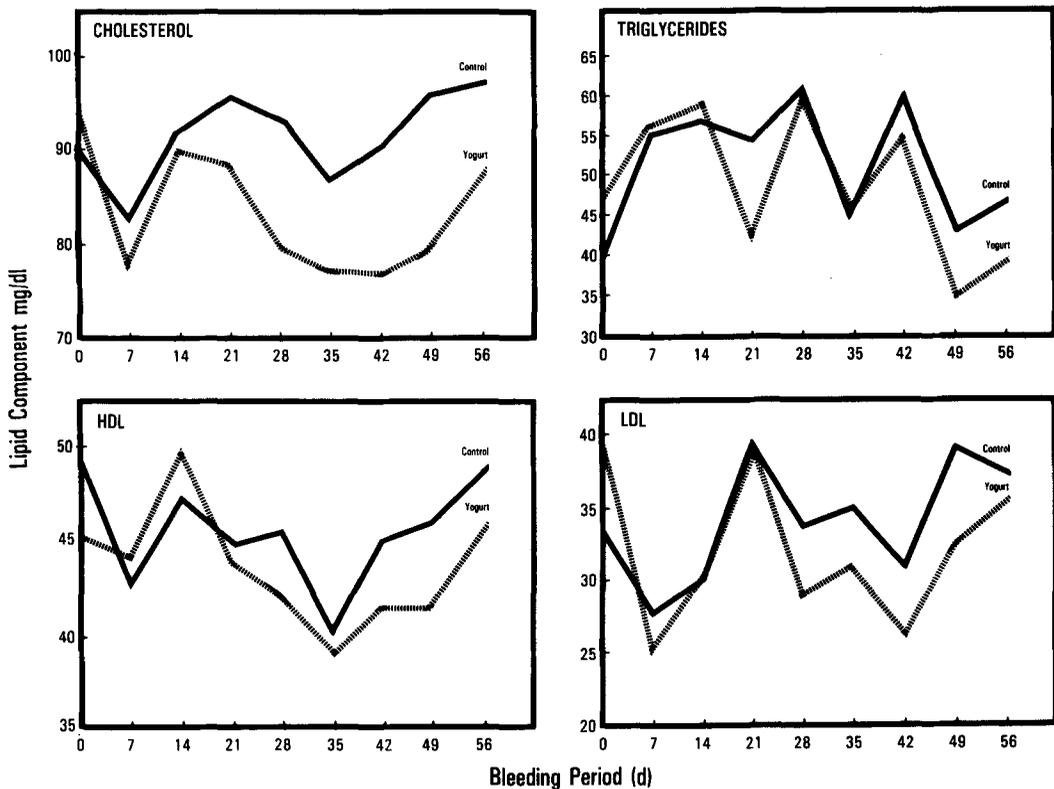


Figure 4. Effect of dietary *acidophilus* yogurt on serum cholesterol (SE = 5.12), triglycerides (SE = 5.61), high density lipoproteins (HDL) (SE = 1.90) and low density lipoproteins (LDL) (SE = 3.19) of mature boars fed a high-cholesterol diet. Control diet = solid line; yogurt diet = dashed lines.

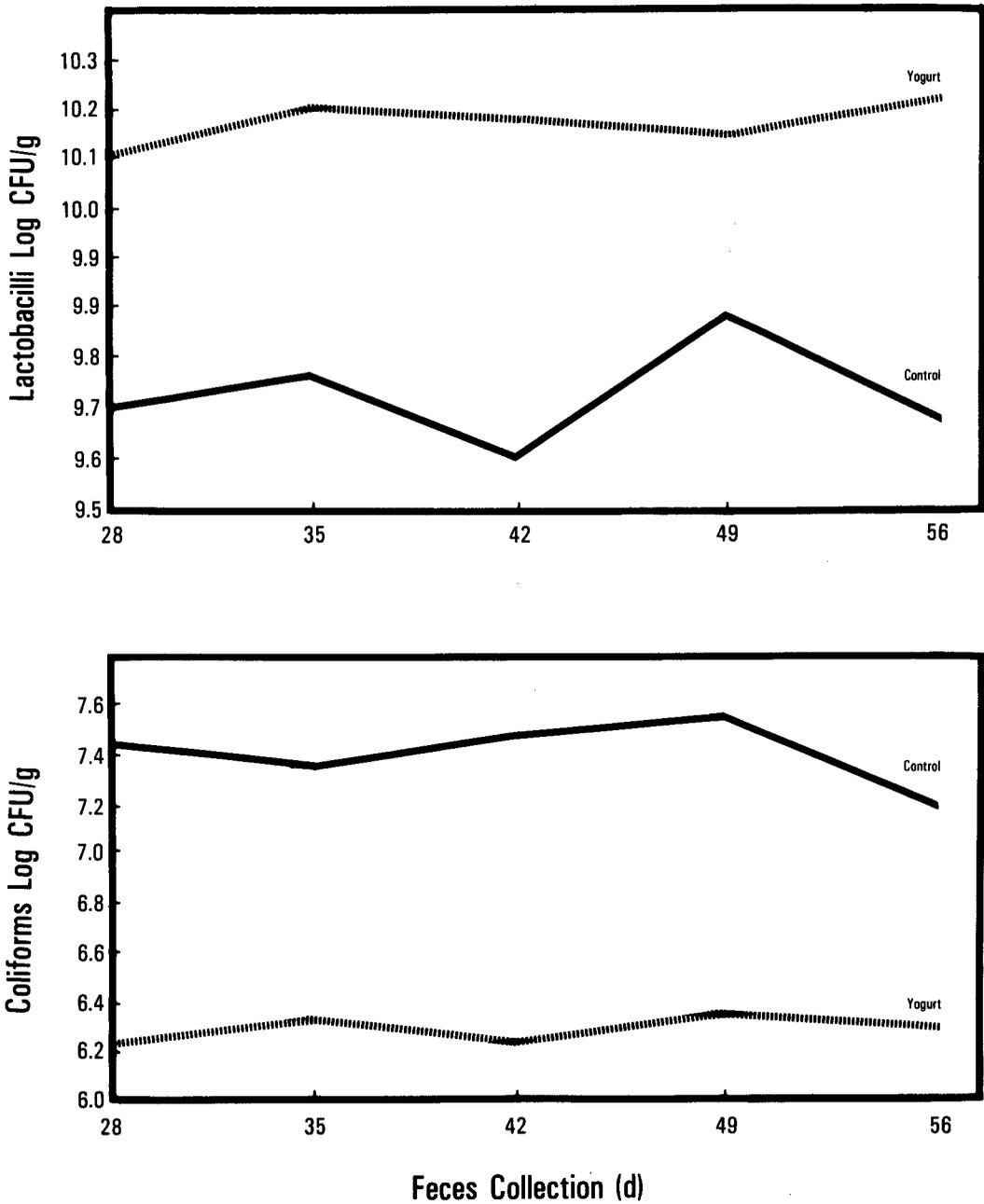


Figure 5. Effect of dietary *acidophilus* yogurt on fecal coliforms ( $P < .0001$ ; pooled SE = 2.97) and lactobacilli ( $P < .0001$ ; pooled SE = 4.92) of mature boars fed a high-cholesterol diet. Control diet = solid lines; yogurt diet = dashed lines.

5 for d 28 to d 56. The log counts for lactobacilli and coliforms were 4% higher and 18% lower, respectively, for the boars fed yogurt than for the control animals over the 28-d span. There were significant time effects

for both fecal coliforms ( $P < .0010$ ) and lactobacilli ( $P < .0288$ ). Even though there was a significant interaction between treatment and time for both fecal coliforms ( $P < .0003$ ) and lactobacilli ( $P < .018$ ), the authors believe that

the significance demonstrated by the main effects is a real observation. The basis for this belief was that the polynomial transformation for time demonstrated a significant ( $P < .0001$ ) cubic effect for fecal coliforms and a quartic effect ( $P < .0031$ ) for fecal lactobacilli.

### Discussion

The latest U.S. census (1986) describes the number-one cause of death in the U.S. as heart disease. When categorizing this disease by sex, males have a greater incidence of heart disease than females. Information published by the Lipids Research Clinics Program (1984) indicates that the higher the serum cholesterol level is in humans, the greater the chance for the development of coronary heart disease. Thus, increased research has sought ways to lower serum cholesterol levels in man.

Using physically mature boars as a model, serum cholesterol and LDL were reduced by feeding porcine *Lactobacillus acidophilus* yogurt in combination with a high-cholesterol diet. This finding agrees with research conducted with other species of animals: rabbits (Thakur and Jha, 1981), humans (Mann, 1977) and rats (Grunewald, 1982). The paradigm of the mechanism of this phenomenon is not universally agreed on. Gilliland et al. (1985) suggested that LA may be able to incorporate steroids into their cellular structure when grown under appropriate conditions. Nelson and Gilliland (1984) suggested that LA has the ability to act directly on cholesterol. Mann (1977) suggests that  $\beta$  hydroxy- $\beta$ -methyl glutaric acid (HGM) is the factor in yogurt that lowers serum cholesterol. Because HGM is present in such meager amounts in yogurt, Thakur and Jha (1981) concluded that some other factor such as Ca also might be responsible. Stahelin et al. (1981) found that casein and lactose are not responsible for this phenomenon; thus, something besides one of these bulk constituents of yogurt causes its cholesterol lowering effect.

Our data when applied to the correlation equations derived from the Framingham study (Truett et al., 1967) indicate that the risk of coronary heart disease would be reduced by 21% if adult males consumed AY in combination with high-cholesterol diets, assuming that the AY were made from strains of LA that assimilate cholesterol.

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