THE EFFECT OF FEEDING SKIM MILK OR SKIM MILK YOGURT ON SERUM CHOLESTEROL AND LIVER LIPIDS IN RATS

by

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INTRODUCTION

The consumption of cultured products and low fat milk increased considerably in the United States from 1967 to 1977 even though the total consumption of fluid, evaporated, condensed milk and butter declined during that period (1). Low fat and skim milk consumption increased by 265% and yogurt by 500%. The increase in the consumption of cultured dairy foods, notably that of yogurt, has been ascribed to their image as wholesome, high-protein, convenient, health, or low fat foods.

Belief in the beneficial effects of yogurt for human health and well-being has existed in many civilizations for a long time. Yogurt may be more nutritious than milk because in its manufacture the protein (2-7), carbohydrates (8-10) and fats (11) are predigested by bacterial cultures to increase the availability of nutrients for use by the body. Several vitamins including thiamin, riboflavin, niacin and folic acid are increased in the conversion of milk to yogurt though vitamin B_{12} and vitamin C are reduced (12,13).

In addition to high nutritional properties, yogurt also possesses considerable therapeutic value (14). The autodigesting feature of yogurt makes it a well-tolerated source of milk for lactase-deficient persons. Another common use of yogurt has been in gastrointestinal disorders such as diarrhea (particularly infantile), gastroenteritis and constipation. Yogurt has been reported to have antibacterial

effects on pathogens resistant to standard antibiotics and to have inhibitory effect on proliferation of some tumor cells.

In 1974, Mann & Spoerry (15) reported a hypocholesterolemic effect in Maasai men who consumed large quantities (up to 8.33 l/man/day) of milk fermented with a wild Lactobacillus culture. That observation has prompted a series of animal and human investigations of the hypocholesterolemic effect of milk and yogurt. A number of workers have confirmed the hypocholesterolemic effect of yogurt in human studies (16-18) and in animal studies (19-21), but others have failed to confirm this effect (22-24). Lactose (25), calcium (26), milk fat membrane (27), hydroxymethylglutarate (28), orotic acid (29,30) and uric acid (31) have been suggested, but not confirmed, as possible hypocholesterolemic factors in yogurt or milk.

This study was aimed at determining if there was a hypocholesterolemic effect when skim milk or skim milk yogurt was substituted for 45% of a chow diet (without or with 0.5% cholesterol added) to provide 20% of total daily calories intake of rats. Also, the amount of orotic and uric acids in the skim milk and skim milk yogurt were determined by high performance liquid chromatography (HPLC).

REVIEW OF LITERATURE

Positive hypocholesterolemic effect of yogurt

Human studies. In 1974, Mann and Spoerry (15) observed that excessive amounts of fermented milk were hypocholesterolemic when fed to Maasai tribesmen in Africa. The original purpose of their study was to examine the hypothesis that adding a surfactant would facilitate absorption of cholesterol from the gut and result in hypercholesterolemia. Twenty-four Maasai males aged 16 to 23 years were divided into two groups of 12 each and fed for 3 weeks a regimen of fermented whole milk 5 days followed by a meat day and a soup day to clean the gut in preparation for milk. The treatment group received a surfactant Tween 20 and the controls received a placebo. Each Maasai male normally consumed about 4 to 5 liters of fermented milk daily containing 660 Kcal and 120 mg cholesterol/liter. As this study progressed, the consumption of milk increased until by treatment day 4, the men were consuming an average of 8.33 liters. The researchers were unable to control the food intake so that the men gained weight and body fat. Men who gained over 5 lb in 3 weeks showed an average fall of cholesterol of approximately 28 mg/100 ml, whereas men gaining less than 5 lb lowered their level of cholesterol only 8 mg/100 ml. These responses suggested that some regulatory factor existing in fermented milk other than the calorie balance or the surfactant had influenced

cholesterolemia in these Maasai men.

Subsequently, Mann (16) confirmed and extended the aforementioned findings with American volunteers. Subjects (20 men and 6 women), aged 24-55 years, were fed large amounts of whole milk yogurt, skim milk yogurt or whole milk for 12 days. In general, there was a reduction in serum cholesterol during the feeding of both whole and skim milk yogurt with a slow return toward normality upon return to their normal diet. A daily intake of 2 or 4 l of whole milk yogurt or 2 l of skim milk yogurt produced significant reductions of cholesterolemia. Two liters daily of whole milk did not significantly affect cholesterolemia. Mann concluded that yogurt contained a "milk factor" which lowered levels of cholesterolemia even while the dietary intake of cholesterol was large (120 mg/l yogurt).

Hepner et al. (17) observed the effect of yogurt on serum cholesterol in volunteers (24 men and 30 women) between 21 and 55 years of age. Serum cholesterol was reduced by 5 to 10% after 1 week of dietary supplementation of 720 ml per day of either nonpasteurized or pasteurized yogurt with 2% fat. The reduction caused by 2% butter fat milk was less than that observed when 2% butter fat yogurt was fed.

Bazzarre et al. (18) compared males and females as separate groups with regard to the effects of yogurt and calcium supplementation at 4 weekly intervals. Subjects (16

females and 5 males) 18-30 years of age consumed their usual food intake supplemented by three 8-ounce servings of Dannon low fat yogurt for one week and at the third week with a calcium carbonate supplement. Total serum cholesterol decreased ($P<0.001$) while HDL-cholesterol and HDL:total cholesterol ratios ($P<0.001$) increased for females following yogurt supplementation. Total cholesterol was lower, but not significantly, following calcium supplementation, while HDL-cholesterol and HDL:total cholesterol ratios were significantly higher for the females. Total cholesterol, HDL-cholesterol and total cholesterol ratios for males after supplementation with yogurt or calcium were not significantly different from the baseline values. Thus, yogurt and calcium altered cholesterol metabolism in females but not in males.

Animal studies. The hypocholesterolemic effect of yogurt also has been demonstrated in animal studies by various researchers. Thakur and Jha (19) fed 45 albino rabbits supplements of cholesterol, milk (20 ml), yogurt (20 ml) and calcium in addition to a normal diet for 16 weeks. Yogurt, calcium and milk reduced serum cholesterol levels in rabbits fed the cholesterol diet. Yogurt caused a greater extent of hypocholesterolemia than milk. Atherosclerotic lesions in the aorta were high in the cholesterol-fed group, but were not observed in the yogurt-fed group.

The anticholesterol activity of yogurt in albino rabbits

was confirmed by Dwivedi et al. (20). After feeding 9 rabbits 0.5 mg cholesterol and 50 ml of yogurt per day, mild anticholesterol activity of the yogurt was observed on the basis of serum cholesterol estimations on days 0, 7, 30, 60 and 90. But angiograms of aortas before death and gross and microscopic pathological changes on post-mortem were indicative of atherosclerotic changes in rabbits fed cholesterol alone or cholesterol with yogurt. They concluded that feeding of yogurt did not prevent arterial damage even though it reduced the level of cholesterol to some extent.

In rats, changes in the concentrations of cholesterol and triglycerides in plasma, liver and aorta, due to full cream cow's milk, curds and acidophilus milk were observed by Chawla and Kansal (21). Plasma cholesterol was reduced by 28.0, 28.3 and 33.6% in male albino rats which received supplements (25g/day) of milk, acidophilus milk and yogurt, respectively for 90 days. In rats given milk/cultured milks, the cholesterol concentration in the liver was 37.3-47.8% and triglycerides 63.8-67.2% of the values observed for control rats. The concentrations of cholesterol in the aorta in rats which received milk/cultured milks were 64.0-65.5% and those of triglycerides were 35.7-39.0% of the values observed for control rats. A slight, but statistically significant, lower concentration of cholesterol was observed in plasma and aorta of rats fed the supplements of yogurt than in those fed milk.

No hypocholesterolemia effect of yogurt

While yogurt has been reported to decrease plasma cholesterol in both humans and in animals, some investigators have failed to confirm this effect. Nine patients (10 to 71 years) were studied by Gold and Samuel (22), three with normal serum cholesterol, four with familial hypercholesterolemia, and two with essential hypercholesterolemia. The daily consumption of 1 to 2 cups of yogurt (8 oz/cup) for 6-22 weeks did not influence serum cholesterol concentrations in those patients.

Rossouw et al. (23) studied schoolboys aged 16 to 18 years whose diets were complemented with 2 l of skim milk, yogurt, or full cream milk daily for 3 weeks. After a fall in all serum lipids during the precomplementation week, serum total cholesterol and low-density lipoprotein cholesterol rose for the first 2 weeks on yogurt or full cream milk. Total cholesterol returned to baseline values during the third week on yogurt or full cream milk. High-density lipoprotein cholesterol and high-density lipoprotein/total cholesterol ratio rose transiently in all three groups. No convincing evidence of a milk factor could be found.

Thompson et al. (24) provided sixty-eight healthy volunteers aged 18-26 years with 1 l supplements of 2% butterfat milk, whole milk, skim milk, yogurt, buttermilk, or sweet acidophilus milk daily for a 3-wk period. Despite

increase in caloric intakes on all supplements, cholesterol values changed by no more than 2.5% for any group except those of the skim milk group fell by 8.5%. However, none of these changes reached the 5% significance level. No significant changes were seen in LDL or HDL cholesterol on any of the supplements.

Massey (32) studied the effect of changing milk and yogurt consumption on human nutrient intake and serum lipoproteins. Thirty-two healthy male college students drank no milk for 3 weeks, then 1500 ml milk with 2% fat daily for 3 weeks, their usual diet for 2 weeks, no milk again for 3 weeks, and 1250 ml nonfat milk daily for 3 final weeks. Similarly, 30 female college students consumed 480 ml lowfat yogurt for 4 weeks, then no yogurt for 4 weeks in a crossover design. Although some serum lipid means differed significantly among some sampling points, there was no effect on total cholesterol, total triglycerides, high density lipoprotein cholesterol, or distribution of electrophoretic lipoprotein fractions that could be attributed to changing milk or yogurt consumption.

Factors in milk and yogurt contributing to the hypocholesterolemic effect

Many factors have been suggested to account for the hypocholesterolemic action of yogurt. Many researchers are trying to establish whether the effect is due to the milk component of yogurt, a chemical modification of one or more

of the constituents of milk, a bacterial metabolite of milk or some other mechanism.

Milk factor. More data from various studies suggested that there was a factor in milk (milk factor) that exerted a hypocholesteremic effect. Malinow and McLaughlin (33) observed a decrease in plasma cholesterol levels of rats on a chow-skim milk diet. From the 15th day after birth, newborn rats were offered rat chow mixed with water or skim milk (1 ml/0.7 g). In the chow-skim milk group, plasm cholesterol was lowered in 43- and 64-day-old males and in 64-day-old females.

Additional observations by Nair and Mann (28) pointed to a hypocholesteremic factor in skim milk. The cholesterolemic responses of rats fed 97% chow, 2.5% corn oil and 0.5% cholesterol were compared with those of rats fed 72% chow, 25% skim milk powder, 2.5% corn oil and 0.5% cholesterol. After 6 and 11 weeks the cholesterolemia was significantly lower for the group receiving milk than for the control group receiving only chow.

Howard (34) confirmed that skim milk lowered cholesterol in healthy human volunteers. Eight men and eight women, aged 22-55, were divided into two equal groups. One group consumed 4 pints of whole milk daily whereas the second group received 4 pints of the reconstituted dried skim milk throughout the three main meals. After the second week, the fall in serum cholesterol was 5% for the whole

milk group and 15% for those on skim milk, One week later, after a normal diet had been resumed, the mean for serum cholesterol still had not reached the pretreatment level.

Calcium. Howard (26) suggested that the hypocholesterolemic factor in milk and milk products might be calcium because milk contained a high content of calcium (2.4 g/4 pints milk) and calcium has been reported to exert a hypocholesterolemic effect in man (35,36). Carlson et al. (37) studied the effect of oral administration of 2 g calcium on serum lipids of 16 hyperlipidemic patients during 8 weeks. The average serum cholesterol value before treatment was 337 $\pm$ 13.5 mg/100 ml and decreased to 321$\pm$12.8 mg/100 ml during placebo treatment and to 303$\pm$12.0 mg/100 ml during treatment with calcium. The decrease was significant at the 0.1% level when the mean value during placebo treatment was compared to the mean value during calcium therapy.

However, when seven healthty volunteers were asked to consume 2.4 g calcium daily as the gluconate, the results were negative for cholesterol (34). Bazzarre et al. (18) studied the effects of yogurt and calcium supplementation on plasma total cholesterol. Mean total cholesterol decreased ($P<0.001$) for females following yogurt supplementation but was not significantly lower following calcium supplementation. Yogurt, calcium and milk reduced serum cholesterol levels in rabbits fed the cholesterol diet (19),

but yogurt caused a greater extent of hypocholesterolemia than milk ($P<0.001$) even though the calcium contents of milk and yogurt are almost equal. It was suggested that calcium was an active factor in yogurt, but that other hypocholesterolemic agents also may be present.

Lactose. Helms (25) suggested that lactose may be the active factor to exert the cholesterol-lowering effect of skim milk. Lactose (100 g/day, equivalent to four pints or about 2.25 l of milk) was found to have a reduction ($p<0.05$) in serum cholesterol of about 7%, but the effect of lactose seemed insufficient to account for the 15% decrease seen with skim milk (38). In further studies (27), the lack of effect of lactose, lactose with calcium and magnesium, and whey from Leicestershire cheese demonstrated that the hypocholesterolemic factor is not lactose alone or in combination with these mineral salts.

Milk fat globule membrane. The finding that butterfat but not cream or cheese (containing the same amount of fat) increased serum cholesterol suggested that the hypocholesterolemic factor may be present in the cell membranes surrounding the fat globules (27). Change in serum cholesterol of volunteers after 2 weeks on butterfat was +9.8% ($P < 0.01$) for the control weeks, while the butter milk produced a change of -6.4% ($P < 0.01$). Thus, the fat-globule membrane, a characteristic component of butter milk, accounted for the cholesterol-lowering factor in milk.

Antila et al. (39) agreed that the cholesterol-lowering effect of buttermilk may be associated with the membrane material. The concentration of membrane material in buttermilk was about five times that in cultured skim milk and butter milk decreased plasma cholesterol of male volunteers more effectively than did cultured skim milk.

However, Hussi et al. (40) have reported that, under carefully controlled conditions, no significant differences could be observed in serum lipids of groups of male prisoners ingesting the control diet and those ingesting the buttermilk diet. No hypocholesterolemic effect of buttermilk was observed in rats (41).

3-hydroxy-3-methylglutaric acid (HMG). Mann (16) administered radioactive acetate to human volunteers and observed that incorporation of the acetate into serum cholesterol was inhibited during the consumption of yogurt indicating decreased cholesterol biosynthesis. He postulated that HMG in the fermented milk inhibited the rate limiting enzyme, hydroxymethyl glutaryl CoA reductase (HMG CoA reductase) in cholesterol biosynthesis. When Nair and Mann (28) added 0.1% HMG to diets with or without cholesterol, the hypocholesteremic effects in rats were similar to those observed for skim milk. The workers, therefore, postulated that HMG was also the hypocholesteremic factor in milk. However, no analyses for HMG in milk were presented to support this speculation.

Orotic acid. Boguslawske and Wrobel (42) established the presence of a heat stable, dialyzable, TCA-soluble factor in unfermented bovine milk which exhibited an inhibitory effect on rat liver cholesterol synthesis both in vitro and in vivo. Ten ul cow's milk or 25 ul of human milk added to 2.6 ml of incubation mixture inhibited completely the incorporation of ^{14}C-acetate and ^{3}H-mevalonate into digitonin-precipitable sterols by liver preparation. The inhibitory activity of cow's or human milk resisted 10 min boiling and was found in the supernatant after protein had been precipitated from cow's milk. Overnight dialysis completely abolished the inhibitory effect of cow's milk. These observations suggested a low molecular weight and probably nonprotein nature for the sterol synthesis inhibitor. In vivo studies indicated that the incorporation of ^{14}C acetate (but not ^{3}H-mevalonate) into sterols by liver slices obtained from rats fed milk for 3 days was about 50% lower than the controls.

Among the many low molecular weight, heat stable compounds found in milk, orotic acid (6-carboxy-2,4-dioxypyrimidine), an intermediate in pyrimidine biosynthesis, has had marked effects on lipid metabolism that may favor it as one of the active components in the hypocholesteremic effect of milk. Windmueller (43) demonstrated the depression of cholesterol, phospholipid and triglyceride of plasma in rats fed a fat-free diet

containing 1% orotic acid. Okonkwo and Kinsella (44) reported that when rats were fed a normal rat ration, the addition of 0.15% orotic acid or skim milk powder containing 0.15% orotic acid enduced fatty livers within 8 days.

The work by Bernstein et al. (29,30) suggested that orotic acid of milk may be involved in its hypocholesteremic effect. They (29) first determined the effect of 50 ul volumes of bovine milk, cultured buttermilk, or orotic acid solution (73 mg/liter) on the incorporation of [1-^{14}C] acetate or [5-^{3}H] mevalonic acid into cholesterol in 3.5 ml rat liver homogenate preparations. An average inhibition of acetate incorporation into cholesterol of 98.4%, 98.3%, or 95.7% was observed for bovine milk, cultured buttermilk, or orotic acid, respectively. Mevalonic acid incorporation was not affected statistically by the presence of bovine milk or orotic acid. This indicated that orotic acid appeared to be the primary component in bovine milk responsible for the inhibition of cholesterol biosynthesis in this in vitro system and the inhibition of cholesterol was prior to the formation of mevalonate.

In their second study on rat liver homogenates (30), bovine milk or orotic acid in solution at the final concentrations varying from 3.3 um to 322 um inhibited the incorporation of [1-carbon 14] acetate into cholesterol up to 72 $\pm$ 10%. Thus, acetyl-coenzyme A synthetase was the affected enzyme. From a Lineweaver-Burk plot of the

inhibition of yeast acetyl-coenzyme A synthetase, orotic acid was shown to be a noncompetitive inhibitor of acetyl-coenzyme A synthetase. Neither raw milk nor pasteurized milk inhibited HMG coenzyme A reductase.

Ahmed et al. (45) isolated two factors in bovine skim milk that affected hepatic cholesterolgensis in rat liver homogenates. One of the inhibitors was in the dialysate of whey and was identified as orotic acid. The other inhibitor, present in the retentate, was not identified. Orotic acid appears to act by inhibiting cholesterol biosynthesis from acetate, whereas the retentate inhibitor exerted its effect beyond the formation of mevalonate in the biosynthetic pathway.

Papa et al. (46) undertook studies to isolate and characterize the retentate inhibitor in bovine skim milk so as to gain understanding of milk's hypocholesteremic effect. This inhibitor was associated with the proteose-peptone fraction of milk, which was derived by proteolysis from B-casein. The association was most likely hydrophobic in nature. The inhibitor appeared to be pyrimidine-like , yet its relative mobilities and retention times did not resemble those of the trimethylsilyl (TMS) derivatives of standards. It reduced the incorporation of both acetate and mevalonate into cholesterol and thus affected the synthetic pathway beyond the formation of mevalonic acid.

Uric acid. While in the bovine milk the 2 inhibitors

were identified as pyrimidines, Ward et al. (31) discussed one of the two inhibitors found in human milk which inhibited the incorporation of both radioactive acetate and mevalonate into cholesterol by rat liver preparation (42). The material was not a nucleoside and its structure was similar to a free purine. By gas liquid chromatograghy, the spectra of the unknown and standard uric acid matched.

Starter culture

Commercial yogurt production generally utilizes a 1:1 mixed starter culture of only Streptococcus thermophilus and Lactobacillus bulgaricus. It is still not known whether the increased hypocholesterolemia activity is due to any specific lactic culture. Rao et al. (47) demonstrated that metabolites produced in milk fermented by Streptococcus thermophilus significantly lowered plasma cholesterol levels in rats. The liver cholesterol levels were lower in the group receiving thermophilus milk than in the group receiving skim milk. Grunewald (48) studied effects of feeding 10% skim milk and 10% milk fermented by L. acidophilus in rats. After 4 weeks, rats receiving the fermented milk had lower ($P<0.005$) serum cholesterol levels (65 mg/dl) than did the water-fed (78 mg/dl) or milk-fed (79 mg/dl) rats. Gilliland et al. (49) found that consumption of L. acidophilus RP32, which was selected for its ability to grow well in the presence of bile and to assimilate

cholesterol from the laboratory medium, inhibited increases in serum cholesterol levels of pigs ($P<0.05$) fed a high-cholesterol diet. Consumption of L. acidophilus P47, which was selected for its ability to grow in the presence of bile and lack of ability to remove cholesterol from the growth medium, failed to have a similar effect. They concluded that only selected strains of L. acidophilus caused reduction in serum cholesterol.

However, another study by Grunewald (50) used male mice which were fed either water, 10% liquid skim milk, 10% nonfermented acidophilus milk or 10% fermented acidophilus milk. There were no significant differences found in the serum cholesterol levels among treatment groups. Hepner et al. (17) also had reported that the hypocholesterolemic effect was not due to a direct alteration of intestinal flora by yogurt with regard to the similarity in results obtained using supplementation with pasteurized versus nonpasteurized yogurt. Pulusani and Rao (51) demonstrated that the distribution of cholesterol among various body pools (whole body lipids, liver lipids and liver cholesterol) was not altered when rats were fed chow with skim milk fermented by thermophilus, bulgaricus and acidophilus milk. Jaspers et al. (52) modified the usual diets of 10 human adults by incorporating 681 g nonfat, unpasteurized yogurt daily throughout three 14-21 day periods. A different set of select culture strains, two

commercial and one patented, were used for yogurt production. Only temporary hypocholesterolemic effect of yogurt consumption was seen between strains.

Fermentation

While the hypocholesterolemic agent has been suggested to be orotic acid or uric acid, several studies have reported measuring the quantity of these agents in milk and yogurt fermented from milk. Okonkwo and Kinsella (53) had determined that commercial milk powders contain from 112 to 134 mg of orotic acid per 100 g powder. They also found that during the preparation of commercial yogurt from milk, the mean concentration of orotic acid decreased from 82 to 46 ppm (54). This decrease was attributed to the fermentation, that is, the metabolic activity of the Lactobacillus species used in preparation of the yogurt. However, Ferreira (55) did not observe a decrease in orotic acid content of yogurt over the same time period. There may be strain differences in the use of orotic acid by culture microorganism. Larson and Hegarty (56) found that orotic acid tended to be more prevalent in milk of 11 ruminant species than in milk of 9 nonruminant species. The concentration was dependent on the amount of soluble whey solids and degree of fermentation in processing. Marsili et al. (57) used an isocratic high preformance liquid chomatograghy (HPLC) technique to analysize the concentrations of orotic acid and uric acid in dairy

products with the result of 83.6 $\pm$ 1.0 ppm and 31.8 $\pm$ 0.0 ppm in whole milk, respectively. Haggerty et al. (58) also used HPLC to measure the concentration of orotic acid, uric acid and HMG in fermentated milks. The concentration of orotic acid found in yogurt ranged from 17.3 to 32.4 ppm. During fermenation orotic acid decreased significantly. Uric acid did not change significantly during fermentation. The concentration of uric acid found in the yogurt was 14.2 to 18.2 ppm. HMG was not found in milk or yogurt. There were no significant differences in yogurt strains in their effects on orotic and uric acid concentration.

MATERIALS AND METHODS

Skim milk and skim milk yogurt preparation

Skim milk. Nonfat dry milk (NFDM) (Carnation) was reconstituted to give a total milk solid content of 28% (w/w). The reconstituted milk was brought to a boil in a microwave oven, cooled in a water bath to 25°C and stored in a refrigerator at 4°C.

Skim milk yogurt. Reconstituted skim milk containing 28% NFDM solids was brought to a boil in a microwave oven and cooled in a water bath to 45°C, then innoculated with 1% starter of nonfat yogurt (Dannon) based on 1 g starter/100 g skim milk, incubated at 45°C until a firm coagulum was produced and the pH value was 4-4.5 (Corning-140 pH meter). The skim milk yogurt was stored in a refrigerator at 4°C.

High performance liquid chromatography (HPLC) analysis for orotic and uric acids (57,58)

Preparation of skim milk and skim milk yogurt extracts. Five grams of reconstituted skim milk or skim milk yogurt were weighed into a centrifuge tube, then 0.5 ml of 5% oxalic acid (w/v) and 10 ml of 95% ethanol were added. The mixture was agitated for 10 min and centrifuged in a IEC Clinical Centrifuge (Internation Equipment Company, Needham HTS, MA) at the highest speed for 15 min at room temperature. The supernatant was filtered through Whatman #4 paper, evaporated in a rotary evaporator (Buchler

Instruments, Fort LEE, NJ), adjusted to a volume of 10 ml with deionized water. The extract was again filtered through a 0.45 um metricel membrane filter (Gelman Sciences Inc., Ann Arbor, MI).

Apparatus and operating conditions. HPLC analysis was conducted using a Beckman Model 110A pump with a Beckman Model 210 Sample Injection Valve (Beckman Instruments, Inc., Fullerton, CA). A Hitachi Model 100-10 ultraviolet/ visible detector (Hitachi, Ltd., Tokyo, Japan) was used to monitor orotic acid and uric acid at a wavelength of 278 nm and 0.0090 N H_2SO_4 was used as the mobile phase. Analysis was performed at a flow rate of 0.7 ml/min at room temperature.

HPLC column and guard column. A 300 mm x 7.8 mm i.d. HPLC organic acid analysis column (Bio-Rad Laboratories, Richmond, CA) was used. The column contained Aminex HPX-87H, a strong cation exchange resin (8% crosslinked and 9 um diameter) which separated organic acids by ion exclusion and partition chromatography. A 4.6 mm ID x 40 mm long Micro-Guard HPLC guard cartridge (Bio-Rad Laboratories, Richmond, CA) was used to prevent HPLC column degradation caused by particulate matter, irreversibly bound material, and aggressive reagents in the sample or the solvents. The guard cartridge was made of type 316 stainless steel and Kel-F fluoropolymer, and was housed in a reusable standard cartridge holder (Bio-Rad Laboratories, Richmond, CA).

Qualitation and quantitation. Qualitative analyses of

orotic acid and uric acid were conducted by comparing retention times of unknowns to those of known standards prepared from high purity chemical standards (Sigma Chemical Co., St. Louis, MO). The orotic and uric acids standards were placed in deionized water and 0.1 N NaOH was added to adjust the pH to 8.00. Peak area and retention time was integrated with an ALTEX Model C-R1A integrator (Shimadzu Corp., Kyoto, Japan). Quantitation was based on the external standard method.

Animal study

Animals and diets. Fifty-four, male weanling (50-60g) Sprague-Dawley rats (Harlan Industries, Madison, WI) were randomly divided into six groups of nine each and fed the diets shown in Table 1. Water was added to diets A and B so that all diets would be similar in calorie density. The composition and calorie density of diets are given in Table 2. Diets were prepared every third day. Skim milk (SM) and skim milk yogurt (SMY) were made one day before diet preparation. A Model A-200-D mixer (Hobart MFG Co., TROY, OH) was used to mix the diets. SM and SMY diets were made finer in a food processor. Diets were stored in the refrigerator at 4°C for 3 days.

TABLE 1

Diet ingredients (g/100 g)

Ingredients	Diet groups					
	A	B	C	D	E	F
Chow[1]	70	69.5	55	54.5	55	54.5
Water	30	30				
Skim milk			45	45		
Skim milk yogurt					45	45
Cholesterol		0.5		0.5		0.5

[1] Rodent Laboratory Chow 5001 (Purina Mills Inc., St. Louis, MO): ground extruded corn, soybean meal, dried beet pulp, fish meal, ground oats, brewer's dried yeast, dehydrated alfalfameal, cane molasses, wheat germ meal, dried whey, meat and bone meal, animal fat preserved with BHA, dicalcium phosphate salt, wheat middlings, calcium carbonate, vitamin B_{12} supplement, DL-methionine, Ca pantothenate, choline chloride, folic acid, riboflavin supplement, thiamin, niacin supplement, pyridoxine hydrochloride, ferrous sulfate, vitamin A supplement, D-activated animal sterol, vitamin E supplement, Ca iodate, cobalt carbonate, copper sulfate, zinc sulfate, zinc oxide.

TABLE 2

Nutrient composition and calorie density of the experimental diets (g/100 gm)

	Diets					
	Chow + Water		Chow + SM		Chow + SMY	
Nutrients	70%	30%	55%	45%	55%	45%
Protein	16.4	-	12.9	4.4	12.9	4.5
Fat	3.2	-	2.3	-	2.3	-
Carbohydrate	34.3	-	27.0	6.7	27.0	6.4
Vitamin	0.7	-	0.6	-	0.6	-
Ash	5.1	-	4.0	0.9	4.0	0.9
Fiber	4.1	-	3.2	-	3.2	-
Moisture	7.0	30.0	5.5	32.4	5.5	32.4
Calories	232		227		226	
Calorie Density	2.3		Kcal/gm 2.3		2.3	

1SM, skim milk; SMY, skim milk yogurt.

Experimental protocol. The animals were housed in stainless steel cages and given food and water ad libitum. Room conditions were maintained at 22 C with a 12-hr light-dark cycle. During an adjustment period of one week, all rats received ground chow (Rodent Laboratory Chow 5001, Purina Mills Inc., St. Louis, MO), after which they were fed the 6 experimental diets for seven weeks. Feed intakes were measured daily and animals were weighed twice per week. At the end of the third, fifth and seventh weeks, 3 rats from each group were sacrificed in the fed state. Rats were anesthetized using sodium pentobarbital injected

intraperitoneally. Blood was collected from the abdominal aorta, and was allowed to clot at room temperature for 1 h and then centrifuged at 2800 rpm for 30 min in a IEC Centra-7R refrigerated centrifuge (International Equipment Company, Needham Heights, MA). Serum obtained was transferred to plastic vials and was stored at -20°C. Livers also were removed, washed in saline solution (0.9% NaCl), weighed and stored at -20°C.

Analytical methods

Serum cholesterol: Total, HDL and LDL cholesterol. Total, HDL, and LDL cholesterol levels were measured using the LDL-Direct-Plus Cholesterol Ratio System (Isolab, Akron, OH). In this assay, total cholesterol was determined by adding serum directly to the cholesterol reagent (enzyme reagent and activator), but for HDL and LDL cholesterol, serum was added to an affinity column, and was eluted sequentially with Alpha agent for HDL cholesterol and Beta agent for LDL cholesterol. The fractions collected were added to cholesterol reagent, mixed and incubated at 37 C for 5 min. The absorbence of the pink chromophore was read at 505 nm with a Spectronic 20 spectrophotometer (Bausch & Lomb, Rochester, NY).

Liver lipid and cholesterol. Liver lipids were determined by a modification of the Folch gravimetric method (59). Livers were homogenized with a polytron homogenizer

(Brinkmann Instruments, Westburg, NY) and were extracted with a 2:1 (v/v) methylene chloride : methanol mixture. Adding sodium chloride to the extraction resulted in two phases: an upper aqueous phase and a lower organic phase. The aqueous phase was discarded and a measured aliquot of the organic layer was analyzed for total cholesterol by the ferric chloride-sulfuric acid method (60). The rest of the organic layer was evaporated, dried and weighed to measure total liver lipids. Full descriptions of these analytical methods are given in the appendix.

Statistical Analysis

A two-factor fixed-effects statistical model was employed with three rats randomly assigned to each of the 18 diet x period combinations. After the serum measurements were obtained, the three analyses of variance dictated by the design were run on the HDL, LDL, total serum cholesterol, liver weights, liver lipids data using a SAS GLM procedure which displayed all of the desired diet, period, and diet x period comparisons along with corresponding measures of their statistic significance. Special attention was paid to differences at two significance levels: $P \leq 0.01$ and $P \leq 0.05$ also was noted.

RESULTS

Orotic acid and uric acid. The analytical results for orotic and uric acid in skim milk (SM) and skim milk yogurt (SMY) after 0 or 3 days storage are reported in Table 3. The chromatograms are illustrated in Fig. 1. After 0 day storage, the concentration of orotic acid was 106.18 ppm in SM and 46.05 ppm in SMY. After fermentation, orotic acid was lower ($P<0.05$) in SMY than in SM. The concentration of uric acid was 23.27 ppm in SM and 20.06 ppm in SMY. Uric acid did not change significantly during fermentation. Diets with 45% of chow replaced with SMY contained about 0.0025% orotic acid and 0.001% uric acid. After 3 days storage, there were no significant changes of orotic acid or uric acid in SM and SMY as compared to those at 0 day storage. No bacterial spoilage had been found in the diets during 3 days storage.

TABLE 3

Concentration (ppm) of orotic acid and uric acid in skim milk and skim milk yogurt after 0 or 3 days storage[1,2]

Diets	Orotic acid	Uric acid
	0 day storage	
Skim milk	106.18[a]	23.27[a]
Skim milk yogurt	46.05[b]	20.06[a]
	3 days storage	
Skim milk	102.35[a]	21.44[a]
Skim milk yogurt	46.87[b]	20.36[a]

[1] Values are means for 6 samples. Standard error of a mean for orotic and uric acids: 3.38 and 0.81.
[2] Values in a column not sharing a common letter are significantly different ($P \leq 0.05$).

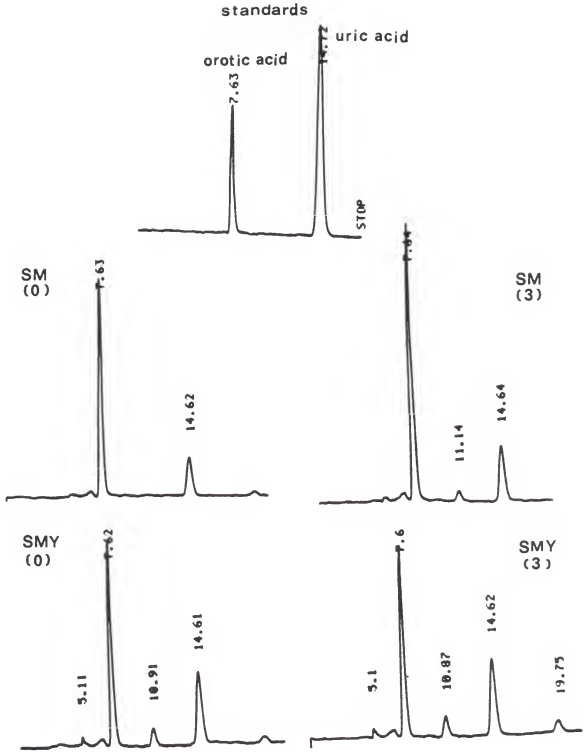

FIGURE 1 CHROMATOGRAMS OF OROTIC ACID AND URIC ACID IN SKIM MILK OR SKIM MILK YOGURT AFTER 0 OR 3 DAYS STORAGE

Weight gain, food intake and feed efficiency. Mean weight gain, food intake and feed efficiency (total weight gain/ total food intake) of animals are given in Table 4. There were no significant diet differences in feed efficienbcy, weight gain and food intake after 3, 5 or 7 weeks. Weight gain had a linear relationship with food intake after either 3, 5 or 7 weeks. When linear adjustment of weight gain for food intake was performed, there still were no significant diet differences in adjusted weight gain. The body weights of animals showed that the growth rates were similar for all diet groups for the entire experimental period (Fig. 2). Data for individual animals are shown in the appendix.

Serum HDL, LDL and total cholesterols. ANOVA procedure was used to determine statistical differences for serum HDL, LDL and total cholesterol variables (Table 5). Significant F values were found not only for the diet x period interaction, but also for main effects of diet and period. Period and diets did have an interaction with serum HDL, LDL and total cholesterol.

TABLE 4

Weight gain (unadjusted), food intake and feed efficiency of rats fed chow, skim milk or skim milk yogurt diets without or with cholesterol addition after 3, 5 and 7 weeks[1,2]

Diets[3]	Weight gain	Food intake	Feed efficiency
	g/day	g/day	Wt gain/total intake
After 3 weeks			
A	6.4	26.0	0.24
B	6.2	25.9	0.24
C	6.3	26.3	0.24
D	6.6	27.2	0.24
E	6.0	26.1	0.23
F	7.0	28.9	0.25
After 5 weeks			
A	5.5	25.5	0.21
B	6.1	25.9	0.23
C	6.0	26.1	0.23
D	5.7	26.5	0.21
E	5.5	25.6	0.22
F	5.8	25.8	0.22
After 7 weeks			
A	4.9	26.0	0.19
B	4.8	26.3	0.18
C	5.0	26.1	0.19
D	4.8	26.7	0.18
E	4.6	25.8	0.18
F	4.6	25.8	0.18

[1] The values are means for 3 animals after 3, 5 and 7 weeks. Standard error of a mean for weight gain, food intake and feed efficiency: 0.30, 0.82 and 0.008.

[2] There were no significant diet differences in feed efficiency, weight gain and food intake after 3, 5 and 7 weeks.

[3] Diets: A(70% chow + 30% water); B(69.5% chow + 30% water + 0.5% cholesterol); C(55% chow + 45% skim milk); D(54.5% chow + 45% skim milk + 0.5 % cholesterol); E (55% chow + 45% skim milk yogurt); F(54.5% chow + 45% skim milk yogurt + 0.5% cholesterol).

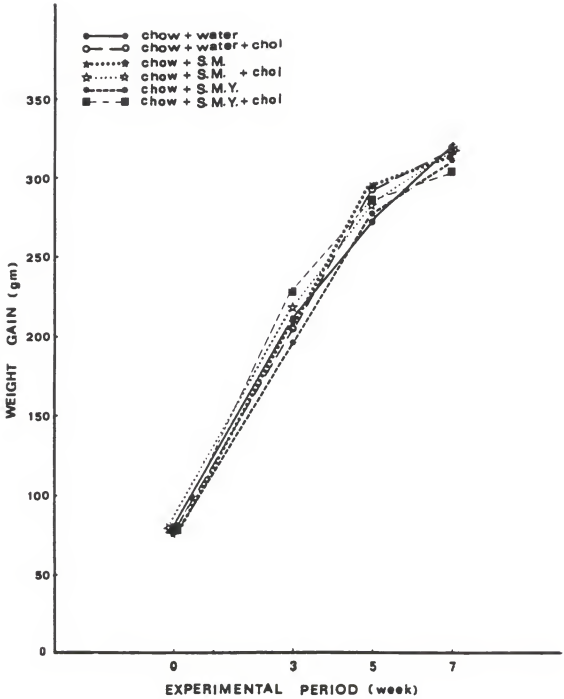

FIGURE 2 GROWTH CURVES OF RATS FED CHOW, SKIM MILK OR SKIM MILK YOGURT DIETS WITHOUT OR WITH CHOLESTEROL ADDITION FOR 3, 5 AND 7 WEEKS.

TABLE 5

Mean squares (MS) and significance in ANOVA procedure for serum HDL, LDL and total cholesterol

		HDL			LDL			Total		
S[1]	DF	MS	F	P	MS	F	P	MS	F	P
Diet	5	62.45	4.54	<.01	620.52	27.62	<.01	227.08	22.77	<.01
Period	2	300.55	21.86	<.01	53.58	2.38	<.11	384.88	38.54	<.01
D x P	10	42.47	3.06	<.01	53.01	2.36	<.03	52.63	5.28	<.01
R: D & P	34	13.75			22.47			9.97		
Total	51									

1 : source of variation.

A comparison of the cholesterolemic responses in group A, receiving the chow diet, with group B, receiving the chow diet with 0.5% added cholesterol, is shown in Fig. 3. Group B had higher levels of total serum cholesterol after 3 ($P<0.01$), 5 ($P<0.01$) and 7 ($P<0.05$) weeks, which was reflected mainly in the increased levels of serum LDL cholesterol after 3 ($P<0.01$), 5 ($P<0.05$) and 7 ($P<0.01$) weeks. Serum HDL cholesterol for rats fed the high cholesterol diet was not significantly different after 3 and 5 weeks, but it decreased ($P<0.01$) after 7 weeks.

Without cholesterol added to diets, serum HDL, LDL and total cholesterol of rats fed chow, skim milk (SM) or skim milk yogurt (SMY) diets are shown in Table 6. A comparison of the chow group (70% chow + 30% water) with the SM group (55% chow + 45% skim milk) and the SMY group (55% chow + 45% skim milk yogurt) is shown in Fig. 4. After 5 weeks, the total serum cholesterol in the SM group was 8.5% ($P<0.05$) lower than that in the chow group. It still was 7.2% lower after 7 weeks but the difference was not significant. Serum HDL cholesterol in the SM group followed the same pattern. It was 16.3% ($P<0.01$) lower than that in the chow group after 5 weeks, while it was 12.3% lower (not significantly) after 7 weeks. LDL cholesterol level was not affected by the consumption of SM throughout the experimental period. In the SMY group , the total serum cholesterol was 9.1% ($P<0.05$) lower than that in the chow group after 5 weeks and

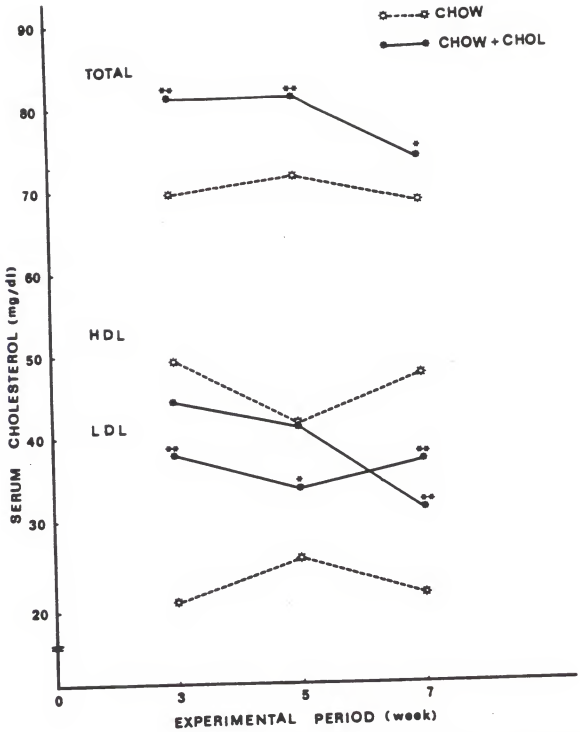

FIGURE 3 SERUM HDL, LDL AND TOTAL CHOLESTEROL OF RATS FED CHOW DIETS WITHOUT OR WITH CHOLESTEROL ADDITION AFTER 3, 5 or 7 WEEKS; ASTERISK INDICATES SIGNIFICANT DIFFERENCE FROM THE CHOW DIET WITHOUT CHOLESTEROL

(**: $P < 0.01$; *: $P < 0.05$)

TABLE 6

HDL, LDL and total serum cholesterol (mg/dl) of rats fed chow, skim milk or skim milk yogurt diets without cholesterol addition after 3, 5 and 7 weeks[1,2]

Diet[3] treatment	Serum cholesterol HDL	LDL	total
After 3 weeks			
Chow	49.05a	20.02a	69.48a
SM	48.38a	23.12a	71.17a
SMY	48.57a	22.71a	70.40a
After 5 weeks			
Chow	41.93a	25.20a	71.53a#
SM	35.11b#	22.57a	65.58b
SMY	41.44a	25.34a	65.03b
After 7 weeks			
Chow	47.24a	20.98a	68.47a
SM	41.44ab	17.39a	63.51a
SMY	36.67b*	23.18a	60.82b*

[1]The values are means for 3 animals of each group after 3, 5 and 7 weeks. Standard error of a mean (SEM) for HDL, LDL and total cholesterol: 2.14, 2.74 and 1.82.

[2]Values in a column at the same experimental period not sharing a common litter are significantly different (*: $P \leq 0.01$; #: $P \leq 0.05$).

[3]Diet treatment: Chow (A) = 70% chow + 30% water; SM (C) = 55% chow + 45% skim milk; SMY (E) = 55% chow + 45% skim milk yogurt.

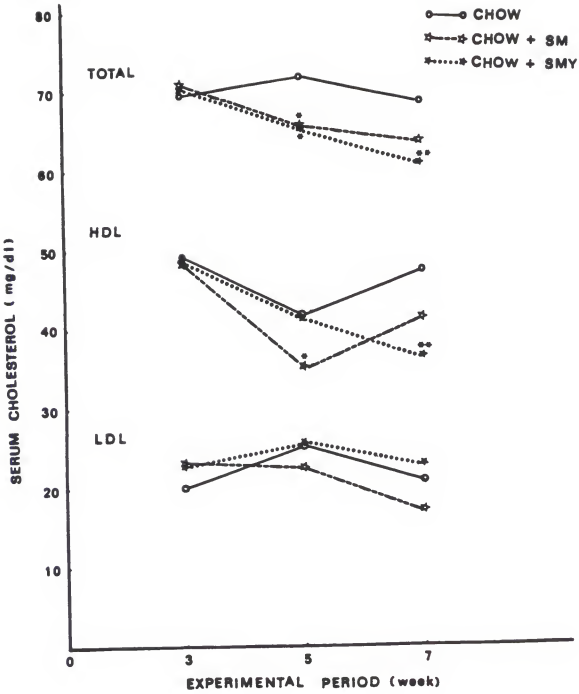

FIGURE 4 SERUM HDL, LDL AND TOTAL CHOLESTEROL OF RATS FED CHOW, SKIM MILK OR SKIM MILK YOGURT DIETS WITHOUT CHOLESTEROL ADDITION AFTER 3, 5 AND 7 WEEKS; ASTERISK INDICATES SIGNIFI CANT DIFFERENCE FROM THE CHOW DIET. (**: $P < 0.01$; *: $P < 0.05$)

continued to be 11.2% ($P<0.01$) lower after 7 weeks. Serum HDL cholesterol in the SMY group was 22.4% ($P<0.01$) lower only after 7 weeks. LDL cholesterol in the SMY group remained almost unchanged throughout the experimendital time with no significant difference from that in the chow group.

With 0.5% cholesterol added to the diets, serum HDL, LDL and total cholesterol of rats fed chow, skim milk (SM) or skim milk yogurt (SMY) diets are shown Table 7. A comparison of the chow group (69.5% chow + 30% water + 0.5% choelsterol) with the SM group (54.5% chow + 45% skim milk + 0.5% cholesterol) and the SMY group (54.5% chow + 45% skim milk yogurt + 0.5% choelsterol) is given in Fig. 5. In the SM group , the total serum cholesterol was 9.7% ($P<0.01$) lower than that in the chow group after 5 weeks, while this effect disappeared after 7 weeks. Serum HDL cholesterol was higher than that in the chow group ($P<0.01$) after 7 weeks. Serum LDL cholesterol was 26.5% ($P<0.05$) lower only after 3 weeks. In the SMY group, total serum cholesterol was 7% ($P<0.05$) higher after 3 weeks, thereafter it was 12% ($P<0.01$) lower after 5 weeks and maintained constantly lower ($P<0.01$) after 7 weeks as compared to that in the chow group. Serum HDL cholesterol in the SMY group did not change significantly for the entire experimental time, while serum LDL cholesterol in the SMY group was higher ($P<0.01$) than that in the chow group only after 5 weeks.

TABLE 7

HDL, LDL and total serum cholesterol (mg/dl) of rats fed chow, skim milk or skim milk yogurt diets with cholesterol addition after 3, 5 and 7 weeks[1,2]

Diet[3] treatment	Serum cholesterol HDL	LDL	total
After 3 weeks			
Chow	44.52ab	37.94a#	81.37a
SM	49.66a	27.90b	81.72a
SMY	40.64b*	40.61a*	87.20b#
After 5 weeks			
Chow	41.44a	33.75a	81.05a*
SM	39.51a	34.14a	73.12b
SMY	41.62a	51.12b*	71.32b
After 7 weeks			
Chow	31.28a	37.13a	73.70a
SM	40.71b*	33.72a	73.71a
SMY	35.64ab	33.70a	65.46b*

[1]The values are mean for 3 animals of each group after 3, 5 and 7 weeks. Standard error of a mean (SEM) for HDL, LDL and total cholesterol: 2.14, 2.74 and 1.82.

[2]Values in a column at the same experimental period not sharing a common letter are significantly different (*: $P \leq 0.01$, #: $P \leq 0.05$).

[3]Diet treatment: Chow (B) = 69.5% chow + 30% water + 0.5% cholesterol; SM (D) = 54.5% chow + 45% skim milk + 0.5% choelsterol; SMY (F) = 54.5% chow + 45% skim milk yogurt + 0.5% cholesterol.

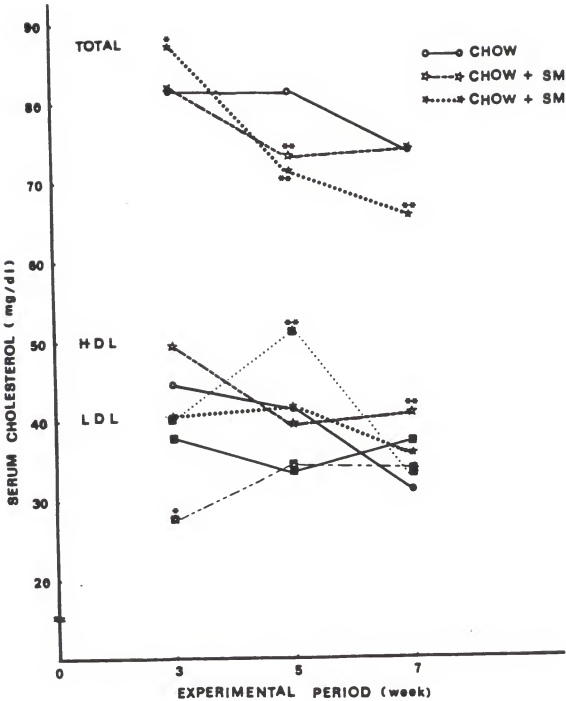

FIGURE 5 SERUM HDL, LDL AND TOTAL CHOLESTEROL OF RATS FED CHOW, SKIM MILK OR SKIM MILK YOGURT DIETS WITH CHOLESTEROL ADDITION AFTER 3, 5 AND 7 WEEKS; ASTERISK INDICATES SIGNIFICANT DIFFERENCE FROM THE CHOW DIET.
(**: $P < 0.01$; * : $P < 0.05$)

Liver weights, liver lipid and cholesterol. ANOVA procedure was used to determine statistical differences for liver lipid and liver cholesterol variables (Table 8). Diets and period did influence both liver lipid and cholesterol. Liver weights, liver lipid and cholesterol of rats fed chow, skim milk (SM) or skim milk yogurt (SMY) diets without or with cholesterol addition are reported in Table 9 and Table 10. Without cholesterol added to the diets (Table 9), there were no significant differences observed in liver weights of rats after 3, 5 and 7 weeks except that after 3 weeks, rats fed the SMY diet had smaller livers than those fed the chow diet. The consumption of SM had no effect on lowering hepatic lipid and cholesterol contents after 3, 5 and 7 weeks. There were no significant differences in liver cholesterol of rats fed the chow diet or the SMY diet after 3, 5 and 7 weeks, while the consumption of SMY significantly reduced the hepatic lipids only after 7 weeks ($P<0.05$). With 0.5% cholesterol added to the diets (Table 10), there were no significant differences in liver weights of rats fed chow, SM or SMY after 3, 5 and 7 weeks. Rats fed the SM diet for 3 weeks had lower levels of liver cholesterol ($P<0.05$) than those fed the chow diet. Feeding SMY had no lowering effect on liver lipid and cholesterol observed after 3, 5 and 7 weeks.

TABLE 8

Mean squares (MS) and significance in ANOVA procedure for liver lipid and liver cholesterol

		Liver lipid			liver cholesterol		
S[1]	DF	MS	F	P	MS	F	P
Diet	5	30.90	4.69	<.01	1.092	5.76	<.01
Period	2	12.89	1.96	<.16	0.461	2.43	<.10
D x P	10	11.58	1.76	<.11	0.188	0.99	<.47
R: D & P	36	6.59			0.190		
Total	53						

1 : source of variation.

TABLE 9

Liver weights, total liver lipid and cholesterol of rats fed chow, skim milk or skim milk yogurt diets without cholesterol addition after 3, 5 and 7 weeks[1,2]

Diet[3] treatment	Liver weight	Liver lipid	Liver cholesterol
	g	mg/g liver	mg/g liver
After 3 weeks			
Chow	11.63a	29.99a	1.04a
SM	10.90ab	34.07a	0.72a
SMY	9.70b	33.58a	0.60a
After 5 weeks			
Chow	11.47a	32.40a	0.81a
SM	12.27a	33.40a	1.02a
SMY	12.40a	34.72a	1.12a
After 7 weeks			
Chow	11.93a	35.12a	0.69a
SM	12.53a	33.53ab	0.43a
SMY	12.50a	30.23b	0.72a

[1] The values are means for 3 animals of each group after 3, 5 and 7 weeks. Standard error of a mean (SEM) for liver weights, liver lipid and cholesterol: 0.67, 1.48 and 0.251.

[2] Values in a column at the same experimental period not sharing a common letter are significantly different ($P \leq 0.05$).

[3] Diet treatment: Chow (A) = 70% chow + 30% water; SM (C) = 55% chow + 45% skim milk; SMY (E) = 55% chow + 45% skim milk yogurt.

TABLE 10

Liver weights, total liver lipid and cholesterol of rats fed chow, skim milk or skim milk yogurt diets with cholesterol addition after 3, 5 and 7 weeks[1,2]

Diet[3] treatment	Liver weight	Liver lipid	Liver cholesterol
	g	mg/g liver	mg/g liver
After 3 weeks			
Chow	11.97a	33.22a	1.65a
SM	12.53a	35.43a	0.72b
SMY	13.40a	34.93a	1.38ab
After 5 weeks			
Chow	12.67a	35.15a	1.38a
SM	12.40a	35.36a	1.25a
SMY	13.00a	38.39a	1.96a
After 7 weeks			
Chow	13.33a	38.32ab	1.45a
SM	13.77a	34.49b	1.24a
SMY	13.10a	38.80a	1.18a

[1]The values are means for 3 animals of each group after 3, 5 and 7 weeks. Standard error of a mean (SEM) for liver weights, liver lipid and cholesterol: 0.67, 1.48 and 0.251.

[2]Values in a column at the same experimental period not sharing a common letter are significantly different ($P \leq 0.05$)

[3]Diet treatment: Chow (B) = 69.5% chow + 30% water + 0.5% cholesterol; SM (D) = 54.5% chow + 45% skim milk + 0.5% cholesterol; SMY (F) = 54.5% chow + 45% skim milk yogurt + 0.5% cholesterol.

DISCUSSION

Our data indicated that both skim milk (SM) and skim milk yogurt (SMY) were hypocholesterolemic for rats fed the diets without or with 0.5% cholesterol addition. This effect may be due to the experimental period, age of the rats, and the amount of SM or SMY consumption.

In the study from Kritchevsky et al. (61), serum cholesterol levels of rats given either chow + whole milk or chow + skim milk for 3 weeks were significantly lower than those of rats fed the control chow + water diet, while the control group showed the greatest weight gain. However, Rao et al. (47) did not find the hypocholesterolemic effect of skim milk when the average weight gains of rats fed chow + SM for 4 weeks was significantly higher than those of rats fed chow + water. They claimed that the initial weights of the rats (328 g) were different from those of rats (179 g) in the study from Kritchevsky et al. (61). In our study, weight gain, food intake and feed efficiency of the rats were not significantly different among treatment groups. The rats fed chow + SM or chow + SMY diets without or with cholesterol addition had significantly lower serum cholesterol levels than did the rats fed chow + water after 5 or 7 weeks. The initial weights of our rats (Fig. 2) were even lighter (65-70 g) than those in the former two studies, that is, younger rats were used in this experiment. Marlett et al. (62) reported that fluid skim milk (FSM) decreased

plasma cholesterol at 1.5 and 3 weeks of feeding in the young rats (42 g); plasma cholesterol levels of the older rats (157 g) were not altered by FSM. There were no significant diets differences on mean body weight of young and older rats fed the control casein diet or FSM diet at 3 weeks. Thus, the hypocholesterolemic effect of feeding SM or SMY may depend on the initial age of rats and the experimental period. Also, equal growth of rats among treatments would be desirable in those observations.

Milk or yogurt was reported to exert an hypocholesterolemic effect when the supplementation provided above 24% (2-8 l) of total daily calories intake of the human subjects (16,17). However, such voluminous intake is not feasible for most of the people. In animal studies, the weanling rats fed SMP providing 25.2% of the calories had significantly lower plasma cholesterol levels than did those in the control group at 1.5 and 3 weeks of feeding (62). Chawla and Kansal (21) provided the supplement of yogurt as about 24% of total daily calories intake of rats. Based on the zero day level, plasma cholesterol in rats rose 10.4% by the 30th day and fell to the minimum of 33.6% by the 90th day. According to our study, SM or SMY was supplied so as to provide about 20% of total daily calorie intake of the rats. The decrease of total serum cholesterol in rats fed SM or SMY either with or without cholesterol addition was significant after 5 weeks. Therefore, our data showed that

feeding SM or SMY to the rats did have a hypocholesterolemic effect on total serum cholesterol even though the consumption of SM or SMY was decreased to 20% of total daily calorie intake of rats.

Adding 0.5% of cholesterol to chow diet B significantly increased total serum cholesterol of rats after 3, 5 and 7 weeks as compared to those of rats fed chow diet A without cholesterol addition. Both SM and SMY reduced serum cholesterol levels of rats on the high cholesterol diet after 5 weeks by 9.7% and 12 %, respectively. SMY consistently reduced serum cholesterol levels by 11.2% after 7 weeks. In the groups without cholesterol addition, the same trend was observed after 5 weeks by 8.5% for SM and 9.1% for SMY. After 7 weeks, 11.2% decrease in serum cholesterol of rats fed SMY, but no significant change in SM. Both diets without or with cholesterol addition showed the similar extent of hypocholesterolemic effect of SM or SMY. SMY had the more consistent cholesterol-lowering effect.

The hypocholesterolemic effect of SM or SMY may mirror the change of lipoprotein cholesterol. In our study, the cholesterol-lowering effect of feeding the SM diet or the SMY diet without cholesterol addition was reflected in the decrease of serum HDL cholesterol, while serum LDL cholesterol was not affected. Our data confirmed the study of Stahelin et al. (63) who used swine as a model (with the

lipoprotein distribution similiar to humans). Serum HDL cholesterol levels of swine were significantly lower by 14% in skim milk group and the skim milk yogurt lowered HDL cholesterol to the same extent as skim milk did. Serum LDL and VLDL cholesterol decreased only in swine fed skim milk. In human studies, no significant changes were found in LDL and HDL cholesterol of healthy human subjects consuming 1 l yogurt for a 3-wk period (24). Rossouw et al. (23) reported that HDL cholesterol decreased during both a baseline diet period and after 7 days of milk or yogurt supplementation. The importance of the HDL cholesterol decrease in those observations is uncertain. Jaspers et al. (52) reported that HDL-C cholesterol of human adults supplied 681 g nonfat yogurt throughout three 14-21 day periods remained unchanged. Whether this decrease of HDL cholesterol in our study was accompanied by decreased HDL_2, which may be antiatherogenic (64,65), needs to be investigated in the future.

Adding 0.5% cholesterol to the diets, HDL and LDL cholesterol of rats fed SM or SMY fluctuated during 3, 5 and 7 weeks. However, rats fed the SMY diet had higher LDL levels ($P<0.01$) than did those fed the chow diet or the SM diet after 5 weeks. Dwivedi et al. (20) found that rabbits fed 0.5% gm cholesterol and 50 ml of curd for 90 days were not prevented from arterial damage even though the level of serum cholesterol was reduced to some extent. Further

studies of aortal cholesterol deposits also would be required to ascertain the role of milk or yogurt in the treatment as well as prophylaxis of atherosclerosis.

Serum HDL cholesterol changes may reflect altered hepatic metabolism induced by the influx of nutrients from the consumption of SM or SMY. Orotic acid is normally found in cow's milk at the concentrations of 69-122 ppm (66) and in yogurt at the range of 34-46 ppm (13,54). By HPLC analysis, the concentrations of orotic in SM or SMY in our study were 106.18 ppm and 46.05 ppm, respectively. Bernstein et al. (29,30) suggested that orotic acid of bovine milk was the factor to inhibit the acetyl-CoA synthetase in cholesterol biosynthesis in rat liver homogenate. Ahmed et al. (45) reported that hepatic cholesterol synthesis in rat liver slices was reduced by orotic acid isolated from skim milk. Orotic acid at the level of 1% in the diet of rats was reported to decrease serum lipid levels but increase the quantities of lipid in the liver (67,68). In our study, feeding the SM or SMY diets containing 0.0025% orotic acid did not show any significant effect on liver cholesterol and liver lipid of rats after 5 weeks. Pulusani and Rao (51) also found that whole body lipids, liver lipids and liver cholesterol were not significantly different when rats were fed chow + water, chow + skim milk, and chow + skim milk fermented by Lactobacillus thermophilus or Lactobacillus bulgaricus. It seems that the quantity of orotic acid in SM

or SMY supplemented to rats was insufficient to alter hepatic cholesterolgenesis. Also, orotic acid probably can not be the "milk factor" enhanced by fermentation since it was found in lesser amount in SMY than in SM.

Ward et al. (31) reported that uric acid isolated from human milk inhibited hepatic cholesterolgenesis in vitro. Uric acid is found in cow's milk at the concentrations of 20-22 ppm (57) and in yogurt at the range of 14-18 ppm. The concentrations of uric acid in SM or SMY in our study were 23.27ppm and 20.06 ppm, respectively. However, no recent studies have been done on its hypocholesterolemic effects when fed to rats orally. Further investigations are needed to examine the role of uric acid on the cholesterol-lowering effect of SM or SMY.

Calcium has been suggested to be an active factor in milk or yogurt to exert this hypocholesterolemic effect on serum cholesterol (17-19,26). The decrease of total cholesterol in females was due to the biliary cholesterol losses with regard to increased binding to calcium salts in the GI tract (69). Thus, calcium may be responsible for this cholesterol-lowering effect of SM or SMY in our study without changing hepatic cholesterolgenesis. However, calcium may not be able to account for this greater extent hypocholesterolemic effect of skim milk yogurt in our study since yogurt and milk contained the same amount of calcium.

Various studies have reported that lactic acid bacteria

in yogurt and their metabolic activities may influence serum cholesterol (16,17). Gilliland et al. (49) demonstrated that certain strains of L. acidophilus can assimilate cholesterol in the GI tract and may be reponsible for reducing serum cholesterol level. Mott et al. observed a decrease in serum cholesterol of pigs fed L. acidophilus cells with an associated increase in the amount of cholesterol detected in fecal material (70). Yogurt production utilizes the mixed starter culture of Streptococcus thermophilus and Lactobacillus bulgaricus. The ingestion of microorganisms might metabolize the cholesterol secreted in the bile to result in greater bile deconjugation and fecal excretion of cholesterol. Further investigations in this area are warranted.

SUMMARY

The concentration of orotic acid and uric acid in skim milk (SM) or skim milk yogurt (SMY) was analyzed by high performance liquid chromatography (HPLC) in order to investigate the "milk factors" which have been hypothesized to exert a hypocholesterolemic effect. The concentration of orotic acid in SM was 106.18 ppm. During fermentation it decreased significantly to 46.05 ppm in SMY. Uric acid did not change significantly during fermentation. The concentration of uric acid in SM or SMY was 23.27 ppm and 20.06 ppm, respectively. After 3 days storage, there were no significant changes in orotic acid or uric acid in SM and SMY as compared to those at 0 day storage.

Serum cholesterol and liver lipids of 54 rats fed chow, SM or SMY diets without or with 0.5% cholesterol addition after 3, 5 or 7 weeks were studied. In the SM or SMY group, SM or SMY replaced 45% of chow to provide 20% of total daily calorie intake of rats. Water was added to the chow diet so that all diets would be similar in calorie density. There were no significant diet difference in feed efficiency, mean weight gain and food intake of rats after 3, 5 or 7 weeks. In the diets without cholesterol addition, total serum cholesterol of rats fed SM or SMY was 8.3% and 9.1% lower ($P<0.05$), respectively, than that of the chow group after 5 weeks. The SMY group also had a lower level of total serum

cholesterol (11.2%, $P<0.01$) after 7 weeks. The hypocholesterolemic effect of feeding SM or SMY was reflected in a significant decrease of serum HDL cholesterol while serum LDL cholesterol was not significantly affected. With 0.5% cholesterol added to the diets, total serum cholesterol of rats fed SM or SMY was 9.7% and 12% lower ($P<0.01$), respectively, than that of the chow group after 5 weeks. The SMY group also had a lower level of serum cholesterol (11.2%, $P<0.01$) after 7 weeks. HDL and LDL cholesterol of rats fed SM or SMY fluctuated during 3, 5 and 7 weeks.

SM or SMY did not have any significant effect on liver cholesterol and liver lipid of rats fed diets without cholesterol after 5 weeks or fed diets with cholesterol after 5 or 7 weeks. However, liver lipid of rats fed the SMY diet without cholesterol addition was lower ($P<0.05$) than that of rats fed the chow diet after 7 weeks.

Feeding SM or SMY to rats on the chow diets without or with 0.5% cholessterol addition did lower serum total cholesterol after 5 weeks. The consumption of SMY consistently exerted this effect after 7 weeks. Serum HDL cholesterol of rats fed the SM or SMY diets without cholesterol addition followed the response of total cholesterol. Liver lipid and liver cholesterol of rats fed the SM or SMY diets without or with cholesterol addition were not significantly different from those of rats fed the

chow diets after 5 weeks. Orotic acid and uric acid may not be the main factors which exerted the hypocholesterolemic effect of SM or SMY.

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APPENDIX

ANALYTICAL METHODS

Serum cholesterol: Total, HDL and LDL cholesterol

Isolab's LDL-Direct Plus Cholesterol Ratio System is available in one kit size: separation columns, Alpha fraction elution agent, Beta fraction elution agent, cholesterol reagent set (enzyme reagent and activator). The column is with a bed of heparin-agarose to separate the lipoprotein fractions. To activate the Cholesterol Reagent, add equal volumes of Enzyme Reagent and Activator to the required total volume.

Procedure

1. Remove first the column's top cap, then the bottom closure. This order of opening is important-otherwise, air will enter the column tip, interfering with free liquid flow.

2. Use the wide end of a Pasteur pipette to push the upper disc down until it contacts the top of the resin bed. Do not compress the bed.

3. Allow the column to drain until the liquid level reaches the top disc, where flow will automatically stop.

4. Equilibrate the column bed by adding 1.0 ml of Alpha fraction elution agent to the column. Allow column to drain. Discard eluate.

5. With the column positioned over a test tube (12x75 mm, 5 ml), add 0.2 ml blood serum to the column, near or on the

upper disc. Collect the eluate.

6. Add 1.0 ml of Alpha fraction elution agent and collect the entire volume in the same test tube, for a total fraction volume of 1.2 ml. Mix well.(Alpha fraction elution)

7. Place the column over a clean 12x75 mm test tube.

8. Add 1.2 ml of Beta fraction agent and collect the entire volume. Mix well.(Beta fraction elution)

9. To activate the cholesterol reagent, add equal volumes of enzyme reagent and activator to the required total volume. Mix well.

10. Prepare 1:6 diluted total blood serum samples (T) and cholesterol standard (C) by adding 200 ul of each to 1.0 ml saline in small test tubes and mixing gently.

11. Add 200 ul saline to reagent blank; 200 ul of fraction A, fraction B and diluted T to appropriate assay tube. Add 200 ul of cholesterol standard to its assay tube. Mix gently.

12. Pipette 1.8 ml of cholesterol reagent into each assay tube, including reagent blank and cholesterol standard.

13. Incubate all mixed tubes for 5 minutes at 37°C.

14. Set spectrophotometer at 505 nm. Zero instrument in accordance with manufacturer's instructions using the reagent blank tube as the blank.

15. Read absorbance of each tube within 20 minutes. If any absorbance is greater than 1.0, dilute original sample with an equal volume of saline and repeat the assay. Multiply the

new absorbance by 2.

16. Calculation

A = absorbance of Alpha cholesterol assay tube

B = absorbance of Beta cholesterol assay tube

T = absorbance of diluted Total cholesterol assay tube

C = absorbance of cholesterol standard assay tube

STD = concentration (mg/dl) of cholesterol standard

Alpha cholesterol conc. = A/C (STD)

Beta cholesterol conc. = B/C (STD)

Total cholesterol conc. = T/C (STD)

% Recovery

= (Alpha conc. + Beta conc.) / Total serum conc. (100)

Determination of total liver lipid

Reagents:

1. Extraction mixture: Methylene chloride:methanol (2:1, v/v).

2. 0.73% NaCl.

Procedure:

1. Homogenize 2.0 g liver sample in 20 ml extraction mixture for 30 s using a polytron high-speed homogenizer (Brinkmann Instruments, Westburg, NY) in 50 ml plastic centrifuge tubes.

2. Shake homogenates 5 min on an automatic shaker.

3. Centrifuge (lab top type) for 10 min at top speed.

4. Filter the supernatant into a fresh 50 ml plastic centrifuge tube. Reextract the sediment by adding 10 ml extraction mixture and proceed with the same steps as described above. The final total volume in the new 50 ml plastic tube would be 30 ml.

5. Add 8 ml 0.73% NaCl to the new tube, shake 5 min on an automatic shaker, and centrifuge (lab top type) tubes for 5 min.

6. Aspirate off and discard top aqueous layer.

7. Pipet 0.2 ml of the bottom organic layer to the glass tube for liver cholesterol analysis. Pour the rest into a dried, pre-weighed aluminum dish.

8. Let the dish set as solvent evaporates.

9. Dry the dish at 103°C for 1 h in an oven.

10. After cooling in a desiccator, weigh the lipid-containing dish to the nearest mg.

11. Calculations:

$$\frac{\text{mg lipid}}{\text{liver}} = \frac{\text{mg lipid in each dish} \times \frac{\text{total volume of organic layer}}{(\text{total volume} - 0.2)\ \text{of organic layer}}}{\text{2.0 g liver sample}}$$

Determination of liver cholesterol

Reagents

1. Concentrated sulfuric acid-iron reagent mixture. 3 ml sulfuric acid (AR grade) to 0.3 ml of a mixture of 2.5 g ferric chloride dissolved (shake slowly to dissolve) in 100 ml phosphoric acid (assay 85%). Store in a glass-stoppered bottle.

2. Glacial acetic acid, AR grade.

3. Isopropyl alcohol, AR grade.

4. Cholesterol standards. Dissolve 100, 200, 300, and 400 mg of cholesterol in isopropyl alcohol and dilute each solution to 100 ml. The 100 mg/100 ml standard is used routinely. All four standards should be used to prepare a standard line.

Procedure:

1. Pipet 0.2 ml of the organic layer (lipid extraction procedure) for total liver cholesterol determination into the bottom of a disposable 15 ml test tube.

2. Pipet 0.2 ml of the standard (100 mg cholesterol/100 ml isopropyl alcohol) into another disposable 15 ml test tube.

3. Add 5 ml of isopropyl alcohol rapidly to each of the tubes from an automatic pipettor, vortex and mix thoroughly.

4. Pipet 1 ml of each supernatant fluid into new 15 ml tubes (screw-type teflon coated cap).

5. Pipet 1 ml of isopropyl alcohol into another 15 ml tube,

which will hold the reagent blank.

6. Add 3 ml of glacial acetic acid to all new tubes, vortex and mix.

7. At 30 s intervals, allow 3.3 ml of the concentrated sulfuric acid-iron reagent mixture to drip slowly down the sides of the centrifuge tubes so that the acid will form a layer underneath the solutions. Cap immediately and slowly invert the tubes 5 times.

8. Allow tubes to stand 10 min at room temperature to cool.

9. Transter solutions to respective cuvettes and tap sides to eliminate any bubbles.

10. Without delay, measure absorbance of standard (A_s) and of the unknown sample (A_u) against the reagent blank at 560 nm (Bausch and Lomb Spectronic 20 Colorimeter).

11. Calculations:

Total liver cholesterol:

$$\frac{\text{mg cholesterol}}{\text{g liver}} = \frac{\frac{A_u}{A_s} \times 100 \text{ mg/dl} \times \frac{\text{total volume of organic layer}}{100}}{2 \text{ g liver sample}}$$

TABLE 3

Concentration of orotic acid and uric acid in skim milk and skim milk yogurt after 0 and 3 days storage

Diets		Orotic acid	Uric acid
		ppm	ppm
0 day storage			
Skim milk	1	100.68	22.82
	2	115.52	26.52
	3	104.40	23.56
	4	101.82	22.56
	5	103.40	20.90
	6	111.24	23.64
Skim milk yogurt	1	37.65	17.47
	2	46.63	21.11
	3	33.53	15.93
	4	58.34	24.66
	5	50.35	21.17
	6	49.80	20.01
3 days storage			
Skim milk	1	113.10	22.36
	2	91.22	18.64
	3	88.92	18.84
	4	107.40	22.68
	5	102.86	23.20
	6	110.62	22.92
Skim milk yogurt	1	44.78	22.00
	2	53.49	25.50
	3	46.62	21.61
	4	46.68	19.85
	5	42.77	17.24
	6	40.89	15.96

TABLE 4

Weight gain (unadjusted), food intake and feed efficiency of rats fed chow, skim milk or skim milk yogurt without or with cholesterol addition after 3 weeks

Diets	Weight gain	Food intake	Feed efficiency
	g/day	g/day	wt gain/total intake
A1	5.5	25.2	0.22
2	6.8	27.2	0.25
3	6.9	25.7	0.24
B1	5.7	23.7	0.24
2	7.2	28.6	0.25
3	5.7	25.4	0.23
C1	6.8	26.2	0.26
2	5.7	25.7	0.22
3	6.4	27.1	0.24
D1	6.8	28.2	0.24
2	6.0	26.1	0.23
3	7.0	27.2	0.26
E1	5.6	24.8	0.23
2	5.5	25.0	0.22
3	6.8	28.6	0.23
F1	6.7	26.9	0.25
2	6.8	28.0	0.24
3	7.8	31.9	0.25

TABLE 4

Weight gains (unadjusted), food intake and feed efficiency of rats fed chow, skim milk or skim milk yogurt without or with cholesterol addition after 5 weeks

Diets	Weight gain	Food intake	Feed effeciency
	g/day	g/day	wt gain/total intake
A4	5.5	25.9	0.21
5	6.0	26.2	0.23
6	5.0	24.5	0.20
B4	5.7	25.3	0.23
5	6.1	25.1	0.24
6	6.4	27.4	0.23
C4	6.0	27.0	0.22
5	6.0	25.7	0.23
6	5.9	25.6	0.23
D4	5.7	26.1	0.22
5	5.7	27.0	0.21
6	5.6	26.4	0.21
E4	5.7	25.0	0.23
5	5.2	25.1	0.21
6	5.7	26.6	0.21
F4	5.9	25.7	0.23
5	5.2	24.1	0.21
6	6.3	27.6	0.23

TABLE 4

Weight gain (unadjusted), food intake and feed efficiency of rats fed chow, skim milk and skim milk yogurt without or with cholesterol addition after 7 weeks

Diets	Weight gain	Food intake	Feed effeciency
	g/day	g/day	wt gain/total intake
A7	4.7	24.9	0.19
8	5.8	27.4	0.21
9	4.1	25.6	0.16
B7	4.4	24.3	0.18
8	5.3	28.1	0.19
9	4.7	26.5	0.18
C7	4.8	24.3	0.20
8	5.1	26.9	0.19
9	5.0	27.0	0.19
D7	4.9	26.7	0.19
8	4.8	26.7	0.18
9	4.7	26.7	0.18
E7	4.8	25.9	0.18
8	4.4	25.3	0.18
9	4.6	26.2	0.18
F7	4.9	25.6	0.19
8	5.0	26.6	0.19
9	4.0	25.1	0.16

TABLE 6, 7, 9, 10

HDL, LDL, total serum cholesterol, liver weights, total liver lipid and cholesterol of rats fed chow, skim milk or skim milk yogurt diets without or with cholesterol addition after 3 weeks

Animal groups	Serum cholesterol			Liver		
	HDL	LDL	Total	weight	lipid	cholesterol
		mg/dl		g	mg/g liver	
A1	50.48	17.91	66.42	10.8	25.24	0.61
2	49.62	14.85	70.09	12.3	32.21	1.37
3	47.05	27.31	71.94	11.8	32.51	1.13
B1	43.67	33.74	81.36	10.7	27.38	1.11
2	59.22	40.32	-	13.8	37.00	2.36
3	45.36	39.77	81.37	11.4	35.27	1.57
C1	51.05	17.91	68.71	11.8	34.40	0.52
2	47.05	27.31	72.87	9.3	37.76	0.82
3	47.05	24.14	71.94	11.6	30.05	0.83
D1	43.70	24.14	78.51	12.9	34.42	0.59
2	53.08	22.57	81.36	10.9	35.72	1.17
3	52.21	37.01	85.29	13.8	36.14	0.78
E1	48.76	20.23	69.17	9.2	33.28	0.46
2	49.91	21.40	71.94	9.1	34.20	0.74
3	47.05	26.51	70.09	10.8	33.25	0.59
F1	37.01	40.32	83.29	13.1	33.22	1.48
2	37.01	43.67	87.18	12.2	35.89	1.21
3	47.91	37.84	91.12	14.9	35.67	1.44

TABLE 6, 7, 9, 10

HDL, LDL, total serum cholesterol, liver weights, total liver lipid and cholesterol of rats fed chow, skim milk or skim milk yogurt diets without or with cholesterol addition after 5 weeks

Animal Groups	Serum cholesterol			Liver		
	HDL	LDL	Total	weight	lipid	cholesterol
	mg/dl			g	mg/g liver	
A4	41.99	24.14	70.70	12.0	30.62	1.00
5	41.90	24.14	70.08	11.8	32.45	1.07
6	41.90	27.31	73.80	10.6	34.14	0.65
B4	43.67	31.31	78.51	12.3	36.18	1.53
5	40.32	32.93	83.29	12.1	34.24	1.31
6	40.32	37.01	81.36	13.6	35.02	1.31
C4	37.01	21.01	64.61	12.3	33.44	0.96
5	31.30	22.57	62.04	12.2	33.38	1.60
6	37.01	24.14	70.08	12.3	33.37	0.54
D4	40.32	32.93	74.73	12.8	35.22	1.52
5	42.83	24.14	63.25	11.4	31.98	0.59
6	35.37	-	81.37	13.0	38.88	1.60
E4	40.32	20.46	64.61	11.8	33.39	0.54
5	40.32	32.86	69.47	13.2	39.62	2.11
6	43.67	22.70	61.01	12.2	31.14	0.70
F4	43.67	59.23	68.25	12.6	41.29	3.17
5	43.36	45.36	71.93	12.6	39.01	1.58
6	37.83	48.76	73.79	13.8	34.87	0.78

TABLE 6, 7, 9, 10

HDL, LDL, total serum cholesterol, liver weights, total liver lipid and cholesterol of rats fed chow, skim milk or skim milk yogurt diets without or with cholesterol addition after 7 weeks

Animal Groups	Serum cholesterol			Liver		
	HDL	LDL	Total	weight	lipid	cholesterol
	mg/dl			g	mg/g liver	
A7	49.16	18.20	64.68	12.1	34.53	0.32
8	41.71	23.51	71.87	14.5	34.44	0.81
9	50.84	21.22	68.85	9.2	36.38	0.87
B7	29.71	39.26	73.69	12.8	38.68	1.26
8	32.07	32.86	71.87	14.8	38.25	1.97
9	32.07	39.26	75.53	12.4	38.03	1.13
C7	40.89	21.01	64.68	12.8	32.65	0.59
8	41.71	13.72	62.04	12.1	34.85	0.38
9	41.71	17.45	63.80	12.7	33.08	0.32
D7	32.86	42.53	70.05	14.2	32.99	1.00
8	39.26	28.92	73.69	13.8	36.65	1.31
9	50.00	29.71	77.38	13.3	33.83	1.42
E7	37.83	22.22	59.76	11.7	29.65	0.79
8	35.83	22.22	63.31	12.6	32.02	0.56
9	36.34	25.09	59.41	13.2	29.02	0.80
F7	31.24	34.35	66.89	12.5	40.12	1.26
8	34.85	33.86	62.60	14.5	39.23	1.15
9	40.83	32.88	66.89	12.3	37.05	1.13

THE EFFECT OF FEEDING SKIM MILK OR SKIM MILK YOGURT ON SERUM CHOLESTEROL AND LIVER LIPIDS IN RATS

by

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The concentration of orotic acid and uric acid in skim milk (SM) or skim milk yogurt (SMY) was analyzed by high performance liquid chromatography (HPLC) in order to investigate the "milk factors" which have been hypothesized to exert a hypocholesterolemic effect. The concentration of orotic acid in SM was 106.18 ppm. During fermentation it decreased significantly to 46.05 ppm in SMY. Uric acid did not change significantly during fermentation. The concentration of uric acid in SM or SMY was 23.27 ppm and 20.06 ppm, respectively. After 3 days storage, there were no significant changes in orotic acid or uric acid in SM and SMY as compared to those at 0 day storage.

Serum cholesterol and liver lipids of 54 rats fed chow, SM or SMY diets without or with 0.5% cholesterol addition after 3, 5 or 7 weeks were studied. In the SM or SMY group, SM or SMY replaced 45% of chow to provide 20% of total daily calorie intake of rats. Water was added to the chow diet so that all diets would be similar in calorie density. There were no significant diet difference in feed efficiency, mean weight gain and food intake of rats after 3, 5 or 7 weeks. In the diets without cholesterol addition, total serum cholesterol of rats fed SM or SMY was 8.3% and 9.1% lower ($P<0.05$), respectively, than that of the chow group after 5 weeks. The SMY group also had a lower level of total serum cholesterol (11.2%, $P<0.01$) after 7 weeks. The hypocholesterolemic effect of feeding SM or SMY was

reflected in a significant decrease of serum HDL cholesterol while serum LDL cholesterol was not significantly affected. With 0.5% cholesterol added to the diets, total serum cholesterol of rats fed SM or SMY was 9.7% and 12% lower ($P<0.01$), respectively, than that of the chow group after 5 weeks. The SMY group also had a lower level of serum cholesterol (11.2%, $P<0.01$) after 7 weeks. HDL and LDL cholesterol of rats fed SM or SMY fluctuated during 3, 5 and 7 weeks.

SM or SMY did not have any significant effect on liver cholesterol and liver lipid of rats fed diets without cholesterol after 5 weeks or fed diets with cholesterol after 5 or 7 weeks. However, liver lipid of rats fed the SMY diet without cholesterol addition was lower ($P<0.05$) than that of rats fed the chow diet after 7 weeks.

Feeding SM or SMY to rats on the chow diets without or with 0.5% cholessterol addition did lower serum total cholesterol after 5 weeks. The consumption of SMY consistently exerted this effect after 7 weeks. Serum HDL cholesterol of rats fed the SM or SMY diets without cholesterol addition followed the response of total cholesterol. Liver lipid and liver cholesterol of rats fed the SM or SMY diets without or with cholesterol addition were not significantly different from those of rats fed the chow diets after 5 weeks. Orotic acid and uric acid may not be the main factors which exerted the hypocholesterolemic

effect of SM or SMY.