Isolation and Screening of Microorganisms for Their Ability to Reduce the Amount of Cholesterol in a Model System

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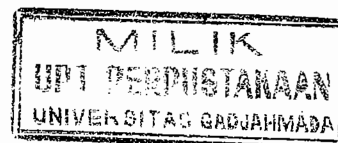
ABSTRACT

The objective of this study was to isolate and screen the ability of microorganisms to reduce the amount of cholesterol in a model system supplemented with cholesterol. The cholesterol-degrading bacteria were isolated from samples of various fats and authentic strains were also used. Analyses were performed to determine the rate of cholesterol degradation in model system, effect of the medium pH on cholesterol reduction, and identification of cholesterol-degrading bacteria. ***Rhodococcus equi*** *ATCC 21107,* ***Rhodococcus equi*** *ATCC 33706,* ***Leuconostoc cremoris, Serratia marcescens*** *ATCC 13880 and several bacterial isolates from fats were capable of degrading cholesterol in a model system supplemented with pure soluble cholesterol. Morphological and biochemical characteristics revealed that some of the bacterial isolates tentatively classified as belonged to the genus* ***Pseudomonas***.

INTRODUCTION

The ability of microorganisms to degrade cholesterol was observed very early (Tak, 1942; Turfitt, 1944; 1948) and has been studied in various fields. This includes the production of starting materials for chemical synthesis of pharmaceutical steroids; transformation of cholesterol to coprostanol and related compounds by intestinal bacteria; and the possibility for reducing cholesterol in food. It has been reported that a large number of microorganisms could degrade cholesterol, but only a few accomplish this without accumulating steroid intermediates (Turfitt, 1944; Arima et al., 1969; Marschek et al., 1972; Owen et al., 1983; Aihara et al., 1986; Watanabe et al., 1986). Arima et al. (1969) examined 1589 microbial strains for their ability to decompose cholesterol and found 236 cultures from actinomycetes, bacteria, yeast and molds that oxidized cholesterol to cholestenone. The complete decomposition of the cholesterol molecule was observed in species of the genera *Arthrobacter, Bacillus, Brevibacteium, Corynebacterium, Microbacterium, Mycobacterium, Nocardia, Protaminobacter, Serratia,* and *Streptomyces* (Arima et al., 1969). Watanabe et al. (1986) isolated organisms capable of degrading cholesterol from foods of animal origin, such as butter, pork, bacon, and chicken fat. Most of the strains isolated belonged to the genus *Rhodococcus*. These organisms degraded cholesterol with little or no accumulation of steroid intermediates as determined by thin layer chromatography (TLC). Ferreira and Tracey (1984) compared the numerical taxonomy of cholesterol-degrading soil bacteria. They found that the majority of the cholesterol degrading bacteria were classified as *Rhodococcus* spp., *Mycobacterium* spp., and *Nocardia* spp.

One potential approach to reducing cholesterol in animal derived foods is through the use of enzyme systems from cholesterol metabolizing microorganisms to degrade the compound to nontoxic metabolites. Many microorganisms have been observed as capable of decomposing cholesterol, but information on their activity in foods is limited. Progress in developing biological systems for the reduction of the cholesterol content in food will depend on the discovery and isolation of efficient enzyme systems. The objective of this study was to isolate and screen the ability of microorganisms to reduce the amount of cholesterol in a model system supplemented with cholesterol.



MATERIALS AND METHODS

Isolation of microorganisms

The cholesterol-degrading bacteria were isolated from samples of various fats by an enrichment culture technique (Watanabe et al., 1986). Portions of 500 mg of chicken fat, beef fat, beef kidney fat, lamb fat, beef tallow, butter and shortening were added to 10 mL of Bennet's medium containing (g/L) : yeast extract, 0.2; beef extract, 0.2; neopeptone, 0.4; and glucose, 0.02 (Johnson and Somkuti, 1990) supplemented with 0.1% polyoxyethanyl-cholesteryl sebacate (water soluble cholesterol, Sigma Chemical Co., St. Louis, MO). The tubes were incubated at 30°C in an incubator shaker (100 rpm) for 6 days. The cultures that developed were reincubated in the same medium for an additional three 72 hr periods by succesively transferring 100 µL of the culture broth to fresh medium. The cultures were then serially diluted and surface plated on trypticase soy agar (TSA) (Difco Laboratories, Detroit, MI). Representative colonies were picked on agar plates and purified by repeated serial platings. The isolates were then maintained on brain heart infusion (BHI) broth (Difco) at 4°C. For preservation, the cultures were grown for 24 hr at 37°C and lyophilized in trypticase soy broth (TB) (Difco) supplemented with 20% (v/v) sterile skim milk.

Cultures maintenance

In addition to the bacterial isolates obtained from fats, several authentic strains were also used. These included *Rhodococcus equi* ATCC 21107, *R. equi* ATCC 33706 provided by Dr. Terrance L. Johnson, U. of North Carolina, Chapel Hill, North Carolina: *Serratia marcescens* ATCC 13880, from the Dept. of Microbiology, Colorado State University (CSU); and laboratory strains of *Lactococcus lactis* ssp. *lactis, Streptococcus thermophilus, Lactococcus lacti*s ssp. *lactis* var. *diacetylactis, lactococcus lactis* ssp. *cremoris, Leuconostoc cremoris, Lactobacillus lactis* and *Lactobacillus bulgaricus* (Department of Animal Sciences, Colorado State University). The isolates were maintained on BHI broth (Difco) at 4°C. For preservation, the cultures were lyophilized in TSB supplemented with 20% (v/v) sterile skim milk. The inoculum was prepared by growing two loopfuls of the cultures in 10 mL of BHI broth suplemented with 10 µg of cholesterol per mL of BHI broth (dissolved in n-propanol at 2mg/mL) for 24 hr at 30°C. Cholesterol (dissolved in n-propanol) was used to grow the inoculum because it has been reported that cells pregrown in the presence of cholesterol reduced more cholesterol than that without cholesterol (Johnson and Somkuti, 1990; Aihara et al., 1986). However, since cholesterol has to be dissolved in n-propanol, polyoxycholesteryl sebacate (water soluble cholesterol) was used for screening. Otherwise a large amount of n-propanol need to be added into the medium, which may inhibit bacterial growth.

Degradation of cholesterol in model system

The test microorganisms were inoculated (1%, v/v) and grown in Bennet's medium supplemented with 0.1% or 0.05% of polyoxyethanyl-cholesteryl sebacate (water soluble cholesterol, Sigma Chemical Co., St. Louis, MO) for screening the authentic strains and fat isolates, respectively. The supplementation of cholesterol was done after autoclaving the medium at 121°C for 15 min, and stirring for 3 hr. After the water soluble cholesterol was completely dissolved, the medium was filter sterilized (0.45µ) and distributed to sterile test tubes (10 mL/tube). The test organisms (24-hr old cultures) were inoculated (0.1 mL/tube) and incubated aerobically at 30°C for 7 days. Cholesterol content, pH values and total colony counts of the samples were analyzed after 0,3 and 7 days.

Effect of the medium pH on cholesterol reduction

Bennet's medium was autoclaved for 15 min at 121°C, supplemented with water soluble cholesterol (0.05% w/v) and stirred until the cholesterol was dissolved. After the cholesterol was completely dissolved, the pH of the medium was adjusted to 6.0, 7.0 or 8.0 with 3N KOH or 4N HCl and filter sterilized (0.45 µ). The medium was distributed to sterile flasks (100 mL each) and inoculated with 24-hr old cultures (1% v/v). After inoculation the medium was incubated at 37°C. Cholesterol content, pH values and total colony counts of the samples were analyzed after 0, 2 and 4 days.

Degradation of cholesterol in phosphate buffer containing beef tallow or ground beef

Ten g portions of beef tallow or ground beef were added to 90 mL of phosphate buffer (pH 7.2) and the mixtures were homogenized by sonication (Sonic 300

Dismembrator, Artek Systems., Farmingdale, N.Y) at a setting of 6 for 15 min. The substrates were incubated under aerobic conditions (in an incubator shaker at 150 gyration per min) at 30°C for 48 hr after addition of 1% (v/v) of the test cultures. Control samples consisted of substrates without the addition of test microorganisms.

Identification of cholesterol degrading bacteria

Some of the most active bacteria in degrading cholesterol in Bennet's medium supplemented with water soluble cholesterol along with authentic strains were tested for: cell and colony morphology, gram stain, motility, acid-fast stain, sugar tube fermentations (glucose, lactose, sucrose, mannitol, maltose, galactose, fructose, sorbitol, arabinose, xylose, rhamnose, innositol, trehalose, and glycerol), litmus milk reaction, hemolysis production, nitrate reduction, starch hydrolysis, casein hydrolysis, lipid hydrolysis, gelatin hydrolysis, catalase and cytochrome oxidase, oxygen requirements, hydrogen sulfide production, and IMViC (Indole, Methyl red, Voges-Proskauer and Citrate) reactions, and urea hydrolysis. The tests were performed by the methods described by Barnett (1989).

Analyses

Cholesterol content was determined by the colorimetric ferric chloride method (Thomas and Stevens, 1960). Test samples were extracted with an equal volume of ethyl acetate and centrifuged at 10,000xg for 10 min for phase separation. Aliquots (1.0 mL) of the ethyl acetate layer were evaporated to dryness. Residues were dissolved in 3 mL of the ferric chloride reagent (ferric chloride dissolved at 70 mg/100 mL in acetic acid) and two mL of concentrated sulphuric acid. The mixtures were vortexed to obtain thorough mixing and allowed to cool to room temperature for 15 min. Absorbance was read at 560 nm with a spectrophotometer (Bausch and Lomb Incorporated, Rochester, N.Y).

The pH values of the medium were determined with a Corning pH meter (Model 125, Medfield, MA) equipped with a Corning combination electrode. Total bacterial colony counts were determined by direct plating on TSA. Serial dilutions of 1 mL samples were made and 0.1 mL portions were spread plated in duplicate onto the agar. The plates were incubated at 37°C for 48 hr under aerobic conditions.

Statistical analysis

The experiments were replicated twice and duplicate measurements were performed in each replicate. The data were analyzed using analysis of variance and treatment means were compared using Duncan's multiple range test (Steel and Torrie, 1980).

RESULTS AND DISCUSSION

Isolation and screening of cholesterol-degrading microorganisms

Thirty three cultures were isolated from chicken fat, beef fat, lamb fat, shortening, processed beef tallow and butter in the preliminary screening. These isolates were further screened for their ability to degrade cholesterol in a model system. Bennet's medium supplemented with 0.1% of water soluble cholesterol was used to screen the cholesterol-metabolizing activities of authentic strains. However, at 0.1% concentration the absorbances were greater than 1.0, and therefore needed to be diluted. To avoid technical errors resulting from dilution, screening of the culture isolates from fats was conducted at the 0.05% concentration. The extent of cholesterol degradation after growing some authentic strains and culture isolates from fats in Bennet's medium supplemented with water soluble cholesterol are shown in Tables 1 and 2. More than 50% of the cholesterol supplied was degraded by *R. equi* ATCC 21107 (52.2%), *R. equi* ATCC 33706 (57.0%) *Leuconostoc cremoris* (53.3%) and *Serratia marcescens* ATCC 13880 (53.1%), after 7 days of incubation at 30°C. Of all the fat isolates tested, 14 strains degraded more than 50% of the cholesterol supplied after 7 days of growth in Bennet's medium. These strains were isolates No. 3 (64.9%), 4 (50.6%), 5 (50.7%) and 6 (59.9%) from chicken fat, isolates No. 2 (52.7%), 4 (70.8%), 6 (53.4%), 7 (53.4%), 10 (56.3%), from beef fat, isolate No. 4 from processed tallow (73.6%), isolates No. 1 (52.2%), 2 (63.6%) and 5 (65.6%) from lamb fat and isolates No. 2 from butter (56.3%) (Table 3.). These results are in agreement with those of previously reported studies (Arima, et al., 1969; Watanabe et al., 1986; Johnson and Somkuti, 1990). Watanabe et al. (1986) isolated approximately fifty strains of bacteria that utilized cholesterol as the sole carbon and energy source from butter, bacon, pork fat, and chicken fat. Among them sixteen strains degraded over 70% of the cholesterol within three days. Most of

them were identified as *Rhodococcus erythropolis* and *R. equi*. Similar results were reported by Johnson and Somkuti (1990) who found that 10 of 27 authentic strains of *Rhodococci*, *Brevibacteria*, *Microbacteria*, and *Nocardia* degraded 80% of cholesterol supplied after 7 days of growth in Bennet's medium. However, only four of these organisms degraded 30% or more of the cholesterol supplied after 3 days of growth. They belonged to ***R. equi***. In a systematic study of the cholesterol-degrading microorganisms, Arima et al. (1969) observed that 101 of 276 bacterial strains and 123 of 132 actinomycete strains were able to oxidize more than 20% of the cholesterol (0.1%) supplied in 7 days. In this present study cholesterol content of control samples decreased 17.5 - 31.8% in 7 days of incubation. It has been shown that cholesterol can slowly autoxidize in the presence of air, heat light and radiation (Hubbard et al., 1989). Type of oxidation depends upon the physical state of cholesterol. In aqueous solution, the 5,6 double bond in the A and B rings is the most reactive site (Maerker, 1987; Hubbard et al., 1989).

Table 1. **Cholesterol reduction (μg cholesterol/mL medium) by several strains of microorganisms in Bennet's medium incubated at 30°C for 7 days under aerobic conditions[1]**

Microorganisms	Incubation at 30°C for 7 days		Reduction
	Before	After	(%)
Control (no microorganisms)	294 ± 46[2]	242 ± 49**	17.5
Lactococcus ssp. *lactis*	300 ± 16	291 ± 31	3.1
Streptococcus thermophillus	310 ± 26	221 ± 60**	28.6*
Lactococcus lactis ssp. *lactis* var. *diacetylactis*	298 ± 20	211 ± 67**	29.4*
Lactococcus lactis ssp. *cremoris*	289 ± 49	204 ± 53**	29.5*
Rhodococcus equi ATCC 21107	280 ± 8	134 ± 22**	52.2*
Rhodococcus equi ATCC 33706	280 ± 15	120 ± 8 **	57.0*
Leuconostoc cremoris	273 ± 48	127 ± 18**	53.3*
Lactobacillus lactis	263 ± 41	191 ± 51**	27.3*
Lactobacillus bulgaricus	238 ± 28	200 ± 51	15.7
Serratia marscescens ATCC 13880	273 ± 9	128 ± 45**	53.1*

[1] The medium was supplemented with water soluble cholesterol (0.1% w/v).
2 Mean ± standard deviation (μg cholesterol/mL medium).
* Significantly (P < 0.05) higher than the control.
** Significantly (P < 0.05) lower than its initial level before incubation.

Table 2. **Cholesterol reduction (μg cholesterol/mL medium) by several fat isolates incubated in Bennet's medium at 30°C for 7 days under aerobic conditions[1]**

Fat Isolates	Incubation at 30°C for 7 days		Reduction
	Before	After	(%)
Control (no isolates)	133 ± 10[2]	91 ± 6**	31.8
Shortening 1	151 ± 9	95 ± 23**	37.5
Chicken fat 1	155 ± 11	92 ± 2**	40.5*
Chicken fat 2	149 ± 15	77 ± 4**	48.4*
Chicken fat 3	153 ± 3	54 ± 6**	64.9*
Chicken fat 4	152 ± 4	75 ± 3**	50.6*
Chicken fat 5	168 ± 18	83 ± 4**	50.7*
Chicken fat 6	135 ± 8	54 ± 4**	59.9*
Beef fat 1	161 ± 13	95 ± 27**	40.5*
Beef fat 2	151 ± 12	71 ± 18**	52.7*
Beef fat 3	154 ± 2	79 ± 4**	48.7*
Beef fat 4	157 ± 9	46 ± 9**	70.8*
Beef fat 5	144 ± 11	83 ± 22**	42.7*
Beef fat 6	137 ± 5	64 ± 6**	53.4*
Beef fat 7	136 ± 4	63 ± 3**	53.4**
Beef fat 8	116 ± 3	80 ± 15**	30.6
Beef fat 9	114 ± 15	64 ± 22**	43.4*
Beef fat 10	109 ± 14	48 ± 7**	56.3**
Beef fat 11	95 ± 3	49 ± 2**	48.2*
Beef kidney fat 1	137 ± 10	78 ± 2**	42.6*
Beef kidney fat 2	143 ± 11	85 ± 5 **	40.4*
Beef kidney fat 3	137 ± 3	76 ± 14**	44.7*
Processed tallow 1	102 ± 2	58 ± 2**	43.1*
Processed tallow 2	103 ± 7	68 ± 4**	34.6
Processed tallow 3	107 ± 9	75 ± 8**	30.1
Processed tallow 4	96 ± 2	25 ± 5**	73.6*
Processed tallow 5	109 ± 10	66 ± 22**	39.0
Lamb fat 1	122 ± 2	58 ± 6**	52.2*
Lamb fat 2	152 ± 9	55 ± 3**	63.6*
Lamb fat 3	108 ± 3	69 ± 6**	36.6
Lamb fat 4	130 ± 4	82 ± 36**	37.5
Lamb fat 5	153 ± 5	53 ± 19**	65.6*
Butter 1	131 ± 26	77 ± 14**	41.1*
Butter 2	128 ± 12	56 ± 20**	56.3*

[1] The medium was supplemented with water soluble cholesterol (0.05% w/v).
2 Mean ± standars deviation (μg cholesterol/mL medium).
* Significantly (P < 0.05) higher than the control.
** Significantly (P < 0.05) lower than its initial level before incubation.

Table 3. **Total colony counts[1] (log CFU/mL medium) of several strains of microorganisms incubated at 30°C for 7 days in Bennet's medium under aerobic conditions[2]**

Microorganisms	Days of incubation at 30°C		
	0	3	7
Control (no microorganisms)	< 1.0	<1.0	<1.0
Lactococcus ssp. *lactis*	7.6 ± 0.3[a]	8.6 ± 0.2[b]	9.0 ± 0.2[c]
Streptococcus thermophillus	7.9 ± 0.2[a]	8.7 ± 0.2[b]	8.9 ± 0.5[b]
Lactococcus lactis ssp. *lactis* var. *diacetylactis*	7.8 ± 0.1[a]	9.6 ± 1.2[b]	9.1 ± 0.5[b]
Lactococcus lactis ssp. *cremoris*	7.7 ± 0.2[a]	8.7 ± 0.2[b]	8.9 ± 0.3[b]
Rhodococcus equi ATCC 21107	6.0 ± 0.0[a]	7.1 ± 0.7[b]	8.1 ± 0.0[c]
Rhodococcus equi ATCC 33706	6.8 ± 1.1[a]	9.2 ± 0.1[b]	8.9 ± 0.4[b]
Leuconostoc cremoris	6.4 ± 0.4[a]	7.5 ± 0.5[b]	7.8 ± 0.6[b]
Lactobacillus lactis	5.6 ± 0.1[a]	7.3 ± 0.3[b]	7.4 ± 0.2[b]
Lactobacillus bulgaricus	6.5 ± 0.8[a]	7.1 ± 0.3[a]	8.3 ± 0.3[b]
Serratia marscescens ATCC 13880	8.0 ± 0.5[a]	9.1 ± 0.0[b]	8.7 ± 0.2[a]

[1] Mean ± standard deviation.

[2] Supplemented with water soluble cholesterol (0.1% w/v).

[a-c] Means with different superscripts within the same row are significantly (P<0.05) different.

Total colony counts and pH after incubation

The total bacterial colony counts of all samples inoculated with authentic strains or bacterial isolates from fats increased significantly (P<0.05) during incubation for 3 days at 30°C (Tables 3 and 4). The total colony counts of most samples were not significantly (P>0.05) different after 7 days of incubation in Bennet's medium at 30°C. The results indicated that after 3 days of incubation the bacterial cultures might reach the stationary phase. Production of cholesterol oxidase by *Rhodococcus* sp. GKI during its primary stage of the stationary phase was reported by Kreit et al. (1992). The stationary phase of *Rhodococcus* sp. GKI occurred after about 100 hr of incubation at 30°C. In this present study no colonies were detected in control samples at 10^{-1} dilution during incubation.

The pH values of the samples inoculated with authentic strains slightly decreased during incubation (Table 5). The pH values of the samples inoculated with all bacterial isolates from fats increased during incubation (Table 6). The pH values of the control samples were relatively constant (7.0 to 7.1) during incubation at 30°C for 7 days.

Table 4. **Total colony counts[1] (log CFU/mL medium) of several fat isolates incubated in Bennet's medium at 30°C for 7 days under aerobic conditions[2]**

Microorganisms	Days of incubation at 30°C		
	0	3	7
Control (no isolates)	<1.0	<1.0	<1.0
Shortening 1	3.4 ± 0.4[a]	4.7 ± 0.4[b]	6.5 ± 0.9[c]
Chicken fat 1	7.3 ± 0.0[a]	9.0 ± 0.1[b]	8.8 ± 0.1[b]
Chicken fat 2	8.4 ± 0.6[a]	9.2 ± 0.6[b]	8.7 ± 0.4[ab]
Chicken fat 3	8.1 ± 0.0[a]	8.9 ± 0.6[b]	8.6 ± 0.6[ab]
Chicken fat 4	8.0 ± 0.2[a]	9.5 ± 0.9[b]	9.3 ± 0.6[b]
Chicken fat 5	5.8 ± 0.8[a]	8.3 ± 0.6[b]	8.6 ± 1.5[b]
Chicken fat 6	8.2 ± 0.2[b]	9.1 ± 1.3[c]	7.9 ± 0.0[c]
Beef fat 1	7.5 ± 0.0[a]	9.2 ± 0.1[b]	8.9 ± 0.1[b]
Beef fat 2	8.4 ± 0.1[a]	9.8 ± 0.4[b]	9.5 ± 0.9[b]
Beef fat 3	7.8 ± 0.4[a]	9.5 ± 0.1[b]	9.3 ± 0.4[b]
Beef fat 4	8.0 ± 0.1[a]	9.3 ± 1.3[b]	9.0 ± 0.1[b]
Beef fat 5	6.4 ± 0.3[a]	9.4 ± 0.8[b]	9.3 ± 0.1[b]
Beef fat 6	7.8 ± 0.0[a]	9.6 ± 0.1[c]	9.0 ± 0.2[b]
Beef fat 7	8.5 ± 0.2[a]	9.4 ± 0.5[b]	9.0 ± 0.4[ab]
Beef fat 8	7.1 ± 0.1[a]	9.0 ± 0.2[b]	8.9 ± 0.4[b]
Beef fat 9	7.8 ± 0.3[a]	9.7 ± 0.2[c]	9.1 ± 0.2[b]
Beef fat 10	7.8 ± 0.1[a]	9.6 ± 1.4[b]	9.0 ± 1.0[b]
Beef fat 11	8.0 ± 0.5[a]	9.8 ± 0.7[b]	9.5 ± 0.5[b]
Beef kidney fat 1	8.0 ± 0.2[a]	9.6 ± 1.1[b]	9.3 ± 0.1[b]
Beef kidney fat 2	7.7 ± 0.5[a]	9.2 ± 1.1[b]	8.7 ± 0.5[ab]
Beef kidney fat 3	7.9 ± 0.3[a]	8.9 ± 0.6[b]	9.2 ± 0.2[b]
Processed tallow 1	8.3 ± 0.1[a]	9.9 ± 0.3[b]	9.4 ± 0.8[b]
Processed tallow 2	6.4 ± 0.1[a]	9.4 ± 0.4[c]	8.8 ± 0.5[b]
Processed tallow 3	8.1 ± 0.2[a]	9.7 ± 0.1[b]	9.7 ± 0.3[b]
Processed tallow 4	4.9 ± 0.0[a]	5.4 ± 0.1[b]	6.1 ± 0.3[c]
Processed tallow 5	7.8 ± 0.4[a]	9.6 ± 0.4[b]	9.4 ± 0.2[b]
Lamb fat 1	8.1 ± 0.5[a]	9.7 ± 0.3[b]	8.4 ± 0.3[b]
Lamb fat 2	7.8 ± 0.0[a]	9.4 ± 0.8[b]	8.7 ± 0.6[b]
Lamb fat 3	7.9 ± 0.2[a]	9.3 ± 1.6[b]	8.7 ± 1.4[ab]
Lamb fat 4	7.8 ± 0.3[a]	9.4 ± 0.5[b]	8.7 ± 0.5[b]
Lamb fat 5	7.9 ± 0.2[a]	9.4 ± 1.1[b]	9.4 ± 0.7[b]
Butter 1	6.5 ± 0.1[a]	7.7 ± 0.0[b]	7.7 ± 0.0[b]
Butter 2	5.2 ± 0.2[a]	7.8 ± 0.0[c]	6.6 ± 0.1[b]

[1] Mean ± standard deviation.

[2] The medium was supplemented with water soluble cholesterol (0.05 % w/v).

[a-c] Means with different superscripts within the same row are significantly (P<0.05) different.

Table 5. **pH[1] Values during incubation of several strains of microorganisms in Bennet's medium at 30°C for 7 days under aerobic conditions[2]**

Microorganisms	Days of incubation at 30°C		
	0	3	7
Control (no microorganisms)	7.0 ± 0.1[a]	7.0 ± 0.1[a]	7.0 ± 0.2[a]
Lactococcus ssp. *lactis*	7.4 ± 0.3[a]	6.2 ± 0.3[b]	6.1 ± 0.4[b]
Streptococcus thermophillus	7.4 ± 0.3[a]	6.0 ± 0.3[b]	5.6 ± 0.3[c]
Lactococcus lactis ssp. *lactis* var. *diacetylactis*	7.4 ± 0.3[a]	5.8 ± 0.2[b]	5.6 ± 0.4[b]
Lactococcus lactis ssp. *cremoris*	7.5 ± 0.3[a]	6.0 ± 0.4[b]	5.8 ± 0.2[b]
Rhodococcus equi ATCC 21107	6.9 ± 0.2[a]	6.2 ± 0.1[b]	6.3 ± 0.1[b]
Rhodococcus equi ATCC 33706	6.9 ± 0.2[a]	6.2 ± 0.2[b]	6.4 ± 0.2[b]
Leuconostoc cremoris	7.7 ± 0.5[a]	6.0 ± 0.1[b]	5.8 ± 0.1[b]
Lactobacillus lactis	7.7 ± 0.4[a]	5.7 ± 0.2[b]	5.8 ± 0.1[b]
Lactobacillus bulgaricus	7.8 ± 0.5[a]	5.9 ± 0.5[b]	5.9 ± 0.2[b]
Serratia marscescens ATCC 13880	7.4 ± 0.2[a]	5.7 ± 0.2[c]	6.0 ± 0.0[b]

[1] Mean ± standard deviation.
[2] The mediun was supplemented with water soluble cholesterol (0.1% w/v).
[a-c] Means with different superscripts within the same row are significantly ($P<0.05$) different.

Table 6. **pH[1] changes during incubatiön of several fat isolates in Bennet's medium at 30°C for 7 days under aerobic conditions[2]**

Microorganisms	Days of incubation at 30°C		
	0	3	7
Control (no isolates)	7.1 ± 0.1[a]	7.0 ± 0.3[a]	7.1 ± 0.0[a]
Shortening 1	7.1 ± 0.2[a]	7.1 ± 0.3[a]	7.3 ± 0.4[a]
Chicken fat 1	6.4 ± 0.1[a]	8.2 ± 0.3[b]	8.5 ± 0.1[b]
Chicken fat 2	7.1 ± 0.1[a]	8.2 ± 0.3[b]	8.7 ± 0.2[c]
Chicken fat 3	7.0 ± 0.1[a]	7.9 ± 0.3[b]	7.7 ± 0.2[c]
Chicken fat 4	7.0 ± 0.1[a]	7.9 ± 0.5[b]	8.1 ± 0.6[b]
Chicken fat 5	7.1 ± 0.0[a]	7.9 ± 0.4[b]	8.4 ± 0.1[c]
Chicken fat 6	7.0 ± 0.0[a]	7.7 ± 0.1[b]	8.2 ± 0.3[c]
Beef fat 1	6.5 ± 0.0[a]	7.8 ± 0.3[b]	8.1 ± 0.2[b]
Beef fat 2	6.5 ± 0.1[a]	7.9 ± 1.1[b]	8.4 ± 0.8[b]
Beef fat 3	7.1 ± 0.1[a]	8.1 ± 0.3[b]	8.5 ± 0.2[b]
Beef fat 4	6.4 ± 0.1[a]	7.7 ± 0.5[b]	8.3 ± 0.5[b]
Beef fat 5	7.1 ± 0.0[a]	7.5 ± 0.2[b]	8.1 ± 0.7[c]
Beef fat 6	7.1 ± 0.1[a]	7.9 ± 0.1[b]	8.5 ± 0.1[c]
Beef fat 7	7.1 ± 0.1[a]	8.0 ± 0.7[b]	8.7 ± 0.4[c]
Beef fat 8	7.1 ± 0.0[a]	8.2 ± 0.1[b]	8.6 ± 0.2[c]
Beef fat 9	7.0 ± 0.1[a]	8.0 ± 0.2[b]	8.5 ± 0.2[c]
Beef fat 10	7.0 ± 0.3[a]	8.0 ± 0.3[b]	8.6 ± 0.1[c]
Beef fat 11	7.0 ± 0.2[a]	7.9 ± 0.4[b]	8.5 ± 0.5[c]
Beef kidney fat 1	6.9 ± 0.0[a]	8.1 ± 0.1[b]	8.6 ± 0.1[c]
Beef kidney fat 2	7.0 ± 0.1[a]	8.1 ± 0.2[b]	8.7 ± 0.4[c]
Beef kidney fat 3	7.1 ± 0.1[a]	8.0 ± 0.1[b]	8.4 ± 0.1[c]
Processed tallow 1	8.3 ± 0.1[a]	9.9 ± 0.3[b]	9.4 ± 0.8[b]
Processed tallow 2	6.4 ± 0.1[a]	9.4 ± 0.4[c]	8.8 ± 0.5[b]
Processed tallow 3	8.1 ± 0.2[a]	9.7 ± 0.1[b]	9.7 ± 0.3[b]
Processed tallow 4	4.9 ± 0.0[a]	5.4 ± 0.1[b]	6.1 ± 0.3[c]
Processed tallow 5	7.8 ± 0.4[a]	9.6 ± 0.4[b]	9.4 ± 0.2[b]
Lamb fat 1	7.1 ± 0.0[a]	7.9 ± 0.3[b]	8.5 ± 0.3[c]
Lamb fat 2	6.9 ± 0.1[a]	8.1 ± 0.1[b]	8.6 ± 0.1[c]
Lamb fat 3	7.1 ± 0.4[a]	7.7 ± 0.2[b]	8.3 ± 0.4[c]
Lamb fat 4	7.0 ± 0.0[a]	7.7 ± 0.1[b]	8.4 ± 0.1[c]
Lamb fat 5	6.9 ± 0.0[a]	7.9 ± 0.5[b]	8.6 ± 0.1[c]
Butter 1	6.8 ± 0.7[a]	7.8 ± 0.2[b]	8.1 ± 0.5[b]
Butter 2	6.8 ± 0.3[a]	7.9 ± 0.3[b]	7.0 ± 0.1[a]

[1] Mean ± standard deviation.
[2] The medium was supplemented with water soluble cholesterol (0.05 % w/v).
[a-c] Means with different superscripts within the same row are significantly ($P<0.05$) different.

Effect of medium pH on cholesterol reduction

The effect of pH (6.0 - 8.0) on the cholesterol degrading activity of some active microorganisms was studied in Bennet's medium incubated with *R. equi* ATCC 21107, *R. equi* 33706. *L. cremoris*, *L. lactis*, *S. marcescens* ATCC 13880, and bacterial isolate No. 1 from chicken, isolate No. 2 from butter, and isolate No.1 from beef fat at 30°C for 2 and 4 days. The greatest cholesterol reduction was observed in the medium with initial pH values of 6.0, after 2 days of incubation (Fig. 1). As the incubation continued, the extent of the degradation changed depending on the type of microorganism. At the initial pH of 7.0 and 8.0 degradation was greater after 4 days of incubation (Fig. 2). The results indicated that under aerobic conditions the microorganisms were capable of degrading cholesterol at pH values of 6.0 - 8.0 within 4 days of incubation. All of the isolates from fats tested had better degrading ability at initial pH values of 6.0. Johnson and Somkuti (1990) reported an optimum pH of 8.0 on cholesterol dissimilation by *Rhodococcus* sonicated cell extracts. Further study, however, indicated that the optimum pH for the secreted enzymes was much broader (6.0 - 8.0) (Johnson and Somkuti, 1991). They reported that the secreted cholesterol oxidases showed a broader range (pH 4-10) of activity than the cellbound enzyme (pH 5 – 10).

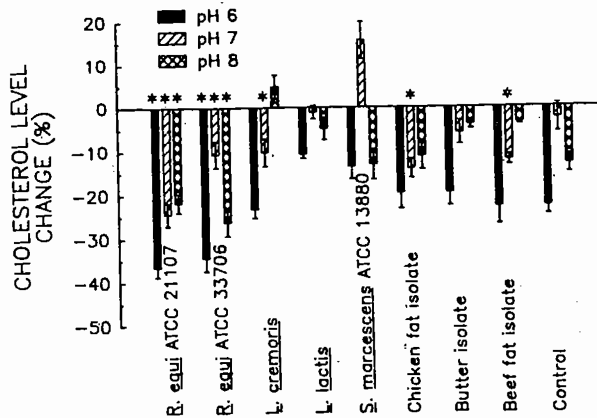


Figure 1. **Effect of intial pH of substrate on cholesterol reduction in Bennet's medium incubated with selected microorganisms at 37°C for 2 days under aerobic conditions. (*) Significantly ($P<0.05$) lower than the corresponding control.**
Initial cholesterol level : 111 - 159 μg/mL.

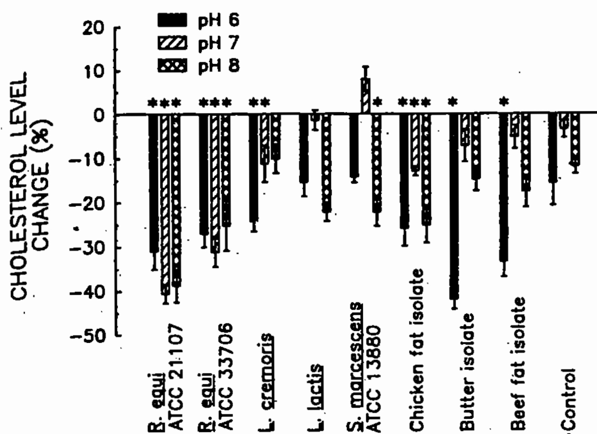


Figure 2. **Effect of initial pH of substrate on cholesterol reduction in Bennet's medium incubated with selected microorganisms at 37°C for 4 days under aerobic conditions. (*) Significantly (P<0.05) lower than the corresponding control. Initial cholesterol level : 111 - 159 µg/mL.**

Degradation of cholesterol in beef tallow or ground beef containing phosphate buffer

In order to study the effect of cholesterol source on substrate degradation, phosphate buffer (pH 7.2) samples containing tallow or ground beef, which was homogenized by sonication, were used to grow test cultures. The experiment studying degradation of cholesterol in tallow and ground beef by the most active microorganisms indicated that *R. equi* ATCC 21107 and *R. equi* ATCC 33706 caused net reductions of 39.1% and 40.1%, respectively, in beef tallow and 58.6% and 38.8% in ground beef (Fig. 3). The percent net reduction was determined by subtracting the percentage cholesterol reduction of the inoculated samples from that in control samples. After 2 days of incubation, the total bacterial counts of all inoculated ground beef and control samples increased (Fig. 4). With the exception of *R. equi* ATCC 21107, the total bacterial counts of all inoculated and control samples decreased after 2 days of incubation of substrates with added tallow. The cholesterol reduction in control samples may have been caused by autooxidation of the cholesterol resulting from sonication. Smith et al. (1991) resported that sonication treatmen of unpasteurized raw milk had market effect on cholesterol oxidation by *P. fluorescens* cholesterol oxidase. Approximately 84% of the initial cholesterol was oxidized in 3 hr at 37°C. The enzyme oxidized no more than 17% of the cholesterol in raw milk without sonication in 3 hr at 37°C. Degradation of cholesterol by the extracellular and cell-bound enzymes from *R. equi* No. 23 in phosphate buffer containing lard was reported by Watanabe et al. (1989). They found that more than 90% of the cholesterol was degraded after incubation

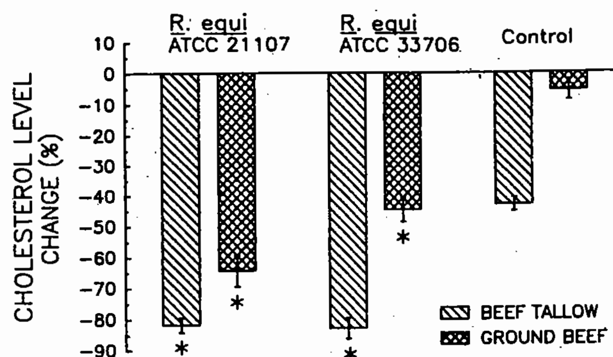


Figure 3. **Cholesterol reduction in phosphate buffer containing ground beef and tallow incubated with *R. equi* ATCC 21107 and ATCC 33706 for 2 days at 30°C under aerobic conditions.**
(*) Significantly (P<0.05) lower than the corresponding control. Initial cholesterol level: 32.5-66.3 µg/mL.

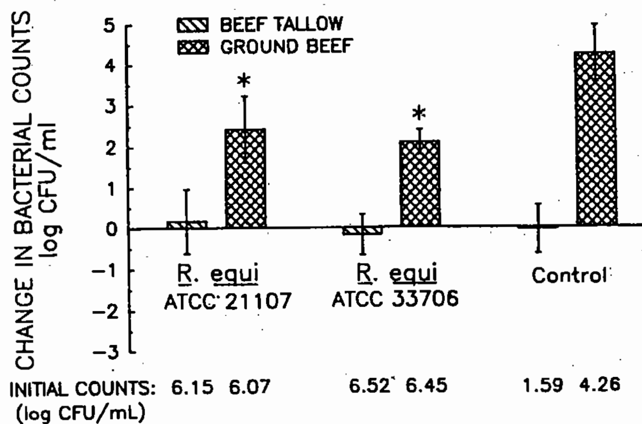


Figure 4. **Change in bacterial counts of *R. equi* ATCC 21107 and ATCC 33706 in phosphate buffer containing tallow and ground beef after incubation at 30°C for 2 days under aerobic conditions (*) Significantly (P<0.05) lower than the corresponding control.**

for 48 hr. Cholesterol degradation in egg yolk by *R. equi* No. 23 was studied by Aihara et al. (1988.a,b). The organism was grown in a culture mediun (10 mL/tube) containing egg yolk at levels of 0.2, 0.4, 0.5, 0.7 and 1.0 g per tube with continuous shaking at 37°C. The corresponding cholesterol levels were 3, 6, 9, 12 and 17 mg, respectively. The cholesterol content decreased with increasing incubation time (whiting 3 days). However, at higher levels (0.7 and 1.0 g egg yolk per tube), more residual cholesterol was observed, compared to lower (0.2; 0.4; and 0.5 g egg yolk per tube) after incubation for 3 days at 37°C.

Identification of cholesterol-degrading bacteria

Among the isolates, which had the ability to degrade over 50% of cholesterol supplied, three of them were chosen for identification along with authentic strains of *R. equi* ATCC 211107 and *R. equi* ATCC 33706. The three most active cultures were bacterial isolate No. 4 from processed tallow, bacterial isolate No. 4 from beef fat and bacterial isolate No. 2 from butter. They degraded 73.6%, 70.8%, and 56.4% of the cholesterol supplied in Bennet's medium during 7 days of incubation at 30°C, respectively. The morphological characteristics of the cultures were observed on cells grown on TSA for 18-24 hr under phase contrast microscopy (Olympus Biological Microscope, Model BHTU, Olympus Optical Co. Ltd., Tokyo, Japan). The results of the biochemical tests and morphological characteristics are shown in Table 7. All the fat isolates tested were rods, Gram negative, aerobic, motile, acid fast negative, catalase, oxidase and α-hemolysis positive, nitrate reduction positive, gelatinase negative, and proteinase negative. The strains except beef fat isolate No. 4 did not utilize glucose, lactose, sucrose, mannose, maltose, galactose, fructose, sorbitol, arabinose, xylose, inositol, glycerol, trehalose and starch. Butter isolate No. 2 and processed tallow isolate No. 4 were negative for IMViC and litmus milk reactions. Hydrogen sulfide was not produced by both strains. Lipid was hydrolyzed by both strains.

Bacterial isolate No. 4 from beef fat differed from the other strains in its ability to utilize starch, glucose, lactose, sucrose, mannose, maltose, galactose, fructose, sorbitol, arabinose, xylose, inositol, glycerol, and trehalose. Gas was produced from glucose, sucrose, mannose, maltose, galactose, fructose, sorbitol, and arabinose. The isolate was positive for litmus milk, Voges-Proskauer, and citrate tests, but negative for indole and methyl red tests. The strain produced hydrogen sulfide, but did not produce lipase. All of the isolates grew well under aerobic and candle jar incubations, but only slightly in anaerobic conditions (Gas Pak Jar, Difco Laboratories, Detroit, MI). This indicated that the organisms were aerobic to facultatively anaerobic. The strains were tentatively classified as belonging to the genus *Pseudomonas* on the basis of the following phenotypic characteristics. All strains were Gram negative rods, aerobic, catalase positive, oxidase positive, nitrate reduction positive, and motile (Mac Faddin, 1976). Some species of the genus *Pseudomonas* which are capable of reducing nitrate can grow anaerobically (Mac Faddin, 1976). Yellow and orange pigmentation were observed in these culture.

Many microorganisms, including representatives of *Pseudomonas* have been reported as capable of degrading cholesterol (Owen et al,. 1983; Lee et al., 1989; Rhee et al., 1991). Lee et al., (1989) isolated *Pseudomonas* sp. from soil that exhibited markedly high activity of cholesterol oxidase in culture broth. The degradation of cholesterol by *Pseudomonas* sp. NCIB 10590 under aerobic conditions was studied by Owen et al. (1983). This organism was highly efficient in the degradation of bile acids and grew well on cholesterol as the sole carbon source. Smith et al. (1991) reported that cholesterol oxidase from *Pseudomonas fluorescens* oxidized 78% of cholesterol in homogenized milk, compared with 20% for the *Rhodococcus* enzymes and 16% for the *Streptomyces* enzyme.

The morphological and biochemical characteristics of the authentic strains of *R. equi* ATCC 21107 and *R. equi* ATCC 33706 indicated that they were Gram positive, pleomorphic rods which differentiated into coccal forms, acid fast stain positive, catalase positive, β-hemolysis positive, and facultative aerobes. The results are in agreement with findings by Ferreira and Tracey (1984) and Watanabe et al. (1986). They reported the following morphological and biochemical characteristics for *Rhodococcus equi*: Gram positive, pleomorphic rod-shaped, non-motile, produced catalase and β-hemolysis, contained meso-diaminopimelic acid, arabinose, and galactose in the cell wall. Casein, adenine, starch and gelatin were not utilized by the organism.

Table 7. **Biochemical and morphological characteristics of the isolates and some authentic strains of *Rhodococcus equi*.**

Types of test	**Beef fat isolate No. 4**	**Butter isolate No. 2**	**Processed tallow isolate No. 4**	**R. equi ATCC 21107**	**R. equi ATCC 33706**
Gram stain	–	–	–	+	+
Shape	Short rod	rod	short rod	pleomorphic	pleomorphic
Acid fast	–	–	–	+	+
Motility	+	+	+	–	–
Catalase production	+	+	+	+	+
Urea hydrolysis	–	–	–	–	–
Starch hydrolysis	+	–	–	–	–
Lipid hydrolysis	–	[illegible]	+	–	–
Case in hydrolysis	–	–	–	–	–
Hemolysis production	+ (α)	+ (α)	+ (α)	+ (β)	+ (β)
Gelatin hydrolysis	–	–	–	–	–
Glycerol	+	–	–	–	–
Litmus milk reactions	+	–	–	–	–
Nitrate reduction	+	+	+	–	–
Oxidase production	+	+	+	+	–
Oxygen requirement	aerob	aerob	aerob	facultative aerob	facultative aerob
Hydrogen sulfide production	+	–	–	–	–
SUGAR FERMENTATION :					
Glucose	+ A/G	–	–	–	–
Lactose	+ A	–	–	–	–
Sucrose	+ A/G	–	–	–	–
Mannose	+ A/G	–	–	–	–
Maltose	+ A/G	–	–	–	–
Galactose	+ A/G	–	–	–	–
Fructose	+ A/G	–	–	–	–
Sorbitol	+ A/G	–	–	–	–
Arabinose	+ A/G	–	–	–	–
Xylose	+ A	–	–	–	–
Rhamnose	–	–	–	–	–
Inositol	+	–	–	–	–
Trehalose	+ A	–	–	–	–
IMViC reactions :					
Indole test	–	–	–	–	–
Methyl red test	–	–	–	–	–
Voges-Proskauer test	+	–	–	–	–
Citrate utilization	+	–	–	–	–

A : Acid production.
A/G : Acid and gas production.

CONCLUSIONS

The results of the study indicated that *R. equi* ATCC 21107, *R. equi* ATCC 33706, *Leuconostoc cremoris*, *Serratia marcescens* ATCC 13880 and several bacterial isolates from fats were capable of degrading cholesterol in a model system supplemented with pure soluble cholesterol or in phosphate buffer supplemented with beef fat and ground beef. The initial pH values of the medium affected the extent of cholesterol degradation. Under aerobic conditions the pH values of 6.0 resulted in greater degradation. Morphological and biochemical characteristics revealed that some of the bacterial isolates (isolate No. 4 from beef fat, isolate No. 2 from butter, and isolate No. 4 from processed tallow) tentatively classified as belonged to the genus *Pseudomonas*. Further study is needed to screen additional microorganisms, to isolate enzymes for increased activity, to optimize condition for cholesterol degradation, and to determine the degradation products.

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