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Amylomyces rouxii Strain CBS 438.76 Affects Cholesterol Metabolism in Cholesterol-Fed Rats

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Summary We examined the effects of *Amylomyces rouxii*, which is a mold found in some fermented foods in Indonesia, on serum cholesterol and hepatic LDL receptor mRNA in rats. Rats were fed a 0.5% cholesterol-enriched diet with (*A. rouxii* group) or without (control group) 30 g/kg *A. rouxii* for 4 wk. There were no significant differences in the body weight, food intake or liver weight among the groups. However, the weight of the cecum in the *A. rouxii*-fed group was significantly higher than that in the control group. The cecal pH in the *A. rouxii*-fed group was significantly lower than that in the control group. Cecal acetic acid, propionic acid and total SCFA concentrations in the *A. rouxii*-fed group were significantly higher than those in the control group. The serum total cholesterol and VLDL+intermediate density lipoprotein (IDL)+LDL-cholesterol concentrations in the control group were significantly higher than those in the *A. rouxii*-fed group at the end of the 4-wk feeding period. There were no significant differences in the HDL-cholesterol or triglyceride concentrations between the groups. The hepatic LDL receptor and cholesterol 7α-hydroxylase mRNA levels in the *A. rouxii*-fed group were significantly higher than those in the control group. The results of this study demonstrate that feeding of *A. rouxii* lowers the serum total cholesterol level by enhancement of the cecal SCFA concentration and the hepatic LDL receptor mRNA.

Key Words: rats, *Amylomyces rouxii* strain CBS 438.76, cholesterol, cholesterol 7α-hydroxylase mRNA, LDL receptor mRNA

It has been reported that numerous fermented milks and yoghurts acting as probiotics or prebiotics have hypocholesterolemic functions in human subjects and in experimental animals (*1–3*). The cholesterol-lowering mechanism depends on the binding of cholesterol to *Lactobacillus acidophilus* (*4*). We also reported previously that a probiotic mixture comprised of *Bacillus*, *Lactobacillus*, *Streptococcus*, *Saccharomyces* and *Candida* improved the intestinal flora balance and lowered HMG-CoA reductase activity in rats (*5*, *6*).

Amylomyces is a monotypic genus containing the single variable species *A. rouxii*, which is closely related to *Rhizopus oryzae*, identifiable from the formation of rhizoids, stolons, and black-pigmented sporangia (*7*). All fermented foods made with *A. rouxii* in Indonesia are slightly alcoholic paste-like products (*8*). *A. rouxii* is a major component of starter cultures for traditional fermented foods, such as tempeh, in Southeast Asia, China, and the Indian subcontinent (*9*) and is not present in the intestine generally. Molds are often mixed with other types of microorganisms, such as specific bacteria or yeasts in order to produce a product with certain characteristics. Linoleic and α-linolenic acid concentrations increase in the fermented foods and vitamin B_{12} in fermented foods such as soybean tempeh increases 26 times as compared with soybeans (*10*). Saito et al. (*11*) have reported that lactic acid fermentation of potato pulp is increased by *A. rouxii* and *R. oryzae*.

SCFA exert a number of general actions on the large bowel, which include lowering of colonic pH and increased electrolyte and fluid absorption, which assists in the prevention of diarrhea (*12*). Individual acids appear to promote colonic muscular activity in a dose-dependent manner (*13*) and large bowel blood flow through relaxation of the vasculature (*14*). Butyrate may be the most important for the maintenance of colonic health. The acid is a major metabolic fuel for normal colonocytes (*15*). Its infusion to the colon may relieve ulcerative colitis (*16*) and its presence can assist in the maintenance of a colonic cell phenotype through

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Abbreviations: GLC, gas-liquid chromatography; IDL, intermediate density lipoprotein; SCFA, short chain fatty acid; TG, triglyceride.

a number of mechanisms (*17*). Propionate may also be of metabolic importance in the colon because it exerts some of the antineoplastic effects of butyrate although at much higher levels (*18*). Elevated concentrations of propionate in the large bowel may also suppress cholesterol synthesis in that viscus (*19*). In this study, we examined the effects of diets containing *A. rouxii* strain CBS 438.76 on cecal SCFA, serum lipids, liver lipids and hepatic mRNAs.

MATERIALS AND METHODS

Animal and diets. Male F344/DuCrj rats (8 wk old) were purchased from Charles River Japan, Inc. (Yokohama, Japan). All rats were housed individually in cages in a facility with a 12 h light-dark cycle. Temperature and humidity were controlled at 23±1°C and 60±5%, respectively. The rats were divided randomly into two groups of five. There were no significant differences in body weight or serum total cholesterol concentrations between the groups at the start of the experimental period. The composition of each 0.5% cholesterol-enriched diet is shown in Table 1. *A. rouxii* strain CBS 438.76 was grown in potato dextrose broth (Difco) at 25°C for 5 d. The mycelia were recovered by filtration and lyophilized. The experimental group was fed for 4 wk the diet that contained 30 g/kg of *A. rouxii* strain CBS 438.76 cell body, while the control group was fed the diet containing 5% cellulose instead of 30 g/kg of *A. rouxii*. The rats were allowed free access to experimental diets and water for 4 wk. Body weight and feed consumption were recorded weekly and every day, respectively. This experimental design was approved by the Animal Experiment Committee of Obihiro University of Agriculture and Veterinary Medicine and all animal procedures described conformed to standard principles in *Guide for the Care and Use of Laboratory Animals* (*20*).

Analytical procedures. Blood samples (1 mL) were collected between 0800 and 1000 h from the jugular veins of food-deprived rats overnight. The samples were taken into tubes without an anticoagulant. After the samples stood at room temperature for 2 h, serum was prepared by centrifugation at 1,500×*g* for 20 min. At the end of the experimental period of 4 wk, all fecal excretions during 2 d were collected. Fecal dry weights did not differ between the groups. The rats were killed by ether inhalation, and their livers were quickly removed, washed with cold saline (9 g NaCl/L), blotted dry on filter paper, and weighed before frozen storage.

Chemical analysis. Total cholesterol, HDL-cholesterol, and triglyceride (TG) concentrations in the serum were determined enzymatically using commercially available reagent kits (assay kits for the TDX system; Abbott Laboratory Co., Irving, TX). The cholesterol content of VLDL+intermediate density lipoprotein (IDL)+LDL was calculated from the difference between total cholesterol and HDL-cholesterol.

Total lipids were extracted from liver and feces by a mixture of chloroform/methanol (2 : 1, v/v) (*21*). The neutral steroid in each total lipid obtained by saponification was acetylated (*22*) and analyzed by gas-liquid chromatography (GLC) using a Shimadzu 14A chromatograph (Kyoto, Japan) with a DB17 capillary column (0.25 mm×30 m; J&W Scientific, Folsom, CA) with nitrogen as the carrier gas. Acidic steroids in feces were measured by GLC following the method of Grundy et al. (*23*). A part of the cecum was taken out into desalting water in a vial without exposure to air, and suspended. The suspension of cecum was deproteinized by perchloric acid (final concentration 50 g/L) cooled in ice, and the supernatant was added to a NaOH solution to precipitate perchloric acid and to form potassium salts of the SCFA. Individual SCFA was measured by GLC with a glass column (2,000 mm×3 mm) packed with 80–100 mesh chromosorb W-AW DMCS with H_3PO_4 (100 mL/L) as the liquid phase after adding H_3PO_4 by the procedure of Hara et al. (*24*).

Table 1. Composition of experimental diets.

Component	Dietary group[1]	
	CD	AD
Casein	25	25
Corn oil	5	5
Mineral mixture[2]	3.5	3.5
Vitamin mixture[3]	1	1
Cellulose powder	5	5
α-Cornstarch	15	15
Amylomyces rouxii	—	3
Cholesterol	0.5	0.5
Choline chloride	0.2	0.2
Sodium cholate	0.125	0.125
Sucrose to	100	100

[1] CD, cholesterol-enriched diet; AD, cholesterol-enriched diet+*Amylomyces rouxii*.
[2] AIN-76 mineral mixture (*40*).
[3] AIN-76 vitamin mixture (*40*).

RNA isolation, RT-PCR and Southern blot analysis. Total RNA was isolated from liver tissue by the acid guanidium-phenol-choloroform method, using Isogen (Nippon Gene, Tokyo, Japan) (*25*). mRNAs encoding the LDL receptor, cholesterol 7α-hydroxylase, and glyceraldehyde-3-phosphate dehydrogenase (GAPDH, used as an invariant control) were analyzed by semiquantitative RT-PCR and subsequent Southern hybridization of the PCR products with each inner oligonucleotide probe. Total RNA samples were treated with DNase RQ1 (Promega, Madison, WI) to remove genomic DNA and subjected to RT-PCR by using Moloney murine leukemia virus reverse transcriptase (GIBCO, Gaithersburg, MD) and EX-Taq polymerase (Takara, Tokyo, Japan) with LDL receptor primers of oligonucleotides (upstream primer, 5′-ATTTTGGAGGATGAGAAGCAG-3′; downstream primer, 5′-CAGGGCGGGGAGGTGT-GAGAA-3′), cholesterol 7α-hydroxylase of oligonucleotides (upstream primer, 5′-GCCGTCCAAGAAATCAAG-CAGT-3′; downstream primer, 5′-TGTGGGCAGCGAG-AACAAAGT-3′), and GAPDH primers of oligonucle-

otides (upstream primer, 5′-GCCATCAACGACCCCTTCATT-3′; downstream primer, 5′-CGCCTGCTTCACCACCTTCTT-3′) (*26*). The reaction mixtures for the PCRs contained 25 pmol of each primer, 1.25 U EX-Taq polymerase, 1×PCR buffer (Takara), and 200 μmol/L dNTP in a 50 μL reaction volume. The expected sizes of DNA fragments amplified with these primers were 931 bp for the LDL receptor, 306 bp for cholesterol 7α-hydroxylase, and 702 bp for GAPDH. Temperature cycling was as follows: first cycle, denaturation at 94°C for 3 min, annealing at 60°C for 1 min, and extension at 72°C for 2 min. Subsequent cycles were denaturation at 94°C for 1 min, annealing at 60°C for 1 min, and extension at 72°C for 2 min. The thermal cycling was completed by terminal extension at 72°C for 10 min. In total, 25 cycles were performed for the LDL receptor amplifications, 30 cycles for cholesterol 7α-hydroxylase, and 20 cycles for GAPDH. Amplification products were electrophoresed on 2% agarose gel, and transferred to a nylon membrane (Biodyne B, Pall Bio-Support, East Hills, NY). Blots were hybridized with an LDL receptor probe of a 54-base oligonucleotide (5′-GTGAACTTGGGTGAGTGGGCACTGATCTGAGGGGCAGGCAGGCACATGTACTGG-3′), cholesterol 7α-hydroxylase probe of a 54-base oligonucleotide (5′-CCCGAAGGCCTGTTTAAGTGATGACTCTCAGCCGCCAAGTGACATCATCCAGTG-3′), and GAPDH probe of a 54-base oligonucleotide (5′-TGATGACCAGCTTCCCATTCTCAGCCTTGACTGTGCCGTTGAACTTGCCGTGGG-3′). The probe was 3′-tailing labeled with digoxigenin, using a DIG oligonucleotide tailing kit (Boehringer Mannheim, Germany). Prehybridization, hybridization and detection were carried out with a DIG luminescent detection kit (Boehringer Mannheim) as recommended by the manufacturer. The relative quantity of mRNA was estimated by densitometry scanning with X-ray film.

Statistical analysis. Data are presented as means±SD. The mean and SD for serum total cholesterol, HDL-cholesterol, VLDL+IDL+LDL-cholesterol and triglyceride for each time point were calculated. Student's *t* test was used to evaluate the difference between the control group and the *A. rouxii* group.

RESULTS

Feed intake, rat growth, liver weight, cecum weight and cecal pH

There were no significant differences in the body weight, feed intake, liver weight or cecum weight between the groups (Table 2). The cecal pH in the control group was significantly higher than that in the *A. rouxii*-fed group (Table 2).

Tissue lipid concentration

The serum total cholesterol and VLDL+IDL+LDL-cholesterol concentrations in the control group were significantly higher than those in the *A. rouxii*-fed group at the end of the 4-wk feeding period (Table 3). There were no significant differences in the HDL-cholesterol or triglyceride concentrations between the groups at the end of the 4-wk feeding period (Table 3). There was no significant difference in the liver cholesterol concentration (control group and *A. rouxii*-fed group:

Table 2. Body weight, food intake, relative liver and cecum weights and cecal pH in rats fed control diet (CD) and *Amylomyces rouxii* diet (AD) for 4 wk[1].

Component	Dietary group	
	CD	AD
Initial body weight (g)	183±5	181±5
Body weight gain (g/4 wk)	60±5	52±9
Food intake (g/4 wk)	416±24	400±29
Liver weight (g/100 g body)	4.6±0.2	4.8±0.2
Cecum weight (g/100 g body)	1.5±0.1	1.8±0.2**
Cecal pH	7.00±0.16	6.67±0.16*

[1] Values are expressed as mean±SD, $n=5$. Mean values are significantly different from those of the control diet: *$p<0.05$, **$p<0.01$.

Table 3. Serum total cholesterol, VLDL+IDL+LDL-cholesterol, HDL-cholesterol and triglyceride concentrations in rats fed control diet (CD) and *Amylomyces rouxii* diet (AD) for 4 wk.[1]

Dietary group	Week 0	1	2	4
		(mmol/L)		
Total cholesterol				
CD	1.66±0.09	3.99±0.48	3.80±0.57	4.91±0.41
AD	1.76±0.11	2.66±0.15**	2.99±0.44*	3.78±0.78*
VLDL+IDL+LDL-cholesterol				
CD	0.59±0.06	2.96±0.39	3.03±0.50	4.16±0.45
AD	0.66±0.05	1.79±0.16**	2.30±0.41*	3.12±0.68*
HDL-cholesterol				
CD	1.07±0.04	1.03±0.10	0.78±0.08	0.74±0.08
AD	1.10±0.07	0.87±0.05*	0.69±0.05	0.66±0.13
Triglyceride				
CD	0.56±0.08	1.34±0.26	1.34±0.23	0.71±0.26
AD	0.63±0.13	1.09±0.19	1.15±0.12	0.62±0.16

[1] Values are expressed as means±SD, $n=5$. Mean values are significantly different from those of the control diet: *$p<0.05$, **$p<0.01$.

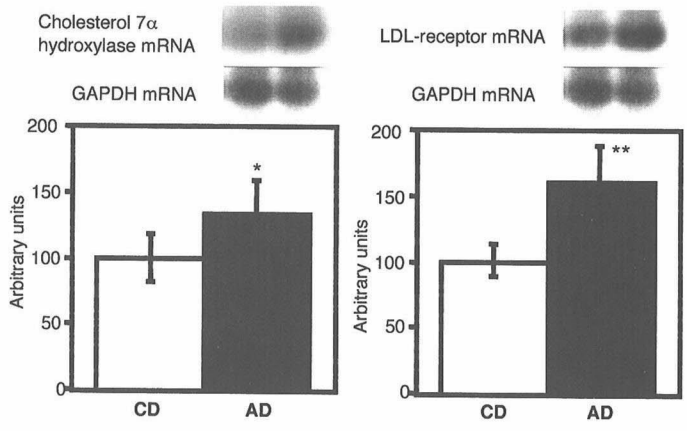

Fig. 1. Hepatic cholesterol 7α-hydroxylase mRNA and LDL receptor mRNA concentrations in rats fed *Amylomyces rouxii* for 4 wk. Each value represents the mean±SD, n=5. Mean values are significantly different from the control diet: $^*p<0.05$, $^{**}p<0.01$. The values of cholesterol 7α-hydroxylase and LDL receptor mRNAs were normalized to the value of GAPDH mRNA, and values for the rats fed the *Amylomyces rouxii* diet are expressed relative to the average values for rats fed the control diet, which was set to 100. Top panel illustrates the representative Southern hybridization of PCR amplified cholesterol 7α-hydroxylase cDNA and LDL receptor cDNA of hepatic RNA.

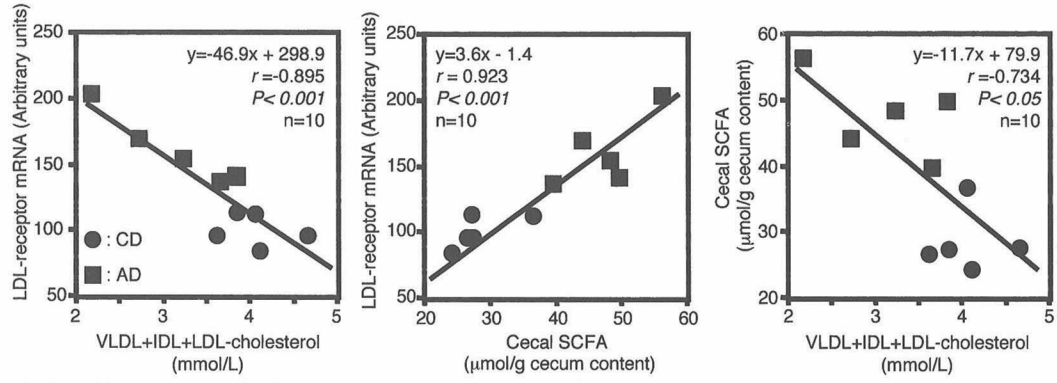

Fig. 2. Relationships between the hepatic LDL receptor mRNA level and serum VLDL+IDL+LDL-cholesterol concentration, the hepatic LDL receptor mRNA level and cecal short-chain fatty acid concentration, and the cecal short-chain fatty acid concentration and serum VLDL+IDL+LDL-cholesterol concentration in rats fed *Amylomyces rouxii* for 4 wk.

16.0±4.1 and 17.1±1.9 μmol/g wet liver, respectively) between the groups at the end of the experimental period.

Hepatic mRNA

The relative quantities of mRNAs were determined by the Southern hybridization of PCR-amplified cholesterol 7α-hydroxylase cDNA and LDL receptor cDNA in the rat liver. The values of cholesterol 7α-hydroxylase and LDL receptor mRNAs were normalized to the value of GAPDH. The values of the *A. rouxii*-fed rats were expressed relative to the average values of the control-diet group, which were normalized to 100. The relative quantities of hepatic LDL receptor and hepatic cholesterol 7α-hydroxylase mRNAs in the *A. rouxii*-fed group were significantly higher than those in the control group ($p<0.05$) (Fig. 1). The hepatic LDL receptor mRNA level correlated negatively ($r=-0.895$, $p<0.001$) with the serum VLDL+IDL+LDL-cholesterol concentration and correlated positively ($r=0.923$, $p<0.001$) with the cecal SCFA concentration (Fig. 2). The serum VLDL+IDL+LDL-cholesterol concentration correlated negatively ($r=-0.734$, $p<0.05$) with the cecal SCFA concentration (Fig. 2).

Cecal SCFA and fecal lipid concentrations

Cecal acetic acid, propionic acid and total SCFA concentrations in the *A. rouxii*-fed group were significantly higher than those in the control group (Table 4). There was no significant difference in the fecal total bile acid excretion (4.33±2.45 and 7.21±3.46 μmol/rat/d vs. the control group and *A. rouxii* fed group) between the groups.

Table 4. Cecal short-chain fatty acid concentration in rats fed control diet (CD) and *Amylomyces rouxii* diet (AD) for 4 wk.[1]

Component	Dietary group	
	CD	AD
	(μmol/g cecum content)	
Total	28.3±4.8	47.7±6.3**
Acetic acid	24.1±4.1	40.1±0.9**
Propionic acid	2.8±0.7	5.4±0.9**
Butyric acid	1.5±0.4	2.2±0.7

[1] Values are expressed as means±SD, n=5. Mean values are significantly different from those of the control diet: $^*p<0.05$, $^{**}p<0.01$.

DISCUSSION

In the present study we examined the effects of *A. rouxii* as a prebiotic on serum cholesterol, hepatic LDL receptor mRNA and hepatic cholesterol 7α-hydroxylase

mRNA levels in cholesterol-fed rats. The serum total cholesterol concentration in the *A. rouxii*-fed group was reduced by 23% compared with the control group. Most of the serum cholesterol in animals fed cholesterol and/or high fat diets is associated with LDL cholesterol (*5*, *27*). Therefore, lowering the LDL-cholesterol level may be an important factor for lowering the serum total cholesterol level in cholesterol-fed rats. In fact, a high correlation was found between the serum VLDL+IDL+LDL-cholesterol concentration and the serum total cholesterol concentration ($r=0.973$, $p<0.001$), and the serum VLDL+IDL+LDL-cholesterol concentration in the *A. rouxii*-fed group was significantly lower than that in the control group in the present experiment. Sonoyama et al. (*28*) reported that a diet containing 150 g/kg sugar beet fiber reduced ileal concentrations of apo A-I and apo A-IV mRNAs in rats as compared with those in rats fed a fiber-free diet. However, in the present study, there was no significant difference in the HDL-cholesterol concentration between both the *A. rouxii*-fed and control groups. Fukushima and Nakano (*6*) have reported that a mixture of microorganisms decreases the serum VLDL+IDL+LDL-cholesterol concentration but does not affect the serum HDL-cholesterol concentration in rats fed a cholesterol diet. This mechanism includes a decrease in HMG-CoA reductase activity, binding of steroids to the organisms in vitro, and so possibly in vivo, and decreased cholesterol micelle formation in vitro, and so possibly in vivo (*6*). There was a significant difference neither in the HMG-CoA reductase mRNA level nor in fecal bile acid concentrations between both the *A. rouxii*-fed and control groups in the present study (unpublished data). Though these results did not agree with the above report, it may be possible that the *A. rouxii* lowers the hepatic cholesterol synthetic rate compared with the control group. Because the LDL receptor mRNA level in the *A. rouxii*-fed group was higher than that in the control group, more cholesterol uptake by the liver or physiologically active matter such as fungal metabolite ML-236B (*29*) may have lowered the synthetic rate in the *A. rouxii*-fed group. However, the mechanism was not clear in the present study.

Hara et al. (*30*) have reported that products of fermentation of sugar-beet fiber by cecal bacteria lower the plasma cholesterol concentration in rats and that SCFA, as fermentation products, suppress cholesterol synthesis in the rat liver and intestine (*19*). We also found that increased SCFA, as fermentation products, suppressed the serum cholesterol concentration in the *A. rouxii*-fed group. It has been reported that dietary fiber and SCFA production elevate hepatic cholesterol synthesis (*31*–*34*). Further, the hepatic LDL-receptor level in the *A. rouxii*-fed group was significantly higher than in the control group. Thus, the hepatic cholesterol concentration in the *A. rouxii*-fed group tended to be promoted as compared with that in the control group. The result for the LDL-receptor mRNA level agreed with our previous results (*35*, *36*). Fukushima et al. (*35*) have reported that mushroom fibers lower the serum VLDL+IDL+LDL-cholesterol level by enhancement of hepatic LDL-receptor mRNA. In the present study, it also was shown that the hepatic LDL receptor mRNA level correlated negatively with the serum VLDL+IDL+LDL-cholesterol concentration and correlated positively with the cecal SCFA concentration. It may be that the hepatic LDL-receptor mRNA level was increased and the serum total cholesterol concentration was decreased by the increased cecal SCFA in the *A. rouxii*-fed group. It has been reported that dietary fish oil elevates hepatic LDL receptor activity in rats (*37*) and high dietary cholesterol and saturated fat intakes suppress hepatic LDL receptor mRNA in African green monkeys (*38*). However, there was no correlation between the plasma LDL cholesterol concentration and hepatic LDL receptor mRNA (*38*). Furthermore, there are few reports available for the relationship between molds, as prebiotics, and hepatic LDL receptor mRNA.

Though the SCFA concentration was elevated in the cecum in rats fed *A. rouxii* in this study, the hepatic cholesterol 7α-hydroxylase mRNA level in the *A. rouxii*-fed group was significantly higher than in the control group. Thus, the feeding of *A. rouxii* tended to promote the fecal total bile acid concentration as compared with the control group. Illman and Topping (*39*) reported that cecal propionate correlated negatively with the plasma cholesterol concentration and positively with cecal neutral steroids and bile acids. In the present experiment, there was no correlation between cecal SCFA and fecal bile acid, ($r=0.599$, $0.05<p<0.1$).

It is known that *A. rouxii* is not present in the intestine generally, and there have been no reported results on cholesterol metabolism in rats fed the *A. rouxii* cell body. The evidence for the presence of *A. rouxii* is unclear in the intestine. Therefore, it may be possible that *A. rouxii* produces more SCFA in the cecum because Oda et al. have confirmed that acetate, propionate, lactate and butyrate in fermented potato pulp were so increased as to lower cecal pH by *A. rouxii* and *R. oryzae* in vitro (unpublished data). Furthermore there is another possibility, sucrose conjugating with the *A. rouxii* cell body, which may have a high water-holding capacity, and might not be absorbed in small intestine. Instead, SCFA could be fermented by intestinal flora in the cecum.

In conclusion, the effect of the *A. rouxii* was most clearly seen when compared with rats fed the control diet. Its effects were to elevate the cecal SCFA concentration, hepatic LDL receptor mRNA and cholesterol 7α-hydroxylase mRNA levels in the *A. rouxii*-fed group. The results demonstrate that the *A. rouxii* lowers serum total cholesterol and VLDL+IDL+LDL-cholesterol concentrations as a prebiotic.

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