



## Some Bovine proteins behave as dietary fibres and reduce serum lipids in rats

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1 **Some Bovine proteins behave as dietary fibres and reduce serum lipids in rats.**

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22 **mRNAs**

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1 **ABSTRACT**

2 We examined the physiological importance of bovine dietary proteins in rats fed diets  
3 prepared from bovine Achilles' tendons and arteries. Rats were fed for 4 weeks, with  
4 20% casein diet (CON), in comparison with two diets containing 15% casein and 5% of  
5 either bovine Achilles' tendons (AC) or arteries (AR) protein preparations. The serum  
6 total cholesterol concentration and non-HDL cholesterol level in the AR fed group were  
7 significantly lower ( $P<0.05$ ) than those in the CON fed group at the end of the 4 week  
8 feeding period. The hepatic mRNAs were measured, and the HMG-CoA reductase  
9 mRNA level was significantly lower ( $P<0.05$ ) in AR fed group compared to CON fed  
10 group. Total hepatic cholesterol concentration, in rats fed AC was significantly  
11 ( $P<0.05$ ) higher than in the CON fed group. The serum triglyceride (TG)  
12 concentration and fatty acid synthase (FAS) mRNA level in AC and AR fed groups were  
13 significantly lower ( $P<0.05$ ) compared to the CON fed group through out the feeding  
14 period. Faecal neutral sterol excretion was significantly ( $P<0.05$ ) higher in the AC and  
15 AR fed groups compared to the CON fed group. The results of this study demonstrate  
16 that the some bovine dietary proteins have similar functions as dietary fibres which  
17 lowered the serum lipid concentration by enhancing faecal neutral sterol excretion, or  
18 suppressing lipid synthesis in the liver. Moreover, favourable amino acid compositions  
19 in AR and AC may also have an effect on low plasma lipid concentration in bovine  
20 protein diets fed groups.

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## 1 **Introduction**

2 At present there is an increasing interest in the capacity of food and food components  
3 to reduce the risk of non infectious diseases related to diet. Dietary fibres have gained  
4 a considerable interest due to its favourable health effects. According to recent studies  
5 it has been proven that dietary fibre intake is inversely correlated with several  
6 cardiovascular disease factors, which supports its protective role against cardio vascular  
7 diseases and recommendations for its increased consumption (Lairon *et al.*2005, Erkkila  
8 & Lichtenstein, 2006).

9 From past studies it has been shown that some plant fibres have hypocholesterolemic  
10 effect; which may be due to fibre-induced alterations of intestinal absorption, intestinal  
11 or pancreatic hormone secretion, lipoprotein metabolism, bile acid metabolism, or  
12 fermentation by-products and their effects on hepatic cholesterol synthesis (Kay 1982).  
13 When animal dietary fibres are concerned, relatively a few studies has been carried out  
14 to investigate the effect on lipid metabolism. Normally, people believe that those who  
15 at a risk of coronary heart disease should consume less red meat due to adverse health  
16 impacts.

17 Bovine Achilles' tendons and arteries are waste products of the beef industry, which  
18 could be used as a potential source of functional protein for possible food applications  
19 due to their favourable amino acid composition and undigestible behaviour. This  
20 hypothesis may be supported by previous findings showing that dietary proteins with  
21 favourable amino acid composition reduced the serum cholesterol level in rats (Morita  
22 *et al.*1997,Gudbrandsen *et al.*2005). Undigestible behaviour of animal protein may be  
23 due to the abound collagen concentration as AC and AR contain abound glycine and  
24 proline concentrations (Schubert *et al.* 1974). In this study, we investigated the effect  
25 of some bovine dietary proteins on serum lipids, liver lipids, faecal lipids, bacterial

1 population in the colon, and hepatic mRNAs in rats.

## 2 **Experimental methods adopted**

### 3 *Animals and diets*

4 Male Crj / Wistar rats (5 weeks old) were purchased from Charles River Japan Inc.  
5 (Yokohama, Japan). They were housed individually in cages with free access to food  
6 and water. The animal facility was maintained on a 12 h light/dark cycle at a  
7 temperature of  $23 \pm 1$  °C and relative humidity of  $60 \pm 5$  %. The rats were randomly  
8 assigned in to three groups ( $n=5$ ). There were no significant differences in body  
9 weights and serum total cholesterol concentrations among groups at the beginning of  
10 the experiment. **The composition of each diet is shown in Table 1.** The experimental  
11 rats were fed for 4 weeks, with 20% casein diet, in comparison with two diets  
12 containing 15% casein and 5% of either bovine Achilles' tendons or arteries protein  
13 preparations. The AC and AR proteins were prepared as follows; first, raw bovine  
14 Achilles' tendons and arteries were washed and boiled to be defatted for 2 hours. The  
15 boiled Achilles' tendons and arteries were freeze-dried and milled at 300 mesh. The  
16 compositions of AC and AR were as follows (mg/g dry weight): moisture, 2.1 and 4.1;  
17 protein: 73.9 and 81.0; Lipid: 23.1 and 8.4; carbohydrate: 0.5 and 5.9; ash: 0.4 and 0.6,  
18 respectively. Total moisture, protein, lipid and carbohydrate content were determined  
19 by the procedure of the Association of Official Analytical Chemists (1990). Dietary  
20 cholesterol level in the AC and AR was determined (g/1kg of diet), and it was 0.1  
21 (0.01%) and 0.06 (0.006%), respectively. The control group consisted of rats fed 200  
22 g/kg of casein. The amino acid compositions of the AC, AR and casein were shown in  
23 the Table 2. The amino acid compositions were determined as follows; first 4 mg of  
24 AC and AR samples were hydrolyzed in 2 ml of 6 N HCL at 110 °C for 24 h, vaccum  
25 dried and then reconstituted with 1 ml of 0.2 N HCl, filtered with 0.45µm diameter filter

1 (W-13-5, Tosoh, Japan) and analysed with a Hitachi-8700 amino acid analyser. The  
2 rats were allowed free access to food and water for 4 weeks. Body weight and food  
3 consumption were recorded weekly and daily, respectively. This experimental design  
4 was approved by the Animal Experiment Committee of Obihiro University of  
5 Agriculture and Veterinary Medicine. All animal procedures conformed to standard  
6 principles described in *Guide for the care and Use of Laboratory Animals* (National  
7 Research Council, 1985).

#### 8 *Analytical procedures*

9 Blood samples (1 mL) were collected between 08.00 and 10.00 hours from the  
10 jugular veins of fasting rats anaesthetized by sodium pentobarbital. The samples were  
11 taken in to tubes without an anticoagulant. After the samples were allowed to stand at  
12 room temperature for 2 h, the sera were separated by centrifugation at 1500 g for 20 min.  
13 All faecal excretions were collected during the last 3 d of the experimental period (4  
14 weeks). Faecal dry weight did not differ among groups. The rats were killed by ether  
15 inhalation, and the livers and caecum quickly removed, washed with cold saline (9g  
16 NaCl/L), blotted dry on filter paper, and weighed before freezing for storage.

#### 17 *Chemical analysis.*

18 Total cholesterol, HDL-cholesterol, and triglyceride (TG) concentrations in the serum  
19 were determined enzymatically using commercially available reagent kits (assay kits for  
20 the TDX system; Abbott laboratory Co., Irving, TX). The non-HDL cholesterol  
21 concentration was calculated as follows: non-HDL cholesterol= total  
22 cholesterol–HDL-cholesterol.

23 Total lipids were extracted from liver and faeces by a mixture of  
24 chloroform/methanol (2:1, vol/vol) (Folch *et al.* 1957). The neutral steroids in each  
25 lipid sample obtained by saponification were acetylated (Matsubara *et al.* 1990) and

1 analysed by gas–liquid chromatography (GLC) using a Shimadzu 14A chromatograph  
2 (Kyoto, Japan) with a DB17 capillary column (0.25mm×30m; J&W Scientific, Folsom,  
3 CA) with nitrogen as the carrier gas. Acidic sterols in faeces were measured by GLC  
4 following the method of Grundy *et al.* (1965). Sterol balance was calculated as sterol  
5 balance = (faecal cholesterol+faecal coprostanol+faecal bile acids)-dietary cholesterol  
6 intake. A part of the caecal content was taken out into desalting water in a vial without  
7 exposure to air, and suspended. The suspension of caecum was deproteinized with  
8 perchloric acid and to form sodium salts of the short chain fatty acids (SCFAs).  
9 Individual SCFA was measured by GLC with a glass column (2000 x 3 mm) packed  
10 with 80–100 mesh chromosorb W-AW DMCS with H<sub>3</sub>PO<sub>4</sub> (100 mL/L) as the liquid  
11 phase after adding H<sub>3</sub>PO<sub>4</sub> by the procedure of Hara *et al.* (1994). Faecal nitrogen  
12 content was determined by Kjeldahl's method. Apparent digestibility of protein was  
13 calculated as apparent protein digestibility = (protein intake-faecal protein)/protein  
14 intake×100.

#### 15 *RNA isolation, reverse transcription-PCR, and Southern blot Analysis*

16 Total RNA was isolated from the liver by the acid guanidium-phenol-choloroform  
17 method using Isogen (Nippon Gene, Tokyo, Japan; Chomczynski & Sacchi,1987).  
18 mRNA encoding the LDL receptor (LDL-R), cholesterol 7 $\alpha$ -hydroxylase (CYP7A1),  
19 fatty acid synthase (FAS), HMG-CoA reductase, scavenger receptor type B1(SR-B1),  
20 sterol regulatory elementary binding protein (SREBP-1c), and GAPDH (used as an  
21 invariant control) were analysed by semi-quantitative RT-PCR and subsequent Southern  
22 hybridization of the PCR products with each inner oligonucleotide probe. Total RNA  
23 samples were treated with DNase RQ1 (Promega, Madison, WI, USA) to remove  
24 genomic DNA and subjected to RT-PCR by using Moloney murine leukemia virus  
25 reverse transcriptase (Gibco-BRL, Gaithersburg, MD, USA) and EX-Taq polymerase

1 (Takara, Tokyo, Japan) with LDL receptor primers of oligonucleotides (upstream primer,  
2 5'-ATTTTGGAGGATGAGAAGCAG-3'; downstream primer,  
3 5'-CAGGGCGGGGAGTGTGAGAA-3'), CYP7A1 primers of oligonucleotides  
4 (upstream primer, 5'-GCCGTCCAAGAAATCAAGCAGT-3'; downstream primer,  
5 5'-TGTGGGCAGCGAGAACAAAGT-3'), FAS primers of oligonucleotides (upstream  
6 primer, 5'-GCTGGAGCCCCTTTTTGTCTT-3'; down stream primer,  
7 5'-ACCCAGCACTGCAGTTTTCT-3'), HMG-CoA reductase primers of  
8 oligonucleotides (upstream primer, 5'-GCGTGCAAAGACAATCCTGGAG-3';  
9 downstream primer, 5'-GTTAGACCTTGAGAACCCAATG-3'), SR-B1 primers of  
10 oligonucleotides (upstream primer, 5'-GTAGGGCCCAGAAGACACCAC-3';  
11 downstream primer, 5'-CGCCTGCTTCACCACCTTCTT-3'), SREBP-1c primers of  
12 oligonucleotides (upstream primer, 5'-GAGCCACAATGAAGACCGCA-3';  
13 downstream primer, 5'-CAAGGACAAGGGGCTACTCT-3'), and GAPDH primers of  
14 oligonucleotides (upstream primer, 5'-GCCATCAACGACCCCTTCATT-3';  
15 downstream primer, 5'-CGCCTGCTTCACCACCTTCTT-3'). The reaction mixtures  
16 for the PCRs contained 25 pmol of each primer, 1.25 U of EX-Taq polymerase, 1 X  
17 PCR buffer (Takara), and 200  $\mu$ M dNTP in a 50- $\mu$ l reaction volume. Temperature  
18 cycling was as follows: first cycle, denaturation at 94°C for 3 min, annealing at 60°C for  
19 1 min, and extension at 72°C for 2 min. Subsequent cycles were denaturation at 94°C  
20 for 1 min, annealing at 60°C for 1 min, and extension at 72°C for 2 min. The thermal  
21 cycling was completed by terminal extension at 72°C for 10 min. In total, 25 cycles  
22 were performed for LDL-R, CYP7A1, 30 cycles for HMG-CoA reductase, SR-B1, 20  
23 cycles for FAS, GAPDH and SREBP-1c. Amplification products were  
24 electrophoresed on 2% agarose gel and transferred to a nylon membrane (Biodyne B,  
25 Pall Bio-Support, East Hills, NY, USA). Blots were hybridized with LDL-R probe of a



1 54-base oligoneucleotide  
2 (5'-CCAGGGTTGGTCGCTTTGCCCTGGAGCTGATCTGTCACCTCCAGCTCTCC  
3 CTC-3'), CYP7A1 probe of a 54-base oligonucleotide  
4 (5'-CCCGAAGGCCTGTTTAAGTGATGACTCTCAGCCGCCAAGTGACATCATCC  
5 AGTG-3'), FAS receptor probe of a 54-base oligonucleotide  
6 (5'-CTGCTCTCTGTGGATAGGACTGAATGCTGTGGCCTTCTGATAGACTCTTCT  
7 GGA-3'), HMG-CoA reductase probe of a 54-base oligonucleotide  
8 (5'-GATCTGTTGTGAACCATGTGACTTCTGACAAGATGTCCTGCTGCCAATGC  
9 TGCC-3'), SR-B1 probe of a 54-base oligoneucleotide  
10 (5'-TGCCGTGTGGACAGTGTGACATCTTGGGGCTCAGGACGTGGCACTGGCG  
11 GGTTG-3'), SREBP-1c probe of a 54-base oligonucleotide  
12 (5'-GCCGGCGTCTGAGGGTGGAGGGGTCAGCGTTTCTACCACTTCAGGTTTCA  
13 TGCC-3'), and GAPDH probe of a 54-base oligonucleotide  
14 (5'-TGATGACCAGCTTCCCATTCTCAGCCTTGACTGTGCCGTTGAACTTGCCG  
15 TGGG-3'). The probe was 3'-tailing labeled with digoxigenin, using a DIG  
16 oligonucleotide tailing kit (Boehringer Mannheim, Mannheim, Germany).  
17 Prehybridization, hybridization, and detection were carried out with a DIG luminescent  
18 detection kit (Boehringer Mannheim, Mannheim, Germany) as recommended by the  
19 manufacturer. The relative quantity of mRNA was estimated by densitometry scanning  
20 with x-ray film.

#### 21 *Growth of bacteria in the caecum*

22 *Coliform* in the caecum was inoculated and grown for 2 days on DHL agar (Eiken  
23 Chemical Co., Ltd, Tokyo, Japan) plates at 37 °C. Anaerobe, *Lactobacillus* and  
24 *Bifidobacterium* in the caecum were incubated for 5 days on GAM agar (Nissui  
25 Pharmaceutical Co., Ltd, Tokyo, Japan), Rogosa agar (Merck KGaA, Darmstadt,

1 Germany) and BL agar (Eiken Chemical Co., Ltd, Tokyo, Japan) at 37 °C by the gaspak  
2 method according to the procedure of Mitsuoka *et al.* (1964,1965,1976).

### 3 *Statistical Analysis*

4 Data are presented as means and standard deviations for serum total cholesterol,  
5 HDL-cholesterol and non-HDL cholesterol at prescribed times. The significance of  
6 differences among treatment groups was determined by ANOVA with Duncan's  
7 multiple range test and student *t*-test (SAS Institute, Cary, NC, USA). Differences  
8 were considered significant at  $P<0.05$ .

### 9 **Results**

10 There was no significant difference in the food intake, body weight gain, liver weight,  
11 caecum weight, faecal dry weight and caecal pH, among groups at the end of the  
12 experimental period (data not shown). Apparent digestibility of AC and AR was  
13 around 92-93%, and which was lower than CON, 96%.

14 Table 3 shows the serum total cholesterol, non-HDL cholesterol, HDL-cholesterol  
15 and triglyceride concentrations of rats. The serum total cholesterol and non-HDL  
16 cholesterol concentration in the AR fed group were significantly lower than the CON  
17 group at the end of the 4 week feeding period. There was no significant difference in  
18 the HDL cholesterol concentration among the groups. The serum triglyceride  
19 concentration was significantly lower in AC and AR fed groups than that in the CON  
20 group through out the feeding period. The liver cholesterol concentrations in rats at  
21 the end of the experimental period are shown in Fig. 1. The liver cholesterol  
22 concentration in rats fed AC was significantly higher than in the CON group at the end  
23 of the 4 week feeding period.

24 Table 4 shows the **dietary cholesterol intake**, faecal neutral steroid and acidic steroid  
25 concentrations of rats. **The bovine dietary cholesterol intake in rats fed AC and AR**

1 diets were  $7.20 \pm 1.03$  and  $6.91 \pm 0.61$   $\mu\text{mol}/\text{rat}/\text{day}$ , respectively. The faecal cholesterol  
2 and coprostanol concentrations were significantly higher in the AC and AR fed groups  
3 than those in the CON group, and those in the AC fed group were the highest among all  
4 the groups. However, the data on faecal coprostanone level was lacking in the Table 4  
5 due to unavailability. Further, there were no significant differences in total bile acid,  
6 cholic acid, chenoceoxycholic acid, deoxycholic acid and lithocholic acid  
7 concentrations among the groups. Sterol balance in rats fed CON, AC and AR were  
8  $14.64$ ,  $26.96$  and  $14.16$   $\mu\text{mol}/\text{rat}/\text{day}$ , respectively. There were no significant  
9 differences in total SCFA, acetic acid, propionic acid and *n*-butyric acid excretions in  
10 caecum among 3 groups (data not shown).

11 The relative quantities of mRNA were determined by the Southern hybridization of  
12 PCR-amplified LDL-R cDNA, CYP7A1 cDNA, HMG-CoA reductase cDNA, SR-B1  
13 cDNA, SREBP-1c cDNA, and FAS cDNA in the rat liver. The values of LDL-R,  
14 CYP7A1, HMG-CoA reductase, SR-B1, SREBP-1c and FAS mRNA were normalized  
15 to 100. There was no significant difference in relative quantity of LDL-R mRNA  
16 among 3 groups and the quantities for CON, AC, AR were 0.856, 809, 0.796  
17 respectively. No significant difference was observed for the relative quantity of  
18 CYP7A1 mRNA among 3 groups (Fig.2). The relative quantity of hepatic HMG-CoA  
19 reductase mRNA in the AR fed group was significantly lower than the CON group  
20 ( $P < 0.05$ ), and that in AC fed group tended to be lower compared to the CON  
21 group (Fig.2). The relative quantity of FAS mRNA level in the AC and AR fed groups  
22 was significantly lower than that in the CON group (Fig.3). No significant difference  
23 was observed for the relative quantity of SR-B1 mRNA level, and the quantities for  
24 CON, AC and AR were 0.68, 0.67 and 0.66, respectively. The SREBP-1c mRNA level  
25 in AR fed group was significantly lower than that in the CON group, and that in the AC

1 fed group tended to be lower compared to the CON group (Fig.3). The FAS mRNA  
2 level was positively correlated with SREBP-1c mRNA level, the correlation coefficient  
3 ( $r$ ) being 0.660 ( $P < 0.01$ ).

4 Table 5 shows the caecal bacterial population in rats. Anaerobe bacterial population  
5 was significantly higher in the AC and AR fed groups compared to the CON group.  
6 *Bifidobacterium* population in the AR fed group was significantly higher compared to  
7 the CON group, and that in the AC fed group tended to be elevated compared to the  
8 CON group. *Coliform* bacterial population was significantly higher in the AC fed  
9 group compared to the CON group, and that in the AR fed group tended to be elevated  
10 compared to the CON group.

## 11 **Discussion**

12 In the present study, we examined the effects of some bovine proteins on serum lipids,  
13 liver lipids, faecal lipids, caecal lipids, bacterial population in the colon, and hepatic  
14 mRNAs in rats. The serum total cholesterol and non-HDL cholesterol concentration in  
15 the AR fed group were significantly lower than those in the CON group at the end of the  
16 4 week feeding period. Several mechanisms have caused to these findings of bovine  
17 proteins. One possible reason may be bovine proteins act as dietary fibres. This  
18 could be supported by the lower digestibility of AC and AR (92-93%) compared to  
19 CON (96%). Jorgensen *et al.* (2003) has reported that increased intake of dietary fibre  
20 was associated with a concomitant loss of protein and energy to faeces. Previous  
21 findings have showed that dietary fibres lower plasma cholesterol levels in animals and  
22 humans compared with casein-based diets (Khosla *et al.*1991; Anderson *et al.*1995).  
23 The factor lowering the cholesterol concentration in the AR fed group was the lowering  
24 of non-HDL cholesterol level, which was in agreement with previous findings  
25 (Fukushima *et al.*2000; Li *et al.*2004). However, there was no significant difference in

1 hepatic LDL receptor mRNA level in rats fed bovine proteins compared to CON group.  
2 Thus, the lower non-HDL cholesterol concentration in the AR fed group may be due to  
3 any other reason. This result was in agreement with previous findings showing that  
4 there was no correlation between plasma LDL cholesterol concentration and hepatic  
5 LDL receptor mRNA level (Sorci-Thomas *et al.*1989). The CYP7A1 mRNA level and  
6 fecal bile acid concentration were not significantly different among the group. The  
7 rate-limiting enzyme in endogenous sterol biosynthesis is HMG-CoA reductase, which  
8 catalyzes the synthesis of mevalonate, and the activity of HMG-CoA reductase is also  
9 regulated by changes in the exogenous cholesterol concentration (Brown & Goldstein,  
10 1997). In this experiment, HMG-CoA reductase mRNA expression was significantly  
11 lower in AR fed group, and tended to be lower in AC fed group compared to the CON  
12 group. This may be another reason for low total serum cholesterol level which was  
13 positively correlated with HMG -CoA reductase mRNA level in AR fed group, the  
14 correlation coefficient being 0.742 (P<0.01). The decrease in HMG-CoA reductase  
15 mRNA is surprising and conflicts with previous reports related to dietary fibre. Lund  
16 *et al.* (1993) and Moundras *et al.*(1997) have reported that dietary fibre increases  
17 hepatic reductase activity. The mechanism of reduction of HMG CoA reductase  
18 activity might be due to the increased liver cholesterol concentration which may inhibit  
19 the hepatic HMG-CoA reductase mRNA level. In rats, most of the serum cholesterol  
20 (60-80%) is transported in HDL and only 5-10% in LDL, since they are lack of lipid  
21 transfer protein (Day *et al.*1979). In contrast, this study showed only 30% of serum  
22 cholesterol was from HDL cholesterol even in rats fed the 0.01% and 0.006% bovine  
23 dietary cholesterol diets. The dietary cholesterol level and 7% soybean oil content  
24 with 0.4% plant sterols in AIN 93G diet may be a reason for lower HDL cholesterol  
25 concentration in AC and AR diets fed groups. However, it has been shown that

1 feeding normal Wistar-Furth or Sprague-Dawley rats with diets containing 1%  
2 cholesterol had negligible effects on serum cholesterol level (Ness *et al.*2004).

3 Amino acid composition of animal protein might be an important factor affecting  
4 behaviour of animal protein. Undigestible behaviour of animal protein may be due to  
5 the abundant collagen concentration as AC and AR contain abundant glycine and proline  
6 concentrations (Schubert *et al.*1974). In addition, it has been suggested that favourable  
7 amino acid composition is at least partially responsible for the suppressive effect on  
8 plasma cholesterol in previous studies (Kayashita *et al.* (1995, 1997), Morita *et al.*1997,  
9 Tomotake *et al.*2000, Gudbrandsen *et al.*2005). Lower methionine level or lower  
10 methionine: glycine (Table 2) ratio (80-85%) in AC and AR diets might have hindered  
11 the transfer of cholesterol from liver in to blood stream (Morita *et al.*1997). In fact,  
12 the lower methionine level may reduce phosphatidyl choline synthesis via phosphatidyl  
13 ethanolamine, leading to depression of apolipoprotein release into circulation (Morita *et*  
14 *al.*1997). Moreover, dietary arginine concentration was comparatively higher in AC  
15 and AR diets, which was negatively correlated with serum non- HDL cholesterol  
16 level( $r= 0.863$ ;  $P<0.01$ ) in a previous study (Eklund & Sjoblom, 1980). These facts  
17 may suggest another mechanism of serum cholesterol lowering in AR fed group. On  
18 the other hand, effect of methionine would give a reason for higher liver cholesterol  
19 level in both AC and AR fed groups. The liver cholesterol concentration was  
20 significantly higher in AC fed group, and tended to be higher in AR fed group, than that  
21 in the CON group. Han *et al.* (2003) also has reported that liver cholesterol  
22 concentration in the groups fed the enzyme resistant fractions of beans was significantly  
23 higher than in the cellulose powder fed group, although the reason for this remained  
24 unclear, it may be speculated that the increase in liver cholesterol concentration was due  
25 to enhanced level of hepatic SR-B1 mRNA level. However, in this study no

1 significant difference was observed in SR-B1 mRNA level among groups. This  
2 finding doesn't agree with previous findings in mice showing that dietary fibres lower  
3 liver lipid concentration (Van Bennekum *et al.*2005). However, the significantly  
4 higher liver cholesterol level in AC fed group may be a compensatory response to the  
5 higher sterol excretion compared to control.

6 The faecal cholesterol and coprostanol concentration in AC and AR groups were  
7 significantly higher than those in the CON group. Neutral sterols occupy a major part  
8 of steroid excretion, and it is noteworthy that the excretions of cholesterol and  
9 coprostanol were brought by feeding AC and AR. This may be another reason for  
10 lower serum cholesterol concentration in AR fed group. This was in agreement with  
11 findings for some dietary plant fibres (Kritchesvsky 1988; Eastwood 1992; Moundras *et*  
12 *al.* 1997). Although, the excretion of neutral sterol was affected by bovine protein,  
13 acid sterols was unaffected compared with casein. This was in contrast with previous  
14 findings showing that some plant fibres lower serum cholesterol by increasing  
15 cholesterol elimination as bile acids and neutral sterols (Fukushima *et al.* 2000; Buhman  
16 *et al.* 1998). Furthermore, Hara *et al.* (1998) reported that sugar-beet fibre reduces  
17 serum cholesterol level by reducing cholesterol synthesis in rats.

18 According to the results of dietary cholesterol intake, faecal sterol excretion and  
19 HMG-CoA reductase mRNA level, the sterol balance was 1.86 folds higher in AC fed  
20 group, and that in AR fed group was not different, compared to the control group.  
21 Whereas, HMG-CoA reductase mRNA level in AC fed group was not significantly  
22 different compared to the control group and that in AR fed group was significantly  
23 lower relative to the control. In this study, AC and AR diets may maintain the body  
24 sterol balance by two different mechanisms.

25 Serum TG concentration was significantly lower in the AC and AR fed groups

1 compared to the CON group. This was in agreement with previous findings showing  
2 that dietary fibres reduce serum triglyceride levels in rats (Li *et al.* 2004). Low TG  
3 level in AC and AR fed groups was accompanied by the reduction in FAS mRNA level  
4 in animal protein fed groups. In fact, the mRNA expression of FAS was significantly  
5 lower in AR fed group, and tended to be lower in AC fed group compared to the CON  
6 group. The FAS is a multiant enzyme complex that catalyzes the synthesis of  
7 long-chain fatty acids from acetyl CoA and malonyl CoA (Wakil *et al.*1983).  
8 Moreover, SREBP-1c mRNA level was significantly lower in AR fed group, and it  
9 tended to be lower in AC fed group compared to the CON group. The correlation  
10 between SREBP-1c and FAS was  $r = 0.660$  ( $P < 0.01$ ). It was suggested that the  
11 bovine protein-dependant decrease in FAS gene expression is due to the suppression of  
12 the gene expression of SREBP-1c, which is the transcriptional factor to generate FAS  
13 mRNA. SREBPs are membrane-bound transcriptional factors which regulate the gene  
14 expression of enzymes in fatty acid and cholesterol biosynthesis (Brown & Goldstein,  
15 1997; Horton & Shinomura, 1999). SREBP-1c preferentially enhances transcription of  
16 genes required for fatty acid synthesis (Horton *et al.*2002).

17 According to results of this study, anaerobe bacteria population of caecum in the  
18 AC and AR fed groups was significantly higher than the CON group. These results  
19 were accompanied by higher fecal coprostanol concentration which may be an index of  
20 the activity of intestinal flora (Arjmandi *et al.*1992). It was suggested that protein was  
21 one of the major substrates for caecal fermentation in rats when animal protein based  
22 diet was fed (Tsukahara & Ushida, 2000). Fatty acids of anaerobic bacteria are a  
23 major source of energy for the colonic mucosa in humans (Roediger, 1980).  
24 *Bifidobacterium* population was significantly higher in the AR fed group, and tended to  
25 be higher in the AC fed group compared to the CON group. In addition, coliform



1 bacteria population was significantly higher in AC fed group and tended to be higher in  
2 AR fed group than that in the CON group. However, there was no significant  
3 difference in caecal pool size of SCFA among the groups. The reason for higher  
4 bifidobacteria and coliform population in bovine protein fed groups remains unclear.  
5 However, the undigested AC and AR proteins might be used as nitrogen sources by  
6 ceacal bacteria.

7 In conclusion, effects of some bovine proteins were most clearly seen when  
8 compared with rats fed casein. Bovine artery protein (AR) reduced the total serum  
9 cholesterol by reducing non-HDL cholesterol, accompanied by lower HMG CoA  
10 reductase mRNA level, and by increasing faecal neutral sterol excretion. Animal  
11 proteins such as bovine Achilles' tendon protein and artery protein, reduced TG  
12 concentration by reducing the fatty acid synthesis in liver accompanied by lower FAS  
13 mRNA level compared to the casein fed group. In view some of these facts; we  
14 conclude that some bovine animal proteins show dietary fibre like properties by virtue  
15 of their low digestibility.

1 Acknowledgements

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6 Education, Culture, Sports, Science, and Technology.

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1 Abbreviations

2 AC, Bovine Achilles`tendon protein diet; AR, Bovine artery protein diet; CON, casein;  
3 GAPDH, glyceraldehyde-3-phosphate dehydrogenase; IDL, intermediate density  
4 lipoprotein; RT-PCR, reverse transcription polymerase reaction; SCFA, short-chain fatty  
5 acid; LDL-R, low density lipoprotein receptor, SR-B1, scavenger receptor class type  
6 1;SREBP-1c,sterol regulatory element binding protein-1c; FAS, fatty acid synthase;  
7 cholesterol 7 $\alpha$ -hydroxylase (CYP7A1);HMG-CoA reductase, hydroxyl methyl  
8 glutaryl-CoA reductase; TG, triglyceride.

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1 **Figure legends**

2 Fig. 1. Liver cholesterol concentration in rats fed animal proteins for 4 weeks.  
3 Values are expressed as means  $\pm$  SD for five rats. Values with different superscript  
4 letters are significantly different ( $P < 0.05$ ).

5 Figure. 2. Hepatic HMG-CoA reeducate mRNA and cholesterol  $7\alpha$  hydroxylase  
6 mRNA expressions in rats fed animal proteins for 4 weeks. Values are expressed as  
7 means  $\pm$  SD for five rats. Mean values with different superscript letters are  
8 significantly different ( $P < 0.05$ ).

9 Figure. 3. Hepatic SREBP-1c and fatty acid synthase mRNA expressions in rats fed  
10 animal proteins for 4 weeks. Values are expressed as means  $\pm$  SD for five rats.  
11 Mean values with different superscript letters are significantly different ( $P < 0.05$ ).

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TABLE 1  
Composition of experimental diets

| Ingredients                                   | Dietary group <sup>1</sup> |                  |          |
|-----------------------------------------------|----------------------------|------------------|----------|
|                                               | CON                        | AC               | AR       |
|                                               |                            | <i>g/kg diet</i> |          |
| Casein                                        | 200                        | 150              | 150      |
| Bovine Achilles' tendon protein (cholesterol) | -                          | 50(0.1)          | -        |
| Bovine artery protein (cholesterol)           | -                          | -                | 50(0.06) |
| Soybean oil                                   | 70                         | 70               | 70       |
| Mineral mixture <sup>2</sup>                  | 35                         | 35               | 35       |
| Vitamin mixture <sup>3</sup>                  | 10                         | 10               | 10       |
| Cellulose powder                              | 50                         | 50               | 50       |
| Sucrose                                       | 100                        | 100              | 100      |
| L-cystine                                     | 3                          | 3                | 3        |
| Choline hydrogen tartrate                     | 2.5                        | 2.5              | 2.5      |
| 3-Butylhydroquinone                           | 0.014                      | 0.014            | 0.014    |
| $\alpha$ -Corn starch                         | 529.486                    | 529.486          | 529.486  |

<sup>1</sup>CON, basal diet; AC, bovine Achilles' tendon diet; AR, bovine artery diet  
<sup>2</sup>AIN-93G mineral mixture  
<sup>3</sup>AIN-93G vitamin mixture

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**TABLE 2**  
**Amino acid compositions of bovine proteins**

| Amino acids<br>(%W/W) | CON   | AC    | AR    |
|-----------------------|-------|-------|-------|
| Asp                   | 6.75  | 6.78  | 4.85  |
| Thr                   | 4.43  | 2.69  | 2.82  |
| Ser                   | 6.24  | 4.79  | 3.63  |
| Glu                   | 18.42 | 10.72 | 7.57  |
| Gly                   | 3.04  | 33.30 | 30.04 |
| Cys                   | 0.26  | 0.14  | 0.49  |
| Val                   | 7.31  | 3.43  | 10.27 |
| Met                   | 2.48  | 0.76  | 0.56  |
| Ile                   | 5.32  | 2.08  | 3.67  |
| Leu                   | 9.13  | 4.69  | 8.20  |
| Tyr                   | 3.93  | 0.95  | 2.60  |
| Phe                   | 3.88  | 2.17  | 3.61  |
| Lys                   | 6.92  | 3.21  | 2.95  |
| His                   | 2.48  | 0.92  | 0.88  |
| Arg                   | 2.70  | 7.14  | 3.75  |
| Ala                   | 4.32  | -     | -     |
| Pro                   | 12.39 | 16.24 | 14.09 |

CON,Casein;AC, bovine Achilles' tendon powder; AR,  
bovine artery powder

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TABLE 3

Serum total cholesterol, VLDL+IDL+LDL-cholesterol, HDL-cholesterol, and triglyceride concentrations in rats fed bovine proteins for 4 weeks<sup>1</sup>

| Dietary group             | Wk0        | Wk2                      | Wk4                     |
|---------------------------|------------|--------------------------|-------------------------|
| Total cholesterol         |            | <i>mmol/L</i>            |                         |
| CON                       | 2.51± 0.15 | 2.53± 0.26 <sup>a</sup>  | 2.29± 0.209             |
| AC                        | 2.37± 0.42 | 2.16± 0.34 <sup>ab</sup> | 1.99± 0.433             |
| AR                        | 2.46± 0.14 | 2.08± 0.30 <sup>b</sup>  | 1.94± 0.248 *           |
| HDL-cholesterol           |            |                          |                         |
| CON                       | 0.77± 0.02 | 0.72± 0.10               | 0.69± 0.08              |
| AC                        | 0.75± 0.15 | 0.68± 0.13               | 0.66± 0.14              |
| AR                        | 0.76± 0.05 | 0.64± 0.10               | 0.64± 0.08              |
| VLDL+IDL+LDL -cholesterol |            |                          |                         |
| CON                       | 1.75± 0.13 | 1.81± 0.18 <sup>a</sup>  | 1.59± 0.16              |
| AC                        | 1.62± 0.29 | 1.47± 0.25 <sup>b</sup>  | 1.33± 0.31              |
| AR                        | 1.70± 0.14 | 1.45± 0.24 <sup>b</sup>  | 1.31± 0.19 *            |
| Triglyceride              |            |                          |                         |
| CON                       | 0.42± 0.15 | 0.85± 0.24 <sup>a</sup>  | 0.76± 0.17 <sup>a</sup> |
| AC                        | 0.34± 0.16 | 0.44± 0.12 <sup>b</sup>  | 0.35± 0.16 <sup>b</sup> |
| AR                        | 0.39± 0.07 | 0.52± 0.08 <sup>b</sup>  | 0.37± 0.16 <sup>b</sup> |

<sup>1</sup>Values are expressed as means ± SD for five rats. \**P* < 0.05 vs. BD by student's *t*-test.

<sup>a,b</sup>Mean values within a row with unlike superscript letters were significantly different by Duncan's multiple range test (*P* < 0.05).

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TABLE 4

**Cholesterol intake, faecal neutral steroid and acidic steroid concentrations in rats fed bovine proteins for 4 weeks<sup>1</sup>**

|                           | Dietary group           |                         |                         |
|---------------------------|-------------------------|-------------------------|-------------------------|
|                           | CON                     | AC                      | AR                      |
|                           |                         | <i>μmol/rat/day</i>     |                         |
| <b>Cholesterol Intake</b> | -                       | 7.20±1.03               | 6.91±0.61               |
| <b>Faecal Cholesterol</b> | 11.62±4.00 <sup>c</sup> | 20.69±5.45 <sup>a</sup> | 12.65±1.72 <sup>b</sup> |
| <b>Faecal Coprostanol</b> | 2.20±0.90 <sup>c</sup>  | 12.69±2.50 <sup>a</sup> | 7.88±2.05 <sup>b</sup>  |
| <b>Faecal LCA</b>         | 0.14±0.07               | 0.13±0.01               | 0.12±0.03               |
| <b>Faecal DCA</b>         | 0.05±0.03               | 0.07±0.02               | 0.05±0.03               |
| <b>Faecal CDCA</b>        | 0.02±0.03               | 0.03±0.01               | 0.02±0.02               |
| <b>Faecal CA</b>          | 0.20±0.11               | 0.16±0.20               | 0.08±0.02               |
| <b>Faecal TBA</b>         | 0.41±0.23               | 0.39±0.10               | 0.27±0.04               |

<sup>1</sup>Values are expressed as means ± SD for five rats. <sup>a,b,c</sup>Means within a row with unlike superscript letters were significantly different (p<0.05). LCA, lithocholic acid; DCA, deoxycholic acid; CDCA, chenodeoxycholic acid; CA, cholic acid; TBA, total bile acid.

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TABLE 5  
Caecal bacterial population in rats fed bovine proteins for 4 weeks<sup>1</sup>

| Dietary group          | CON                                | AC                      | AR                      |
|------------------------|------------------------------------|-------------------------|-------------------------|
|                        | log <sub>10</sub> cfu/g wet caecum |                         |                         |
| <i>Bifidobacterium</i> | 5.97±0.83 <sup>b</sup>             | 6.30±0.49 <sup>ab</sup> | 6.65±0.32 <sup>a</sup>  |
| <i>Lactobacillus</i>   | 5.73±0.64 <sup>a</sup>             | 6.12±0.61 <sup>a</sup>  | 5.67±0.83 <sup>a</sup>  |
| <i>Coliform</i>        | 3.91±0.35 <sup>b</sup>             | 4.91±1.36 <sup>a</sup>  | 4.61±0.79 <sup>ab</sup> |
| Anaerobe               | 6.84±0.33 <sup>b</sup>             | 7.48±0.35 <sup>a</sup>  | 7.31±0.27 <sup>a</sup>  |

<sup>1</sup>Values are expressed as means±SD for five rats. <sup>a,b</sup>Means within the same rows bearing different superscript roman letters were significantly different (p<0.05 ).

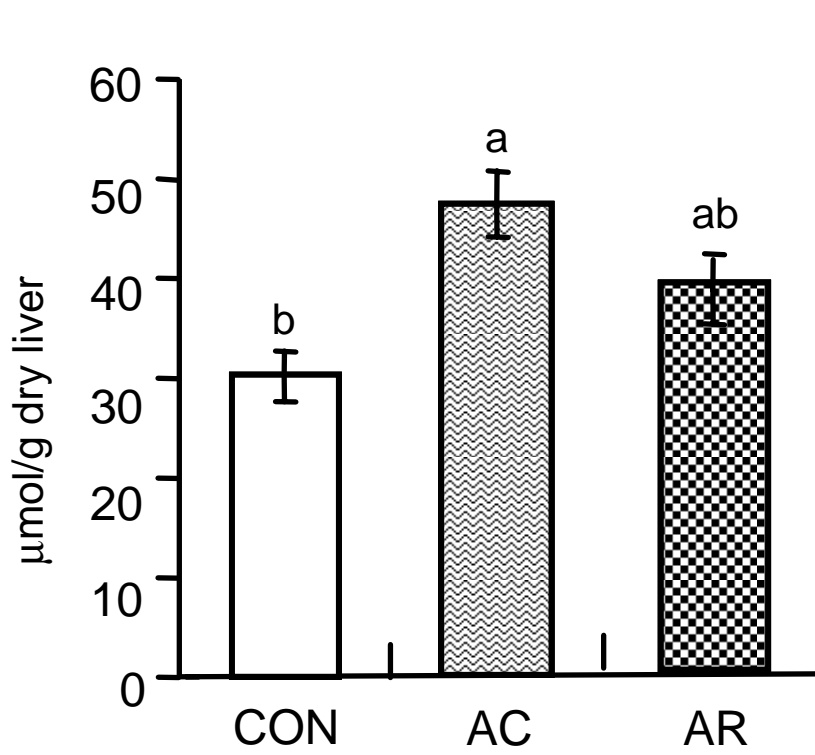
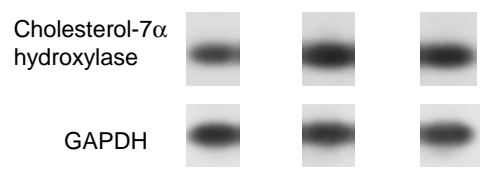
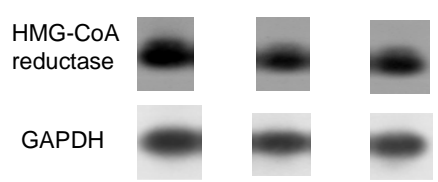


Figure:1 Liyanage Ruvini

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HMG-CoA reductase

Cholesterol 7α-hydroxylase

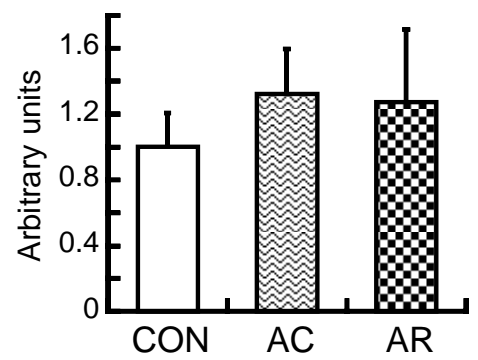
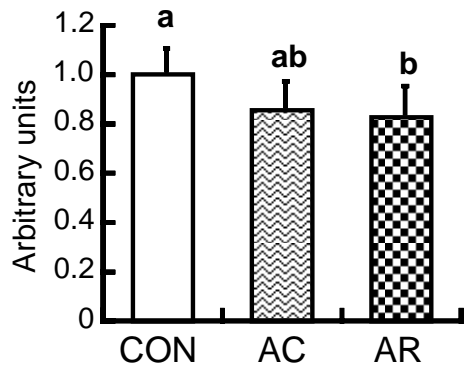


Figure:2 Liyanage Ruvini



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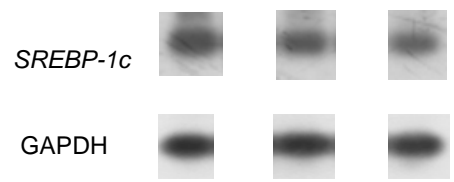
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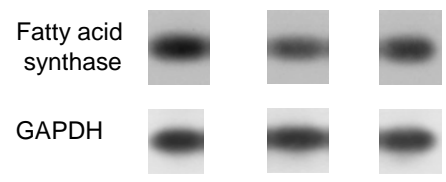
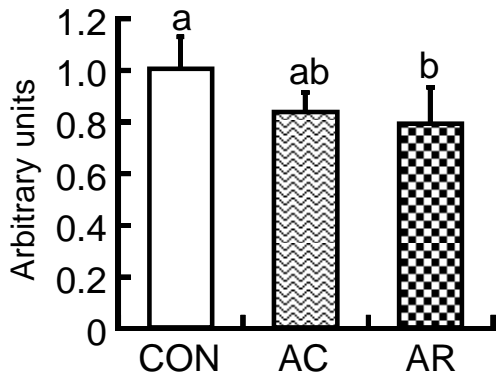
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**SREBP-1c**



**Fatty acid synthase**

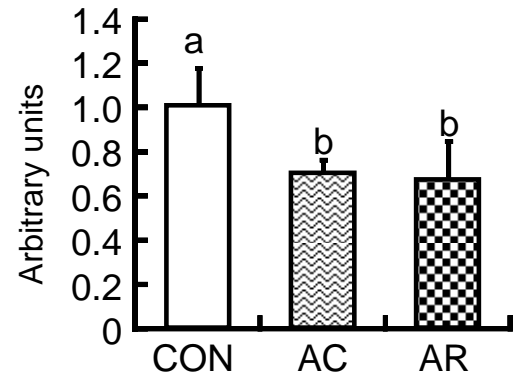


Figure:3 Liyanage Ruvini