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Effects of milk fermented by *Lactobacillus gasseri* SBT2055 on adipocyte size in rats

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Despite adequate scientific evidence of the potential benefits of probiotics to human health or disease prevention, their contribution to the growth of adipose tissue remains to be established. Four-week-old male Sprague-Dawley rats were fed a diet containing skim milk (control diet) or skim milk fermented by *Lactobacillus gasseri* SBT2055 (LGSP diet) for 4 weeks. Their body weight gain, adipose tissue weight, adipocyte size distribution profile, blood and hepatic lipids, and serum leptin, glucose and adiponectin levels were determined. There was a significant reduction in average adipocyte size in mesenteric white adipose tissue ($P=0{\cdot}004$). Moreover, the rats fed the LGSP diet displayed greater numbers of small adipocytes from mesenteric and retroperitoneal adipose tissues than did those on the control diet. Whereas adiponectin concentrations did not differ between the groups, serum leptin concentrations were decreased to 32 % in the LGSP diet group compared with the control group. Concentrations of serum glucose and lipids, and liver lipids, except for the liver TAG level, were similar in the two groups. These results indicate a possible role for a fermented milk product in the regulation of adipose tissue growth.

***Lactobacillus gasseri*: Probiotics: Leptin: Adipocyte size**

Probiotics were first used by Fuller and are defined as live microbial feed supplements which have a beneficial effect on human health through the gastrointestinal tract[1]. Most probiotic products such as yoghurts contain lactic acid bacteria[2]. Their effects include the prevention or amelioration of diarrhoea[3], prevention of cancer[4], anti-metabolic syndrome actions[5] and so on. *Lactobacillus gasseri*, in the genus of *Lactobacillus acidophilus*, is a major species of the human flora. Many healthy effects of *L. gasseri* have been reported. For example, inhibitory activity against some pathogenic and food-spoilage species[6], lowering of serum cholesterol concentrations[7], adjustments of the immune system[8] and an enhancement of intestinal functions[9].

Recently, the antilipolytic effect of probiotics has attracted the attention of the public[5,7]. In the prevention of metabolic syndrome, reduction of obesity is important. Recent studies revealed that adipose tissue secretes cytokines referred to as adipocytokines. Adipocytokines, such as leptin and adiponectin, are known to act as a regulator of energy homeostasis[10,11]. Generally, the concentration of leptin in serum is positively associated with increases in the weight of adipose tissue and adipocyte size[12] while the serum adiponectin level is negatively associated with adipose tissue weight[13]. Leptin functions as part of a feedback mechanism that suppresses appetite through its receptor at the hypothalamus[14]. Adiponectin has an important role in carbohydrate and lipid metabolism[11]. In studies of probiotics, there are few reports about their effect on adipose tissue and these adipocytokines.

From the perspective of preventing metabolic syndrome, it is important to consider lipid metabolism and its regulation. It has already been reported that the administration of *L. gasseri* has a beneficial effect on serum cholesterol concentrations in hypercholesterolaemic rats[7]. However, there is no information about the effect of *L. gasseri* on serum and liver TAG metabolism which underlies the excess accumulation of TAG in adipose tissue. Furthermore, leptin has been reported to be involved in TAG accumulation in the liver[15]. Therefore, we investigated the effect of a milk product fermented by *L. gasseri* SBT2055 (LGSP) on serum lipid and adipocytokine concentrations and adipocyte size in white adipose tissues of rats.

Materials and methods

Preparation of the milk product fermented by Lactobacillus gasseri SBT2055

LGSP was prepared as described previously[16]. Briefly, skim milk powder (Snow Brand Milk Products Co. Ltd, Tokyo, Japan) was hydrated with deionized water, added to a yeast

Abbreviations: cfu, colony-forming units; LGSP, *Lactobacillus gasseri* fermented milk product.

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extract, and sterilized at 95°C for 30 min. After inoculating *L. gasseri* (Snow Brand Milk Products Co.), skim milk was incubated at 37°C for 16 h. The fermented products containing the bacterial cells were freeze dried and used for the subsequent experiments. Skim milk powder was also treated in a similar manner without inoculating *L. gasseri*. The chemical composition of either skim milk (34·7 % protein, 0·9 % fat, 52·6 % carbohydrate, 7·9 % ash and 3·9 % moisture) or fermented skim milk (35·4 % protein, 0·9 % fat, 52·6 % carbohydrate, 7·7 % ash and 3·4 % moisture) was almost the same. The latter also contained 11·8 g lactic acid/100 g. In addition, the concentration of viable *L. gasseri* in the final LGSP-containing diet was 6×10^7 colony-forming units (cfu)/g diet. According to Ouwehand & Salminen[17], probiotic microorganisms should be viable and abundant (at least 10^7 cfu/g) in the fermented milk to exert positive effects.

Animal and diets

Four-week-old male Sprague-Dawley rats were obtained from Kyudo Co. (Kumamoto, Japan). The animals were housed individually in stainless steel cages in an air-conditioned room (21–24°C, lights on 08.00–20.00 hours). Experiments were carried out according to the Guidelines for Animal Experiments of the Faculty of Agriculture and the Graduate Course, Kyushu University, Fukuoka, Japan, and Law No. 105 and Notification No. 6 of the Government of Japan.

Before the experiments, all rats were allowed free access to commercial chow for a week, and then divided into two groups. Experimental diets were prepared according to the AIN-76 formula[18] with some modifications, containing in g/kg: 100 fat (90 lard and 10 maize oil), 200 skim milk powder or fermented milk powder (LGSP), 125 casein, 150 α-maize starch, 50 cellulose, 3 DL-methionine, 35 mineral mixture (AIN-76), 10 vitamin mixture and sucrose to 1000 g. The diets containing skim milk powder and fermented milk powder were designated the skim milk diet and LGSP diet, respectively. The quantity of protein supplied from the fermented and non-fermented skim milk powder was 70·8 and 69·4 g/kg diet, respectively. Therefore, the total protein content in the skim milk (194 g/kg diet) and LGSP (195 g/kg diet) diets was similar. These experimental diets were given to rats for 4 weeks with pair-feeding. The rats were killed without fasting after the feeding period by withdrawing blood from the abdominal aorta under diethyl ether anaesthesia. The liver and white adipose tissues (mesenteric, perirenal, retroperitoneal and epididymal) were excised and weighed. The livers were kept at −20°C until the analyses.

Methods of analysis

Analyses of lipids, glucose and cytokines. The serum TAG, total cholesterol, HDL-cholesterol, phospholipid and glucose levels were measured using enzyme assay kits (Triglyceride E test, Phospholipids C test and Glucose C test from Wako Pure Chemicals, Osaka, Japan; Determiner TC555 from Kyowa Medix, Tokyo, Japan). Serum adiponectin and leptin concentrations were measured using ELISA kits (Mouse/rat adiponectin ELISA kit from Otsuka Pharmaceutical, Tokyo, Japan; rat leptin ELISA from Yanaihara Institute, Shizuoka, Japan). Liver lipids were extracted by the method of Folch *et al.*[19]. The TAG level was determined by the method of Fletcher[20], the total cholesterol level by the method of Sperry & Webb[21] and the phospholipid level by the method of Wootton[22].

Measurement of adipocyte cell size. Adipocyte cell sizes were measured as described elsewhere[23]. In short, mesenteric, retroperitoneal and epididymal white adipose tissues rinsed with saline solution were fixed in 10 % neutral formalin buffered solution, embedded in paraffin, cut into 10 μm sections and stained with haematoxylin. Cell sizes were measured by NIH-image (100 cells/rat).

Statistics

The data were expressed as means with standard errors and analysed using Student's *t* test, taking the statistical difference to be $P<0{\cdot}05$. The analysis was carried out with Excel 2002 (Microsoft, Redmond, WA, USA).

Results

Body and organ weights as well as several metabolic and morphometric parameters were determined in the two rat groups (Table 1). Body weight gain was similar between the two groups. There was no effect of the LGSP-containing diet on the adipose tissue and liver weights. However, there was a tendency for mesenteric adipose tissue to be lighter in the LGSP-fed group ($P=0{\cdot}104$). There was no significant effect of the LGSP-containing diet on serum lipid and glucose levels. There was no significant effect of LGSP on liver lipid levels except for a significant decrease in liver TAG levels ($P=0{\cdot}009$). Serum leptin concentrations were drastically decreased (32 %, $P=0{\cdot}031$) in the LGSP-fed group compared with the control group. By contrast, there was no significant effect of the LGSP-containing diet on adiponectin concentrations.

Moreover, the LGSP-containing diet affected average adipocyte size (Table 1) and its distribution in white adipose tissues (Fig. 1). Feeding on the LGSP-containing diet was associated with a reduction in total adipocyte size in mesenteric (22 %, $P=0{\cdot}004$) and retroperitoneal (18 %, $P=0{\cdot}053$) adipose tissues compared with the control group. Furthermore, the number of smaller adipocytes was increased in mesenteric, retroperitoneal and epididymal adipose tissues, while the number of large adipocytes was decreased in mesenteric and retroperitoneal adipose tissues when rats were fed the LGSP-containing diet (Fig. 1).

Discussion

Several studies have shown the health-promoting effects of fermented milks[2–5]. In the present study, when skim milk was fermented by *L. gasseri* (LGSP), there was no significant difference in adipose tissue weight between the rats fed the LGSP-containing diet and those fed the skim milk-containing diet. However, the LGSP-containing diet led to a greater decrease in adipocyte size in the mesenteric and retroperitoneal adipose tissues than did the skim milk-containing diet. This is the first report that a fermented milk product containing *L. gasseri* regulates the size of adipocytes. Likewise in the fermented milk group, there was a decrease in the number of

Table 1. Effect of diets containing skim milk (SM) and fermented milk (LGSP) on morphometric and metabolic parameters (Mean values with their standard errors for seven rats per group)

	SM		LGSP		
	Mean	SE	Mean	SE	*P*
Food consumption (g/d)	22·6	0·7	21·9	0·6	0·424
Body weight gain (g)	219	8	199	10	0·136
Liver mass (g)	18·3	0·5	16·7	0·8	0·122
Mesenteric fat mass (g)	7·5	0·7	5·9	0·6	0·104
Perirenal fat mass (g)	2·1	0·3	1·8	0·4	0·542
Retroperitoneal fat mass (g)	6·1	0·6	5·4	0·5	0·347
Epididymal fat mass (g)	7·6	0·5	6·4	0·7	0·141
Mesenteric adipocyte size ($\mu m^2 \times 10^3$)	3·06	0·16	2·39	0·11	0·004
Retroperitoneal adipocyte size ($\mu m^2 \times 10^3$)	5·10	0·32	4·21	0·27	0·053
Epididymal adipocyte size ($\mu m^2 \times 10^3$)	4·26	0·24	3·59	0·36	0·153
Serum TAG (mg/dl)	666	110	494	116	0·304
Serum phospholipids (mg/dl)	224	17	209	15	0·506
Serum total cholesterol (mg/dl)	69·3	4·1	73·9	3·8	0·420
Serum HDL-cholesterol (mg/dl)	20·7	1·8	26·7	4·8	0·266
Serum glucose (mg/dl)	247	11	215	13	0·100
Serum leptin (ng/ml)	1·39	0·15	0·94	0·12	0·031
Serum adiponectin (μg/ml)	4·01	0·32	4·12	0·17	0·768
Liver TAG (mg/g)	44·6	5·2	25·6	3·3	0·009
Liver phospholipids (mg/g)	26·8	0·5	27·7	0·7	0·325
Liver total cholesterol (mg/g)	2·9	0·2	3·0	0·3	0·920

larger adipocytes (except in epididymal tissue) with an increase in the number of smaller adipocytes in all white adipose tissues. The present findings raise the possibility of an anti-obesity effect of LGSP, since adipose tissue mass is closely linked to either the number or the size of fat cells. In fact, the decrease in adipocyte size is considered to help in preventing obesity due to the inhibition of hypertrophy[23] and hyperplasia[24]. Furthermore, Marques *et al.*[25] suggested that enlarged adipocytes secrete growth factors that trigger adipogenesis through preadipocyte differentiation. Alternatively, adipocyte size in the subcutaneous abdominal depot was identified to be a significant predictor for the future development of diabetes mellitus type 2[26]. In this context, it is worth determining if the fermented product plays a role in regulating the development of diabetes mellitus type 2.

One of the differences between the fermented milk and non-fermented milk in the present study was the presence of live bacteria in the former. The amount of *L. gasseri* SBT2055 was 6×10^7 cfu/g diet which appeared to be enough to exert a positive effect[17]. A recent report revealed that *L. gasseri* SBT2055 has the ability to survive in the gastrointestinal tract and alters the composition and metabolism of the intestinal microflora[27]. In our preliminary study with an animal model of obesity using rats, there was a significant difference in faecal NEFA content (26·1 (SE 1·2) and 30·3 (SE 1·5) mg/d for skim milk and fermented skim milk diets, respectively, $P=0·03$). Although the mechanism involved has not been fully clarified, live *L. gasseri* has been reported to bind directly to cholesterol, thereby interrupting its absorption in the intestine[7]. Therefore, it is unclear if *L. gasseri*-containing fermented products disrupt the degradation and absorption of TAG in the intestine. Alternatively, it remains unexplored whether fermented products influence the degradation of otherwise indigestible components such as dietary polysaccharides, thereby having an impact on the energy balance. Turnbaugh *et al.*[28] presented evidence that the distal gut microbiota of genetically obese mice has an increased capacity to harvest energy from diet. The result identifies the gut microbiota as an additional factor contributing to the pathophysiology of obesity.

Lactic acid might have antiperoxidative action in rats fed the lactic acid-containing diet[29]. In addition, oral administration of a diet based on wheat bread baked with lactic acid improved glucose tolerance in rats compared with a diet of wheat bread alone[30]. These previous findings might have some relationship with the lowering effect of LGSP on both adipocyte size and the level of TAG in liver in the present study. However, we cannot explain how the antiperoxidative action of dietary lactic acid has a beneficial effect.

The adipocyte hormone leptin is a cytokine, which elicits a pro-inflammatory immune response[31]. In the present study, serum leptin concentrations were lower when the rats were fed the LGSP-containing diet. According to studies in rodents[12] and man[32], the expression and release of leptin depend on adipocyte size. Therefore, it is likely that a greater proportion of smaller adipocytes in the rats fed the fermented product than in the control diet-fed rats is responsible for the lower concentration of leptin. Adiponectin, which is exclusively produced by adipocytes, is considered to be anti-inflammatory[33]. Plasma levels of adiponectin are generally reduced in obese individuals[13]. In contrast to leptin, there was no significant difference in the concentration of adiponectin in serum between the LGSP-fed rats and the control diet-fed rats. One possible reason why the response of these adipokines to the dietary fermentation product differs may be due to the amount produced by the adipocytes, because the release of chemokines from freshly isolated adipocytes is much greater for adiponectin (ng/μm^2) than leptin (pg/μm^2)[34]. Alternatively, the response of the serum adiponectin level to the size of adipocytes may not be as sensitive as that of the leptin level. In fact, the secretion of leptin from cultured adipocytes was positively correlated with cell size before as well

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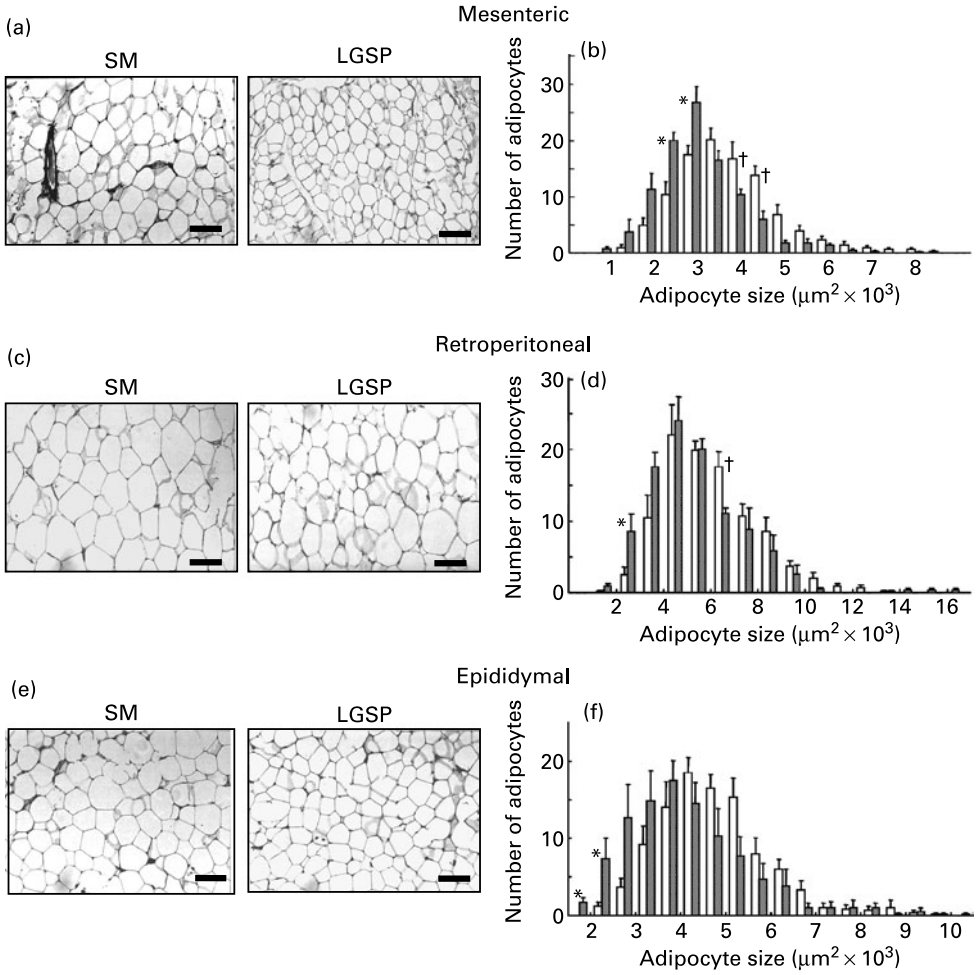

Fig. 1. The effect of dietary skim milk (SM) and fermented milk (*Lactobacillus gasseri* fermented milk product, LGSP) on cell size in white adipose tissues. Adipocytes in paraffin sections of mesenteric (a), retroperitoneal (c) and epididymal (e) white adipose tissues (scale bar: 100 μm). Profile of the distribution of cell size for adipocytes from mesenteric (b), retroperitoneal (d) and epididymal (f) white adipose tissues (□, SM; ▨, LGSP). Values are means with their standard errors depicted by vertical bars for seven rats per group. Mean values for small adipocytes were significantly higher in the LGSP group than SM group: *$P<0{\cdot}05$. Mean values for large adipocytes were significantly lower in the LGSP group than SM group: †$P<0{\cdot}05$.

as after the correction of cell surface area. However, adiponectin that correlated with adipocyte cell size lost its association after the correction of cell surface area[35].

Leptin exerts anorectic effects through its hypothalamic receptor[36]. However, several lines of evidence indicate that leptin's actions are not the result of its anorectic effects alone. For instance, leptin may have a central role in preventing the accumulation of hepatic TAG through the regulation of fat synthesis and its distribution and by modulating hepatic β-oxidation[37]. In the present study, the LGSP-containing diet resulted in a greater decrease in the level of liver TAG than did the control diet though there was no significant difference in the serum TAG levels. Therefore, the present results suggest that the decreased leptin level in the rats fed the LGSP-containing diet does not have an adverse effect on liver and serum lipid metabolism.

In summary, the present results indicate that the fermented milk product containing *L. gasseri* SBT2055 may exert a beneficial effect on the onset of obesity by influencing the size of the cells from visceral adipose tissues. The effect of LGSP on adipose tissue growth should be evaluated under various conditions including a high-fat diet and in an obese animal model.

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