Health-promoting effects of lactic acid bacteria and probiotics

―Their antiobesity and lipid lowering effects―

Yasunori Yonejima

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Table of content

Chapter 5 General discussion and conclusion

Chapter 1
General introduction

1-1 Role of intestinal microbiota in host health

Large numbers of bacteria, as much as 10^{12} CFU of bacteria in 1g of digesta, are present in the human intestine. They constitute a complex microbial community that interacts with host intestinal tissues to secrete protective molecules such as IgA and defensin peptides (Masuda *et al*., 2011). Indigenous microbiota have the potential to inhibit colonization of pathogens (Stecher and Hardt, 2008). Indeed, traveller's diarrhea is a typical result of imbalanced intestinal microbiota (Mathewson *et al*., 1985).

In addition to host defense, intestinal microbiota can attenuate host nutrition with the modification of hormone secretions (Cani *et al*., 2009). Since intestinal tissues secrete several peptide hormones that influence movement of the digestive tract as well as the appetite of the host (Naslund *et al*., 1999), the connection between intestinal microbiota and energy homeostasis of the host is increasingly recognized. Some studies have indicated that altered intestinal microbiota are associated with obesity. For example, the number of Firmicutes was increased, whereas the number of Bacteroidetes was reduced, in obese mice and humans compared with lean individuals (Ley *et al*., 2005; Turnbaugh *et al*., 2006; Ley *et al*., 2006; Furet *et al*., 2010). Children becoming overweight by 7 years of age had lower levels of bifidobacteria and higher levels of *Staphylococcus aureus* during the first year of life than infants maintaining a healthy weight (Kalliomäki *et al*., 2008).

In this context, a certain modification of intestinal microbiota can deteriorate or improve human health. The existence of a better composition of intestinal microbiota has been supposed to be true. Restoration or remodification of intestinal microbiota is therefore believed to be effective to prevent or even cure the deteriorated status of host health.

1-2 Probiotic for enteric disorders

A bacterium that provides specific health benefits when consumed as a food component or supplement has been referred to a probiotic. Typical probiotics are those belonging to genera *Lactobacillus* and *Bifidobacterium*. Probiotics have the potential to modify the composition of intestinal microbiota (Ohashi *et al*., 2001). Accordingly, it is widely recognized that probiotics can restore deteriorated microbiota. *Lactobacillus rhamnosus* GG can cure traveller's diarrhea (Oksanen *et al*., 1990; Sazawal *et al*., 2006). In other cases, probiotics can prevent infection of some enteric pathogens (Table 1-1).

The probiotic bacterium *L. rhamnosus* GG has been applied to rotavirus-infected children to cure or prevent diarrhea by stimulation of specific IgA responses to the rotavirus (Majamaa *et al*., 1995).

On the other hand, non-pathogenic and non-life-threatening enteric disorders such as severe constipation can be cured or prevented by probiotics (Table 1-2). *Enterococcus faecalis* EC12 enhances chloride secretion from the intestine through induction of Na^+-K^+-$2Cl^-$ co-transporter 1 (NKCC1) in enterocytes to increase the water content of digesta, which improves the constipation (Inoue *et al*., 2007).

In addition to enteric disorders, probiotics attract attention for their preventive effect on allergies (Table 1-3).

1-3 Relation between intestinal microbiota and obesity

Obesity is defined as the excess accumulation of visceral adipose tissue (WHO, 2011) which is induced by a lack of exercise and improper dietary habits. Visceral fat accumulation is a major risk factor for the development of diabetes, hyperlipidemia, hypertension, and arteriosclerosis. Intestinal microbiota have a role in the host's metabolic homeostasis (Cani *et al.*, 2009). According to Cani *et al.*, an altered composition of the intestinal microbiota is associated with type 2 diabetes mellitus and obesity. Since obesity is inevitably associated with low-grade inflammation, a bacterial compound, which is an inducer of inflammation, might act as a triggering factor in the development of obesity. Cani suggested that this bacterial compound might be lipopolysaccharide (LPS), a component of the cell wall of gram-negative bacteria, because the mice lacking the major LPS co-receptor CD14 were resistant to high-fat-diet-induced inflammation and metabolic diseases. Additionally, in high-fat-diet-fed mice, dietary modulation of intestinal microbiota to increase bifidobacteria reduced endotoxemia as well as reducing inflammation development. This finding suggests that intestinal microbiota contribute to the pathophysiological regulation of endotoxemia (Cani *et al.*, 2007).

On the other hand, short-chain fatty acids have been proposed to bind to specific G-protein-coupled receptors (GPR43), which contributes to an increase in adipocyte differentiation and inhibits lipolysis in the adipose tissue. So short-chain fatty acids (SCFA) produced by probiotic bacteria could act as metabolic regulators of energy uptake in adipose tissue (Kimura *et al.*, 2013).

1-4 Aim of this study

As discussed above, the potential of probiotics as health promoters is now widely

accepted, evidenced by the huge variety of commercial products with a successful sales record. Probiotics are composed mainly of lactic acid bacteria; as they are common in traditional fermented foods, the safety of the products is quite obvious. This situation may have helped to increase the market for probiotics.

However, the mode of its action has not been clarified so far in many cases. This is because probiotics show effects on disorders not only directly but also indirectly. Interaction with the host's natural immune system is defined as direct action of probiotics. Therefore, involvement of a Toll-like receptor (TLR) in the health-beneficial effect of probiotics has been discussed frequently. Th1 and Th2 cytokine balance was once discussed (Shida *et al*., 1998), and an induction of T-regulatory cells was the focus of recent probiotic studies (Torii *et al*., 2007). Secretion of beneficial products such as SCFA or growth promotion of other indigenous health-beneficial bacteria is defined as indirect action of probiotics (Ohashi *et al*., 2001).

The aims of this study were to investigate (1) the effect of a tablet containing probiotic lactobacilli and medicinal herbs on the fecal microbiota in humans, (2) the effect of a probiotic, *L. gasseri* NT, on lipid metabolism in normal mice, (3) in an obese model, and to make clear the targeted organs of affection.

Table 1-1 Improvement of pathogen-induced diseases by probiotics in human or mice model

Probiotic species	Dose	Human/ mice	Pathogens	Results	References
L. rhamnosus GG	Fermented milk and powder (10^{10}-10^{11})/day	Human	RV	Period of diarrhea ↓	Isolauri *et al*., 1991
L. rhamnosus GG	Fermented milk and powder (10^{10}-10^{11})/day	Human	RV	Period of diarrhea ↓	Kaila *et al*., 1992
B. bifidum *S. thermophilus*	1.9×10^{8} CFU/g 1.4×10^{7} CFU/g	Human	RV	Morbidity of diarrhea ↓	Saavedra *et al*., 1994
L. rhamnosus GG	1×10^{10} CFU/day	Human	RV	Period of diarrhea ↓	Isolauri *et al*., 1994
L. rhamnosus GG	1.9×10^{8} CFU/g		RV	Period of diarrhea ↓, IgA ↑	Majamaa *et al*., 1995
L. reuteri	10^{7} or 10^{10} CFU/g	Human	RV	Period of diarrhea ↓	Shornikova *et al.*, 1997
B. breve YIT4064	5×10^{10} CFU/day		RV	Infection of RV ↓	Araki *et al.*, 1999
L. acidophilus *B. infantis*	10^{9} viable/day 10^{9} viable/day	Human	RV	Period of diarrhea ↓	Lee *et al.*, 2001
L. rhamnosus *L. reuteri*	10^{10} CFU/day 10^{10} CFU/day	Human	RV	Period of diarrhea ↓	Rosenfeldt *et al.*, 2002
C. butyricum	10^{8} CFU/day	Mice	EHEC	Survival rate ↑ Shigera toxin ↓	Takahashi *et al.*, 2004
B. longum BB536	NR	GF mice	EHEC	Survival rate ↑ EHEC ↓	Namba *et al.*, 2003

RV: rotavirus; EHEC: enterohemorrhagic *E. coli*

Table 1-2 Effects of probiotics on constipation in humans

Probiotic species	Dose	Results	References
B. longum BB536	Fermented milk (2×10^9/day)	Improvement defecation frequency ↑	Yaeshima *et al.*, 1997
L. casei Shirota	Beverage (6.5×10^9/day)	Improvement	Koebnick *et al.*, 2003
B. lactis DN-173	Fermented milk (1.25×10^{10}/day)	Improvement	Yang *et al.*, 2008

Table 1-3 Preventive effects on allergies by probiotics

Probiotic species	Dose	Type of allergy	Results	References
L. paracasei	Fermented milk (2×10^9 CFU/day)	Perennial allergic rhinitis	Improved quality of life	Wang *et al.*, 2004
B. longum BB536	Powder (5×10^{10} CFU/day)	Cedar pollen	Reduced ocular symptom scores	Xiao *et al.*, 2007
L. acidophilus L-55	Yogurt	Cedar pollen	Reduced ocular symptom scores, IgE ↓	Kimura *et al.*, 2012
L. casei Shirota	Fermented milk	Seasonal allergic rhinitis	IgE ↓ , IgG ↑	Ivory *et al.*, 2008
L. rhamnosus GG	Powder in capsule (1×10^{10} CFU/day)	Atopic disease	Morbidity of atopy ↓	Kalliomäki *et al.*, 2001
L. fermentum VRI-033	1×10^9 CFU/day	Atopic disease	Improvement of SCORAD	Weston *et al.*, 2005
B. breve M-16V	Powder	Atopic disease	Bifidobacterium ↑ Improvement of SCORAD	Taniuchi *et al.*, 2005

Chapter 2
Effect of a tablet containing probiotic bacteria and stomachic herbs on human fecal microbiota

2-1 Introduction

Probiotic bacteria, namely lactobacilli and bifidobacteria, are now well recognized for their health promoting effects (Macfarlane *et al.,* 1999). Administration of probiotics is often realized orally by a single dose, although sometimes they are dosed with medicinal plant materials. Lactic acid bacteria react with gut epithelial tissues to affect the mucosal immunity through the stimulation of toll-like receptors (TLRs). Lactic acid bacteria affect the host physiology through chloride secretion from the gut, to increase water contents of the digesta (Inoue *et al.,* 2007) , and modification of the sympathetic and parasympathetic nervous systems, to reduce blood pressure and blood sugar levels (Tanida *et al.,* 2005). Probiotics also strongly affect the host's indigenous intestinal microbiota (Ohashi *et al.,* 2001, Ohashi *et al.,* 2006). Indeed, they promote different colonic fermentation patterns (Ohashi *et al.,* 2004). Probiotics, more precisely fermented milk, may affect the gastric emptying, reducing the digesta transit (Ohashi *et al.,* 1997). Digesta kinetics have strong influences on the digestion, therefore the administration of probiotics can modulate the amount of material flowing into the large intestine. The availability of probiotics should modify the composition of the microbiota. Medicinal plants such as swertia, cinnamon, and fennel, have long been administered to patients with digestive troubles. They engage with the host's digestive physiological systems, though the mechanisms are undefined. The introduction of these medicinal plants into probiotic therapies may modulate the effects of probiotics. In this chapter, the effect of a tablet containing both probiotic and medicinal herbs on the fecal microbiota is shown in human volunteers.

2-2 Materials and Methods

Six healthy volunteers (4 men and 2 women, aged from 24 to 55 years old, average age : 36) were recruited from Nitto Pharmaceutical Industries, Ltd. to test a tablet containing probiotics and medicinal plants. Experiments were conducted in accordance with the Declaration of Helsinki. All subjects were informed of the details of the present study and gave their consent to participate. No restrictions of food choice were imposed on the subjects throughout the study, except prohibition of ingestion of fermented milk, fermented soybeans and pickles the day before the sampling. The tablet is a probiotic product, the Guard (Kowa Shinyaku Co. Ltd. Japan) and contains *Bacillus natto*

NT(*Bacillus natto*)(10^8 cfu/9 tablets), *Lactobacillus acidophilus* NT (Lactomin) (10^9 cfu/9 tablets), dimethylpolysiloxane, powdered swertia herb, powdered cinnamon bark, powdered fennel, methyl methionine sulfonium chloride, precipitated calcium carbonate, magnesium carbonate (Table 2-1). The 6 healthy subjects took 3 tablets, 3 times a day after meals for 10 days. Stool samples were collected before and after the administration period. An additional sampling was made 7 days after the end of administration. Fresh feces were transferred into sterile plastic tubes (50 ml) containing 30 ml of ethanol to maintain an ethanol concentration in excess of 70%. Samples of feces were kept at 5°C until the analyses, which were performed within three months of collection. After homogenization, a portion of the suspension was centrifuged (6,700×g, for 10 min, 4°C) and the pellets were collected. Approximately 0.2 g of pellets were washed twice with 1×phosphate-buffered saline (PBS). Bacterial DNA was extracted from the pellets using ISOFECAL for Beads Beating kit (NIPPON GENE Co. Ltd) and FastPrep FP120 (BIO101 Co. Ltd). All individual samples of bacterial 16S rRNA gene were subjected to temperature gradient gel electrophoresis (TGGE) analysis as described elsewhere (Inoue *et al.,* 2003, Muyzer *et al.,* 1993). The gel image was taken by a densitometer (GS-800; Bio-Rad, California, USA). The profiles were analyzed using a software package for imaging and analyzing electrophoresis gels (Quantity one, Bio-Rad, California, USA), and cluster analysis of the TGGE band profiles was performed using hierarchical clustering analysis with Euclidean square distances using the Microsoft Excel macro program developed by S. Aoki (Gunma University, Maebashi, Japan; program available at http://aoki2.si.gunma-u.ac.jp/lecture/stats-by-excel/vba/html/clustan.htm). A corresponding dendrogram was constructed with Ward linkages. In this study, very weakly stained bands with an optical density (OD) of <0.1 as determined by the software were ignored, because on occasion such bands cannot be identified automatically by the software and this could result in artifacts in the analysis. The density of the bands, as determined by the software, was not used in the analysis. If a lane had a band at a certain position, an assignment of "1"was made; if there was no band at the same position, an assignment of "0"was made. A mixture of 16S rRNA gene of known bacteria (Inoue *et al.,* 2003) was applied to the TGGE gels as standard markers. *B.natto* NT and *L. acidophilus* NT were also used as standards. Twenty one bands on the TGGE gel were excised and put into 20μl of sterile water. The excised gel was left in water overnight at 4℃ to elute the DNA (Ohashi *et al.,* 2004). These bands (indicated in Fig. 2-3) were selected because their densities had changed remarkably between before and after the probiotic administration. After the extraction step was performed, a portion of eluted DNA solution was amplified with primers 968F, which

do not contain the 40 bases GC clump and univ-R. The PCR conditions were principally the same as described elsewhere (Inoue *et al.,* 2003) except for the cycle number: 30 cycles. The size and amount of the PCR products were confirmed by analyzing all samples by 1.5% agarose gel (wt/vol) electrophoresis and ethidium bromide staining. The obtained bands were purified using Wizard SV Gel and PCR Clean-UP System (Promega, Tokyo, Japan). Purified products were sequenced after TA-cloning, as described elsewhere (Ohashi *et al.,* 2004). Briefly, PCR amplicons were ligated into pGEM-T vector (Bio-Rad, Tokyo, Japan) and *Escherichia coli* JM109 was transformed. Clones harboring amplified DNA were selected by blue-white screening, and 8 white clones were randomly selected from each plate (one plate for each TGGE band). Colony PCR using SP6 and T7 primers was performed to check the size of inserted DNA with r-Taq polymerase. The following thermal cycle was applied: 94°C for 3 min of initial denaturation followed by 25 cycles of 94°C for 30 sec, 48°C for 30 sec, and 72°C for 50 sec, with a final elongation at 72°C for 3 min. Restriction fragment length polymorphism (RFLP) analyses of PCR products were performed with *Hae* Ⅲ(Toyobo, Tokyo, Japan), *Hha* Ⅰ (Toyobo), and *Rsa* Ⅰ (Toyobo) according to the manufacturer's instructions to group the obtained *E.coli* clones. Although several clone groups were recognized for each excised TGGE band, the most abundant clone group was selected and the insert of one randomly selected clone from the clone group was sequenced. Sequencing was performed at Shimadzu Genomic Research, Co. Ltd. (Kyoto, Japan). Sequences were compared via the BLAST program at NCBI (http://www.ncbi.nlm.nih.gov/blast) with those registered in databases (such as DDBJ/EMBL/ GenBank) to suggest possible taxonomic names. The relative abundance of bifidobacterial 16S rRNA gene in fecal samples was quantified with the Light Cycler system (Roche Diagnostics, Tokyo, Japan).The FastStart DNA Master SYBR Green I was used for PCR. The reaction mixture (20μl) contained 3 mM $MgCl_2$, 2μl of the 10×Mastermix, 20 ng of fecal DNA, and 0.5μM of each primer. For quantification of total bifidobacterial 16S rRNA gene, the primers g-Bifid-F (5'-CTCCTGGAAACGGGTGG-3') and g-Bifid-R (5'-GGTGTTCTTCCCGATATCTACA-3') were used (Matsuki *et al*., 1998). The thermal program consisted of initial denaturation at 95°C for 30 sec followed by 40 cycles of 95°C for 4 sec and 62°C for 25 sec. Dilutions of the genomic DNA from *Bifidobacterium longum* JCM7050 of known amount of viable count were used to construct calibration curves. These calibration curves were applied to the calculation of the total count of *Bifidobacterium* in fecal samples.

2-3 Results

The bacterial species, which may typically contribute to the change in the diversity of the intestinal microbiota, appearing as TGGE bands were detected. The detected bands are shown in Fig. 2-1.

The band profiles differing between before and after intake as evidenced by cluster analysis are shown in Fig. 2-2. According to the dendrogram created from the TGGE band profiles, profiles from D-after and E-after constructed one cluster. Others tended to construct one cluster for each subject such as A- before with A-after. According to the clustering analysis (Fig. 2-2), Subjects A, B, C and F formed one group, and subject D clustered with subject E. This result indicates that the intestinal microbiota of subjects D and E were relatively similar to each other and that the composition of microbiota was modified in a similar manner by the mixture of probiotics and herbs.

There was a band with increased density which was commonly shared by 4 subjects (A, D, E, F) below the bottom markers (Fig. 2-1). On the other hand, there were no remarkable changes in the bands which existed at the same position in lane *L. acidophilus* NT and *B. natto* NT for each subject. Twenty-one TGGE bands, indicated in Fig. 2-3, whose density changed remarkably were identified. Table 2-2 shows the results of sequence analyses of these TGGE bands. *Bifidobacterium*-like sequences were determined for band 1 to band 10; *Haemophilus*-like sequences were determined for band 11 and band 12; a *butyrate-producing bacterium*-like sequence was determined for band 13; an *Acidaminococcus*-like sequence was determined for band 14; *Ruminococcus*-like sequences were determined for band 15 and band 16; a *butyrate-producing bacterium*-like sequence was determined for band 17; a *Clostridium colinum*-like sequence was determined for band 18; a *Ruminococcus*-like sequence and a *Clostridium orbiscidens*-like sequence were determined for band 19; and *Megamonas*-like sequences were determined for band 20 and band 21.

In the case of subject A, the density of the bands identified as *Bifidobacterium* increased after the probiotic tablet administration. In the case of subject B, the density of the bands identified as *Haemophilus* and *butyrate-producing bacterium* decreased and the density of the band identified as *Acidaminococcus* increased after the administration. In the case of subject C, the density of the bands identified as *Ruminococcus* and *Bifidobacterium* decreased after the administration. In the case of subject D, the density of the bands identified as *butyrate-producing bacterium* and *Bifidobacterium* increased after the administration. In the case of subject E, the density of the bands identified as *Bifidobacterium* increased after the administration. In the case of subject F, the density of the bands identified as *Bifidobacterium*, *Megamonas* and

Ruminococcus or *Clostridium orbiscidens* increased and the density of the band identified as *Clostridium colinum* decreased after the administration.

2-4 Discussion

The estimated total cell numbers of bifidobacteria are listed in Table 2-3. The number of bifidobacteria increased after administration of the probiotic tablet in four subjects (A, D, E and F), decreased a little in one subject (C), and decreased in one subject (B).The TGGE results showed common and individual changes of intestinal microflora among the six subjects. In four cases out of six, the density of the bands identified with *Bifidobacterium* increased after administration of the probiotic tablet. This was confirmed by real-time PCR. The results indicate that intake of the probiotic tablet affected colonic environment and had a bifidogenic effect on at least four of the six subjects with different magnitudes. Individual differences in response to the tablet administration may reflect individual differences in composition of intestinal microflora. A similar observation was reported by Ohashi *et al* (Ohashi *et al.,* 2004) about the cecal fermentation pattern.

In the present experiment, for example, subjects D and E, both had relatively similar intestinal microflora as evidenced by the cluster analysis of their TGGE profiles. Bifidobacteria increased by some 30 to 40% after administration of the probiotic tablet. Subject F showed a similar level of increase (45%) in the size of the bifidobacterial population. Among the four positively responding subjects in terms of bifidobacteria, subject A showed a notable increase of 380%. Interestingly, subject A showed the smallest bifidobacterial population before the administration of the tablets. At the moment we do not know of any specific component of intestinal microflora other than bifidobacteria which would result in an increase in bifidobacteria.

Another particular modification in intestinal microbiota was the stimulation of lactate-utilizing bacteria such as *Acidaminococcus*, *butyrate-producing bacterium*, and *Megamonas*. These bacteria produced butyric acid or propionic acid from lactic acid. An increase in bifidobacteria may help higher lactate production, but it is important to convert lactate into short-chain fatty acids by lactate-utilizers, because butyrate and propionate have particular importance for the host health (Kameue *et al.,* 2006, Kishimoto *et al.,* 2006).

This type of human study needs a certain level of subject numbers due to the tremendous heterogenesity of human intestinal microbiota. Indeed, each person has his(her) own composition of microbiota. However, even with the limited number of subjects, there was no general tendency of modification of intestinal bacteria. In this

experiment, since only 6 subjects were available, the sample number was too small to draw a definitive conclusion. Individual differences in intestinal microbiota may be large enough to react with probiotics differently, because the interaction between indigenous intestinal bacteria and probiotic strains differ from one individual to another (Ohashi *et al.,* 2004, Ohashi *et al.,* 2006). In the case of rodent models, the effects of intestinal modifiers such as dietary prebiotics show a defined tendency as evidenced by clearly separated clusters (*i.e.,* administer and non-administer) (Maekawa *et al.,* 2005).

This is because the rodent model has a relatively well defined and simplified intestinal microbiota (Yanabe *et al.,* 2001). Such a condition cannot be achieved in human experiments. Therefore, substantial numbers of subjects are needed to draw a general conclusion about the effects of probiotics or prebiotics in human cases. This experiment was indeed a preliminary one. However, there were some changes about band profiles in all subjects and particularly stimulation of bifidobacteria was detected in four cases out of six. This can be considered as an indication of the potential of this mixture of probiotics and medicinal herb plants.

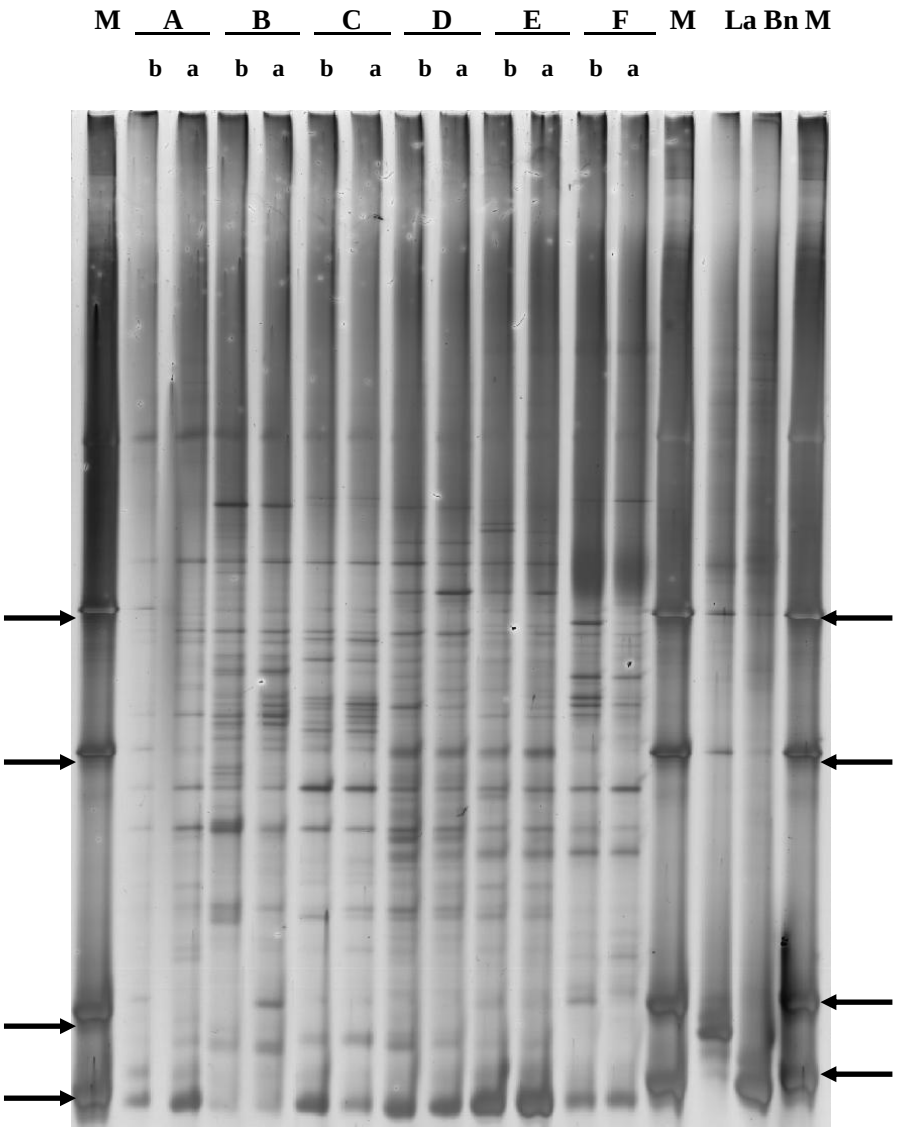


Fig. 2-1. Gel image of TGGE of bacterial 16S rRNA gene PCR product (rDNA) (developed by silver staining)

A～F : Subjects; La : *Lactobacillus acidophilus* NT 16S rDNA; Bn : *Bacillus natto* NT 16S rDNA; M : marker; b : Before administration; a : After administration. The markers consisted of four bacterial 16S rDNA prepared in our laboratory. The four horizontal arrows along with the standard marker lanes indicate the 16S rDNA bands corresponding those of *Ruminococcus hydrogenotrophicus* (Accession number of GenBank : X95624, homology 91%), *Clostridium sp*. (AF157053, homology 85%), *Escherichia coli* (A14565, homology 100%), and *Lactobacillus gasseri* strain KC5a (AF243165, homology 97%) from top to bottom.

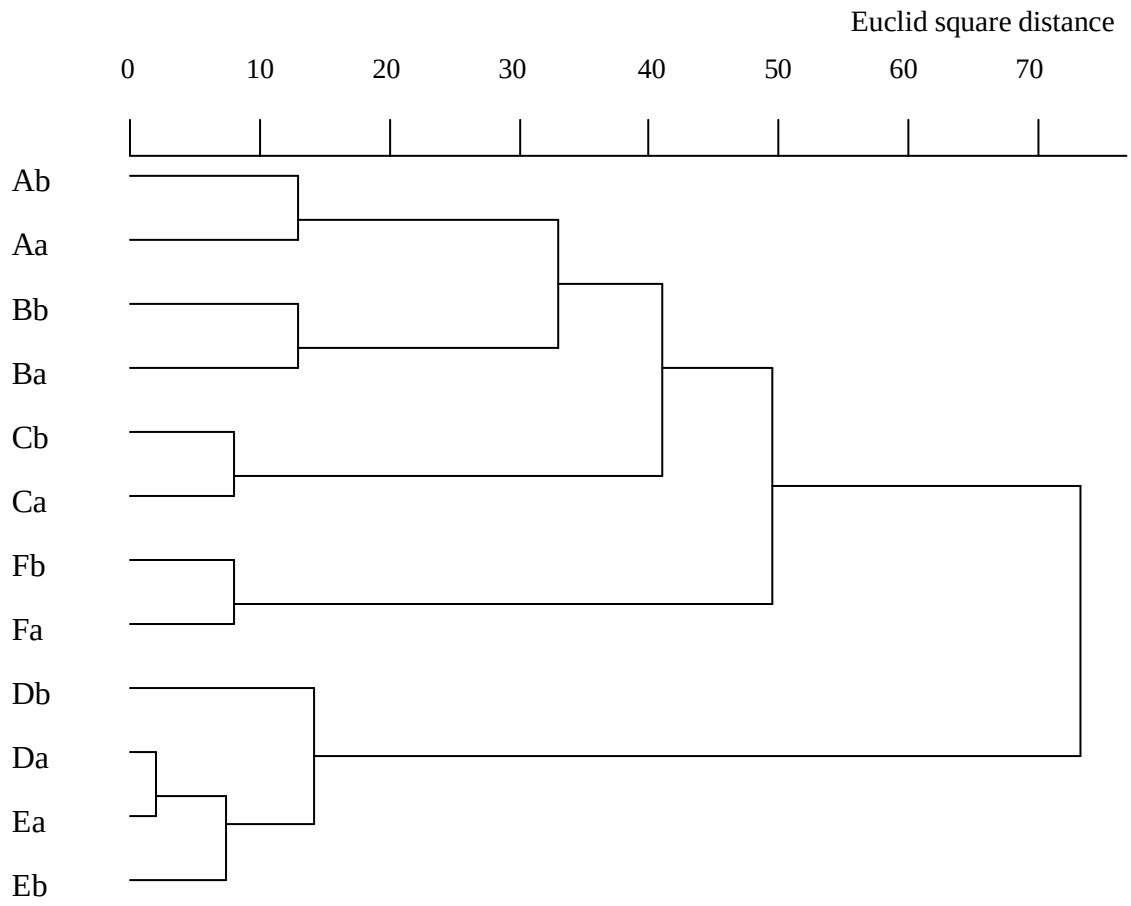


Fig. 2-2. Dendrogram obtained from hierarchical clustering of the TGGE band profiles.
A～F：Subjects; b：Before administration; a：After administration

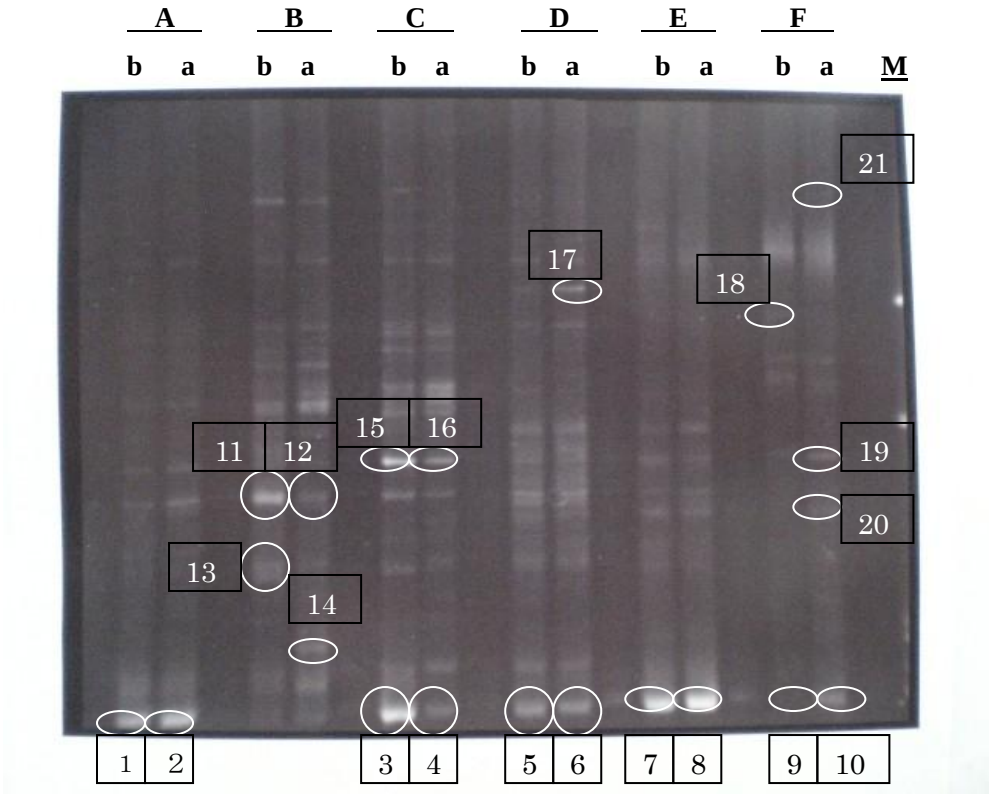


Fig. 2-3. Gel image of TGGE of bacterial 16S rRNA gene PCR product (developed by ethidium bromide staining). A～F : Subjects; M : marker; b : Before administration; a : After administration

Table 2-2.Results of BLAST searches for partial sequences of bacterial 16S rRNA gene PCR product

Subjects	Band No.	First match with known bacterium (Accession No.)	Identity (%)
A	1	*Bifidobacterium sp.* (AF306789)	100
		Collinsella aerofaciens (AJ245920)	99
	2	*Bifidobacterium longum* (EF589112)	100
		Lactobacillus sp (AB158766)	96
B	11	*Haemophilus parainfluenzae* (EU083530)	99
	12	*Haemophilus parainfluenzae* (EU083530)	99
	13	*Butyrate-producing bacterium* (AJ270469)	100
	14	*Acidaminococcaceae bacterium* (AB298734)	93
C	3	*Bifidobacterium* sp (AF306789)	99
	4	*Bifidobacterium* sp (AF306789)	100
		Bifidobacterium longum (EF589112)	100
	15	*Ruminococcus sp* (EF036467)	100
	16	*Ruminococcus sp* (EF036467)	100
D	5	*Bifidobacterium adolescentis* (AP009256)	98
		Bifidobacterium longum (EF589112)	99
		Clostridium sp (DQ100445)	91
	6	*Bifidobacterium adolescentis* (AP009256)	99
		Collinsella aerofaciens (AJ245920)	100
	17	*Butyrate-producing bacterium* (AJ27042)	94
E	7	*Bifidobacterium adolescentis* (AY305304)	99
	8	*Bifidobacterium longum* (EF589112)	100
F	9	*Bifidobacterium longum* (EF589112)	100
	10	*Bifidobacterium longum* (EF589112)	100
	18	*Clostridium colinum* (X76748)	92
	19	*Ruminococcus* sp (EF036467)	99
		Clostridium orbiscidens (Y18187)	98
	20	*Megamonas sp* (EU346729)	99
	21	*Megamonas sp* (EU346729)	97

Table 2-1. Ingredients of the tablet per nine tablets

Ingredient	dosage
Bacillus natto	10 mg
Lactomin	30 mg
Dimethylpolysiloxane	84.6 mg
Powdered Swertia Herb	30 mg
Powdered Cinnamon Bark	30 mg
Powdered Fennel	30 mg
Methyl Methionine Sulfonium Chloride	30 mg
Precipitated Calcium Carbonate	300 mg
Magnesium Carbonate	300 mg

Table 2-3. The relative abundance (log_{10}cell/g feces) of each operational taxonomic unit (OTU) related to bifidobacteria in the feces

Subjects	Before	After	Post
A	8.71	9.28	10.08
B	9.97	8.74	9.57
C	9.70	9.28	9.85
D	10.38	10.48	10.29
E	10.43	10.57	10.03
F	9.75	9.92	9.38

Before：Before administration; After：After administration; Post：Post-administrat

Chapter 3

Effect of lactic acid bacteria on lipid metabolism and fat synthesis in mice fed a high-fat diet

3-1 Introduction

Obesity is defined as the excess accumulation of visceral adipose tissue (WHO 2011) because of a lack of exercise and improper food habits. Visceral fat accumulation is a major risk factor for the development of several diseases, including diabetes, hyperlipidemia, hypertension and arteriosclerosis. Therefore, stimulation of lipolytic activity in adipose tissue or inhibition of fat synthesis is one way to prevent these serious diseases. For example, a dietary fiber, gum arabic, helps reduce body fat deposition by enhancing fat utilization in adipose tissues (Ushida *et al*., 2011). Higher lipolytic activity may be related to the higher expression level of beta 3 adrenaline receptor (ADRB3) in visceral adipose tissue. A decline in ADRB3 is, in fact, believed to contribute to the development of obesity (Fisler *et al*., 2008). On the other hand, the lipid composition of animal cells is controlled by SREBPs, transcription factors released from membranes by sterol-regulated proteolysis (Sakai *et al*., 1998). SREBPs are also known as a master regulator that participates in insulin resistance (Ide *et al*., 2004).

Probiotics, namely, lactobacilli and bifidobacteria, are now well recognized for their health-promoting effects (Macfarlane *et al*., 1999). In many cases, lactic acid bacteria react with the mucosal immunity of the gut to exert a physiological effect (Ohashi *et al*., 2009). For example, lactic acid bacteria stimulate chloride secretion from the gut to increase the water content of the digesta (Inoue *et al*., 2007) and adjust the sympathetic and parasympathetic nervous systems to reduce blood pressure and blood sugar levels (Tanida *et al*., 2005). The clearest effect was obtained in the improvement of hypercholesterolemia (Kondo *et al.*, 2010). The anti-obesity effect of lactic acid bacteria has also been reported. For example, Usman and Hosono (Usman 1999) indicated the capability to remove cholesterol from a culture medium with *Lactobacillus gasseri* strains. Portugal *et al*. (Portugal *et al*., 2006) suggested that the effect of *L. delbrueckii* on cholesterol metabolism was through Apolipoprotein E (ApoE) which is a cholesterol transporter. These health-promoting effects may be related to the anti-obesity effects of lactic acid bacteria. Indeed, the anti-obesity effects of lactic acid bacteria have been reported. Kadooka *et al.* (Kadooka *et al.*, 2010) indicated that administration of the probiotic bacterium *L. gasseri* in fermented milk reduced adiposity and body weight in obese adults, possibly by reducing lipid absorption and inflammatory status.

In this chapter, we evaluated the effects of the administration of lactic acid bacteria

on lipid metabolism and fat synthesis in a mouse high-fat-diet model focusing on visceral fat. In addition, the use of an *ex-vivo* test to measure ADRB3-dependent fat mobilization is introduced to better determine the responsiveness of adipose tissue to adrenergic stimulation, since ADRB3 expression is an indirect indicator of fat mobilization.

3-2 Materials and Methods

Animal experiments

Thirty 12-week-old female Balb/c mice were purchased from Japan SLC (Shizuoka, Japan). They were housed in five plastic cages (each containing six mice) in a room kept at 25±1°C with a 12-h light and dark cycle. The mice were divided into the following four groups: LaboMR (six mice fed a conventional crude and low fat diet, Labo MR Stock, Nihon Nosan Kogyo, Tokyo, Japan), 10%FD (six mice fed 10 kcal% fat diet, D12450B, Research Diets, Inc., New Brunswick, NJ, USA), 45%FD (nine mice fed 45 kcal% fat diet, D12451, Research Diets, Inc., New Brunswick, NJ, USA) and 45%FD+Lg (nine mice fed 45 kcal% fat diet, D12451, Research Diets, Inc.) including *Lactobacillus gasseri* NT(10^9 CFU/g). *L. gasseri* NT was originally isolated from human feces. The composition of each diet is shown in Table 3-1. The mice had free access to their diet and drinking water for 13 weeks. On the last day of feeding, the body weight and periovarian fat weight were measured. Periovarian fat, regarded as visceral adipose tissue, was removed through a midline incision. A portion of the periovarian fat was collected in RNAlater solution (Sigma, Japan) for mRNA analyses and in Hanks' Balanced Salt Solutions for the *ex vivo* test for lipolysis. A portion of the liver was removed and fixed in the RNAlater solution for mRNA analyses. Blood samples were collected from the inferior vena cava to measure the free fatty acid (FFA), triglyceride (TG) and insulin concentrations.

The experiments were approved by the Animal Experiment Committee of Kyoto Prefectural University.

Ex vivo test for lipolysis of visceral adipose tissue

The collected fat tissues were cut into pieces of ca. 20 mg and cultured in 96-well plates with a basal medium. The basal medium was composed of DMEM/Ham's F12 (Nacalai Tesque, Kyoto, Japan) containing 10% (v/v) bovine fetal serum (Zhang *et al.*, 2000) and 1% (w/v) Pen-Strep Solution (10,000 units/mL penicillin and 10,000 ug/mL streptomycin in 0.85% NaCl) (Thermo Fisher Scientific, Kanagawa, Japan). After three hours of incubation, glycerol released into media was measured with the use of a

Glycerol Assay Kit (Cayman Chemical Company, Ann Arbor, MI, USA).

Blood test

Serum FFA and TG levels were determined using a NEFA C-test (Wako, Japan) and TG E-test (Wako, Japan), respectively. Serum insulin was measured using a Mouse Insulin Kit (Morinaga Institute of Biological Science, Yokohama, Japan).

Determination of mRNA expression level

Extraction of RNA from the periovarian adipose tissue and the liver and subsequent cDNA synthesis was carried out as reported elsewhere (Yoshikawa *et al.*, 2009). The expression levels of each gene were evaluated by the real-time PCR approach using a LightCycler 480 Real-Time PCR System (Roche Applied Science, Tokyo, Japan). PCR was performed with a thermal cycle program with an initial denaturation at 95°C for 5 min followed by 50 cycles of 95°C for 10 s and 60°C for 20 s. In this analysis, the β-actin gene was used as the housekeeping gene. The genes and the oligonucleotide primer sets together with TaqMan probes were as follows: beta actin (GeneBank accession number: NM007393), 5'-agagggaaatcgtgcgtgac-3' (forward) and 5'-caatagtgacctggccgt-3' (reverse), Roche probe No.101; beta 3 adrenaline receptor (*ADRB3*; GeneBank accession number: NM013462), 5'-cagccagccctgttgaag-3' (forward) and 5'-ccttcatagccatcaaacctg-3' (reverse), Roche probe No.13; tumor necrosis factor alpha (*TNF-α*; GeneBank accession number: NM013693), 5'-ttgtcttaacgctgatttggt-3' (forward) and 5'-gggagcagaggttcagtgat-3' (reverse), Roche probe No.64; monocyte chemoattractant protein 1 (*MCP-1, CCL2*; GeneBank accession number: NM011333), 5'-catccacgtgttggctca-3' (forward) and 5'-gatcatcttgctggtgaatgagt-3' (reverse), Roche probe No.62; insulin receptor (*INSR*; GeneBank accession number: NM010568), 5'-agcacagtttgggagagtgg-3' (forward) and 5'-ccagctgccacacaatacag-3' (reverse), Roche probe No.4; sterol regulatory element-binding protein (*SREBP*; GeneBank accession number: NM011480), 5'-ggttttgaacgacatcgaaga-3' (forward) and 5'-cgggaagtcactgtcttggt-3' (reverse), Roche probe No.78; and fatty acid synthase (*FAS*; GeneBank accession number: NM007988), 5'-gctgctgttggaagtcagc-3' (forward) and 5'-agtgttcgttcctcggagtg-3' (reverse), Roche probe No.58. Delta Ct was calculated by subtraction of the crossing point cycle of the housekeeping gene from those of the other genes analyzed. All data for mRNA expression level, except for Fig. 3-1A and Fig. 3-3, are presented as a ratio relative to the control.

Statistical analyses were performed with JMP 10 (SAS Institute Japan) and Excel

Toukei 2010 (Social Survey Research Information, Tokyo, Japan).

3-3 Results

Body weight and visceral fat

Body weight differed little between the 45%FD and 45%FD+Lg groups throughout the experiments. However, the weight of the periovarian fat was significantly lower in group 45%FD+Lg than in group 45%FD ($p < 0.05$). The relative weight (% in body weight) of the periovarian fat of group 45%FD+Lg was nearly the same as that of group 10%FD (Table 3-2).

Gene expression in the periovarian adipose tissue and the liver

The relative expression levels of *ADRB3* in the periovarian fat decreased with the increase in periovarian fat weight ($p < 0.01$) (Fig. 3-1A). However, there were no differences between group 45%FD+Lg and group 45%FD (Fig. 3-1B), contrary to our expectations. The relative expression of *TNF-α* mRNA in the periovarian fat was lower in group 45%FD+Lg than in group 45%FD ($p < 0.05$) (Fig. 3-4A). The MCP-1 expression level in the periovarian fat was significantly enhanced by feeding a high fat diet (45%FD). However, there was no significant difference between groups 45%FD and 45%FD+Lg (Fig. 3-4B).

The relative expression of hepatic *SREBP* mRNA was lower in group 45%FD+Lg than in group 45%FD ($p < 0.05$). The relative expression of hepatic *FAS* mRNA was lower in group 45%FD+Lg than in group 45%FD ($p < 0.05$). The relative expression of hepatic *INSR* mRNA was higher in group 45%FD+Lg than in group 45%FD ($p < 0.01$) (Fig. 3-5).

Glycerol release from the periovarian fat

A portion (ca. 20 mg) of the periovarian adipose tissue released around 0.30-1.31 umol of glycerol in 3 hours. These values are considered to represent the range of the basal lipolytic rate. Diet significantly affected the lipolytic rate, as shown in Fig. 3-2A and Fig. 3-2B.

The relationship between the glycerol release and periovarian fat weight is also shown in Fig. 3-2A. Glycerol release decreased with the increase in periovarian fat weight ($p < 0.05$), but there was no difference between group 45%FD and group 45%FD+Lg (Fig. 3-2B). A similar relationship was observed for the effect of *L. gasseri* NT, as shown in Fig. 3-1B.

Blood FFA, TG, and insulin

As shown in Table 3-3, blood FFA was significantly lower in the 45%FD+Lg group than in the 45%FD group ($p < 0.05$).

3-4 Discussion

We evaluated the effects of the administration of lactic acid bacteria on lipid metabolism and fat synthesis in a mouse high-fat-diet model, focusing on visceral fat.

Lipid mobilization from the adipose tissue releases FFA and glycerol. This mobilization is mediated by the sympathetic nervous system; norepinephrine is the most potent regulator of lipid mobilization in adipocytes (Commins *et al*., 1999). This stimulation is mediated by the β3 adrenaline receptor (Susulic *et al.*, 1995). For this reason, mRNA expression of *ADRB3* was used to evaluate lipolysis (Ushida *et al*., 2011, Zhang *et al*., 2008, Rayner *et al*., 2001). In this context, an *ex vivo* test is a beneficial way to evaluate directly lipid mobilization because *ADRB3* expression is an indirect indicator of fat mobilization. It was shown in this experiment that the heavier visceral adipose tissue had a lower expression of *ADRB3*. Therefore, the negative correlation between adipose tissue weight and *ADRB3* expression level suggests the involvement of *ADRB3* in the development of obesity, as indicated by earlier studies (Cao *et al*., 2011). Declines in adrenergic receptor expression may result in lower lipid mobilization ($p < 0.05$) (Fig. 3-3), which leads to the development of obesity. However, this was not the case when we compared groups 45%FD+Lg and 45%FD. The periovarian fat weight in the former group was significantly lower than that in the 45%FD group without a significant difference in *ADRB3* expression and glycerol release. This suggests that the anti-obesity effects of *L. gasseri* NT may have other mechanisms. Lipid mobilization is induced by excited sympathetic nerves. Indeed, stimulation of autonomic nerve systems by intraduodenal administration of lactic acid bacteria was observed in the study of Nagai *et al.* (Nagai *et al*., 2009), in which decline of renal sympathetic nerve activity was suggested.

Obesity leads to infiltration of macrophages in adipose tissues with a concomitant increase in pro-inflammatory cytokines, such as TNF-α (Uysal *et al*., 1997). Since the level of TNF-α in adipose tissue correlates with the degree of adiposity and insulin resistance (Uysal *et al*., 1997), TNF-α, therefore, is a key molecule that exacerbates the lifestyle-related diseases caused by obesity. In this experiment, a high-fat diet did not enhance *TNF-α* expression significantly. Therefore, it was suggested that the level of inflammation in adipose tissue in the 45%FD group was not severe. Administration of *L. gasseri* NT significantly decreased *TNF-α* expression in the periovarian fat tissue in this

experiment. It is known that *L. rhamnosus* GG decreased TNF-α production in a murine macrophage cell line, RAW 264.7 (Pena *et al*., 2003). Inactivation of macrophages by lactobacilli may be a common phenomenon for probiotic lactobacilli (Inoue *et al*., 2007). For example, *L. casei* Shirota suppressed the production of TNF-α by macrophages (Yasuda *et al*., 2008).

If macrophages are the main target of lactic acid bacteria to reduce TNF-α expression in adipose tissue, their infiltration may be affected by *L. gasseri* NT. Monocyte chemoattractant protein 1 (MCP-1) and its receptor, CCR2, play pivotal roles in the development of inflammatory responses. Macrophage infiltration in adipose tissue induced by feeding on a high-fat diet for normal wild-type mice was not observed in CCR2-KO mice, and there was a significant reduction in TNF-α mRNA expression (Weisberg *et al*., 2003, Weisberg *et al*., 2006, Kanda *et al*., 2006). Accordingly, it is reasonable to conclude that most TNF-α in adipose tissues is induced by infiltrated macrophages. The increase in MCP-1 expression in adipose tissue in the 45%FD group suggests the pro-inflammatory status of these mice. *L. gasseri* NT did not significantly alter the expression of MCP-1. Together with the reduction in TNF-α expression in the adipose tissue, it is suggested that *L. gasseri* NT has at least a preventive effect against inflammation induced by fat accumulation (Fig. 3-4).

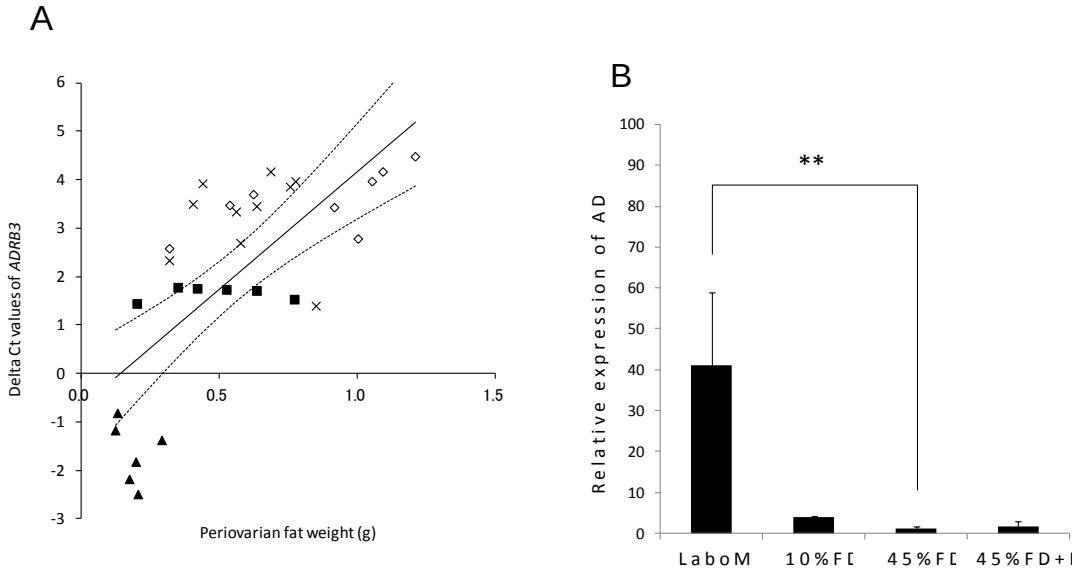
The intestine is an important immune organ, and the intestinal microbiota plays a crucial role in the development of local and systemic immunity. Stimulation of mucosal immune tissue by probiotic lactobacilli, indeed, inactivates macrophages (Inoue *et al*., 2007).

Hyperinsulinemia induces SREBP-1c expression, leading to the transcriptional activation of all lipogenic genes in the liver (Shimomura *et al*., 1999). SREBP has also been known to play a critical role in the development of beta-cell dysfunction in the pancreas caused by elevated FFA (Wang *et al*., 2003). In this study, the mRNA expression levels of *SREBP* and *FAS* were significantly lower in group 45%FD+Lg than in group 45%FD (Fig. 3-5). Blood serum FFA was significantly lower in group 45%FD+Lg than in group 45%FD (Table 3-3). These results indicate that suppression of *SREBP* and *FAS* in the liver leads to a decrease in fatty acid synthesis and FFA. In a previous study, lactic acid bacteria reduced hepatic lipogenesis and particularly reduced in the expression of SREBP and FAS (Kitawaki *et al*., 2009). Administration of lactic acid bacteria, therefore, demonstrates anti-lipogenic effects at least in the liver.

In this experiment, the blood insulin level was not high enough to be judged as demonstrating insulin resistance (Table 3-3). In addition to this, there was no significant difference in insulin level between groups 45%FD+Lg and 45%FD. Therefore, the

involvement of insulin in the effects of *L. gasseri* NT on lipid metabolism of mice is still hard to prove.

In conclusion, oral administration of *L. gasseri* NT did not enhance lipid mobilization in adipose tissue but could reduce fat synthesis in the liver, which suggests its potential to prevent the development of obesity and obesity-related disorders. It is not clear in which pathways the stimulatory signal caused by orally administered freeze-dried *L. gasseri* NT was transmitted to the effective sites in the liver and the adipose tissue. Further studies are required to clarify this point.



Bioscience of Microbiota, Food and Health. 2013. ***32***: 51-58.

Fig. 3-1. ADRB3 mRNA expression of periovarian fat. A, Relation between the delta Ct values of *ADRB3* of periovarian fat and periovarian fat weight. y=4.87x-0.70 (r=0.71) (y, delta Ct values of *ADRB3* of periovarian fat; x, periovarian fat weight; r, correlation coefficient) was obtained. Delta Ct was calculated by subtraction of the crossing point cycle of the β-actin gene from the ADRB3 gene. The broken lines represent 95% confidence lines. A significant positive correlation ($p < 0.01$) is shown between the Delta Ct values of *ADRB3* and periovarian fat weight. This means that negative correlation is shown between the expression levels of *ADRB3* and periovarian fat weight. B, Relative expression of ADRB3 mRNA for each group. The data were assessed by Dunnett's multiple comparison tests. The mean value of the 45%FD group was set to 1. ▲, LaboMR; ■, 10%FD; ◇, 45%FD; ×, 45%FD+Lg ** $p < 0.01$

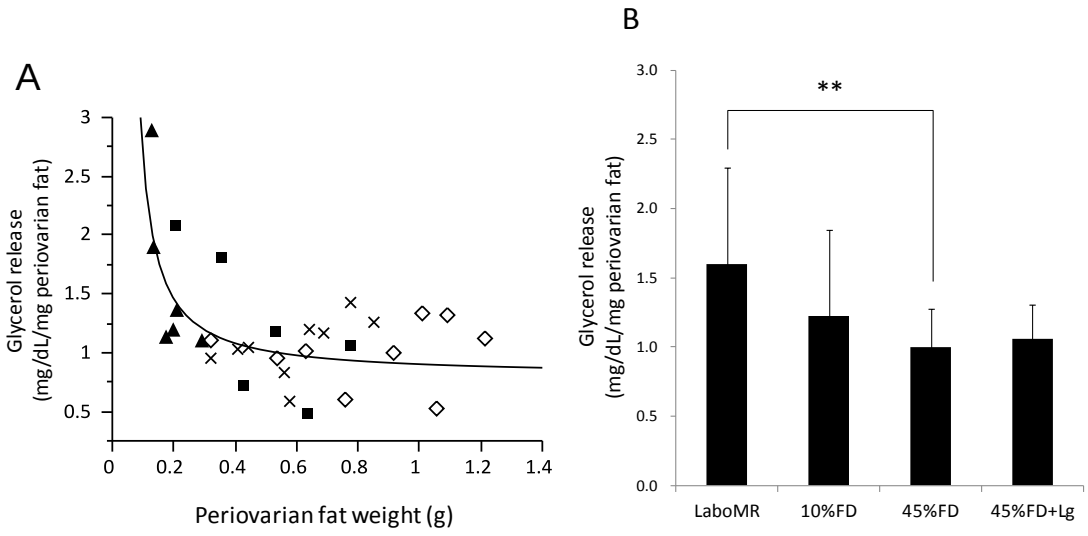


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Fig. 3-2. Glycerol release from periovarian fat. A, Relation between glycerol release from periovarian fat and periovarian fat weight. Log(y)=-0.22+0.12/x (y, glycerol release; x, periovarian fat weight) was obtained. A negative correlation ($p < 0.05$) is shown between the glycerol release and periovarian fat weight. B, Glycerol release from periovarian fat in each group. The data were assessed by Dunnett's multiple comparison tests. The mean value of the 45%FD group was set to 1.

▲, LaboMR; ■, 10%FD; ◇, 45%FD; ×, 45%FD+Lg ** $p < 0.01$

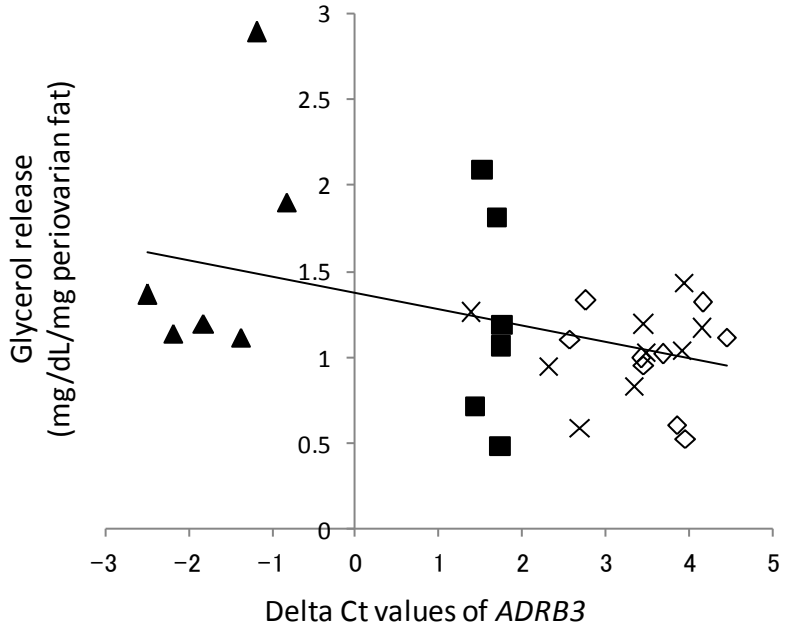

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Fig. 3-3. Relation between glycerol release from periovarian fat and the delta Ct values of *ADRB3* of periovarian fat. y=-0.10x-1.37 (r=0.41) (y, glycerol release from periovarian fat; x, delta Ct values of *ADRB3* of periovarian fat; r, correlation coefficient) was obtained. Delta Ct was calculated by subtraction of the crossing point cycle of the β-actin gene from that of the ADRB3 gene. A significant negative correlation ($p < 0.05$) is shown between glycerol release from periovarian fat and the delta Ct values of *ADRB3* of periovarian fat. This means that positive correlation is shown between between glycerol release from periovarian fat and the expression levels of *ADRB3*.
▲, LaboMR; ■, 10%FD; ◇, 45%FD; ×, 45%FD+Lg

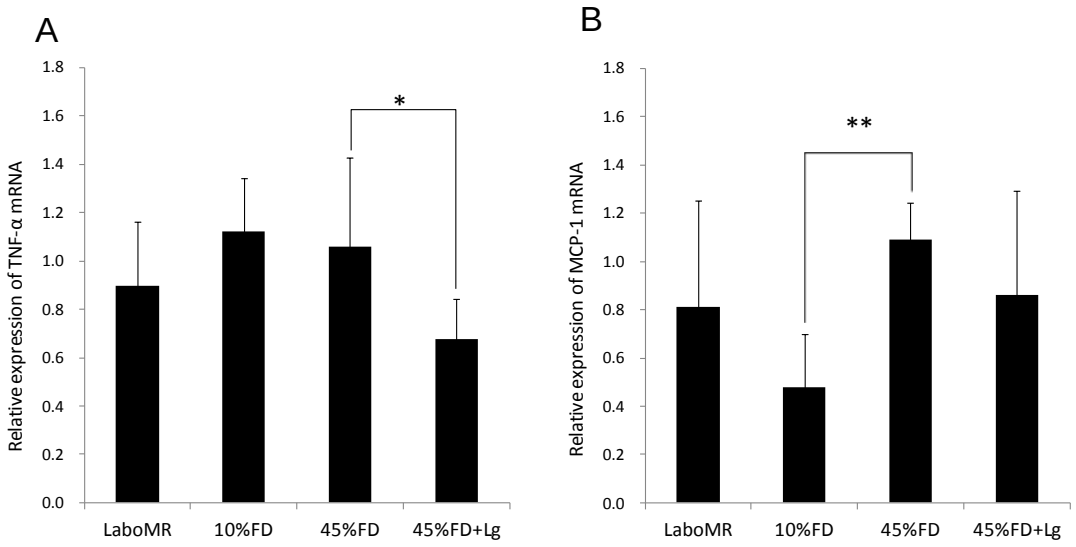

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Fig. 3-4. Relative expression of proinflammatory marker gene in periovarian fat. A, Relative expression of TNF-α mRNA in periovarian fat. The data were assessed by Dunnett's multiple comparison tests. The mean value of the 45%FD group was set to 1. B, Relative expression of MCP-1 mRNA in periovarian fat. The data were assessed by Dunnett's multiple comparison tests. The mean value of the 45%FD group was set to 1. $^{**}p < 0.01$; $^{*}p < 0.05$

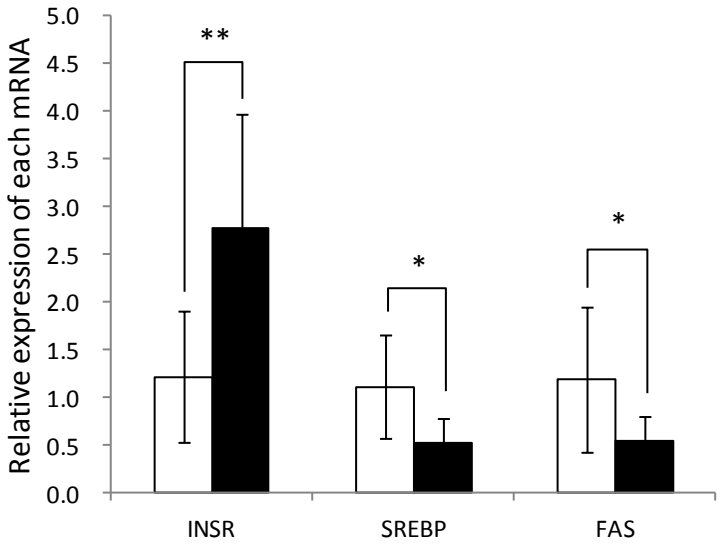

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Fig. 3-5. Relative expression of insulin receptor (*INSR*), sterol regulatory element-binding protein (*SREBP*) and fatty acid synthase (*FAS*) mRNA in the liver. Differences were evaluated using the Student's *t*-test.
□, 45%FD; ■, 45%FD+Lg $^{**}p < 0.01$; $^{*}p < 0.05$

Table 3-1. The composition of each diet

Ingredient	Labo MR Stock	D12450B	D12451
Protein	18.80 %	19.20 %	23.70 %
Carbohydrate	54.70 %	67.30 %	41.40 %
Fat	3.90 %	4.30 %	23.60 %
Calories	2.31 kcal/g	3.87 kcal/g	4.73 kcal/g
Calories from fat	0.351 kcal/g	0.385 kcal/g	2.12 kcal/g

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Table 3-2. Effects of *L. gasseri* NT on body weight and periovarian fat weight

	LaboMR	10%FD	45%FD	45%FD+Lg
Body weight (g)	$24.2 \pm 1.19^{**}$	$24.9 \pm 1.77^{**}$	29.1 ± 2.06	28.7 ± 1.86
Periovarian fat (g)	$0.189 \pm 0.061^{**}$	$0.487 \pm 0.204^{**}$	0.837 ± 0.294	$0.585 \pm 0.175^{*}$
Periovarian fat (%)	$0.775 \pm 0.217^{**}$	$1.921 \pm 0.719^{*}$	2.832 ± 0.881	$2.030 \pm 0.559^{*}$

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Each value is the mean±SD. The data were assessed for statistical significance compared with the 45%FD group by Dunnett's multiple comparison tests. A significant difference in periovarian fat between the 45%FD group and 45%FD+Lg group was observed ($p < 0.05$). $^{**}p < 0.01$; $^{*}p < 0.05$

Table 3-3. Effects of *L. gasseri* NT on serum lipid and insulin

	45%FD	45%FD+Lg
TG (mg/dL)	108.8 ± 41.0	91.4 ± 6.3
FFA (mEq/L)	1.103 ± 0.315	0.835 ± 0.121*
Insulin (pg/mL)	3800 ± 1968	2773 ± 841

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Each value is the mean±SD. Differences were evaluated using the Student's *t*-test. A significant difference in FFA between the 45%FD group and 45%FD+Lg group was observed ($p < 0.05$). $^{*}p < 0.05$

Chapter 4

Lactobacillus gasseri NT decreased visceral fat through enhancement of lipid excretion in feces of KK-A^y mice

4-1 Introduction

There are as many as 10^{12} CFU bacteria in 1 g of digesta in the human intestine. They constitute a complex microbial community that interacts with host intestinal tissues and have roles not only in nutrition, but also in immunological defense against harmful agents. Thus, the intestinal microbial community contributes to nutritional efficiency and the maintenance of host health (Guarner *et al*., 2003). In addition to host defense, intestinal tissues secrete several peptide hormones that influence the movement of the digestive tract as well as the appetite of the host. The intestinal microbial community can attenuate host nutrition by modification of hormone secretion (Cani *et al*., 2009). The connection between gut microbiota and the energy homeostasis of the host is increasingly recognized. For example, dietary modulation of gut microbiota to increase the bifidobacterial population significantly reduced endotoxemia and improved glucose tolerance (Cani *et al*., 2007). This modulation also alleviates inflammation in mice fed a high fat diet (Cani *et al*., 2007). In addition to glucose metabolism, some preliminary studies have indicated the importance of gut microbiota in the lipid metabolism of the host (Cani *et al*., 2009). In this context, prebiotics and probiotics, which are recognized as intestinal flora modifiers, attract attention from those working on weight control and obesity prevention. In former chapter, it was confirmed that administration of *L. gasseri* NT reduced periovarian fat and serum-free fatty acid (FFA) in BALB/c mice fed a high-fat diet. These effects were induced by repression of fat synthesis (Yonejima *et al*., 2013), but the mechanisms by which probiotics affect lipid metabolism must be evaluated, because BALB/c mice do not consume an excessively high-fat diet, which means that they are not good models for obesity due to excessive food consumption. Accordingly, next study was conducted using KK-A^y mice, a model for diabetes characterized by hyperphagia, hyperglycemia, hyperinsulinemia, and insulin resistance (Moussa *et al*., 1999).

In this chapter, the effects of a probiotic, *L. gasseri* NT, on lipid digestion and metabolism in KK-A^y mice was evaluated by a pair-feeding approach. The reason for pair feeding was to eliminate the effect of *L. gasseri* NT on food consumption.

4-2 Materials and Methods

The experiments were approved by the Animal Experiment Committee of Kyoto

Prefectural University. Chemicals were purchased from Nacalai Tesque (Kyoto, Japan) unless otherwise stated. Four-week-old male KK-A^y mice were purchased from CLEA Japan (Tokyo, Japan). They were kept in individual cages in a room kept at 25 ± 1°C under a 12-h light and dark cycle. They were divided into the following groups: HFD (eight mice fed a 45kcal%Fat diet, D12451, Research Diets, New Brunswick, NJ, USA, 4.73 kcal/g) and HFD+Lg (eight mice fed D12451 supplemented with freeze-dried *L. gasseri* NT at 10^9 CFU/g, 4.73 kcal/g). The energy intake of the mice was adjusted by pair feeding for 5 weeks. Thus the food intake levels of the groups were nearly the same. Average daily food intakes were 4.79 ± 0.40 g and 4.73 ± 0.28 g, and total calorie intakes were 725 ± 60 kcal and 716 ± 42 kcal for the HFD and HFD+Lg groups respectively. The levels were slightly lower than consumption in ad libitum feeding (about 5 g for KK-A^y mice at 40 g of body weight). Body weight was measured daily. At the end of the experiment, the mice were dissected under general anesthesia to remove periovarian fat and the liver. The periovarian fat was considered to represent visceral adipose tissue. After removal, a portion (about 500 mg) of the periovarian fat was collected in RNAlater® solution (Sigma, Tokyo, Japan) for mRNA analysis. A portion (about 500 mg) of the liver was also collected in RNAlater® solution for mRNA analysis. These subsamples were stored at -30ºC after overnight fixation in RNAlater®. The other portion (about 500 mg) of the liver was freeze-dried for lipid extraction. The entire intestine was removed, and the distal portion of the ileum, 1 cm from the ileocecal valve, was collected in RNAlater® solution for mRNA analysis. At the same time, the entire ileal digesta were collected and immediately stored at -30ºC. Blood samples were collected from the inferior vena cava. Sera were stored at -30ºC until analysis. Fecal samples were collected at the time of dissection and stored at -30ºC until biochemical analysis. Portions of the feces were further freeze-dried for lipid extraction. The serum levels of triglycerides (TG), total cholesterol (T-cho), free fatty acid (FFA), and glucose were determined by TG E-test (Wako), Cholesterol E-test Wako (Wako), NEFA C-test (Wako), and Glutest Every® (Sanwa Kagaku Kenkyusyo, Aichi, Japan) respectively. Total RNA was extracted from the periovarian adipose tissue and the liver, and cDNA was synthesized as reported in a previous study (Yoshikawa *et al.*, 2009). The expression levels of the target genes were determined by the real-time PCR approach using by the use of LightCycler® 480 (Roche Applied Science, Tokyo, Japan). PCR was performed with a thermal cycle program with initial denaturation at 95°C for 5 min, followed by 50 cycles of 95°C for 10 s and 60°C for 20 s. In this analysis, the β-actin gene was used as housekeeping gene. The target genes with their GenBank accession numbers were as follows: β-actin (GenBank accession number: NM007393),

Cholesterol 7α-hydroxylase (CYP7) (GenBank accession number: NM007824.2), Monocyte chemo-attractant protein 1 (MCP-1, CCL2) (GenBank accession number: NM011333), Fatty acid transport protein 2 (FATP2, Slc27a2) (GenBank accession number: NM011978.2), and Fatty acid transport protein 4 (FATP4, Slc27a4) (GenBank accession number: NM011989.4). The primers and probes used in this study were designed by means of software (https://www.roche-applied-science.com/). Delta Ct was calculated by subtracting the crossing point cycle of the housekeeping gene (beta-actin) from those of the other genes analyzed. All data for mRNA expression are presented as the ratio of the level for HFD+Lg relative to that in the control, HFD. Extraction of total lipids from the freeze-dried liver and feces was carried out by the ordinary method (Forch *et al*., 1956). Briefly, portions of the liver (about 30 mg) or feces (about 10 mg) were extracted by chloroform-methanol solution. The total lipids of the extracts were measured as constant weight after evaporation of the solvent. The TG concentration in the extract resolved by 2-propanol (1 mL) was further determined by TG E-test. Determination of the bile acid concentrations in the feces and digesta was carried out as reported by Higaki *et al*. (Higaki *et al*., 2006). A portion of the feces (about 10 mg) or the ileal digesta (about 10 mg) was extracted with 90% ethanol, and extracts were further subjected to the Total Bile Acid Test (Wako, Osaka, Japan). Determination of the lipase activity of the ileal digesta was carried out by means of Lipase Kit S (DS Pharma Biomedical, Osaka, Japan). Digesta were diluted 40-hold with distilled water before analysis. Activities were expressed as values relative to that of 10 U/mL of porcine pancreatic lipase. Student's *t*-test was done after an *F*-test, which confirmed homoscedasticity. Statistical analyses were performed with Excel Toukei 2010 (Social Survey Research Information, Tokyo).

4-3 Results

The body weight of mice was nearly the same for the HFD and HFD+Lg groups throughout the experiment, but the weight (g) and relative weight (% of body weight) of the periovarian ($p < 0.01$) fat and the liver ($p < 0.05$) were both significantly lower in the HFD+Lg group than in the HFD group (Table 4-1). In addition, total lipids and TG in the liver were significantly lower in the HFD+Lg group than in the HFD group ($p < 0.01$) (Table 4-3).

There was no significant difference in serum TG levels between the two groups, but HFD+Lg mice showed significantly lower T-cho ($p < 0.01$), FFA ($p < 0.05$), and glucose ($p < 0.05$) than the HFD mice (Table 4-2). The HFD+Lg mice showed significantly higher fecal TG and intestinal bile acid than the HFD mice ($p < 0.05$) (Table 4-3).

The HFD+Lg mice showed higher relative expression of *CYP7* mRNA in the liver than the HFD mice ($p < 0.05$), but the HFD+Lg mice showed lower periovarian fat *MCP-1* mRNA expression and jejunal *FATP2* and *FATP4* mRNA than the HFD mice ($p < 0.05$) (Fig. 4-1).

The relative lipase activity of the ileal digesta was lower in the HFD+Lg group than in the HFD group ($p < 0.05$) (Fig. 4-2).

4-4 Discussion

In this experiment, oral administration of *L. gasseri* NT significantly increased the fecal TG concentration, which suggests that lipid digestion and/or absorption was repressed by feeding of *L. gasseri* NT. Decreased lipid absorption might explain the reduction in visceral fat weight and blood FFA, in that ileal lipase activity decreased 10% and mRNA expression of fatty acid transport proteins in the small intestine was down regulated by feeding of *L. gasseri* NT. Significantly lower serum cholesterol confirms reduced lipid digestion and/or absorption in the small intestine.

Recently, Matsumura (Matsumura *et al*., 2010) reported that *L. gasseri* NLB367 inhibited pancreatic lipase *in vitro*, though he did not describe in detail the mechanisms of inhibition. There are several possible mechanisms for lactobacillal inhibition of lipase: that *L. gasseri* NLB367 reacts directly with lipase to prevent lipase from binding with its substrate, or *L. gasseri* NLB367 inhibits micelle formation. Matsumura stressed in his report that a wide variety of lactobacilli inhibit lipase. Accordingly, it is plausible that its inhibitory effect on lipase is not species- or strain-specific. The latter possibility, inhibition of micelle formation, is more plausible as an inhibitory mechanism.

On the other hand, the bile acid concentration of the ileal digesta increased. Indeed, the color of the ileal digesta changed visually, becoming redder. It is interesting to consider why the bile acid concentration was elevated when the lipase activity was decreased by feeding of *L. gasseri* NT. Increased hepatic expression of *CYP7* mRNA might explain the increased bile acid concentration in the small intestinal digesta, because Cholesterol 7α-hydroxylase, *CYP7*, is the enzyme that catalyzes the synthesis of bile acid from cholesterol. In considering the fecal bile acid concentration, which did not differ between the groups of animals, the size of the bile acid pool should have increased in the jejunum and the ileum. If micelle formation was significantly inhibited by *L. gasseri* NT under the high-fat diet, more bile acid might be secreted to support fat digestion and absorption. Inhibition of micelle formation occurres when bile acid is absorbed by hydrophobic materials such as soy peptide (Nagaoka *et al*., 2010). A range of lactic acid bacteria absorb and retain bile acids within the cell (Yokota *et al*., 2000), (Kurdi *et al*.,

2000), which may inhibit the micelle formation. Moreover, the solubility of bile acid can be affected indirectly by *L. gasseri* NT, because bile acids become insoluble when the intestinal pH is lower than 6.5. If the pH condition in the intestine becomes low due to acids produced by lactic acid bacteria, bile acid becomes inactive as to forming micelles (Narusawa *et al*., 1998).

Lowered lipase activity might also explain the increase in bile acid pool in the small intestine. This is consistent with the finding that the administration of chitosan, which inhibits pancreatic lipase activity, induced bile acid excretion in a rat model (Gallaher *et al*., 2000).

A negative correlation has been found between the LDL-cholesterol concentration in blood and bile acids in the intestine (Furuya *et al.*, 2006). A reduction in bile acid biosynthesis might increase hepatic cholesterol and oxysterol levels, which should influence the function of the lipogenic SREBP-1c by attenuating processing and activation of it (Watanabe *et al*., 2013). SREBPs, family members of membrane-bound transcription factors, regulate lipid homeostasis by controlling enzymes such as fatty acid synthase (FAS). Indeed, overexpression of nSREBP-1c in the liver produces a triglyceride-enriched fatty liver (Horton *et al*., 2002). This might decrease hepatic TG production.

At the transcriptional level, bile acids, which are endogenous ligands of the hepatic farnesoid X receptor (FXR), activate the transcription of several hepatic genes that can modulate TG levels, such as the atypical nuclear receptor small heterodimer partner (SHP) (Goodwin *et al*., 2000) and PPARα (Torra *et al*., 2003). PPAR-α is a nuclear receptor that regulates hepatic lipid metabolism by up regulating carnitine palmitoyl transferase 1 (CPT-1), which oxidizes fatty acids (Brandt *et al*., 1998).

Since bile acids have many regulatory functions in lipid metabolism, the effect of *L. gasseri* NT on lipid metabolism may be elicited by induction of bile acid secretion. This aspect should be examined in further studies. The serum bile acid could not be determined in this experiment due to the blood sample's limited quantity. Moreover, not all feces were collected in this experiment. The limited fecal sample also limited the discussion of the bile acid balance. Additional study that focuses on bile acid metabolism should be considered.

In conclusion, oral administration of *L. gasseri* NT decreased visceral fat weight through inhibition of lipase and, plausibly, reduction of bile acid activity in the intestine.

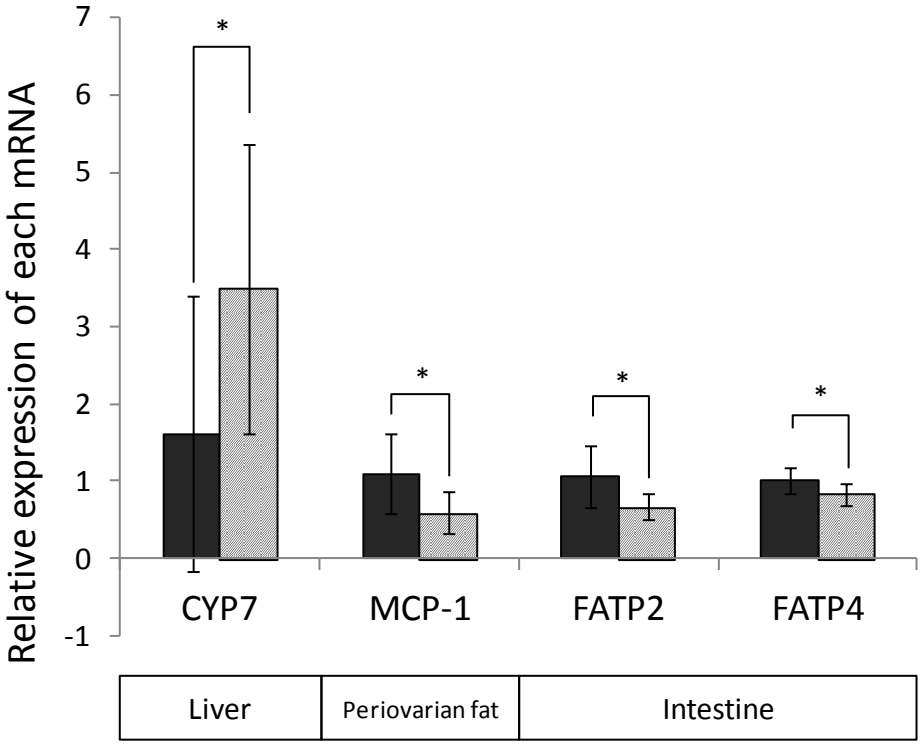

Fig. 4-1. Relative expression of *CYP7* mRNA in the liver, *MCP-1* mRNA in the periovarian fat, and *FATP2* and *FATP4* in the intestine. Values are mean ± SD. Differences were evaluated by Student's *t*-test.

■, HFD; ▨, HFD+Lg * $p < 0.05$

Modified from Yonejima *et al*., (2013)

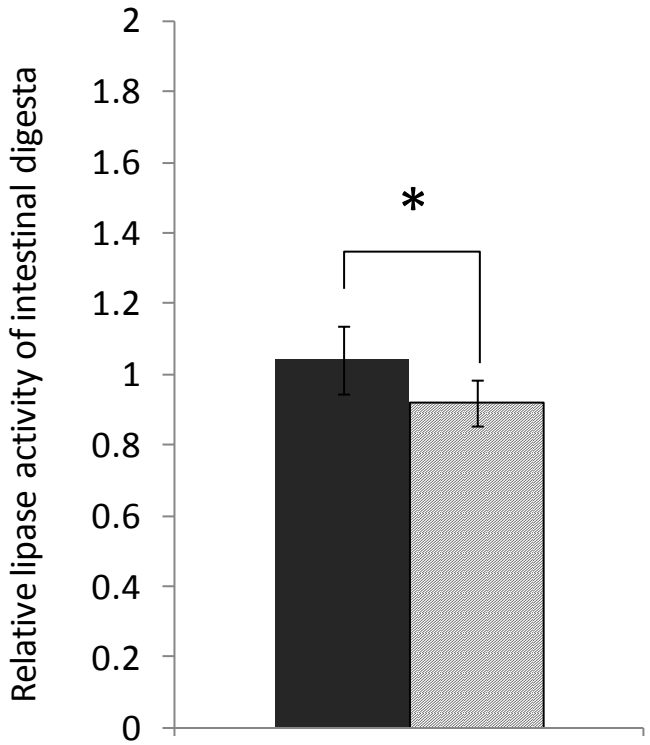

Fig. 4-2. Relative lipase activity of intestinal digesta to porcine pancreatic lipase (10 U/mL). Intestinal digesta was diluted with water by 40-hold. Values are mean ± SD. Differences were evaluated by Student's *t*-test.

■, HFD; ▨, HFD+Lg * $p < 0.05$

Modified from Yonejima *et al.*, (2013)

Table 4-1. Effects of *L. gasseri* NT on body weight, periovarian fat weight and liver weight

	HFD	HFD+Lg
Body weight (g)	44.6 ± 2.3	43.0 ± 2.3
Periovarian fat weight (g)	3.26 ± 0.48	2.60 ± 0.36**
Periovarian fat weight (g/100g body weight)	7.28 ± 0.75	6.03 ± 0.69**
Liver weight (g)	2.50 ± 0.26	2.14 ± 0.23*
Liver weight (g/100g body weight)	5.62 ± 0.50	4.98 ± 0.53*

HFD, group fed high-fat diet (45kcal%Fat diet); HFD+Lg, group fed high-fat diet (45kcal%Fat diet) supplemented with *L. gasseri* NT. Values are mean ± SD.
Differences were evaluated by Student's *t*-test. $^{**}p < 0.01$, $^{*}p < 0.05$
Modified from Yonejima *et al*., (2013)

Table 4-2. Effects of *L. gasseri* NT on serum lipids, blood glucose level

	HFD	HFD+Lg
Serum TG (mg/dL)	229 ± 141	115 ± 76
Serum T-cho (mg/dL)	163 ± 22	130 ± 20**
Serum FFA (mEq/L)	1.77 ± 0.83	0.86 ± 0.20*
Blood glucose level (mg/dL)	526 ± 88	397 ± 103*

HFD, group fed high-fat diet (45kcal%Fat diet); HFD+Lg, group fed high-fat diet (45kcal%Fat diet) supplemented with *L. gasseri* NT. TG, triglyceride; T-cho, total cholesterol; FFA, free fatty acid. Values are mean ± SD. Differences were evaluated using the Student's *t*-test. $^{**}p < 0.01$, $^{*}p < 0.05$

Modified from Yonejima *et al*., (2013)

Table 4-3. Effects of *L. gasseri* NT on lipids and TG in the liver, TG and bile acid in the feces, and bile acid in digesta

	HFD	HFD+Lg
Liver		
Total lipids (g/100g freeze-dried hepatic tissue)	21.0 ± 6.0	14.6 ± 3.5*
Total lipids (mg liver)	212 ± 72	111 ± 17**
TG (g/100g freeze-dried hepatic tissue)	11.7 ± 3.3	5.4 ± 1.6*
TG (mg liver)	119 ± 41	42 ± 13**
Feces		
TG (g/100g freeze-dried feces)	18.4 ± 5.0	26.3 ± 11.0*
Bile acid concentration (μmol/g wet feces)	2.26 ± 0.68	1.82 ± 0.46
Small intestinal digesta		
Bile acid (μmol/g wet digesta)	19.8 ± 5.4	25.8 ± 7.0*
Total bile acid (μmol)	2489 ± 1318	2971 ± 1442
Weight (mg)	125 ± 58	128 ± 76
Weight (mg dry water)	31.1 ± 14.7	32.1 ± 19.4

HFD, group fed high-fat diet (45kcal%Fat diet); HFD+Lg, group fed high-fat diet (45kcal%Fat diet) supplemented with *L. gasseri* NT. TG, triglyceride. Values are mean ± SD. Differences were evaluated using the Student's *t*-test. $^{**}p < 0.01$, $^{*}p < 0.05$
Modified from Yonejima *et al*., (2013)

Chapter 5
General discussion and conclusion

5-1 General discussion

In this study, I have determined the effect of probiotics on indigenous intestinal microbiota and their effects on lipid metabolism in both normal and obese models under high-fat-diet feeding.

Probiotics used in chapter 2 contained either *B. natto* NT or *L. acidophilus* NT.

It was confirmed that administration of the tablet for 10 days increased bifidobacterial population together with their symbiotic bacteria, such as *Acidaminococcus* spp., Megamonas spp., and an unknown butyrate-producing bacterium.

The increase in bifidobacteria by probiotics was the major observation throughout the study. As indicated by Ohashi *et al.* (2001), probiotics may have the potential to modify indigenous intestinal microbiota in general, even though they do not colonize in the intestine. Our observation seems to provide another example to explain the effect of probiotics that do not colonize in the intestine. At present, the mechanisms for such growth promotion of indigenous bifidobacteria or other intestinal bacteria remain unclear. However, the probiotic strains should have grown to some extent in order to show such growth promotion for the indigenous bacteria. It is noteworthy that other bacteria affected by probiotics were the acid-utilizing bacteria. Probiotic strains were lactic acid bacteria, which produce lactate and acetate (or only lactate if they are homo fermenters). These acids can be converted into butyric acid by butyrate producers, such as *Acidaminococcus* and *Megasphaera elsdenii*, or other lactate-utilizing butyrate producers (Tsukahara *et al.*, 2002; Duncan *et al.*, 2007). It is plausible that growth promotion of these acid-utilizing bacteria may help further the growth of indigenous lactate producer, such as bifidobacteria or lactobacilli. Accordingly, the indirect effect of probiotics seems to be quite visible: a stimulation of the indigenous bifidobacteria or lactobacilli through the growth of indigenous acid-utilizing bacteria.

Regarding enteric disorders, bifidobacteria can protect from enteropathogenic infection through the production of acetate (Fukuda *et al.*, 2011). Bifidobacteria was also effective in reducing constipation (Yaeshima *et al.*, 1997; Yang *et al.*, 2008). Probiotics composed of bifidobacteria were also effective to prevent allergies (Xiao *et al.*, 2007; Taniuchi *et al.*, 2005) and obesity (Kondo *et al.*, 2010; Yin *et al.*, 2010). These beneficial health effects must be caused by the increase in indigenous bifidobacteria unless the probiotic strain grows well in the intestine in the case of *B. animalis* (Ishizuka *et al.*, 2012).

In this study, the effects of the probiotic strain on obesity were focused. As noted above, many studies have been conducted to demonstrate the positive effect of probiotics on obesity (Kitawaki *et al*., 2009; Kadooka *et al*., 2010). Since obesity causes diabetes, hyperlipidemia, hypertension, and arteriosclerosis, effective remedies for obesity are still needed (Norman *et al*., 1989).

Studies conducted in this decade have revealed that obesity is caused by a coordinated mechanism involving several organs and tissues (Das *et al*., 2010). Possible target organs and/or tissues for the treatment of obesity are presented in Fig. 5-1.

In adipose tissue, the adrenergic receptor (ADRB) plays an important role in metabolism, which mediates the catecholamine-induced lipolysis. Thus, the adrenergic system plays a key role in regulating energy balance through thermogenesis and lipid metabolism (Deram *et al*., 2009). In addition, adipose tissue secretes many biologically active substances: adipocytokines such as leptin, adiponectin, tumor necrosis factor α (TNF-α), and monocyte chemoattractant protein 1 (MCP-1). TNF-α and MCP-1 are up-regulated, but adiponectin is down-regulated in obese adipose tissue. It is widely accepted that inflammatory cytokines accumulated within the adipose tissue critically contribute to the development of many aspects of the metabolic syndrome resulting in diabetes and atherosclerosis (Matsuzawa *et al*., 1999; Hotamisligil *et al*., 1993; Sartipy *et al*., 2003; Takahashi *et al*., 2010).

In the liver, sterol regulatory element binding proteins (SREBPs), family members of membrane-bound transcription factors, regulate lipid homeostasis by controlling enzymes such as fatty acid synthase (FAS). Indeed, overexpression of nSREBP-1c in the liver produces a triglyceride-enriched fatty liver (Horton *et al*., 2002). On the other hand, the peroxisome proliferator-activated receptor α (PPAR-α) is a nuclear receptor that regulates liver lipid metabolism by up-regulating its target enzyme of FA oxidation, carnitine palmitoyl transferase 1 (CPT-1) (Brandt *et al*., 1998).

Regarding mitochondria, energy combustion is controlled by peroxisome PPAR-α-regulated mitochondrial and peroxisomal fatty acid β-oxidation systems. Stimulation of mitochondrial fatty acid β-oxidation by PPAR-α activator in the liver and muscle contributes to reducing plasma triglycerides (Kim *et al*., 2012).

Finally, in the brain, the hypothalamus has a key role in the control of both body fat content and glucose metabolism. The neuronal system, which regulates energy intake, energy expenditure, and endogenous glucose production, senses and responds to input from hormonal and nutrient-related signals that convey information regarding both body energy stores and current energy availability (Schwartz *et al*., 2000).

New pathways related to lipid metabolism have been continuously discovered, and

these novel pathways became potential targets for drug development in the treatment of obesity. Probiotics can also affect these targets to prevent obesity; therefore, we have conducted a series of experiments using normal mice and spontaneous diabetic mice under high-fat-diet feeding.

In chapter 3, we evaluated the effects of probiotic *L. gasseri* NT on lipid metabolism of the visceral adipose tissues in Balb/c mice under high-fat-diet feeding. Balb/c mice was chosen because this strain was normal and one of the most widely used strains. In this experiment, *L. gasseri* NT did not stimulate lipolytic activity in the visceral adipose tissue (Figs. 3-1B and 3-2B). This finding was a bit unexpected because probiotics can enhance sympathetic stimulation (Nagai *et al*., 2009), which is received by ADRB3. Modification of intestinal microbiota may help to reduce the LPS burden, which down-regulates ADRB3 in adipose tissue (Ushida *et al*., 2011), because the ADRB3 of adipose tissue is down-regulated by TNF-α released by infiltrated monocytes in response to LPS. *L. gasseri* NT did not up-regulate ADRB3 in the visceral adipose tissues. However, *L. gasseri* NT decreased the mRNA expression of SREBP and its target gene, FAS, in the liver (Fig. 3-5). The induction of hepatic SREBP was observed in Hepatitis C virus (HCV) infection through TNF-α activation (Li *et al*., 2013); accordingly, *L. gasseri* NT reduced the hepatic LPS burden. As shown by the previous study, an increase in bifidobacteria cured the endotoxemia (Cani *et al*., 2007). *L. gasseri* NT probably decreased the portal LPS burden, which may have reduced TNF-α response. The reduction in the portal LPS burden may not be enough to reduce inflammatory responses in tissues or peripheral organs other than the liver. Measurement for blood LPS is not an easy task due to the low level of concentration. In recent experiment, however, the daily supply of portal LPS was significantly decreased by the feeding of gum arabic to mice (Iida *et al*., personal communication).

In this study, I have evaluated the effect of a probiotic, *L. gasseri* NT, on lipid digestion and metabolism in KK-*Ay* mice with a pair-feeding approach. In the former study, BALB/c mice did not consume a high-fat diet excessively, which means that this strain is not a good model for the obesity induced by excessive food intake. Accordingly, a study using KK-*Ay* mice was conducted, which are the model for diabetes characterized by excessive food intake.

Oral administration of *L. gasseri* NT decreased the visceral fat and total lipids in the liver (Tables 4-1 and 4-3). The reduction was caused by suppression of lipid absorption through the inhibition of intestinal lipase and, plausibly, by a reduction in bile acid activity in the intestine (Fig. 5-2).

Probiotics can interact with bile acids to reduce the bioavailability of bile acids

(Al-Salami *et al*., 2012). Probiotics absorb or even hydrolyze bile acids. *L. gasseri* NT can bind cholesterol onto the cell wall (Usman and Hosono, 1999). Bile salt hydrolases (BSHs, EC. 3.5.1.24) are another determinant originating from probiotics in bile acid metabolism. BSHs are detected in a range of probiotic strains (Tanaka *et al*., 1999). The presently used *L. gasseri* NT also has bile salt hydrolases. The literature supports our expectation regarding interaction between *L. gasseri* NT and bile acids.

The mechanism of interaction between bile acids and intestinal microbiota is still under debate (Pavlovic *et al*., 2012). Further studies are required to define the effect of probiotics on bile acid-related lipid metabolism.

5-2 Conclusion

The present study demonstrated the beneficial function of probiotics against obesity. The first study demonstrated that oral administration of a tablet containing *L. acidophilus*, *B. natto*, and stomachic herbs can affect the human intestinal microbiota by increasing indigenous bifidobacteria. The second and the third studies demonstrated that administration of *L. gasseri* NT did not enhance lipid mobilization but reduced fat synthesis and lipid absorption. *L. gasseri* NT retain bile acids within the cell, which may inhibit the micelle formation and induce the secretion of additional bile acids. Bile acid is a key factor for prevention of fat accumulation. These results demonstrate the potential of probiotics to prevent development of obesity and obesity-related disorders.

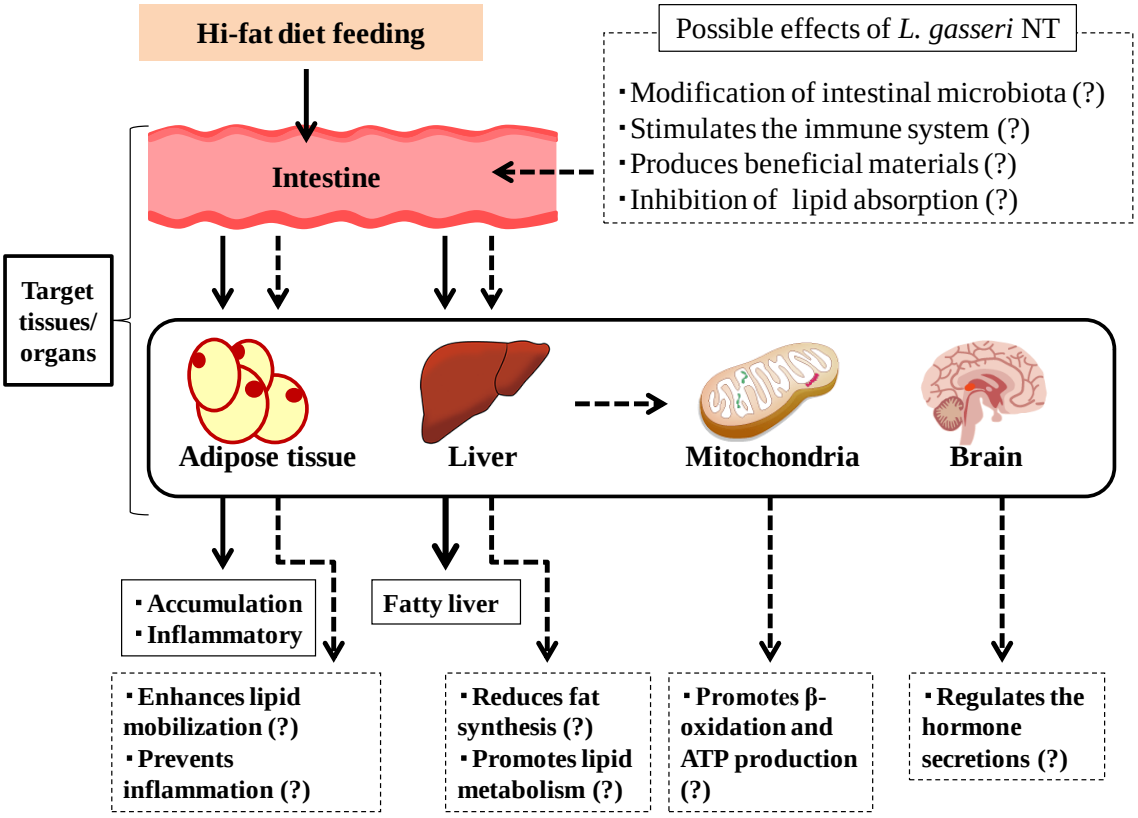

Fig. 5-1. Possible target organs/tissues for obesity treatment and possible effects of *L. gasseri* NT. The solid arrows represent the pathway induced by high-fat-diet feeding. The broken arrows represent the pathways induced by administration of *L. gasseri* NT.

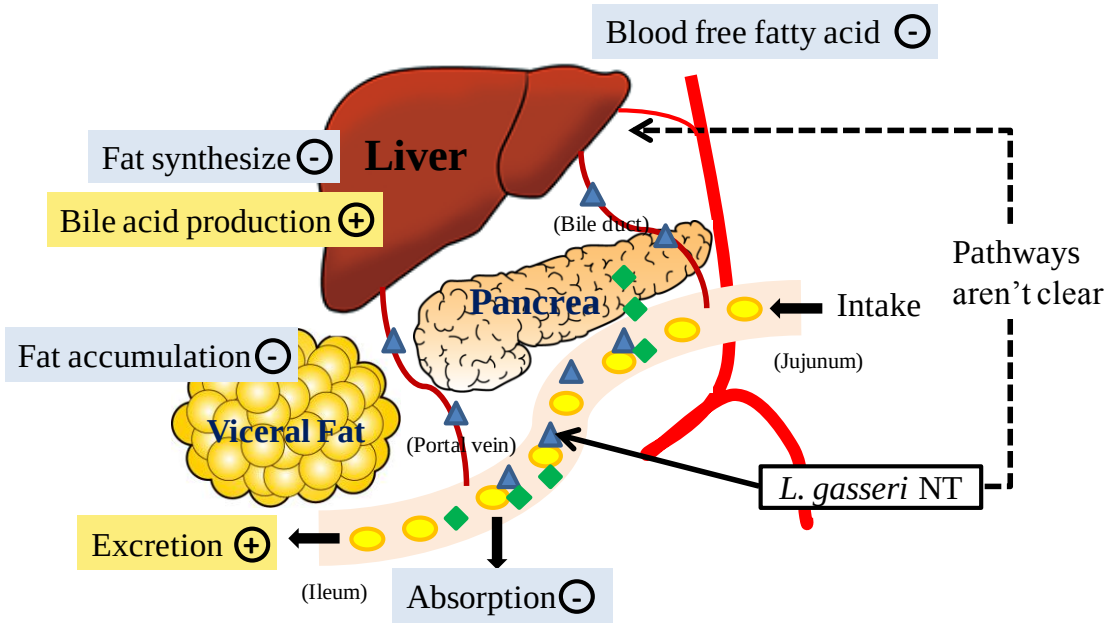

Fig. 5-2. Possible mechanism of anti-obesity effects by *L. gasseri* NT.
▲, Bile acid; ●, Lipid; ◆, Lipase; ⟶, Inhibition of lipase; ⊕, Up-regulation by *L. gasseri* NT; ⊖, Down-regulation by *L. gasseri* NT.

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Summary

Chapter 1

The intestinal microbial community contributes to the nutritional efficiency and maintenance of host health. Probiotic bacteria, namely lactobacilli and bifidobacteria, are now well recognized for their health promoting effects. Several diseases are related in intestinal microbiata. For example, obesity are associated with low-grade inflammation and an altered composition of the gut microbiota, a bacterial compound might act as a triggering factor in the development of obesity, diabetes mellitus and inflammation induced by a high-fat diet. The aims of this study were to investigate the effect of tablet containing both probiotic and medicinal herbs on the fecal microbiota in human, and to evaluated the effect of a probiotic, *L. gasseri* NT, on lipid metabolism in normal and obese mice model.

Chapter 2

We evaluated the effects of a tablet containing *Bacillus natto* and *Lactobacillus acidophilus* as probiotics supplemented with three stomachic herbs on human fecal microbiota. Six healthy subjects ingested 3 tablets, 3 times a day after meals for 10 days. Fecal samples were collected before and after the administration period, and 1 week post administration. As shown by the TGGE images, the density of the bands identified with *Bifidobacterium* increased in four cases. This increase was confirmed by real-time PCR. It was observed that the densities of the bands identified with *Haemophilus* decreased, *Ruminococcus* decreased, *Clostridium colinum* decreased, *Acidaminococcus* increased and *Megamonas* increased individually.

Chapter 3

Visceral fat accumulation is a major risk factor for the development of obesity-related diseases, including diabetes, hyperlipidemia, hypertension, and arteriosclerosis. Stimulation of lipolytic activity in adipose tissue or inhibition of fat synthesis is one way to prevent these serious diseases. Lactic acid bacteria have an anti-obesity effect, but the mechanisms are unclear. Therefore, we evaluated the effect of the administration of lactic acid bacteria (*Lactobacillus gasseri* NT) on lipid metabolism and fat synthesis in a mouse high-fat-diet model, focusing on visceral fat. Balb/c mice were fed a 45 kcal% fat diet for 13 weeks with and without a freeze-dried preparation of *L. gasseri* NT (10^9 CFU/g). An *ex vivo* glycerol assay with periovarian fat revealed that *L. gasseri* NT did not stimulate lipolytic activity. However, *L. gasseri* NT decreased the mRNA

expression of sterol regulatory element-binding protein (SREBP) and its target gene fatty acid synthase (FAS) in the liver and decreased free fatty acid (FFA) in the blood. In conclusion, these findings indicated that administration of *L. gasseri* NT did not enhance lipid mobilization but can reduce fat synthesis, suggesting its potential for improving obesity-related diseases.

Chapter 4

Dietary supplementation with *Lactobacillus gasseri* NT significantly decreased visceral fat weight and triglyceride (TG) in the liver in KK-*Ay* mice on a high-fat diet, but increased fecal TG. A decrease in lipase activity and down regulation of fatty acid transport proteins in the small intestine was involved in fat accumulation by *L. gasseri* NT.

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