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ORIGINAL ARTICLE

POSSIBLE RELATIONSHIP BETWEEN PERCENTAGE OF BODY FAT AND *LACTOBACILLALES* IN GUT MICROBIOTA: RESULTS FROM A COMMUNITY-BASED STUDY

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Abstract In recent years, *Lactobacillales* in gut microbiota have been suggested to be in association with obesity. The aim of this study was to investigate a relationship among obesity, serum cholesterol and gut microbiota in Japanese community inhabitants.

Subjects included 613 inhabitants (249 males and 364 females), who participated in the Iwaki Health Promotion Project in 2007. The gut mircoflora extracted from faeces were investigated using terminal restriction fragment length polymorphism (T-RFLP) method and allocated to 28 operational taxonomic units (OTUs). Proportions of OUT332 (*Lactobacillales*) in gut microbiota were compared among strata of percentages of body fat and serum cholesterol after the subjects were stratified by them. These comparisons were performed in the subjects who were ≥65 years old and <65 years old, respectively.

In women who were ≥65 years old, proportions of OTU332 (*Lactobacillales*) in the lowest stratum of body fat, serum level of total and LDL cholesterol were the highest in their strata.

A large proportion of *Lactobacillales* in gut microbiota might reduce absorption of cholesterol, leading to a decrease in body fat of them.

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Key words: Gut microbiota; *Lactobacillales*; body fat percentage; LDL cholesterol.

原　著

一般住民の腸内細菌叢における乳酸菌と肥満の関連について

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抄録　近年，腸内の乳酸菌が肥満に関与する可能性が指摘されている．本研究の目的は，腸内細菌中の乳酸菌割合と体脂肪率および血中コレステロールの関係を明らかにすることである．

2007年に岩木健康増進プロジェクトに参加した一般住民617名(男249名，女364名)について調査を行った．対象を65歳以上と未満に分け，それぞれの群における腸内細菌中の乳酸菌割合と体脂肪率および血中コレステロールを比較検討した．腸内細菌叢の解析にはTerminal restriction fragment length polymorphism法を用い，構成細菌を28のOperational Taxonomic Units (OTUs)に分類した．

65歳以上の女性において体脂肪率25％未満群はそれ以上の群よりOTU332(乳酸菌優勢群)の割合が高かった．またLDLコレステロール100 mg/dL未満の群はそれ以上の群よりOTU332の割合が高かった．

腸内の乳酸菌割合の増加は脂質代謝に影響を与え，これにより体脂肪率が減少する可能性が示唆された．

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キーワード：腸内細菌；乳酸菌；体脂肪率；LDLコレステロール．

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Introduction

The prevalence of clinical obesity, diabetes mellitus, high blood pressure and dyslipidemia has been increasing in developed countries including Japan[1]. Impaired lifestyles, abnormality of hereditary, endocrinal and other physical conditions have been recognised as causes of obesity, but *Lactobacillales* in gut microbiota is not noticed in relation to obesity widely.

There are some basic research in relation to gut microbiota and obesity. Some previous studies have shown that components of gut microbiota are different between identical twins with or without obesity[2]. Body weight loss in obese people has been reported to alter their components of gut microbiota to that of non-obese[3]. These studies have suggested that gut microbiota concerns difference of host's physique, such as obese or non-obese. A causal relationship that gut microbiota has an influence on host's physique has been also presumed in works in animals. Body fat of mice given gut microbiota of obese mice has been demonstrated to become higher than that of mice given that of non-obese mice[4]. In terms of individual species of bacteria, intake of *Lactobacillales* has been found effective to prevent or improve not only obesity but also obesity-related diseases, such as hypertension, hyperglycaemia and dyslipidaemia [5-8].

In human, the association between *Lactobacillales* in gut microbiota and obesity has, however, been still remained unclear. Santacruz et al[10] have reported that weight reduction by dietary control with physical exercise increases an amount of *Lactobacillales* in gut microbiota. By contrast, Armourgon et al[11] and Million et al[12] have shown that the amount of *Lactobacillales* in gut microbiota is greater in the obese than that in the non-obese, and Karlsson et al[13] have reported that the amount of faecal *Lactobacillales* does not differ between obese and non-obese pre-school children[13]. The controversy may result from limited number of subjects studied and limited detectability in analysing method for gut microbiota[14].

In recent years, Terminal restriction Fragment Length Polymorphism (T-RFLP) method has been used to assess microbiota. Nagashima et al [15, 16] has combined T-RFLP analysis to 16SrDNA clone library method, allowing us to assess a composition ratio of whole gut microbiota at family-genus level in a short period of time. The aim of this study was to examine a relationship between obesity and gut microbiota in Japanese community inhabitants. We could emphasise two valuable points, i.e. a novel method for assessing microbiota and a large number of subjects from general population, in this study.

Subjects and Methods

1. Subjects

Subjects of this study were 814 adults who participated in the Iwaki Health Promotion Project in 2007. This project was aimed to promote health of residents of Iwaki district, Hirosaki City, which locates in northern Japan, thus prolonging lifespans of them.

Those with medical histories of malignancy, esp. digestive system, and/or those who had received certain kinds of drugs, esp. antibiotics, anti-dyslipidaemic agents and laxatives, were eliminated from this study. Those with missing values in parameters were also ineligible. Finally, 613 subjects (249 males and 364 females) were eligible for our analyses.

Methods and purposes of this study were thoroughly explained to the subjects and written informed consents were obtained from all of them prior to the investigation. The Iwaki Health Promotion Project and this study were approved by the Ethics Committee of Hirosaki University Graduate School of Medicine.

2. Lifestyle and physical measurements of the subjects

Self-questionnaires were given to the subjects prior to the study and were collected on the investigation day after individual interviews by trained staffs. They consisted of sex, age and medical histories of the subjects. Smoking status (never-smoking, current smoking) and presence of habitual drinking were asked regarding their lifestyle. Frequencies, kinds and amounts of tobacco and/or alcohol drinks were also inquired when they were smokers and/or habitual drinkers. Then Brinkman index and consumed pure alcohol quantity per day were calculated for each subject. Percentages of body fat were determined by impedance method using a multi-frequency body composition analyser (TANITA, MC-190).

3. HDL and LDL cholesterol

Blood samples were collected on the day of investigation when the subjects were under fasting conditions. Serum levels of total cholesterol, high-density lipoprotein (HDL) and low-density lipoprotein (LDL) cholesterol were measured by Mitsubishi Medience Co., Ltd. after the blood samples were centrifuged to extract serums.

4. Analysis of gut microbiota

(a) Extraction of deoxyribonucleic acid (DNA) of bacteria in gut microbiota from faeces

To collect faeces of the subjects, commercial containers (TechnoSuruga Laboratory Co., Ltd., Shizuoka, Japan) were used. The collecting containers were substituted for contents liquids GTC solution (100mM Tris-HCl [pH 9.0], 40mM Tris-EDTA [pH 8.0], 4M Guanidine Thiocyanate). Samples are stored −83 ℃ until DNA extraction.

About 800 μL faecal GTC buffer suspensions were moved to test tubes with zirconium beads (NIPPON GENE CO., Ltd., Tokyo, Japan) and then they were beaten in room temperature for 5m/s 3 minutes FastPrep FP100A Instrument (MP Biomedicals, Irvine, CA). After cooled, they were centrifuged in 5,000 rpm for 1 minute. Thereafter, DNA was extracted from the bead-treated suspension using Magtration System 12GC and GC series MagtrationMagaZorb DNA Common Kit 200N (Precision System Science, Chiba, Japan), and the final concentration of each DNA sample was adjusted to 10 ng/μL.

(b) Polymerase Chain Reaction (PCR) Amplification

27F [5'-AGAGTTTGATCCTGGCTCAG-3'], or 516f [5'-TGCCAGCAGCCGCGGTA-3'] and 1492R [5'-GGTTACCTTGTTACGACTT-3'] were used to amplify the 16S rRNA genes. The 5' ends of the forward primer 27F were labelled with 6'-carboxyfluorescein (6-FAM), which was synthesized by Applied Biosystems Japan (Tokyo, Japan). The 5' ends of the forward primer516F were labelled with HEX, which was synthesized by Applied Biosystems Japan (Tokyo, Japan). PCR amplification of the DNA samples (10 ng of each DNA) was as follows; (1) preheating at 95 ℃ for 15 min, (2) 30 cycles of denaturation at 95 ℃ for 35 sec, (3) annealing at 50 ℃ for 30 sec, (4) extension at 72 ℃ for 10 min. The amplified 16S rRNA genes were purified using MultiScreen FB plates (Millipore, Tokyo, Japan) and redissolved in −40 μL distilled water.

The purified FAM-labelled PCR products (2 μL) were digested with 10 U of either HhaI or MspI or AluI at 37 ° C for 3 hours. The purified Hex-labelled PCR products (2 μL) were digested with 10 U of either BslI (New England BioLabs, Inc. Ipswich, USA) at 55 ° C for 3 hours.

(c) T-RFLP analysis

The length of terminal restriction fragment fragments was determined with an ABI PRISM 3100 genetic analyzer (Applied Biosystems,

Tokyo, Japan) in GeneScan mode. The standard size markers, such as GS-2500 ROX (Applied Biosystems, Tokyo, Japan), were used. The fragment sizes were estimated using local Southern method in GENESCAN 3.1 software (Applied Biosystems, Tokyo, Japan). The major T-RFs were identified by computer stimulation, which was performed using T-RFLP analysis programme, such as the phylogenetic assignment database for T-RFLP analysis of human colonic microbiota (PAD-HCM), and Microbiota Profiler (Infocom T-RFLP Database & Analysis Software, Infocom Co., Tokyo, Japan). The distances were calculated to determine any similarity among the samples and were represented graphically by constructing a dendrogram. Pearson's similarity coefficient analysis and unweighted pair-group methods with arithmetic means (UPGMA) were used to establish types of dendrogram. The T-RFs were quantified as percentage of individual T-RF peak area per total T-RF peak areas, and this was expressed as a percentage of the area under the curve (%AUC).

(d) Operational taxonomic units (OTUs)

The T-RFs were allocated to 28 operational taxonomic units (OTUs) according to their fragment sizes. Proportions of OTU332 calculated from %AUC of T-RFs were treated as proportions of *Lactobacillales* in gut microbiota because OTU332 corresponded to it according to human colonic microbiota (PAD-HCM), and Microbiota Profiler (Infocom T-RFLP Database & Analysis Software, Infocom Co., Tokyo, Japan).

(e) Statistical analysis

The subjects were stratified by sexes and ages (≥ 65 years old and < 65 years old) before analyses. Then a percentage of body fat was stratified into 4 strata (<15%, 15-20%, 20-25% and >25% in males, <25%, 25-30%, 30-35% and >35% in females) and the subjects were allocated to them.

Proportions of OTU332 were compared among 4 body fat strata using Bonfferoni method after plausible confounding factors, such as Brinkman index and consumed pure alcohol quantity per day, were adjusted using analysis of covariance (ANCOVA).

Additional analyses were needed for the female subjects who were ≥65 years old following the analyses regarding percentages of body fat. They were stratified into 4 strata according to their serum total cholesterol levels (<180 mg/dl, 180-200 mg/dl, 200-220 mg/dl and >220 mg/dl), serum HDL cholesterol levels (<50 mg/dl, 50-60 mg/dl, 60-70 mg/dl and >70 mg/ml) and serum LDL cholesterol levels (<100 mg/dl, 100-120 mg/dl, 120-140 mg/dl and >140 mg/dl). Then percentages of OTU332 were compared among these three kind of strata using Bonferroni method after adjustment of the plausible confounding factors above mentioned using ANCOVA.

All p-values were two-tailed and considered to be statistically significant when they were less than 0.05. Data analyses were performed using PASW Statistics 18 (IBM, Armonk, NY, USA).

Results

Characteristics of the subjects are shown in Table 1. The subjects included many middle-aged and elderly ones in both sexes and age difference between males and females was not provided. Percentages of body fat and serum cholesterol levels (tolat, LDL, HDL) of females were significantly higher than those of males. The male subjects of this study consumed alcohols and smoked more than the female subjects did.

Adjusted means of percentages of OTU332 according to body fat strata are given in Figure 1. We could not detect any significant

Table 1 Characteristics of the subjects

	Males (N=249)	Females (N=364)	
Age (years old)	57.3 ± 0.8	57.7 ± 0.7	
Percentage of body fat (%)	20.0 ± 0.3	30.2 ± 0.3	**
Total cholesterol (mg/dL)	198.0 ± 1.9	204.4 ± 1.6	*
HDL cholesterol (mg/dL)	58.4 ± 1.0	64.2 ± 0.7	**
LDL cholesterol (mg/dL)	115.9 ± 1.8	123.7 ± 1.5	**
Consumed pure alcohol quantity per day (g)	45.9 ± 3.2	5.5 ± 1.0	**
Brinkman index (stick/year)	324.8 ± 25.1	29.5 ± 5.7	**

Mean ± SD
HDL: high-density lipoprotein
LDL: low-density lipoprotein
* : $p<0.05$, ** : $p<0.01$, P-values were calculated by un-pared t-test.

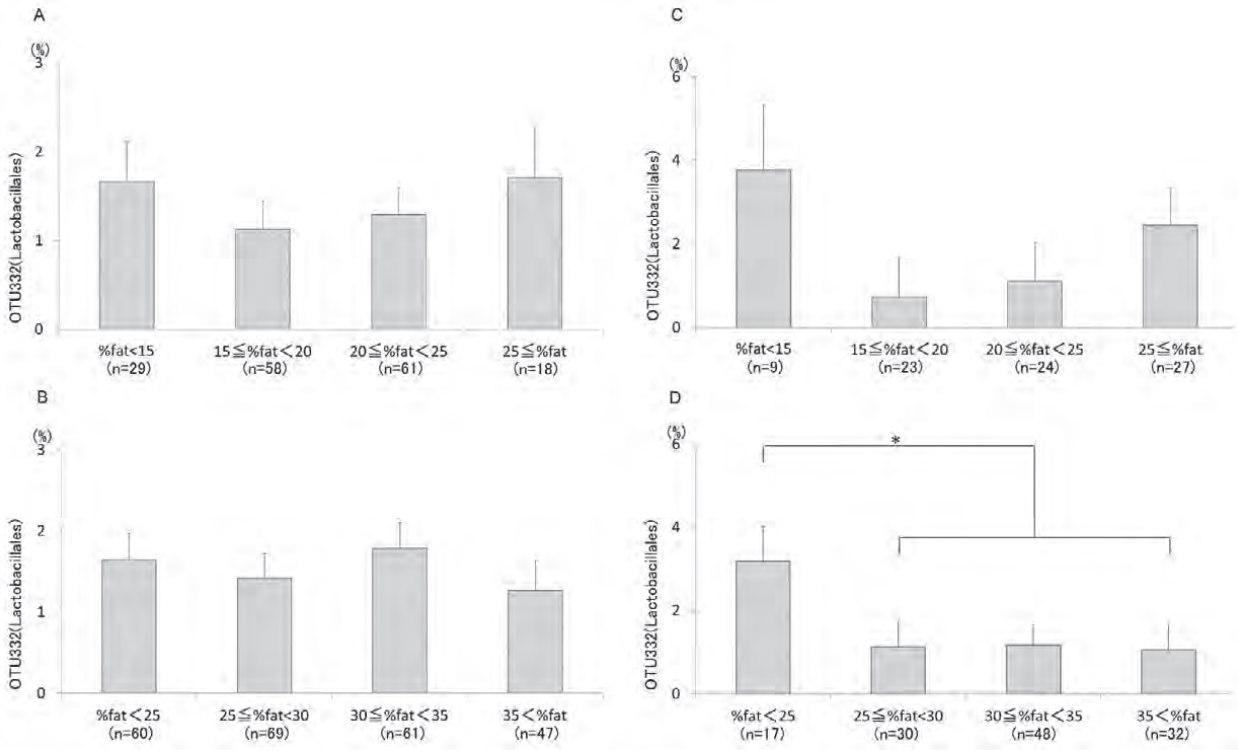

Figure 1 Percentage of OTU332 and body fat in males and females
Error bars represent standard errors of the means
Significant differences between stratum of %fat<25 and other strata were represented as *.
*: $p<0.05$
(A) Male,age<65y. (B) Male,age≧65y. (C) Female,age<65y. (D) Female,age≧65y

differences of percentages of OTU332 among these four strata in males. On the other hand, adjusted mean of percentages of OUT332 in the subjects whose percentages of body fat <25% was the highest among the subjects who were ≥65 years old although there were not any relationships between percentages of OTU332 and body fat in the subjects who were <60 years old in females.

Figure 2 presents a relationship between percentage of OTU332 and three kinds of serum cholesterol strata in the female subjects who were ≥65 years old. In serum total and LDL cholesterol strata, the lowest stratum showed

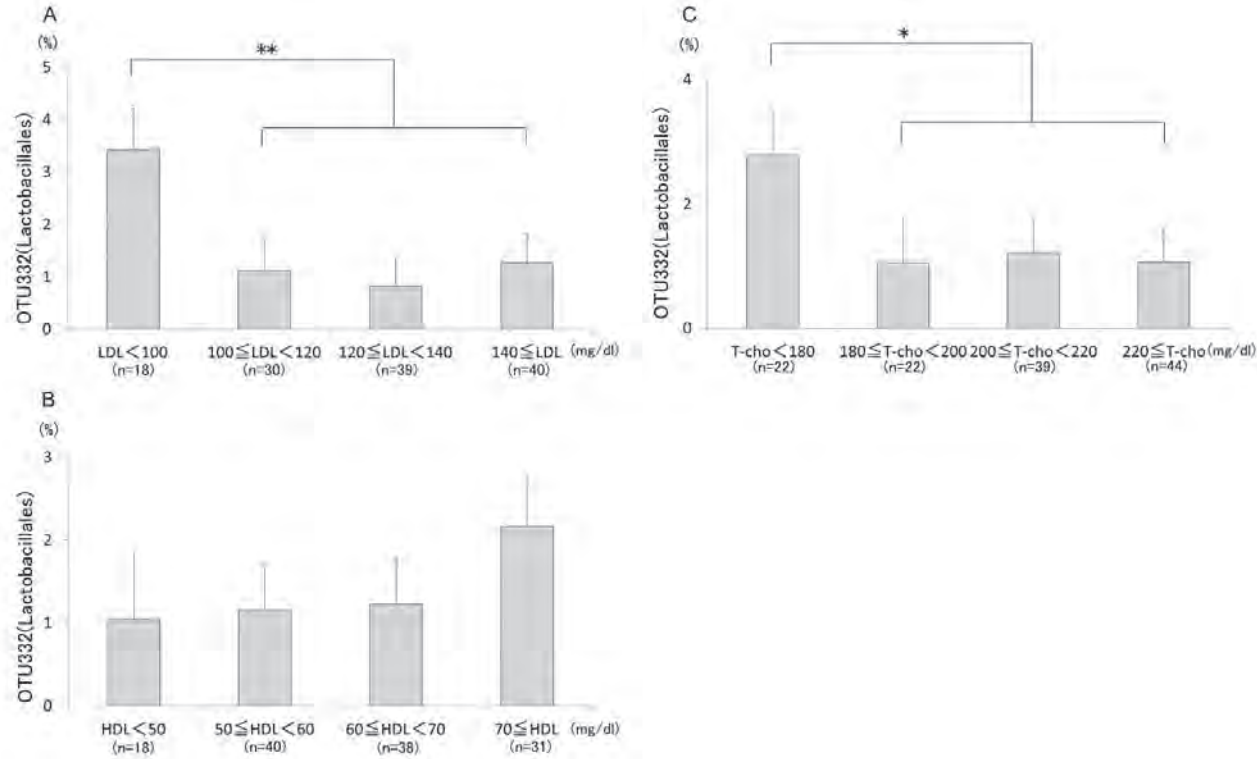

Figure 2 Percentage of OTU332 and serum cholesterol in women who were ≥65 years old
Error bars represent standard errors of the mean.
LDL: low density lipoprotein cholesterol
HDL: high density lipoprotein cholesterol
T-cho: total cholesterol;
Significant differences between the lowest stratum and other strata were represented as *.
*: $p<0.05$
**: $p<0.01$

the highest percentages of OTU332 and there were not any significant differences among other three strata. Serum HDL cholesterol did not show an association with percentages of OTU332.

Discussion

The present study was, to the best of our knowledge, a first community-based study that investigated the association between the amount of faecal *Lactobacillales* and obesity using T-RFLP analysis. There have been some previous studies which have discussed gut microbiota in the field of community-based studies, but they could observe only a part of it to assume proportions of all kinds of bacteria.

Santacruz et al. showed an increase in an amount of faecal *Lactobacillales* following body weight loss[10], but several studies have reported the opposite results[11, 12, 13]. In the present study, the subjects who were ≥65 years old with percentages of body fat <25% were found to have the highest proportion of *Lactobacillales* in body fat strata among females. In elderly females, a category "percentage of body fat <25%" are considered "average or less than average" according to criteria of the American College of Sports Medicine (ACSM)[17] as well as the Japanese Society for the Study of Obesity[18]. Therefore, our study suggested that high proportion of *Lactobacillales* in faeces was associated with non-obese physique in elderly women.

One of the reasons why *Lactobacillales* plays a role in prevention and improvement of obesity is considered that it reduces reabsorption and accelerates excretion of cholesterols from digestive tracts, thus lowering serum level of total cholesterol[9,19-21]. In this context, the amount of *Lactobacillales* in faeces could be related to body fat through cholesterol absorption. We revealed that high proportion of *Lactobacillales* in faeces was associated with the lowest stratum of total and LDL cholesterol in elderly women. Given that *Lactobacillales* in gut microbiota has an important effect on cholesterol absorption, decrease in *Lactobacillales* could be a risk factor of obesity for elderly women.

In contrast to elderly women, the proportion of *Lactobacillales* in gut microbiota did not have any relationship with body fat among males and young women. Serum levels of female hormone, which has an accelerative effect on excretion of cholesterol from digestive tracts[23], among post-menopausal women have been reported to be lower than those among men and young women[22]. Moreover, post-menopausal females with low levels of female hormone are easy to become obese through an increase in serum levels of cholesterol[24, 25]. We recognised that the association of body fat with Lactobacillales in gut microbiota among elderly women in this study was led by low levels of female hormone. Decline in lipid and glucose metabolism caused by aging should enhance the association [26-31].

There are some limitations in this study. Firstly, a major section of digestion and absorption is small intestine although gut microbiota is in colon and rectum. Our hypothesis that gut microbiota is associated with body fat would be acceptable when clear relationship between small intestine and gut microbiota is revealed. Secondary, bacterial genes in faeces include genes of gut microbiota and genes of bacteria ingested from mouth with foods. Other hypothesis that body fat is associated with bacteria which have adhered to foods could be denied. Intake frequency of fermented foods, which is rich in *Lactobacilles*, should have been asked in self-questionnaire. It is also possible that lifestyle with frequent intake of fermented foods provides lean physique. Tertiary, reasons why the relationship between *Lactobacillales* and body fat is limited in elderly women was not discussed sufficiently. Serum levels of female hormone in the subjects were not determined in this study. An investigation whether reduced levels of serum cholesterol would lead to lose body fat in elderly women is also needed.

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