

1 **Title:** Association analysis between type 2 diabetes and the gut microbiota: a community-
2 based cross-sectional study in Japan

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11 **Running head:** Association between type 2 diabetes and the gut microbiota

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16 **Keywords:** gut microbiota, type 2 diabetes, *Lactobacillales*, community-based cross-
17 sectional study

18 **Abstract:** There are a few studies on the association between type 2 diabetes (T2D) and
19 the gut microbiota but no community-based cross-sectional study. In the Iwaki Health
20 Promotion Project (Hirosaki, Japan), we surveyed medical information and lifestyle and
21 analyzed the gut microbiota using the terminal restriction fragment length
22 polymorphism technique or next-generation sequencing. Based on these data,
23 multivariable logistic regression analysis was performed with or without T2D as the
24 dependent variable. Consequently, it was suggested that the *Lactobacillales* population
25 in the gut microbiota was positively associated with T2D, consistent with the results of
26 case-control studies.

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INTRODUCTION

28 Type 2 diabetes (T2D) is caused by insulin resistance or impaired insulin secretion.
29 T2D is related to genetic factors and lifestyle, such as overeating or lack of exercise. In
30 Japan, the sum of patients with T2D and potential diabetics was 20 million in 2016 (The
31 National Health and Nutrition Survey in Japan, 2018). The prevalence is higher in males
32 and with increasing years.

33 Research on the association between various diseases and the gut microbiota has
34 advanced [1] and become increasingly important. Also, case-control studies on the
35 association between T2D and the gut microbiota have been reported [2-10]. These studies
36 commonly indicated an increased *Lactobacillales* population in the fecal microbiota in
37 T2D, so *Lactobacillales* have the potential to be a novel target for prevention and therapy
38 of T2D.

39 Community-based health examination surveys (in Hirosaki City, Aomori, Japan)
40 have been performed for many years, called “the Iwaki Health Promotion Project” [11].
41 In this project, about ten hundreds of people participate every year, and data from more
42 than 2000 items related to vital and medical information, dietary habits, and lifestyle
43 are collected. These also include data on the gut microbiota obtained using the terminal
44 restriction fragment length polymorphism (T-RFLP) technique [12, 13] or next-
45 generation sequencing (NGS) [14].

46 There has been no community-based cross-sectional study on the association
47 between T2D and the gut microbiota. Therefore, in this study, multivariable logistic
48 regression analysis was performed with or without T2D as the dependent variable using
49 Iwaki Health Promotion Project data.

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MATERIALS AND METHODS

52 *The Iwaki Health Promotion Project*

53 This survey was intended for the participants of the Iwaki Health Promotion
54 Project (UMIN ID: UMIN000040459) in 2012, 2014, and 2016. The number of subjects
55 by sex and age is shown in Table 1, in which those with ameliorated T2D were excluded
56 because of the difficulty of attributing to either of the binary variables. Subjects were
57 inquired about sex, age, medical history, drug intake, smoking, alcohol drinking, exercise,
58 etc. Weight and height were measured to calculate the body mass index (BMI). The
59 results of inquiries to the subjects with or without T2D are shown in Table 2. This study
60 was approved by the Ethics Committee of Hirosaki University Graduate School of
61 Medicine (No. 2012-050, 2014-014, 2016-028). Written informed consent was obtained
62 from all participants.

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64 *Microflora analysis*

65 Fecal DNA was prepared as described previously [15]. Feces were collected into a
66 4 M guanidine thiocyanate solution, followed by bead-beating and extraction using
67 magnetic beads. The fecal microbiota was analyzed by T-RFLP [12, 13] or NGS [14],
68 targeting the 16S rRNA gene.

69 *In silico* T-RFLP using bacterial 16S rRNA gene sequences obtained by random
70 cloning analysis of the fecal microbiota provided correspondence between operational
71 taxonomic units (OTUs) in T-RFLP and phylogenetic bacterial groups [12, 13]. This study
72 classified these OTUs into eight bacterial functional groups (B-1–B-8; see Table 2) based
73 on the organic acid-producing activity, which refers to the report of Kettle et al. [16].
74 Conversion equations are given below.

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$$B1 = OTU366 + OTU469 \times 0.97 + OTU853$$

76 $B2 = OTU317$

77 $B3 = OTU106 + OTU168 + OTU338 + OTU369 \times 0.33 + OTU494 \times 0.84 + OTU650 +$
78 $OTU657 \times 0.08 + OTU754 \times 0.52 + OTU919 \times 0.80 + OTU940 \times 0.28 + OTU955$
79 $\times 0.92$

80 $B4 = OTU110 \times 0.04 + OTU124$

81 $B5 = OTU332 + OTU520 + OTU657 \times 0.92$

82 $B6 = OTU369 \times 0.33 + OTU494 \times 0.14 + OTU505 \times 0.5 + OTU517 + OTU640 +$
83 $OTU749 + OTU754 \times 0.10 + OTU940 \times 0.24 + OTU955 \times 0.08$

84 $B7 = OTU110 \times 0.96 + OTU369 \times 0.17 + OTU469 \times 0.03$

85 $B8 = OTU369 \times 0.17 + OTU494 \times 0.02 + OTU505 \times 0.5 + OTU754 \times 0.38 + OTU919$
86 $\times 0.20 + OTU940 \times 0.48 + OTU990$

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88 *Multivariable logistic regression analysis*

89 Multivariable logistic regression analysis (forward selection method) was
90 performed with or without T2D as the dependent variable. The number of subjects with
91 or without T2D is shown by sex in Table 2. The functional bacterial groups in Table 3 in
92 T-RFLP or seven orders, including *Bacteroidales*, *Bifidobacteriales*, *Clostridiales*,
93 *Coriobacteriales*, *Enterobacteriales*, *Lactobacillales*, and *Selenomonadales*, in NGS were
94 the independent variables. The sequences derived from these seven orders accounted for
95 >90% of the total. Age, BMI, smoking, alcohol drinking, and exercise were used as
96 moderators in the regression analysis. In 2016, when subjects were inquired about drug
97 intake, α -glucosidase inhibitor (α -GI), a diabetes drug, was also used as a moderator.
98 JUSE-StatWorks/V5E (The Institute of JUSE, Tokyo, Japan) was used for statistical
99 analysis. The significance level was set at $p < 0.05$.

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RESULTS

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In Table 2, the T2D ratio in subjects was 3.6% to 4.2% in females and 7.2% to 7.7% in males, which is almost the same level as the ratio of outpatients with T2D to the population (>20 years old in 2016) in Aomori Prefecture: 3.1% in females and 5.4% in males. Moreover, differences between subjects with and without T2D in age, BMI and habits of life were shown.

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This study used multivariable logistic regression to assess the association between T2D and the gut microbiota (OTUs) in the Iwaki Health Promotion Project participant population for 3 years. A summary of the results is presented in Table 4. The functional bacterial group B-5 (*Lactobacillales*, refer to Table 3) was commonly extracted as a significant variable in males and females in case of 2012 and 2014, in which T-RFLP analysis was performed, with odds ratios [OR; 95% confidence intervals (95% CIs)] was 1.062 (1.025–1.100), 1.068 (1.019–1.20), 1.085 (1.032–1.142), and 1.069 (1.020–1.121), respectively. In case of 2016, in which NGS analysis was performed, *Lactobacillales* was a significant variable in males with ORs (95% CIs) of 1.174 (1.019–1.351), but it was an insignificant variable in females, with p-value of a slightly over 10% ($p = 0.109$). The functional bacterial group B-4 (*Bifidobacterium* spp.) was also a significant variable in some cases, that is, males and females in 2012 and males in 2014 and 2016, with ORs (95% CIs) of 1.150 (1.064–1.244), 1.075 (1.007–1.146), 1.053 (1.013–1.095), and 1.074 (1.024–1.125), respectively. As for the rest, the functional bacterial group B-3 (*Clostridium* clusters IV, XI, XIVa, and XVIII) in females in 2014, *Bifidobacterium* and *Coriobacteriales* in males in 2016, and *Bacteroidales* in females in 2016 were significant variables.

DISCUSSION

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126 Although previous studies on the association between T2D and the gut microbiota
127 were performed in various countries (Austria, Iran, Denmark, Japan, and Sweden) with
128 various techniques (NGS, quantitative polymerase chain reaction, and T-RFLP), a
129 positive association with *Lactobacillales* was indicated in any studies, suggesting a
130 considerably high certainty of the results. In this study, a multivariable logistic
131 regression analysis was performed to assess the association between T2D and the gut
132 microbiota in a cross-sectional study intended for people in Hirosaki, Japan. Similar
133 results to previous studies were obtained.

134 This and previous studies suggested the causal relationship between T2D and
135 *Lactobacillales*, but the cause or result is unknown. First, whether *Lactobacillales* are
136 innate bacteria or bacteria originating from foods, such as yogurt, was discussed. Sato et
137 al. [6] and Adachi et al. [9] considered that these bacteria are innate because the number
138 of subjects who consumed yogurt was not significantly different between the control and
139 T2D groups and significantly fewer in the latter group. Furthermore, the number of
140 probiotic bacteria taken from supplements or foods was $\sim 10^{10}$ cells/day. These bacteria
141 cannot colonize and proliferate in the gut to remain a minor population. Therefore, the
142 idea that these bacteria are innate is deemed to be appropriate.

143 Morita et al. [17] indicated that lactate and pyruvate, produced in the small
144 intestinal in a bacteria-dependent manner, enhanced immune responses by inducing
145 dendrite protrusion of intestinal CX3CR1⁺ mononuclear cells. These findings speculated
146 a possible causal relationship as follows: an excess of bacteria, including lactic acid
147 bacteria in the small intestine, induces the overstimulation of immune responses,
148 followed by chronic inflammation, resulting in the T2D onset (18, 19). Moreover, Koh et
149 al. [20] have described that imidazole propionate produced by fecal microbiota belonging

150 to the order *Lactobacillales*, including *Streptococcus spp.* and *Lactobacillus spp.* impairs
151 insulin signaling at the level of insulin receptor substrate, which may result in the
152 pathogenesis of T2D.

153 If that is the case, why do bacteria such as lactic acid bacteria proliferate
154 abnormally in the small intestine? Usually, gastric acid strongly prevents bacterial entry
155 to the small intestine from the oral cavity so that almost no bacterial proliferation occurs.
156 Therefore, reduced secretion of gastric acid by aging or by taking the medicine for gastric
157 hyperacidity that is induced by stress, disturbed eating habit and so on, may result in
158 the proliferation of these bacteria in the small intestine followed by their subsequent
159 increase in the large intestine [21-24]. Yuan et al. [25] and Ciardullo et al. [26] have
160 reported that regular and prolonged use of proton pump inhibitors (PPIs) was associated
161 with a higher risk of type 2 diabetes. In the Iwaki Health Promotion Project in 2016, we
162 also found that the ratio of subjects prescribed PPIs was significantly greater in
163 population with T2D than in that without T2D (Table 2). These findings seem to provide
164 partial support to the above-mentioned speculation.

165 On the one hand, some studies suggested that α -GI increases populations of
166 *Bifidobacterium spp.* and *Lactobacillales* in the fecal microbiota [6, 9, 10]. In this project
167 in 2016, the number of subjects with T2D who did or did not take α -GI was 12 (10 males
168 and 2 females) and 45 (23 males and 22 females), respectively. *Bifidobacterium spp.*
169 population in the fecal microbiota in males was higher in subjects with T2D taking α -GI
170 than those not taking α -GI [mean \pm standard deviation (SD), 24.3 \pm 7.9 vs. 9.3 \pm 11.0; p
171 = 0.003, Wilcoxon rank-sum test]. Similarly, in *Lactobacillales* in males, the population
172 was higher in subjects with T2D taking α -GI (mean \pm SD, 29.4 \pm 14.6 vs. 8.5 \pm 14.9; p <
173 0.001). No significant difference was observed in females. From these facts, here we
174 accessed using α -GI as an additional moderator for the regression analysis in 2016, in
175 which subjects were inquired about drug intake.

176 On the other hand, α -GI is not involved in the other studies that indicate the
177 positive association between T2D and *Lactobacillales* [5, 7, 8]. Furthermore, the
178 *Lactobacillales* population in the fecal microbiota also increased in a mouse model of
179 insulin resistance [27]. Considering these findings, α -GI is deemed to have only limited
180 effectiveness in this and previous studies on the association between T2D and the gut
181 microbiota.

182 Taken together, multivariable logistic regression analysis was performed in the
183 community-based cross-sectional study to assess the association between T2D and the
184 gut microbiota. It was suggested that the *Lactobacillales* population was positively
185 related to T2D, as indicated in previous studies. It is expected that future analyses will
186 be performed with more subjects, and studies on the elucidation of the causal
187 relationship are making progress. As such, novel techniques for prevention and therapy
188 targeted to the bacteria are being developed.

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AUTHER CONTRIBUTIONS

195 K.N. played a role in the investigation, conceptualization and writing original
196 draft. T.H. played a role in the data curation, formal analysis and investigation. J.M.,
197 T.M. and Y.T. played a role in the project administration and reviewing. S.N. played a
198 role in the project administration and supervision.

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CONFLICT OF INTEREST

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The authors declare no competing interests.

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DATA AVAILABILITY STATEMENT

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The data presented in this study are available under the agreement with

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Innovation Center for Health Promotion, Hirosaki University.

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301 model of insulin resistance. *Exp Ther Med* 18: 857-866.

Table 1. The number of subjects by sex and age each year

Year		2012		2014		2016	
No. of subjects		936		1,103		1,088	
Sex		Male	Female	Male	Female	Male	Female
	~20	16	19	27	43	24	34
	30	51	68	73	84	81	96
	40	56	86	78	101	74	111
Age	50	73	131	76	130	82	125
	60	95	166	103	196	106	177
	70	45	104	48	107	43	95
	80~	12	14	14	23	17	23
Sum		348	588	419	684	427	661

The subjects with ameliorated T2D are excluded.

Table 2. Results of inquiries to subjects with or without T2D by sex each year

Year		2012			2014			2016			
		With T2D	Without T2D	<i>p</i>	With T2D	Without T2D	<i>p</i>	With T2D	Without T2D	<i>p</i>	
Male	No. of subjects, n (%)	25 (7.2)	323 (92.8)		31 (7.4)	388 (92.6)		33 (7.7)	394 (92.3)		
	Age	67 (77-62)	55 (64-42.5)	0.000	66, 77-63	52 (64-39)	0.000	67 (76-63)	52 (64-38)	0.000	
	BMI	24.2 (26.3-22.5)	23.2 (25.3-21.2)	0.000	23.9, 25.6-22.9	23.3 (25.0-21.5)	0.062	23.8 (25.7-22.2)	23.6 (25.5-21.7)	0.594	
	No. of subjects, n (%)	With smoking habit	3 (12.0)	107 (33.1)	0.027	6 (19.4)	125 (32.2)	0.162	4 (12.1)	117 (29.7)	0.042
		with drinking habit	18 (72.0)	226 (70.0)	1.000	18 (58.1)	269 (69.3)	0.228	21 (63.6)	275 (69.8)	0.440
		With exercise habit	13 (52.0)	95 (29.4)	0.025	12 (38.7)	122 (31.4)	0.426	10 (30.3)	111 (28.2)	0.841
		Prescribed PPIs	ND	ND		ND	ND		6 (18.2)	18 (4.6)	0.007
	Female	No. of subjects, n (%)	21 (3.6)	567 (96.4)		29 (4.2)	655 (95.8)		24 (3.6)	637 (96.4)	
		Age	69.5 (72.5-64)	58 (67-46)	0.000	70, 74-64	58 (66-42.5)	0.000	68 (72.3-57)	57 (66-43)	0.000
		BMI	24.4 (25.9-22.8)	22.1 (24.1-20.0)	0.000	24.0 (27.6-22.5)	21.7 (23.9-19.6)	0.000	23.8 (26.6-22.0)	21.9 (24.2-19.8)	0.016
No. of subjects, n (%)		With smoking habit	1 (4.8)	33 (5.8)	1.000	2 (6.9)	51 (7.8)	1.000	0 (0.0)	64 (10.1) ^a	0.156
		with drinking habit	1 (4.8)	137 (24.2)	0.037	2 (6.9)	179 (27.3)	0.016	5 (20.8)	176 (27.8) ^b	0.642
		With exercise habit	11 (52.4)	168 (29.6)	0.032	15 (51.7)	213 (32.5)	0.043	7 (29.2)	155 (24.3)	0.629
		Prescribed PPIs*	ND	ND		ND	ND		6 (25.0)	44 (6.9)	0.007

Age and BMI are expressed as median (interquartile range). The number of subjects in ^a and ^b are 636 and 634, respectively.

P values were calculated by using the Wilcoxon rank sum test and the Fisher's exact test.

*The number of subjects that were prescribed PPIs within three months before the date of inquiry. PPIs, proton pump inhibitors; ND, no data

Table 3. Classification of OTUs in T-RFLP into the bacterial functional groups

Bacterial functional groups	Phylogenetic bacterial groups	Organic acid producing activity	OTUs in T-RFLP *
B-1	<i>Bacteroides</i>	Acetate, Propionate, Succinate	366, 469 (97), 853
B-2	<i>Prevotella</i>	Acetate, Propionate, Succinate	317
B-3	<i>Clostridium</i> cluster IV, XI, XIVa, XVIII	Acetate, Succinate	106, 168, 338, 369 (33), 494 (84), 650, 657 (8), 754 (52), 919 (80), 940 (28), 955 (92)
B-4	<i>Bifidobacterium</i>	Acetate, Lactate	110 (4), 124
B-5	<i>Lactobacillales</i> (<i>Enterococcus</i> , <i>Lactobacillus</i> , <i>Streptococcus</i>)	Lactate	332, 520, 657 (92)
B-6	<i>Clostridium</i> cluster IV, XI, XIVa, XVIII	Butyrate, Lactate, Formate	369 (33), 494 (14), 505 (50), 517, 640, 749, 754 (10), 940 (24), 955 (8)
B-7	<i>Clostridium</i> cluster IX	Acetate, Propionate	110 (96), 369 (17), 469 (3)
B-8	<i>Clostridium</i> cluster XIVa	Acetate, Butyrate, Formate Lactate, Acetate → Butyrate	369 (17), 494 (2), 505 (50), 754 (38), 919 (20), 940 (48), 990

* In silico T-RFLP using the 16S rRNA gene sequences obtained by the random cloning analysis of fecal microbiota provides a correspondence between the OTUs in T-RFLP and the phylogenetic bacterial groups (12, 13). In this research, these OTUs were further classified into the 8 bacterial functional groups (B-1 to B-8) on the basis of the organic acid producing activity. Even if the clones from the bacterial 16S rRNA gene belong to the same OTU, they are allocated to the different functional group in some cases because of the different organic acid producing activity. Numbers in parentheses indicate the allocation ratios (%), which were calculated from the number of clones belonging to each functional group.

Table 4. Multivariable logistic regression analysis for the presence of T2D

Year	Sex	Variables	B	SE	P	OR	95% CI		
							Lower	Upper	
2012	Male	B-4 (<i>Bifidobacterium</i>)	0.140	0.040	0.00045 *	1.150	1.064	1.244	
		B-5 (<i>Lactobacillales</i>)	0.060	0.018	0.00075 *	1.062	1.025	1.100	
		Constant	-15.195	2.810	0.00000				
	Female	B-5 (<i>Lactobacillales</i>)	0.066	0.024	0.00603 *	1.068	1.019	1.120	
		B-4 (<i>Bifidobacterium</i>)	0.072	0.033	0.03159 *	1.075	1.007	1.146	
		B-6 (<i>Clostridium</i>)	0.057	0.032	0.07192	1.059	0.994	1.127	
		Constant	-16.395	2.974	0.00000				
	2014	Male	B-5 (<i>Lactobacillales</i>)	0.082	0.026	0.00163 *	1.085	1.032	1.142
			B-4 (<i>Bifidobacterium</i>)	0.052	0.020	0.00788 *	1.053	1.013	1.095
			Constant	-11.749	2.397	0.00000			
Female		B-3 (<i>Clostridium</i>)	-0.094	0.029	0.00128 *	0.910	0.860	0.964	
		B-5 (<i>Lactobacillales</i>)	0.067	0.024	0.00498 *	1.069	1.020	1.121	
		B-8 (<i>Clostridium</i>)	-0.053	0.033	0.10939	0.948	0.889	1.012	
		B-4 (<i>Bifidobacterium</i>)	0.024	0.018	0.19678	1.024	0.989	1.061	
Constant	-11.465	2.269	0.00000						

continued

		<i>Bifidobacteriales</i>	0.071	0.024	0.00382 *	1.074	1.024	1.125
	Male	<i>Coriobacteriales</i>	0.160	0.072	0.02529 *	1.174	1.019	1.351
		<i>Lactobacillales</i>	0.036	0.018	0.04820 *	1.037	1.001	1.074
2016		Constant	-8.968	1.352	0.00000			
		<i>Bacteroidales</i>	-0.063	0.025	0.01043 *	0.939	0.894	0.986
	Female	<i>Lactobacillales</i>	0.033	0.021	0.10876	1.034	0.992	1.077
		Constant	-4.960	1.609	0.00206			

Refer to Table 2 with respect to the variables of B-3, B-4, B-5, B-6 and B-8.

Age, BMI, the habits of smoking, alcohol drinking and exercise, and the medication of α -GI (only in the case of 2016) were used as the moderator variable.

The variables with $p < 0.2$ were presented. * indicate $p < 0.05$.

SE, standard error; OR, odds ratio; CI, confidence interval.