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Comparative Analysis of Gastrointestinal Microbiota Between Normal and Caudal-Related Homeobox 2 (*Cdx2*) Transgenic Mice

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Background/Aims: Caudal-related homeobox 2 (*Cdx2*) is expressed in the human intestinal metaplastic mucosa and induces intestinal metaplastic mucosa in the *Cdx2* transgenic mouse stomach. Atrophic gastritis and intestinal metaplasia commonly lead to gastric achlorhydria, which predisposes the stomach to bacterial overgrowth. In the present study, we determined the differences in gut microbiota between normal and *Cdx2* transgenic mice, using quantitative reverse transcription-polymerase chain reaction (qRT-PCR). **Methods:** Twelve normal (control) and 12 *Cdx2* transgenic mice were sacrificed, and the gastric, jejunal, ileac, cecal and colonic mucosa, and feces were collected. To quantitate bacterial microbiota, we used real-time qRT-PCR with 16S rRNA gene-targeted, species-specific primers. **Results:** The total numbers of bacteria in the gastric, jejunal, ileac, cecal, and colonic mucosa of the *Cdx2* transgenic mice were significantly higher than those of the normal mice. The *Bacteroides fragilis* group and also *Prevotella* were not detected in the stomach of the normal mice, although they were detected in the *Cdx2* transgenic mice. Moreover, the *Clostridium coccoides* group, *Clostridium leptum* subgroup, *Bacteroides fragilis* group, and *Prevotella* were not detected in the jejunum or ileum of the normal mice, although they were detected in the *Cdx2* transgenic mice. The fecal microbiota of the normal mice was similar to that of the *Cdx2* transgenic mice. **Conclusions:** Our results showed the differences in composition of gut microbiota between normal and *Cdx2* transgenic mice, which may be caused by the development of gastric achlorhydria and intestinal metaplasia in *Cdx2* transgenic mice. **(Intest Res 2015;13:39-49)**

Key Words: Microbiota; *Cdx2*; Intestinal metaplasia; Gastric achlorhydria; 16S rRNA

INTRODUCTION

Caudal-related homeobox 2 (CDX2), a caudal-related homeobox transcription factor, is selectively localized in the nuclei of fetal and adult mucosal epithelial cells in the small and large intestines of humans and mice.[1] CDX2 is important for the transcriptional regulation of intestinal genes and the differentiation of intestinal cells *in vitro*.[2,3] Although normal gastric mucosa does not express CDX2, nuclear immunoreactivity for CDX2 is detected in the human gastric intestinal metaplastic mucosa.[4-8] We previously established *Cdx2* transgenic mice that exclusively expressed CDX2 in their gastric epithelium,[9] and demonstrated that their gastric fundic mucosa was completely morphologically changed into intestinal metaplastic mucosa. Moreover, we previously showed that expression of CDX2 may be an early event that

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triggers the development of intestinal metaplasia in the human gastric mucosa.[5] These findings suggest that CDX2 has a regulatory role in the development and differentiation of the intestinal metaplasia.

In humans, chronic gastritis due to *Helicobacter pylori* infection commonly leads to loss of parietal and chief cells, diminution of the gastric glands, and thus atrophy or thinning of the mucosa. In addition, chronic atrophic gastritis often leads to the development of intestinal metaplasia. In this situation, humans develop gastric achlorhydria.[10] Gastric achlorhydria also occurs in association with intestinal metaplasia in *Cdx2* transgenic mice.[9] Gastric achlorhydria predisposes the stomach to bacterial overgrowth. To date, the few studies that have explored the microbiota of the stomach with either negative or positive *H. pylori* status have used molecular methods.[11-13] However, to the best of our knowledge, there have been no studies published that focus on characterization of the gastric microbiota in severe atrophic gastritis or intestinal metaplasia.

Identification of bacterial strains by conventional culture-based methods provides an incomplete and biased picture of the biodiversity of intestinal microbiota, because many species cannot be cultivated *in vitro*.[14] Therefore, culture-independent molecular methods, based on 16S rRNA genes, such as fluorescent *in situ* hybridization,[15] denaturing gradient gel electrophoresis,[16] and cloning and sequencing of rDNA,[14] have been introduced to obtain a better understanding of the gut microbiota. Recently, Matsuda et al.[17] developed a quantitative RT-PCR (qRT-PCR) method with 16S rRNA-gene-targeted, species-specific primers for species that were designed for analysis of human intestinal microbiota.

The aim of the present study was to determine the differences in gut microbiota between normal and *Cdx2* transgenic mice, using this qRT-PCR method.

METHODS

1. Mice

We used *Cdx2* transgenic mice with stomach-specific expression of *Cdx2* under the control of the rat H^+/K^+-ATPase β-subunit gene promoter.[9] The *Cdx2* transgenic mice were originally from a C57BL/6J background. The transgenic mice and normal mice (C57BL/6J) had ad libitum access to standard laboratory chow (CE-2; CLEA Japan, Tokyo, Japan) and drinking water, and were maintained on a 12-hour light/dark cycle. Twelve normal mice and 12 *Cdx2* transgenic mice (six male and six female, seven weeks old) were sacrificed, and the mucosae of the stomach, jejunum, ileum, cecum, and colon were scraped off with a spatula and collected in 1 mL RNAlater (Life Technologies, Carlsbad, CA, USA), an RNA stabilization solution, prior to bacteriological analysis. Feces were also collected in 1 mL RNAlater and the preparations were incubated for 10 minutes at room temperature.

All experiments were carried out in a humane manner after receiving approval from the Institutional Animal Experiment Committee of the Jichi Medical University, and in accordance with the Institutional Regulation for Animal Experiments and Fundamental Guidelines for Proper Conduct of Animal Experiment and Related Activities in Academic Research Institutions under the jurisdiction of the Ministry of Education, Culture, Sports, Science and Technology of Japan.

2. Total RNA Isolation

For RNA stabilization, an aliquot of the fecal homogenate (200 μL) was added to 1 mL sterilized phosphate buffer solution, and centrifuged at 5,000 × g for 10 minutes. The supernatant was discarded, and the pellet was stored at –80°C until used for the extraction of RNA. RNA was isolated using a modified method of acid guanidinium thiocyanate–phenol–chloroform extraction.[17,18] Finally, the nucleic acid fraction was suspended in 1 mL nuclease-free water (Ambion, Austin, TX, USA).

3. qRT-PCR

qPT-PCR was conducted according to previous reports.[17-23] It was conducted in a one-step reaction, using a Qiagen OneStep RT-PCR kit (Qiagen GmbH, Hilden, Germany). Each reaction mixture (10 μL) was composed of 1× Qiagen OneStep RT-PCR buffer, 0.5× Q-solution, each dNTP at a concentration of 400 μmol/L, a 1:100,000 dilution of SYBR Green I (Life Technologies, Carlsbad, CA, USA), 0.4 μL Qiagen OneStep RT-PCR enzyme mix, each specific primer (Table 1) at a concentration of 0.6 μmol/L (except for g-Bfra-F2/g-Bfra-R at 1.2 μmol/L and sg-Lsak-F/sg-Lsak-R at 2.4 μmol/L), and 5 μL template RNA. The reaction mixture was incubated at 50°C for 30 minutes for reverse transcription. The continuous amplification program consisted of one cycle at 95°C for 15 minutes; 40 cycles at 94°C for 20 seconds, 50, 55, or 60°C (Table 1) for 20 seconds, and 72°C for 50 seconds; and finally, one cycle at 94°C for 15 seconds. Fluorescent products were detected in the last step of each cycle. Melting curve

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Table 1. 16S or 23S rRNA Gene-Targeted Primers Used in This Study

Target	Primer	Sequence (5′–3′)	Product size (bp)	Annealing temp (°C)	Refs
Clostridium coccoides group	g-Ccoc-F	AAATGACGGTACCTGACTAA	440	55	19
	g-Ccoc-R	CTTTGAGTTTCATTCTTGCGAA			
Clostridium leptum subgroup	sg-Clept-F	GCACAAGCAGTGGAGT	239	55	19
	sg-Clept-R3	CTTCCTCCGTTTTGTCAA			
Bacteroides fragilis group	g-Bfra-F2	AYAGCCTTTCGAAAGRAAGAT	495	50	20
	g-Bfra-R	CCAGTATCAACTGCAATTTTA			
Bifidobacterium	g-Bifid-F	CTCCTGGAAACGGGTGG	552	55	19
	g-Bifid-R	GGTGTTCTTCCCGATATCTACA			
Atopobium cluster	c-Atopo-F	GGGTTGAGAGACCGACC	190	55	19
	c-Atopo-R	CGGRGCTTCTTCTGCAGG			
Prevotella	g-Prevo-F	CACRGTAAACGATGGATGCC	513	55	19
	g-Prevo-R	GGTCGGGTTGCAGACC			
Clostridium perfringens	s-Clper-F	GGGGGTTTCAACACCTCC	170	60	17
	ClPER-R	GCAAGGGATGTCAAGTGT			21
Lactobacillus gasseri subgroup	sg-Lgas-F	GATGCATAGCCGAGTTGAGAGACTGAT	197	60	17
	sg-Lgas-R	TAAAGGCCAGTTACTACCTCTATCC			
Lactobacillus brevis	s-Lbre-F	ATTTTGTTTGAAAGGTGGCTTCGG	289	55	17
	s-Lbre-R	ACCCTTGAACAGTTACTCTCAAAGG			
Lactobacillus casei subgroup	sg-Lcas-F	ACCGCATGGTTCTTGGC	296	60	17
	sg-Lcas-R	CCGACAACAGTTACTCTGCC			
Lactobacillus fermentum	LFer-1	CCTGATTGATTTTGGTCGCCAAC	414	55	22
	LFer-2	ACGTATGAACAGTTACTCTCATACGT			
Lactobacillus fructivorans	s-Lfru-F	TGCGCCTAATGATAGTTGA	452	55	17
	s-Lfru-R	GATACCGTCGCGACGTGAG			
Lactobacillus plantarum subgroup	sg-Lpla-F	CTCTGGTATTGATTGGTGCTTGCAT	54	60	17
	sg-Lpla-R	GTTCGCCACTCACTCAAATGTAAA			
Lactobacillus reuteri subgroup	sg-Lreu-F	GAACGCAYTGGCCCAA	289	60	17
	sg-Lreu-R	TCCATTGTGGCCGATCAGT			
Lactobacillus ruminis subgroup	sg-Lrum-F	CACCGAATGCTTGCAYTCACC	182	60	17
	sg-Lrum-R	GCCGCGGGTCCATCCAAAA			
Lactobacillus sakei subgroup	sg-Lsak-F	CATAAAACCTAMCACCGCATGG	303	60	17
	sg-Lsak-R	TCAGTTACTATCAGATACRTTCTTCTC			
Enterobacteriaceae	En-lsu-3F	TGCCGTAACTTCGGGAGAAGGCA	428	60	18
	En-lsu-3′R	TCAAGGACCAGTGTTCAGTGTC			
Enterococcus	g-Encoc-F	ATCAGAGGGGGATAACACTT	337	55	17
	g-Encoc-R	ACTCTCATCCTTGTTCTTCTC			
Streptococcus	g-Strept-F	AGCTTAGAAGCAGCTATTCATTC	306	60	23
	g-Strept-R	GGATACACCTTTCGGTCTCTC			
Staphylococcus	g-Staph-F	TTTGGGCTACACACGTGCTACAATGGACA	79	60	17
	g-Staph-R	AACAACTTTATGGGATTTGCWTGA			
Pseudomonas	PSD7F	CAAAACTACTGAGCTAGAGTACG	215	60	18
	PSD7R	TAAGATCTCAAGGATCCCAACGGCT			

Specific primer sets were developed by using 16S rRNA gene sequences, except for En-lsu-3F/En-lsu-3′R, which target 23S rRNA genes.

analysis was performed after amplification to distinguish the targeted from nontargeted PCR products. The melting curve was obtained by slow heating at temperatures from 60°C to 95°C at a rate of 0.2°C/s, with continuous fluorescent collection. Amplification and detection were performed in 384-well optical plates on an ABI PRISM 7900HT sequence detection system (Applied Biosystems, Foster City, CA, USA).

4. Determination of Bacterial Count by qRT-PCR

The standard curve was generated with qRT-PCR data, cycle threshold values, and the corresponding cell count, which was determined microscopically with the 4',6-diamidino-2-phenylindole (DAPI) staining method upon the dilution series of the following standard strains (appendix). For the determination and quantification of the target bacteria present in samples, three serial 10-fold dilutions of extracted RNA samples (corresponding to 1/2,000, 1/20,000, and 1/200,000 of the amount of RNA extracted from 20-mg samples) were applied to qRT-PCR, and cycle threshold values in the linear range of the assay were applied to the standard curve generated in the same experiment to obtain the corresponding bacterial count in each nucleic acid sample. The results were converted into the count per sample. Total bacteria were calculated as a total population of 12 bacterial groups.

5. Statistical Analysis

We used SPSS Statistics 14.0 software (Nihon IBM Inc., Tokyo, Japan). A non-parametric Mann–Whitney *U* test was performed to determine the significance levels for the differences in mean bacterial number for normal and *Cdx2* transgenic mice. The significance of the difference in numbers of mice positively testing for each bacterium between normal and *Cdx2* transgenic mice was determined with Fisher's exact test. A value of $P<0.05$ was regarded as significant.

RESULTS

1. Gut Microbiota in Normal Mice

The compositions of both the predominant and subdominant bacterial populations were analyzed by qRT-PCR, with lower detection limits in the range of 10^2–10^4 cells/g feces, and the mean±SD for the total population of these 12 bacterial groups and one species was $\log_{10}$ 10.3±0.5 cells/g feces (Table 2, Fig. 1–6). *Lactobacillus* was the dominant bacterial genus in normal mouse feces and was detected in almost all gut mucosa in normal mice (Table 2, Fig. 1–6). *Lactobacillus* was the dominant bacteria in the normal mouse gut. The *C. coccoides* group, *C. leptum* subgroup, and *Enterococcus* were detected in the normal mouse stomach. However, the detection rates were under 50% (Table 2, Fig. 1). Only *Lactobacillus* was detected in the jejunum of normal mice (Table 2, Fig. 2). Some other bacterial groups such as *Enterococcus*, *Streptococcus*, and *Bifidobacterium* were detected in the normal mouse ileum. However, the detection rates were under 50% (Table 2, Fig. 3). Various bacterial groups detected in the normal mouse feces were also detected in the cecum and the colon of normal mice, in contrast to the jejunum and the ileum, although the bacterial counts and detection rates were lower than those in normal mouse feces (Table 2, Fig. 4–6).

2. Comparison of Gut Microbiota of Normal and *Cdx2* Transgenic Mice

The average numbers of total bacteria in the gastric, jejunal, ileac, cecal, and colonic mucosa of *Cdx2* transgenic mice were significantly higher than those in normal mice (Table 2, Fig. 1–5). The *Bacteroides fragilis* group and *Prevotella* were not detected in the stomach, jejunum, or ileum of normal mice but they were detected in *Cdx2* transgenic mice (Fig. 1–3). Moreover, the detection rates of the *C. coccoides* group, *C. leptum* subgroup, and *Enterococcus* in the intestine of normal mice were significantly lower than those of *Cdx2* transgenic mice (Table 2). In contrast, there was no significant difference between the average number of total bacteria in the feces of *Cdx2* transgenic mice and that of normal mice (Fig. 6). However, presence of the *B. fragilis* group in the feces of *Cdx2* transgenic mice was significantly higher than that for normal mice, and the number of *Bifidobacterium* in the feces of *Cdx2* transgenic mice was significantly lower than that for the normal mice.

DISCUSSION

To our knowledge, this report represents the first quantitative examination of bacterial populations in intestinal metaplasia, without culture. We clarified that gastric achlorhydria due to intestinal metaplasia increases the diversity of gut microbiota. Moreover, this effect was observed not only in the stomach but also in the intestine.

Recently, the sequencing approach using 16S rRNA gene clones has extensively been conducted to investigate the composition of human gastric microbiota.[11,12,24] These stud-

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Table 2. Bacterial Number, Determined by Real-Time Quantitative RT-PCR of the Gut Flora of Normal and Caudal-Related Homeobox 2 (*Cdx2*) Transgenic (Tg) Mice

Bacterial species	Stomach				Jejunum				Ileum				Cecum				Colon				Feces			
	Cdx2 Tg mice		Normal mice		Cdx2 Tg mice		Normal mice		Cdx2 Tg mice		Normal mice		Cdx2 Tg mice		Normal mice		Cdx2 Tg mice		Normal mice		Cdx2 Tg mice		Normal mice	
Total bacteria	7.7±0.4	100	5.3±1.0*	100	6.7±0.7	100	3.4±1.2*	42*	6.3±0.4	100	4.9±0.8*	100	7.6±0.3	100	6.6±0.4*	100	7.6±0.3	100	5.4±1.3*	100	10.5±0.310	0	10.3±0.3	100
Obligate anaerobes																								
Clostridium coccoides group	4.7±0.4	75	5.3±0.2	33	5.1±0.6	67	<3.0	0*	4.6±0.6	100	<3.0	0*	7.1±0.4	100	5.8±0.5*	67	7.2±0.4	100	5.5±0.5*	25*	9.8±0.3	100	9.7±0.6	100
Clostridium leptum subgroup	4.9±0.8	92	5.2±1.4	50	5.4±2.0	100	<3.0	0*	4.6±0.6	100	<3.0	0*	7.1±0.4	100	5.8±0.4*	100	6.9±0.2	100	<3.0	0*	9.3±0.4	100	9.5±0.5	100
Bacteroides fragilis group	4.2±0.2	58	<3.0	0*	5.3±1.2	42	<3.0	0*	4.4±0.7	58	<3.0	0*	6.1±0.5	100	<3.0	0*	5.4±0.7	100	<3.0	0*	8.6±0.9	100	6.5±0.3*	100
Bifidobacterium	<3.0	0	5.0	8	4.3±0.3	25	<3.0	0	<3.0	0	4.3±0.2	33	3.6±0.3	50	5.2±0.7*	42	3.9±0.8	50	5.4±1.0	33	6.9±0.4	50	8.5±0.2*	100*
Atopobium cluster	<3.0	0	<3.0	0	5.6±1.1	25	<3.0	0	3.9±0.3	25	<3.0	0	4.9±0.5	100	5.2±0.9	50*	5.8±1.2	83	5.0±0.9	33*	7.6±0.6	100	8.7±0.3*	100
Prevotella	4.3±0.3	50	<3.0	0*	4.4±0.9	58	<3.0	0*	4.7±0.4	67	<3.0	0*	6.2±0.6	100	4.7±0.3*	25*	6.1±0.4	100	5.5±0.8	42*	9.3±0.4	100	8.9±0.2	100
Clostridium perfringens	<1.7	0	<1.7	0	<1.7	0	<1.7	0	<1.7	0	<1.7		<1.7	0	<1.7	0	<1.7	0	<1.7	0	<1.7	0	2.2	17
Facultative anaerobes																								
Total *Lactobacillus*	7.7±0.4	100	4.9±0.4*	100	6.2±0.6	100	3.4±1.2*	42*	6.2±0.4	100	4.8±0.8*	100	6.7±0.4	100	6.3±0.4*	100	6.5±0.5	100	5.1±1.2*	100	10.2±0.3	100	9.9±0.2*	100
Enterobacteriaceae	4.6±0.3	100	<3.3	0	4.0±0.5	100	<3.3	0*	4.6±0.5	100	4.7	8*	5.2±0.4	100	<3.3	0*	5.3±0.4	100	<3.3	0*	7.9±0.5	100	5.1±0.6*	83
Enterococcus	4.8±0.5	100	3.5±0.4*	42*	4.1±0.8	100	<2.0	0*	4.2±0.5	100	3.9±0.7	50*	5.1±0.5	100	4.5±0.3*	92	4.8±0.5	100	5.0±0.4	42*	7.9±0.3	100	8.2±0.3	100
Streptococcus	<2.9	0	<2.9	0	<2.9	0	<2.9	0	<2.9	0	4.2±0.4	42*	<2.9	0	4.9±0.6	75*	<2.9	0	5.7±0.4	33	6.9±0.7	100	7.2±0.6	100
Staphylococcus	3.2	17	<2.3	0	4.2±0.7	42	<2.3	0*	3.2±0.1	25	<2.3	0	2.9±0.4	92	3.5±0.5	33*	3.7±0.5	100	3.3±0.3	33*	5.5±0.6	100	5.2±0.3	100
Obligate aerobe																								
Pseudomonas	<2.0	0	<2.0	0	4.9	8	<2.0	0	2.7	17	<2.0	0	3.1	17	<2.0	0	3.0	17	<2.0	0	3.5	17	<2.0	0

Average bacterial number ±SD/g feces (log) detection ration (%).
Average bacterial number: non-parametric Mann-Whitney *U* test.
Detection ratio: Fisher's exact test.
*$P<0.05$.

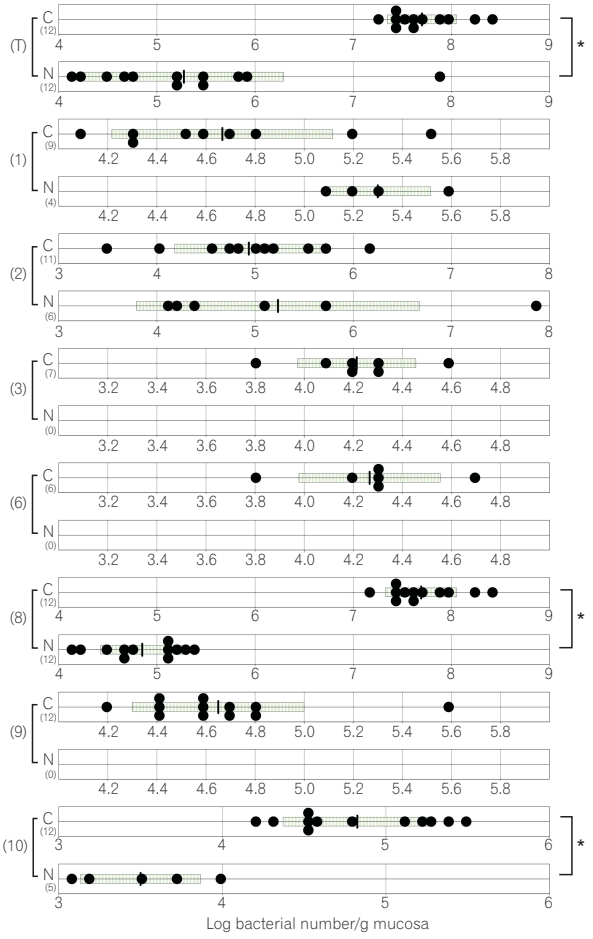

Fig. 1. Number of gastric bacteria for normal and caudal-related homeobox 2 (*Cdx2*) transgenic mice. C corresponds to results for *Cdx2* transgenic mice, N corresponds to results for normal mice. (T) Number of total bacteria; (1) number of *Clostridium coccoides* group; (2) number of *Clostridium leptum* subgroup; (3) number of *Bacteroides fragilis* group; (6) number of *Prevotella*; (8) number of total *Lactobacillus*; (9) number of *Enterobacteriaceae*, (10) number of *Enterococcus*. The SD value of the bacterial number for each group is shown as a bar. *P<0.05.

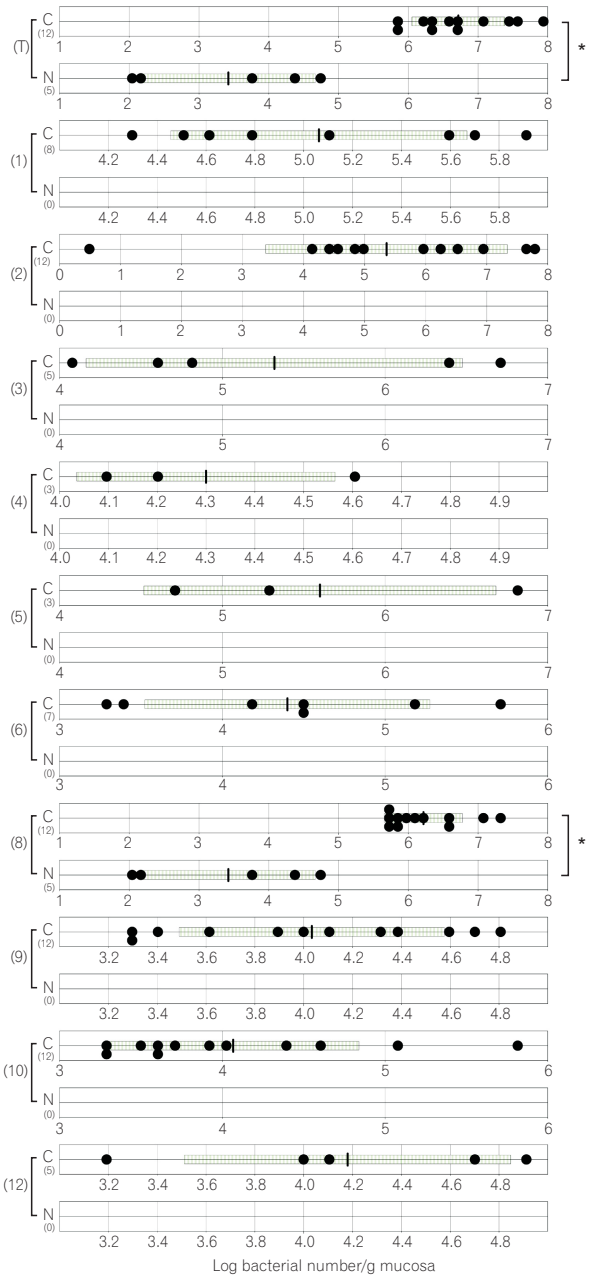

Fig. 2. Number of jejunal bacteria in normal and caudal-related homeobox 2 (*Cdx2*) transgenic mice. C corresponds to results for *Cdx2* transgenic mice, N corresponds to results for normal mice. (T) Number of total bacteria; (1) number of *Clostridium coccoides* group; (2) number of *Clostridium leptum* subgroup; (3) number of *Bacteroides fragilis* group; (4) number of *Bifidobacterium*; (5) number of *Atopobium* cluster; (6) number of *Prevotella*; (8) number of total *Lactobacillus*; (9) number of *Enterobacteriaceae*; (10) number of *Enterococcus*; (12) number of *Staphylococcus*. The SD value of the bacterial number for each group is shown as a bar. *P<0.05.

ies have demonstrated that the gastric community is highly dominated by *Proteobacteria*, *Firmicutes*, *Actinobacteria*, and *Bacteroidetes* in individuals with negative or positive *H. pylori* status. Although such studies have described the diversity of human gastric microbiota and its abundance in good detail, especially concerning predominant bacteria, sequence information about subdominant bacteria is limited and is not adequate for the accurate determination of subdominant bacteria.

The rRNA-targeted qRT-PCR method can detect targeted, viable bacterial populations in the range of 10^2–10^3 cells/g mucosa or more, including subdominant bacteria, with high resolution, and it has several advantages such as sensitivity, rapidity, and accuracy.[17-23] These aspects of the qRT-PCR method contribute to obtaining an accurate under-

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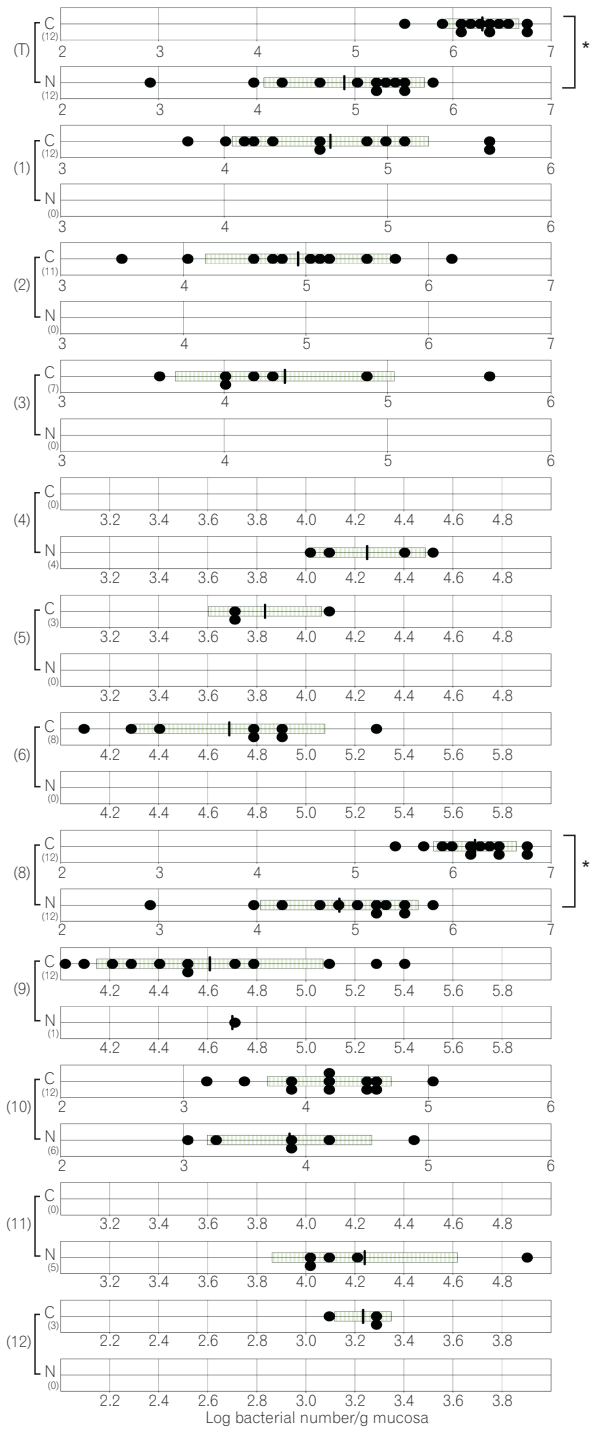

Fig. 3. Number of ileal bacteria in normal and caudal-related homeobox 2 (*Cdx2*) transgenic mice. C corresponds to results for *Cdx2* transgenic mice, N corresponds to results for normal mice. (T) Number of total bacteria; (1) number of *Clostridium coccoides* group; (2) number of *Clostridium leptum* subgroup; (3) number of *Bacteroides fragilis* group; (4) number of *Bifidobacterium*; (5) number of *Atopobium* cluster; (6) number of *Prevotella*; (8) number of total *Lactobacillus*; (9) number of *Enterobacteriaceae*; (10) number of *Enterococcus*; (11) number of *Streptococcus*; (12) number of *Staphylococcus*. The SD value of the bacterial number for each group is shown as a bar. *P<0.05.

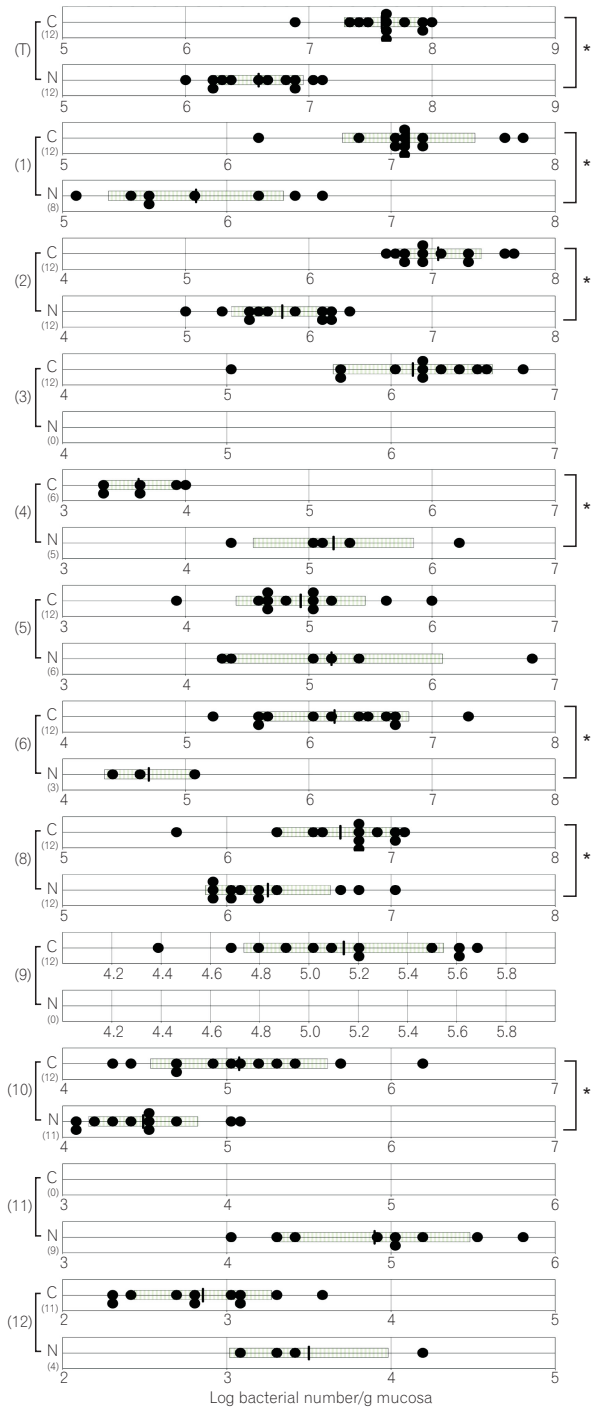

Fig. 4. Number of cecal bacteria in normal and caudal-related homeobox 2 (*Cdx2*) transgenic mice. C corresponds to results for *Cdx2* transgenic mice; N corresponds to results for normal mice. (T) Number of total bacteria; (1) number of *Clostridium coccoides* group ; (2) number of *Clostridium leptum* subgroup; (3) number of *Bacteroides fragilis* group; (4) number of *Bifidobacterium*; (5) number of *Atopobium* cluster; (6) number of *Prevotella*; (8) number of total *Lactobacillus*; (9) number of *Enterobacteriaceae*; (10) number of *Enterococcus*; (11) number of *Streptococcus*; (12) number of *Staphylococcus*. The SD value of the bacterial number for each group is shown as a bar. *P<0.05.

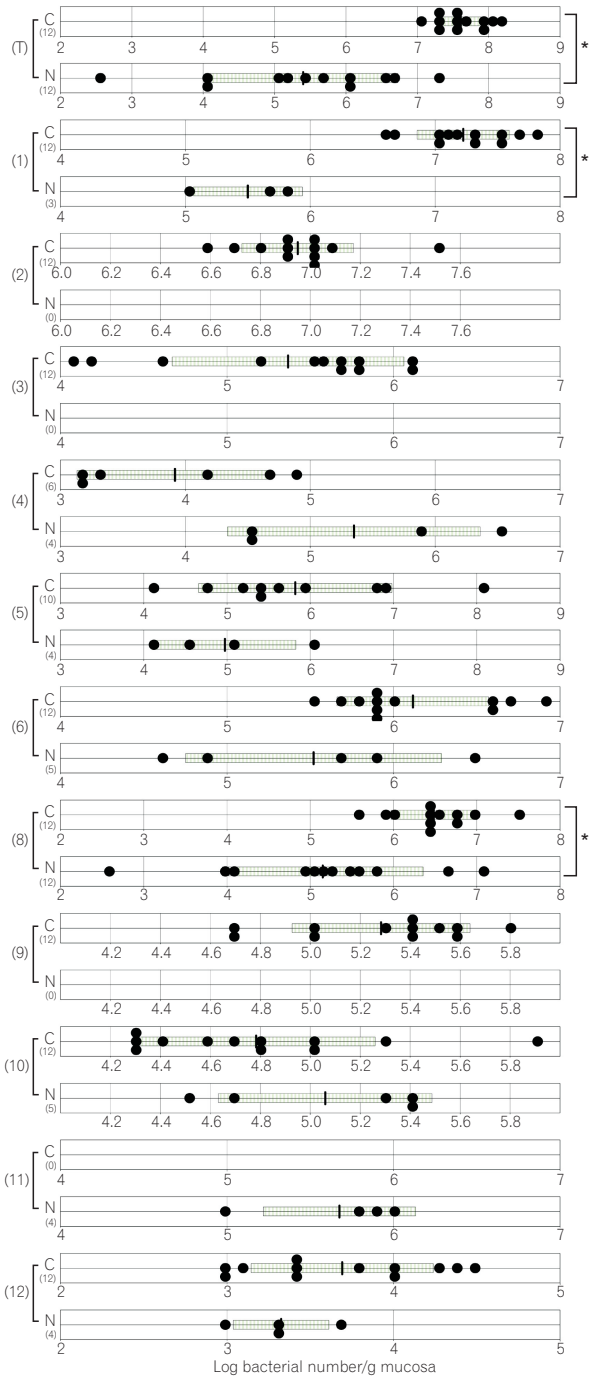

Fig. 5. Number of colonic bacteria in normal and caudal-related homeobox 2 (*Cdx2*) transgenic mice. C corresponds to results for *Cdx2* transgenic mice; N corresponds to results for normal mice. (T) Number of total bacteria; (1) number of *Clostridium coccoides* group; (2) number of *Clostridium leptum* subgroup; (3) number of *Bacteroides fragilis* group; (4) number of *Bifidobacterium*; (5) number of *Atopobium* cluster; (6) number of *Prevotella*; (8) number of total *Lactobacillus*; (9) number of *Enterobacteriaceae*; (10) number of *Enterococcus*; (11) number of *Streptococcus*; (12) number of *Staphylococcus*. The SD value of the bacterial number for each group is shown as a bar. *P<0.05.

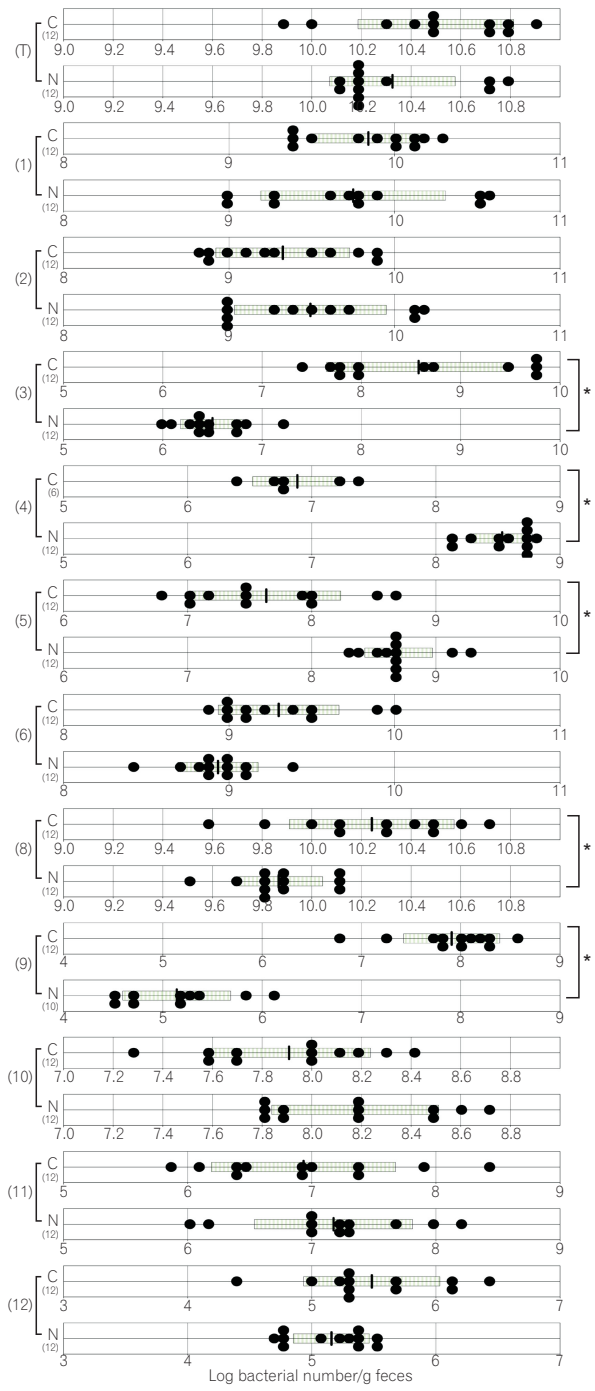

Fig. 6. Number of fecal bacteria in normal and caudal-related homeobox 2 (*Cdx2*) transgenic mice. C corresponds to results for *Cdx2* transgenic mice; N corresponds to results for normal mice. (T) Number of total bacteria; (1) number of *Clostridium coccoides* group; (2) number of *Clostridium leptum* subgroup; (3) number of *Bacteroides fragilis* group; (4) number of *Bifidobacterium*; (5) number of *Atopobium* cluster; (6) number of *Prevotella*; (8) number of total *Lactobacillus*; (9) number of *Enterobacteriaceae*; (10) number of *Enterococcus*; (11) number of *Streptococcus*; (12) number of *Staphylococcus*. The SD value of the bacterial number for each group is shown as a bar. *P<0.05.

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standing of the relationships between viable bacteria and their hosts. However, qRT-PCR can only analyze bacteria for which the proper primers and absolute calibration curves are constructed. For example, *Fusobacterium*, *Veillonella*, and *Neisseria*, which are the dominant bacteria in the oral airway, were not analyzed in the present study. Osaki et al. have reported that *Fusobacterium* and *Veillonella* were not detected in the stomach of Mongolian gerbils.[13] Moreover, previous reports have indicated that the mothers' intestinal microbiota influence gut colonization in their infants.[25] Thus, the observed differences in microbiota between normal mice and *Cdx2* transgenic mice might be influenced by their mothers' intestinal microbiota.

The bacterial counts and detection rates of the *C. coccoides* group, *C. leptum* subgroup, and *Atopobium* cluster in normal mouse feces were similar to those in human feces.[17] These bacteria were the predominant species in human feces and their population was in the range of 10^9–10^{10} bacteria/g feces. However, the population levels of *Bifidobacterium* and the *B. fragilis* group in normal mice were smaller than those in humans, and these are also predominant bacteria in human feces. The bacterial count of *Lactobacillus* in normal mice was higher than that in humans. *Lactobacillus* was the dominant bacteria in normal mouse feces.

The total numbers of bacteria in the gastric, jejunal, ileac, cecal, and colonic mucosa of *Cdx2* transgenic mice were significantly higher than those of normal mice. However, corresponding total numbers in the feces of *Cdx2* transgenic mice were not significantly higher those of normal mice. One of the reasons for this is that feces are formed after water is absorbed in the rectum. Therefore, the bacteria in feces would be more concentrated than those in the mucosa. Another possible reason is that the composition of bacteria might not be the same between the surface of the mucosa and the lumen of the intestine. In fact, *B. fragilis* was not detected in any of the mucosa of *Cdx2* transgenic mice, even though it was detected in the feces of all *Cdx2* transgenic mice in the present study. Further studies would be needed to clarify these issues.

The average total number of bacteria in the gastric mucosa of *Cdx2* transgenic mice was >100 times higher than that for normal mice. Osaki et al. have reported that there was no significant difference in the numbers of bacteria in the total microbiota between *H. pylori*-positive and -negative Mongolian gerbils.[13] However, the stomach pH and the gastric pathological findings were not mentioned in their report. Therefore, the stomachs of *H. pylori*-positive Mongolian gerbils might have not progressed to severe atrophic gastritis or intestinal metaplasia. We have previously reported that the gastric fundic mucosa of *Cdx2* transgenic mice was completely changed morphologically to intestinal metaplastic mucosa, and the pH of the stomach was 7.8±0.2 at the age of 37 days.[9] The gastric acid provides an effective barrier, killing most bacteria that enter the gastrointestinal tract. However, gastric achlorhydria due to intestinal metaplasia provides an opportunity for foreign microbes to enter and colonize the stomach. Furthermore, the effect may extend to intestinal microbiota. We revealed that the total number of intestinal microbiota in *Cdx2* transgenic mice was significantly increased. Gastric achlorhydria due to intestinal metaplasia affects not only small intestinal microbiota but also large intestinal microbiota.

In the present study, the dominant bacteria in the mouse gastric mucosa were *Lactobacillus*, the *C. coccoides* group, *C. leptum* subgroup, and *Enterococcus*. Similarly, another study also reported that the dominant bacteria in the mouse gastric mucosa were *Lactobacillaceae* and *Bacteroidales*.[26] Mice have a habit of autocoprophagy, i.e., eating their own feces; therefore, bacteria in the murine stomach may originate chiefly from their own intestinal microbiota. Bacteria that have a tolerance to acid, or spore-forming ability, might be dominant bacteria. We reported that intestinal-type adenocarcinoma developed from intestinal metaplastic mucosa in the stomach of *Cdx2* transgenic mice without *H. pylori* infection.[27] Moreover, Lofgren et al.[28] reported that *H. pylori*-infected transgenic insulin-gastrin (INS-GAS) mice, with complex gastric microbiota, had more severe gastritis and early onset of gastrointestinal intraepithelial neoplasia compared to germ-free and *H. pylori* monoinfected INS-GAS mice. These results suggest a role of microbiota in the chronic inflammation and carcinogenesis of the stomach. Dicksved et al.[29] reported that the gastric microbiota from 10 patients with gastric cancer was dominated by different species of corresponding genera such as *Streptococcus*, *Lactobacillus*, *Veillonella*, and *Prevotella*. Certain bacteria may be linked with carcinogenesis, although this requires further investigation for confirmation.

In conclusion, intestinal metaplasia affects not only gastric microbiota but also intestinal microbiota. A better understanding of the resident microbial communities in intestinal metaplasia should shed light on the pathogenesis, diagnosis, and treatment of gastrointestinal illnesses.

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Appendix. Standard strains used in this study

Standard strain	Target bacteria
Ruminococcus productus JCM 1471^T	*Clostridium coccoides* group
Feacalibacterium prausnitzii ATCC 27768^T	*Clostridium leptum* subgroup
Bacteroides vulgatus ATCC 8482^T	*Bacteroides fragilis* group
Bifidobacterium adolescentis ATCC 15703^T	*Bifidobacterium*
Bifidobacterium breve ATCC 15700^T	*Bifidobacterium breve*
Colinsella aerofaciens DSM 3979^T	*Atopobium* cluster
Prevotella melaninogenica ATCC 25845^T	*Prevotella*
Clostridium perfringens JCM 1290^T	*Clostridium perfringens*
Escherichia coli JCM 1649^T	*Enterobacteriaceae*
Lactobacillus casei ATCC 334^T	*Lactobacillus casei* subgroup
Lactobacillus acidophilus ATCC 4356^T	*Lactobacillus gasseri* subgroup
Lactobacillus plantarum ATCC 14917^T	*Lactobacillus plantarum* subgroup
Lactobacillus reuteri JCM 1112^T	*Lactobacillus reuteri* subgroup
Lactobacillus ruminis JCM 1152^T	*Lactobacillus ruminis* subgroup
Lactobacillus sakei subsp. sakei JCM 1157^T	*Lactobacillus sakei* subgroup
Lactobacillus brevis ATCC 14869^T	*Lactobacillus brevis*
Lactobacillus fermentum ATCC 14931^T	*Lactobacillus fermentum*
Lactobacillus fructivorans JCM 1117^T	*Lactobacillus fructivorans*
Enterococcus faecalis ATCC 19433^T	*Enterococcus*
Streptococcus mutans IFO13955^T	*Streptococcus*
Staphylococcus aureus ATCC 12600^T	*Staphylococcus*
Pseudomonas aeruginosa IFO 12689^T	*Pseudomonas*