Establishment and analysis of a mouse stomach model transplanted from human gastric microbiota

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Establishment and analysis of a mouse stomach model transplanted from human gastric microbiota

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<TABLE OF CONTENTS>

ABSTRACT·····1
INTRODUCTION·····3
II. MATERIALS AND METHODS ····· 5
1. PATIENTS····· 5
A. SELECTION OF MOUSE-INOCULATION SOURCE·····5
B. TRANSPLANTATION OF HUMAN GASTRIC MICROBIOTA INTO GERM-FREE MOUSE·····5
C. METAGENOME DNA EXTRACTION AND SEQUENCING·····7
D. MICROBIOTA PROFILING AND STATISTICAL ANALYSIS·· 7
E. INOCULATION OF HUMAN GASTRIC JUICE AND TISSUES INTO GERM FREE MICE·····9
III. RESULTS·····10
1.OVERALL EXPERIMENTAL DESIGN·····10
2. CHARACTERIZATION OF THE HUMAN MUCOSAL AND GASTRIC JUICE MICROBIOTA·····11
3. BACTERIAL DIVERSITY OF THE HUMAN MUCOSAL AND GASTRIC JUICE MICROBIOTA·····19
4. A PROCEDURE TO CONSTRUCT HUMANIZED MOUSE STOMACH MODEL ·····28

5. CHARACTERIZATION OF THE HUMAN DONOR AND MOUSE RECIPIENT GASTRIC MICROBIOTA·····33

6. BACTERIAL DIVERSITY OF GASTRIC MICROBIOTA OF THE HUMAN DONORS AND MOUSE RECIPIENTS·····39

7. DIFFERENCES OF GASTRIC MICROBIOTA WITHIN THE HUMAN DONORS AND WITHIN MOUSE RECIPIENTS·····41

8. HISTOPATHOLOGICAL RESULTS AFTER HUMAN GASTRIC TISSUE INOCULATION·····46

IV. DISCUSSION·····48

V.CONCLUSION·····55

REFERENCES ·····56

ABSTRACT(IN KOREAN)·····61

LIST OF FIGURES

Figure 1. Overview of experimental design.

Figure 2. The composition of (A) phylum and (B) genus in gastric mucosal tissues and gastric fluid.

Figure 3. Diversity index (Observed OTUs, Shannon, Inverse Simpsion; InvSimpsion) of gastric microbes in clinical patients.

Figure 4. Multidimensional scaling of weighed UniFrac distance of gastric microbial communities.

Figure 5. Specific microbial taxa are associated with sampling sites by linear discriminant analysis (LDA) effect size (LEfSe).

Figure 6. Specific microbial taxa are associated with disease states by linear discriminant analysis effect size.

Figure 7. Schematic of experimental design for human gastric-microbiota transplantation.

Figure 8. Bacterial community structure in donor humans and recipient mice in the gastric microbiota transplantation experiment.

Figure 9. Bacterial community structure in donor humans and recipient mice in the gastric microbiota transplantation experiment.

Figure 10. Multidimensional scaling of weighed UniFrac distances of gastric microbial communities in humans.
Figure 11. Specific microbial taxa associated with disease states in human gastric tissues.
Figure 12. Multidimensional scaling of weighed UniFrac distances of gastric microbial communities in mice.
Figure 13. Histopathological results of germ free mouse according to human gastric tissues.

LIST OF TABLES

Table 1. Clinical data summary for gastric patients
Table 2. Detailed patients' clinical data
Table 3. The order of relative abundance of genus of gastric musical samples
Table 4. Clinical patient characteristics
Table 5. Detailed patients' clinical data according to different disease states
Table 6. The order of relative abundance of human and gastric microbes

ABSTRACT

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Background and aim: A humanized mouse stomach model provides a well-controlled system to understand the biology and effect of gastric microbiota. This study aimed to characterize gastric microbiomes and develop a humanized mouse model as a research tool for stomach cancer development based on the host-microorganism interactions.

Methods: Gastric mucosal tissue was obtained from 15 patients (chronic superficial gastritis (CSG; n=5), intestinal metaplasia (IM; n=5), and gastric cancer (n=5)). The obtained gastric antral and body mucosal tissues were independently inoculated into different germ-free mice. Microbial community analysis of gastric tissues from patients and mice was performed using 16S rRNA gene amplicon sequencing.

Results: *Helicobacter* were the most dominant bacteria in all three groups. Lachnospiraceae, Lactobacillus, and Streptococcus were the second relatively abundant genera in the 3 human groups (CSG, IM, and gastric cancer group, respectively). However, Firmicutes dominated the relative abundance ratio of mouse samples. Alpha diversity showed significantly decreased number of OTUs in mouse gastric samples than in humans. Community evenness was also much lower in mouse samples. A weighted UniFrac-based comparison demonstrated that distance between samples did not depend on relationships between the donor and recipient. Turicibacter and Hungatella were the most successful taxa forming the majority of the mouse gastric mucosa although they constitute a very small percentage of human microbiota.

Conclusion: Human gastric microbiota exhibited selective colonization in mouse gastric tissue. Our data may form the basis of a system allowing improved understanding of human gastric microbiota and the microbial population in a germ-free mouse model.

Key words : gastric microbiota, humanized mouse model, gastric cancer

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I. Introduction

The human gut, colonized by complex communities of microorganisms, plays essential roles in digestion, nutrient absorption, stimulation of intestinal epithelial regeneration, and immune reactions [1-3]. Before the discovery of *Helicobacter pylori* (*Hp*), the human stomach environment was considered sterile due to its acidic gastric environment suppressing the microorganisms from the oral cavity. However, after the groundbreaking discovery of *Hp,* numerous other microorganisms were also detected in the human stomach. Previous studies found that the human stomach is colonized by complex microbiota mainly including *Proteobacteria, Firmicutes, Actinobacteria,* and *Fusobacterium* phyla [4-6]. This complex microbiota may also modulate the intensity and type of inflammatory and immune responses in the gastric mucosa.

Hp infection is one of most well known risk factors for gastric cancer, causing mucosal atrophy, intestinal metaplasia, and dysplasia. However, among 50% of the global population infected with *Hp,* only 1–2% develop gastric tumors, whereas most infected individuals experience chronic gastritis [4, 7, 8]. Recent human studies have shown a significant increase in the abundance of non-*Hp* bacteria colonizing the stomach, and inflammatory cytokine levels associated with a greater risk of atrophic gastritis in dyspeptic *Hp* infected patients treated with acid-suppressive drugs [9-14]. These findings emphasize the potential involvement of *Hp* and other microbes in gastric carcinogenesis and imply the importance of other factors in gastric carcinogenesis.

To better understand the biology of gastric microbiota both in animal and human hosts, a humanized mouse stomach model can provide a well-controlled system to assay the functional properties of gut communities harvested from humans with different phenotypes, and for conducting clinical trials to show how a host of factors may influence human microbiota and how in turn, the human microbiota shapes disease predispositions. However, no humanized mouse stomach model has been developed for bacterial community analysis in the context of gastric cancer development. Therefore, this study aimed to characterize and compare gastric microbiomes in patients with gastric cancer and a high risk of gastric cancer, and a control group using high throughput next generation sequencing and to develop a humanized mouse model as a research tool for stomach cancer development based on the host-microorganisms interaction.

II. Materials and methods

1. Patients

A. Selection of mouse-inoculation source

Gastric biopsies and gastric juice samples were collected from 13 patients (>19 years old) who underwent standard endoscopy to screen for premalignant or malignant gastric mucosal lesions or who received endoscopy for health check-up. Gastric mucosal tissues were taken in the antrum (2 biopsy specimens) and body (2 biopsy specimens). Gastric juice was taken at about 3 to 10 cc per patient. Six patients with early gastric adenocarcinoma and 6 patients with gastric dysplasia who were confirmed by pathologic findings were enrolled. One healthy patient with chronic superficial gastritis (CSG) without symptoms was enrolled. We excluded patients who had a history of eradication of *Hp*, previous gastric surgery, or other severe comorbidities.

B. Transplantation of human gastric microbiota into germ-free mouse

In total, we enrolled 15 patients to obtain gastric mucosal tissue. Chronic superficial gastritis (CSG) group (n = 5) included healthy patients with only CSG findings by endoscopy without symptoms for the recent 3 months. The intestinal metaplasia (IM) group (n = 5) included patients who were confirmed IM by pathologic findings, without dysplasia or gastric cancer. The gastric cancer group (n = 5) included patients with early gastric adenocarcinoma

confirmed by pathologic findings. We excluded patients who had a history of *Hp* eradication, previous gastric surgery, or other severe comorbidities.

Gastric mucosal (antrum and body) biopsies and blood samples were obtained from each patient during endoscopy. Biopsy specimens per subject for each location were obtained to perform *Hp* tests (1 biopsy specimen), immunohistochemistry (2 biopsy specimens), real-time PCR (2 biopsy specimens), and pyrosequencing (3 biopsy specimens). The biopsy specimens were assessed for the presence of *Hp* and for the presence of IM (hematoxylin and eosin staining). To avoid contamination, the endoscopes were washed and disinfected by immersing in a detergent solution containing 7% proteolytic enzymes and 2% glutaraldehyde. Sterilized gastroscopy forceps were used when obtaining another biopsy from the same patient. The biopsies were stored at -80℃. In patients who had clear gastric fluid, the gastric juice was obtained through a catheter connected to a 5 mL tube during endoscopy.

Positivity of *Hp* was confirmed using conventional tests for *Hp* infection: 1) Rapid urease test (Campylobacter-like organism (CLO) test), 2) Histologic examination (modified Giemsa staining), 3) serum *Hp* immunoglobulin G (IgG). If all tests were negative, we regarded the subject as *Hp*-negative. Serum concentrations of pepsinogen (PG) I and II were evaluated, which are known to be associated with the severity of gastric atrophy. Serum PG status was defined

as “atrophic” when the serum PG I level was ≤ 70 ng/mL, and the PGI/II ratio was simultaneously ≤ 3.0 [15].

C. Metagenome DNA extraction and sequencing

Approximately 20 mg of human or mouse mucosal samples and 2ml of human gastric juice samples were employed as an input for DNA extraction with the DNeasy Blood&Tissue kit (Qiagen, Germany) following the manufacturer’s instructions. The V3-V4 hypervariable regions of the 16S rRNA gene were targeted using a universal primer set (5’-CCTACGGGNGGCWGCAG and 5’-GACTACHVGGGTATCTAATCC) with sequencing barcodes. Sequencing libraries were generated according to the manufacturer’s recommendations (Illumina, CA, U.S.A.) and sequencing was performed on an Illumina MiSeq platform with a 2×250 bp paired-end protocol yielding pair-end reads (Macrogen, Korea). For comparison between human mucosal and gastric fluid, we generated a total of 1,997,818 high-quality sequences with a median sequence number of 49,848 sequences per sample ranging from 22,471 to 92,575. The humanized mouse construction experiment yielded a total of 16,652,287 high-quality sequences with a median sequence number of 190,692 sequence ranging from 50,493 to 290,905.

D. Microbiota profiling and statistical analysis

We largely followed the QIIME2 pipeline for bacteria profiling based on 16S rRNA [16]. DADA2 was selected as a tool for sequence quality control [17]. SILVA

database was chosen as the taxonomic reference database (version 132; https://www. arb- silva. de). A rarefied OTU table from output files was used for downstream analyses using QIIME 2 View or a visualization toolkit also developed at the CMMR named ATIMA (Agile Toolkit for Incisive Microbial Analyses, R Core Team, 2014). ATIMA is a software suite that combines publicly available packages (i.e., APE and VEGAN) [18, 19] and purpose uses written code to import sample data and identify trends in taxa abundance, alpha-diversity, and beta-diversity using sample metadata. Alpha-diversity was determined by the value of clustered observed OTUs (operation taxonomic units), Shannon index, and the inverse Simpson index. Significance of categorical variables was determined using the non-parametric Mann-Whitney test for two category comparisons or the Kruskal-Wallis test when comparing three or more categories. Beta diversity was assessed by weighted UniFrac distance matrices. Correlation between two continuous variables was determined with linear regression models, where p values indicate the probability that the slope of the regression line is zero. PCoA plots employ the Monte Carlo permutation test to estimate p values. All p values are adjusted for multiple comparisons with the FDR algorithm.

We imported results into the R statistical environment (The R Foundation, https://cran.r-project.org/) for further analysis with the bioconductor package phyloseq [20]. Multivariate Analyses of Variance (PERMANOVA, 999

permutations)[21] with the vegan function Adonis were performed to test whether community composition was significantly different by the features associated with sequencing data [19].

E. Inoculation of human gastric juice and tissues into germ free mice

We use two styles of soft isolators (CBC, USA) to maintain germ-free mice. For a one-patient experiment, small isolators (experimental isolators) that have a single port and a single pair of gloves are used. Large isolators (breeding isolators) are used for mice production. These are primarily used to house our breeding colony. To verify the germ-free environment of the isolator and mice, weekly anaerobic culture with mouse feces and inside mold trap samples are performed.

Three groups of 8 week-old C57BL6 background germ-free mice were used in this study. Each mouse was bred in germ-free breeding isolators and experimental isolators. Each group of germ-free mice was transplanted with 3 different human disease groups (CSG, IM and gastric cancer group, respectively). Therefore, 10 mice in each group were transplanted with human gastric antrum (n = 5) and body (n = 5) tissues. One germ-free mouse was kept in the germ-free status in the breeding isolator as the control group. Germ-free mice in experimental isolators were inoculated orally with human gastric tissue once per day for 3 consecutive days using a metal gavage needle.

After one month, the germ-free mice were sacrificed and used for mouse gastric

microbiota analysis and to measure immune cell markers (Ki67, Mist1, hydrogen potassium adenosine triphosphatase (H/K ATPase)).

III. Results

1. Overall experimental design

At the start of this study, to establish a humanized mouse stomach model, a reliable inoculum that could transfer the human microbial community to the mouse model needed to be determined. The inoculum should be able to transfer colonized microbial communities from the human gastric mucosa into a mouse model, rather than simply passing through the stomach. However, since the delivery of gastric juice to mice is less expected to undergo external contamination and much easier for oral injection compared to transfer of mucosa that has to undergo various treatments, we considered the possibility of injecting gastric juice as an inoculum. To this end, we investigated whether microbial populations in human gastric juice could represent those of gastric tissues. After selecting the inoculum source, human microbial communities were inoculated into germ-free mice. We then compared the gastric microbial community structure between the human and mouse stomach. Finally, we observed the immunological changes occurring in the established mouse models upon inoculation of the human gastric microbial community into mice (Figure 1).

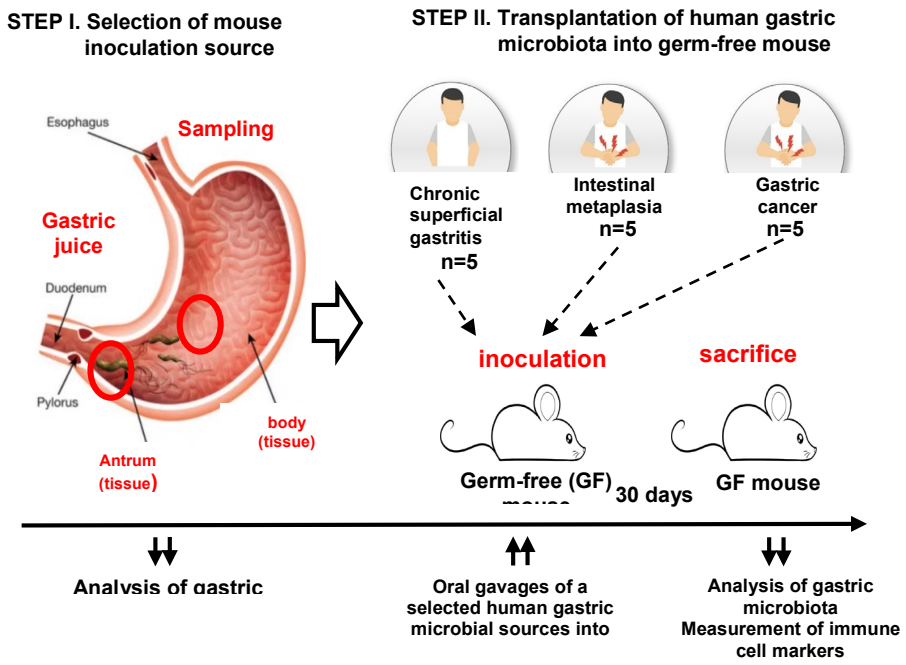


Figure 1. Overview of experimental design.

2. Characterization of the human mucosal and gastric juice microbiota

To compare the microbial community structure between gastric mucosal tissue and gastric juice, tissue and gastric fluid samples were collected from 13 patients undergoing clinically indicated upper endoscopy. The baseline clinical patient characteristics and detailed clinical data are shown in Table 1&2.

Table 1. Clinical data summary for gastric patients

Category		Value (%)
Patients, n		13
Male, n (%)		7 (53.8)
Age, years		56 (27-83)
CLO positive, n (%)		8 (61.5)
Hp lgG positive, n (%)		11 (84.6)
Hp-negative		2 (15.4)
Pepsinogen I		57.9 (5.7-132.4)
Pepsinogen II		19.2 (7.8-32.4)
Pepsinogen I/II		3.2 (0.5-8.5)
Diagnosis	AWD, n (%)	4 (30.77)
	AMD, n (%)	2 (13.38)
	HGD, n (%)	1 (7.69)
	LGD, n (%)	5 (38.46)
	CSG, n (%)	1 (7.69)

CLO, Campylobacter-like organism; *Hp, Helicobacter pylori;* lgG, Immunoglobulin G; AWD, well differentiated adenocarcinoma; AMD, moderate differentiated adenocarcinoma; HGD, high grade dysplasia; LGD, low grade dysplasia; CSG, chronic superficial gastritis

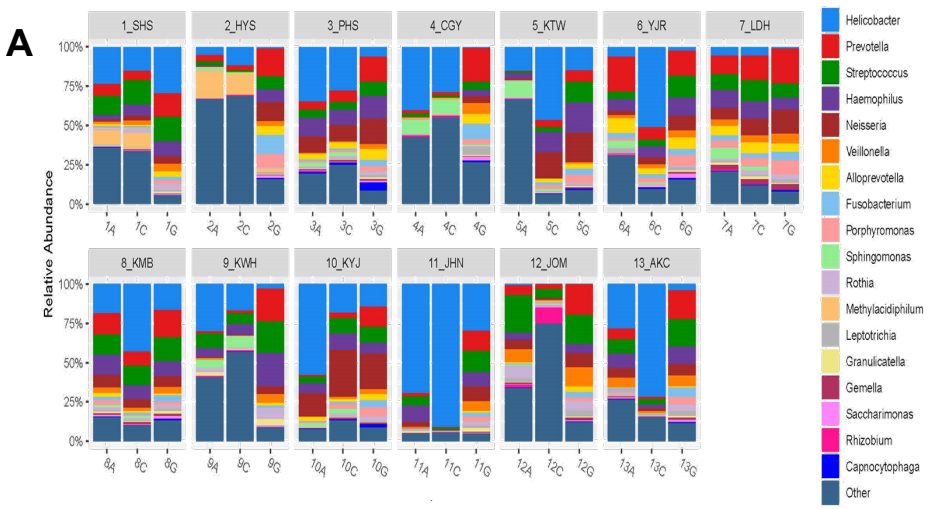
Table 2. Detailed patients′ clinical data

No.	Age (years)	Gender	CLO	*Hp* biopsy	*Hp* IgG	Diagnosis	Pepsinogen I	Pepsinogen II	Pepsinogen I/II
1	52	M	+	−	+	AWD	70.5	32.4	2.2
2	50	F	−	−	-	LGD	35.3	7.8	4.5
3	52	M	+	+	+	LGD	42.5	21.0	2
4	27	F	+	+	+	CSG	56.1	12.1	4.6
5	42	M	−	−	+	AMD	105.8	26.9	3.9
6	60	M	+	+	+	LGD	33.2	16.4	2
7	62	M	−	−	+	LGD	5.7	12.4	0.5
8	60	F	+	+	+	HGD	71.7	32.0	2.2
9	54	M	−	−	+	AWD	43.1	13.1	3.3
10	56	F	+	+	+	LGD	43.3	13.6	3.2
11	83	F	+	−	+	AWD	60.5	24.3	2.5
12	82	F	−	−	−	AMD	132.4	15.5	8.5
13	62	M	+	−	+	AWD	52.2	22.6	2.3

Hp, Helicobacter pylori; CLO, Campylobacter-like organism; lgG, Immunoglobulin G; PG, pepsinogen; AWD, well differentiated adenocarcinoma; AMD, moderate differentiated adenocarcinoma; HGD, high grade dysplasia; LGD, low grade dysplasia; CSG, chronic superficial gastritis

Among 13 patients, 54% were male and their median age was 56 years. Eighty percent of the patients were infected by *Hp*. Six patients (46.2%) had gastric cancer and six (46.2%) had dysplasia. One patient (7.6%) was CSG without a pathology of gastric malignancy or dysplasia.

In particular, mucosal tissues were collected independently from the antrum and body parts of the stomach. Microbial communities were analyzed using bacterial 16S rRNA gene amplicon sequencing. All samples were found to harbor a diverse microbial community. On average, samples were dominated by members of the phyla *Epsilonbacteraeota*, *Proteobacteria*, *Firmicutes*, and *Bacteroidetes*. Other prominent phyla included *Fusobacteria* and *Actinobacteria* (Figure 2A). At genus level, most phyla *Epsilonbacteraeota* were composed of genus *Helicobacter*. The majority of *Proteobacteria* were genus *Neisseria* and *Haemophilus*. Genus *Streptococcus* and *Veillonell* were found to be major contributors to the formation of *Firmicutes* (Figure 2B).



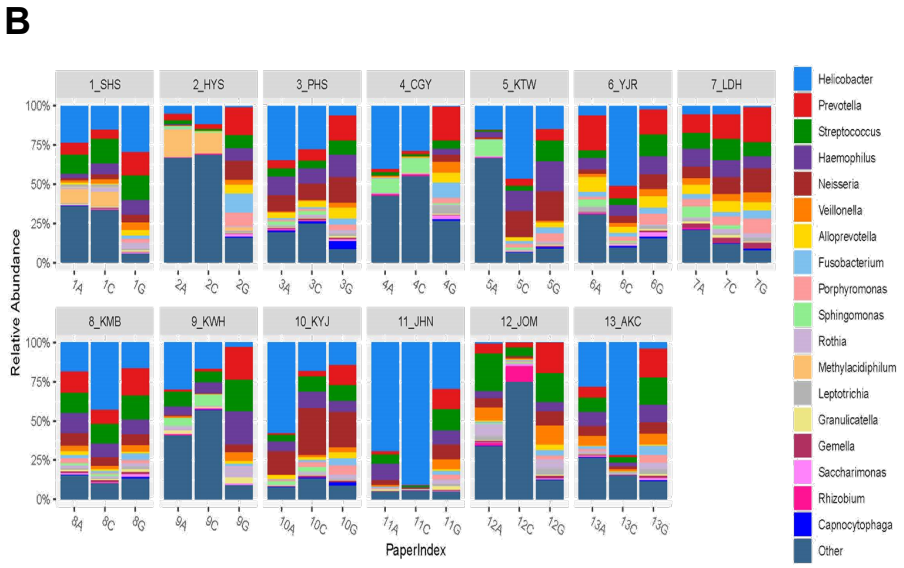


Figure 2. The composition of (A) phylum and (B) genus in gastric mucosal tissues and gastric fluid. The numbers are indicated for patient classification. (A, antral mucosa; C, body (corpus) mucosa; G, gastric fluid)

Considering the levels of CLO and *Hp* IgG, only 2 patients showed a Hp negative response among the 13 patients. Based on these criteria, we analyzed the microbial community structure as *Hp*-positive and -negative response patient groups.

In *Hp*-positive patients, the relative abundance of the genus *Helicobacter* was dominant in both the antrum (29.97%) and body (38.21%) tissues, but in their gastric juice, this genus showed the second-highest relative abundance (10.86%) following the genus Streptococcus. *Hp*-negative patient samples also harbored

extremely low levels of the genus *Helicobacter* only in the tissues (antrum, 2.94%; body, 2.80%) (Table 3).

Table 3. The order of relative abundance of genus in gastric musical samples

***H. pylori* positive (n=11)**						
Order of genus	Antrum	Mean (%)	Body	Mean (%)	Gastric Juice	Mean (%)
1	*Helicobacter*	29.97	*Helicobacter*	38.21	*Streptococcus*	12.99
2	*Streptococcus*	6.28	*Neisseria*	6.47	*Helicobacter*	10.86
3	*Neisseria*	4.41	*Streptococcus*	5.70	*Neisseria*	10.48
4	*Prevotella 7*	3.84	*Haemophilus*	3.33	*Prevotella 7*	8.40
5	*Sphingomonas*	3.40	*Actinobacillus*	2.49	*Haemophilus*	7.05
6	*Haemophilus*	3.38	*Sphingomonas*	2.47	*Prevotella*	6.28
7	*Actinobacillus*	2.90	*Methylacidiphilum*	2.10	*Fusobacterium*	4.92
8	*Methylacidiphilum*	2.44	*Prevotella 7*	2.06	*Veillonella*	4.63
9	*Alloprevotella*	1.92	*Prevotella*	1.42	*Porphyromonas*	4.16

10	*Veillonella*	1.56	*Alloprevotella*	1.25	*Alloprevotella*	3.76
11	*Prevotella*	1.38	*Veillonella*	1.17	*Actinobacillus*	3.75
12	*Fusobacterium*	0.93	*Porphyromonas*	1.04	*Rothia*	2.52
13	*Porphyromonas*	0.86	*Fusobacterium*	0.93	*Leptotrichia*	1.75
14	*Rothia*	0.83	*Rothia*	0.83	*Capnocytophaga*	1.14
15	*Anaerolineaceae*	0.73	*Bacteroides*	0.64	*Saccharimonadaceae*	1.12
16	*Bacteroides*	0.65	*Prevotella 1*	0.64	*Granulicatella*	1.10
17	*Marinobacter*	0.65	*Vibrio*	0.63	*Moraxella*	1.06
18	*Entomoplasmatales*	0.58	*Entomoplasmatales*	0.62	*Treponema 2*	0.73
19	*Leptotrichia*	0.56	*Anaerolineaceae*	0.55	*Gemella*	0.71
20	*Rhodanobacter*	0.55	*Lactobacillus*	0.47	*Campylobacter*	0.69

***H. pylori* negative (n=2)**						
Order of genus	Antrum	Mean (%)	Body	Mean (%)	Gastric Juice n=11	Mean (%)
1	*Streptococcus*	17.40	*Streptococcus*	9.42	*Streptococcus*	13.80

2	*Neisseria*	6.63	*Neisseria*	6.41	*Prevotella 7*	13.14
3	*Haemophilus*	6.42	*Prevotella 7*	5.24	*Neisseria*	12.30
4	*Veillonella*	6.27	*Allorhizobium*	5.15	*Veillonella*	9.10
5	*Prevotella 7*	4.75	*Haemophilus*	4.20	*Haemophilus*	6.17
6	*Rothia*	4.37	*Alloprevotella*	3.73	*Prevotella*	5.82
7	*Sphingomonas*	3.80	*Prevotella*	3.36	*Porphyromonas*	5.43
8	*Prevotella*	3.48	*Helicobacter*	2.80	*Fusobacterium*	4.62
9	*Alloprevotella*	3.37	*Porphyromonas*	2.70	*Rothia*	4.40
10	*Helicobacter*	2.94	*Veillonella*	2.53	*Alloprevotella*	4.32
11	*Porphyromonas*	2.49	*Ruminococcaceae*	2.47	*Leptotrichia*	3.36
12	*Fusobacterium*	2.06	*Cutibacterium*	2.17	*Gemella*	2.60
13	*Gemella*	1.88	*Fusobacterium*	1.78	*Prevotella 6*	1.28
14	*Leptotrichia*	1.85	*Sphingomonas*	1.67	*Actinomyces*	1.08
15	*Bacteroides*	1.78	*Ruminococcaceae*	1.67	*Oribacterium*	0.84
16	*Actinobacillus*	1.39	*Rothia*	1.64	*Lachnoanaerobaculum*	0.83
17	*Allorhizobium*	1.38	*Flavobacteriaceae*	1.61	*Moraxella*	0.81
18	*Actinomyces*	0.99	*Gemmatimonadaceae*	1.58	*Actinobacillus*	0.66
19	*Oribacterium*	0.94	*Gemella*	1.53	*Granulicatella*	0.64

20	*Prevotella 6*	0.70	*Actinobacillus*	1.31	*Campylobacter*	0.63

3. Bacterial diversity of the human mucosal and gastric juice microbiota

Microbial alpha diversity and beta diversity were measured to assess changes in human gastric microbial structures. As shown in Figure 3, alpha diversity values including a number of observed OTUs, Shannon index, and Simpson index in samples were calculated according to various criteria. By measuring the observed OTUs, we found that gastric juice had significantly decreased bacterial diversity compared to gastric mucosal tissues (Figure 3A). Presence of *Hp* decreased the diversity in terms of richness and evenness (Figure 3B). When we calculated alpha diversity in terms of disease classification, the smaller the number of OTUs observed, the greater was the degree of disease progression from CSG to adenocarcinoma (Figure 3D). Beta diversity was calculated using quantitative UniFrac phylogenetic distance matrices and was visualized in PCoA plots. The total diversity captured by the principal coordinates was 47.3%.

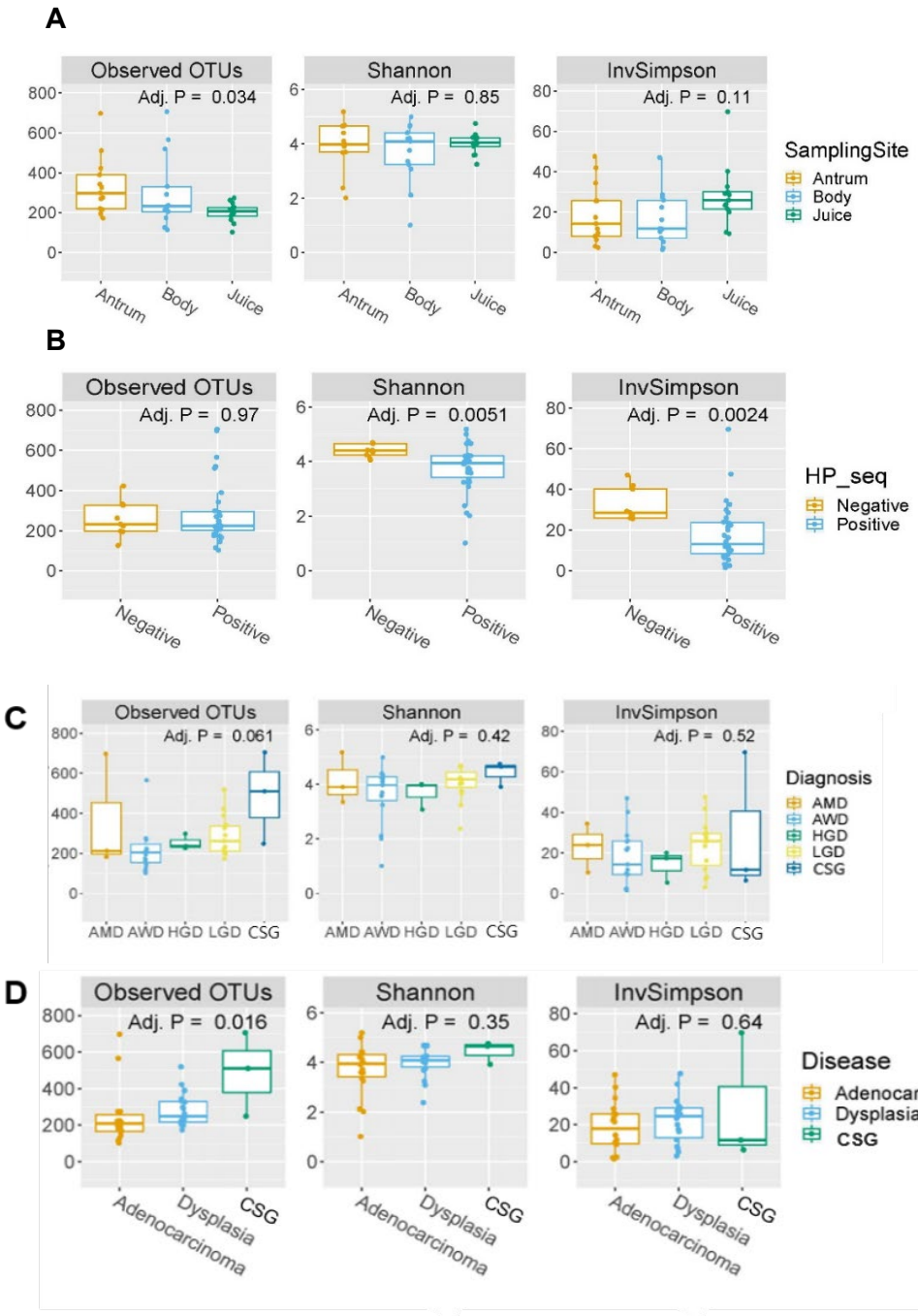
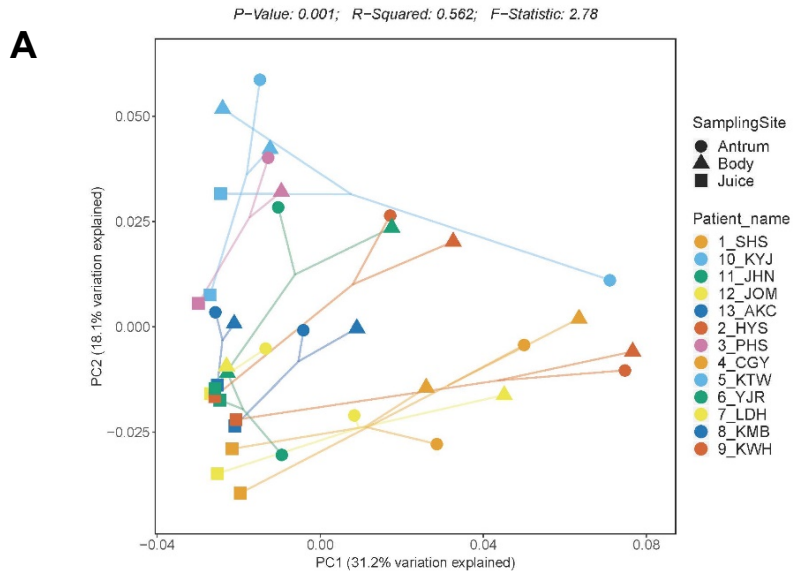


Figure 3. Diversity index (Observed OTUs, Shannon, Inverse Simpsion; InvSimpsion) of gastric microbes in clinical patients. (A) Samples were grouped based on mucosal biopsy sites or gastric fluid (antrum, body, gastric fluid (juice), n=13). (B) Samples were grouped based on *Helicobacter pylori* infection. (C) Samples were classified according to pathological diagnosis. (D) Samples were classified according to disease.

The microbiota composition of samples was classified according to whether the sample was derived from the mucosa or gastric juice, not from the same patient's stomach (Figure 4). In particular, the properties of both PCoA (Figure 4B) and a hierarchical plot (Figure 4C) showed that the distance of microbial community structure between gastric juice samples was much closer than the distance between tissue samples.



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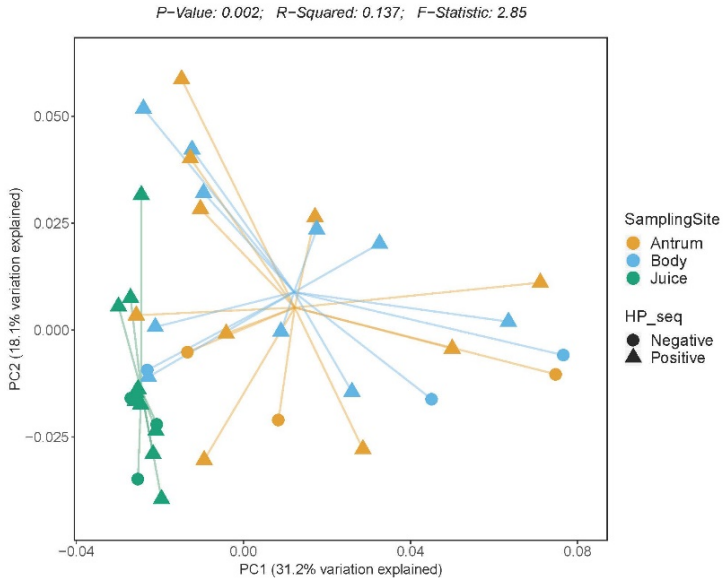

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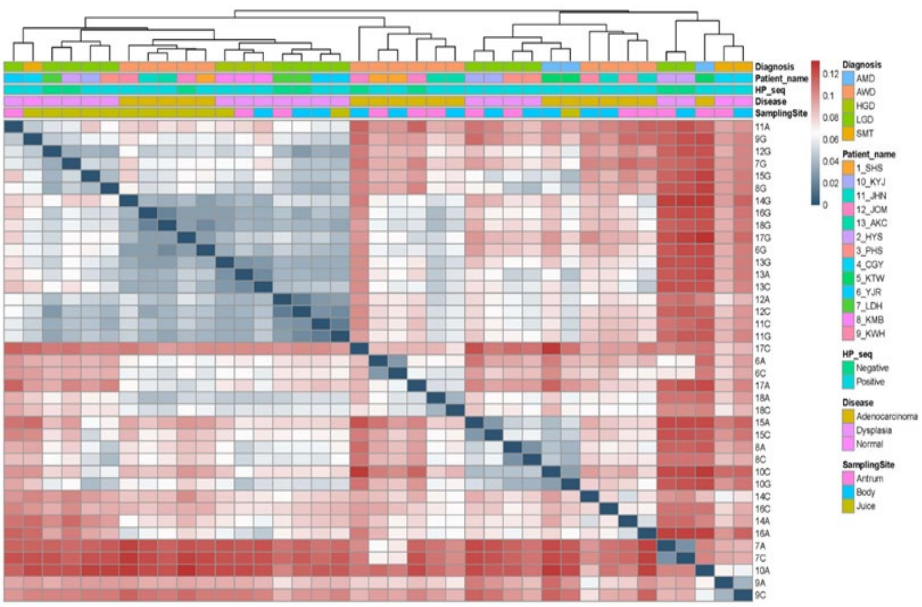

Figure 4. Multidimensional scaling of weighed UniFrac distance in gastric microbial communities. Samples are grouped and color-coded based on (A) patients or (B) sampling sites, and shaped on (A) sampling sites or (B) *Helicobacter pylori* infection. (C) Hierarchical clustering of microbial community composition in human and murine mucosal samples.

To determine the specific microbial taxa associated with sampling sites and disease states, we adopted the linear discriminant analysis (LDA) effect size (LEfSe) method. In mucosal tissues, enrichment of the genera *Helicobacter* and Sphingomonas was observed. In gastric juice, Prevotella, Neisseria, Fusobacterium, and Streptococcus were enriched. (Figure 5). According to pathologic diagnosis, the genera Neisseia, Alloprevotella, Gemella, and Porpyromonas were dominant in dysplasia, whereas Streptococcus was enriched in adenocarcinoma (Figure 6). However, there was no statistically significant difference in the relative abundance among all the genera. Based on these results, we decided to use mucosal tissues as inoculum to generate the humanized microbiota mouse model.

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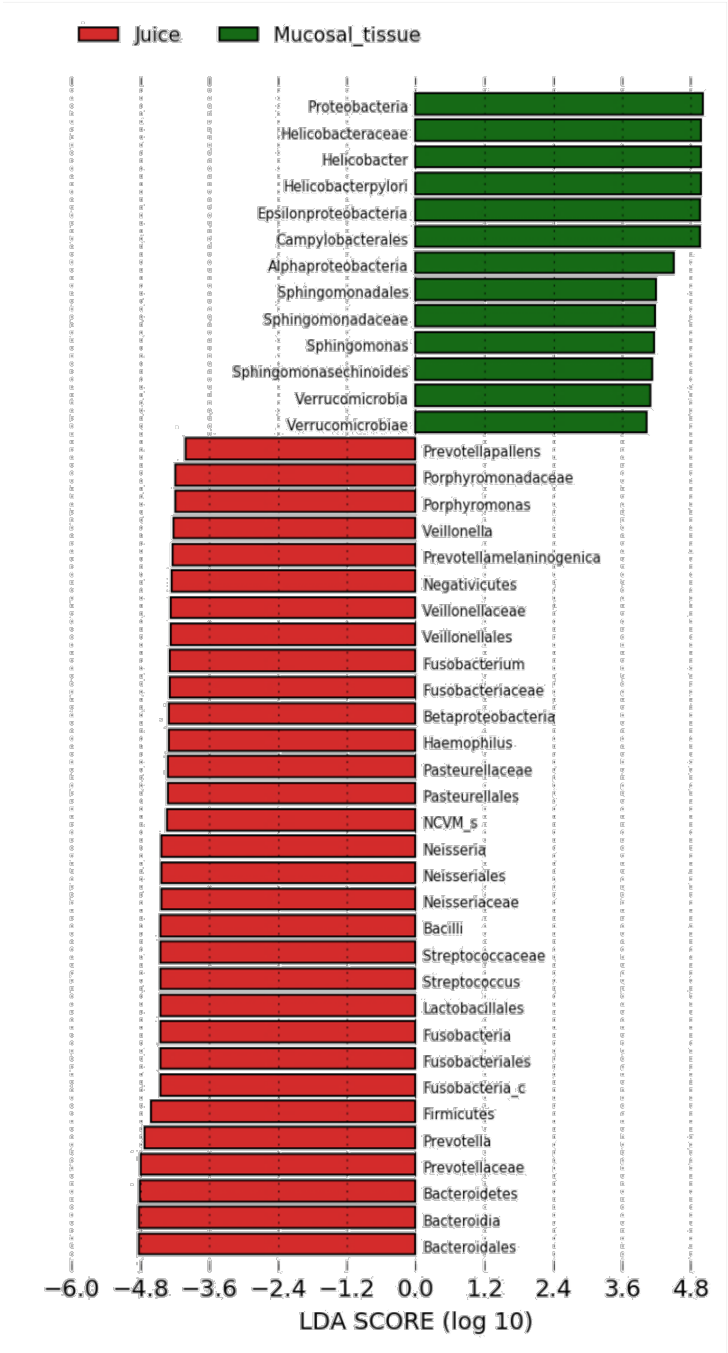

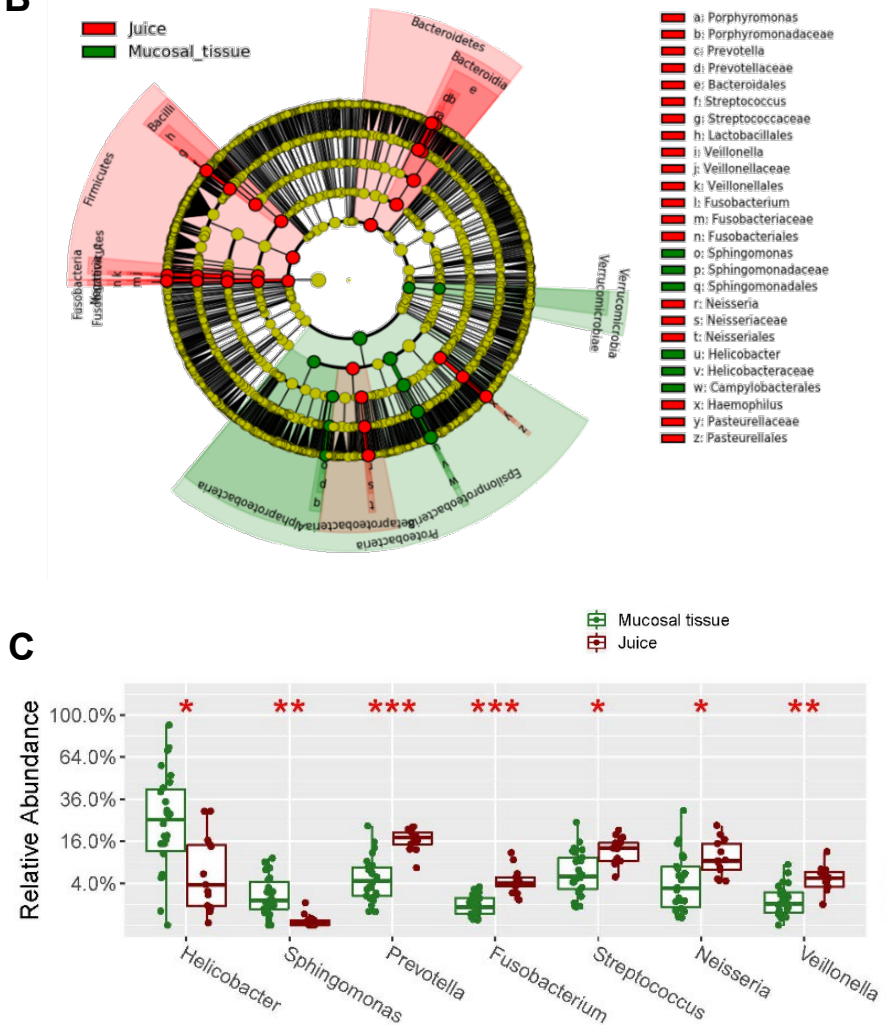

Figure 5. Specific microbial taxa are associated with sampling sites by linear discriminant analysis (LDA) effect size (LEfSe). (A) The list of taxa that are differential among sampling sites with statistical significance and (B) mapping of the differences to taxonomic trees. (C) Relative abundances of taxa that are differential among sampling sites.

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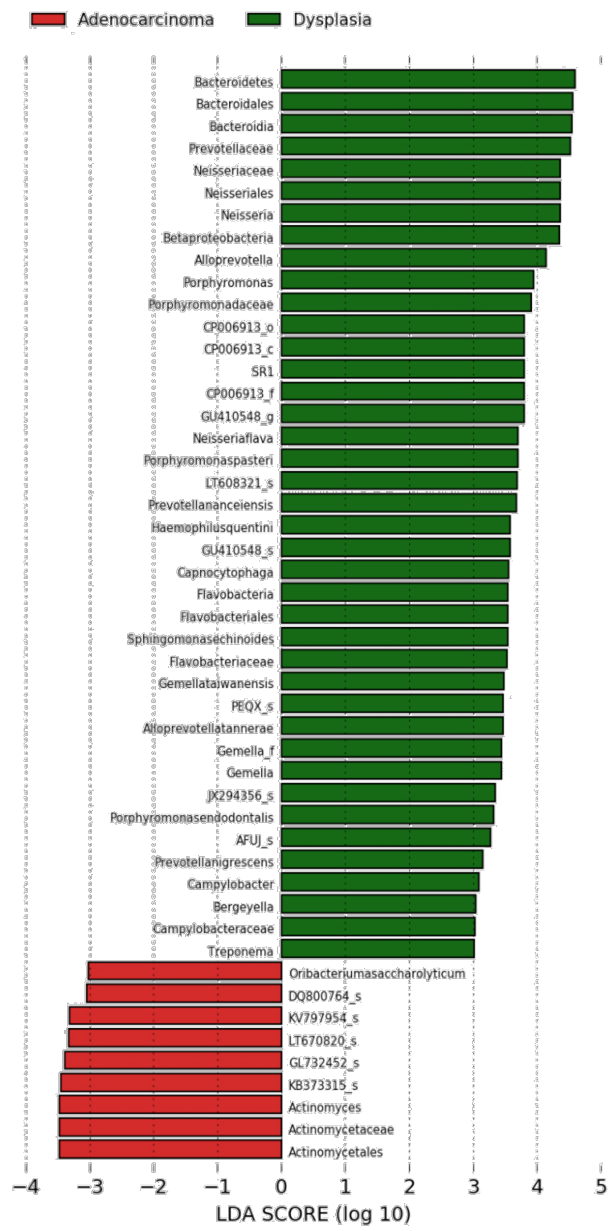

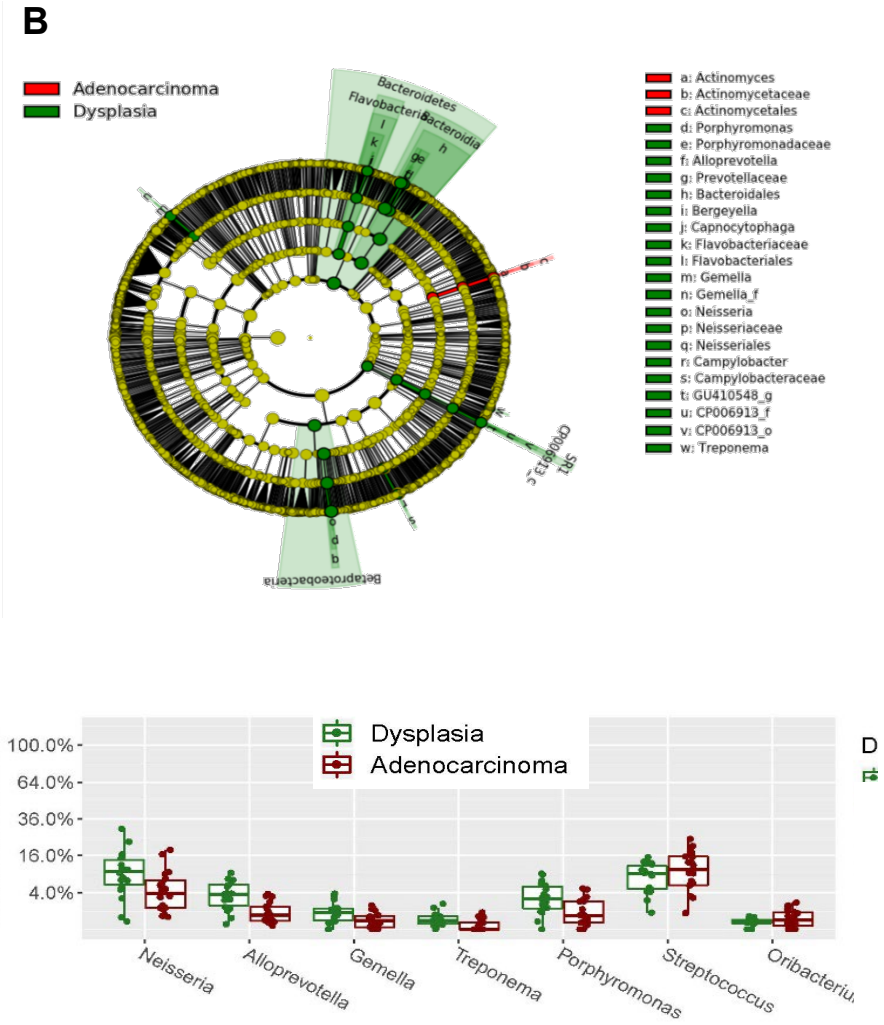

Figure 6. Specific microbial taxa are associated with disease states by linear discriminant analysis effect size. (A) The list of taxa that are differential among disease states with statistical significance and (B) mapping of the differences to taxonomic trees. (C) Relative abundances of taxa that are differential among disease states.

4. A procedure to construct a humanized mouse stomach model

Human gastric microbiota was transplanted into germ-free mice by inoculating gastric tissue. To establish a more diverse human stomach mouse model, 5 patients per group with different disease states including chronic superficial gastritis (CSG), intestinal metaplasia (IM), and gastric cancer (GC) were selected as gastric microbiota donors. The median age of all patients was 58.6 years and male patients formed 40%. Total positivity rate of *Hp* was 53.3%. In each of the three groups, *Hp* infection rates were 20%, 20%, and 80% in CSG, IM, and gastric cancer group, respectively). According to the serum PG levels, only the IM group showed 60% of atrophic changes (Table 4& 5).

Table 4. Clinical patient characteristics

Group	Total	CSG	IM	Gastric cancer
Patients, n	15	5	5	5
Age, years	58.6 (41-75)	59 (51-67)	45 (41-63)	69 (52-75)
Male, n (%)	6 (40)	1 (20)	2 (40)	3(60)
CLO positive, n (%)	5 (30)	1 (20)	3 (60)	1 (20)
Hp lgG positive, n (%)	7 (46.7)	1 (20)	4 (80)	2 (40)
Hp-negative	7 (46.7)	3 (80)	1 (20)	3 (60)
PGI	49.7	48.9	56	37.3

	(26.8-90.3)	(26.8-70.2)	(34.9-73.2)	(29.4-90.3)
PGII	14 (6.3-35.5)	10.1 (6.3-12)	21.2 (7.6-35.5)	8.8 (7.7-18.2)
PGI/II	4.0 (1.9-5.9)	4.9 (4.1-5.9)	2.7 (1.9-4.6)	3.8 (3.6-5)
Atrophic change*	3 (20)	0	3 (60)	0

*Atrophic change defined as PG I level was ≤70 ng/mL, and the PGI/II ratio simultaneously ≤3.0. CSG, chronic superficial gastritis; IM, Intestinal metaplasia; CLO, Campylobacter-like organism; *Hp,*; *Helicobacter pylori;* lgG, Immunoglobulin G; PG, Pepsinogen

Table 5. Detailed patients′ clinical data according to different disease states

No.	Age (Years)	Gender	CLO	*Hp* IgG	PGI	PGII	PGI/II	Atrophic change*
CSG group								
1	63	M	-	-	70.2	12	5.9	N
2	59	F	-	-	26.8	6.3	4.3	N
3	51	F	+	+	42.6	10.3	4.1	N
4	59	F	-	-	49.2	10.1	4.9	N
5	67	F	-	-	48.9	9.3	5.3	N
IM group								
1	42	F	+	+	57.6	21.2	2.7	Y
2	63	F	-	-	34.9	7.6	4.6	N
3	45	F	+	+	73.2	35.5	2.1	N
4	55	M	-	+	41.8	21.8	1.9	Y
5	41	M	+	+	56	18.8	2.9	Y
Gastric cancer group								
1	68	M	-	+	33	8.8	3.8	N
2	70	M	-	-	29.4	8.1	3.6	N
3	52	M	+	+	54.1	14.7	3.7	N
4	69	F	-	-	37.3	7.7	4.8	N
5	75	F	-	-	90.3	18.2	5	N

*Atrophic change defined as PG I level was ≤70 ng/mL, and the PGI/II ratio simultaneously ≤3.0.

Hp, Helicobacter pylori; IgG,immunoglobulin G; CLO, Campylobacter-like organism; PG, pepsinogen

Ground gastric antral and body mucosal tissues of donors were independently inoculated three times into different germ-free mouse subjects, respectively. After 30 days, the mice were autopsied. Metagenome DNA was extracted from the germ-free mouse stomach from the antrum and fundus regions respectively for microbiota analysis. Simultaneously, microbial community analysis using bacterial 16S rRNA gene amplicon sequencing was also performed on gastric mucosal tissues of human donors. The body mucosa of the mouse stomach was also used in experiments to monitor immune cell markers (Figure 7).

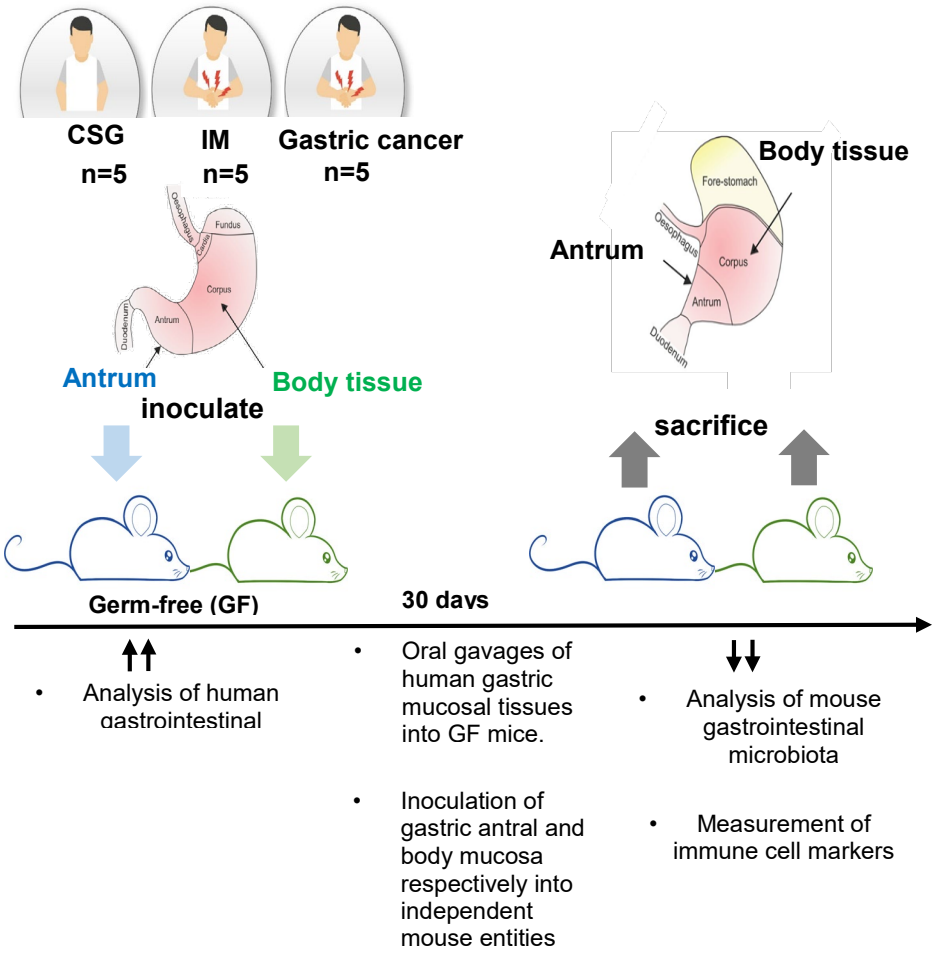

Figure 7. Schematic of experimental design for human gastric-microbiota transplantation into mouse stomach.

5. Characterization of human donor and mouse recipient gastric microbiota

The microbial community structure showed that Firmicutes and Epsionbacteraeota significantly dominated the total microbiota. The human microbial community showed that *Helicobacter* are most dominant bacteria in all three groups. Lachnospiraceae, Lactobacillus, Streptococcus were the second relatively abundant genera in each of the 3 patient groups (CSG, IM, and gastric cancer group, respectively) (Table 6). The relative abundances ratio of germ-free mouse samples was mostly dominated by Firmicutes. Other prominent phyla in gastric microbiota-recipients were Proteobacteria, Bacteroidetes, and Actinobacteria. Meanwhile, Epsionbacteraeota was highly detected in microbiota-donor samples, which show *Hp* positive responses (Figure 8A). According to the alpha diversity analysis, the number of observed OTUs and the value of Shannon index was significantly decreased in mouse gastric samples compared to that of humans. Further, the diversity indicators representing community evenness were observed to be much lower in mouse samples (Figure 8B). When comparing the diversity of patients and mice according to the disease-status of donors, all groups had similar tendencies of alpha diversity (Figure 8C).

Table 6. The order of relative abundance of human and gastric microbes.

CSG				
Order of genus	Human	Mean (%)	Mouse	Mean (%)
1	Helicobacter	19.13	Turicibacter	29.98
2	Lachnospiraceae	7.01	Hungatella	8.98
3	Pediococcus	5.42	Bacteroides	4.01
4	Bacteroides	3.74	Streptococcus	3.12
5	Bifidobacterium	3.28	Blautia	2.61
6	Muribaculaceae	3.07	Granulicatella	2.52
7	Akkermansia	2.62	Lactobacillales	2.42
8	Ruminococcaceae	2.58	Pediococcus	1.86
9	Streptococcus	2.21	Lachnospiraceae	1.85
10	Lactobacillus	1.95	Staphylococcus	1.68
11	Burkholderiaceae	1.56	Prevotella 9	1.52
12	Clostridium	1.43	Acinetobacter	1.39
13	Romboutsia	1.19	Lactobacillus	1.16
14	Blautia	1.15	Faecalibacterium	0.94
15	Muribaculaceae	0.96	Enterococcus	0.86
16	Ruminococcus	0.95	Veillonella	0.67
17	Faecalitalea	0.85	Dialister	0.64
18	Alloprevotella	0.85	Prevotella	0.63
19	Escherichia-Shigella	0.83	Enhydrobacter	0.61
20	Veillonella	0.75	Alistipes	0.59

IM				
Order of genus	Human	Mean (%)	Mouse	Mean (%)
1	Helicobacter	39.15	Turicibacter	25.31
2	Lactobacillus	8.35	Streptococcus	11.11
3	Lachnospiraceae	3.78	Veillonella	8.63
4	Bacteroides	3.48	Hungatella	5.82
5	Ruminococcaceae	2.59	Bacteroides	3.38
6	Actinobacillus	2.37	Haemophilus	2.85
7	Muribaculaceae	2.18	Granulicatella	2.14
8	Streptococcus	2.13	Pediococcus	2.06
9	Neisseria	1.62	Acinetobacter	1.85
10	Bifidobacterium	1.61	Lachnospiraceae	1.75
11	Alistipes	1.06	Blautia	1.48
12	Blautia	0.98	Lactobacillales	1.37
13	Faecalibacterium	0.82	Prevotella	1.31
14	Ruminococcus	0.81	Lactobacillus	1.13
15	Eubacterium	0.76	Faecalibacterium	1
16	Rothia	0.71	Staphylococcus	0.83
17	Muribaculaceae	0.66	Enhydrobacter	0.77
18	Haemophilus	0.62	Rhodococcus	0.67
19	Burkholderiaceae	0.56	Lachnospiraceae	0.62
20	Prevotella	0.53	Fusobacterium	0.57

Gastric cancer				
Order of genus	Human	Mean (%)	Mouse	Mean (%)
1	Helicobacter	18.17	Turicibacter	28.68
2	Streptococcus	7.37	Hungatella	20.46
3	Lachnospiraceae	5.83	Lactobacillus	6.32
4	Lactobacillus	3.55	Blautia	5.25
5	Muribaculaceae	3.4	Enterobacteriaceae	5.01
6	Bacteroides	3.29	Veillonella	4.36
7	Ruminococcaceae	2.79	Streptococcus	2.28
8	Veillonella	2.5	Pediococcus	2.24
9	Prevotella 7	2.4	Bacteroides	1.22
10	Bifidobacterium	1.18	Staphylococcus	1.05
11	Rothia	1.12	Burkholderiaceae	0.95
12	Ruminococcus	1.06	Lachnospiraceae	0.79
13	Actinomyces	1.01	Granulicatella	0.77
14	Faecalibacterium	0.95	Enterococcus	0.63
15	Pediococcus	0.94	Acinetobacter	0.57
16	Muribaculaceae	0.9	Lactobacillales	0.55
17	Leptotrichia	0.85	Pseudomonas	0.45
18	Alloprevotella	0.82	Cutibacterium	0.44
19	Escherichia-Shigella	0.71	Prevotella	0.41
20	Eubacterium	0.71	Faecalibacterium	0.36

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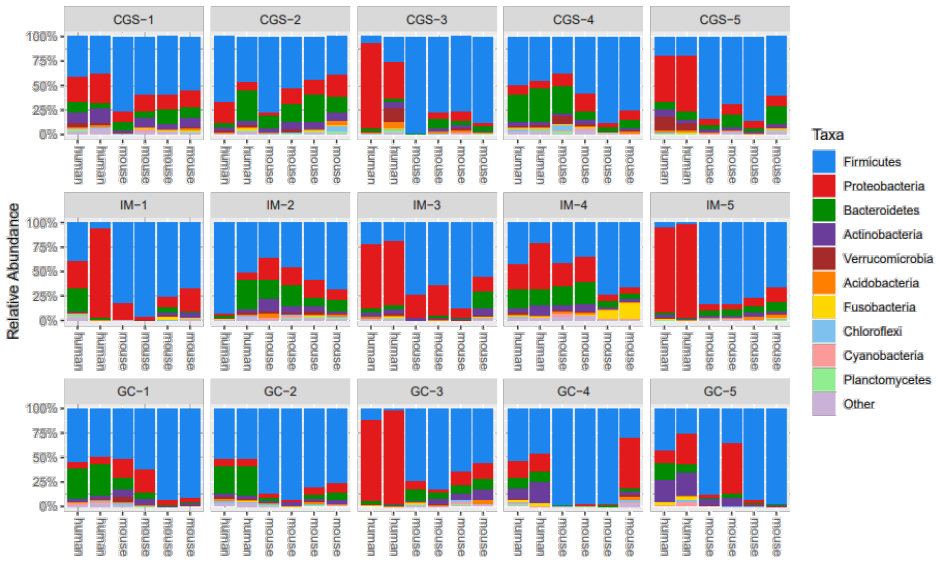

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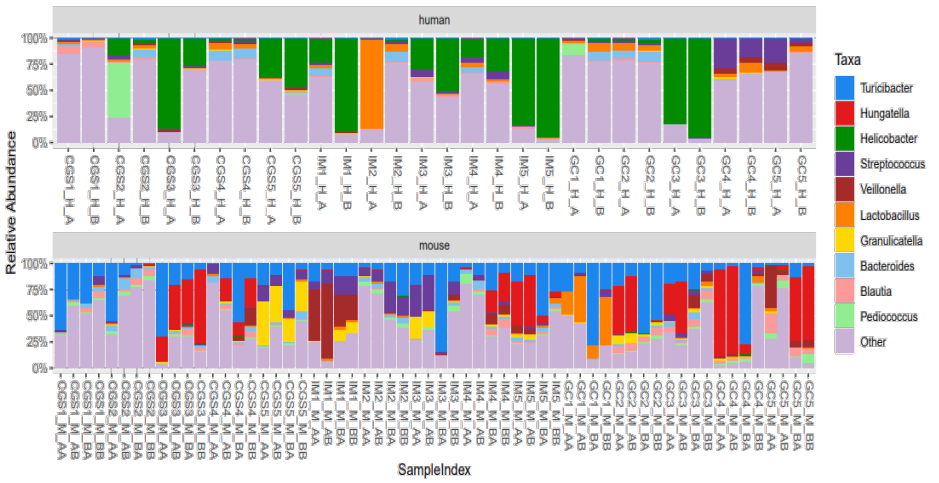

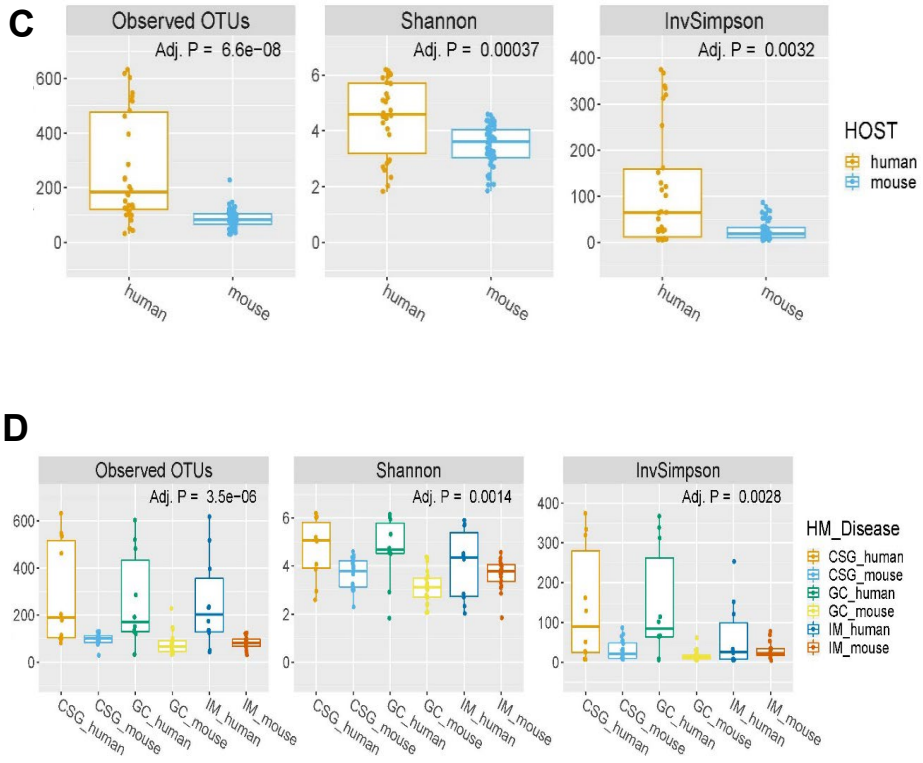

Figure 8. Bacterial community structure in donor humans and recipient mice from the gastric microbiota transplantation experiment. Microbial community structure (phylum level (A) and genus level (B)) in donor humans and recipient mice in the gastric microbiota transplantation experiment. (C) Diversity indices (Observed OTUs, Shannon, Inverse Simpsion; InvSimpsion) of human and murine gastric microbes. Samples were grouped by hosts. (D) Samples were grouped based on the host and the clinical diagnosis of patients.

6. Bacterial diversity of gastric microbiota of human donors and mouse

A weighted UniFrac-based comparison of the human and mouse gastric mucosal-associated microbiota indicated that the overall diversity in microbial composition was mainly differentiated by host type, but the disease of the human donor was not strong enough to cluster the samples. Further, the distance between samples did not depend on the relationships between donors and recipients. (Figure 9A, B). We identified specific microbial taxa associated with hosts by (ANCOM) which is incorporated in Qiime2 pipeline. *Helicobacter pylori*, Bifidobacterium animalis (Actinobacteria), Akkermansia muciniphila (Verrucomicrobia), and Chlostridum disporicum were enriched in humans. Otherwise, Turicibacter, Hungatella effluvia, Pediococcus pentosaceus, Blautia that belong to the Firmicutes, Rhodococcus that are belongs to the Actinobacteria, and Acinetobacter that belongs to the Proteobacteria were enriched in mouse samples (Figure 9C). *Helicobacter* was detected only in human-originated samples and could not colonize the gastric mucosa of a germ-free mouse at all. On the contrary, Turicibacter and Hungatella, belonging to phylum Firmicutes, were the most successful taxa to form the majority of the mouse gastric mucosa although they make up a very small percentage of human microbiota.

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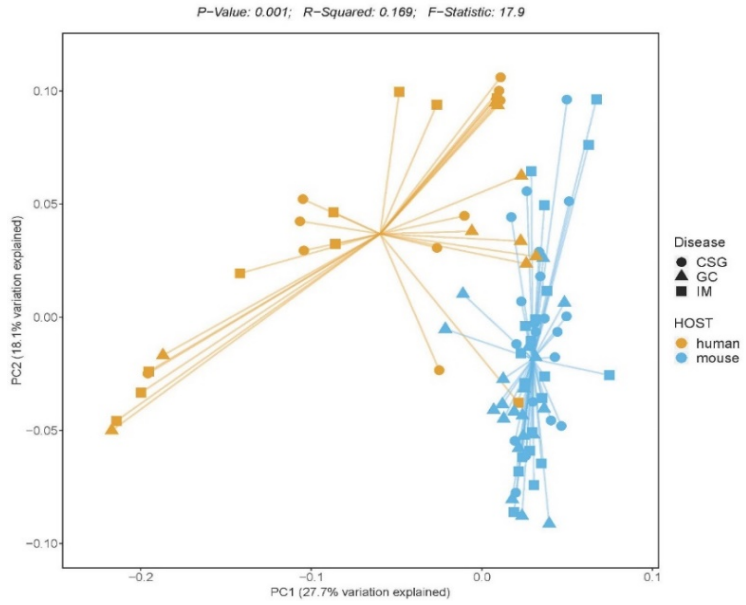

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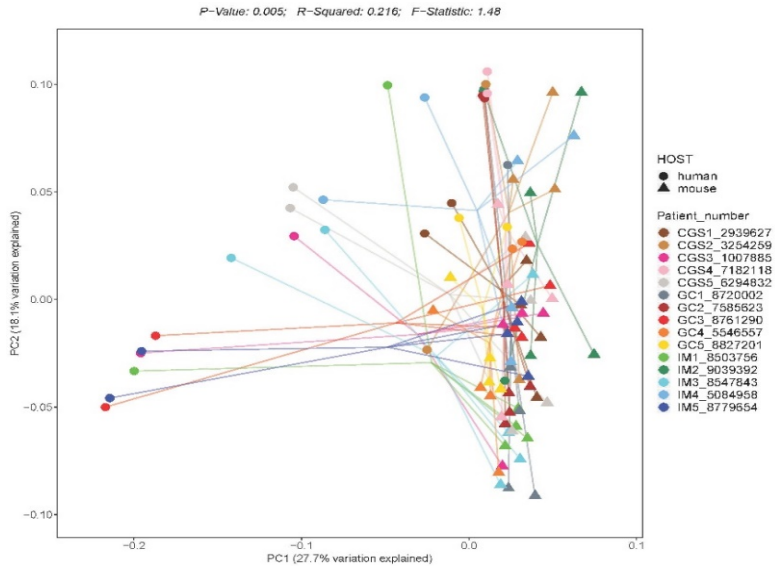


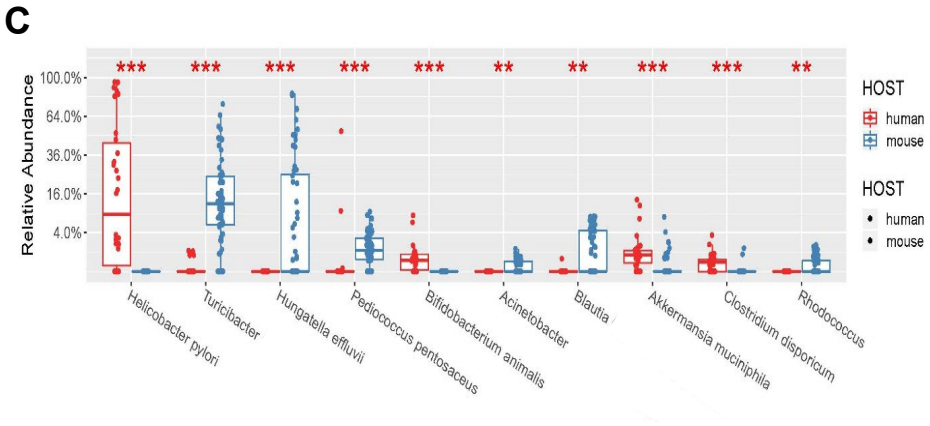
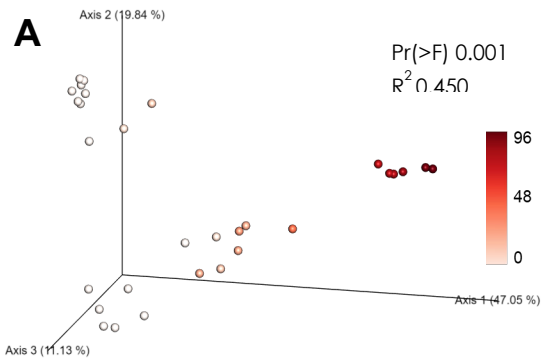


Figure 9. Bacterial community structure in donor humans and recipient mice in the gastric microbiota transplantation experiment. (A) The composition of phyla in gastric mucosal tissues. Diversity indices (Observed OTUs, Shannon, Inverse Simpsion; InvSimpsion) of human and murine gastric microbes. (B) Samples were grouped by hosts. (C) Identification of specific microbial taxa associated with hosts by analysis of the composition of microbiomes (ANCOM)

7. Differences in gastric microbiota within human donors and within mouse recipients

Figure 10 shows PCoA plots based on the Unifrac distance between human gastric samples. Dots represent each human gastric sample, and are colored based on the relative abundance of *Helicobacter pylori* disease status and sampling sites. In Figure 10A, red indicates a high abundance of *Helicobacter*

pylori, and white indicates a low amount of *Helicobacter pylori*. According to samples are aligned in accordance with their *Helicobacter pylori* abundance, the relative amount of *Helicobacter pylori* is a factor that significantly affects the gastric microbial structure in human samples. This is the major factor that significantly affects the gastric microbial structure in human samples rather than factors of disease states or sampling sites. Figure 11 showed specific microbial taxa associated with disease states in human gastric tissues by the LEfSe method. By comparing between CSG and gastric cancer, gastric cancer and IM, CSG and IM, Akkermansia, Longicatena, and, Leuconostoc genus were enriched in the CSG samples. The genus Haemophilus was highly existed in IM samples, whereas Prevotella, Veillonella, and Acinetobacter genus were highly observed in gastric cancer samples. Figure 12 shows the PCoA plots based on unifrac distance between mouse samples. The relative amounts of the most abundant taxa were observed for Hungatella and Turicibacter and this dominant bacterial factor affects the gastric microbial structure in mouse samples rather than clinical factors of disease states or sampling sites.



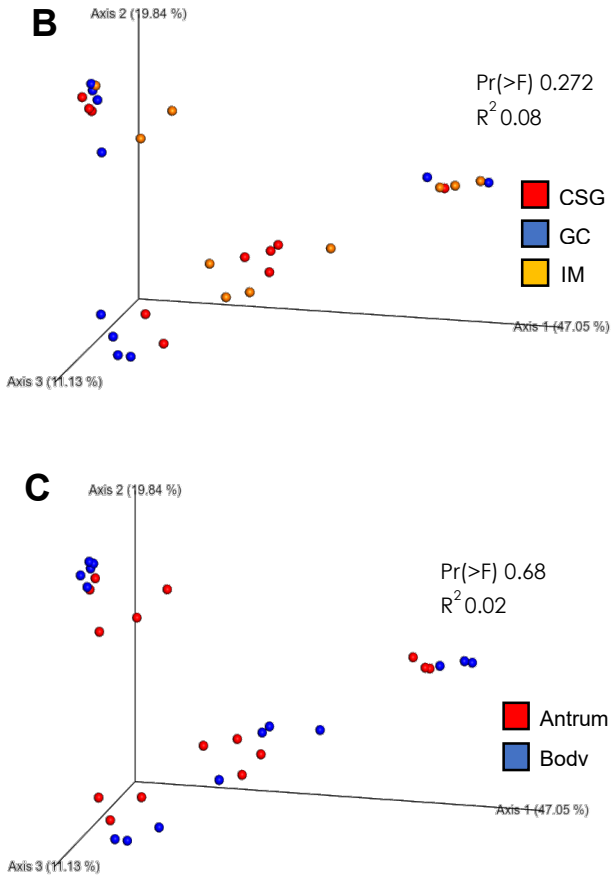


Figure 10. Multidimensional scaling of weighed UniFrac distances of gastric microbial communities in humans. Color coded and shaped on relative abundance of (A) *Helicobacter pylori* (B) disease states, and (C) sampling sites

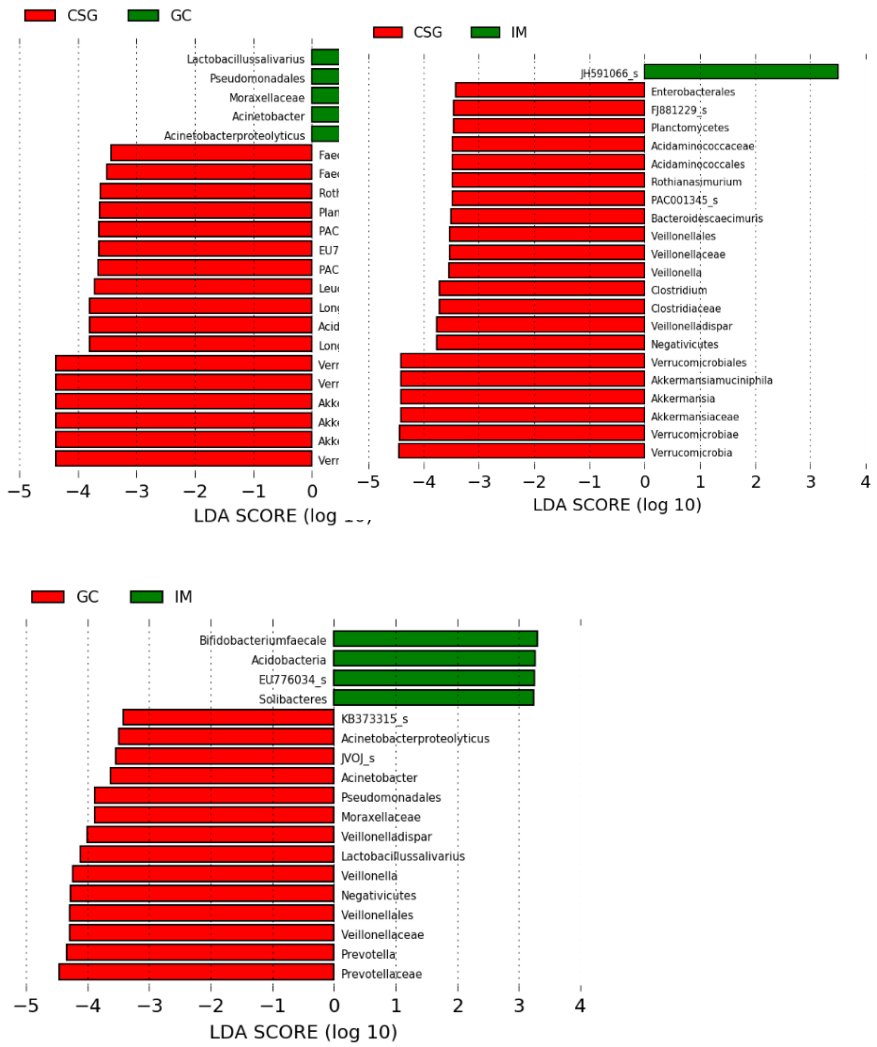
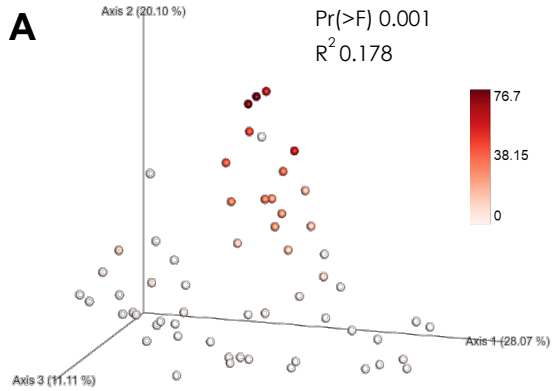
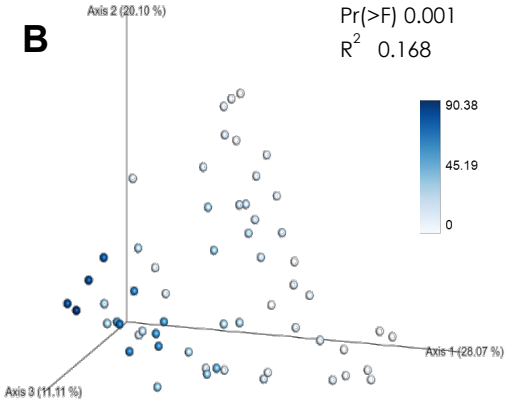
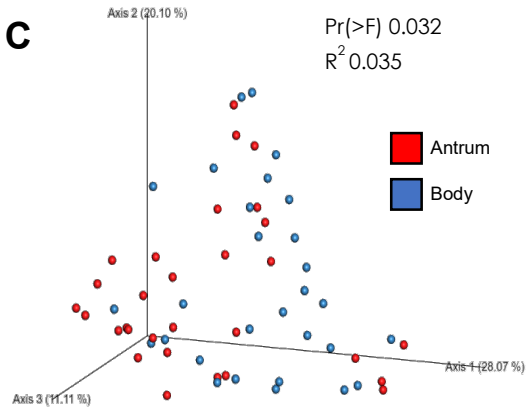


Figure 11. Specific microbial taxa associated with disease states in human gastric tissues







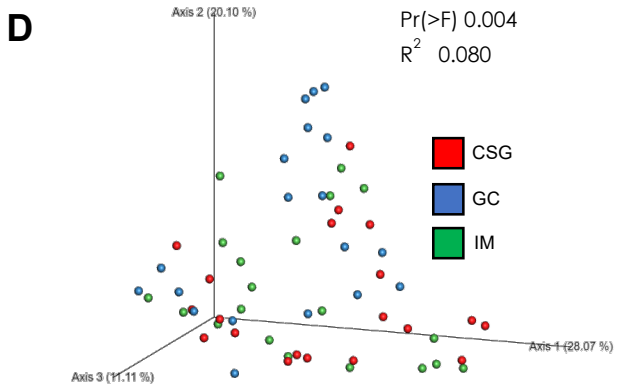
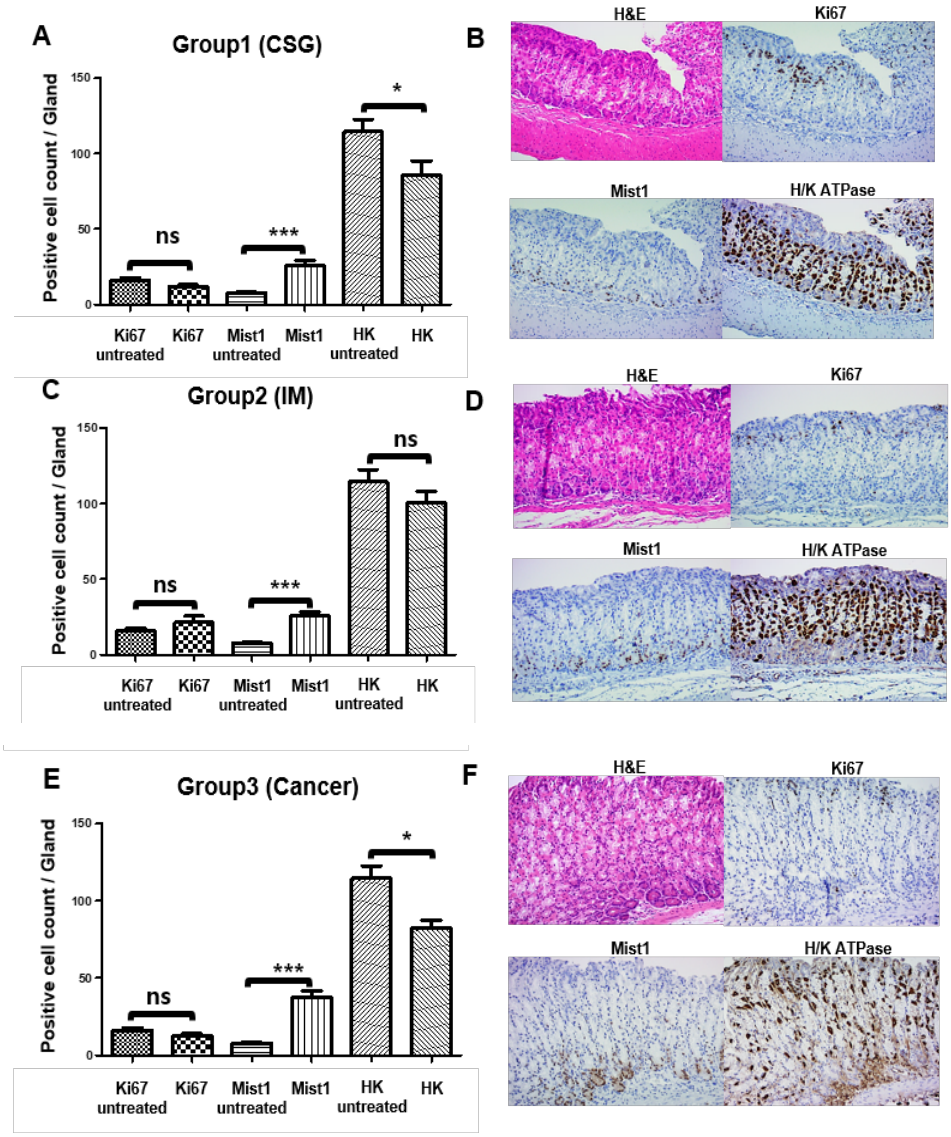


Figure 12. Multidimensional scaling of weighed UniFrac distances of gastric microbial communities in mice. Color coded and shaped on relative abundances of genus (A) *Hungatella*, (B) *Turicibacter*, (C) sampling sites, and (D) donor's disease states.

8. Histopathological results after human gastric tissue inoculation

Histopathological data of germ-free mice demonstrated that Mist1 was significantly increased after transplantation of CSG human gastric tissues, and H/K ATPase in germ-free mice was significantly reduced after transplantation (Figure 13A & B). Mist1 was also significantly increased after transplantation of the IM group of human gastric tissues. However, H/K ATPase did not show any difference after transplantation (Figure 13C & D). Histopathological data from germ-free mice showed that Mist1 was significantly increased after transplantation of human gastric cancer tissues whereas H/K ATPase was

significantly decreased after transplantation with human gastric cancer tissue (Figure 13E & F).



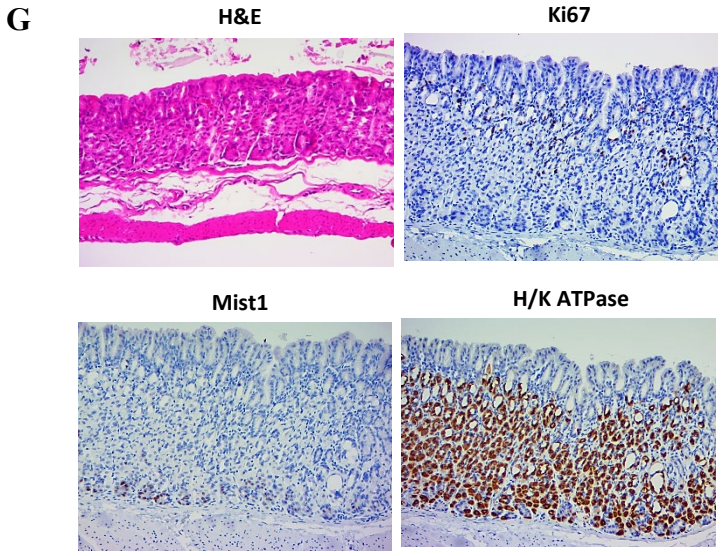


Figure 13. Histopathological results of germ-free mice according to human gastric tissues. (A, B) Positive cell markers and pathologic pictures of germ-free mice after inoculation of human CSG gastric tissues, (C, D) Positive cell markers and pathologic pictures of germ-free mouse after inoculation of human IM gastric tissues, (E,F) Positive cell markers and pathologic pictures of germ-free mouse after inoculation of human gastric cancer tissues. (G) Untreated germ-free mouse (control).

IV. Discussion

Gastric cancer is globally the third leading cause of cancer-related death in men and the fourth and fifth leading cause of death in men and women, respectively with almost 990,000 cases detected annually[22]. Although gastric cancer survival

rates have improved with the use of endoscopy screening, the majority of patients with gastric cancer still present locally advanced or metastatic disease. Until now, *Hp* infection is the most well-known risk factor of gastric cancer. Based on the hypothesis of gastric carcinogenesis, long-term *Hp* infection is associated with gastric atrophy, IM and increased gastric cancer risk. In recent days, many studies have shown that human gastric microbiota has diverse species of bacteria and it has been proposed that gastric microbiota plays a role in the development of gastric cancer. However, there has been scanty data regarding bacteria other than *Hp* with a potential impact on gastric carcinogenesis. Moreover, changes in gastric microbial composition are associated with the gastric carcinogenesis cascade and the role of bacteria other than *Hp* is yet to be established.

To better understand the effect of gastric microbiota in human hosts, making a humanized mouse stomach model can provide a well-controlled system for both understanding the mechanism of gastric carcinogenesis and to overcome clinical limitations. Humanized mice as pre-clinical models for the in vivo study of human cells and tissues have been under development for over 30 years. Theses humanized mice have facilitated novel insights into human disease by in vivo studies without putting patients at risk. In this study, we attempted to generate a humanized mouse gut model as a research tool for stomach cancer development based on the interaction between microorganisms and the host,

and to characterize the gastric microbiomes in gastric cancers based on high-throughput next generation sequencing.

First, to determine whether to use between gastric mucosal tissue of gastric juice for making the humanized mouse model, we decided to obtain some information about gastric microbiota in both the gastric mucosa and gastric juice. We performed a preliminary study for comparing the human gastric microbiota with mucosal tissues and juice. Our preliminary study demonstrated that no correlation with human gastric mucosal tissue and gastric juice. This is because bacteria recently swallowed through the mouth and throat can influence stomach microbiota. Microbiota from the oral cavity and esophagus can make it difficult to detect the true pathogens in the stomach. Another hypothesis to consider is that bacteria that cannot attach to the gastric mucosa and pass through stomach fluids. A previous study also supports our hypothesis [23]. Therefore, analyze the stomach microbiota, we decided to transplant human gastric mucosal tissues into germ-free mice.

Table 3 shows that the most dominant bacteria were *Helicobacter*, Streptococcus, Neisseria, and Prevotella in *Hp*-infected human gastric mucosal tissues. Especially, the relative abundance of genus Helicobacter was dominated in both the antrum and body tissues, but in gastric juice, genus *Helicobacter* showed the second-highest relative abundance following genus Streptococcus. This result is similar to other gastric microbiota studies. Several

recent studies have also demonstrated that bacteria including members of Proteobacteria, Firmicutes, Actinobacteria, and Fusobacteria phyla, can be regularly detected in gastric mucosa [5, 24-26]. *Hp*-negative patient samples showed Neisseria, Haemophilus as the most dominant bacteria. Even though clinical data classified them as non-*Hp* infected patients, we observed that extremely low levels of the genus *Helicobacter* were also detected in the gastric tissues but not in gastric juice. However, we could not distinguish whether this *Helicobacter* genus came from transcriptionally active, inactive, or dead bacteria. This is one of limitation of the clinical diagnosis method in *Hp* infection study.

By measuring the observed OTUs, we found that gastric juice had significantly decreased bacterial diversity compared to gastric mucosal tissues, and the presence of *Hp* decreased the diversity in the aspect of richness and evenness. Moreover, the smaller the number of OTUs observed, the greater the degree of disease progression from CSG to adenocarcinoma (Figure 3D). In particular, the properties of both PCoA and a hierarchical plot (Figure 4B &C) showed that the distance of microbial community structure between gastric juice samples was much closer than the distance between the tissue samples. These microbial analyses suggest that gastric juice cannot accurately represent the microbial community of the stomach tissue. Therefore, it is not appropriate to inject gastric juice to create a human stomach mouse model mimicking the

microbial communities that colonize human gastric mucosa.

In the gastric microbiota transplantation experiment, human microbial community structure showed that Firmicutes and Epsionbacteraeota significantly dominated the total microbiota. However, according to the diversity analysis, human gastric microbiota settles very selectively in the murine stomach, and in all human disease group (CSG, IM, and gastric cancer) recipient mice showed similar tendencies of alpha diversity. Moreover, even if we transplanted *Hp* infected human gastric mucosa into germ-free mice, *Hp* could not survive in the gastric mucosa of germ-free mice . Therefore, in this study, we could not demonstrate that transplantation of human donor-microbiota was successful despite the use of germ-free mice. To the best of our knowledge, there has been no report on a humanized gastric mouse model using germ-free mice. Therefore, it is difficult to compare with other studies for the success of *Hp* transplantation. However, our study demonstrates that human gastric status is quite different from the mouse gastric environment, and so the composition of settled gastric microbiota could also be different.

Interestingly, there were several dominant species in the colonized microbial population in the germ-free mouse model. The most abundant genera in germ-free mouse stomachs were Trucibacter and Hungutella, which was the most significant difference compared to human gastric microbial communities.

This extremely dominant occupation of two genera make it difficult to evaluate

the correlation between mouse immunity markers and the microbial population. Further, there might be a high possibility of masking the effect of interaction between various microbial communities that may contribute to altering murine immune responses. Therefore, we performed multidimensional scaling of weighed UniFrac distance in gastric microbial communities based on immune cell markers except dominant microorganisms. The result showed that microbiota compositional profiles of germ-free mouse stomach were related to the concentration of Ki67, Mist1, and H/K-ATPase cell numbers among the measured immune markers.

Figure 13 shows the histopathologic changes in the positive cell number of immune cell markers. Even though there was no statistically significant difference in the IM transplanted group due to the small sample number, H/K-ATPase was significantly decreased in both the CSG and gastric cancer transplanted group. As gastric H/K-ATPase is well known as a proton pump of the stomach and exists in parietal cells. Therefore, we assumed that the parietal cells of germ-free mice might be decreased after human gastric microbiota transplantation.

According to gastric carcinogenesis, intestinal-type gastric cancer typically arises in the setting of chronic gastritis and develops through the intermediate stages of atrophic gastritis, intestinal metaplasia, dysplasia, finally leading to cancer. This process, known commonly as the “Correa pathway”, is triggered

by Hp infection and depends on the sustained chronic inflammation of gastric mucosa [27]. Loss of parietal cells is the initial histologic change of IM in the gastric Correa pathway. Therefore, our histologic data showed the possibility of changing germ-free naive gastric tissue into the process of a cascade for gastric carcinogenesis.

Interestingly, Mist1 was significantly increased in all three germ-free mouse groups after human tissue transplantation. This phenomenon is difficult to explain clearly; however, it seems that Mist1, which is known as a stem cell marker is a compensating reaction after mucosal damage.

Our study has several limitations. First, although the next generation sequencing molecular modality is powerful and provides a complete view of overall microbiota, it is unable to distinguish between living and dead bacteria. Second, the possibility of contamination in the biopsy channel with throat bacteria could not be completely ruled out. It is difficult to overcome this problem with the currently available clinical methods. The last limitation is small number of enrolled patients in the human disease group.

Despite these limitations, our data may form the basis of a system that can allow us to better understand the human gastric microbiota and microbial population in germ-free mouse models. Although complete transplantation of human donor-microbiota to germ-free mouse was not successful, this study characterized and compared gastric microbiomes among the gastric cancer

group, high risk of gastric cancer group, and control group. Most of all, this is the first study attempting to generate a humanized mouse stomach model as a research tool for stomach cancer development.

V. Conclusion

Human gastric juice cannot accurately represent the microbial community of the stomach mucosa. Human gastric microbiota exhibit selective colonization ability in mouse gastric tissue. Our data may form the basis of a system that can allow better understanding of the human gastric microbiota and microbial population in a germ-free mouse model.

References

1. Delgado S, Cabrera-Rubio R, Mira A, Suarez A, Mayo B. Microbiological survey of the human gastric ecosystem using culturing and pyrosequencing methods. Microb Ecol 2013;65:763-72.
2. Rakoff-Nahoum S, Paglino J, Eslami-Varzaneh F, Edberg S, Medzhitov R. Recognition of commensal microflora by toll-like receptors is required for intestinal homeostasis. Cell 2004;118:229-41.
3. Mazmanian SK, Liu CH, Tzianabos AO, Kasper DL. An immunomodulatory molecule of symbiotic bacteria directs maturation of the host immune system. Cell 2005;122:107-18.
4. Bik EM, Eckburg PB, Gill SR, Nelson KE, Purdom EA, Francois F, et al. Molecular analysis of the bacterial microbiota in the human stomach. Proc Natl Acad Sci U S A 2006;103:732-7.
5. Jo HJ, Kim J, Kim N, Park JH, Nam RH, Seok YJ, et al. Analysis of Gastric Microbiota by Pyrosequencing: Minor Role of Bacteria Other Than Helicobacter pylori in the Gastric Carcinogenesis. Helicobacter 2016;21:364-74.
6. Nardone G, Compare D. The human gastric microbiota: Is it time to rethink the pathogenesis of stomach diseases? United European Gastroenterol J 2015;3:255-60.
7. Smith MG, Hold GL, Tahara E, El-Omar EM. Cellular and molecular

aspects of gastric cancer. World J Gastroenterol 2006;12:2979-90.

8. Wu MS, Chen CJ, Lin JT. Host-environment interactions: their impact on progression from gastric inflammation to carcinogenesis and on development of new approaches to prevent and treat gastric cancer. Cancer Epidemiol Biomarkers Prev 2005;14:1878-82.

9. Stearns JC, Lynch MD, Senadheera DB, Tenenbaum HC, Goldberg MB, Cvitkovitch DG, et al. Bacterial biogeography of the human digestive tract. Sci Rep 2011;1:170.

10. Maldonado-Contreras A, Goldfarb KC, Godoy-Vitorino F, Karaoz U, Contreras M, Blaser MJ, et al. Structure of the human gastric bacterial community in relation to Helicobacter pylori status. Isme j 2011;5:574-9.

11. Roos S, Engstrand L, Jonsson H. Lactobacillus gastricus sp. nov., Lactobacillus antri sp. nov., Lactobacillus kalixensis sp. nov. and Lactobacillus ultunensis sp. nov., isolated from human stomach mucosa. Int J Syst Evol Microbiol 2005;55:77-82.

12. Ryan KA, Jayaraman T, Daly P, Canchaya C, Curran S, Fang F, et al. Isolation of lactobacilli with probiotic properties from the human stomach. Lett Appl Microbiol 2008;47:269-74.

13. Sanduleanu S, Jonkers D, De Bruine A, Hameeteman W, Stockbrugger RW. Non-Helicobacter pylori bacterial flora during acid-suppressive

therapy: differential findings in gastric juice and gastric mucosa. Aliment Pharmacol Ther 2001;15:379-88.

14. Mowat C, Williams C, Gillen D, Hossack M, Gilmour D, Carswell A, et al. Omeprazole, Helicobacter pylori status, and alterations in the intragastric milieu facilitating bacterial N-nitrosation. Gastroenterology 2000;119:339-47.

15. Miki K, Ichinose M, Ishikawa KB, Yahagi N, Matsushima M, Kakei N, et al. Clinical application of serum pepsinogen I and II levels for mass screening to detect gastric cancer. Jpn J Cancer Res 1993;84:1086-90.

16. Bolyen E, Rideout JR, Dillon MR, Bokulich NA, Abnet CC, Al-Ghalith GA, et al. Reproducible, interactive, scalable and extensible microbiome data science using QIIME 2. Nat Biotechnol 2019;37:852-857.

17. Callahan BJ, McMurdie PJ, Rosen MJ, Han AW, Johnson AJ, Holmes SP. DADA2: High-resolution sample inference from Illumina amplicon data. Nat Methods 2016;13:581-3.

18. Paradis E, Claude J, Strimmer K. APE: Analyses of Phylogenetics and Evolution in R language. Bioinformatics 2004;20:289-90.

19. Oksanen J, Blanchet FG, Friendly M, Kindt R, Legendre P, McGlinn D, et al. Vegan: Community Ecology Package. R package Version 2.4-3. 2017.

20. McMurdie PJ, Holmes S. phyloseq: an R package for reproducible interactive analysis and graphics of microbiome census data. PLoS One 2013;8:e61217.

21. JM A. A new method for non-parametric multivariate analysis of variance. Austral Ecology 2008;26:32-46.

22. Jemal A, Bray F, Center MM, Ferlay J, Ward E, Forman D. Global cancer statistics. CA Cancer J Clin 2011;61:69-90.

23. Sung J, Kim N, Kim J, Jo HJ, Park JH, Nam RH, et al. Comparison of Gastric Microbiota Between Gastric Juice and Mucosa by Next Generation Sequencing Method. J Cancer Prev 2016;21:60-5.

24. Aviles-Jimenez F, Vazquez-Jimenez F, Medrano-Guzman R, Mantilla A, Torres J. Stomach microbiota composition varies between patients with non-atrophic gastritis and patients with intestinal type of gastric cancer. Sci Rep 2014;4:4202.

25. Eun CS, Kim BK, Han DS, Kim SY, Kim KM, Choi BY, et al. Differences in gastric mucosal microbiota profiling in patients with chronic gastritis, intestinal metaplasia, and gastric cancer using pyrosequencing methods. Helicobacter 2014;19:407-16.

26. Dicksved J, Lindberg M, Rosenquist M, Enroth H, Jansson JK, Engstrand L. Molecular characterization of the stomach microbiota in patients with gastric cancer and in controls. J Med Microbiol

2009;58:509-16.

27. Correa P. Helicobacter pylori and gastric carcinogenesis. Am J Surg Pathol 1995;19 Suppl 1:S37-43.

ABSTRACT(IN KOREAN)

인체 위 마이크로바이오타를 이식한 마우스 모델의 확립 및 분석

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배경: 위암과 인간 위 미생물 균총의 생물학적 역할과 상호관계를 이해하려면 인간화된 마우스 위 모델을 만드는 것이 통제된 시스템을 구축하기 위한 가장 좋은 방법 중 하나이다. 이 연구는 미생물과 숙주 간의 상호 작용을 기반으로 위암 개발을 위한 연구 도구로서 위 미생물 균총을 이용한 인간화 마우스 모델 개발을 목표로 하였다.

방법: 위 점막 조직을 얻기 위해 15 명의 환자 (만성 표재성 위염; n = 5, 장상피화생; n = 5 및 위암 n = 5)를 등록하였다. 획득 된 인간 위의 전정부와 위체부 점막 조직을 서로 다른 무균 마우스에 독립적으로 이식하였다. 이식 30일 이후 인간과 마우스의 위 조직의 미생물 균총을 16S rRNA 유전자 시퀀싱을 이용하여 분석하여 비교하였다.

결과: 미생물 분석을 통해 인간 3 그룹 모두에서 *Helicobacter* 균주가 가장 우세한 박테리아임을 확인할 수 있었으며, *Lachnospiraceae, Lactobacillus, Streptococcus*들이 두번째로 상대적으로 풍부하게 분포하고 있었다. 알파 다양성 분석에 의하면 미생물 Operational Taxonomic Unit이 인간의

것보다 무균마우스 위의 조직에서 현저히 감소하였으며, 무균마우스 위 조직에서 미생물 균일성이 더 낮은 것으로 관찰되었다. Weighted UniFrac 비교분석에서도 샘플 간의 거리가 인간과 마우스 그룹에서 확연히 차이가 있음을 보여주었다. 특히, 무균마우스 위점막 미생물 균총분석에서는 *Turicibacter*와 *Hungatella* 두 균종이 대부분을 형성하는 우점종으로 확인되었다. 무균 마우스로부터의 확인된 조직 병리학적 결과에서는 인간 위암 조직 이식 후 H/K ATPase가 유의하게 감소되었음이 확인되었다.

결론: 인간 위 미생물 균총은 무균마우스 위 조직에서 선택적 집락 능력을 나타냈으며 인간 위암 조직을 이식 받은 무균 마우스에서 조직 병리학적 변화는 위 점막 손상의 초기 단계를 보여주었다.

핵심되는 말 : 미생물 균총, 인간화 마우스 모델, 위암