Title: Characteristics of intestinal microflora and dysbiosis in relation to the disease duration in patients with Meniere's disease

Short title: Dysbiosis in patients with Meniere’s disease

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Abstract

Meniere's disease is an intractable disease characterized by paroxysms of intense rotatory dizziness, hearing loss, and other auditory symptoms. It is believed to be caused by endolymphatic edema of the vestibule and cochlea. The mechanism underlying endolymphatic edema is believed to be stress-related. Endolymphatic edema identification using cochlear contrast-enhanced MRI (hybrid of reversed image of positive endolymph signal and native image of positive perilymph signal [HYDROPS]) is an objective test for Meniere's disease, enabling reliable diagnosis of this disease. Since dysbiosis of the intestinal microflora has been reported in stress-induced depression and irritable colitis, a similar abnormality may also occur in stress-triggered Meniere's disease. Therefore, we investigated the gut microbiota of 10 patients (6 males and 4 females; mean age, 49.6 ± 8.1 years) diagnosed with Meniere's disease and marked endolymphatic edema on HYDROPS. These 10 patients underwent intestinal microbiota examination, and the results of the examination were evaluated in relation to the duration of the disease, the results of auscultation of the affected side, and Dizziness Handicap Inventory (DHI) scores. A significant negative correlation was found between the disease duration and Shannon diversity index and Faith's phylogenetic diversity, which indicated dysbiosis of the intestinal microflora. No correlation was found between the indicators of microbial

diversity and the results of audiometry or the DHI on the affected side. Dysbiosis of the intestinal microbiota worsened with increasing duration of Meniere's disease. Moreover, *Akkermansia muciniphila* was not detected in any patient with Meniere's disease. Thus, despite the small number of cases in this study (n = 10), the findings indicate the possibility of abnormalities of the intestinal microflora in Meniere's disease.

Introduction

Meniere's disease is an intractable disorder characterized by paroxysms of intense rotatory vertigo and auditory symptoms such as ear closure and hearing loss. Although the pathogenesis of Meniere's disease is not fully understood, it is speculated to be caused by endolymphatic edema of the vestibule and cochlea. The diagnostic criteria for Meniere's disease were defined by the Rosaceae Society (Fig 1). Objective tests for evaluation of Meniere's disease traditionally included the furosemide test and cochleography, but imaging studies using cochlear contrast-enhanced MRI (hybrid of reversed image of positive endolymph signal and native image of positive perilymph signal [HYDROPS]) have made it possible to visually evaluate endolymphatic edema. Treatment of Meniere's disease consists of daily lifestyle guidance and medication for mild cases and surgical approaches, such as endolymphatic sac release, for moderate to severe cases.

Fig 1. Diagnostic Criteria for Meniere's disease in 2015 as defined by the Rosaceae Society.

DNA testing with next-generation sequencing has made it possible to investigate the

52 intestinal microbiota in fecal material in great detail. As a result, the changes in the
53 intestinal microbiota have been tested in relation to various disease groups. In this regard,
54 various studies have investigated the intestinal microbiota of patients with depression and
55 irritable bowel syndrome, in which stress factors are associated with the induction of
56 pathological conditions. We hypothesized that the gut microbiota of patients with
57 Meniere's disease, in whom stressors trigger seizures, may also differ from that of healthy
58 controls. To evaluate this hypothesis, we investigated the gut microbiota of 10 patients
59 diagnosed with Meniere's disease and marked endolymphatic edema by using HYDROPS.
60

61 # Materials and Methods

62 ## Participants

63 Ten patients (6 males, 4 females; mean age, 49.6 ± 8.1 years) with Meniere's disease
64 diagnosed according to the diagnostic criteria of the Barany Society (Fig 1) and showing
65 significant endolymphatic edema on the affected side on HYDROPS of the inner ear were
66 included in this study. The HYDROPS images are presented in Fig 2. The patients did not
67 have any psychiatric, diabetic, or gastrointestinal diseases that may have caused dysbiosis.
68

69 **Fig 2. HYDROPS image in case 1.** The short arrows indicate the cochlea, while the

long arrow indicates the vestibule.

Methods

The 10 participants received a fecal collection kit and obtained fecal samples during a period with no abnormal bowel movements. Intestinal microflora testing was performed by an external contractor (SAIKINSO Co., Tokyo, Japan). The intestinal microflora was analyzed as follows.

Fecal sampling, DNA extraction, and sequencing

Fecal samples were collected using Cykinso fecal collection kits containing guanidine thiocyanate solution (Cykinso Inc., Tokyo. Japan) and stored at 4°C. DNA extraction from the fecal samples was performed using an automated DNA extraction machine (GENE PREP STAR PI-480; Kurabo Industries Ltd., Osaka). The V1-V2 region of the 16S rRNA gene was amplified using a forward primer (16S_27Fmod: TCG TCG GCA GCG TCA GAT GTG TAT AAG AGA CAG AGR GTT TGA TYM TGG CTC AG) and reverse primer (16S_338R: GTC TCG TGG GCT CGG AGA TGT GTA TAA GAG ACA GTG CTG CCT CCC GTA GGA GT) with the KAPA HiFi Hot Start Ready Mix (Roche). To sequence the 16S amplicons using the Illumina MiSeq platform, dual index adapters were

88 attached using the Nextera XT Index kit. The DNA concentration of the mixed libraries
89 was quantified by qPCR using KAPA SYBR FAST qPCR. The master mix (KK4601,
90 KAPA Biosystems) was used with primer 1 (AAT GAT ACG GCG ACC ACC) and primer
91 2 (CAA GCA GAA GAC GGC ATA CGA). Library preparations were performed
92 according to the Illumina 16S library preparation protocol (Illumina, San Diego, CA,
93 USA). Libraries were sequenced using the MiSeq Reagent Kit v2 (500 cycles) and 250-
94 bp paired ends.
95

96 **Taxonomy assignment based on 16S rRNA gene sequences**

97 The paired-end reads of the partial 16S rRNA gene sequences were analyzed using QIIME
98 2 (version 2020.8). The steps for data processing and assignment based on the QIIME 2
99 pipeline were as follows: (1) DADA2 was used for joining paired-end reads, filtering, and
100 denoising; (2) taxonomic information was assigned to each ASV by using a naive Bayes
101 classifier in the QIIME 2 classifier with the 16S gene of the V1-V2 region data of SILVA
102 (version 138) to determine the identity and composition of the bacterial genera. The
103 reliability of these tests has been reported previously [1,2].
104 Shannon diversity index (Shannon index) and Faith's phylogenetic diversity (FPD)
105 were used as indicators of dysbiosis in the gut microbiota. These indices were averaged

over 10000 reads per sample. The Dizziness Handicap Inventory (DHI) was used to assess dizziness symptoms. We performed statistical analysis of the obtained gut microbiota data, age, BMI, duration of Meniere's disease, DHI results, and hearing test results (quadrant method) by using Spearman's correlation coefficient values. The hearing test and DHI evaluation performed closest to the intestinal microbiota tests were used for analyses. Excel with add-in software Stacel4 (OMS, Tokyo, Japan) was used as statistical software.

Results

Table 1 shows the age, sex, BMI, duration of Meniere's disease, DHI results, hearing test results, and dysbiosis index of intestinal bacteria. A significant negative correlation was observed between disease duration and the Shannon index (r = -0.72, p = 0.02) and FPD (r = -0.89, p = 0.0005) (Figs 3 and 4). However, no significant correlation was found between age and Shannon index (r = 0.16, p = 0.64) or FPD (r = -0.35, p = 0.31), BMI and the Shannon index (r = -0.21, p = 0.55) and FPD (r = -0.14, p = 0.68), the DHI results and the Shannon index (r = -0.42, p = 0.22) and FPD (r = -0.08, p = 0.81), and between the hearing test results and the Shannon index (r = -0.03, p = 0.94) and FPD (r = -0.28, p = 0.42).

124 **Detailed data on Meniere's patients**

125

No	Age	BMI	Sex	Disease period (year)	DHI	Hearing test (dB)	Shannon's index	FPD
1	54	22.31	Male	3	46	7.5	6.34	18.41
2	39	26.78	Male	13	56	26.3	5.34	11.17
3	53	26.37	Female	0.67	16	31.3	6.58	19.69
4	60	19.81	Female	6	20	58.8	6.25	14.48
5	54	35.11	Male	7	8	26.3	6.02	15.83
6	47	25.6	Male	5	20	13.8	5.9	13.55
7	36	23.38	Female	10	54	36.3	5.77	12.48
8	61	25.65	Female	6	86	22.5	5.14	14.45
9	46	20.28	Male	0.58	62	21.3	6.7	21.12
10	46	22.91	Male	6	38	35	5.3	12.88
Average	49.6	24.82		5.725	40.6	27.91	5.934	15.406

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128

129 The TAXON BAR showing the intestinal bacterial layer in each case is shown in Figs

130 4 and 5. The organisms identified by phylum, class, order, family, genus, and species are

shown in Fig 4 (the description by bacterial species is too voluminous to be included), and those identified by phylum, class, and order are shown in Fig 5. *Akkermansia muciniphila* was not detected in the gut microbiota of any of the patients.

Discussion

The use of gut microbiota testing to analyze various disease groups is a fairly recent approach. Recognition of the interrelationship between the brain and intestines via intestinal bacteria, which constitute the so-called microbiome-gut-brain axis, and the development of analytical scientific methods such as metagenomic analysis using next-generation sequencers and metabolome analysis have together promoted rapid accumulation of knowledge about the mechanisms underlying the microbiome-gut-brain axis.

In particular, the intestinal microbiota have been extensively investigated in patients with irritable bowel syndrome (IBS) [3], and dysbiosis has also been reported to be induced in the gut microbiota of depressed patients, showing a pattern similar to that of IBS [4]. Stress is one of the factors that induce Meniere's disease attacks. These disease groups show dysbiosis of the intestinal microbiota in comparison with healthy individuals [5]. In animal studies, when the gut microbiota of patients with depression was

149 transplanted into gut microbiota-depleted mice, the transplanted mice exhibited
150 characteristics of depression, both at the behavioral and physiological levels. Thus,
151 dysbiosis of the gut microbiota was considered as a factor in the pathogenesis of
152 depression [6]. In fact, multiple reports have described the effects of stress and depression
153 on Meniere's disease. Patients with refractory Meniere's disease are prone to depressive
154 symptoms, with 40% reported to show neurotic symptoms and 60% showing depressive
155 symptoms [7]. Adrenocorticotropic hormone and blood cortisol levels have been shown
156 to be elevated due to stress in patients with Meniere's disease, and the levels of oxytocin
157 and vasopressin, which are known as stress hormones, are also elevated in patients with
158 Meniere's disease [8,9].

159 This study showed that dysbiosis of the intestinal microbiota progresses as the duration
160 of Meniere's disease increases. As mentioned above, dysbiosis of the intestinal microbiota
161 may have progressed with increasing exposure to stress, the etiological factor, as the
162 duration of illness increased. In this study, patients with a history of psychiatric disorders
163 or complications were excluded. Thus, dysbiosis of the gut microbiota may be a latent
164 event even if it does not cause depression or neurological symptoms in patients with
165 Meniere's disease. The microbial diversity indices showed no correlation with DHI and
166 hearing test results. Meniere's disease is known to cause exacerbation and amelioration,

167 but there may have been no association between the degree of disability due to Meniere's
168 disease and dysbiosis of the gut microbiota. However, this study was conducted on a
169 limited number of cases, and future comparisons based on multiple examinations in the
170 same patients and statistical analyses with a larger number of cases are necessary in this
171 regard. We could not find any reports on the association between Meniere's disease and
172 intestinal microflora, or between Meniere's disease and IBS. This may be due to the fact
173 that doctors who treat Meniere's disease are typically otorhinolaryngologists and do not
174 often conduct abdominal examinations or interview patients regarding abdominal
175 conditions.

176 Dysbiosis of the intestinal microbiota has been verified to occur first or at the onset of
177 various diseases. For example, rheumatoid arthritis is triggered by an immune response
178 to specific intestinal bacteria [10]. In the present study, the longer the duration of
179 Meniere's disease, the stronger the dysbiosis, suggesting that a prolonged Meniere's
180 disease status may have caused dysbiosis. Future studies should aim to evaluate a larger
181 number of cases and investigate whether a specific intestinal bacterium is a trigger for
182 Meniere’s disease.

183 Notably, none of the patients with Meniere's disease had *Akkermansia muciniphila,* a
184 gram-negative enterobacterium belonging to the phylum *Verrucomicrobia* that has been

identified in the feces of healthy individuals. *Akkermansia muciniphila* is capable of growing on mucin covering the gastrointestinal mucosa as a single source of nutrition [11]. It is a commensal bacterium characterized by its degradative ability and accounts for 1%-4% of the human intestinal microflora [12].

It is noteworthy that aerobic exercise is commonly recommended as an initial lifestyle intervention for patients with Meniere's disease, and aerobic exercise has been suggested to induce an increase in *Akkermansia muciniphila,* which may be effective for early treatment for Meniere's disease. In addition, intestinal colonization of *Akkermansia muciniphila* may improve the symptoms of Meniere's disease.

In this study, the mean BMI of the patient group was 24.8, and although some obese patients were included in the study population, most patients had BMI within the standard range, so it is unlikely that obesity had any effect.

Only 10 patients were evaluated in this study, and future studies should aim to accumulate data from more cases. However, the fact that dysbiosis of the intestinal microbiota depended on the duration of Meniere's disease and that *Akkermansia muciniphila* was not detected in all cases suggests that the intestinal microbiota in patients with Meniere's disease is different from that in normal individuals. Future investigation of the intestinal microbiota in Meniere's disease may lead to elucidation of the

pathogenesis and development of new treatments. Further studies will be required to accumulate data from more cases.

Conclusions

Ten patients with a definite diagnosis of Meniere's disease and marked endolymphatic edema on HYDROPS underwent examinations of the intestinal microflora. A significant negative correlation was found between the duration of Meniere's disease and dysbiosis of intestinal microbiota. None of the patients harbored *Akkermansia muciniphila.* However, this study was limited by the small number of patients. Nevertheless, the relationship between Meniere's disease and the intestinal microbiota has not been reported previously, and these findings may help researchers determine the pathogenesis of Meniere's disease and propose new treatments for it in the future.

The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

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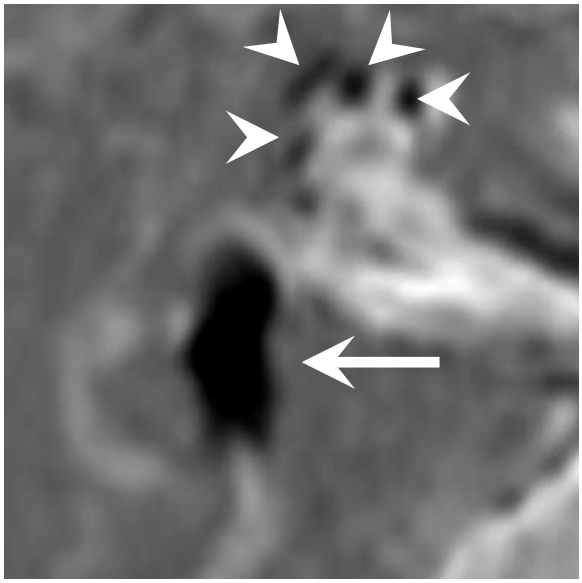
Definitive Meniere disease[*]	
A.	Two or more spontaneous episodes of vertigo, each lasting 20 minutes to 12 hours
B.	Audiometrically documented low to medium frequency sensorineural hearing loss in one ear, defining the affected ear on at least one occasion before, during, or after one of the episodes of vertigo
C.	Fluctuating aural symptoms (hearing, tinnitus, and fullness) in the affected ear
D.	Not better accounted for by another vestibular diagnosis
Probable Meniere disease	
A.	Two or more episodes of vertigo or dizziness, each lasting 20 minutes to 24 hours
B.	Fluctuating aural symptoms (hearing, tinnitus, or fullness) in the affected ear
C.	Not better accounted for by another vestibular diagnosis

[*]No serological or genetic marker is considered for the diagnosis of MD.

Fig 1. Diagnostic Criteria for Meniere's disease in 2015 as defined by the Rosaceae Society.



Short arrow is cochlea, Long arrow is vestibular

Fig 2. HYDROPS image in case 1. The short arrows indicate the cochlea, while the long arrow indicates the vestibule.

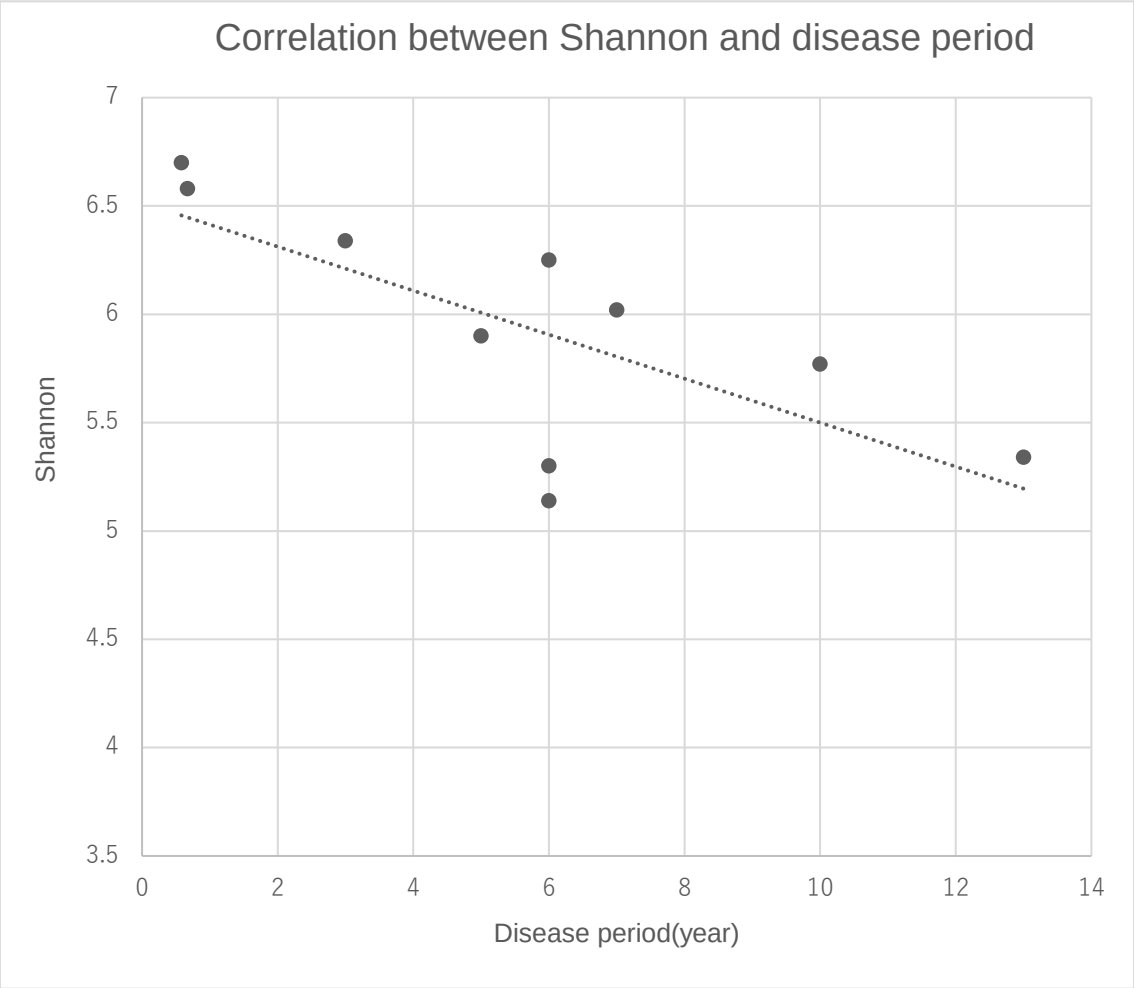


Fig 2.

r = -0.72, p = 0.02

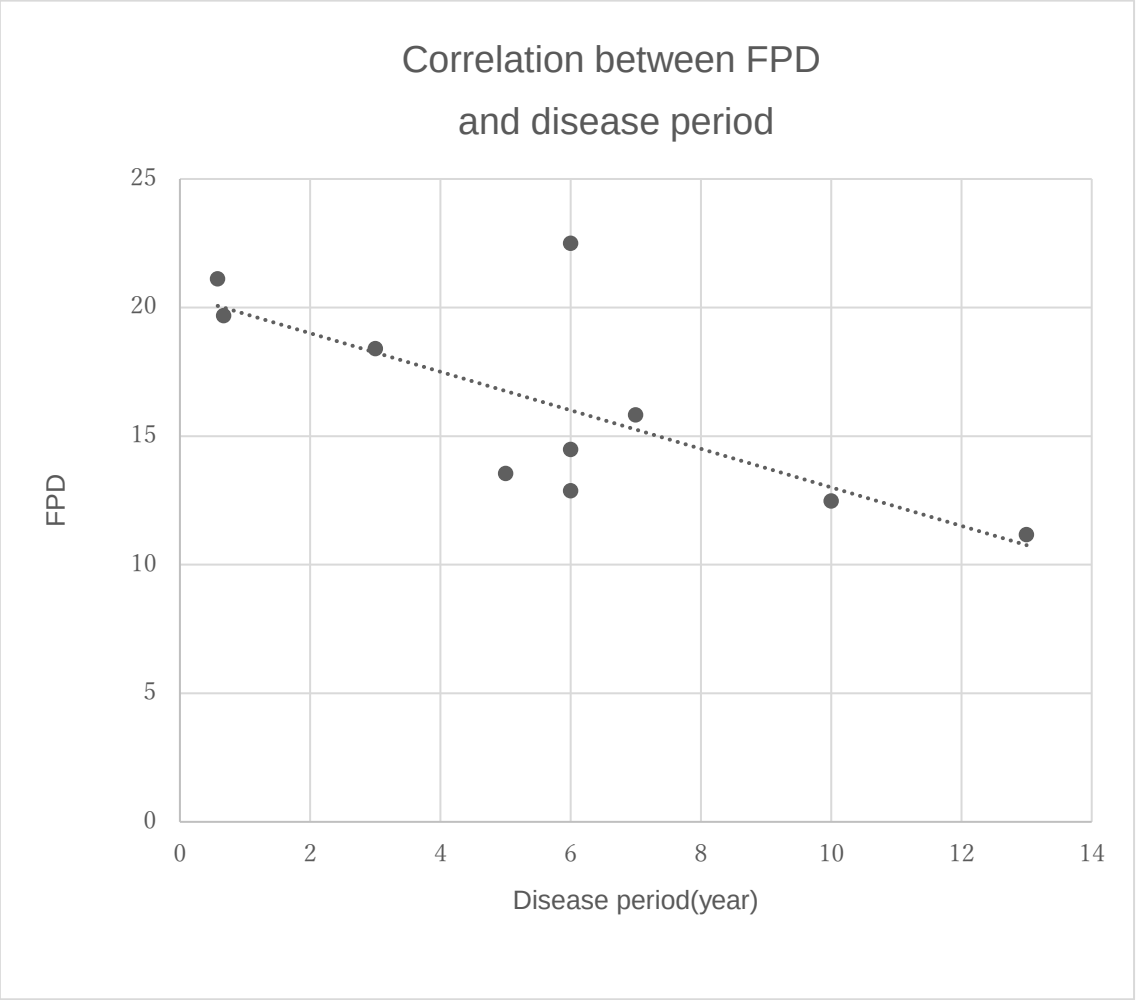


Fig 3.

r = -0.89, p = 0.0005

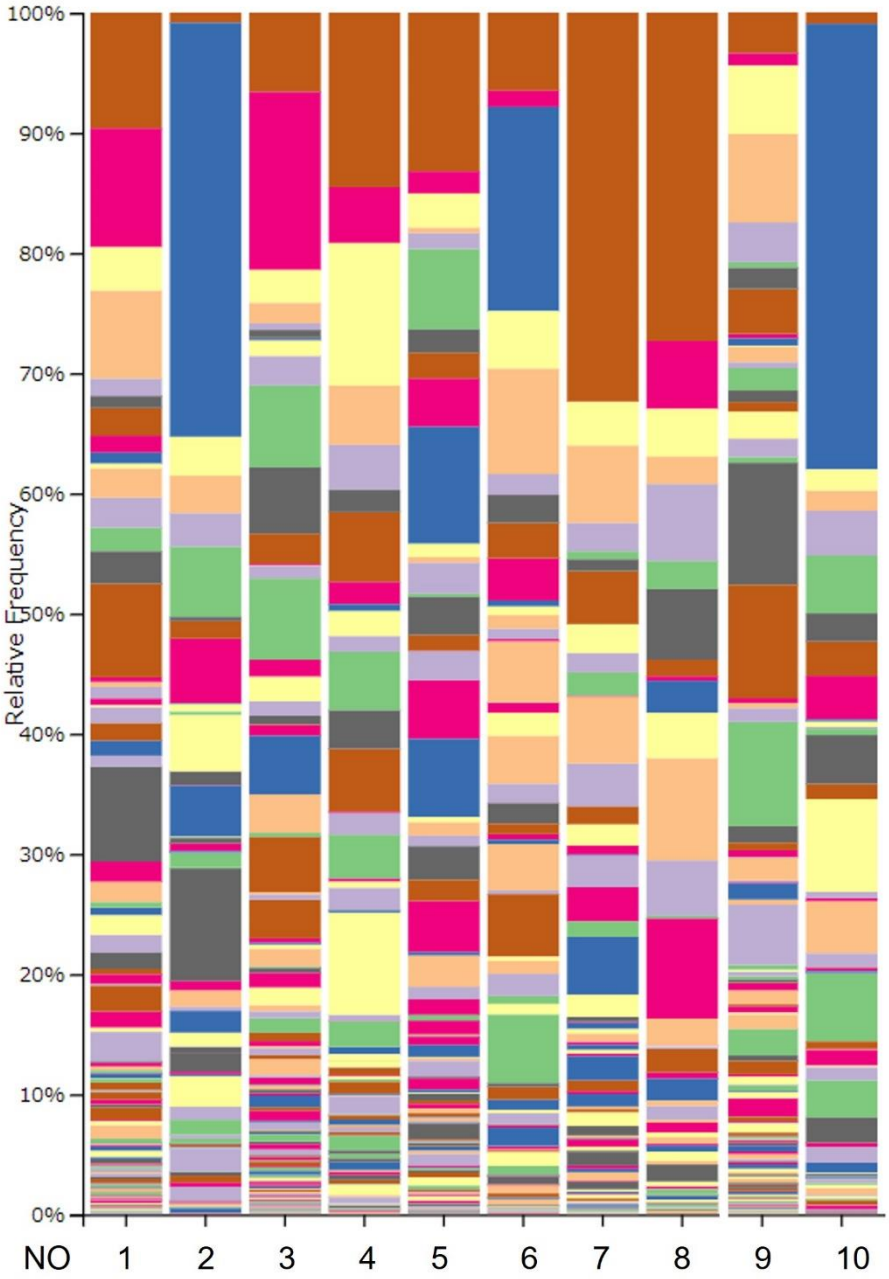


Fig 4. Identification by phylum, class, order, family, genus, and species.

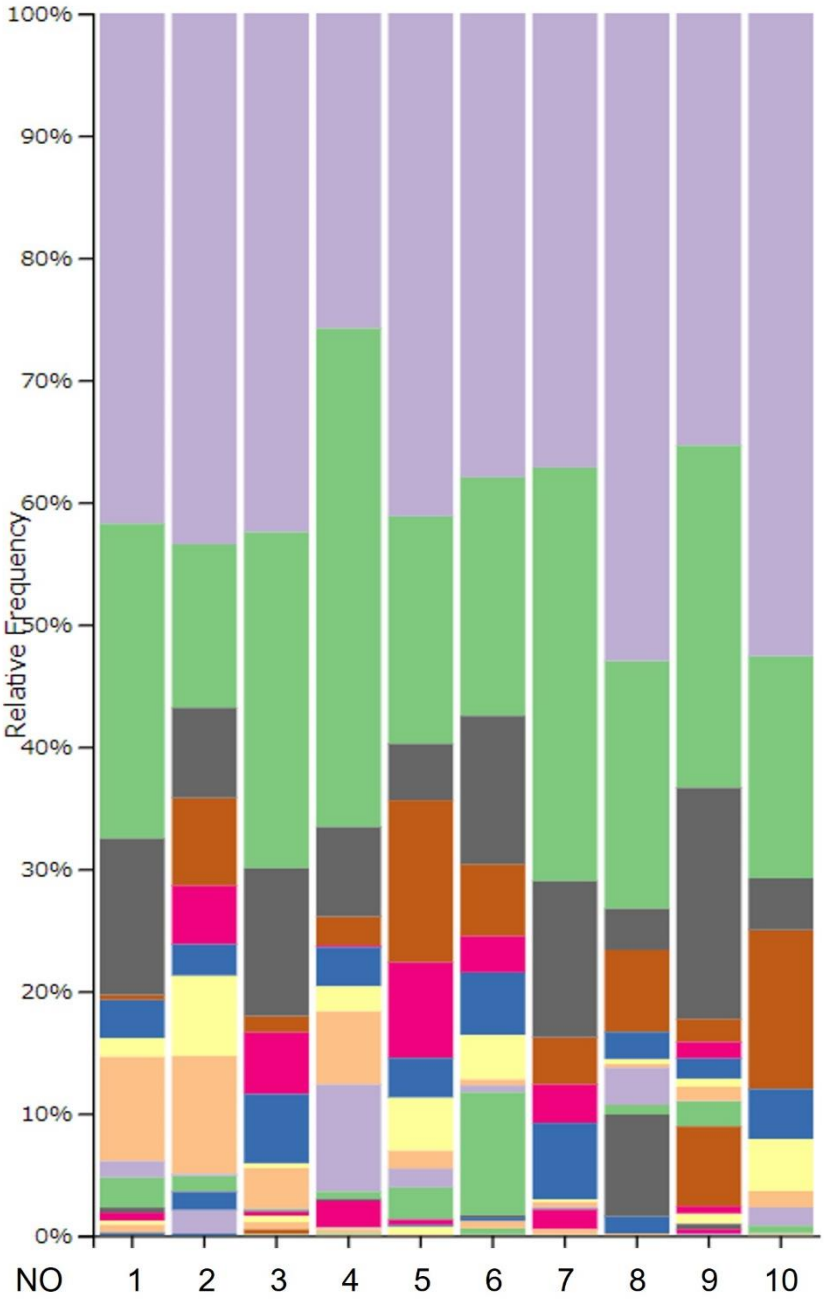


Fig 5. Identification by phylum, class, and order.

D_0__Bacteria;D_1__Bacteroidota;D_2__Bacteroidia;D_3__Bacteroidales
D_0__Bacteria;D_1__Firmicutes;D_2__Clostridia;D_3__Lachnospirales
D_0__Bacteria;D_1__Firmicutes;D_2__Clostridia;D_3__Oscillospirales
D_0__Bacteria;D_1__Actinobacteriota;D_2__Actinobacteria;D_3__Bifidobacteriales
D_0__Bacteria;D_1__Firmicutes;D_2__Negativicutes;D_3__Veillonellales-Selenomonadales
D_0__Bacteria;D_1__Proteobacteria;D_2__Gammaproteobacteria;D_3__Burkholderiales
D_0__Bacteria;D_1__Actinobacteriota;D_2__Coriobacteriia;D_3__Coriobacteriales
D_0__Bacteria;D_1__Firmicutes;D_2__Bacilli;D_3__Erysipelotrichales
D_0__Bacteria;D_1__Firmicutes;D_2__Bacilli;D_3__Lactobacillales
D_0__Bacteria;D_1__Firmicutes;D_2__Negativicutes;D_3__Acidaminococcales
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D_0__Bacteria;D_1__Desulfobacterota;D_2__Desulfovibrionia;D_3__Desulfovibrionales
D_0__Bacteria;D_1__Firmicutes;D_2__Clostridia;D_3__Peptostreptococcales-Tissierellales
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D_0__Bacteria;D_1__Firmicutes;D_2__Clostridia;D_3__Eubacteriales
D_0__Bacteria;D_1__Proteobacteria;D_2__Gammaproteobacteria;D_3__Pasteurellales
D_0__Bacteria;D_1__Firmicutes;D_2__Clostridia;D_3__Clostridia UCG-014
D_0__Bacteria;D_1__Firmicutes;D_2__Clostridia;D_3__Christensenellales
D_0__Bacteria;D_1__Actinobacteriota;D_2__Actinobacteria;D_3__Actinomycetales
D_0__Bacteria;D_1__Patescibacteria;D_2__Saccharimonadia;D_3__Saccharimonadales
D_0__Bacteria;D_1__Firmicutes;D_2__Clostridia;D_3__Peptococcales
D_0__Bacteria;D_1__Firmicutes;D_2__Bacilli;D_3__Staphylococcales
D_0__Bacteria;D_1__Cyanobacteria;D_2__Cyanobacteriia;D_3__Chloroplast
D_0__Bacteria;D_1__Campilobacterota;D_2__Campylobacteria;D_3__Campylobacterales
D_0__Bacteria;D_1__Bacteroidota;D_2__Bacteroidia;D_3__Flavobacteriales
D_0__Bacteria;D_1__Actinobacteriota;D_2__Actinobacteria;D_3__Micrococcales

Definitive Meniere disease[*]	
A.	Two or more spontaneous episodes of vertigo, each lasting 20 minutes to 12 hours
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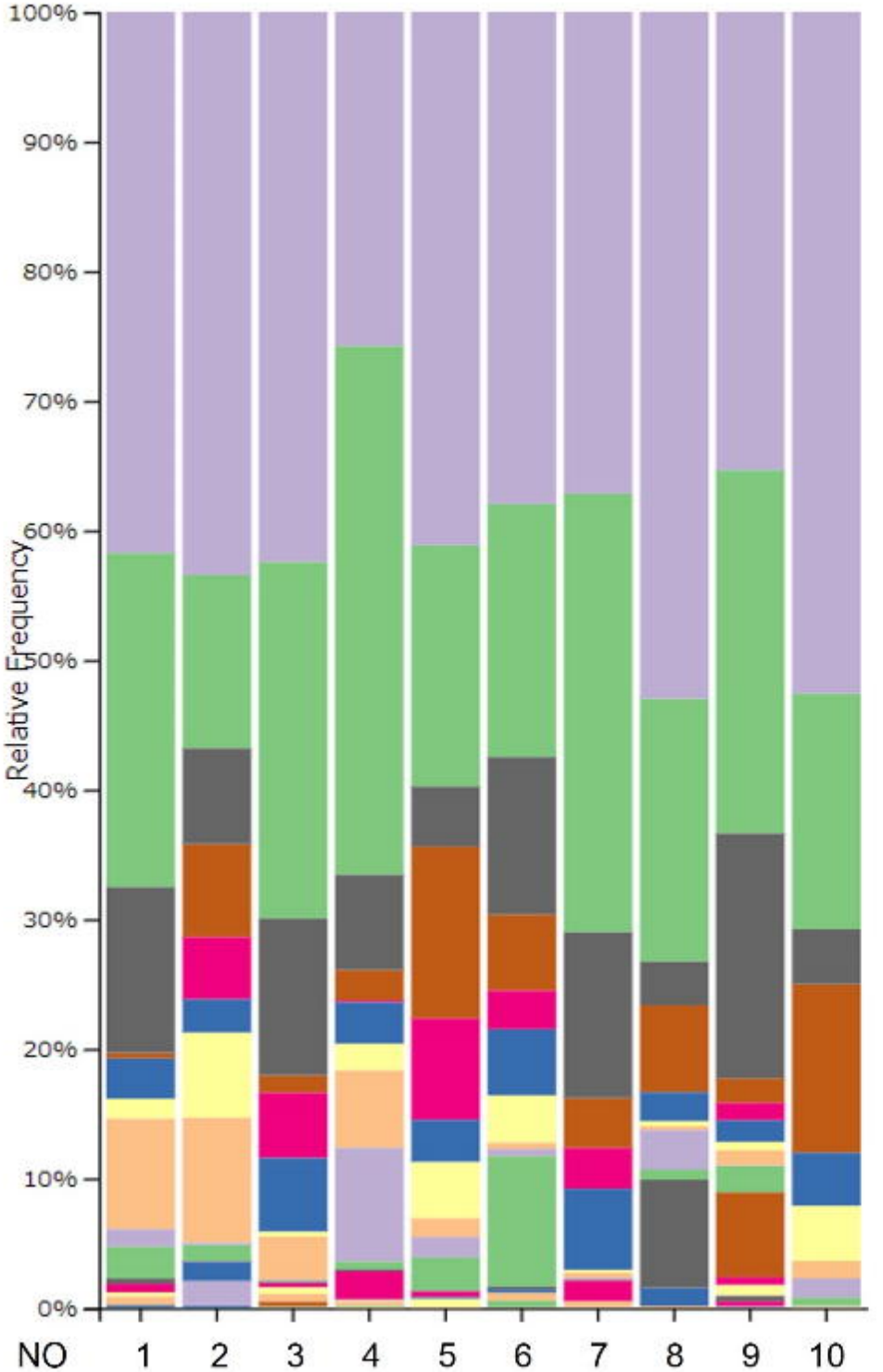
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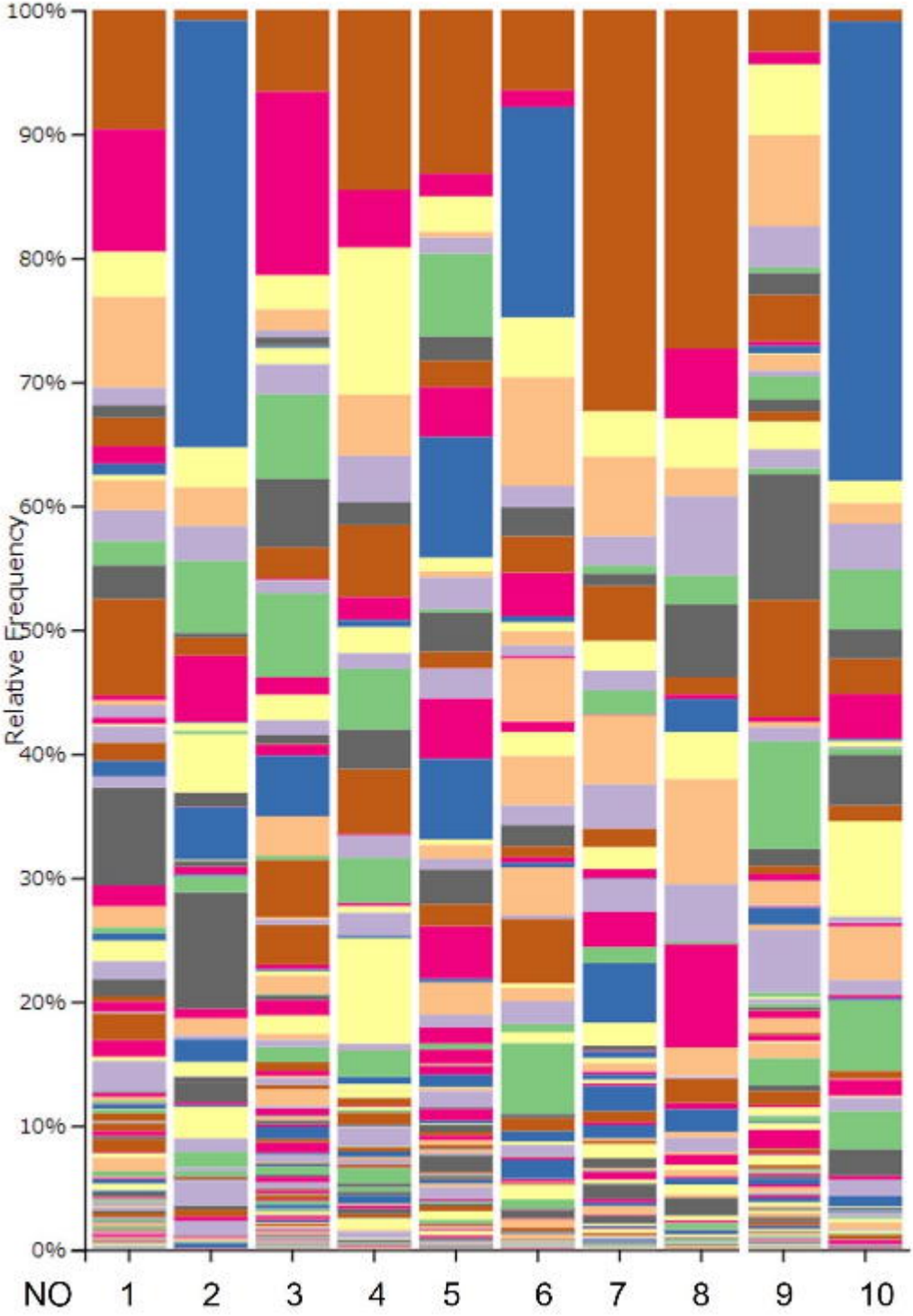
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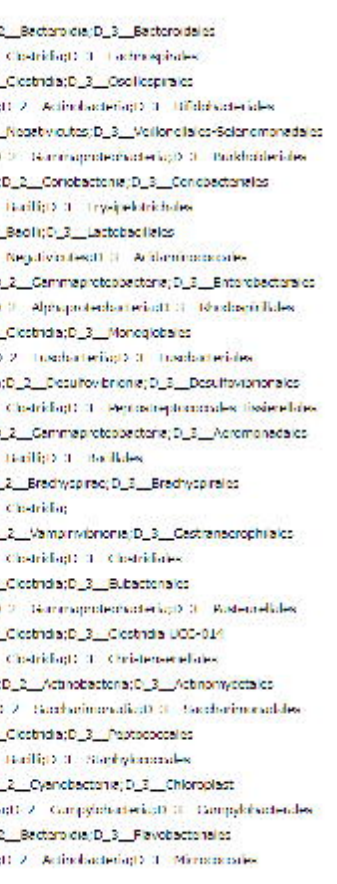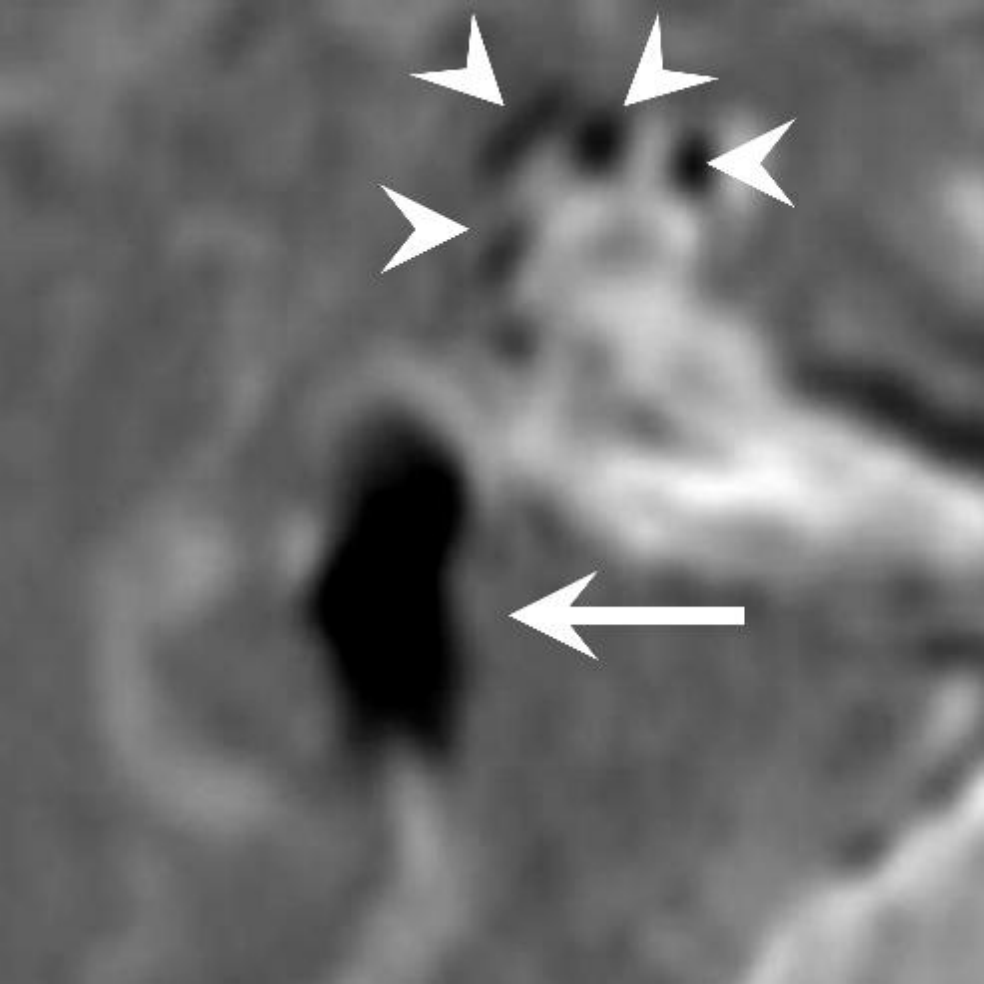
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D_0__Bacteria;D_1__Firmicutes;D_2__Clostridia;D_3__Oscillospirales
D_0__Bacteria;D_1__Firmicutes;D_2__Clostridia;D_3__Oscillospiraceae
D_0__Bacteria;D_1__Actinobacteriota;D_2__Coriobacteriia;D_3__Coriobacteriales
D_0__Bacteria;D_1__Firmicutes;D_2__Negativicutes;D_3__Veillonellales-Selenomonadales
D_0__Bacteria;D_1__Proteobacteria;D_2__Gammaproteobacteria;D_3__Burkholderiales
D_0__Bacteria;D_1__Actinobacteriota;D_2__Coriobacteriia;D_3__Eggerthellales
D_0__Bacteria;D_1__Firmicutes;D_2__Bacilli;D_3__Lactobacillales
D_0__Bacteria;D_1__Firmicutes;D_2__Negativicutes;D_3__Acidaminococcales
D_0__Bacteria;D_1__Proteobacteria;D_2__Gammaproteobacteria;D_3__Enterobacterales
D_0__Bacteria;D_1__Proteobacteria;D_2__Alphaproteobacteria;D_3__Rhodospirillales
D_0__Bacteria;D_1__Firmicutes;D_2__Clostridia;D_3__Monoglobales
D_0__Bacteria;D_1__Desulfobacterota;D_2__Desulfovibrionia;D_3__Desulfovibrionales
D_0__Bacteria;D_1__Proteobacteria;D_2__Gammaproteobacteria;D_3__Aeromonadales
D_0__Bacteria;D_1__Firmicutes;D_2__Bacilli;D_3__Bacillales
D_0__Bacteria;D_1__Spirochaetota;D_2__Brachyspirae;D_3__Brachyspirales
D_0__Bacteria;D_1__Firmicutes;D_2__Clostridia;
D_0__Bacteria;D_1__Cyanobacteria;D_2__Vampirovibrionia;D_3__Gastranaerophilales
D_0__Bacteria;D_1__Firmicutes;D_2__Clostridia;D_3__Christensenellales
D_0__Bacteria;D_1__Firmicutes;D_2__Clostridia;D_3__Butyricicoccales
D_0__Bacteria;D_1__Proteobacteria;D_2__Gammaproteobacteria;D_3__Pasteurellales
D_0__Bacteria;D_1__Firmicutes;D_2__Clostridia;D_3__Clostridia UCG-014
D_0__Bacteria;D_1__Firmicutes;D_2__Clostridia;D_3__Christensenellales
D_0__Bacteria;D_1__Actinobacteriota;D_2__Actinobacteria;D_3__Actinomycetales
D_0__Bacteria;D_1__Proteobacteria;D_2__Gammaproteobacteria;D_3__Cardiobacteriales
D_0__Bacteria;D_1__Firmicutes;D_2__Clostridia;D_3__Peptococcales
D_0__Bacteria;D_1__Firmicutes;D_2__Bacilli;D_3__Staphylococcales
D_0__Bacteria;D_1__Cyanobacteria;D_2__Cyanobacteria;D_3__Chloroplast
D_0__Bacteria;D_1__Proteobacteria;D_2__Gammaproteobacteria;D_3__Campylobacterales
D_0__Bacteria;D_1__Bacteroidota;D_2__Bacteroidia;D_3__Flavobacteriales
D_0__Bacteria;D_1__Actinobacteriota;D_2__Actinobacteria;D_3__Micrococcales