

1 **Comparison of the human gastric microbiota in hypochlorhydric states arising**
2 **as a result of *Helicobacter pylori*-induced atrophic gastritis, autoimmune**
3 **atrophic gastritis and proton pump inhibitor use**

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28 **ABSTRACT**

29 **Objective:** Several conditions associated with reduced gastric acid secretion confer
30 an altered risk of developing a gastric malignancy. *Helicobacter pylori*-induced
31 atrophic gastritis predisposes to gastric adenocarcinoma, autoimmune atrophic
32 gastritis is a precursor of type I gastric neuroendocrine tumours, whereas proton
33 pump inhibitor (PPI) use does not affect stomach cancer risk. We hypothesised that
34 each of these conditions was associated with specific alterations in the gastric
35 microbiota and that this influenced subsequent tumour risk.

36 **Design:** 95 patients (in groups representing normal stomach, PPI treated, *H. pylori*
37 gastritis, *H. pylori*-induced atrophic gastritis and autoimmune atrophic gastritis) were
38 selected from a cohort of 1400. RNA extracted from gastric corpus biopsies was
39 analysed using 16S rRNA sequencing (MiSeq).

40 **Results:** Samples from normal stomachs and patients treated with PPIs
41 demonstrated similarly high microbial diversity. Patients with autoimmune atrophic
42 gastritis also exhibited relatively high microbial diversity, but with samples dominated
43 by *Streptococcus*. *H. pylori* colonisation was associated with decreased microbial
44 diversity and reduced complexity of co-occurrence networks. *H. pylori*-induced
45 atrophic gastritis resulted in lower bacterial abundances and diversity, whereas
46 autoimmune atrophic gastritis resulted in greater bacterial abundance and equally
47 high diversity compared to normal stomachs. Pathway analysis suggested that
48 glucose-6-phosphate-1-dehydrogenase and D-lactate dehydrogenase were over
49 represented in *H. pylori*-induced atrophic gastritis versus autoimmune atrophic
50 gastritis, and that both these groups showed increases in fumarate reductase.

51 **Conclusion:** Autoimmune and *H. pylori*-induced atrophic gastritis were associated
52 with different gastric microbial profiles. PPI treated patients showed relatively few
53 alterations in the gastric microbiota compared to healthy subjects.

54

55 **SIGNIFICANCE OF THIS STUDY**

56 **1. What is already known about this subject?**

- 57 • Some conditions which result in reduced gastric acid secretion and
58 hypochlorhydria are associated with an increased risk of gastric tumourigenesis.
- 59 • This risk is different in patients with *H. pylori*-induced atrophic gastritis,
60 autoimmune atrophic gastritis and chronic proton pump inhibitor use.
- 61 • Hypochlorhydria and *H. pylori* infection cause alterations in the composition of
62 the gastric microbiota.

63 **2. What are the new findings?**

- 64 • We used 16S rRNA sequencing to characterise the microbiota in gastric corpus
65 biopsies from a well characterised cohort of patients.
- 66 • The gastric microbiota was different in patients who were hypochlorhydric as a
67 result of *H. pylori*-induced atrophic gastritis, autoimmune atrophic gastritis and
68 proton pump inhibitor use.
- 69 • Biochemical pathways associated with gastric carcinogenesis such as the
70 fumarate reductase pathway were predicted to be altered in patients with
71 atrophic gastritis.

72 **3. How might it impact on clinical practice in the foreseeable future?**

- 73 • Understanding how the microbiota that colonise the hypochlorhydric stomach
74 influence gastric carcinogenesis may ultimately permit stratification of patients'
75 subsequent tumour risk.
- 76 • Interventions that alter the composition of the gastric microbiome in
77 hypochlorhydric patients with atrophic gastritis should be tested to investigate
78 whether they alter the subsequent risk of developing gastric malignancy.

79 **INTRODUCTION**

80 Gastric adenocarcinoma is the third most common cause of cancer related mortality
81 worldwide[1] and most cases are associated with chronic *Helicobacter pylori*
82 infection. Gastric cancer usually develops via the premalignant condition of gastric
83 atrophy, which is associated with the loss of acid-secreting parietal cells[2]. The
84 resulting hypochlorhydria potentially leads to alterations in the composition of the
85 gastric microbiota by providing a more favourable environment for colonisation. It is
86 currently unclear to what extent the non-*H. pylori* gastric microbiota contributes
87 towards gastric carcinogenesis. Although the hypochlorhydria associated with
88 autoimmune atrophic gastritis also increases the risk of developing gastric
89 adenocarcinoma[3], it is more frequently associated with the development of another
90 tumour, the type I gastric neuroendocrine tumour (NET)[4]. However,
91 hypochlorhydria does not always increase the risk of gastric tumour development, as
92 observed following chronic proton pump inhibitor (PPI) use[5]. Therefore, factors in
93 addition to hypochlorhydria affect gastric cancer risk and one of these could be the
94 gastric microbiota.

95 Although originally thought to be sterile, several bacterial communities have been
96 shown to survive in the normal human stomach[6]. Differences have also been
97 observed depending upon *H. pylori* status[6]. There is now overwhelming evidence
98 that certain bacteria influence cancer development. Potential mechanisms include
99 altering the host immune system, exacerbating inflammation, or converting dietary
100 nitrates to produce carcinogens such as N-nitrosamines and nitric oxide[7, 8, 9, 10,
101 11, 12, 13].

102 We therefore hypothesised that three stimuli which result in hypochlorhydria, namely
103 *H. pylori*-induced atrophic gastritis, autoimmune atrophic gastritis and proton pump

104 inhibitor use cause specific changes to the composition of the gastric microbiota. In
105 addition, the gastric microbiota that is present in these conditions contributes
106 towards the specific gastric tumour risk that is associated with each of these
107 hypochlorhydric states. We have used 16S rRNA sequencing to determine the
108 gastric mucosal microbiota profiles in patients with these causes of hypochlorhydria
109 and have compared these with samples obtained from healthy subjects and from
110 patients with *H. pylori*-induced gastritis, but no evidence of gastric atrophy.

111 **METHODS**

112 **Ethics**

113 Acquisition of the biopsies used in this study was approved by Liverpool
 114 (08/H1005/37) and Cambridge East (10/H0304/51) Research Ethics Committees as
 115 previously described[14, 15]. All patients gave written informed consent.

116 **Patients**

117 One hundred gastric biopsy samples in 5 different groups were selected from a
 118 cohort of 1400 prospectively recruited patients who underwent diagnostic upper
 119 gastrointestinal endoscopy at Royal Liverpool University Hospital[14] and from 8
 120 patients with type I gastric NETs who had been recruited to a clinical trial[15, 16]
 121 (Table 1).

122 **Table 1.** Summary of patient group characteristics

| | Group | Total no. of samples | Number of females | Age (years) | | BMI | | <i>H. pylori</i> serology +ve | <i>H. pylori</i> histology /urease +ve | PPI use | Atrophic gastritis | Serum gastrin (pM) | | Anti GPC/IF antibody |
|---|----------------------------|----------------------|-------------------|-------------|-----------|--------|-----------|-------------------------------|--|---------|--------------------|--------------------|-----------|----------------------|
| | | | | Median | IQR | Median | IQR | | | | | Median | IQR | |
| 1 | Normal | 20 | 13 | 46 | 30.5-58.7 | 24.5 | 21.2-26.8 | 0 | 0 | - | - | 22.5 | 17.5-28.5 | - or ND |
| 2 | PPI treated | 19 | 13 | 60 | 46-67 | 28.5 | 26.1-33.2 | 0 | 0 | + | - | 140 | 94-200 | - or ND |
| 3 | <i>H. pylori</i> gastritis | 22 | 11 | 57.5 | 47.7-64 | 27.4 | 23.3-27.1 | 22 | 22 | - | - | 21.5 | 17.4-28 | - or ND |
| 4 | <i>H. pylori</i> atrophy | 23 | 15 | 65 | 55-72 | 25.9 | 24.6-31.9 | 23 | 6 | - | + | 100 | 64-260 | - or ND |
| 5 | Autoimmune atrophy | 11 | 6 | 67 | 60-76 | 28.6 | 23.7-35 | 2 | 0 | - | + | 800 | 470-1050 | + |

123

124 ND= Not done

125 IQR= Interquartile range, 25% and 75%

126

127

128 Patients in the normal stomach group had a normal endoscopy, no evidence of *H.*
129 *pylori* infection by histology, rapid urease test or serology, were not taking a PPI and
130 were normogastrinaemic. Patients belonging to the *H. pylori* gastritis group were
131 positive for *H. pylori* by rapid urease test, histology and serology, had no histological
132 evidence of atrophic gastritis, were not taking a PPI and were normogastrinaemic.
133 Patients in the *H. pylori*-induced atrophic gastritis group showed histological
134 evidence of corpus atrophic gastritis and/or intestinal metaplasia, had no dysplasia
135 or cancer, were positive for *H. pylori* by serology, were not taking a PPI and were
136 hypergastrinaemic. Six out of the 23 patients in this group were also *H. pylori*
137 positive by urease test and/or histology. Patients in the autoimmune atrophic gastritis
138 group had histological evidence of atrophic gastritis, no evidence of *H. pylori*
139 infection by rapid urease test or histology, positive anti-gastric parietal cell and/or
140 intrinsic factor antibodies, were markedly hypergastrinaemic and 8 out of 11 also had
141 grade 1 type I gastric NETs. Patients in the PPI-treated group were currently taking
142 PPIs, had no evidence of *H. pylori* infection by serology, rapid urease test or
143 histology, had no histological evidence of atrophic gastritis and were
144 hypergastrinaemic (suggesting significant hypochlorhydria).

145 **Samples**

146 At least two biopsies per site were obtained from the gastric antrum and corpus for
147 histopathology. Eight additional corpus biopsies were stored in RNA later
148 immediately after removal and were extracted using a modified Tri- reagent
149 protocol[17]. Briefly, samples were thawed and separated from RNA later, before
150 being homogenised in Tri-Reagent® (Sigma-Aldrich, Gillingham, UK). Chloroform
151 was added and the resulting clear aqueous layer was combined with isopropanol
152 before centrifugation to produce a precipitated RNA pellet. This was washed with

153 75% and 100% ice cold ethanol before being allowed to dry and then resuspended in
154 diethylpyrocarbonate (DEPC)-treated water (Sigma-Aldrich, Gillingham, UK). RNA
155 was stored in ethanol at -80°C. Ethanol was removed and pellets were resuspended
156 in DEPC-water prior to reverse transcription.

157 **Gastrin assays**

158 Serum gastrin concentrations were measured by radioimmunoassay (RIA) as
159 previously described[18, 19]. Fasting serum gastrin concentrations were all <40pM in
160 normogastrinaemic subjects and >40pM (with the majority >100pM) in
161 hypergastrinaemic subjects.

162 **Reverse Transcription**

163 Samples and random primers were denatured together for 5 minutes at 65°C before
164 Proto reaction mix and Proto enzyme from a ProtoScript® II First Strand cDNA
165 Synthesis kit (NEB, E6560L) were added. Samples were then incubated at 25°C for
166 5 minutes, 42°C for 20 minutes, and 80°C for 5 minutes. Newly synthesised cDNA
167 was then measured using a Qubit high sensitivity assay (ThermoFisher Ltd, Paisley,
168 UK).

169 **16S rRNA Sequencing**

170 The 16S rRNA gene was targeted using V1-V2 (27F and 388R) primers[20] with
171 slight modifications: forward primer 5'ACACTCTTTCCCTACACGACGC
172 TCTTCCGATCTNNNNNAGAGTTTGATCMTGGCTCAG'3, reverse primer
173 5'GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCTGCTGCCTCCCGTAGGAGT'
174 3. Primers were validated using a mock community described in supplementary
175 methods. The following cycling conditions were used: initial denaturation 94°C for 5

176 minutes, followed by 10 cycles of denaturation at 98°C for 20 seconds, annealing at
177 60°C for 15 seconds, and elongation at 72°C for 15 seconds, followed by a final
178 elongation step of 72°C for 1 minute. PCR amplicons were purified to remove excess
179 primers, nucleotides, salts, and enzymes using the Agencourt® AMPure® XP
180 system (Beckman Coulter Ltd, High Wycombe, UK). Purified amplicons were used in
181 a second PCR reaction with the same conditions except with 20 cycles. This second
182 step was used to add dual index barcodes. The PCR amplicons were purified as
183 above. All PCR reactions used Kapa HiFi HotStartStart 2x master mix (Anachem
184 Ltd, Bedfordshire, UK) and all primers were used at 10µM. Amplicon sizes were
185 checked using a fragment analyser (Advanced Analytical, Ankeny, USA) and size
186 selection was performed using a Pippin prep (Sage Science, Beverly, USA). The
187 quantity and quality of the samples in the final libraries were checked using a SYBR
188 Green qPCR assay and the Illumina Library Quantification kit (Kapa) on a Roche
189 Light Cycler LC480II, according to the manufacturer's instructions.

190 Prior to loading samples onto the MiSeq, PhiX was added (10-15%) to increase
191 diversity, and samples were then denatured with NaOH according to the Illumina
192 MiSeq protocol. ssDNA library fragments were diluted to a final concentration of
193 8pM. 600µl of ssDNA library was loaded into a MiSeq Reagent Cartridge and a 500–
194 cycle PE kit v2 was used. Paired-end sequencing was performed according to the
195 manufacturer's instructions (Illumina, SanDiego, CA, USA). Sequence analysis
196 methodology is described in the supplementary methods. Reads were submitted to
197 EBI short-read archive accession-PRJEB21104.

198 **Statistical analysis**

199 Details are described in the supplementary methods.

200 **RESULTS**

201 **Patient characteristics**

202 Patients were selected from the larger cohort according to criteria defined above.

203 Characteristics of the selected patients are shown in table 1 and figure 1A. One

204 sample from the normal stomach group and four from the PPI-treated group were

205 subsequently excluded because sequencing showed the presence of >15% *H. pylori*

206 despite this organism being undetected by conventional clinical tests (most likely due

207 to the higher sensitivity of 16S rRNA sequencing compared to routine clinical tests).

208 Ninety-five samples were therefore analysed. Negative extracts from the RNA

209 extraction procedures, a water sample in the first PCR and a mock bacterial

210 community were also sequenced.

211

212 **Detection of Operational Taxonomic Units (OTUs)**

213 In total 10,386 OTUs were identified. Extraction controls contained fewer OTUs than

214 the patient samples, whilst the mock communities (and a random selection of 10

215 gastric samples – data not shown) showed consistency between MiSeq runs (Figs 1-

216 3). Despite the negative extracts being theoretically sterile, as expected they

217 generated 16S signals due to known background reagent contamination[21].

218 Samples from the autoimmune atrophic gastritis group contained the largest number

219 of OTUs, whilst all other patient groups were comparable (Fig 1B). Mock

220 communities demonstrated the expected bacterial ratios (Fig 3B).

221

222

223 **Bacterial diversity and abundance in the different hypochlorhydric states**

224 Twenty-three known phyla were identified, mainly Proteobacteria, Firmicutes,
225 Bacteroidetes, Actinobacteria, Fusobacteria and Cyanobacteria. Bacteroidetes,
226 followed by Proteobacteria and Firmicutes were most common in normal stomachs,
227 whereas samples from PPI-treated patients contained slightly more Firmicutes and
228 fewer Bacteroidetes. The *H. pylori* gastritis and *H. pylori* atrophic gastritis samples
229 were dominated by Proteobacteria (as *Helicobacter* itself is a member of this
230 phylum), whilst biopsies from patients with autoimmune atrophic gastritis contained
231 the largest proportion of Firmicutes compared to all other patient groups.

232 *Alpha diversity*

233 Diversity indices demonstrated that the microbiota in normal stomachs was
234 significantly more diverse than in the stomachs of all other patient groups except for
235 the patients who had autoimmune atrophic gastritis (Fig 2). Evaluation of evenness
236 (by Pielou's evenness and Simpson) suggested that the samples from normal
237 stomachs and from the stomachs of patients taking PPIs contained bacterial
238 communities that were more equal in abundance than those in the other patient
239 groups, which were more skewed (Fig 2). Calculations based on richness indicated
240 that the samples from normal stomachs also contained the greatest number of
241 different bacterial species compared to all other groups, whilst the two *H. pylori*
242 infected groups (*H. pylori*-induced gastritis and atrophy) contained significantly fewer
243 species.

244

245

246 *Beta diversity*

247 When beta diversity was explored using nonmetric distance scaling (NMDS), patient
248 groups clustered predominantly by bacterial abundance (Fig 4). When *H. pylori* was
249 removed from the analysis however, *H. pylori* gastritis patients no longer clustered
250 separately by abundance from subjects with normal stomachs (Fig 4). Despite this,
251 following removal of *H. pylori*, there was a significant difference in abundance for *H.*
252 *pylori*-induced atrophic gastritis patients compared to normal stomachs, suggesting
253 strongly that there are differences in the proportions of non-*H. pylori* bacteria in these
254 subjects compared with others (Fig 4). Samples from patients who had autoimmune
255 atrophic gastritis displayed the only significant differences in terms of the presence
256 or absence of specific bacteria compared to other groups (Fig 4). This suggests that
257 changes in the gastric bacterial community during hypochlorhydria usually involve
258 changes in the relative proportions of bacteria that are already present, and only
259 rarely involve the loss or gain of specific bacterial genera.

260

261 **Comparisons between the microbiota profiles in the different patient groups**
262 **and healthy controls**

263 *Normal stomach versus PPI treated patients*

264 Patients receiving PPIs showed similar bacterial profiles to those found in the
265 stomachs of normal subjects, despite having significantly higher serum gastrin
266 concentrations (suggesting the presence of hypochlorhydria) (Figs 1A, 3 & S1).
267 Nonetheless there were differences in the ranks of most abundant bacterial families.
268 In normal (control) patients Prevotellaceae were the most abundant bacterial family

269 (23%), followed by Streptococcaceae (10%), Paraprevotellaceae (7%) and
270 Fusobacteriaceae (5%); amongst PPI-treated individuals Streptococcaceae (17%)
271 outranked Prevotellaceae (11%), Campylobacteraceae (5%) and Leptotrichiaceae
272 (4%; Fig 3A). The only significant differences at genus level between these groups
273 were decreases in *Actinobacillus* and *Tannerella* in the PPI-treated stomachs (Table
274 S1).

275 Very few differences were identified in co-occurrence network analyses when the
276 microbiota in normal stomachs was compared to PPI-treated stomachs. The only
277 observed difference was a negative correlation between *Helicobacter* and
278 *Acinetobacter* in the PPI-treated samples, whereas this relationship was positively
279 correlated in normal stomach biopsies (Figs 5A, S2A & Table S2A). Predicted
280 pathway analysis showed no significantly different biochemical pathways between
281 these two groups (Table S3).

282

283 *Normal stomach versus H. pylori-induced gastritis*

284 Unsurprisingly, the microbiota in the stomachs of patients who had *H. pylori*-induced
285 gastritis consisted almost entirely of Helicobacteraceae (97%) (Fig 3). When
286 compared to normal patients, *H. pylori*-induced gastritis patients showed a greater
287 number of differences at the genus level than all other patient groups (Table S1).

288 The majority of these differences resulted from reductions in the proportions of
289 several bacterial genera within the *H. pylori* gastritis group. To ensure that the
290 dominance of *H. pylori* did not skew the proportions of the other bacteria in a
291 misrepresentational way, *H. pylori* OTUs were removed from the abundance table
292 followed by differential expression analysis on the remaining raw abundances. This

293 analysis resulted in almost identical results to when *H. pylori* remained (Table S1).
294 Due to the dominance of *H. pylori* in these patients, very few co-occurrence networks
295 were identified, but positive correlations were observed between *Kocuria* and
296 *Skermanella* in both groups (Figs 5A & B). Predicted pathway analysis suggested a
297 reduction in several dehydrogenases in the stomachs of patients who had *H. pylori*
298 gastritis (Table S3).

299

300 *Normal stomach versus H. pylori-induced atrophic gastritis*

301 The stomachs of patients who had *H. pylori*-induced atrophic gastritis were also
302 dominated by Helicobacteraceae (62%), followed by Streptococcaceae (5%),
303 Fusobacteriaceae (2%) and Prevotellaceae (2%) (Fig 3). At the genus level, several
304 differences were observed between normal stomachs and the stomachs of patients
305 with *H. pylori*-induced atrophic gastritis. These included decreases in the proportions
306 of *Tannerella* (*E. coli*/*Shigella*/*Salmonella*), *Treponema*, and *Prevotella* in the *H.*
307 *pylori*-induced atrophic gastritis group. The vast majority of these differences
308 remained when *H. pylori* was removed from the analysis (Table S1). Prevotellaceae
309 were generally lower in all patient groups compared to normal stomachs (Figs S1
310 and 3). As with the *H. pylori* gastritis group, the majority of these changes reflected
311 decreases in the proportions of various bacterial genera within the *H. pylori*-induced
312 atrophic gastritis group, with the only increase being in *Helicobacter* itself.

313 Co-occurrence networks were more complicated in *H. pylori*-induced atrophic
314 gastritis patients compared to those subjects who had *H. pylori*-induced gastritis
315 (Figs 5B & D). Clear negative relationships were observed between *Helicobacter* and
316 genera such as *Streptococcus*, whilst *Campylobacter*, *Prevotella*, *Haemophilus* and

317 *Veillonella* were amongst the most well-connected and influential bacteria observed
318 in the stomachs of *H. pylori* atrophic gastritis patients (Fig 5 and Table S2B).
319 Predicted pathway analysis showed that several pathways were under-represented
320 in the *H. pylori*-induced atrophic gastritis group, including succinate dehydrogenase
321 (Table S3). Over-represented pathways included fumarate reductase (Table S3).

322

323 *Normal stomach versus autoimmune atrophic gastritis*

324 Streptococcaceae (38%) were the most dominant group identified in the stomachs of
325 patients who had autoimmune atrophic gastritis, followed by Prevotellaceae (9%),
326 Flavobacteriaceae (7%), Campylobacteraceae (7%), Enterobacteriaceae (5%) and
327 Pasteurellaceae (5%). The stomachs of autoimmune atrophic gastritis patients
328 contained a higher proportion of Streptococcaceae than all other patient groups (Fig
329 3) and were the only samples that showed complete loss or gain of bacteria rather
330 than simply changes in bacterial proportions (Fig 4D). For example, the stomachs of
331 autoimmune atrophic gastritis patients were colonised by *Gemella* and *Bosea* unlike
332 any other patient group. Alterations in the relative proportions of other bacteria were
333 also found in the stomachs of patients with autoimmune atrophic gastritis. These
334 included increases in the proportions of *Streptococcus*, *Campylobacter* and
335 *Haemophilus* (Table S1).

336 Few co-occurrence networks were identified, presumably due to the dominance of
337 Streptococcaceae, although *Stenotrophomonas* and *Delftia*; and *Selenomonas* and
338 *Pseudomonas* showed strong positive correlations (Fig 5D and Table S2B).

339 Predicted pathway analysis suggested that several pathways were over- or under-

340 represented in the stomachs of patients who had autoimmune atrophic gastritis
341 (table S3).

342

343 *H. pylori* gastritis versus *H. pylori*-induced atrophic gastritis

344 We investigated whether the microbiota in the stomachs of patients who had *H.*
345 *pylori*-induced atrophic gastritis (which were likely to be hypochlorhydric as indicated
346 by hypergastrinaemia) differed from that in patients who had *H. pylori* gastritis,
347 normal gastric acid secretion and normogastrinaemia. No significant differences
348 were identified at the genus level. However, several OTUs belonging to *H. pylori*
349 were found more frequently in the *H. pylori*-induced atrophic gastritis group, possibly
350 suggesting the presence of particular strains within this group (Table S4A).
351 Interestingly, only two other OTUs differed in abundance between these groups,
352 *Streptococcus mitis* and *Neisseria mucosa*. However, these did not remain
353 significant once *H. pylori* was removed from the analysis. This suggests that the
354 presence of atrophy does not result in extensive changes to bacterial communities in
355 the stomach relative to the simple presence of *H. pylori*, but may result in specific
356 differences in individual bacterial strains.

357

358 **Comparisons between the gastric microbiota of individuals with** 359 **hypochlorhydria of different aetiologies**

360 Patients with *H. pylori*-induced atrophic gastritis and those receiving PPIs had similar
361 fasting serum gastrin concentrations (median 100pM and 140pM respectively),
362 possibly suggesting similar degrees of hypochlorhydria (although *H. pylori* infection

363 may have directly contributed to the hypergastrinaemia in the former group). In
364 contrast patients with autoimmune atrophic gastritis were associated with higher
365 fasting serum gastrin concentrations (median 800pM; Table 1). No direct association
366 between fasting serum gastrin concentration and bacterial taxa was observed
367 between the different groups (PERMANOVA Unifrac P=0.512, weighted Unifrac
368 P=0.721 and Bray-Curtis P=0.556). This is reflected in the evidence that patients
369 with *H. pylori*-induced atrophic gastritis and those receiving PPIs exhibited marked
370 differences in 16S rRNA microbiota profiles, co-occurrence networks and predicted
371 pathways, despite similar gastrin levels. And that patients with autoimmune atrophic
372 gastritis showed similarities to individuals with *H. pylori*-induced atrophic gastritis by
373 predicted pathway analysis, despite markedly different serum gastrin concentrations
374 (Table S3).

375 Samples from patients with autoimmune atrophic gastritis contained significantly
376 more *Streptococci* than all other groups (Fig 3 & Table S1). *Streptococcus* did not
377 appear to be similarly increased in *H. pylori*-induced atrophic gastritis; this may have
378 been due to the negative relationship observed between *Helicobacter* and
379 *Streptococcus* identified in co-occurrence networks (Fig 5C).

380 Gastric biopsies from patients with autoimmune atrophic gastritis and those on PPIs
381 both showed greater bacterial diversity than was observed in the stomachs of
382 patients with *H. pylori*-induced atrophic gastritis (Fig 2). At the genus level, patients
383 with autoimmune atrophic gastritis showed significant increases in *Tannerella*,
384 *Dorea*, *Streptococcus*, *Fusobacterium* and *Campylobacter* compared to the patients
385 with *H. pylori*-induced atrophic gastritis (Table S4B). The stomachs of PPI-treated
386 patients also contained significantly higher proportions of *Fusobacterium* and
387 *Campylobacter* than the stomachs of *H. pylori*-induced atrophic gastritis patients.

388 Furthermore, patients receiving PPI treatment showed significantly higher
389 proportions of *Flavisolibacter* and *Dermacoccus* in their stomachs than autoimmune
390 atrophic gastritis patients, but significantly less *Paludibacter*, *Granulicatella*,
391 *Streptococcus*, and *Neisseria*.

392 Patients who had atrophic gastritis due to *H. pylori* or an autoimmune aetiology both
393 showed over-representation of several mutual pathways compared to controls (Table
394 S3). However, differences between the two groups were also observed. For
395 example, glucose-6-phosphate 1-dehydrogenase and D-lactate dehydrogenase
396 pathways were over-represented in the stomachs of patients who had *H. pylori*-
397 induced atrophic gastritis compared to those who had autoimmune atrophic gastritis
398 (Table S3).

399

400

401

402 **DISCUSSION**

403 Gastric samples obtained from subjects who had a normal stomach, no evidence of
404 *H. pylori* infection and normogastrinaemia had the highest levels of microbial
405 diversity. This is consistent with other reports of healthy populations showing more
406 microbial diversity[22, 23, 24]. These samples also contained the greatest proportion
407 of Prevotellaceae (23%) which corroborates previous research that reported normal
408 stomachs contained 37% *Prevotella*, reducing to 28% in dyspeptic patients[25]. In
409 general, the microbiota, co-occurrence networks and predicted pathways in samples
410 from PPI-treated patients were similar to those in normal stomachs. This agrees with
411 other reports that PPIs do not significantly influence the gastric microbiota[26, 27]. At
412 the OTU level however, samples from PPI-treated patients contained significantly
413 more *Streptococcus*. This has also been observed in gastric[27] and faecal samples
414 from twins discordant for PPI use[28].

415 *H. pylori* gastritis, and to some extent *H. pylori*-induced atrophic gastritis samples
416 were dominated by *H. pylori*. This observation may have been exacerbated by our
417 use of RNA as opposed to DNA for sequencing, unlike many other publications.
418 When these two techniques were directly compared, *H. pylori* abundance was found
419 to be 19.9 times higher in RNA compared to DNA from gastric fluid samples, and
420 was also more dominant in biopsies than gastric fluid[26, 29]. The use of RNA
421 ensured that only viable bacteria were included in the analysis, giving a better
422 indication of the taxa that are likely to be influencing the gastric environment. *H.*
423 *pylori* colonisation was associated with a decrease in gastric bacterial diversity, and
424 dominance of this organism, which is highly adapted to the gastric environment, has
425 also been reported previously[6, 30, 31].

426 The majority of changes observed in *H. pylori* gastritis and *H. pylori*-induced atrophic
427 gastritis samples were due to reductions in non-*H. pylori* bacteria. *H. pylori*-induced
428 atrophic gastritis samples showed complex co-occurrence networks, unlike *H. pylori*
429 gastritis which showed few connections, presumably related to the dominance of *H.*
430 *pylori* itself in those samples. *Campylobacter*, *Prevotella*, *Haemophilus* and
431 *Veillonella* were amongst the most influential genera in *H. pylori*-induced atrophic
432 gastritis samples. These bacteria have been previously identified in oral and gastric
433 samples[29]. The only differences found between the two *H. pylori* patient groups at
434 the OTU level were increased abundances of specific *H. pylori* OTUs (possibly
435 suggesting specific bacterial strains) and increased proportions of *Streptococcus*
436 *mitis* and *Neisseria mucosa* in the atrophic group. The former species and latter
437 genus have been identified from oral microbiota as potential biomarkers for
438 pancreatic cancer[32]. *Neisseria* has been shown to produce large amounts of
439 alcohol dehydrogenase, which produces the carcinogen acetaldehyde, and along
440 with *H. pylori*'s high production of this enzyme, may also contribute to gastric
441 carcinogenesis[33]. Some strains of Streptococcaceae have previously been shown
442 to affect the outcomes of *H. pylori* infection. For example, *S. mitis* induces a coccoid
443 state in *H. pylori*[34] and this may lead to unsuccessful antibiotic treatment and false
444 negative diagnostic test results. Moreover, this coccoid form has been suggested to
445 be more associated with gastric adenocarcinoma development than the spiral
446 form[35, 36].

447 The stomachs of patients with autoimmune atrophic gastritis (who probably had the
448 most profound reductions in acid secretion, as suggested by higher fasting serum
449 gastrin concentrations), showed high bacterial diversity. Samples from this group
450 also showed significantly higher proportions of *Streptococcus* than any of the other

451 groups. They also contained *Ruminococcus* and *Gemella* unlike any other patient
452 group except *H. pylori*-induced atrophic gastritis, although they did not contain
453 genera such as *Arthrobacter*, *Cupriavidus* and *Sneathia*. Therefore, bacterial
454 communities were both lost and gained in this condition. Co-occurrence networks
455 appeared to be disrupted by the overabundance of Streptococcaceae resulting in
456 few connections.

457 The microbial profiles in the stomachs of patients with *H. pylori*-induced atrophic
458 gastritis and autoimmune atrophic gastritis were quite different. In addition, pathways
459 such as glucose-6-phosphate 1-dehydrogenase and D-lactate dehydrogenase were
460 over-represented in the stomachs of patients with *H. pylori*-induced atrophic gastritis
461 compared to autoimmune atrophic gastritis. Overexpression of these pathways has
462 been associated with poorer prognoses in gastric cancer[37, 38]. Conversely,
463 several other metabolic pathways such as fumarate reductase were increased in
464 representation in patients with both autoimmune and *H. pylori* associated atrophic
465 gastritis. Fumarate reductase is involved in the metabolism of some bacteria and is
466 essential for colonisation by *H. pylori* in the mouse stomach[39, 40, 41]. Interestingly,
467 succinate dehydrogenase (which has an opposite action to fumarate reductase) was
468 found to be decreased in both atrophic gastritis groups compared to both the normal
469 and PPI-treated samples. Lower levels of succinate dehydrogenase have previously
470 been found in gastrointestinal tumours and parietal cells[42, 43]. PPI-treated patients
471 showed more similarities in microbial diversity and abundance to the patients who
472 had autoimmune atrophic gastritis, than to the patients who had *H. pylori*-induced
473 atrophic gastritis.

474

475 **Conclusion**

476 Our findings indicate that *H. pylori* colonisation and hypochlorhydria result in
477 changes in gastric bacterial abundance and only rarely in loss/gain of bacteria. PPI
478 treatment did not significantly alter the gastric microbiota from that of a normal
479 stomach, despite serum gastrin concentrations being comparable to those found in
480 patients with *H. pylori*-induced atrophic gastritis. Autoimmune atrophic gastritis
481 resulted in a different, more diverse microbial pattern than that observed in the
482 stomachs of patients who had *H. pylori*-induced atrophic gastritis. This may be due
483 to differences in acid secretion between these conditions or other factors such as
484 different immune profiles. Several biochemical pathways were represented in similar
485 fashions in both atrophic gastritis groups. In particular, gastric-atrophy was
486 associated with changes in the citric acid cycle (biochemical pathway that is known
487 to be associated with gastric carcinogenesis) and our findings suggest that the
488 microbiota may be an important contributor to this.

489

490

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497 studies via grants from National Institute for Health Research (NIHR) via the
498 Liverpool Biomedical Research Centre (BRC) and Trio Medicines Ltd.

499

500 **CONFLICT OF INTERESTS**

501 DMP has previously received research funding from Trio Medicines Ltd to investigate
502 the treatment of gastric neuroendocrine tumours using Netazepide. None of the
503 other authors has any conflict of interests.

504

505 **AUTHOR CONTRIBUTIONS**

506 BNP contributed to, analysis, interpretation and acquisition of data, drafting and
507 critically appraising the manuscript. UZI performed and designed analysis, performed
508 statistical analysis, interpreted data and critically appraised the manuscript. RD'A
509 designed methodology, acquired data and critically appraised the manuscript. MDB
510 contributed to analysis and interpretation of data, statistical analysis and critically
511 appraising the manuscript. RE, CAD and ARM contributed to methodology,
512 acquisition of data and critically appraising the manuscript. LT provided pathology
513 expertise, interpretation of data and critically appraised the manuscript. AV, NH and
514 DMP formulated conception of the study, its design, secured funding, interpreted

515 data and critically appraised the manuscript. All authors approved the final version of
516 the manuscript and agree to accountability for all aspects of the work.

517

518 **FIGURE LEGENDS**

519 **Table 1.** Summary of patient group characteristics. ND = Not done, IQR =

520 Interquartile range, 25% and 75%.

521 **Figure 1.** (A) Median fasting serum gastrin concentrations (pM) in patient groups.

522 Kruskal-Wallis test with Dunn's comparison, plotted using Tukey's method *=P<0.05,

523 and ****=P<0.0001 vs control. (B) Mean number of OTUs identified within each

524 patient group, 1-way ANOVA and Tukey's multiple comparison test *=P<0.05,

525 **=P<0.01, Control vs Autoimmune atrophic gastritis P=0.059, Control vs Neg

526 P=0.061 and Hp-induced atrophic gastritis vs Neg P=0.059.

527 **Figure 2.** Five different diversity indices of human gastric microbiota (Fisher alpha:

528 parametric index of diversity that models species as logseries distribution; Pielou's

529 evenness: how close in numbers each species is; Richness: number of species per

530 sample; Shannon: a commonly used index to characterise species diversity; and

531 Simpson: which takes into account the number of species present, as well as their

532 relative abundance). Pair-wise ANOVA was performed between different groups and

533 if significant (P<0.001), the p-values have been drawn on top. Atrophy=H. pylori

534 associated atrophy, Auto=autoimmune atrophic gastritis, Control=normal stomach,

535 HP Gast=H. pylori associated gastritis, PPI=proton pump inhibitor and Neg=

536 extraction control.

537 **Figure 3.** Relative abundances of taxa found within (A) groups and (B) individual

538 human gastric biopsies. Hp=H. pylori, IM=intestinal metaplasia, IM+At=intestinal

539 metaplasia and atrophy, PPI=proton pump inhibitor, EC=extraction controls (one of

540 the EC samples was included in a run with more H. pylori dominant samples),

541 H=H₂O and M=mock community (which showed consistent findings on two runs as

542 shown). All *H. pylori* atrophic gastritis samples were positive for *H. pylori* by serology,
543 + indicates whether these samples were also positive by histology/rapid urease test.
544 Autoimmune atrophic gastritis samples recorded as 's' were also positive for *H. pylori*
545 by serology.

546 **Figure 4.** Nonmetric distance scaling (NMDS) demonstrating clustering of patient
547 groups using (A) unweighted Unifrac distance (pair-wise distance between samples
548 is calculated as a normalised difference in cumulative branch lengths of the
549 observed OTUs for each sample on the phylogenetic tree without taking into account
550 their abundances in samples), (B) Bray-Curtis distance (abundance of OTUs alone
551 and not considering the phylogenetic distance) and (C) weighted Unifrac
552 (unweighted unifrac distance weighted by abundances of OTUs). Serum gastrin
553 concentration indicated by size of each point. Ellipses represent 95% CI of standard
554 error for a given group. Dotted ellipses represent the 95% CI of standard error when
555 *H. pylori* were removed from the analysis. Atrophy=*H. pylori* associated atrophic
556 gastritis, Auto=autoimmune atrophic gastritis, Control=normal, HP Gastr=*H. pylori*
557 associated gastritis and PPI=proton pump inhibitor. PERMANOVA (distances against
558 groups) suggests significant differences ($P < 0.001$ for all three distances) in microbial
559 community explaining the following variations (R^2) between groups: 10% (8.6%
560 without *H. pylori* when using Unweighted Unifrac; 58% (14.5% without *H. pylori*)
561 when using Weighted Unifrac; and 15% when using Bray-Curtis distance. No
562 significant explanation was observed ($P > 0.05$) for age, BMI, or serum gastrin
563 concentration in the PERMANOVA test. (D) Data from betadisper plots (a mean to
564 compare the spread/variability of samples for different groups) representing
565 difference in distances (Bray-Curtis, Unweighted and weighted Unifrac) of group
566 members from the centre/mean of individual groups after obtaining a reduced-order

567 representation of abundance table using Principle Coordinate Analysis. The pair-
568 wise differences in distances from group centre/mean were then subjected to
569 ANOVA and if significant ($P < 0.001$), the p-values were drawn on top.

570 **Figure 5.** Co-occurrence network analysis between different genera (OTUs collated
571 together at genus level) when considering samples for (A) normal stomach, (B) *H.*
572 *pylori* gastritis, (C) *H. pylori*-induced atrophic gastritis and (D) autoimmune atrophic
573 gastritis. The genera were connected (Blue: positive correlation; Red: negative
574 correlation) when the pair-wise correlation values were significant ($P_{\text{adj}} < 0.05$) after
575 adjusting the P values for multiple comparisons. Furthermore, subcommunity
576 detection was performed by placing the genera in the same subcommunity
577 (represented by colour of nodes) when many links were found at correlation values
578 > 0.75 between members of the subcommunity. The size of the nodes represent the
579 degree of connections.

580

581 **Supplementary Figure and Table Legends**

582 **Figure S1.** Relative abundances of taxa found within human gastric biopsies after
583 removal of Helicobacteraceae. Hp=*H. pylori*, IM=intestinal metaplasia,
584 IM+At=intestinal metaplasia and atrophy, PPI=proton pump inhibitor. All *H. pylori*
585 atrophy samples were positive for *H. pylori* by serology.

586 **Figure S2.** Co-occurrence network analysis between different genera (OTUs
587 collated together at genus level) when considering samples for (A) PPI. The genera
588 were connected (Blue: positive correlation; Red: negative correlation) when the pair-
589 wise correlation values were significant ($P_{\text{adj}} < 0.05$) after adjusting the P values for

590 multiple comparisons. Furthermore, subcommunity detection was performed by
591 placing the genera in the same subcommunity (represented by colour of nodes)
592 when many links were found at correlation values >0.75 between members of the
593 subcommunity. The size of the nodes represent the degree of connections (B)
594 network-wide statistics by degree, closeness, betweenness and eigenvalue centrality
595 for *H. pylori* atrophic gastritis cases. The nodes (coloured with respect to
596 subcommunity they are part of) were placed on concentric circles with values
597 increasing from center to the periphery. A high betweenness for a node suggests
598 many connections, whereas a high eigenvalue centrality suggests that those
599 connections, in turn, are all well connected. On average a high betweenness and at
600 the same time low eigenvalue centrality for a subcommunity suggests a
601 keystone/important subcommunity.

602

603 **Table S1.** Significantly different genera identified between normal stomach samples
604 and PPI, autoimmune atrophic gastritis, *H. pylori*-induced atrophic gastritis and *H.*
605 *pylori* gastritis. The most significant species are identified at the top. Differential
606 expression analysis based on the Negative Binomial (Gamma-Poisson) distribution
607 and were corrected for multiple comparisons. * indicates a genus no longer
608 significant when *H. pylori* was removed from the analysis.

609 **Table S2A.** Stable bacterial populations and correlations in PPI patients compared
610 to other groups (if the correlation between two genera were consistently positive or
611 negative in different groups). PPI versus *H. pylori*-induced atrophic gastritis in table
612 S2B. No significant comparisons were found between PPI and autoimmune atrophic
613 gastritis groups.

614 **Table S2B.** Stable bacterial populations and correlations in *H. pylori*-induced
615 atrophic gastritis patients compared to other groups.

616 **Table S3.** The top most significant predicted pathways found for each group
617 comparison.

618 **Table S4A.** Significant bacterial species identified between *H. pylori* atrophic gastritis
619 and *H. pylori* gastritis. The most significant species are identified at the top.
620 Differential expression analysis based on the Negative Binomial (Gamma-Poisson)
621 distribution. Streptococcus identified by BLAST as *S. mitis* with 98% coverage, 99%
622 identity and *Neisseria mucosa* had 98% coverage and 100% identity. None of these
623 OTUs remained significant when *H. pylori* was removed from the analysis.

624 **Table S4B.** Significant bacterial genera identified between autoimmune atrophic
625 gastritis and *H. pylori*-induced atrophic gastritis. The most significant species are
626 identified at the top. Differential expression analysis based on the Negative Binomial
627 (Gamma-Poisson) distribution. NB when *H. pylori* was removed from the analysis
628 these genera remained significant, with an additional genus *Desulfobulbus* also
629 reaching significance.

630

631

632

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634

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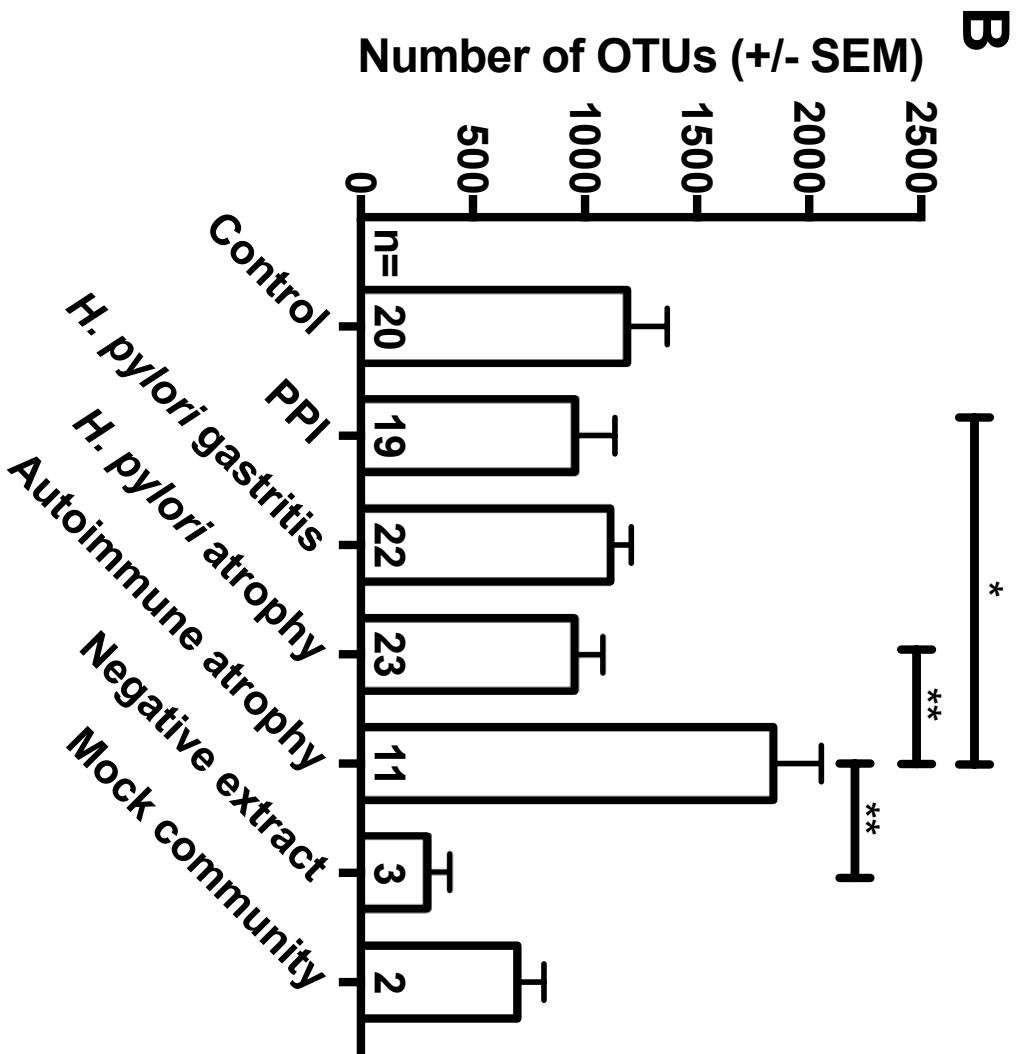
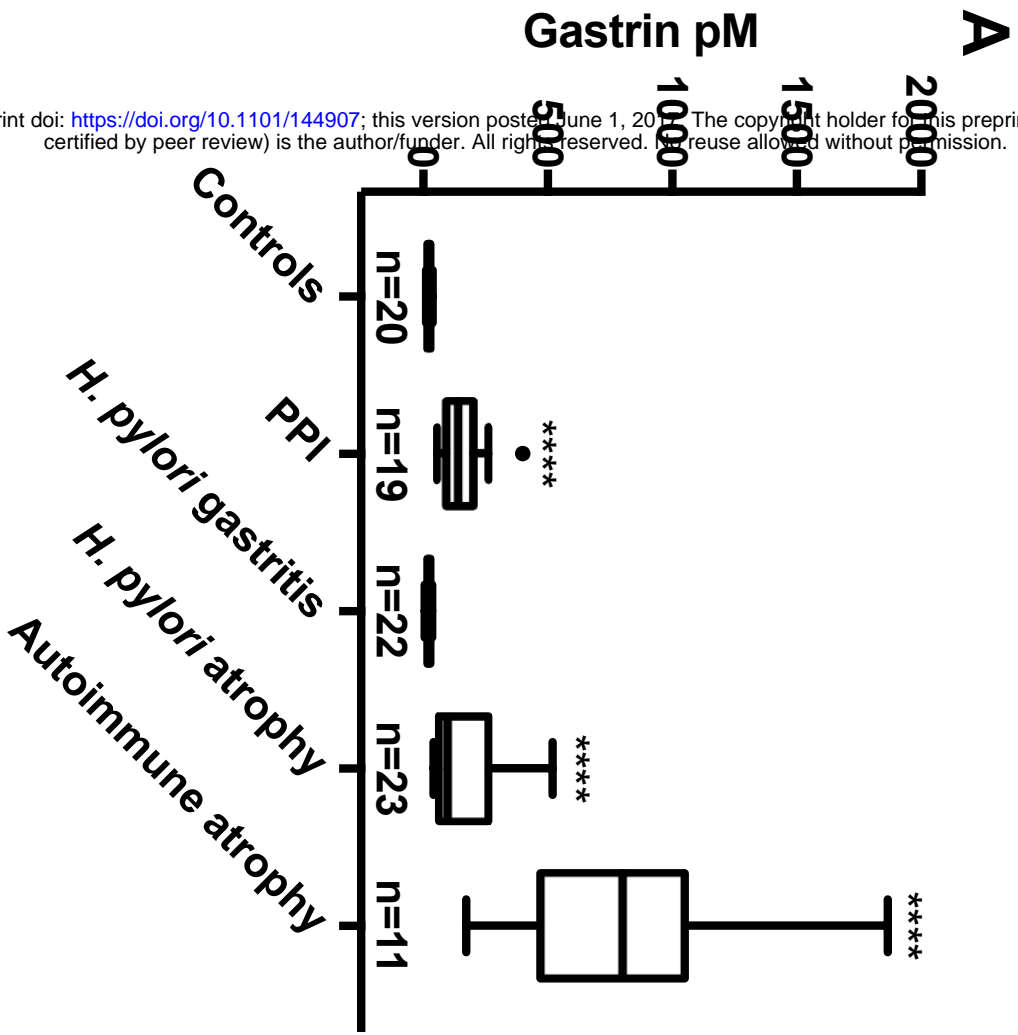
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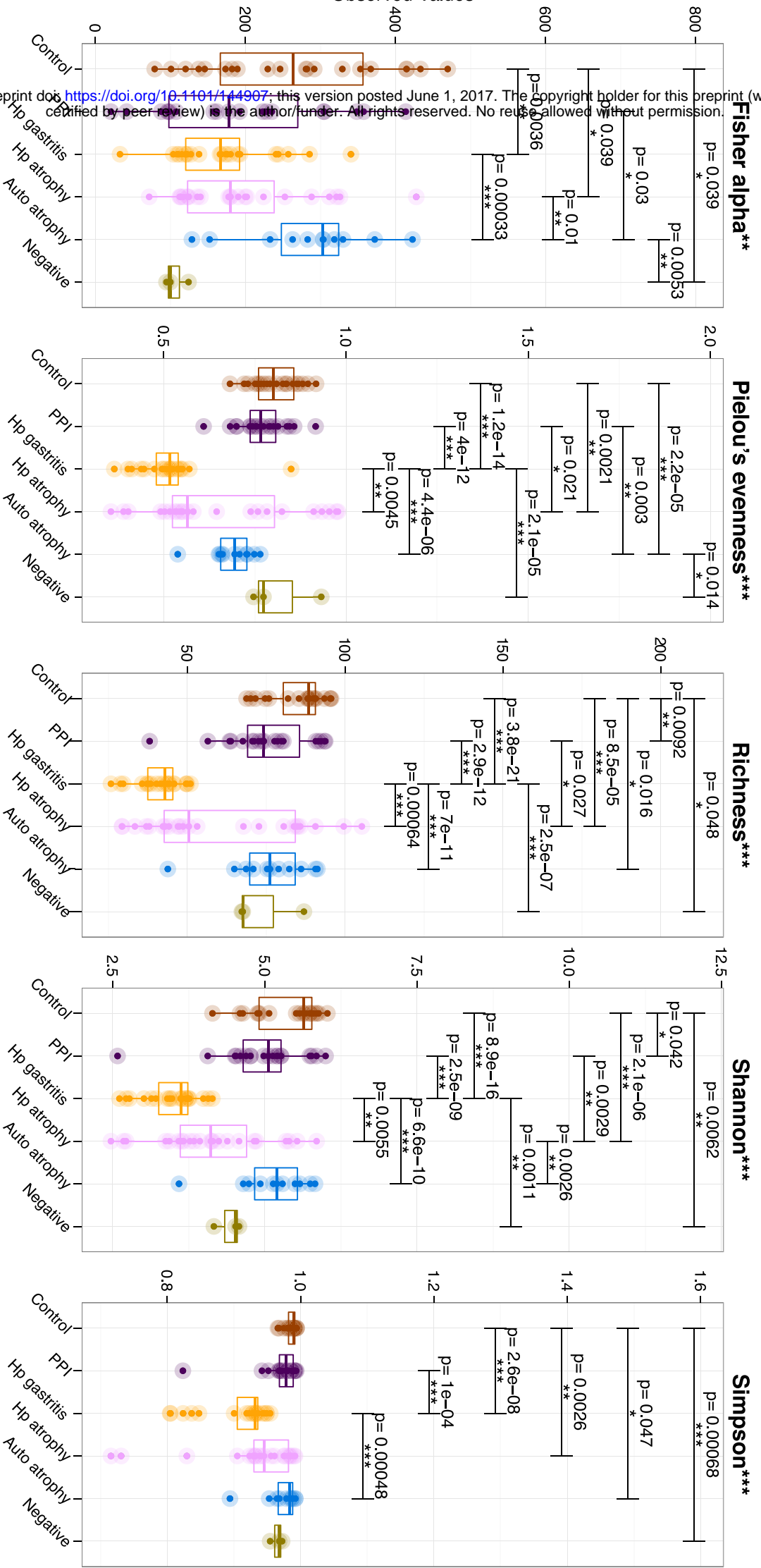
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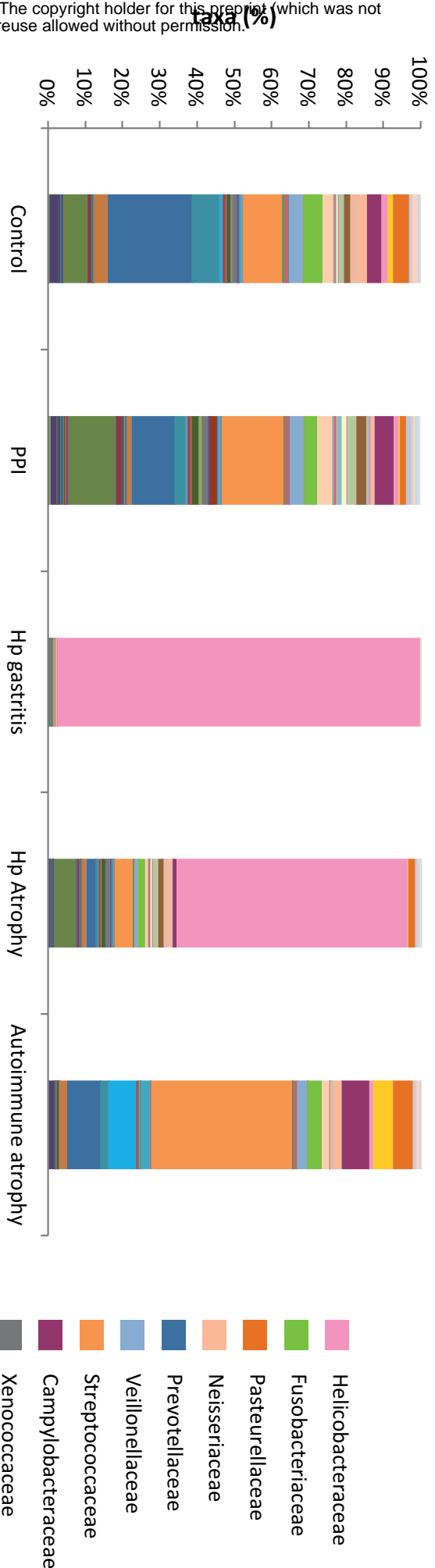
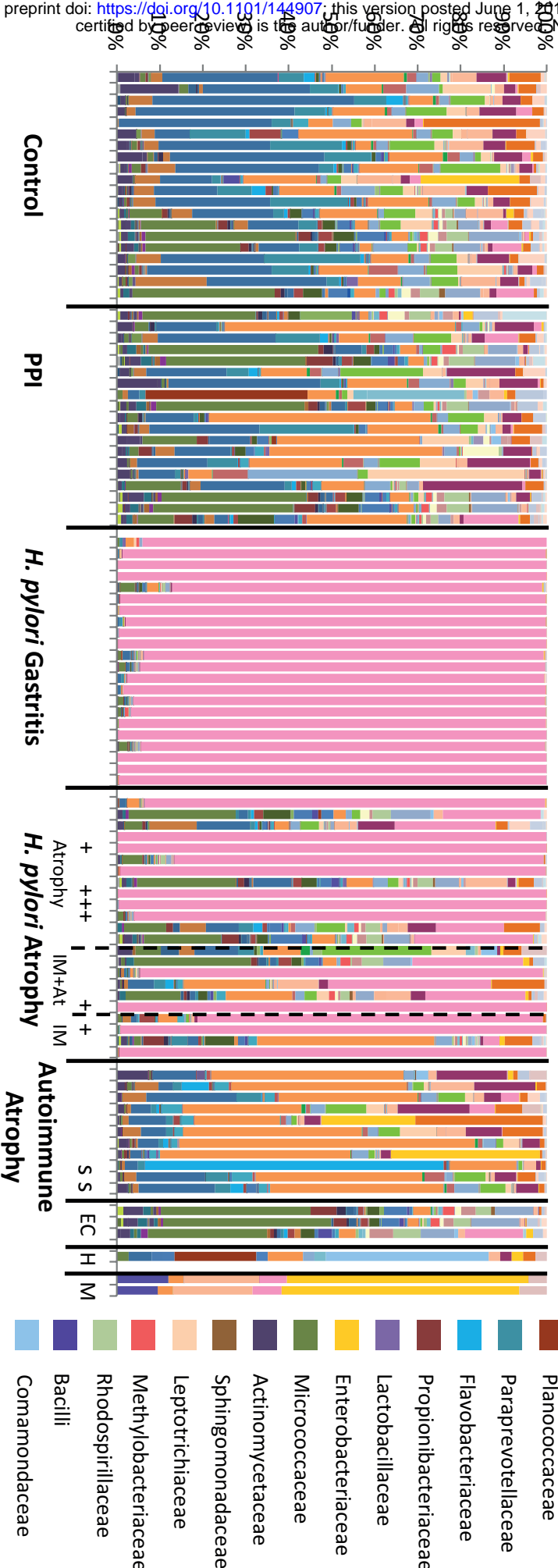
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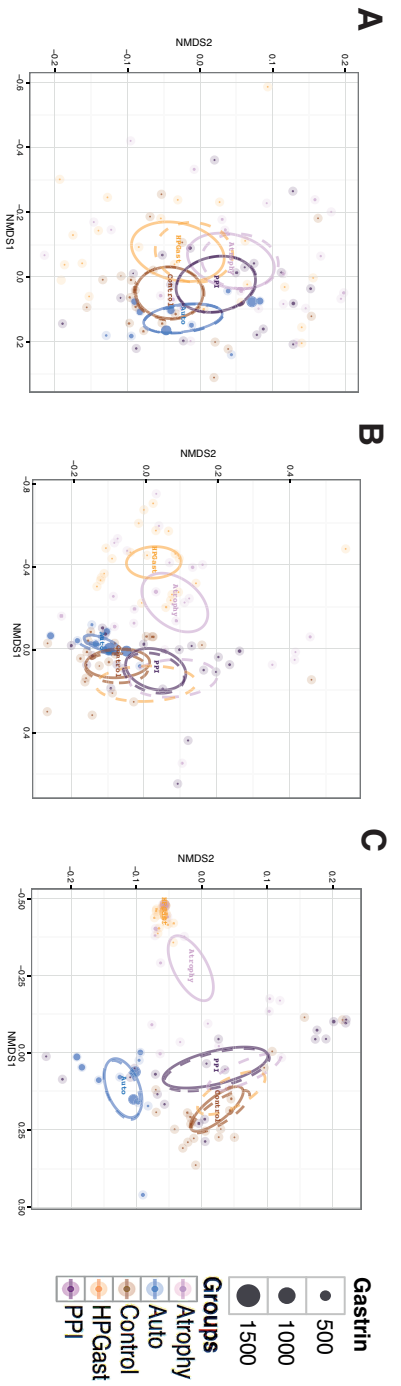


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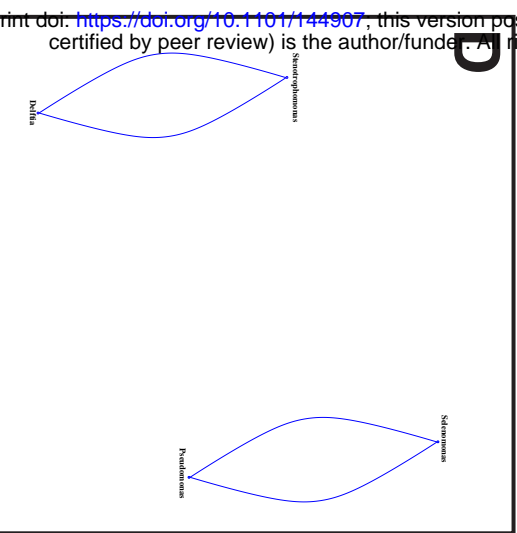
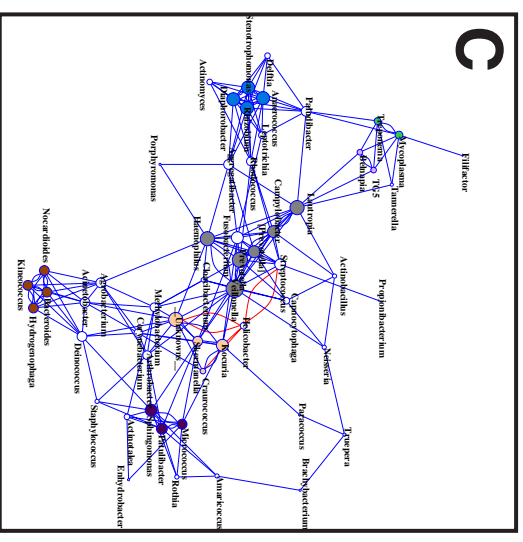
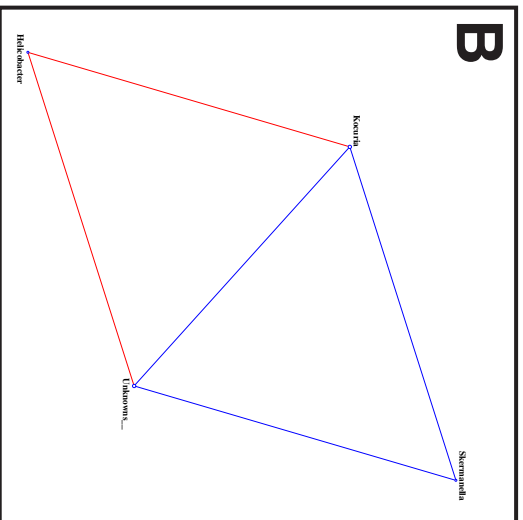
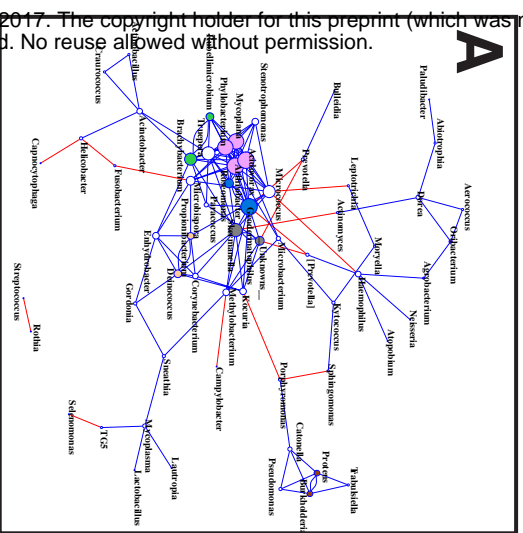
A**Average relative abundance of taxa (%)****Relative abundance of taxa (%)**

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D

| <i>H. pylori</i> included in analysis | | | <i>H. pylori</i> removed from analysis | | | |
|---------------------------------------|---|--------------------------------------|--|---|--------------------------------------|------------------------------|
| Group Comparison | Unifrac significance (presence/absence) | Bray-Curtis significance (abundance) | Weighted Unifrac (abundance) | Unifrac significance (presence/absence) | Bray-Curtis significance (abundance) | Weighted Unifrac (abundance) |
| Control | PPI | ns | 0.0116 * | ns | ns | 0.0132 * |
| | Hp Gastritis | ns | 1.3741×10 ⁻¹² *** | ns | ns | ns |
| | Hp Atrophy | 0.059 | ns | ns | 0.0193* | ns |
| PPI | Autoimmune | 0.0638 | ns | 0.0564 | ns | ns |
| | Hp Gastritis | ns | 9.9989×10 ⁻²⁵ *** | ns | ns | ns |
| | Hp Atrophy | ns | ns | ns | ns | ns |
| Hp Gastritis | Autoimmune | 0.0019 ** | 0.0037 ** | 0.0018 ** | 0.0043 ** | 0.0034 ** |
| | Hp Atrophy | ns | ns | ns | ns | ns |
| | Autoimmune | 0.0060 ** | 0.0080 ** | 2.5084×10 ⁻⁹ *** | 0.0090 ** | 0.0249* |
| Hp Atrophy | 3.159×10 ⁻⁵ *** | ns | 0.0568 | 1.191×10 ⁻⁴ *** | 3.765×10 ⁻⁴ *** | 0.0227* |



Autoimmune atrophy