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# Gastric microbes associated with gastric inflammation, atrophy and intestinal metaplasia 1 year after *Helicobacter pylori* eradication

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## ABSTRACT

**Objective** *Helicobacter pylori* is associated with gastric inflammation, precancerous gastric atrophy (GA) and intestinal metaplasia (IM). We aimed to identify microbes that are associated with progressive inflammation, GA and IM 1 year after *H. pylori* eradication.

**Design** A total of 587 *H. pylori*-positive patients were randomised to receive *H. pylori* eradication therapy (295 patients) or placebo (292 patients). Bacterial taxonomy was analysed on 404 gastric biopsy samples comprising 102 pairs before and after 1 year *H. pylori* eradication and 100 pairs before and after 1 year placebo by 16S rRNA sequencing.

**Results** Analysis of microbial sequences confirmed the eradication of *H. pylori* in treated group after 1 year. Principal component analysis revealed distinct microbial clusters reflected by increase in bacterial diversity ( $p < 0.00001$ ) after *H. pylori* eradication. While microbial interactions remained largely unchanged after placebo treatment, microbial co-occurrence was less in treated group. *Acinetobacter lwoffii*, *Streptococcus anginosus* and *Ralstonia* were enriched while *Roseburia* and *Sphingomonas* were depleted in patients with persistent inflammation 1 year after *H. pylori* eradication. A distinct cluster of oral bacteria comprising *Peptostreptococcus*, *Streptococcus*, *Parvimonas*, *Prevotella*, *Rothia* and *Granulicatella* were associated with emergence and persistence of GA and IM. Probiotic *Faecalibacterium prausznii* was depleted in subjects who developed GA following *H. pylori* eradication. Functional pathways including amino acid metabolism and inositol phosphate metabolism were enriched while folate biosynthesis and NOD-like receptor signalling decreased in atrophy/IM-associated gastric microbiota.

**Conclusion** This study identified that gastric microbes contribute to the progression of gastric carcinogenesis after *H. pylori* eradication.

## INTRODUCTION

Independent epidemiological studies have confirmed that infection with *Helicobacter pylori* is the most important acquired aetiological agent for gastric cancer,<sup>1</sup> a global leading cause of cancer-related deaths. The chronic inflammation induced by *H. pylori* induces several histopathological changes in the gastric epithelium and maintains a constant production of a cascade of cytokines which in turn attracts immune cells that generate oxidative

## Significance of this study

### What is already known on this subject?

- *Helicobacter pylori* infection is associated with gastric inflammation, precancerous gastric atrophy and intestinal metaplasia.
- Less than 3% of *H. pylori*-infected individuals develop gastric cancer, and about 20% of individuals with chronic gastritis are *H. pylori* negative; other organisms may induce gastric inflammation and gastric carcinogenesis.

### What are the new findings?

- Microbial co-occurrence was reduced and characterised by a distinct cluster of oral bacteria 1 year after eradication of *H. pylori*, while gastric microbial ecology remained largely unchanged 1 year after placebo treatment.
- One year after *H. pylori* eradication, *Acinetobacter lwoffii*, *Streptococcus anginosus* and *Ralstonia* were enriched while *Roseburia* and *Sphingomonas* were depleted in patients with persistent inflammation.
- A distinct cluster of oral bacteria comprising *Peptostreptococcus*, *Streptococcus*, *Parvimonas*, *Prevotella*, *Rothia* and *Granulicatella* were associated with emergence and persistence of atrophy and intestinal metaplasia, while probiotic *Faecalibacterium prausznii* was depleted in subjects who developed atrophy 1 year after *H. pylori* eradication.
- Amino acid metabolism and inositol phosphate metabolism were enriched while folate biosynthesis and NOD-like receptor signalling decreased in subjects with emerged intestinal metaplasia following *H. pylori* eradication.

### How might it impact on clinical practice in the foreseeable future?

- This study demonstrated the contribution of gastric microbes in the development and perpetuation of precancerous gastric lesions after *H. pylori* eradication.
- The identified microbes that associated with progression of gastric inflammation, atrophy or intestinal metaplasia are potential therapeutic targets in gastric cancer prevention.



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radicals with the potential to damage host DNA.<sup>2</sup> The mechanism employed by *H. pylori* in promoting the emergence of pre-neoplastic gastric lesions (atrophy and intestinal metaplasia) is predominantly chronic inflammation. Treatment regimens targeted at *H. pylori* eradication have been demonstrated to be effective in preventing the progression of pathological changes in the gastric mucosa.<sup>3,4</sup> However, despite the reported effectiveness of eradication therapy, some patients do continue to develop pre-neoplastic gastric lesions including gastric atrophy (GA) and intestinal metaplasia (IM).<sup>3</sup> Notably, less than 3% of *H. pylori*-infected individuals develop gastric cancer,<sup>5</sup> and about 20% of individuals with chronic gastritis are *H. pylori* negative, suggesting that other organisms may induce gastric inflammation and even gastric carcinogenesis.<sup>6</sup>

It is possible that, while *H. pylori* initiate the inflammatory process in the stomach, other gastric microbes with pro-inflammatory potential play an important role in maintaining the progression of inflammation and dysplastic changes leading to development of gastric cancer. The loss of acid-secreting parietal cells induced by chronic *H. pylori* infection in the gastric epithelium may provide a favourable environment for colonisation by non-*H. pylori* microbes to induce further pro-carcinogenic process. Moreover, the 'point of no return' in the cascade of events leading to gastric cancer is reportedly associated with patients with IM and dysplasia, independent of *H. pylori* status.<sup>2</sup>

The relevance of non-*H. pylori* gastric microbes in the development of gastric cancer was previously demonstrated by using transgenic INS-GAS mice, overexpressing gastrin. Antibiotic-treated INS-GAS mice colonised with *H. pylori* had delayed onset of gastric cancer compared with control mice infected with *H. pylori* without antibiotic treatment,<sup>7</sup> suggesting that gastric microbes could enhance the effects of *H. pylori* in gastric carcinogenesis. Single-nucleotide polymorphisms in host genes that influence bacterial pattern recognition receptors in gastric cancer patients have also been reported,<sup>8</sup> underscoring the active role of bacteria-host cross-talk in gastric tumourigenesis. It is therefore pertinent to investigate bacteria that are associated with inflammation, atrophy and IM after the eradication of *H. pylori* to establish a surveillance programme based on gastric cancer risk stratification specific to non-*H. pylori*-infected patients

Here, we carried out a retrospective study involving a 1-year follow-up of *H. pylori*-positive subjects who received *H. pylori* eradication therapy compared with those who received placebo. We identified gastric microbes that are associated with gastric inflammation, atrophy and IM after *H. pylori* eradication.

## MATERIALS AND METHODS

### Patients

A total of 587 *H. pylori*-positive patients residing in Yantai county of Shandong Province, China, with incidence of 50 gastric cancer cases per 100 000 population, were randomised as described before,<sup>4</sup> of which 295 *H. pylori*-positive patients received a 1-week course of omeprazole (20 mg), amoxicillin (1 g) and clarithromycin (500 mg) (OAC treatment) twice daily, while 292 *H. pylori*-positive subjects received placebo (placebo treatment). Subjects underwent endoscopy with biopsy with sterile forceps at baseline and after 1 year from the same sites of both the antrum and corpus. Histology and urease test were used to confirm the eradication of *H. pylori*. Tissue biopsies were also obtained during endoscopy and frozen immediately at -80°C.

### Histological assessment

Severity of gastric inflammation, GA and IM was graded according to the updated Sydney classification by a pathologist who was unaware of the treatment condition. Progression and regression were defined as increase or decrease, respectively, of inflammation, GA or IM scores after 1 year.

### Sample sequencing

Purification of DNA for 16S rRNA gene sequencing was performed on a total of 404 gastric biopsy samples, comprising 100 pairs before and after 1 year of placebo treatment, and 102 pairs before and after eradication of *H. pylori* by OAC treatment (online supplementary table 1). Gastric biopsies tissues were digested using lysozyme and mutanolysin enzymes (Sigma, Hong Kong), followed by bead beating and DNA purification with QIAamp DNA Mini Kit. Illumina Primer pair 515 f, 5'-GTGC-CAGCMGCCGCGGTAA-3' and 806 r, 5'-GGACTACHVGG-GTWTCTAAT-3' targeted across the V4 hypervariable regions of the 16S rRNA gene was used for sequencing on Illumina MiSeq platform. Invitrogen SequelPrep Normalization Plate Kit was used for Library clean-up and normalisation.

### Quality control and annotation of sequences

Mothur software<sup>9</sup> suite was used for sequence curation and analysis. Briefly, Needleman-Wunsch alignment algorithm was used to merge paired-end reads into contigs with default parameters. This was followed by alignment against SILVA 16S rRNA sequence database (V.123) with nearest alignment space termination (NAST) algorithm.<sup>10</sup> Contigs that mapped within V4 region were trimmed and merged with sequences having a difference of most two nucleotide bases, followed by chimeric sequences screening using de novo Uchime.<sup>11</sup> Greengenes database (V.13.8) was used for taxonomy assignment. Reads assigned as non-bacterial or unknown kingdoms were discarded. Average neighbour clustering algorithm was used to cluster the resulting sequences into operational taxonomic units (OTUs), with 97% identity cut-off. The functional potential of the gastric microbial communities was estimated by the PICRUSt<sup>12</sup> algorithm. Kyoto Encyclopedia of Genes and Genomes (KEGG) database was used for pathway enrichment analysis.

### Statistical analyses

Differentially abundant non-*H. pylori* microbes and predicted microbiota functional alterations associated with inflammation, GA and IM were analysed using Lefse algorithm.<sup>13</sup> Differences with log<sub>10</sub> linear discriminant analysis scores (absolute values) >2.0 and p value <0.05 were considered significant. Co-occurrence and co-exclusion relationships between bacteria with relative abundance greater than 0.1%, and with *Helicobacter* at the genus level were estimated using SparCC algorithm,<sup>14</sup> known for its robustness to gut microbiome data compositionality. P values less than 0.05 after multiple comparisons correction using false discovery rate method were considered significant. Cytoscape V.3.0.4 was used for visualisation of bacteria co-occurrence. Network parameters including topological coefficient, closeness centrality and stress were estimated using the network analyser algorithm of Cytoscape V.3.0.4 with default parameters, and compared using Mann-Whitney U test.

## RESULTS

### Eradication of *H. pylori* with OAC treatment

Acute and chronic gastritis decreased in both antrum and corpus of the stomach in patients after *H. pylori* eradication. In the

**Table 1** Demographic of study subjects

Number of subjects	OAC-treated group (n=102)	Placebo group (n=100)
Inflammation at baseline	102	100
Inflammation at after 1 year	16	91
Atrophy at baseline	17	15
Atrophy after 1 year	37	40
Intestinal metaplasia at baseline	49	45
Intestinal metaplasia after 1 year	24	29

OAC, omeprazole, amoxicillin and clarithromycin.

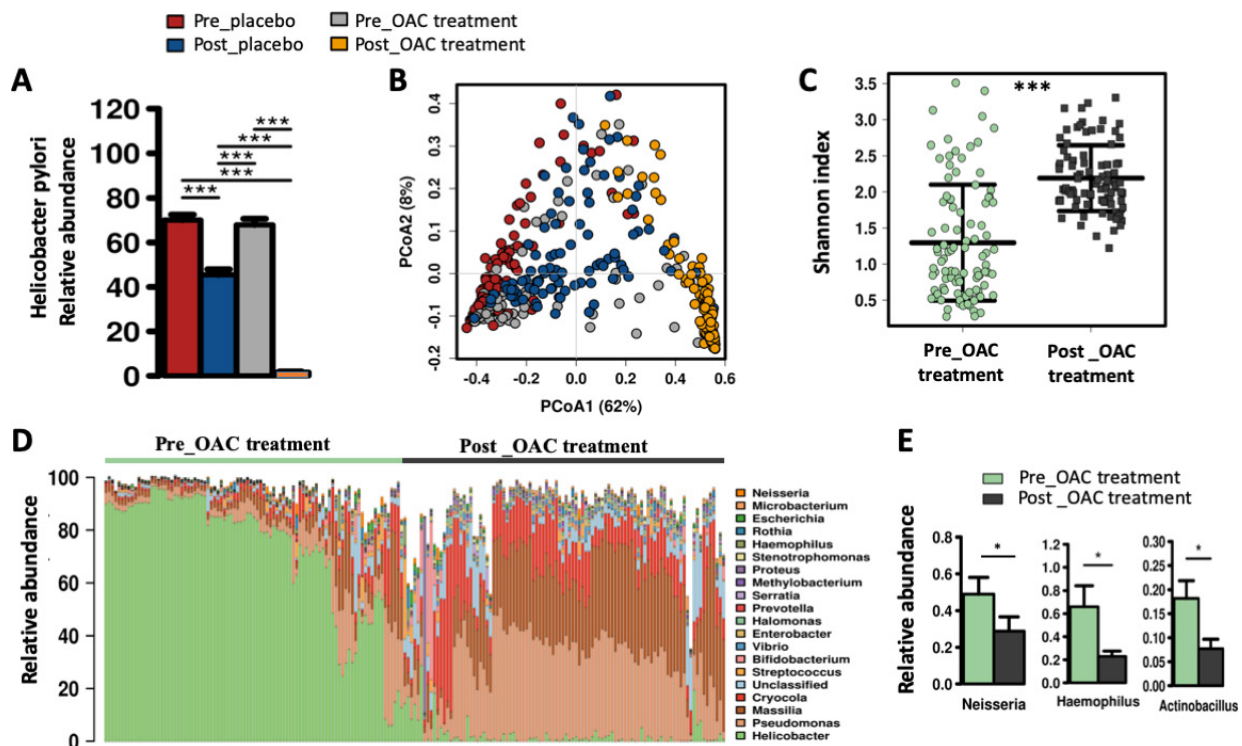
placebo group, antral biopsy specimens had more pronounced acute gastritis and a marginal increase in atrophy compared with OAC-treated group. Changes in IM were, however, similar between placebo and OAC-treated group.<sup>3</sup>

Analysis of 16S rRNA gene sequences was performed on a total of 404 gastric biopsy samples, comprising 100 pairs before and after 1 year of placebo treatment, and 102 pairs before and 1 year after OAC treatment. Demographic details of subjects are presented in table 1. The eradication of *H. pylori* was confirmed in the OAC-treated group with relative abundance of *H. pylori* of  $0.016 \pm 0.0018$  compared with pre-treatment ( $0.68 \pm 0.027$ ), pre-placebo ( $0.67 \pm 0.025$ ) and post-placebo ( $0.46 \pm 0.023$ ) ( $p < 0.00001$ ) groups (figure 1A). Principal component analysis revealed a distinct gastric microbiota cluster for OAC-treated group (figure 1B). Eradication of *H. pylori* was associated with increase in bacterial diversity ( $p < 0.00001$ ) (figure 1C), reflected by proliferation of several bacterial species (figure 1D). However, with the exclusion of *H. pylori*, the increase in bacteria diversity was no longer observed, indicating that the increased diversity

resulted from the dominant abundance of *H. pylori* before OAC therapy. The most abundant bacteria families after *H. pylori* eradication with relative abundance greater than 1% are *Pseudomonadaceae*, *Oxalobacteriaceae*, *Microbacteriaceae*, *Enterobacteriaceae*, *Lachnospiraceae*, *Vibrionaceae* and *Halomonadaceae* (online supplementary figure 1). They have increased respectively by 3.9, 4.6, 4.2, 2.3, 1.1, 6.9 and 5.7 folds 1 year after *H. pylori* eradication. In addition to *Helicobacter*, *Haemophilus* ( $p=0.0061$ ), *Actinobacillus* ( $p=0.0019$ ) and *Neisseria* ( $p=0.0056$ ) were observed to be reduced after OAC treatment (figure 1E) and were represented by *Haemophilus influenzae*\_Otu42, *Actinobacillus parahaemolyticus*\_Otu100 and *Neisseria subflava* at OTU level (online supplementary table 2). Samples with  $>1\%$  *H. pylori* relative abundance in the OAC-treated group were regarded as being positive for *H. pylori* infection as previously described<sup>15</sup> and were excluded from downstream analysis.

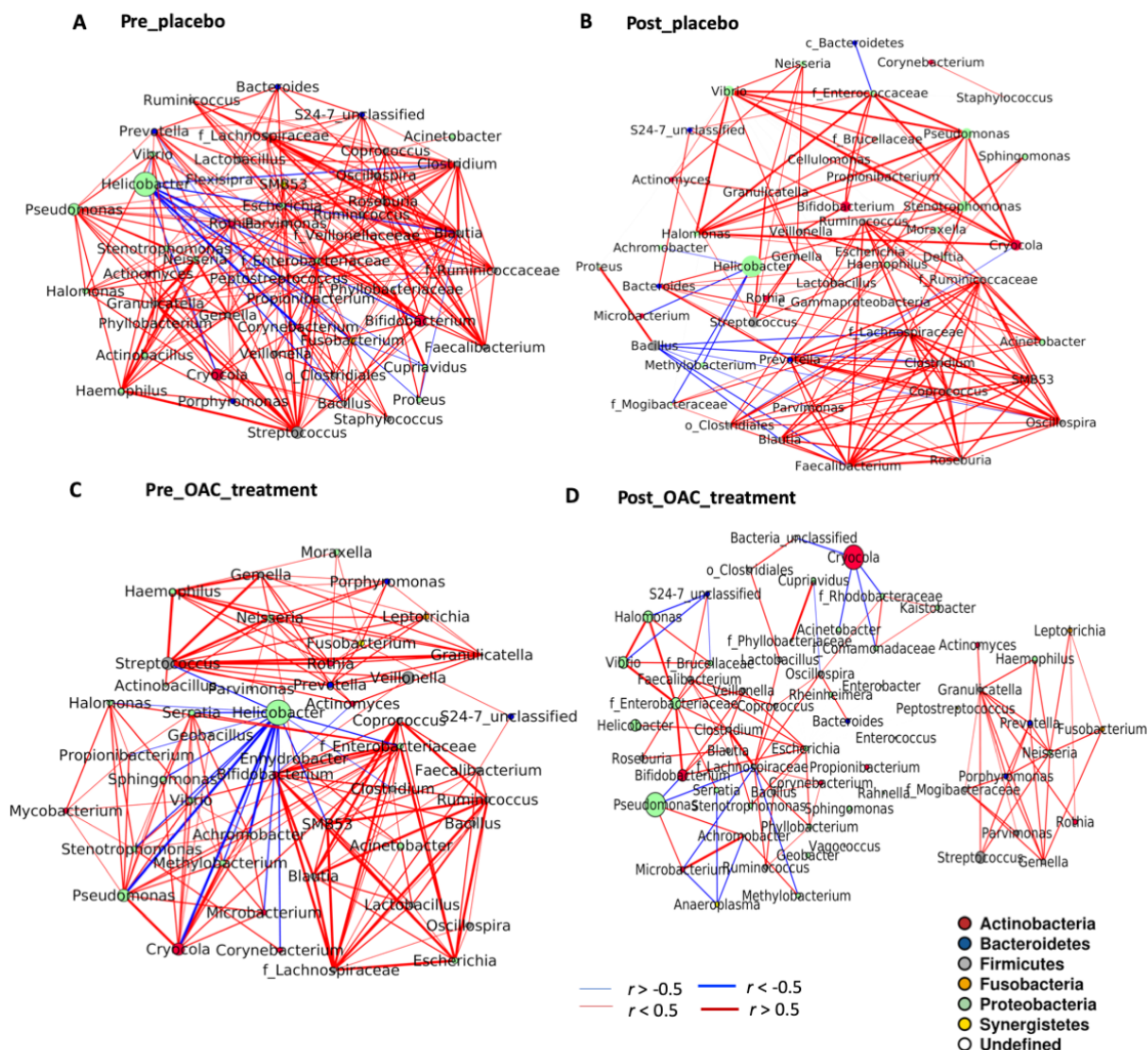
### Microbiota ecology is altered after *H. pylori* eradication

Gastric microbial co-occurrence remained largely unchanged in the placebo-treated group (figure 2A,B, online supplementary tables 3–4). Much less microbial co-occurrence was, however, observed after a year following the eradication of *H. pylori* by OAC treatment (figure 2C,D, online supplementary tables 5–6). Network topology analysis showed that topological coefficient ( $p=0.023$ ) closeness centrality ( $p=0.016$ ) and stress ( $p=0.01$ ) of the bacteria nodes were statistically different in the microbial ecological network in the absence of *H. pylori* compared with the *H. pylori*-infected placebo group. In addition, *H. pylori* had predominantly co-exclusive association with other gastric microbes in the *H. pylori*-infected groups. In the OAC-treated



**Figure 1** Eradication of *Helicobacter pylori* with omeprazole, amoxicillin and clarithromycin (OAC) treatment. (A) Relative abundance of *H. pylori* in Pre\_placebo, Post\_placebo, Pre\_OAC treatment and Post\_OAC treatment groups. (B) Principal component analysis plot showing a distinct cluster for Post\_OAC treatment group. (C) Bacterial diversity estimated by Shannon index for Pre\_OAC treatment and Post\_OAC treatment groups. (D) Top 20 bacterial genera before and after 1 year of OAC treatment. (E) Relative abundance of *Neisseria*, *Haemophilus* and *Actinobacillus* 1 year after OAC treatment. \*Adjusted  $p < 0.05$ , \*\*adjusted  $p < 0.01$ , \*\*\*adjusted  $p < 0.001$ .





**Figure 2** Microbiota ecology is altered after *Helicobacter pylori* eradication. Ecological co-occurrence network in (A) Pre\_placebo, (B) Post\_placebo, (C) Pre\_OAC treatment and (D) Post\_OAC treatment groups. Significant ( $Q > 0.05$ ) SparCC algorithm was used to infer co-occurrence among top (0.1% relative abundance) bacterial genera and with *Helicobacter*.

group, a distinct cluster of oral bacteria including *Peptostreptococcus*, *Parvimonas*, *Fusobacterium*, *Haemophilus*, *Neisseria*, *Gemella*, *Granulicatella*, *Rothia*, *Streptococcus* and *Porphyromonas* was observed (figure 2D). The change in gastric microbial ecology after *H. pylori* eradication indicates that *H. pylori* impacts the interactions of other gastric microbes which may contribute to inflammation and development of cancerous lesions in infected patients.

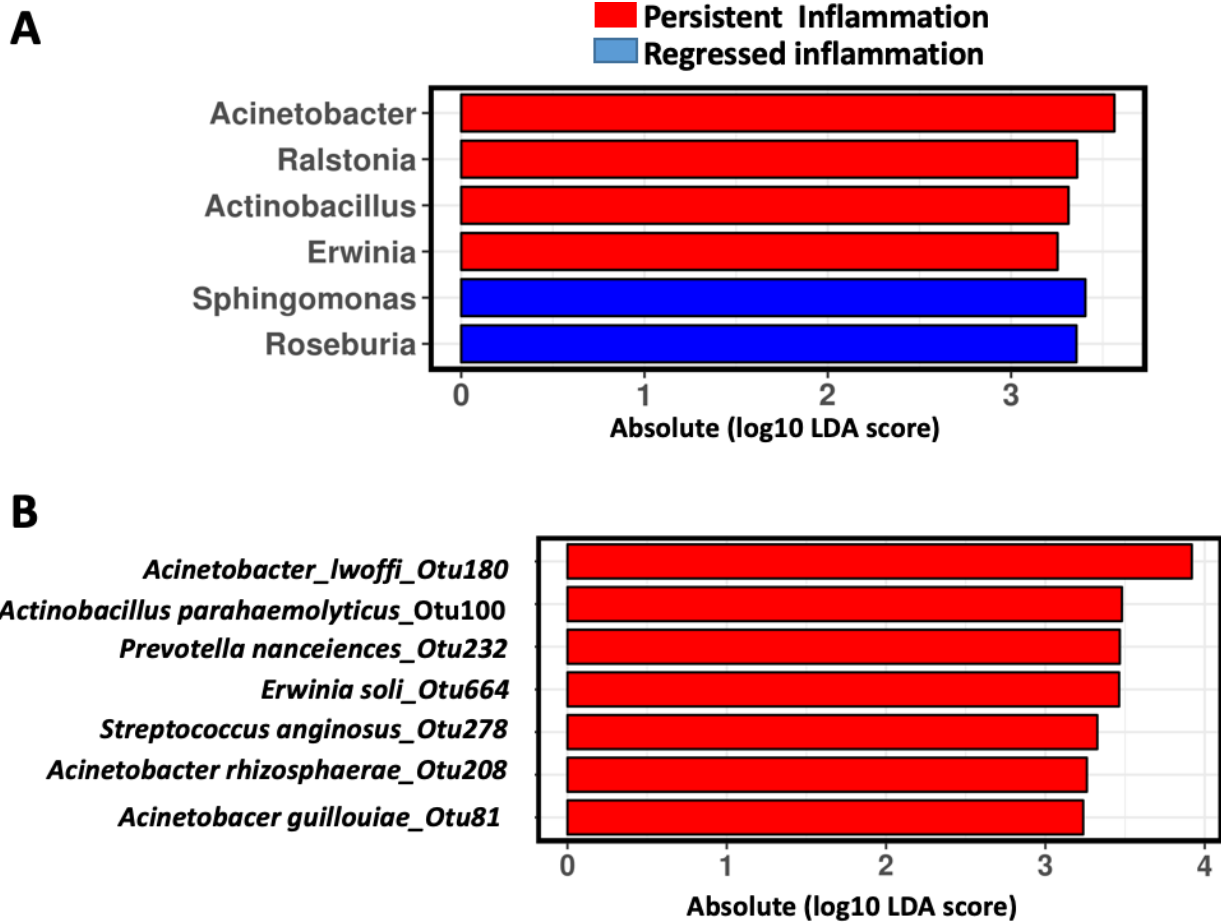
### Bacteria associated with inflammation after *H. pylori* eradication

The intensities of polymorphonuclear infiltration in acute inflammation decreased in 84.1% of patients after *H. pylori* eradication.<sup>3</sup> We then determined the bacteria that may potentially contribute to the persistent inflammation in patients after *H. pylori* eradication. Using linear discriminant analysis with effect size (Lefse), we identified *Acinetobacter*, *Ralstonia*, *Actinobacillus* and *Erwinia* as enriched while *Sphingomonas* and *Roseburia* as depleted bacterial genera in patients with

inflammation in the absence of *H. pylori* (figure 3A). At the OTU level, *Acinetobacter iwoffii*\_Otu180, *Actinobacillus para-haemolyticus*\_Otu100, *Prevotella nancei*\_Otu232, *Erwinia soli*\_Otu664, *Streptococcus anginosus*\_Otu278, *Acinetobacter rhizosphaerae*\_Otu208 and *Acinetobacter guillouiae*\_Otu81 were enriched in patients with persistent inflammation 1 year after *H. pylori* eradication (figure 3B).

### Non-*H. pylori* bacteria associated with gastric atrophy

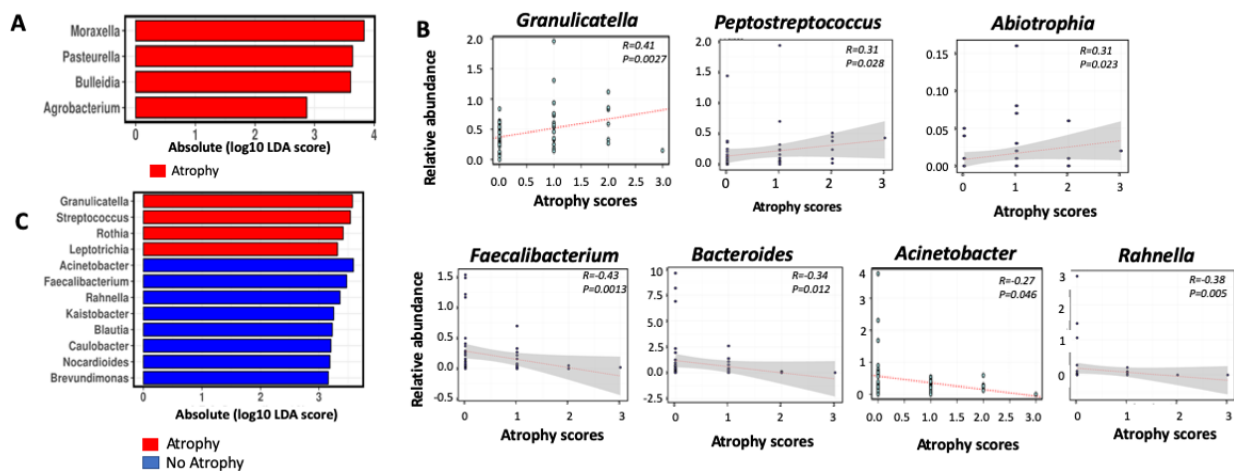
Histological changes induced by chronic *H. pylori* infection may be aggravated by gastric microbes. We investigated differentially abundant bacteria associated with atrophy before and after OAC treatment. Our analysis revealed that *Moraxella*, *Pasteurella*, *Bulleidia* and *Agrobacterium* were enriched in patients with atrophy before *H. pylori* eradication therapy (figure 4A). After *H. pylori* eradication treatment, atrophy scores decreased in 20.8% of subjects.<sup>3</sup> Correlation analysis on atrophy scores and bacteria abundance in OAC-treated group revealed that *Granulicatella* ( $R=0.41$ ,  $p=0.0027$ ), *Abiotrophia* ( $R=0.31$ ,  $p=0.023$ )



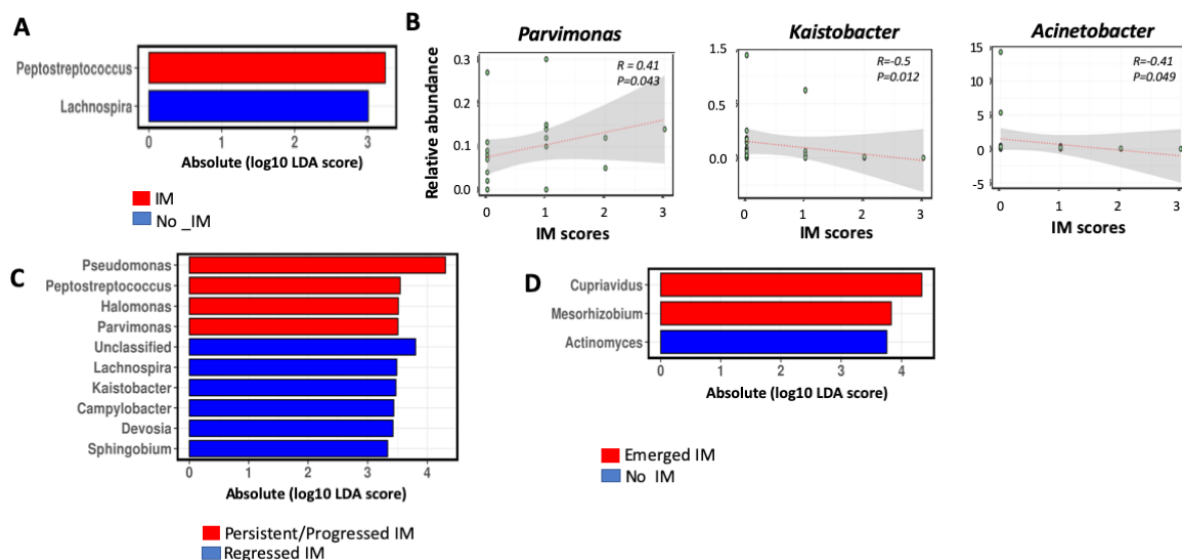
**Figure 3** Bacteria associated with inflammation after *Helicobacter pylori* eradication. Linear discriminant analysis (LDA) with effect size (Lefse) showing (A) bacterial genera and (B) bacterial OTU associated with inflammation 1 year after *H. pylori*. OTU, operational taxonomic unit.

and *Peptostreptococcus* ( $R=0.31$ ,  $p=0.028$ ) were positively correlated, while *Faecalibacterium* ( $R=-0.43$ ,  $p=0.0013$ ), *Rahnella* ( $R=-0.38$ ,  $p=0.005$ ), *Bacteroides* ( $R=-0.34$ ,  $p=0.012$ ), *Clostridium* ( $R=-0.29$ ,  $p=0.035$ ) and *Acinetobacter* ( $R=-0.27$ ,  $p=0.046$ ) were negatively correlated with atrophy scores (figure 4B). We further observed the enrichment

of *Granulicatella*, *Streptococcus*, *Rothia* and *Leptotrichia* in subjects with emerged atrophy 1 year after *H. pylori* eradication (figure 4C). *Acinetobacter*, *Faecalibacterium*, *Rahnella*, *Kaistobacter*, *Blautia*, *Caulobacter*, *Norcardioides* and *Brevundimonas* were more abundant in subjects with no atrophy development following *H. pylori* eradication.



**Figure 4** Bacteria associated with atrophy before and after *Helicobacter pylori* eradication. (A) Linear discriminant analysis (LDA) with effect size (Lefse) showing bacterial genera enriched in patients with atrophy before *H. pylori* eradication. (B) Spearman correlation of bacterial genera with atrophy scores after *H. pylori* eradication. (C) LDA with effect size (Lefse) showing bacterial genera enriched or depleted in patients with emerged atrophy 1 year after *H. pylori* eradication.



**Figure 5** Bacteria associated with intestinal metaplasia (IM) before and after *Helicobacter pylori* eradication. (A) Linear discriminant analysis (LDA) with effect size (Lefse) showing bacterial genera enriched or depleted in patients with IM before *H. pylori* eradication. (B) Spearman correlation of bacterial genera with IM scores after *H. pylori* eradication. (C) Linear discriminant analysis (LDA) with effect size (Lefse) showing bacterial genera enriched or depleted in patients with persistent IM or (C) emerged IM 1 year after *H. pylori* eradication.

### Non-*H. pylori* bacteria associated with intestinal metaplasia

We further investigated the association of non *H. pylori* microbes with IM. We found the enrichment of *Peptostreptococcus* and depletion of *Lachnospira* in patients with IM before OAC treatment (figure 5A). IM showed very little changes after receiving placebo or OAC.<sup>3</sup> Correlation analysis determined that *Parvimonas* ( $R=0.41$ ,  $p=0.043$ ) was positively correlated with IM scores while *Kaistobacter* ( $R=-0.5$ ,  $p=0.012$ ) and *Acinetobacter* ( $R=-0.41$ ,  $p=0.049$ ) were negatively correlated in patients with IM prior to *H. pylori* eradication (figure 5B). Among subjects with IM at baseline, *Pseudomonas*, *Peptostreptococcus*, *Halomonas* and *Parvimonas* were enriched in those with progressed or persisted IM, while *Lachnospira*, *Kaistobacter*, *Campylobacter*, *Devosia* and *Sphingobium* were enriched in subjects with regressed IM (figure 5C). Among subjects with no IM at baseline, *Mesorhizobium* and *Cupriavidus* were enriched while *Actinomyces* was depleted in those with emerged IM following *H. pylori* eradication (figure 5D). We observed that *Peptostreptococcus* was consistently positively associated with IM before and after OAC treatment.

### Functional features of gastric microbiota associated with inflammation, atrophy and IM after *H. pylori* eradication

The contribution of gut microbiota to pathological conditions are mediated by their functional capabilities. We used PICRUSt to infer the metagenome functional content based on the microbial community profiles obtained from 16S rRNA gene sequences. We found that the inflammation-associated gastric microbiota was enriched in oxidative phosphorylation, methane metabolism, chaperones and folding catalysis, energy metabolism, porphyrin and chlorophyll metabolism, as well as lipid biosynthesis functions, in the absence of *H. pylori* (figure 6A). This observation suggests an increase in energy generation and stress adaptation in the inflammation-associated gastric microbiota. Indeed, stress adaptation of the inflammation-associated microbiota was supported by the enrichment of K03088 which

encodes bacterial extracytoplasmic stress response protein RNA polymerase sigma-70 factor.<sup>16</sup> Other KEGG ontologies such as K09687, K09686, K02005 and K03413 encoding multidrug-resistant proteins which are bacterial virulent factors were enriched in inflammation-associated predicted metagenome. Several protein transport encoding genes were reduced in inflammation-associated gastric microbiota (figure 6B).

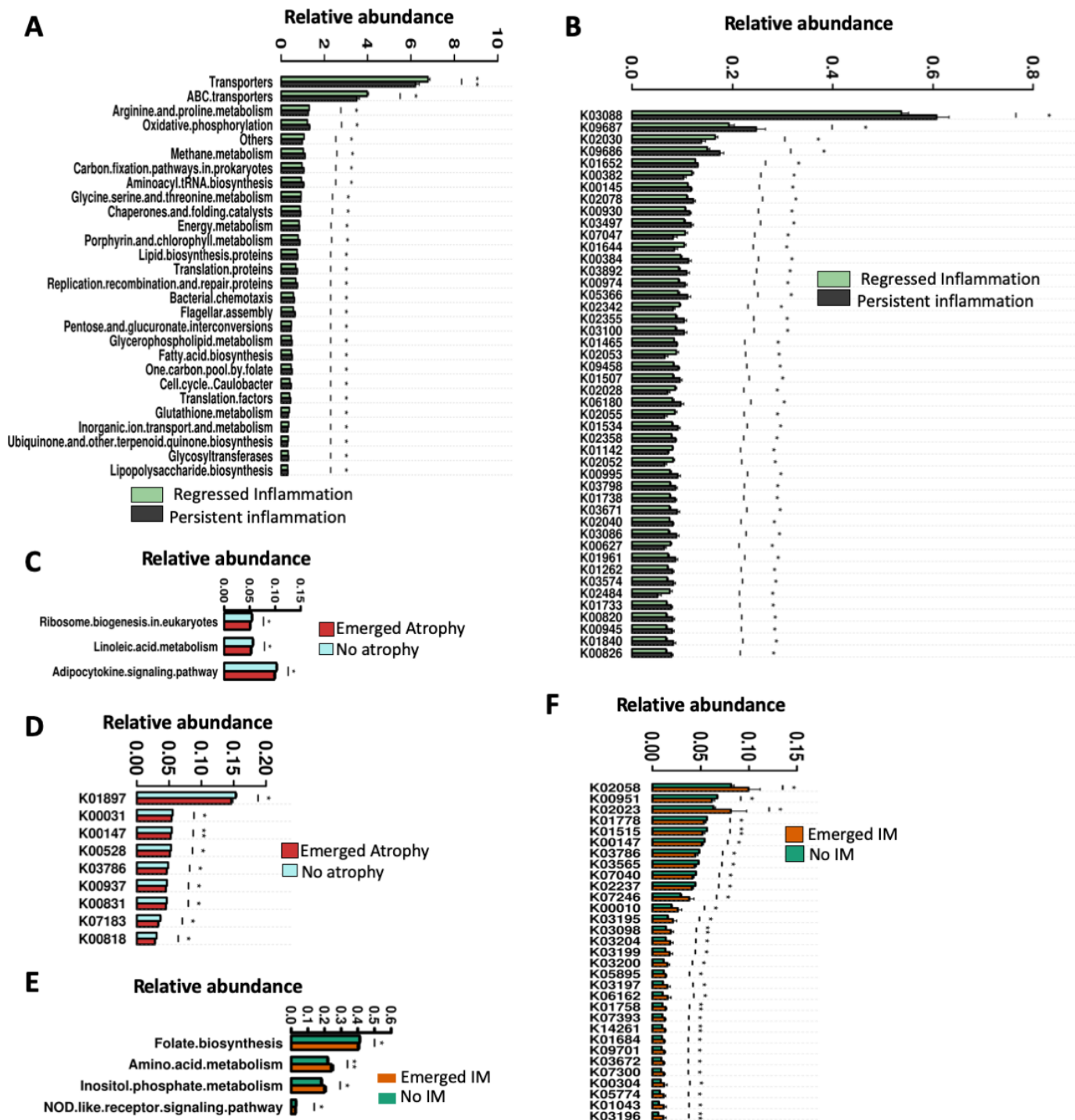
We also observed a reduction in ribosome biogenesis, linoleic acid metabolism and adipocyte signalling pathways (figure 6C) encoded by predicted genes K01897; long chain acyl coA synthetase, K00528; ferredoxin-NADP+ reductase, K03786; 3-dehydroquinate dehydratase, K07183; response regulator NasT, K00937; polysphosphate kinase, K00831; phosphoserine aminotransferase, K00818; acetylornithine aminotransferase, K00147; glutamate-5-semialdehyde dehydrogenase and K00031; isocitrate dehydrogenase in aggravated, persistent or emerged atrophy-associated microbiota compared with regressed atrophy following *H. pylori* eradication (figure 6D).

Amino acid metabolism and inositol phosphate metabolism were enriched while folate biosynthesis and NOD-like receptor signalling decreased in subjects whose IM emerged after *H. pylori* eradication (figure 6E). Several KEGG genes encoding virulence bacterial type IV secretion system known to be important in genetic exchange and delivery of effector molecules to eukaryotic target cells<sup>17</sup> were found enriched in subjects with emerged IM. These include K03195 (VirB10), K03204 (VirB9), K03199 (VirB4) and K03196 (VirB11) (figure 6F). Decrease of K00147; glutamate-5-semialdehyde dehydrogenase was also associated with emergence of IM as observed with atrophy, suggesting its protective importance against the progression of gastric precancerous lesions.

### DISCUSSION

Infection with *H. pylori* is an important risk factor in gastric cancer development, according to Correa's model.<sup>18</sup> Nevertheless, the risk of gastric cancer reduces only by less than 50%





**Figure 6** Functional features of gastric microbiota associated with inflammation, atrophy and IM after *H. pylori* eradication. (A) Predicted microbiota functional changes and (B) corresponding KEGG ontologies in persistent inflammation-associated gastric microbiota. (C) Predicted microbiota functional changes and (D) corresponding KEGG ontologies in GA-associated gastric microbiota. (E) Predicted microbiota functional changes and (F) corresponding KEGG ontologies in persistent IM-associated gastric microbiota. GA, gastric atrophy; IM, intestinal metaplasia; KEGG, Kyoto Encyclopedia of Genes and Genomes.

after *H. pylori* eradication therapy.<sup>19</sup> As earlier shown by us and others,<sup>3,19</sup> *H. pylori* eradication therapy alone does not preclude the development of precancerous gastric lesions. We therefore carried out this retrospective study and identified gastric microbes and associated functions that are associated with inflammation, atrophy and IM 1 year after *H. pylori* therapy.

Combined OAC treatment therapy for 1 week effectively eradicated *H. pylori*,<sup>3</sup> leading to significantly increased bacterial

diversity after 1 year. In addition to increased diversity, we observed a significant change in bacteria co-occurrence and a distinct cluster of oral microbes in the absence of *H. pylori*. *Haemophilus*, *Neisseria* and *Actinobacillus* were observed to be significantly reduced after OAC treatment. Coinfection of *H. pylori* and *N. subflava* has been reported to be associated with lymphoid follicle formation in human stomach.<sup>20</sup> *N. subflava* induced the production of interleukin 8 in gastric epithelial

cell line and may accelerate the development of gastric lesion in hypochlorhydric stomach.<sup>21</sup> The depletion of *Haemophilus*, *Neisseria* and *Actinobacillus* as observed in this study may be associated with the consequent reduction in inflammation in the OAC-treated group.

We identified high abundance of pathogenic microbes in subjects with persistent inflammation following *H. pylori* eradication. The enriched bacteria in subjects with inflammation included *Acinetobacter lwoffii*, *S. anginosus*, *Ralstonia*, *Erwinia* and *Prevotella*. A previous study carried out in a mouse model showed that infection with *A. lwoffii* induced gastrin production, proliferation of gastric G cells and parietal cell and similar gastric inflammation as *H. pylori* infection, corroborating our observation in this study.<sup>8</sup> Moreover, *Streptococcus* was isolated more frequently from the gastric juice of gastritis compared with healthy stomachs.<sup>22</sup> We and others have shown the increased abundance of *Prevotella* and *S. anginosus* in the mucosa of patients with gastric and oesophageal cancer.<sup>14 23 24</sup> In particular, *S. anginosus* is a human pathogen whose oral presence trended with higher risk of gastric cancer.<sup>25</sup> *Ralstonia* is a gram-positive bacterium with increasing incidence in respiratory infections.<sup>26–28</sup> It was enriched in the gastric mucosa of patients with gastric cancer before subtotal gastrectomy.<sup>29</sup> Taken together, the enrichment of these bacteria in subjects with inflammation in this study highlights their potential pathogenic role in promoting gastric inflammation.

As in other gut diseases such as IBD, colitis and colorectal cancer,<sup>30 31</sup> we found depletion of potentially gut-protective bacteria *Roseburia* and *Sphingomonas* in patients with inflammation. *Sphingomonas* is an aerobic and metabolically versatile bacterial genus that was characterised as being depleted in breast cancer tissues compared with healthy breast tissues.<sup>32</sup> *Roseburia* is associated with the formation of butyrate, a short-chain fatty acid reportedly protective against gut neoplasia.<sup>33</sup> It is important to note that short-chain fatty acids are also produced and absorbed in the stomach<sup>34</sup> and might be playing protective roles as described in the intestine.<sup>35</sup> The potential role of probiotics in alleviating gastric inflammation after *H. pylori* eradication has been previously demonstrated,<sup>36 37</sup> further supporting our findings in this study and suggest that the prescription of probiotics to patients receiving *H. pylori* eradication therapy may prevent gastric inflammation triggered by other gastric microbes.

The development of gastric atrophy and IM leads to loss of acid-secreting parietal cell.<sup>38</sup> This may favour the proliferation of gastric microbes or colonisation of the stomach by oral microbes.<sup>39</sup> We recognised that the emergence of atrophy or IM was associated with increased abundance of *Granulicatella*, *Actinomyces*, *Rothia*, *Peptostreptococcus*, *Streptococcus*, *Abiotrophia* and *Parvimonas*, in the absence of *H. pylori*. These are predominantly oral microbes and form a distinct cluster in the microbial ecology of the OAC-treated patients. Their co-occurrence may be important to their neoplasia-promoting effect and subsequent development of gastric cancer as we previously reported.<sup>26</sup> *Granulicatella* is gram-positive facultative anaerobe linked to root canal infection and was isolated from the peritoneal fluid of a patient with gastric cancer,<sup>40 41</sup> while *Abiotrophia* was highlighted as a potential risk element for gastric cancer,<sup>25</sup> further supporting their potential involvement in development of gastric atrophy. *Mesorhizobium*, a nitrogen-fixing bacterium, which was characterised as being able to invade human cells,<sup>42</sup> was enriched in patients whose IM progressed within 1 year following *H. pylori* eradication. Bacteria producing carcinogenic nitroso compounds through the conversion of nitrates are thought to be important drivers of gastric neoplasia.<sup>43</sup>

Interestingly, *Faecalibacterium*, *Kaistobacter* and *Rahnella* were depleted in subjects whose atrophy emerged following *H. pylori* eradication. Anticarcinogenic role has been demonstrated for *F. prausnitzii* in GI diseases including IBD, colitis-associated colorectal cancer and hepatocellular carcinoma.<sup>44 45</sup> We identify its potential protective role in gastric neoplasia for the first time in this study. Notably, we observed that different sets of bacteria are associated with atrophy and IM, before and after *H. pylori* eradication. The significance of this is that the presence of *H. pylori* changes the gastric milieu which might influence the microflora of the stomach. It would be interesting to follow the effects of different microbiota in the process of gastric carcinogenesis in the future.

Moreover, we demonstrated that energy generation and adaptation to stress was upregulated in the gut microbiota of subjects with gastric inflammation. This observation is further supported by the enriched bacterial extracytoplasmic stress response protein RNA polymerase sigma-70 factor<sup>16</sup> in inflammation-associated gastric microbiota. Our finding is compatible with decline of host antioxidant defenses and consequent physiological and oxidative stress that characterise the stomach during gastric inflammation.<sup>46</sup> Moreover, folate biosynthesis and Nod-like receptor signalling had significantly higher predicted abundances in the gastric microbiota of subjects with IM after *H. pylori* eradication. Folate biosynthesis by the gut microbiota is a source of the water-soluble tetrahydrofolates which play an essential role in human metabolism.<sup>47</sup> Inadequate status of folate has been implicated in increased risk of colon cancer especially if precancerous lesions exist.<sup>48</sup> This may be applicable to gastric carcinogenesis, given our observation in this study. Optimisation of bacterial production of folate may reduce the risk of IM development.<sup>48</sup> Nucleotide oligomerisation domain containing proteins (NOD) are conserved pattern recognition receptors that are stimulated by pathogen-associated molecular patterns (PAMPS). NOD-like receptors (NLR) can regulate innate and adaptive immune response and may participate in carcinogenesis by regulating cell apoptosis.<sup>49</sup> The observed reduction in NLR signalling by IM-associated gut microbiota may represent a means through which immune response to PAMPS are dampened, consequently promoting the development of gastric lesions. Several predicted genes encoding virulence bacterial type IV secretion system, namely, VirB10, VirB9, VirB4 and VirB11, known to be important in genetic exchange and delivery of effector molecules to eukaryotic target cells<sup>17</sup> were found enriched in subjects with emerged IM. These data suggest that the gastric microbes reduce folate biosynthesis, dampen host immunity and deliver effector molecules into host cells in promoting IM development.

In conclusion, this study identified gastric pathogenic microbes that are associated with persistent inflammation (*A. lwoffii*, *S. anginosus* and *Ralstonia*) and atrophy/IM (*Granulicatella*, *Actinomyces*, *Rothia*, *Peptostreptococcus*, *Streptococcus*, *Abiotrophia* and *Parvimonas*) in patients 1 year following *H. pylori* eradication, which are predominantly oral microbes and form a distinct cluster in the microbial ecology in the absence of *H. pylori*. In keeping with the pathogenic ecology, functional pathways including amino acid metabolism and inositol phosphate metabolism were enriched while folate biosynthesis and NOD-like receptor signalling decreased in atrophy/IM-associated gastric microbiota. Thus, our longitudinal study highlights the potential contribution of gastric microbes in the development and perpetuation of precancerous gastric lesions in the absence of *H. pylori*, which may serve as therapeutic targets for the prevention of gastric carcinogenesis.



**Contributors** JJYS designed, supervised the study, performed the clinical trial, collected the tissue samples and edited the manuscript. OOC performed bioinformatics analyses and drafted the manuscript. EC, CHS, STYL and HCHL performed laboratory experiments. JY supervised the study and wrote the manuscript.

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