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Research article

Influence of *helicobacter pylori* on composition and function of gastric microbiota in patients with chronic non-atrophic gastritis

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ABSTRACT

Objective: *Helicobacter pylori* (*H. pylori*) plays a major role in causing and advancing gastrointestinal illnesses. Our aim is to analyze the unique makeup and functional changes in the gastric microbiota of patients with chronic non-atrophic gastritis (CNAG), regardless of the presence of *H. pylori*, and to determine the potential signaling pathways.

Methods: We performed metagenomic sequencing on gastric mucosa samples collected from 17 individuals with non-atrophic gastritis, comprising 6 cases were infected with *H. pylori* (*H. pylori*-infected case group) and 11 cases without (control group). The species composition was evaluated with DIAMOND software, and functional enrichment was assessed utilizing the Kyoto Encyclopedia of Genes and Genomes (KEGG) database. We analyzed antibiotic resistance patterns using the Comprehensive Antibiotic Resistance Database as a reference (CARD).

Results: The presence of *H. pylori* colonization in CNAG patients was associated with increased diversity in the gastric microbiota. The Phylum Firmicutes was found to be less prevalent, while the Phylum Proteobacteria showed an increase. Functionally, pathways associated with metabolic pathways, including vitamins, auxiliaries, amino acid residue, carbon hydrate, and metabolic energy pathways, were enriched in CNAG patients with *H. pylori* infection. Additionally, antibiotic resistance genes correlated with antibiotic efflux pump were enriched.

Conclusions: From a holistic genomic perspective, our findings offer fresh perspectives into the gastric microbiome among CNAG patients carrying *H. pylori*, which is valuable for future research on CNAG.

1. Introduction

Chronic non-atrophic gastritis (CNAG) involves persistent inflammation of the stomach lining due to various pathogenic factors, distinguishing it as a subtype of persistent gastric inflammation [1]. The stomach lining is predominantly infiltrated by lymphocytes and plasma cells and may exhibit erosion and bile reflux [2]. The main goal of CNAG treatment is to improve clinically related symptoms, remove etiology, and protect gastric mucosa, in order to enhance patients' life satisfaction [3]. Moreover, to prevent the progress of CNAG and reduce or prevent persistent gastric atrophy, intestinal lining cells transformation, intraepithelial neoplasia and

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stomach cancer is also imperative [4].

The stomach, as a specialized organ, harbors a unique bacterial composition due to its distinct environment shaped by gastric acid, peristaltic movements, and mucus thickness. This specialized gastric environment maintains relative sterility within the gastric cavity by inhibiting bacterial colonization through high acidity [5]. However, a certain portion of acid-resistant bacteria are capable of residing in the gastric mucosa, such as the Streptococcus, Lactobacillus, and Neisseria [6]. It has been well-recognized that the induction of gastrointestinal diseases is closely correlated with several factors, such as genetic polymorphisms, environmental factors, lifestyle habits, as well as infection pathogens [7]. Among these pathogens, *Helicobacter pylori* (*H. pylori*) is a notable pathogen that has been associated with a range of gastrointestinal diseases such as peptic ulcers, chronic gastritis, stomach cancer, and mucosa-associated lymphoma *Helicobacter pylori* (*H. pylori*), a notable pathogen, is linked to various gastrointestinal disorders including peptic ulcers, chronic gastritis, stomach cancer, and lymphoma of mucosal origin [8,9]. Increasing evidence suggests that *H. pylori* can cause changes to the gut and stomach microbiota [5,10]. A recent study proposed that eradicating of *H. pylori* might reestablish the gastric "microbiome to a condition resembling that of uninfected individuals [11]. The exact impact of *H. pylori* on the gastric mucosa microbiota in CNAG patients remains incompletely understood.

In recent years, advancements in advanced genomic analysis technologies have facilitated comprehensive exploration of the microbiota within the gut, intestines, and stomach [12,13]. This investigation involved gathering gastric mucosa tissue specimens obtained from CNAG patients infected with *H. pylori* and employed metagenomics to analyze the microbiota composition in these mucosal tissues. By comparing them with a control group, we identified the metabolic signaling pathways and antibiotic resistance genes associated with *H. pylori* infection (short as HPI) in CNAG.

2. Methods

2.1. Study design

20 gastric mucosa samples were collected in this study, all obtained from patients with CNAG who underwent urease activity testing for *H. pylori*, at the Department of Gastroenterology, Peking University Shougang Hospital. The endoscopic identification of CNAG adhered to the classification and grading standards established by the Chinese Society of Digestive Endoscopy [9]. Subsequently, 17 samples were chosen for additional microbiota examination, considering the appropriateness of DNA quality and quantity for metagenomics sequencing and analysis. Among these samples, 6 had as HPI case group while 11 were not (control group, short as C). Participant information is presented in Table 1. The Ethics Committee of Peking University Shougang Hospital approved the present research. [IRBK2019-009-01] and exemption from informed consent was approved by the Ethics Committee of Peking University Shougang Hospital. Experimental procedures adhered to pertinent regulations and guidelines.

2.2. Library construction and sequencing

Genomic DNAs were extracted and randomly truncated into DNA fragments. After repairment, the DNA fragments were railed and ligated with Illumina adapters. After quantification with Qubit and quantitative real-time PCR, the assembled DNA pool was examined for gene expression and size profile. Subsequently, based on the adequate pool density and data requirements, the libraries were pooled and analyzed using Illumina sequencing systems.

Table 1
Patient information.

Sample number	Age	Gender	Urease	Clinical diagnosis
H1	68	Female	+	chronic non-atrophic gastritis
H2	26	Male	+	chronic non-atrophic gastritis
H3	33	Male	+	chronic non-atrophic gastritis
H4	67	Female	+	chronic non-atrophic gastritis
H5	49	Male	+	chronic non-atrophic gastritis
H6	39	Male	+	chronic non-atrophic gastritis
C1	64	Female	–	chronic non-atrophic gastritis
C2	32	Female	–	chronic non-atrophic gastritis
C3	57	Female	–	chronic non-atrophic gastritis
C4	21	Male	–	chronic non-atrophic gastritis
C5	63	Female	–	chronic non-atrophic gastritis
C6	56	Male	–	chronic non-atrophic gastritis
C7	33	Female	–	chronic non-atrophic gastritis
C8	37	Male	–	chronic non-atrophic gastritis
C9	66	Male	–	chronic non-atrophic gastritis
C10	53	Female	–	chronic non-atrophic gastritis
C11	57	Female	–	chronic non-atrophic gastritis

H: patients infected with *H. pylori*.
C: patients do not infect with *H. pylori*.

2.3. Data processing

Utilizing the Readfq tool (Version 8, accessible at https://github.com/cjfields/readfq), the Illumina HiSeq sequencing system processed the raw data to generate Clean Data suitable for further analysis. The detailed steps involved in the processing were as follows: a) Sequencing data containing low-grade nucleotides, identified by a quality criterion value of less than 38, were eliminated, and exceeded a certain proportion (with a default length set at 40 bp); b) Removing reads where the N base composition met a defined proportion cutoff (default length set at 10 bp).; c) Excluding analysis with substantial adapter sequence overlap. (with the default length set at 15 bp). To tackle potential host contamination, the Clean Data underwent alignment with a host database using standard Bowtie2.2.4 software (available at http://bowtiebio.sourceforge.net/bowtie2/index.shtml) to filter out host-derived reads [14].

2.4. Metagenome assembly

Using MEGAHIT software (version 1.0.4-beta), the clean data underwent merging. This merging process involved the interruption of assembled Scaftigs at points of N connection, resulting in Scaftigs that were free from N bases. Subsequently, using Bowtie2.2.4 software, the Clean Data of each specimen were individually aligned with the respective Scaffolds to identify and collect Paired-End (PE) reads not utilized in the initial assembly.

2.5. Gene prediction and abundance analysis

The merging Scaffolds, originating from separate assemblies and exceeding 500 bp in length, underwent open reading frame (ORF) identification via the MetaGeneMark tool (Version 2.10, http://topaz.gatech.edu/GeneMark/). Subsequently, the ORF predicted were processed to remove any sequences with lengths below 100 nt, employing the default settings of the MetaGeneMark software. The CD-HIT software (The version is 4.5.8, http://www.bioinformatics.org/cd-hit) was utilized with specific parameter settings (the value of -c is 0.95, the value of -G is 0, the value of -aS is 0.9, the value of -g is 1, the value of -d is 0) to curate an initial non-redundant gene set. The preliminary gene inventory was mapped with Clean Data from each sample using Bowtie 2.2.4. This step facilitated the quantification of reads aligning to each gene across the samples. Genes with a read count of less than two in any sample were subsequently removed, culminating in the final gene catalogue known as Unigenes, which was utilized for subsequent analyses. Gene abundance for each sample was calculated by matching reads with genes and factoring in the length of the genes. This information formed the basis for various analyses, including pan-genome evaluations, statistical summaries, cross-specimen relationship analyses, and Gene quantities illustrated through Venn diagrams, all predicated on the gene abundance within each sample's catalogue [15].

2.6. Taxonomy prediction

The DIAMOND tool (The version is 0.9.9, available at https://github.com/bbuchfink/diamond/) was utilized to conduct a BLAST search of the assembled transcripts against an extensive sequence database comprising Bacteria, Fungi, Archaea, and Viruses, all sourced from the NCBI NR database. (Version: 2018-01-02, https://www.ncbi.nlm.nih.gov/). Given that a single sequence may yield multiple alignment outcomes, the process involves selecting an alignment where the E-value is less than ten times the smallest E-value encountered. This chosen alignment is then subjected to the Lowest Common Ancestor (LCA) algorithm, which is integrated within the MEGAN software for systematic classification. The application of the LCA algorithm ensures the accurate species annotation of the sequences under analysis [16].

2.7. Differential expression analysis

Utilizing version 1.18.0 of the DESeq R package, a study was conducted to investigate the variations in gene expression among two distinct groups. DESeq offers a suite of statistical methods designed to identify variations in digital gene expression, using a model based on a negative binomial distribution. Following the completion of the study, we applied the Benjamini and Hochberg correction to the P-values to manage the false discovery rate. The genes flagged with differential expression detected by DESeq showed a significant corrected P-value of less than 0.05. Prior to commencing the study of variations in gene expression, sequence count normalization for each experimental dataset was conducted utilizing a consistent scaling factor, facilitated by the edgeR program package.

Additionally, utilizing version 1.20.0 of the DEGSeq R package, an analysis was executed to compare gene expression levels across two conditions. The statistical significance (P-values) from this analysis were adjusted utilizing the Benjamini-Hochberg method. The thresholds for considering a gene as significantly differentially expressed were set at a threshold of adjusted P-value less than 0.005 and a minimum absolute log2 fold change of 1 [17].

2.8. KEGG pathway enrichment analysis

KEGG (http://www.genome.jp/kegg/) functions as an extensive database repository that facilitates the comprehension of the high-order functions and operational aspects of biological systems, including the cellular, organism and ecosystem levels. This understanding is derived from molecular-level information, with a particular focus on large-scale molecular datasets that are produced through genomic analysis and other state-of-the-art large-scale experimental methods. To perform the enrichment analysis, we

employed the KOBAS software to statistically evaluate the overrepresentation of differentially expression genes within the context of the KEGG pathways [18].

2.9. Resistance gene annotation

The Unigenes were aligned against the Comprehensive Antibiotic Resistance Database (CARD) utilizing the Resistance Gene Identifier (RGI) tool, available at https://card.mcmaster.ca/. This process aimed to identify antibiotic resistance genes (AROs). The prevalence of the detected AROs was assessed using the alignment results. Subsequently, a series of graphical representations were created to visualize the data: bar charts illustrating the abundance of AROs, heatmaps depicting the clustering of abundance patterns, and comparative analyses highlighting the variations in resistance gene counts across different groups. Furthermore, the distribution of resistance genes across each sample was examined, along with a species attribution analysis to classify the resistance genes. Additionally, an investigation into the underlying resistance mechanisms was conducted to better understand the genetic basis of the observed resistance traits [19].

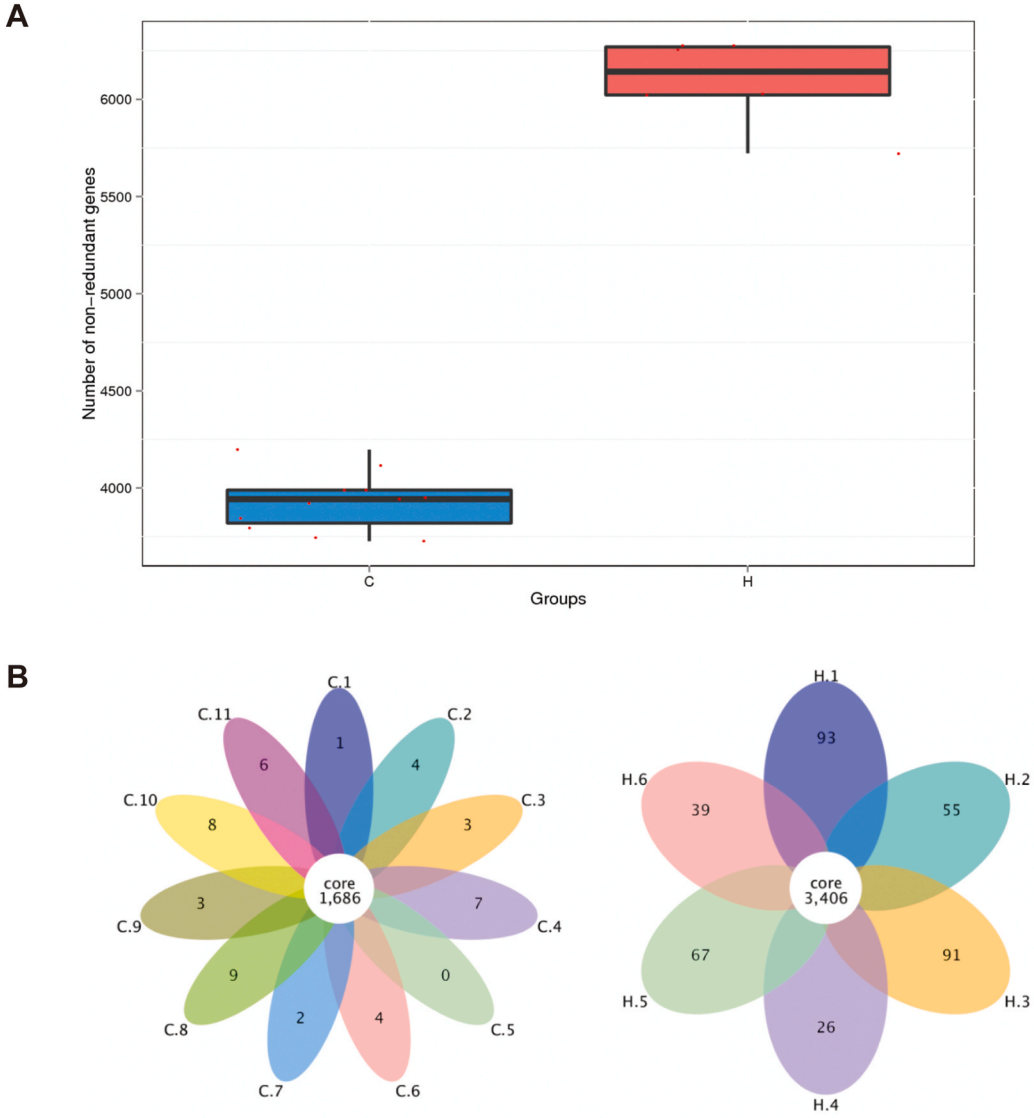

Fig. 1. Analysis of differences on gene numbers. (A) The abundant genes in gastric mucosa samples from non-infected (short as C) and *H. pylori*-infected (short as H) groups. (B) The shared genes in samples of each group were shown in Venn graphs.

2.10. Statistics

Paired difference tests and multiple comparisons were performed to evaluate differences between each group. The statistical assessments were performed with the Wilcoxon signed-rank test in R software.

3. Results

To investigate how *H. pylori* affects the mucosal microbiota in stomach mucosa of patients with CNAG, we obtained gastric mucosal samples from 17 individuals diagnosed with non-atrophic gastritis. Among these, there were 6 cases with presence of *H. pylori* (referred to as the HPI case group, abbreviated as H), while 11 cases were not infected (control group, short as C). All mucosal samples underwent preparation for shotgun metagenomics sequencing. Subsequently, we conducted an analysis of microbial compositions and identified specific functional pathways associated with the altered microbiota spectrum.

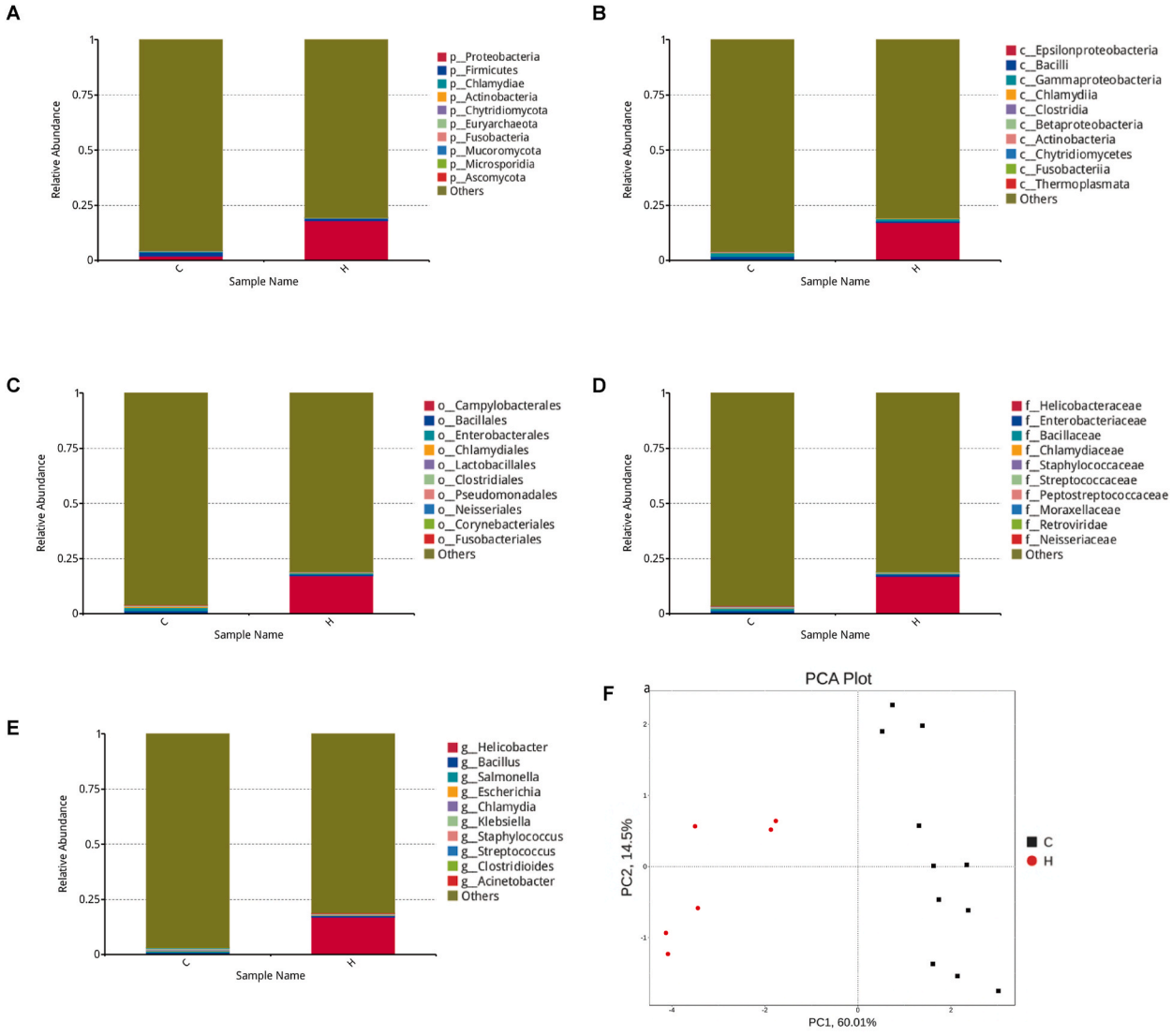

Fig. 2. Analysis of microbiota composition. The top 10 species from species annotation results with maximum relative abundance at (A) phylum level, (B) class level, (C) order level, (D) family level, and (E) genus level. (F) PCA (Principal Component Analysis) of species abundance at phylum level. Red points represent *H. pylori*-infected samples; Black points represent non-infected samples. C: patients do not infect with *H. pylori*. H: patients infected with *H. pylori*. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

3.1. Analysis of differences on gene numbers

To compare the gene counts for both the H and C groups, we generated a box plot. (Fig. 1A). The gene counts of HPI specimens was markedly exceeding those in the non-infected group, suggesting a potentially abundant microbial species upon HPI. The common and unique gene information in samples of each group was analyzed and shown as Venn diagrams (Fig. 1B), which indicated that the HPI samples shared more common genes than the non-infected patients.

3.2. Analysis of microbiota composition

We next performed a taxonomic analysis of gastric mucosal microbiomes to assess which metagenomic species set the two groups apart. We visually displayed the top 10 species from species annotation (sorted from the most to the least abundant in biopsies) with

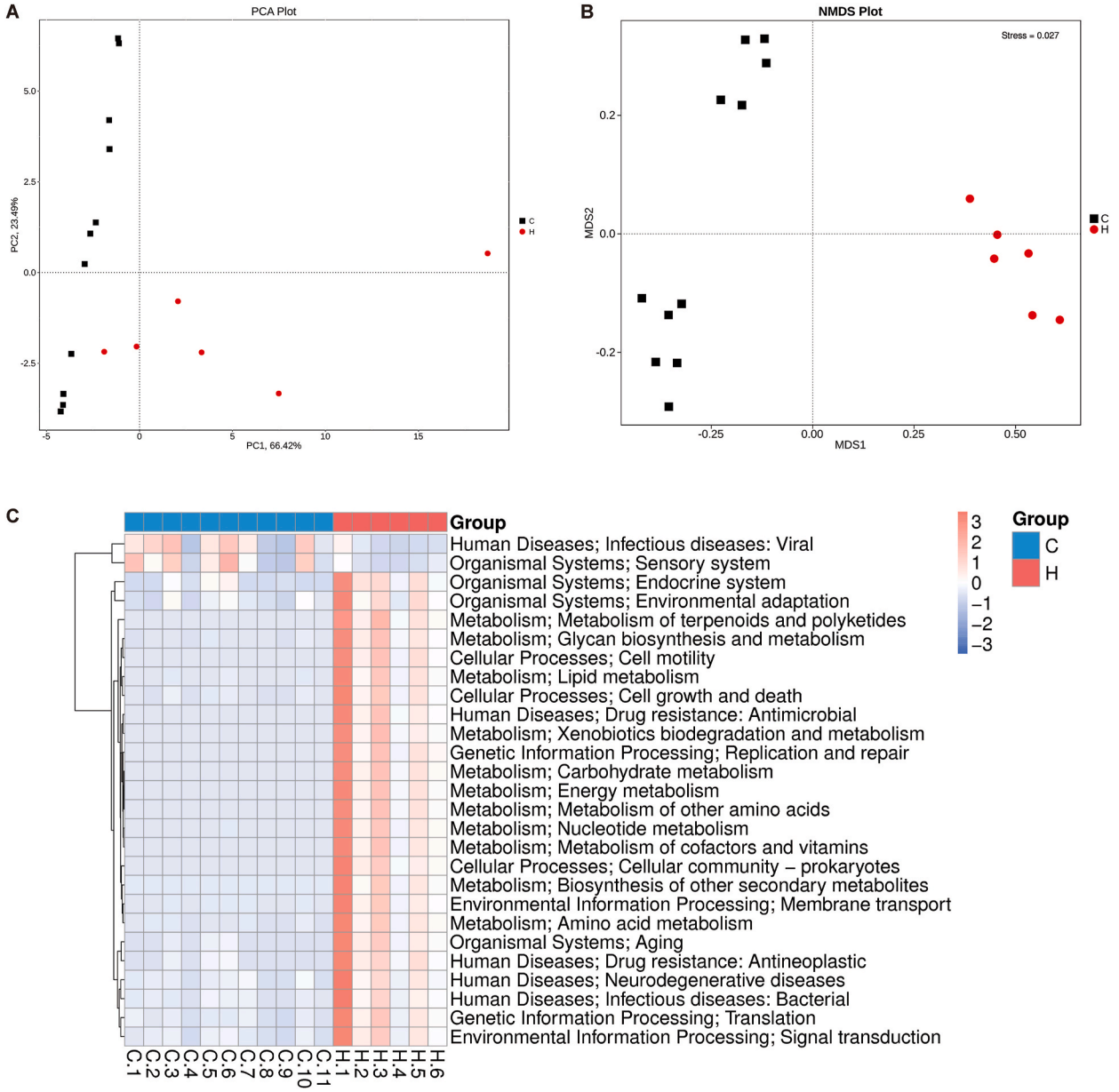

Fig. 3. Functional analysis by KEGG signaling pathways and genome-scale metabolic models. (A) PCA and (B) NMDS of signaling pathway enrichment. Red points represent *H. pylori*-infected samples; Black points represent non-infected samples. (C) Heatmap of enriched signaling pathways. C: patients do not infect with *H. pylori*. H: patients infected with *H. pylori*. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

maximum relative abundance at various taxonomic levels (order, family et al.) in each specimen. The Phylum Proteobacteria and Phylum Firmicutes were the predominant ones in both groups illustrated in Fig. 2A. The proportion of Phylum Proteobacteria was notably elevated and the proportion of Phylum Firmicutes was decreased in HPI samples, compared with the non-infected ones (Fig. 2A). Further classification of the enriched phylum highlighted the Class Epsilon Proteobacteria (Fig. 2B), Order Campylobacter (Fig. 2C), Family Helicobacteraceae (Fig. 2D), and Genus Helicobacter (Fig. 2E) as the predominant genera abundant in HPI gastric mucosa. Principal Component Analysis (PCA) was conducted *using species within phy*la to evaluate the differences in species abundance. The significant distance between points from the two groups indicated the differed species abundance in HPI and non-infected gastric

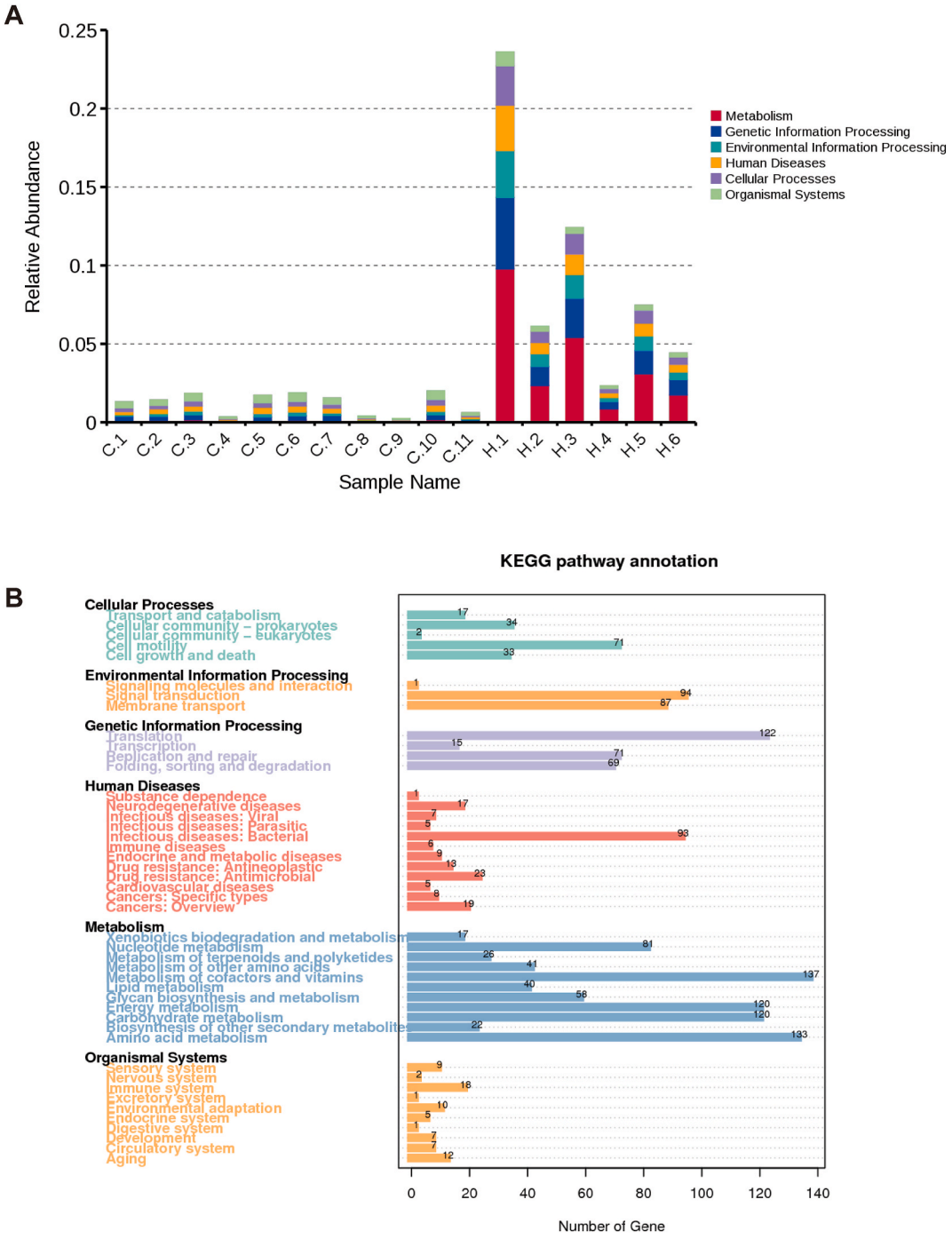

Fig. 4. Functional analysis of genome-scale metabolic signaling. (A) Bar graph of relative abundant pathways in *H. pylori*-infected (short as H) and non-infected groups (short as C). (B) Annotation of KEGG signaling pathways.

mucosa (Fig. 2F).

3.3. Functional analysis by KEGG signaling pathways and genome-scale metabolic models

To explore the deeper meanings of the roles played by the altered dominant microbiota species in gastric mucosa, we performed KEGG functional analysis. The PCA and NMDS (Non-Metric Multi-Dimensional Scaling) analysis (Fig. 3A and B) revealed significantly differed pathway abundance between HPI and non-infected gastric mucosa. We displayed the enriched signaling using the heatmap which showed that the differed genes mainly enriched in signaling pathways correlated with metabolism, as well as genetic information processing (Fig. 3C). Moreover, we annotated the 6 major metabolic pathways to analyze the enrichment of differently expressed genes (Fig. 4A). Analysis of annotated unigenes indicated that the pathways with the most significant enrichment were those pertaining to metabolism of vitamins,coenzymes, carbohydrates, amino acids, and energy, as well as other pathways (Fig. 4B, Fig. S1, and Fig. S2).

3.4. Antimicrobial resistance genes distribution in HPI gastric mucosa

We then utilized the Resistance Gene Identifier (RGI) tool to compare the Unigenes against the CARD database, aiming to assess the prevalence of antimicrobial genes associated with antimicrobial resistance in the two groups (Table S1). We identified that the gene family of the major facilitator superfamily MFS (shown as hp1181) is remarkably enriched in samples with HPI (Fig. 5A). As for resistance mechanism, we identified that this gene family is correlated with antibiotic efflux pump and resistance to antibiotics including fluoroquinolone, tetracycline, and nitroimidazole antibiotic (Table S1). Furthermore, we systematically categorized the modes of resistance exhibited by the listed genes in the CARD database and provided detailed descriptions of their interactions. between the operational mechanisms of these identified resistance genes and the different species within the microbiota. As shown in Fig. 5B, the function of antibiotic efflux pump is correlated with the Phylum Proteobacteria, which is identified to be highly abundant in HPI gastric mucosa as manifested in Fig. 2A.

4. Discussion

HPI is highly associated with chronic atrophic gastritis [20,21]. This study focuses on patients with CNAG, considered the pre-state of stomach cancer, and divided them into HPI and -noninfected groups to analyze the differences in gastric mucosal flora using the Metagenome method. Metagenomics was first reported by Handelman [22] to directly analyze all genome information of microbial populations. Later, Kevin and colleagues [23] defined Metagenomics, as the discipline of "bypassing the extraction and incubation of individual microorganisms and applying genomics techniques to analyze the microbial communities in natural environment". By

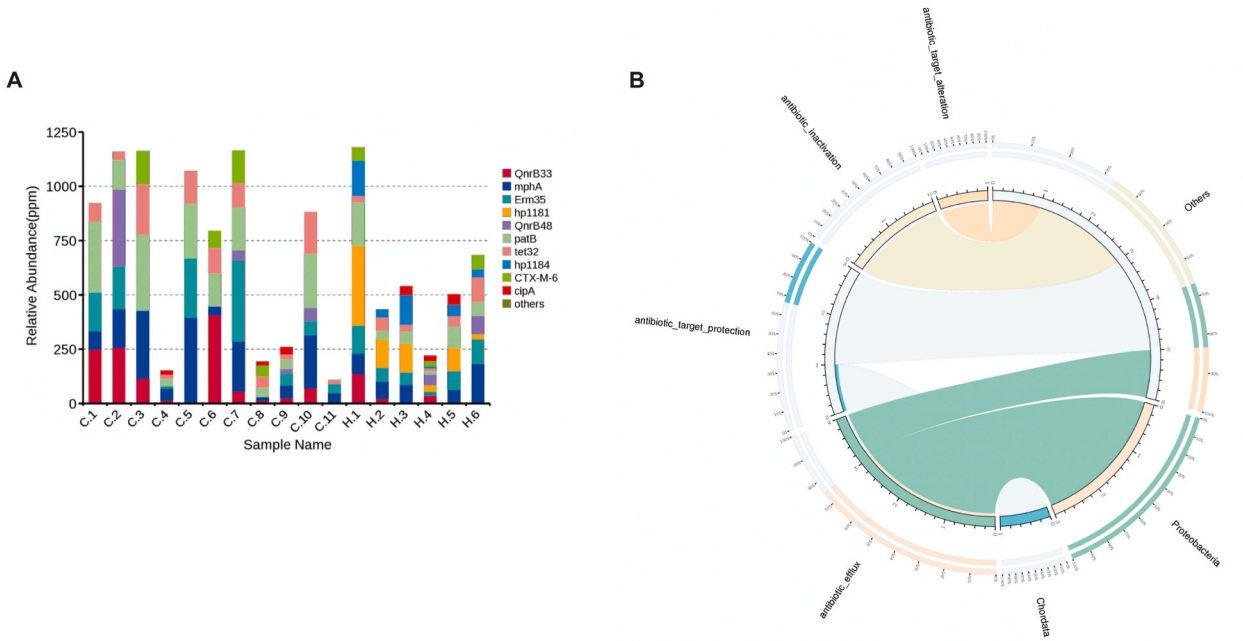

Fig. 5. Antimicrobial resistance genes distribution in *H. pylori*-infected gastric mucosa. (A) Abundance histogram of antimicrobial resistance genes in *H. pylori*-infected (short as H) and non-infected samples (short as C). (B) Circle diagram of resistance mechanisms and microbiota species (on the right is phylum level species information and on the left is resistance mechanism information).

bypassing the extraction and incubation stages of microorganisms, this method presents a more effective strategy for examining non-separable microorganisms. It offers a more accurate depiction of microbial composition and interaction within the sample. It also aids in the molecular-level investigation of microbial metabolism and genomic activities [24].

Increasing number of studies have adopted Metagenomics for better analysis of microbiota various diseases. For example, a recent research described changes in gut microbiota following gastrectomy by performing shotgun metagenomics sequencing and suggested its association with postoperative comorbidities [25]. A study of 276 stomach cancer patients without preoperative chemotherapy showed a reduction in bacterial content within both tumoral and peritumoral niches. Furthermore, the intricate network of gastric bacteria was found to be less complex within the tumor environment [26]. Zhang and colleagues reported that the most representative microbiota species abundant in stomach cancer corresponded to opportunistic or commensal microbiota that commonly reside in the oral cavity, such as genera Neisseria, Aggregatibacter, and Alloprevotella [27]. Here, we identified that CNAG patients with HPI are abundant in Phylum Proteobacteria compared with the *H. pylori*-negative patients. Research indicates that abnormal gut microbiota often attributes to a sustained elevation in the abundance of the phylum Proteobacteria, which is usually minor in the natural human gut flora [28,29]. Furthermore, Proteobacteria not only function in intestinal diseases but also in extraintestinal diseases [30].

Subsequently, we analyzed the signaling pathways that enriched from the microbial species by performing KEGG orthology analysis. According to the KEGG, biochemical pathways are classified into six categories: Human Diseases, Metabolism, Cellular Processes, Environmental Information Processing, Genetic Information Processing and Organismal Systems. We observed an overall elevation of biological pathways in the HPI tissues, with the metabolism pathways being the most notably enriched, including the metabolism of vitamins, coenzymes, carbohydrates, amino acids, and energy.

Resistance genes are frequently found in both environmental and human intestinal bacteria [31,32]. The overuse of antibiotics leads to permanent changes in microbial community in both the environment and the human microbiome, posing threats to human health and damaging the ecological balance [33,34]. Hence, studies on resistance genes have attracted great attention of multiple researchers [35]. In recent years, the CARD has risen to prominence as a favored database for resistance genes, known for its comprehensive data, user-friendly interface, and regular updates [36]. Additionally, we normalized the microbiota richness to analyze the differences in antibiotic resistance capacity for both *H. pylori*-positive and *H. pylori*-negative CNAG patients. We found that the major facilitator superfamily (MFS) antibiotic efflux pump, linked to resistance against various antibiotics including fluoroquinolone, tetracycline, and nitroimidazole antibiotics, is significantly prevalent among *H. pylori*-positive CNAG patients. The classification of resistance mechanisms of the resistant gene showed its correlation with Phylum Proteobacteria, consistent with the results from enriched microbiota species.

5. Conclusions

The application of metagenomics analysis effectively and comprehensively illustrated the alterations in microbiota species among CNAG patients whether they have HPI. In contrast to previous publications on CNAG utilizing 16S sequencing, this study employed metagenomics to investigate the distribution of microflora and their genes in CNAG patients from a metabolic perspective. Functional analysis of the metagenomics data revealed significant metabolic changes following HPI. Our study offers novel insights into the gastric microbial composition associated with HPI in CNAG patients, providing valuable information for future investigations on CNAG. However, besides the small sample size, certain limitations exist in our current study, such as the need for further validation of the speculated regulatory mechanisms using both in vivo and in vitro studies.

Ethics approval and consent to participate

This study received ethical approval from the Institutional Review Board (IRB) of Peking University Shougang Hospital [IRBK2019-009-01] and the Ethics Committee of Peking University Shougang Hospital also approved the exemption from informed consent.

Consent for publication

Not applicable.

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Data availability statement

Data will be made available on request.

CRediT authorship contribution statement

Fan Zhao: Writing – review & editing, Writing – original draft, Supervision, Resources, Project administration, Investigation, Formal analysis, Data curation, Conceptualization. **Linlin Yana:** Writing – original draft, Methodology, Formal analysis, Data curation.

Pengfei Wang: Validation, Methodology, Formal analysis, Data curation. **Ke Zhang:** Validation, Software, Methodology, Formal analysis, Data curation. **Shoukui Hu:** Writing – review & editing, Resources, Funding acquisition, Data curation, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgements

Not applicable.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.heliyon.2024.e31472.

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