Effect of *Helicobacter pylori* Infection on the Composition of Gastric Microbiota in the Development of Gastric Cancer

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Abstract

**Background:** Gastric cancer is one of the most common cancer types worldwide. In China, gastric cancer has become one of the major threats for public health, ranking second on incidence and third on cause of cancer death. Despite the common risk factors that promote the development of gastric cancer, the huge quantity of microorganism colonies within the gastrointestinal tract, particularly *Helicobacter pylori* infection, demonstrates a correlation with chronic inflammation and gastric carcinogenesis, as epidemiological studies have determined that *H. pylori* infection confers approximately 75% of the attributable risk for gastric cancer. **Summary:** The current article draws an overview on the correlation between the microbiota, inflammation and gastric tumorigenesis. *H. pylori* infection has been identified as the main risk factor as it triggers epithelial barrier disruption, survival signaling as well as genetic/epigenetic modulation. Apart from *H. pylori*, the existence of a diverse and complex composition of microbiota in the stomach has been identified, which supports a role of microbiota in the development of gastric cancer. Moreover, metagenomics studies focused on the composition and function of the microbiota have associated microbiota with gastric metabolic diseases and even tumorigenesis. Apart from the gastric microbiota, inflammation is another identified contributor to cancer development as well. **Key Message:** Though *H. pylori* infection and the non-*H. pylori* microbiota play a role in gastric cancer, the properties of gastric microbiota and mechanisms by which they participate in the genesis of gastric cancer.
are still not clearly depicted. Moreover, it remains to be understood how the presence of microbiota along with *H. pylori* infection affects the progress from gastric disease to cancer. **Practical Implications:** This article summarized a clue of the current studies on microbiota, *H. pylori* infection and the progression from gastric disease to cancer.

**Introduction**

Stomach cancer still remains the most common cancer burden in the world. Aged people are at high risk for gastric cancer, as most people diagnosed with stomach cancer are between their late 60s and 80s. Despite a major decline in incidence and mortality, it is estimated that in 2012 there were 951,594 people diagnosed with gastric cancer and about 723,027 died from this disease, making it the fifth cause of new cancer cases and the third cause of cancer mortality [1]. When comparing among nations, the incidence of gastric cancer is particularly high in Japan, China, Southern and Eastern Europe as well as South and Central America, whereas the lowest rates are observed in North America and most parts of Africa [2]. In China, the incidence of gastric cancer ranks second among all cancer types for both sexes, while it ranks third in men after lung and liver cancer and in women after breast and lung cancer [3]. The world-adjusted mortality rate for gastric cancer in China is the highest worldwide, both for men and women (table 1) [4], with the highest mortality geographically occurring in the northern and mid-western provinces. Comparing with the urban areas in China, the rural areas, especially in Gansu, Henan, Hebei, Shanxi and Shaanxi Provinces in the mid-western part of China, present a high risk [5]. While the etiology of gastric cancer is multifactorial and yet to be fully understood, a number of important risk factors have been revealed, including older age, male sex, *Helicobacter pylori* infection, tobacco smoking, diet and family history [6].

*H. pylori* is a Gram-negative bacterial species that specifically colonizes the gastric epithelium. Most people are colonized by *H. pylori* and hence develop co-existing gastritis for decades. Previous studies provide evidence that *H. pylori* co-evolved with humans at least since their migration out of East Africa nearly 60,000 year ago [7]. *H. pylori* is hence regarded as a component of the human normal gastric microbiota. Ever since the first discovery of *H. pylori* in the early 1980s by Dr. Barry J. Marshall and Dr. J. Robin Warren [8], *H. pylori* has been identified as the main cause of chronic gastritis and peptic ulcer [9]. Depending on the prevalence of *H. pylori*, the attributable risk of gastric cancer conferred by *H. pylori* increases from 75% to >90% [10]. *H. pylori* infection is hence the strongest known risk factor for gastric cancer when compared with other ones. For decades, researchers have dedicated their efforts to depicting the mechanisms modulating the biological interactions between *H. pylori* and its hosts and whether it contributes to carcinogenesis.

Apart from *H. pylori*, the stomach harbors $10^3 – 10^4$ bacteria, and this huge number of microorganisms can cause pathology under some condition, such as immunodeficiency [11] or microbiota dysbiosis. Bacteria from the upper digestive track, respiratory tract and small intestine can enter the stomach, as the stomach is in the central position that connects the esophagus and oral cavity on the upper side and the duodenum on the lower side [12]. Though its low-pH environment was believed unsuitable for bacterial colonization, the existence of acid-resistant bacterial strains was confirmed by previous studies [13]. With the application of high-throughput sequencing technology in microbiology, the existence of huge numbers of bacteria other than *H. pylori* in the stomach was proved and a more detailed stomach flora constitution was obtained.

However, few studies on the gastric microbiota have been performed to fully understand the constitution and diversity of the microbiota under health and disease situations as well
as the function of microbiota in the pathogenesis of various digestive diseases. Therefore, the current article provides an overview of the recent findings regarding the relationship between gastric microbiota, microenvironment and gastric tumorigenesis.

**Community Composition and Biodiversity of Gastric Microbiota**

For a long time, the stomach was believed unsuitable for bacteria colonization. Due to the limitations of traditional culture-based methods, early microbiological studies failed to demonstrate the overall microbial community structure, which includes the uncultured microorganisms. The major risk factor for gastric cancer, *H. pylori*, was isolated from the stomach by Marshall et al. in 1982 [8]. With the application of metagenomics and high-throughput sequencing technology in microbiology, the identification of the stomach flora has increased. Although the composition of the stomach microbiota has not been clearly profiled and documented, 130–260 phylotypes from up to 13 phyla have been detected [14–16]. On the phylum level, Proteobacteria (to which *H. pylori* belongs) seems to be the most abundant in the normal gastric microbiome, quickly followed by Firmicutes and then by Bacteroidetes, Actinobacteria and Fusobacteria. The Proteobacteria mainly consists of *Helicobacter*, the genera *Haemophilus*, *Actinobacillus* and *Neisseria*. Firmicutes is mainly composed of species from the genera *Streptococcus* and *Bacillus*, while Prevotella species contribute to the dominance of Bacteroidetes. *Rothia*, *Actinomyces* and *Micrococcus* species are the dominant Actinobacteria [14–17]. Later, Delgado et al. [18] analyzed stomach mucosa biopsies of 12 healthy persons and confirmed that Firmicutes and Proteobacteria were the most abundant phyla followed by members of the phylum Actinobacteria, while Deinococcus-Thermus, Bacteroidetes and Gemmatimonadetes were represented in small numbers (<3%). The sequences were grouped into 59 families (represented by Streptococcaceae, Lactobacillaceae and Propionibacteriaceae) and 69 genera, revealing wide bacterial diversity. Among all, 19 genera were found to compose 35–54% of the total genera detected per sample, including *Lactobacillus*, *Lactococcus*, *Propionibacterium*, *Staphylococcus*, *Streptococcus* and *Brevibacterium*, *Methylobacterium*, *Pseudomonas*, *Serratia*, *Stenotrophomonas*, *Veillonella* and *Vibrio* bacteria (fig. 1).

**Differences in Microbiota Composition between Cancer and Non-Cancer Tissue**

Although what represents normal microbiota in the stomach has not been well established, differences in microbiota composition have been detected between patients suffering from digestive diseases and healthy subjects as well as among groups of healthy human subjects. Concerning variations in the microbiota, Aviles-Jimenez et al. [19] found that bacterial diversity showed a trend to diminish from non-atrophic gastritis to intestinal metaplasia and to gastric cancer, with a decrease in *Porphyromonas*, *Neisseria*, TM7 group and *Streptococcus sinensis* as well as an increase in *Lactobacillus coleohominis* and Lachnospiraceae, which might favor development of gastric cancer. In contrast to previous findings that in the gastric mucosa the most abundant families are Streptococcaceae and Prevotellaceae, Aviles-Jimenez et al. detected Lachnospiraceae as the most abundant (representing almost 20% of the microbiota) in all patients by G3 PhyloChip™. Additionally, the genus *Pseudomonas* is one of the taxa that were significantly more abundant in gastric cancer than in non-atrophic gastritis. The analysis of microbial communities between normal and paired cancer samples from given *H. pylori* carriers displayed that normal gastric mucosa had larger populations of *Propionibacterium* spp., *Staphylococcus* spp. and *Corynebacterium* spp. than that of
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The microbial composition analysis of gastric mucosa from patients with chronic gastritis, intestinal metaplasia and gastric cancer demonstrated that Epsilonproteobacteria (containing *H. pylori*) appeared to be the most prevalent and Epsilonproteobacteria in most of the enrolled patients, especially in *H. pylori*-positive patients by conventional methods, were predominantly composed of *H. pylori* species [16, 20, 21]. Thus, *H. pylori* is the most dominant organism, representing >50% of all bacterial cells in the human stomach in *H. pylori*-positive subjects when detected by conventional means, including culture or biochemical assays, tissue histology and host serological responses [16, 20, 21]. However, in the same patients carrying *H. pylori*, while *Clostridium* and *Prevotella* were denser in gastric cancer mucosa than in normal mucosa of the stomach [17].

### Table 1. Estimated incidence and mortality of stomach cancer worldwide in 2012 [4] (estimated numbers, ×1,000)

|                      | Men cases | Men deaths | Women cases | Women deaths | Both sexes cases | Both sexes deaths |
|----------------------|-----------|------------|-------------|--------------|-----------------|------------------|
| World                | 631       | 469        | 320         | 254          | 925             | 723              |
| More developed regions| 175       | 107        | 99          | 68           | 275             | 175              |
| Less developed regions| 465       | 362        | 21          | 186          | 677             | 548              |
| United States        | 13        | 7          | 8           | 5            | 21              | 12               |
| European Union (EU-28)| 51        | 35         | 31          | 23           | 82              | 58               |
| China                | 283       | 221        | 419         | 104          | 405             | 325              |
| India                | 43        | 41         | 31          | 18           | 63              | 59               |
*H. pylori*, the microbial profile showed that the abundance of *H. pylori* in normal gastric mucosa was higher than that of the paired gastric cancer patients. This decrease in the *H. pylori* population in cancer tissue was associated with the hypochlorhydric mucosal environment, which is not suitable for *H. pylori* colonization [17].

### Impact of *H. pylori* on Gastric Microbiota

Chronic *H. pylori* infection is likely to cause gastric microbiota shift due to the *H. pylori*-associated changes in gastric physiology and immunology, e.g. reduced gastric acidity, perturbed nutrient availability and local innate responses [22]. In mouse models, the mouse genetic background, the strain of *H. pylori* and the *H. pylori* infection period may affect the ability of *H. pylori* to alter the gastric microbiota. Colonization by *H. pylori* in specific pathogen-free female BALB/c mice leads to reduced abundance of *Lactobacillus* species in the gastric microflora when compared with non-infected mice [23]. In the transgenic, insulin-gastrin (INS-GAS) mouse model, the *H. pylori*-infected specific pathogen-free male mice showed significant differences at the phylum level compared with the control mice, with marked reductions in Bacteroidetes and marked increases in Firmicutes [24]. In female C57BL/6N mice, *H. pylori* colonization included decreased Firmicutes (class Bacilli), Bacteroidetes and Proteobacteria and increased Firmicutes (class Clostridia), Proteobacteria (genus Helicobacter) and Verrucomicrobia, but did not substantially change the overall stomach microbiota composition [25]. Osaki et al. [26] determined the differences in the gastric microbiota between *H. pylori*-positive and -negative Mongolian gerbils, indicating that the number of *Bifidobacterium* spp. in *H. pylori*-positive gerbils increased compared to that in the *H. pylori*-negative group, while *Eubacterium cylindroides* and *Prevotella* spp. were detected only in *H. pylori*-negative gerbils.

There are also a limited number of studies reporting the effect of *H. pylori* on the microbiota of the human stomach. The gastric microbial profiles of *H. pylori*-positive patients recorded enriched relative abundances of non-*Helicobacter* bacteria from Proteobacteria, Spirochetes and Acidobacteria [27], specifically from the families Bradyhizobiaceae, Caulobacteraceae, Lactobacillaceae and Burkholderiaceae by comparison with *H. pylori*-negative patients. For *H. pylori*-negative patients, class Alpha-, Beta-, Gamma-Proteobacteria, Bacilli, Bacteroidia, Clostridia, Flavobacteria, Fusobacteria or Negativicutes were found as major taxa [20]. Another study demonstrated that patients positive for *H. pylori* culture showed significantly increased colonization of Proteobacteria and a decrease in Actinobacteria [19].

The above studies suggested that infection with *H. pylori* was closely correlated with the remaining stomach flora. However, due to the limitations of animal models and case studies, the role of the human stomach microbiota in intragastric colonization of *H. pylori* has not been comprehensively understood.

### Gastric Microbiota Alteration following Eradication of *H. pylori* and Gastric Cancer Development

Although eradication of *H. pylori* before the onset of atrophy demonstrated the possibility to protect against gastric cancer [28–30], the antibiotics used affected the commensal microbiota. The few studies that have been performed on gastrointestinal microbiota alteration following eradication of *H. pylori* were based on standard culture methods instead of the current high-throughput metagenomics approach. In one study, 14 patients received 20 mg omeprazole, 1,000 mg amoxycillin plus 400 mg metronidazole (OAM), while another 16...
Patients were given 20 mg omeprazole, 250 mg clarithromycin plus 400 mg metronidazole (OCM), both twice daily for 7 days. Concerning the intestinal microbiota, for both OAM and OCM treatment, there was also a significant increase in the numbers of Enterococci, Enterobacteria and Peptostreptococci. Anaerobic bacteria such as Bifidobacteria and Clostridia were significantly suppressed. These alterations returned to normal 4 weeks after treatment in the OAM group, but persisted in the OCM group [31]. Researchers in Germany observed a decreased colonization in non-spore-forming anaerobic bacteria and an increased colonization with yeasts including Candida albicans, and with Clostridia, after treatment by OCM on the intestinal microflora of 57 patients and 21 controls [32].

Due to the lack of experimental support and the limitation of previous studies, the way gastric microbiota shift and what role the microbiota alteration plays in gastric cancer development is yet to be explored.

**Roles of H. pylori in the Development of Gastric Cancer**

Gastric cancer is classified as diffuse and intestinal type. The development of the intestinal type follows the sequential events from superficial gastritis to chronic atrophic gastritis to intestinal metaplasia, then to dysplasia and finally to gastric adenocarcinoma (fig. 2). During gastric carcinogenesis, genetic predisposition, infection and diet are identified as part of a complex interaction, among which the ongoing local chronic inflammatory induced by H. pylori is likely to be one of major factors for gastric lymphoma development. However, only a small percentage of colonized individuals develop clinically apparent sequelae, though all persons carrying H. pylori have coexisting gastric inflammation [33].

![Fig. 2. The sequential histological events in the progression from superficial gastritis (a), atrophic gastritis (b), intestinal metaplasia (c), low-grade (d) and high-grade gastric dysplasia (e) to gastric adenocarcinoma (f) [82].](image)
Molecular Mechanisms of Epithelial Barrier Disruption by H. pylori

The majority of H. pylori exist as free-living microorganisms in the hosts, while there is about 20% binding to gastric epithelial cells, which are the first barrier against pathogenic microbes [34]. Thus, intimate interactions between H. pylori and epithelial cells are likely to contribute to pathogenesis. Studies have revealed that highly virulent H. pylori strains harbor pathogenicity factors, including Cag pathogenicity island, cytotoxin-associated gene A (CagA), vacuolating cytotoxin [35], OipA, peptidoglycan, iceA and several H. pylori-expressing adhesins. Cag pathogenicity island encodes a type 4 secretion system to inject the bacterial CagA.

In normal epithelium, intercellular junctions and interactions, including tight junctions (TJs), adherens junctions (AJs) and focal adhesions, exhibit tumor-suppressive and/or anti-metastasis properties. CagA is key factor leading to chronic gastritis and ulceration, mucosa-associated lymphoid tissue lymphoma and gastric cancer in humans [34, 36–38]. Injected CagA causes depolarization and disruption of the tight junction barrier function in epithelial cells by recruiting the scaffolding protein ZO-1 to the H. pylori attachment sites [39–41] or by binding to Par1b to form the CagA-Par1b complex which mislocalizes to TJs and apical membranes [40, 42]. Massive injection of CagA into host cells results in the disruption of AJs, as the transfected CagA physically interacts with E-cadherin, a key molecule of AJs, impairing the interaction between E-cadherin and β-catenin and then the stability of the AJ complex [43]. CagAPY (tyrosine-phosphorylated at Glu-Pro-Ile-Tyr-Ala [EPIYA] motifs) translocates and can interact with Crk adaptor proteins to form the CagAPY-Crk complex, which stimulates the breakdown of AJs [44]. Apart from CagA, the highly virulent H. pylori strains also harbor VacA, which is secreted by the bacteria and binds to receptors RPTPα and RPTPβ (receptor protein tyrosine phosphatase α and β) on epithelial cells. After binding to RPTPα and RPTPβ, VacA induces pores and vacuoles, along with the occurrence of VacA internalization [45]. BabA and BabB, SabA as well as AlpA and AlpB are important adhesins, secreted by H. pylori for establishing host-cell contact [46]. As soluble factors, VacA and adhesins could directly open TJs and AJs in a mild way via activation of cellular factors [47]. Cytotoxin-associated gene L (CagL), a specialized adhesin on the type 4 secretion system pilus, binds to and activates integrins, which trigger the delivery of CagA across the host-cell membrane [48].

H. pylori has also been reported to trigger various pathways in the host cell after adherence, including JAK/STAT3, NF-κB, PI3K/Akt, Wnt/β-catenin and Ras/Erk, thus inducing carcinogenesis. As mentioned previously, CagA, the virulence factor of H. pylori, can be injected into the host cells following the activation of the signaling cascades for tumorigenesis. Once translocated into epithelial cells, CagA is phosphorylated by members of the Src family of kinases at the Glu-Pro-Ile-Tyr-Ala (EPIYA) motifs [49–52]. CagL was also reported to activate FAK and Src early in H. pylori infection to guarantee phosphorylation of CagA directly at the injection site [48]. Phospho-CagA subsequently activates a eukaryotic phosphatase (SHP-2) as well as ERK, a member of the MAPK family, leading to elevation of expression of a newly identified oncoprotein CIP2A in AGS cells [53, 54]. Moreover, H. pylori cag+ strains activate heparin-binding EGF in vitro, thus triggering the transactivation of EGF receptor and subsequently inducing the early growth response gene Egr-1 via Ras-mediated activation of ERK1/2 [55, 56]. However, the EGF receptor has also been demonstrated to be activated by the cag-independent way, in which bacterial γ-glutamyltranspeptidase has been identified as a potential upregulator of heparin-binding EGF in gastric epithelial cells [57]. Additionally, deletion of H. pylori OipA affected the phosphorylation status of FAK [58].

The transactivation of epidermal growth factor receptor (EGFR) [59] by H. pylori also induces PI3K/AKT cascade activation in gastric epithelial cells. Upon ligand stimulation, EGFR forms dimers and phosphorylates PI3K, leading to activation of the PI3K/AKT pathway and β-catenin nuclear localization [56, 60, 61]. Transactivation of EGFR induced by H. pylori has also been shown to enhance PI3K/AKT signaling in a Src-dependent manner, which
involved the cag secretion system and peptidoglycan in *H. pylori* [60]. The CRPIA motif in non-phosphorylated CagA was involved in interacting with activated Met, leading to sustained activation of PI3K/Akt signaling in response to *H. pylori* infection [62]. In addition to CagA, the type 4 bacterial secretion system also injects *H. pylori* peptidoglycan into gastric epithelial cells, resulting in activation of the PI3K pathway [63]. VacA was reported to induce activation of the PI3K/Akt signaling pathway and subsequent inhibition of GSK3β when incubated with AZ-521 cells in vitro [64].

Once phosphorylated by AKT, GSK3β gets inactivated and then releases β-catenin, a downstream component of the Wnt signal transduction pathway, for nuclear translocation. Apart from CagA, *H. pylori* constituents such as VacA and the adhesion molecule OipA have been proved to mediate β-catenin nuclear localization. VacA translocates the β-catenin into the nucleus by inactivating GSK3β [64], while the inactivation of OipA decreases β-catenin nuclear localization [65].

**H. pylori and Genetics/Epigenetics**

*H. pylori* infection mediates epigenetic regulations in the gastric epithelium, consequently causing gastric carcinogenesis. It has been reported that some miRNAs expression levels varied significantly between *H. pylori*-positive and -negative subjects, including miR-21, miRNA-223, miR-146a, miR-155 and let-7 [66–68]. Specifically, CagA triggered aberrant epigenetic silencing of let-7 expression, thus increasing Ras expression [68]. Moreover, miR-370 expression was suppressed by *H. pylori* and CagA inhibited, which led to upregulation of FoxM1, inhibition of p27^Kip1^ and cell proliferation [69]. In several diseases characterized by chronic inflammation, hypermethylation of the promoter region CpG islands is associated with transcriptional inactivation of tumor suppressor genes. *H. pylori* has been reported to induce DNA methylation, including Sat α and E-cadherin, leading to the development of gastric cancer [70–73]. Direct adhesion of *H. pylori* to the host cells induces aberrant activation-induced cytidine deaminase expression, resulting in double-strand breaks in host cell genomic sequences [74, 75]. Either introduction of bacterial virulence factors into host cells or induction of inflammatory responses by *H. pylori* contributes to activation-induced cytidine deaminase expression [76, 77].

**Chronic Inflammation**

The ongoing chronic inflammation aroused by *H. pylori* infection is a major step for the initiation and development of gastric cancer. The gastric inflammatory response consists of neutrophils, lymphocytes (T and B cells), plasma cells and macrophages, along with varying degrees of epithelial cell degeneration and injury. *H. pylori* adhesion to epithelial cells is associated with brisk activation of NF-κB and enhanced level of cytokine interleukin-1β (IL-1β), IL-2, IL-6, IL-8 and TNF-α, triggering inflammation in gastric epithelial cells [33]. Cyclooxygenase-2 (COX-2), correlated with gastric carcinogenesis, is also involved in *H. pylori*-induced inflammatory responses; its expression is most strongly increased in the epithelium of malignant and dysplastic glands. The pro-inflammatory prostaglandin PGE2 is subsequently induced after the upregulation of COX-2. Prostaglandins play an important role in the growth and stimulation of inflammation-associated gastric carcinogenesis [78].

**Non-*H. pylori* Microbiota and Gastric Cancer Development**

Findings from current studies support the role of non-*H. pylori* microbiota in the development of gastric cancer. Studies with INS-GAS mice have revealed that male mice with intestinal microbiota developed gastric pathology from chronic gastritis to atrophy and dysplasia.
independent of *H. pylori* infection. Besides, the presence of commensal microbiota accelerated the progression to gastric intraepithelial neoplasia, and gastric intraepithelial neoplasia became invasive in *H. pylori*-infected INS-GAS mice [24, 79]. Furthermore, in male INS-GAS mice with *H. pylori* infection, colonization with artificial mouse intestinal microbiota (Altered Schaedler’s Flora, including ASF356 *Clostridium* species, ASF361 *Lactobacillus murinus* and ASF519 *Bacteroides* species) increased the incidence of gastric intraepithelial neoplasia to 69.0% and promoted the development of cancer [29, 79]. On the other hand, antibiotic treatments significantly delayed the onset of gastric neoplasia in *Helicobacter*-free and specific pathogen-free INS-GAS mice [28]. In C57BL/6N mice, pre-infection with gastric microbial populations affects the subsequent immune response to *H. pylori*, contributing to the diverse outcomes of *H. pylori* infection [25]. After treatment with acid-suppressive drugs, risk of atrophy development is only seen in *H. pylori*-positive subjects [80], while no increased risk has been observed in *H. pylori*-negative individuals or after *H. pylori* eradication, suggesting that the microbiota acquired cannot cause atrophy on their own but could enhance atrophy development together with *H. pylori* [81].

Although studies have proved the existence of complex microbiota in the stomach, the importance of *H. pylori* and the possible impact of the non-*H. pylori* microbiota in the pathogenesis of atrophic gastritis and gastric cancer are to be further investigated.

**Summary**

The incidence of gastric cancer remains highly prevalent in Asia when compared to the West, and *H. pylori* infection is the one gastric cancer risk factor. During the past decades, studies of *H. pylori* have identified several virulence factors contributing to the development of gastric cancer. To date, the application of high-throughput sequencing technology and metagenomics have revealed the existence of a diverse and complex composition of microbiota in the stomach. Additionally, findings from current studies support a role of microbiota in the development of gastric cancer. However, due to the limitation of experimental methods or sample numbers, the properties of gastric microbiota and the mechanisms by which they participate in the genesis of gastric cancer are still not clearly depicted. Moreover, it remains to be understood how the presence of microbiota along with *H. pylori* infection accelerate the progress from gastric disease to cancer.

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