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ORIGINAL ARTICLE

Prognostic effects of the gastric mucosal microbiota in gastric cancer

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Abstract

Gastric cancer (GC) is one of the most common malignant tumors with a high incidence and mortality. Microbiota play a significant role in human health and disease. We aimed to investigate the prognostic value of the gastric microbiota in different stomach microhabitats. We used our previously published 16S rRNA gene sequence data. We retrospectively enrolled a cohort of 132 patients with GC with complete prognostic information and selected 78 normal tissues, 49 peritumoral tissues, and 112 tumoral tissues for microbiota analysis. Patients with different prognoses showed different gastric microbiota compositions and diversity. The association network of the abundant gastric microbiota was more complicated in patients with poor prognoses. In the peritumoral microhabitat of patients with good prognoses, *Helicobacter* was significantly increased, whereas *Halomonas* and *Shewanella* were significantly decreased relative to that in the peritumoral microhabitat of patients with poor prognoses. PiCRUSt analysis revealed that the peritumoral microbiota had more different Kyoto Encyclopedia of Genes and Genomes pathways than did the tumoral and normal microbiota. This study evaluated the long-term prognostic value of the gastric mucosal microbiota in patients with GC. These findings suggested that the characteristic alterations of the gastric mucosal microbiota may be markers for clinical outcomes in these patients.

KEYWORDS
biomarkers, gastric cancer, gastric microbiota, prognosis, tumor microenvironment

Abbreviations: ACE, abundance-based coverage estimator; AJCC, American Joint Committee on Cancer; BMI, body mass index; CFU, colony-forming units; FDR, false discovery rate; GC, gastric cancer; *H. pylori*, *Helicobacter pylori*; IL, interleukin; KEGG, Kyoto Encyclopedia of Genes and Genome; LDA, linear discriminant analysis; LEfSe, linear discriminant analysis effect size; OTU, operational taxonomic unit; PCoA, principal coordinate analysis; PPI, proton pump inhibitors; SD, standard deviation; SparCC, Sparse compositional correlation; STAMP, Statistical Analysis of Metagenomic Profiles software package.

1 | INTRODUCTION

Gastric cancer (GC) is one of the most common malignant tumors worldwide and greatly threatens human health. In China, GC is the third most commonly diagnosed cancer (10.6%) and the second most common cause of cancer-related death (13.6%).[1] Recognized risk factors for GC include *Helicobacter pylori* infection, high salt intake, alcohol consumption, and smoking.[2–4] No obvious symptoms are evident in the early stages of GC; therefore, GC is typically diagnosed at an advanced stage and with a poor prognosis. Thus, more effective methods of diagnosis and treatment are urgently needed worldwide.

There is a growing appreciation that microbiota play important roles in health and disease. Intestine microbiota are the most intensely studied microhabitat, and the bacterial load is 10^{10}-10^{12} colony-forming units (CFU)/ml.[5] There is a lower bacterial load in the human stomach (10^2-10^4 CFU/ml) than in the intestines.[5] Recently, an increasing number of studies have begun focusing on the gastric microbiota. Gastric microbiota dysbiosis is associated with gastrointestinal diseases, particularly GC. Several studies have investigated the differences in gastric microbiota between patients with GC and controls without GC.[6–10] Our previous studies revealed immune and microbiota changes in the tumoral, peritumoral, and normal microhabitats of patients with GC.[11,12] Most previous studies observed only alterations of the gastric microbiota in patients with GC. However, studies on correlations between the long-term prognoses of patients with GC and the gastric mucosal microbiota are lacking.

Here, we retrospectively collected prognostic data; then, we integrated prognostic data and published 16S rRNA gene sequencing data.[12] We aimed to assess diversity and composition of the gastric microbiota across the tumoral, peritumoral, and normal microhabitats in GC patients with different prognoses. This research will enable better understanding of the associations between the gastric mucosal microbiota of patients with GC and their prognoses. Our study will provide clinical value for predicting the prognoses of patients with GC.

2 | MATERIALS AND METHODS

2.1 | Data source

We obtained 16S rRNA gene sequence data from previous articles published by our team.[12]

2.2 | Patients

In this study, we recruited patients with primary GC between March 2009 to August 2013 from the First Affiliated Hospital, School of Medicine, Zhejiang University. All patients were followed up by telephone and outpatient visits. The follow-up deadline was August 1, 2021. Gastric samples were collected from patients undergoing gastrectomy. Peritumoral tissue was defined as 2-5 cm distant from the margins of the tumor, and normal tissue was located more than 5 cm away from the tumor tissue. According to Lauren, GC can be divided into adenocarcinomas of diffuse and intestinal types.[13] Tumor stage was categorized according to the 7th edition American Joint Committee on Cancer (AJCC) guideline.[14] Tumor stage was classified as follows: stage I, invades mucosa or submucosa; stage II, invades muscularis mucosa; stage III, invades adventitia; stage IV, invades adjacent structures. In this study, early-stage GC was defined as AJCC pathologic stage I and II, and late-stage GC was defined as AJCC pathologic stage III and IV.[15] The pathological characteristics of all the samples were confirmed by pathologists after operation. Survival time was calculated as the time between the date of surgery and the date of GC-related death or date of the latest follow-up. The exclusion criteria were: body mass index (BMI = body mass (kg)/height $(m)^2$) > 30; use of antibiotics, probiotics, prebiotics, or synbiotics in the previous month; preoperative chemotherapy, radiotherapy, or other biological treatment before gastrectomy; no postoperative chemotherapy, death of other causes, lost to follow-up. This study was approved by the Ethics Committee of the First Affiliated Hospital, School of Medicine, Zhejiang University, and written informed consent was obtained from all participants. Detailed sample collection, processing, and bioinformatic analysis methods are provided in the Appendix. The list of taxa and relative abundance for each sample is provided in Table S1.

2.3 | Statistical analysis

For continuous variables, independent *t* test, White's nonparametric *t* test, and Mann-Whitney *U* test were applied. For categorical variables between groups, Pearson's chi-square or Fisher's exact test was used, depending on assumption validity. Survival analysis was performed by log-rank (Mantel-Cox) test. For taxa among subgroups, ANOVA test was applied (Tukey-Kramer post-hoc test was used; effect size was Eta-squared). For correlation analyses, Spearman's rank correlation test was used. False discovery rate (FDR) was calculated according to Benjamini-Hochberg; FDR-corrected *P* values were denoted as Q_{FDR} and were used when performing all untargeted screening analyses of different taxa. Statistical analysis was performed using SPSS V19.0 (SPSS Inc.) and STAMP V2.1.3.[16] GraphPad Prism version 6.0 was used for preparation of graphs. All tests of significance were two sided, and $p < 0.05$ or corrected $p < 0.05$ was considered statistically significant.

3 | RESULTS

3.1 | Patient characteristics

Characteristics of included and excluded patients are summarized in Table 1. A total of 132 patients were included in this study: 47 intestinal-type GC, 28 diffuse-type GC, and 57 mixed-type GC patients. All

TABLE 1 Patient characteristics

Characteristics	Included patients ($n = 132$)	Non-included patients ($n = 144$)	*P* value
Age (year, means ± SD)	61.62 ± 11.87	60.40 ± 11.93	ns
Gender (female/male)	40/92	41/103	ns
Weight (kg, means ± SD)	61.67 ± 11.01	59.78 ± 9.70	ns
Height (cm, means ± SD)	165.2 ± 7.30	164.4 ± 6.56	ns
BMI (means ± SD)	22.54 ± 3.36	21.91 ± 2.92	ns
Complications, no.			
Hypertension	38	36	ns
Diabetes mellitus	12	5	ns
Tumor localization, no.			
Proximal stomach	18	17	ns
Body/fundus	49	60	
Antrum	65	67	
Tumor differentiation, no.			
High differentiated	2	0	ns
Moderately/poor differentiated	130	144	
Lauren typing, no.			
Intestinal type	47	49	ns
Diffuse type	28	28	
Mixed type	57	67	
Tumor stage, no.			
Early stage (I + II)	63	79	ns
Late stage (III + IV)	69	65	
Antibiotics use (within 1 mo), no.	0	0	ns
PPI use, no.	132	144	ns
Preoperative chemotherapy, no.	0	0	ns
Smoking, no.	64	63	
Drinking, no.	48	51	

Abbreviations: BMI, body mass index; no., number; ns, nonsignificant; PPI, proton pump inhibitors; SD, standard deviation.

patients had received postoperative chemotherapy. In total, 78 normal tissues, 49 peritumoral tissues, and 112 tumoral tissues were selected for microbiota analysis. More than 90% of the cancer tissues were moderately/poorly differentiated. The median follow-up period was 99 months (range, 3-147 months). As the 5-year survival rate is one of the most commonly used prognostic indicator for patients with cancer, 5-year survival time was chosen as cutoff value to group the patients in this study. Based on the postoperative survival time, patients were divided into the poor-prognosis group (survival time <5 years) or the good-prognosis group (survival time ≥5 years).

3.2 | Alterations of the gastric mucosal microbiota in patients with different survival times

We obtained 4,578,104 high-quality reads with an average of 19,155 reads per sample (Table 2). The high Good's coverage estimator of coverage (nearly 100%) indicates that the identified reads represented the majority of the bacterial sequences present in the stomach. We used multiple metrics to assess the alpha diversity of each sample. The changing trends in diversity indices, including the Shannon, Simpson, and Invsimpson indices, were concordant between the two groups; however, the good-prognosis group exhibited more significant changes (Figure 1A-C). The changes in richness indices, including abundance-based coverage estimator (ACE), Sobs, and Chao, were more significant in the good-prognosis group than in the poor-prognosis group and were decreased in the peritumoral microhabitats in both groups (Figure 1D-F). The rank abundance curve demonstrated that the normal microhabitats in both groups showed high species abundance and evenness (Figure 1G). The good-prognosis group exhibited more operational taxonomic units (OTUs) than did the poor-prognosis group (Figure 1H). Principal coordinate analysis (PCoA) was used to assess beta diversity. Because of the high

TABLE 2 Data information

Group	No. of samples	No. of clean reads	No. of OTUs[a]	Coverage (%)
Good-prognosis group				
Normal	51	729,010	3777	99.151
Peritumor	34	711,040	1440	99.740
Tumor	69	1,558,439	3203	99.455
Poor-prognosis group				
Normal	27	322,994	3107	98.764
Peritumor	15	347,204	1114	99.599
Tumor	43	909,417	2338	99.498

[a]The operational taxonomic units (OTUs) were defined at the 97% similarity level.

degree of interindividual variation, the microbiotas could not be divided into different clusters (Figure S1).

The dominant families of the gastric microbiota are shown in Figure 2A. Linear discriminant analysis (LDA) effect size (LEfSe) analysis identified the taxa with the greatest differences in abundance across different gastric microhabitats (Figure 2B-F). In the good-prognosis group, *Deinococcus* was significantly increased in the normal microhabitat; *Mycobacteriales* was significantly increased in the peritumoral microhabitat; and *Uudibacterium*, *Mycobacteriaceae*, *Erwiniaceae*, and *Phycisphaerae* were significantly increased in the tumoral microhabitat, compared with the poor-prognosis group (LDA score>2; p <0.05). The distributions of *Helicobacter*, *Halomonas*, *Shewanella*, *Selenomonas*, *Veillonella*, and *Streptococcus* differed

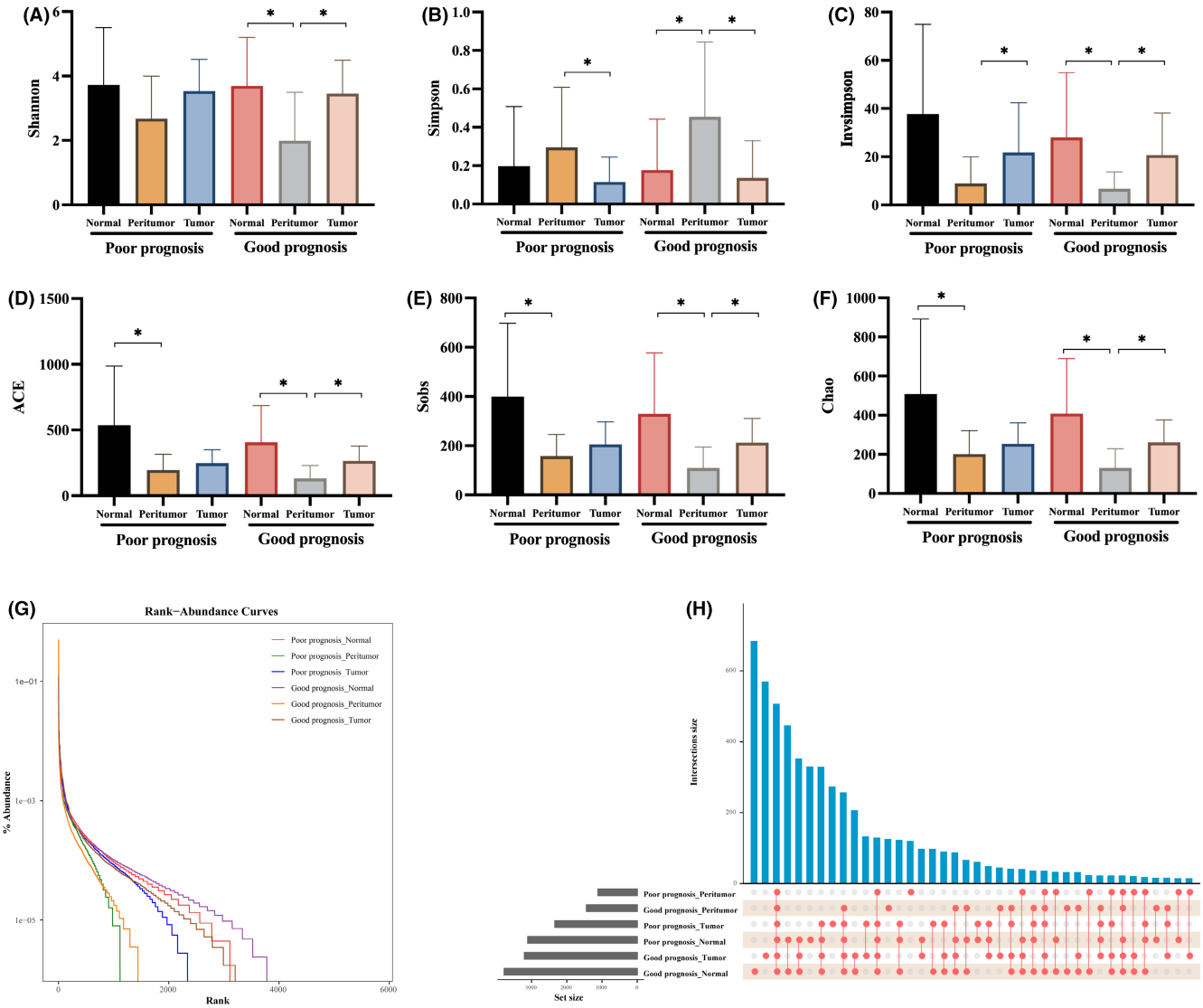

FIGURE 1 Diversity and richness of the gastric microbiota in patients with different survival times. The diversity indices, such as Shannon (A), Simpson (B), and Invsimpson (C), and the richness indices, such as abundance-based coverage estimator (ACE) (D), Sobs (E), and Chao (F), were used to evaluate the overall structure of the gastric microbiota in patients with different survival times. Mann-Whitney *U* tests were used to analyze the diversity and richness of the gastric microbiota. *p <0.05. (G), Rank abundance curve was used to estimate the species abundance and evenness of the gastric microbiota. (H), UpSet plot represented the distribution of the gastric microbiota and displayed the intersection of multiple microhabitats

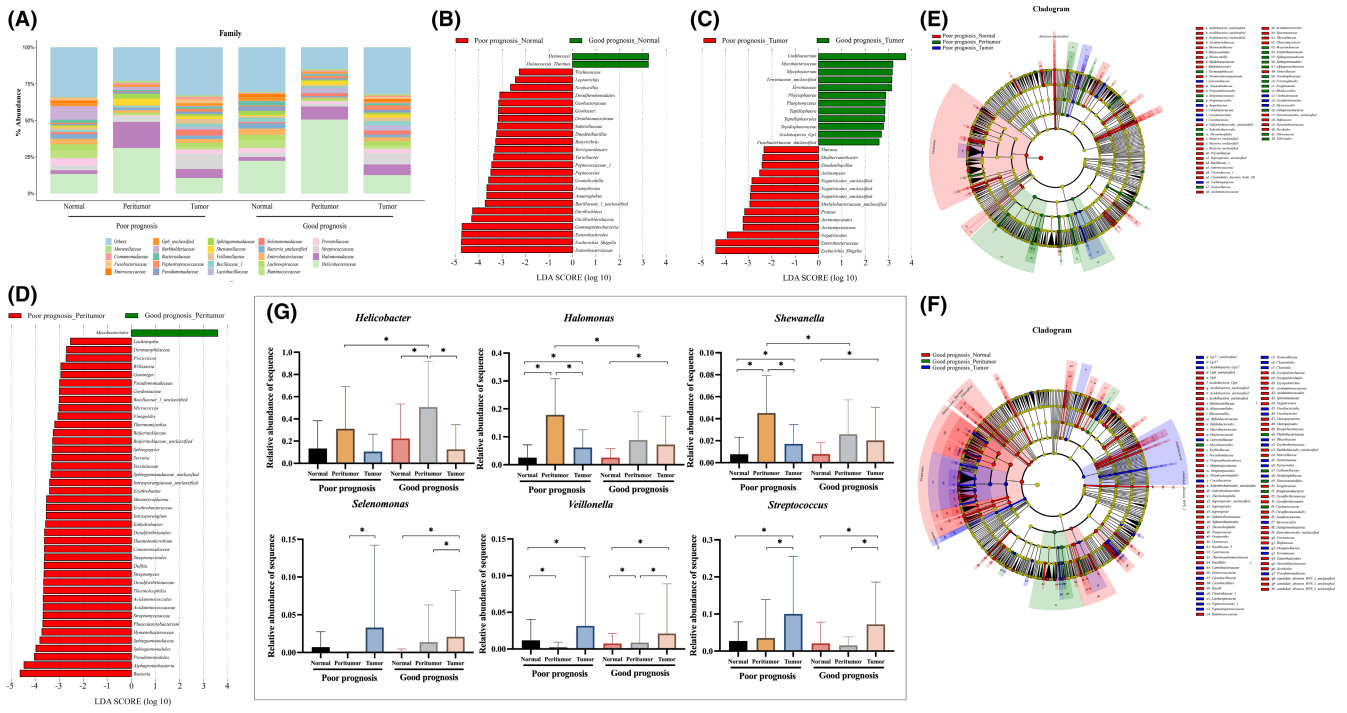

FIGURE 2 A, Profiles of the gastric microbiota in patients with different prognoses. The relative abundance of dominant bacterial taxa at the level of bacterial family was analyzed. B-F, Linear discriminant analysis (LDA) effect size (LEfSe) identifies the taxa with the greatest differences in abundance among the three stomach microhabitats. Only the taxa meeting a significant LDA threshold value of >2 are shown. G, Six differentially abundant bacteria were identified among three microhabitats of patients with different prognoses. Data are presented as mean ± standard deviation. Mann-Whitney *U* tests were used to analyze variation among different microhabitats. $^*p < 0.05$

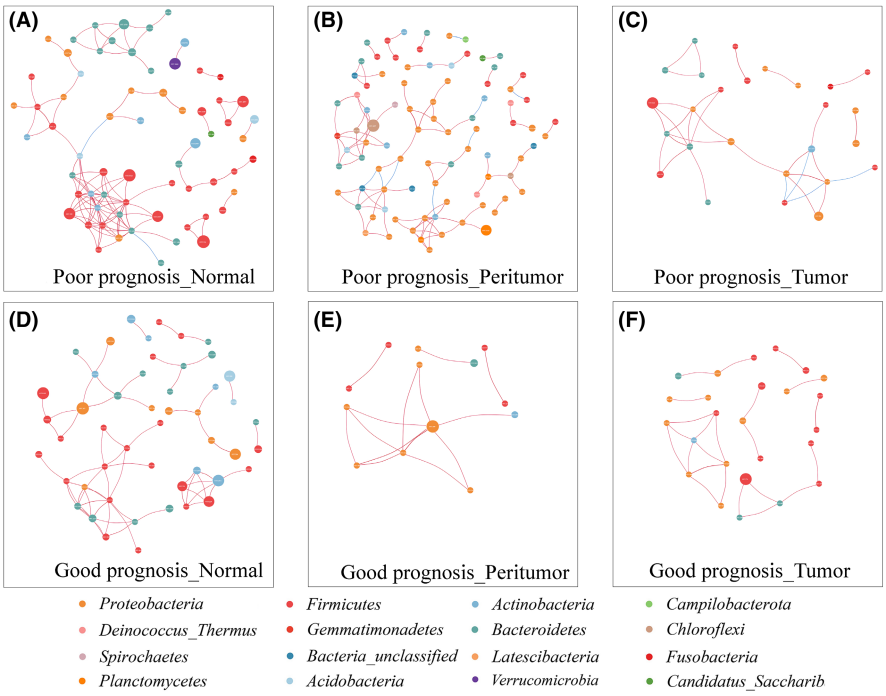

FIGURE 3 Association network of the gastric microbiota in different stomach microhabitats of patients with different prognoses. Association network of the abundant gastric microbiota in normal microhabitats (A, D), peritumoral microhabitats (B, E), and tumoral microhabitats (C, F) in patients with poor and good prognoses, respectively. Red and blue lines represent positive and negative correlations, respectively. Each node in the network represents a single operational taxonomic unit (OTU). Node size indicates the mean abundance of each OTU. Taxonomic information of each OTU is available in Table S1

significantly across the microhabitats (Figure 2G). The *Helicobacter* abundance was highest in the peritumoral microhabitat in both groups, and within this microhabitat, *Helicobacter* was significantly higher in the good-prognosis group than in the poor-prognosis group ($p = 0.0412$; Mann-Whitney *U* tests). However, the *Halomonas* ($p = 0.0153$; Mann-Whitney *U* tests) and *Shewanella* ($p = 0.0331$; Mann-Whitney *U* tests) abundances in the peritumoral microhabitat were significantly lower in the good-prognosis group than in the poor-prognosis group. *Selenomonas*, *Veillonella*, and *Streptococcus* had different distribution trends between the groups, but the difference was not significant. The heatmap in Figure S2 shows the most abundant genus-level microorganisms in the gastric microbiota of both groups.

To explore the possible interactions between differentially abundant microbes, SparCC was used to construct association networks (Figure 3). Table S2 provides annotation information for each OTU. Different bacterial clusters were identified in the two groups. The poor-prognosis group had a more complex interaction network than did the good-prognosis group, especially in the peritumoral microhabitat. In the poor-prognosis group, the peritumoral microhabitat showed a more complicated network than did the tumoral and normal microhabitats. In the normal microhabitat, the interaction network was dominant within or between the *Firmicutes* and *Bacteroidetes* phyla, whereas in the peritumoral microhabitat, the interaction network was dominant within or between the *Proteobacteria* and *Firmicutes* phyla. In the good-prognosis group, the normal microhabitat exhibited a more complex network than did the peritumoral and tumoral microhabitats. The interaction occurred mostly within or between the *Bacteroidetes* and *Proteobacteria* phyla in the normal microhabitat. Most of the significant correlations within the clusters were positive. Alterations in predicted gastric microbial functions are displayed in Figure S3.

3.3 | Alterations of the gastric mucosal microbiota in patients at different tumor stages

We subsequently examined the gastric mucosal microbiota compositions in patients at different tumor stages. We enrolled 63 patients with early-stage GC and 69 with late-stage GC. Finally, 39 normal tissues, 24 peritumoral tissues, and 51 tumoral tissues were used for analysis in the early-stage group; and 39 normal tissues, 25 peritumoral tissues, and 61 tumoral tissues were used for analysis in the late-stage group. Survival times differed significantly between these two cohorts ($p < 0.0001$; log-rank test; Figure 4A). Alpha diversity did not significantly differ between the groups (Figure 4B). The peritumoral microhabitat of both groups showed significant decreases in diversity and richness. Microbiota alterations could not be distinguished between the groups based on unweighted and weighted UniFrac PCoAs (Figure 4C). The late-stage group contained more OTUs, especially in the normal microhabitat (Figure 4D). Figure S4 shows the relative abundances of the dominant bacterial taxa at the family level. The composition of gastric microbiota was not significantly altered between the groups (Figure 4E-K). Interestingly, the abundance of *Helicobacter* was highest in the peritumoral microhabitat in both groups. The *Veillonella* distribution across the microhabitats showed a different pattern between the groups. The abundances of *Halomonas*, *Prevotella*, *Shewanella*, *Streptococcus*, and *Selenomonas* differed among all microhabitats at the same tumor stage. LEfSe analysis showed alterations in the gastric microbiota among the three early-stage and late-stage microhabitats (Figures S5–S7).

3.4 | Gastric mucosal microbiota alterations in patients with different prognoses and tumor stages

We combined the previous groupings for further analysis. We analyzed six normal, two peritumoral, and eight tumoral tissues in the poor-prognosis early-stage group; 21 normal, 13 peritumoral, and 35 tumoral tissues in the poor-prognosis late-stage group; 33 normal,

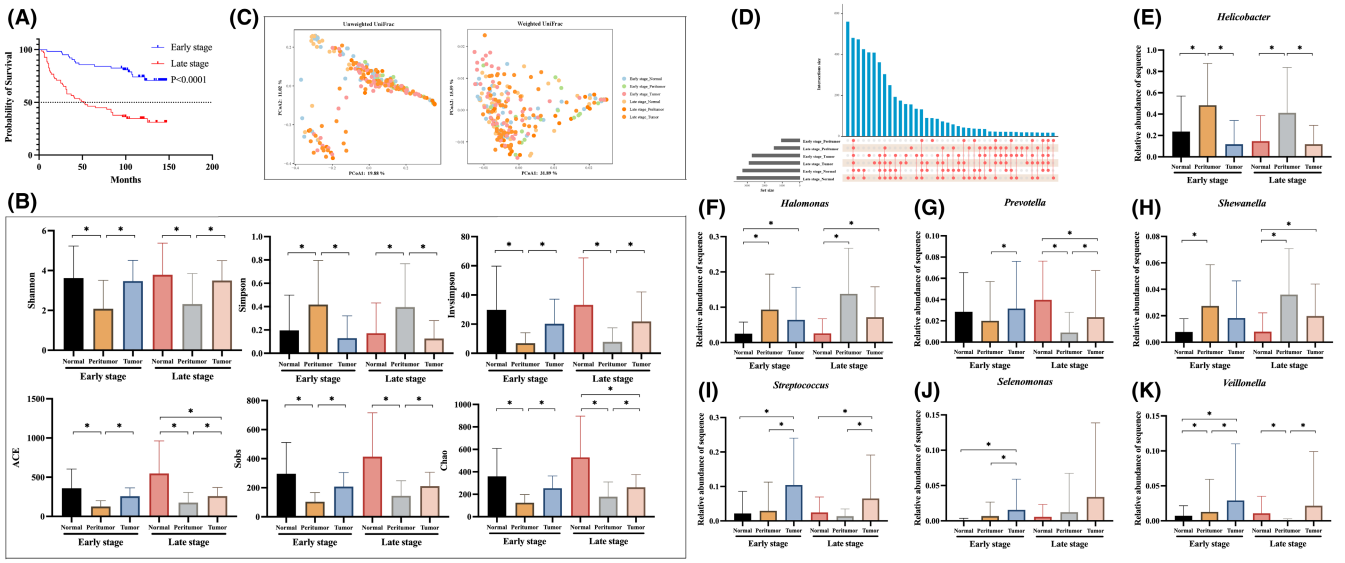

FIGURE 4 A, Profiles of the gastric microbiota in patients with different tumor stages. Significant difference was found in survival time of these two cohorts of patients (log-rank test). B, The alpha diversity indices were used to evaluate the overall structure of the gastric microbiota in patients with different tumor stages (Mann-Whitney *U* tests). C, Plots of principal coordinate analysis (PCoA) of the gastric microbiota in the early-stage and late-stage groups based on the unweighted UniFrac distance and weighted UniFrac distance. D, UpSet plot represented the distribution of the gastric microbiota and displayed the intersection of multiple microhabitats. E-K, Seven differentially abundant bacteria were identified among three microhabitats of patients with different tumor stages (Mann-Whitney *U* tests). Data are presented as mean ± standard deviation. *$p < 0.05$

22 peritumoral, and 43 tumoral tissues in the good-prognosis early-stage group; and 18 normal, 12 peritumoral, and 26 tumoral tissues in the good-prognosis late-stage group. The good-prognosis early-stage group exhibited significantly decreased microbial diversity and richness in the peritumoral microhabitat relative to those in the normal and tumoral microhabitats (Figure 5A). The microbiota changes in these groups could not be distinguished based on unweighted and weighted UniFrac PCoAs (Figure 5B). The differences in microbial community compositions among these groups are shown in Figure 5C. The abundances of *Halomonas*, *Helicobacter*, *Prevotella*, and *Shewanella* differed significantly between these groups (Figure 5D-G). *Halomonas* was altered primarily in the poor-prognosis late-stage and good-prognosis early-stage groups, with significant reductions in the normal microhabitat (Figure 5D). In the peritumoral microhabitat of the late-stage patients, *Halomonas* was significantly increased in the poor-prognosis group relative to that in the good-prognosis group. In the good-prognosis group, the abundance of *Halomonas* was significantly increased in the normal and tumoral microhabitats of patients with early-stage GC compared with that of patients with late-stage GC. The distribution of *Helicobacter* for each group showed similar trends across the three microhabitats (Figure 5E). *Helicobacter* was altered primarily in patients with good prognoses, regardless of stage. In the peritumoral and tumoral microhabitats of patients with late-stage GC, the abundance of *Helicobacter* was significantly decreased in the poor-prognosis group compared with that in the good-prognosis group. All groups except for the poor-prognosis early-stage group had significantly lower abundance of *Prevotella* in the peritumoral microhabitat (Figure 5F). In the peritumoral microhabitat of the late-stage groups, the abundance of *Prevotella* was elevated in patients with poor prognoses relative to that in patients with good prognoses. The abundance of *Shewanella* in the tumoral microhabitat of patients with good prognoses was significantly higher in the early-stage group than in the late-stage group (Figure 5G). LEfSe analysis showed the gastric microbiota alterations among these groups (Figures S8–S11). In addition to this, a history of alcohol consumption and smoking history can also affect gastric mucosal microbiota; the relevant information is provided in Figures S12–S13.

3.5 | Gastric microbiota–based signature in the peritumoral microhabitats discriminated patients with different prognoses

Due to the significant differences in abundance of *Helicobacter*, *Halomonas*, and *Shewanella* in the peritumoral microhabitats of patients with different survival times, we next evaluated the prognostic values of these bacteria. The differential features of these bacteria are presented in Figure 6A–C. Receiver-operating characteristic (ROC) curves of each differential bacterium were constructed and areas under the curves (AUCs) were calculated. The AUCs of *Helicobacter*, *Halomonas*, and *Shewanella* in the peritumoral microhabitats were 0.684, 0.718, and 0.691, respectively (Figure 6D). Among these, *Halomonas* had the best predictive capacity to reflect the prognoses of patients with GC, with a best cutoff value of 6.2725%. Combination of three bacteria significantly improved the ability to predict the prognoses of patients with GC (AUC: 0.749). The predictive values of the ratio of *Halomonas*/*Helicobacter* (H/H, range from 0 to 2656) and *Shewanella*/*Helicobacter* (S/H, range from 0 to 687) were also assessed. The predictive ability of the ratio of H/H (AUC: 0.696; Figure 6E) was almost the same as that of the ratio of S/H (AUC: 0.697; Figure 6F). Based on the best cutoff values, the patients were divided into high–relative-abundance groups and low–relative-abundance groups. The patients with low relative abundance of *Halomonas* ($p = 0.0188$; log-rank test; Figure 6H) and *Shewanella* ($p = 0.0196$; log-rank test; Figure 6I) had longer survival time than patients in the high–relative-abundance groups. However, the patients with low relative abundance of *Helicobacter* ($p = 0.0165$; log-rank test; Figure 6G) and combination of three bacteria ($p = 0.0001$; log-rank test; Figure 6J) had shorter survival time than patients in high–relative-abundance groups. The patients with low ratios of H/H ($p = 0.0067$; log-rank test; Figure 6K) and S/H ($p = 0.0067$; log-rank test; Figure 6L) had longer survival time than patients with high ratios. Therefore, *Helicobacter*, *Halomonas*, and *Shewanella* in the peritumoral microhabitats can be used as potential biomarkers to predict the prognoses of patients with GC.

4 | DISCUSSION

Gastric cancer has a significant negative impact on human health and the social economy. Prognostic factors such as lymphovascular invasion, TNM stage, and tumor location have been described for patients with GC.[17,18] Many studies have reported gastric microbiota changes in patients with GC compared with those of healthy controls and with non-GC.[19–22] However, no previous study has evaluated the prognostic value of the gastric mucosal microbiota; therefore, we evaluated this in the present study. To our knowledge, this is the first study to report the prognostic value of the gastric mucosal microbiota for long-term outcomes of patients with GC. We classified patients according to postoperative survival time and analyzed the gastric mucosal microbiota alteration in these patients with different prognoses. We observed similar patterns in alpha and beta diversity between the groups. Remarkable changes of microbial community compositions and predicted functions were found, particularly in the peritumoral microhabitats. Our previous study indicated the correlation between the gastric microbiota and immune cells in different microhabitats.[23] Interactions between the gastric microbiota and immune cells in different microhabitats may modulate the tumor microenvironment, thus affecting the long-term clinical outcomes of patients with GC.

Helicobacter was enriched in the peritumoral microhabitats, regardless of prognosis or tumor stage, which is consistent with our previous findings.[12] Notably, the peritumoral microhabitats of GC patients with good prognoses had higher abundance of *Helicobacter* than did patients with poor prognoses. Moreover, the abundance of

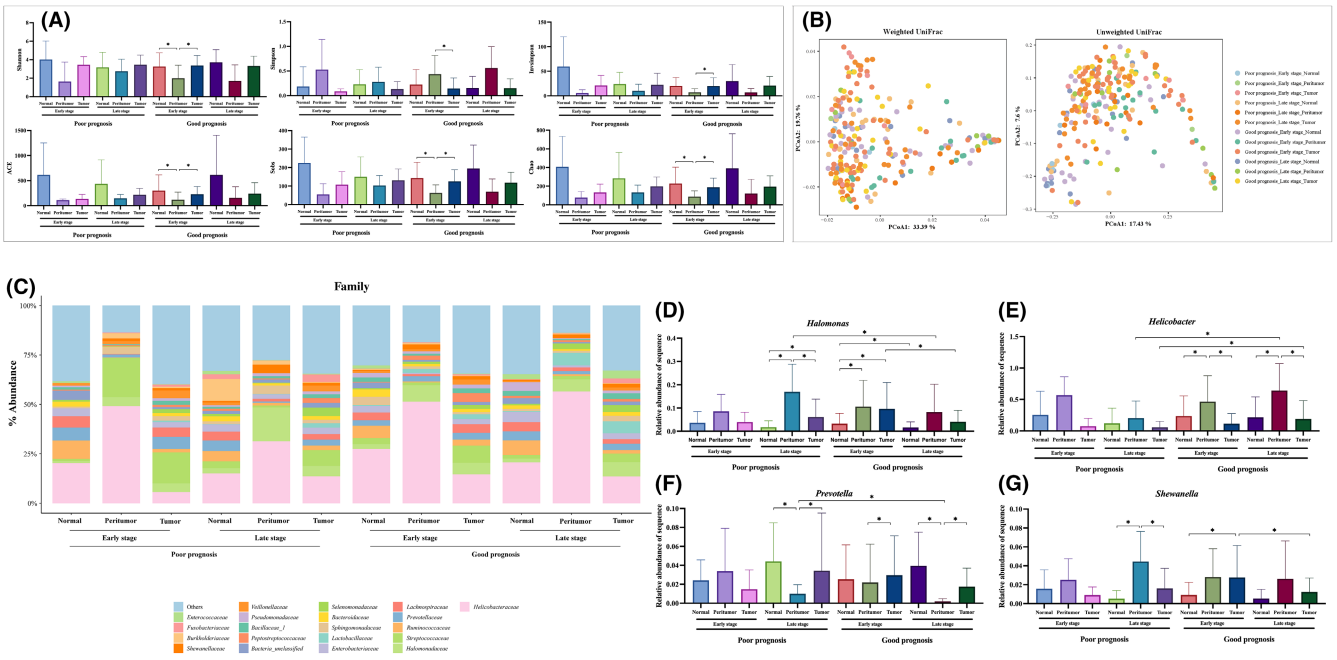

FIGURE 5 A, Profiles of the gastric microbiota in patients with different prognoses and tumor stages. The alpha diversity indices were used to evaluate the overall structure of the gastric microbiota in patients with different prognoses and tumor stages (Mann-Whitney *U* tests). B, Plots of principal coordinate analysis (PCoA) of the gastric microbiota in different groups based on the unweighted UniFrac distance and weighted UniFrac distance. C, The relative abundance of dominant bacterial taxa at the level of bacterial family was analyzed. D-G, Four differentially abundant bacteria were identified among different groups (Mann-Whitney *U* tests). $^{*}p < 0.05$

Helicobacter was significantly elevated in both the peritumoral and tumoral microhabitats of stage III/IV patients with good prognoses. The *Helicobacter* genus consists of 55 species, 46 of which have been published, and of these, *H. pylori* is most commonly related to GC.[24] The carcinogenic *H. pylori* effect has been widely studied in recent decades. The relationship between *H. pylori* and the prognoses of GC patients remains controversial. Some studies reported positive associations,[25–30] whereas others found no relationships.[31,32] *H. pylori* infection induces genome instability in gastric epithelial cells, which plays an important role in gastric carcinogenesis.[33] However, some studies present the opposite opinion. Wu et al. used BAT-26 as a marker of the mutator phenotype in GC and found that alterations in BAT-26 were highly correlated with higher *H. pylori* infection rate, better prognosis, and fewer lymph node metastases.[34] Fang et al. found that GC patients with *H. pylori* infection had fewer PI3K/AKT pathway genetic mutations, less tumor recurrence, and better survival than did *H. pylori*–negative patients and suggested that *H. pylori* infection is an independent prognostic factor of overall survival and disease-free survival.[28] PI3K/AKT pathway activation is common in many tumor types, including GC, prostate cancer, and breast cancer.[35–37] The PI3K/AKT pathway is thought to be involved in tumorigenesis, invasion, and metastasis.[37–39] GC patients with PI3K/AKT pathway genetic mutations exhibited more metastases than did those without these mutations.[28] *H. pylori* infection may regulate GC metastasis through PI3K/AKT pathway genetic mutations to affect survival. Additionally, immune responses have non-negligible contributions. Our previous study showed no relationship between *Helicobacter* and immunosuppressive cells such as BDCA2^{+} plasmacytoid dendritic cells and Foxp3^{+} regulatory T cells.[11] Tumor-specific immune responses were downregulated in *H. pylori*–negative patients, and *H. pylori* infection likely promoted immune responses.[26] Cytotoxin-associated gene A of *H. pylori* reportedly promotes inflammatory responses via the c-Met-PI3K/Akt-mTOR signaling pathway.[40] Thus, we postulate that increased *Helicobacter* might inhibit tumor metastasis and induce stronger immune responses, thus prolonging survival. However, this scenario requires further confirmation. Interestingly, the abundance of *Prevotella* showed the opposite pattern to that of *Helicobacter* in peritumoral microhabitats, which were significantly reduced in stage III/IV patients with good prognoses compared with those in stage III/IV patients with poor prognoses. This finding is in accordance with previous studies. Guo et al. found that strong coexcluding interactions between *Helicobacter* and *Prevotella* presented only in patients with chronic atrophic gastritis and intestinal metaplasia/dysplasia but were absent in normal/superficial gastritis.[41] The decreased *Prevotella* was correlated with immunosuppression in the gastric fluid.[42] *Prevotella copri* is reported to flourish in a proinflammatory environment and can promote inflammation to its own advantage.[43] Therefore, the high abundance of *Prevotella* may modulate immune and inflammatory responses, thus leading to poor prognoses of patients with GC.

In addition to that of *Helicobacter*, the distribution patterns of other bacteria were concerning. *Halomonas* and *Shewanella* were enriched in peritumoral microhabitats regardless of prognosis but were higher in patients with poor prognoses than in those with good prognoses. No previous studies have explored the functions

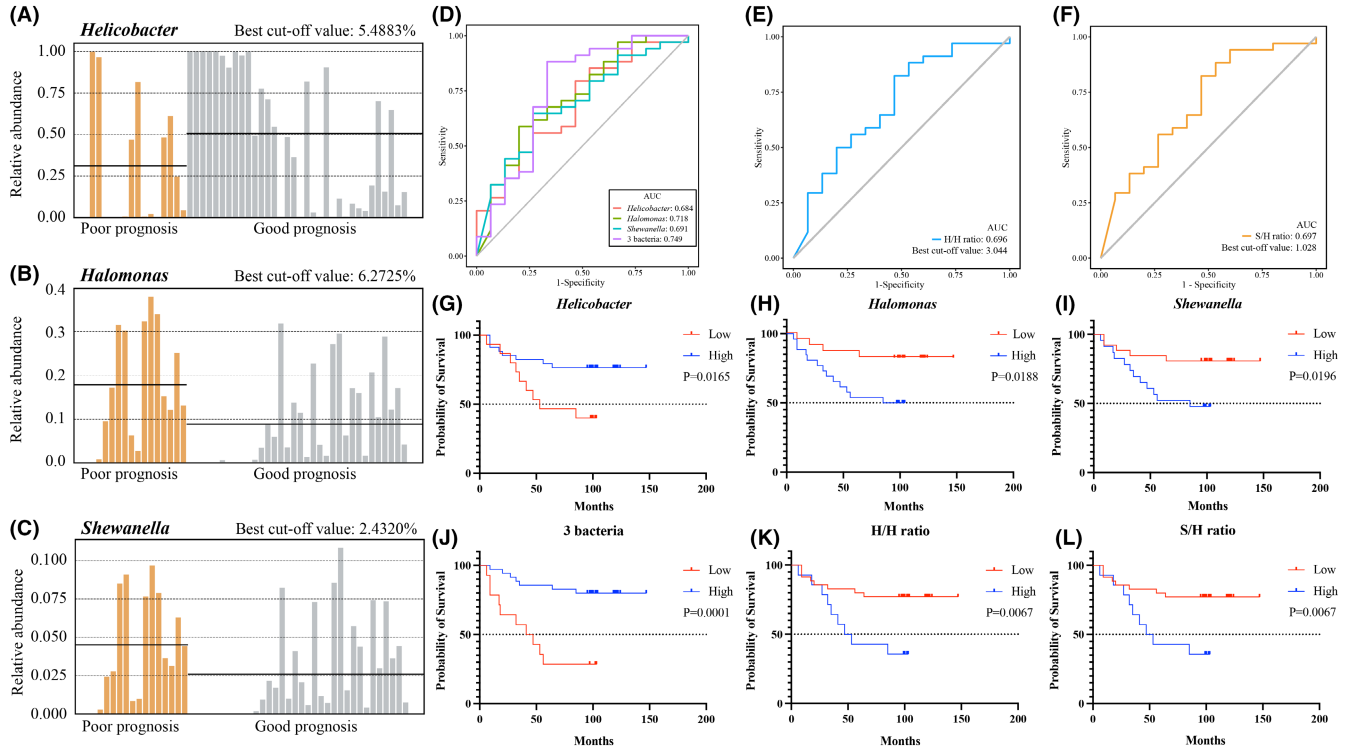

FIGURE 6 Differential genera in peritumoral microhabitats as gastric cancer prognostic markers. The relative abundance and best cutoff values of the differential general such as *Helicobacter* (A), *Halomonas* (B), and *Shewanella* (C) in each peritumoral sample. Receiver-operating characteristic (ROC) curves for the differential genera alone or for the combination of three genera (D), *Halomonas*/*Helicobacter* (E), and *Shewanella*/*Helicobacter* (F) were used to discriminate patients with different prognoses. Survival curves of patients with low and high relative abundance of *Helicobacter* (G), *Halomonas* (H), *Shewanella* (I), combination of three bacteria (J), and with low and high ratios of *Halomonas*/*Helicobacter* (K) and *Shewanella*/*Helicobacter* (L). AUC, area under the ROC curve

of *Halomonas* and *Shewanella* in GC. *Halomonas* is a gram-negative halophilic bacterium. Extracts of *Halomonas* isolated from marine and soil environments exert anticancer effects by inducing apoptosis and inhibiting proliferation in many tumors such as hepatocellular carcinoma, acute lymphoblastic leukemia, and breast cancer.[44–46] No unified understanding exists regarding how *Halomonas* affects immunity. Wang et al. found that an extracellular polysaccharide of marine *Halomonas sp.* 2E1 exhibits immune-enhancing activity by increasing the production of nitric oxide, cyclooxygenase 2, tumor necrosis factor-α, interleukin (IL)-1β, and IL-6 by activating the MAPK and NF-κB pathways in RAW264.7 macrophages.[47] Lipid A of *Halomonas magadiensis* inhibits enteric lipopolysaccharide-induced human monocyte activation.[48] *Shewanella* is considered a human pathogen, causing skin and soft tissue infections, bacteremia, and otitis media.[49] In colorectal adenoma, increased *Shewanella algae* was observed in lesioned mucosa.[50] *Shewanella* was also found in colorectal cancer tissues.[51] In patients with prostate cancer, *Shewanella* was significantly overabundant in malignant tissues compared with benign prostate tissue, and malignant samples harboring high *Shewanella* counts were associated with downregulated toll-like receptor signaling pathways and decreased dendritic cell enrichment, leading to inhibition of immune activity in the malignant tumors.[52] *Halomonas* and *Shewanella* coexist in different human tissues possibly because *Shewanella* has sodium permeation–changing capability and can increase extracellular salt levels to create a suitable environment for *Halomonas*.[53,54] Thus, overabundance of *Shewanella* may increase *Halomonas* and inhibit immune activity leading to shorter survival times among patients with GC.

This study had some limitations. First, this was a single-center study with relatively few patients, and whether the results can be generalized to wider populations of patients with GC is uncertain. Second, because of financial constraints, no specimens were obtained from patients during follow-up. We cannot exclude gastric microbiota alterations in patients who had different lifestyles or received antibiotic therapy and other clinical care during follow-up, thus affecting the final outcomes. Third, although we observed significant alterations of the gastric microbiota, whether the gastric microbiota have a direct role in long-term outcomes is uncertain. Accordingly, the results reveal only correlation rather than a cause-effect relationship. Detailed prospective studies are needed to confirm the clinical impact of the gastric microbiota.

In summary, we investigated the relationship between the gastric microbiota and prognoses of patients with GC. Our results indicated gastric microbiota alterations in different microhabitats of GC patients with different prognoses. We observed a significantly altered composition, correlation network, and gastric mucosal microbiota function in peritumoral microhabitats. Interestingly, in peritumoral microhabitats, the abundance of *Helicobacter* was significantly increased, whereas *Halomonas* and *Shewanella* were significantly decreased in GC patients with good prognoses. These

findings suggested that characteristic alterations of the gastric mucosal microbiota, such as *Helicobacter*, *Halomonas*, and *Shewanella*, in patients with GC may be markers for clinical outcomes. These observations provide new insight into therapeutic strategies for GC. However, the specific roles of different bacteria in the gastric microbiota of patients with different prognoses remain unknown. Further studies are required to reveal the underlying mechanism.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

DATA AVAILABILITY STATEMENT

All data generated or analyzed during this study are included in this published article and its Appendix S1 files.

ETHICS STATEMENT

Approval of the research protocol by an Institutional Reviewer Board: The protocol was approved by the Ethics Committee of the First Affiliated Hospital, School of Medicine, Zhejiang University.

Informed Consent: Informed consent was obtained from patients.

Registry and the Registration No. of the study/trial: N/A.

Animal Studies: N/A.

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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