AMERICAN SOCIETY FOR MICROBIOLOGY | mSphere®

RESEARCH ARTICLE

Gastric Non-*Helicobacter pylori* Urease-Positive *Staphylococcus epidermidis* and *Streptococcus salivarius* Isolated from Humans Have Contrasting Effects on *H. pylori*-Associated Gastric Pathology and Host Immune Responses in a Murine Model of Gastric Cancer

Zeli Shen,[a] JoAnn Dzink-Fox,[a] Yan Feng,[a] Sureshkumar Muthupalani,[a] Anthony J. Mannion,[a] Alexander Sheh,[a] Mark T. Whary,[a] Hilda R. Holcombe,[a] Blanca M. Piazuelo,[b] Luis E. Bravo,[c] Christine Josenhans,[d] Sebastian Suerbaum,[d] Keith T. Wilson,[b] Richard M. Peek,[b] Timothy C. Wang,[e] James G. Fox[a]

[a]Division of Comparative Medicine, Massachusetts Institute of Technology, Cambridge, Massachusetts, USA
[b]Division of Gastroenterology, Vanderbilt University Medical Center, Nashville, Tennessee, USA
[c]Department of Pathology, Universidad del Valle, Cali, Colombia
[d]Medical Microbiology and Hospital Epidemiology, Max von Pettenkofer-Institute, Ludwig Maximilians University, Munich, Germany
[e]Division of Gastroenterology and Irvine Cancer Research Center, Columbia University, New York, New York, USA

ABSTRACT In populations with similar prevalence of *Helicobacter pylori* infection, cancer risk can vary dramatically. Changes in composition or structure of bacterial communities in the stomach, either at the time of exposure or over the course of *H. pylori* infection, may contribute to gastric pathology. In this study, a population of 37 patients from the low-gastric-cancer-risk (LGCR) region of Tumaco, Colombia, and the high-gastric-cancer-risk (HGCR) region of Túquerres, Colombia, were recruited for gastric endoscopy. Antral biopsy specimens were processed for histology and bacterial isolation. Fifty-nine distinct species among 26 genera were isolated by aerobic, anaerobic, and microaerobic culture and confirmed by 16S rRNA analysis. Urease-positive *Staphylococcus epidermidis* and *Streptococcus salivarius* were frequently isolated from gastric biopsy specimens. We asked whether coinfection of *H. pylori* with urease-positive *S. salivarius* and/or *S. epidermidis* had a demonstrable effect on *H. pylori*-induced gastritis in the germfree (GF) INS-GAS mouse model. Coinfections with *S. salivarius* and/or *S. epidermidis* did not affect gastric *H. pylori* colonization. At 5 months postinfection, GF INS-GAS mice coinfected with *H. pylori* and *S. salivarius* had statistically higher pathological scores in the stomachs than mice infected with *H. pylori* only or *H. pylori* with *S. epidermidis* ($P < 0.05$). *S. epidermidis* coinfection with *H. pylori* did not significantly change stomach pathology, but levels of the proinflammatory cytokine genes *Il-1β*, *Il-17A* , and *Il-22* were significantly lower than in *H. pylori*-monoinfected mice. This study demonstrates that non-*H. pylori* urease-positive bacteria may play a role in the severity of *H. pylori*-induced gastric cancer in humans.

IMPORTANCE Chronic infection with *H. pylori* is the main cause of gastric cancer, which is a global health problem. In two Colombian populations with high levels of *H. pylori* prevalence, the regional gastric cancer rates are considerably different. Host genetic background, *H. pylori* biotype, environmental toxins, and dietary choices are among the known risk factors for stomach cancer. The potential role of non-*H. pylori* gastric microbiota in gastric carcinogenesis is being increasingly recognized. In this study, we isolated 59 bacterial species from 37 stomach biopsy samples of Colombian patients from both low-gastric-cancer-risk and high-gastric-cancer-risk regions. Urease-positive *S. epidermidis* and *S. salivarius* commonly cultured from the stomachs, along with *H. pylori*, were inoculated into germfree INS-GAS mice. *S. salivarius* coinfection with *H. pylori*

Editor Vincent B. Young, University of Michigan—Ann Arbor

Address correspondence to James G. Fox, jgfox@mit.edu.

The authors declare no conflict of interest.

Received 20 September 2021
Accepted 3 January 2022
Published 9 February 2022

induced significantly higher gastric pathology than in *H. pylori*-monoinfected mice, whereas *S. epidermidis* coinfection caused significantly lower *H. pylori*-induced proinflammatory cytokine responses than in *H. pylori*-monoinfected mice. This study reinforces the argument that the non-*H. pylori* stomach microflora play a role in the severity of *H. pylori*-induced gastric cancer.

KEYWORDS *H. pylori*, microbiome, *S. epidermidis*, *S. salivarius*, INS-GAS mice

Recent studies in humans indicate that gastric colonization by non-*Helicobacter pylori* bacteria, many of which normally colonize the lower bowel and oral cavity, could modulate the risk for gastric adenocarcinoma (GAC) (1, 2). Dyspeptic *H. pylori* patients treated with acid-suppressive drugs have significant increases in non-*H. pylori* bacteria colonizing the stomach and higher levels of inflammatory cytokines, which confer a greater risk of atrophic gastritis. These data suggest that non-*H. pylori* bacteria colonize the stomach and promote *H. pylori* gastric carcinogenesis (3–5).

Inhabitants of Túquerres in the Colombian Andes have a 25-fold higher risk of gastric cancer than inhabitants of the coastal town of Tumaco, despite a similar prevalence of *H. pylori* infection (6). We recently published a paper describing the gastric microbiome in *H. pylori*-infected individuals living in the low- and high-risk areas of Colombia (7). Twenty individuals from each town, matched for age and sex, were selected, and gastric microbiota analyses were performed by deep sequencing of amplified 16S rRNA. In parallel, analyses of *H. pylori* status, detection of carriage of the *cag* pathogenicity island, and assignment of *H. pylori* to phylogeographic groups were performed to test for correlations between *H. pylori* strain properties and microbiota composition (7). Multiple operational taxonomic units (OTUs) were detected exclusively in either Tumaco or Túquerres. Two OTUs, *Leptotrichia wadei* and *Veillonella* spp., were significantly more abundant in Túquerres, and 16 OTUs, including a *Staphylococcus* species closely related to *Staphylococcus epidermidis*, were significantly more abundant in Tumaco. Four members of the genus *Streptococcus* were correlated with multifocal atrophic gastritis with intestinal metaplasia (MAG-IM). There was no significant correlation between *H. pylori* phylogeographic population or carriage of the *cag* pathogenicity island (PAI) and microbiota composition (7).

Studies have demonstrated that selected bacterial species have a direct effect on growth inhibition of *H. pylori* (8). In particular, two strains of *S. epidermidis* inhibited growth of all *H. pylori* strains (27 strains in one assay and 35 strains in another assay) (8). *Streptococcus bovis/Streptococcus gallolyticus* has been associated with malignant gastrointestinal diseases, especially colon cancer, and has been detected in the stomachs of patients with gastric cancer (5). These findings suggested the importance of non-*H. pylori* bacteria in patients with *H. pylori*-associated gastric diseases. In this study, we used culture-dependent methods to isolate non-*H. pylori* bacteria from gastric biopsy samples collected from high-gastric-cancer-risk (HGCR) and low-gastric-cancer-risk (LGCR) patients with or without *H. pylori* infection. Because urease-positive bacteria, like *H. pylori*, have a selective advantage in colonizing the acidic milieu of the stomach, we tested these bacterial isolates for urease activity. Two urease-positive, Gram-positive organisms, *Streptococcus salivarius* (isolated from high-risk patients) and *Staphylococcus epidermidis* (commonly isolated from LGCR patients), were inoculated into germfree (GF) INS-GAS mice infected with *H. pylori* to ascertain whether these bacteria could influence *H. pylori*-associated pathogenesis.

RESULTS

Bacterial species identification in gastric antral biopsy specimens. No bacterial growth was detected in two of the 37 biopsy samples using three different culture conditions. Of the remaining 35 biopsy specimens, 59 distinct species from 26 genera and 4 phyla were identified by culture and confirmed by 16S rRNA sequence analysis. Seven genera in *Actinobacteria*, one genus in *Bacteroidetes*, 11 genera in *Firmicutes*, and seven genera in *Proteobacteria* were identified (Table 1). The prevalence of

TABLE 1 Bacterial genera isolated from biopsy samples

Genus	Total no.[a]	HGCR		LGCR	
		HP$^+$ (*n*/10)	HP$^-$ (*n*/9)	HP$^+$ (*n*/10)	HP$^-$ (*n*/8)
Actinomyces	4	4			
Aerococcus	1		1		
Arthrobacter	1	1			
Atopobium	3	2	1		
Bacillus	18	3	4	6	5
Campylobacter	1	1			
Citrobacter	1		1		
Enterococcus	1				1
Escherichia	1		1		
Gemella	1	1			
Gordonia	2		1	1	
Helicobacter	17	7		10	
Lactobacillus	2	1			1
Mogibacterium	1	1			
Moraxella	1	1			
Neisseria	2	1		1	
Paenibacillus	1		1		
Prevotella	2	1		1	
Propionibacterium	2		1	1	
Rothia	2	2			
Solobacterium	1	1			
Staphylococcus	14	2	2	4	6
Streptococcus	17	6	5	2	4
Streptomyces	2	2			
Veillonella	7	4		1	2
Xanthomonas	1		1		

[a]Number of biopsy specimens with the indicated bacterial genus. *n*, number of patients; HP$^+$, *H. pylori*-positive patients; HP$^-$, *H. pylori*-negative patients.

bacterial species isolated from the biopsy samples from the HGCR and LGCR populations is presented in Table S1 in the supplemental material.

The isolated bacterial species from LGCR and HGCR regions were also used to determine differences in diversity using community ecology metrics. The results suggested that the LGCR and HGCR regions had statistically different ecological communities based on culture results. The Shannon diversity indexes for HGCR and LGCR regions were 2.027 and 1.664, respectively, indicating an increased diversity in HGCR (Fig. 1A). Dividing the bacteria into subsets based on region and the patient's *H. pylori* status, *H. pylori* infection increased the diversity of culturable organisms compared to that in uninfected individuals from the same geographic location. This finding can potentially signify that *H. pylori* infection can modify the types of bacteria that are cultured using standard clinical microbiological techniques.

For individual biopsy samples, 0 to 9 different bacterial genera were identified. The median number of genera identified was 2, and 85% of samples had fewer than 5 bacterial genera identified. One sample from an *H. pylori*-positive patient from the HGCR group had 9 bacteria genera identified. There was no significant difference in the numbers of bacterial genera isolated from different regions (Fig. 1B). The five most commonly isolated non-*H. pylori* genera from patients were *Bacillus* ($n = 18$), *Streptococcus* ($n = 17$), *Staphylococcus* ($n = 14$), *Veillonella* ($n = 7$), and *Actinomyces* ($n = 4$). The prevalence of several bacterial genera in LGCR was higher. *Bacillus* spp. were isolated from 65% of LGCR biopsy samples but from only 39% of HGCR biopsy samples. Among *Bacillus* spp., *Bacillus pumilus* was isolated from 41% of the biopsy samples in the LGCR region but only 5% of the biopsy samples in the HGCR region. *Staphylococci* spp. were isolated from 59% of LGCR biopsy samples and 22% of HGCR samples. *S. epidermidis* was isolated from 53% of the LGCR biopsy samples but only 17% of the HGCR biopsy samples. *Streptococcus* spp. were isolated from 61% of biopsy samples from HGCR

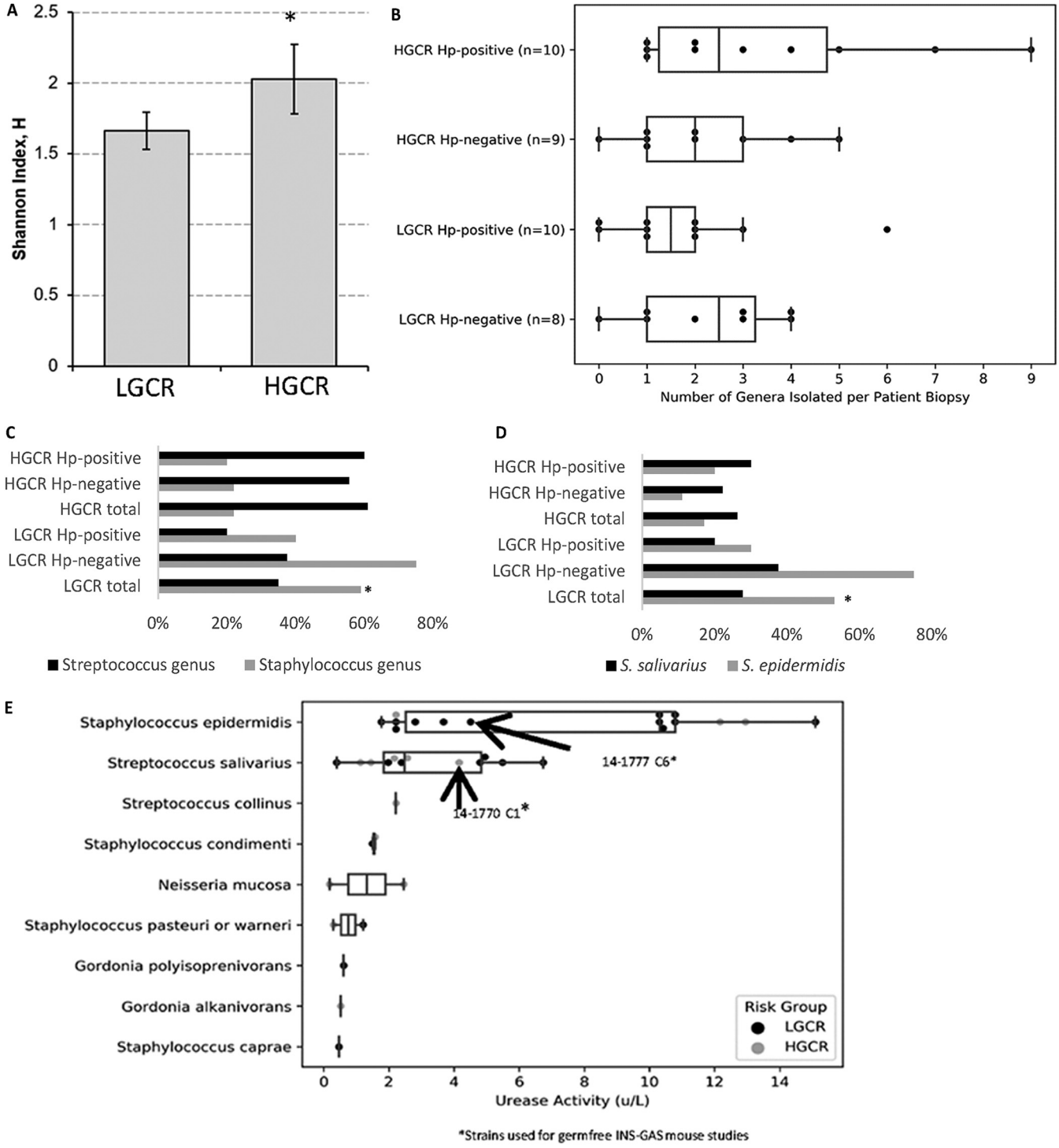

FIG 1 (A) Shannon diversity index for the LGCR and HGCR samples. *, $P < 0.0001$ (implies that HGCR samples have more diversity in bacterial richness and evenness). (B) Non-*H. pylori* bacterial genera isolated from biopsy samples. (C) Prevalence of *Streptococcus* and *Staphylococcus* in LGCR and HGCR populations. *Staphylococcus* was significantly higher in LGCR patients. *, $P < 0.05$. (D) Prevalence of *S. salivarius* and *S. epidermidis* in LGCR and HGCR populations. *, $P < 0.05$. (E) Urease activities of non-*H. pylori* isolates cultured from gastric biopsy specimens. *H. pylori* strain SS1 was used as a positive control and had a urease activity of 262 U/L; *Escherichia coli* ATCC 11775 was used as a negative control. *, strain used in germfree INS-GAS mouse studies.

individuals and only 35% of samples from LGCR individuals. Both *Staphylococcus* spp. and *Streptococcus* spp. were present in 7 biopsy specimens. (Fig. 1C and D). We used Fisher's exact test to determine if it was more likely to isolate each of these species from patients in the LGCR or HGCR region. A statistically significant increase in *Staphylococcus*-positive

TABLE 2 Germfree INS-GAS mouse experiments

Infection status	No. of mice in expt[a]:		
	1	2	3
Controls (uninfected)	7 M	10 M/6 F	6 M/7 F
H. pylori SS1	5 M/2 F	10 M/10 F	12 M/8 F
H. pylori SS1 and *S. salivarius* A and *S. epidermidis* A	4 M/5 F		
S. salivarius A and *S. epidermidis* A	3 M/4 F		
H. pylori SS1 and *S. salivarius* A		11 M/9 F	
H. pylori SS1 and *S. epidermidis* A		9 M/12 F	
H. pylori SS1 and *S. epidermidis* B			10 M/10 F
S. salivarius A		5 M/4 F[b]	
S. epidermidis B			8 M

[a]M, male mice; F, female mice.
[b]Experiment conducted on a different date.

LGCR samples was observed compared to HGCR samples ($P < 0.05$), and the prevalence of *S. epidermidis*-positive samples was significantly higher in LGCR than HGCR samples ($P < 0.05$).

Urease-positive gastric bacteria. Of the 20 patients who were considered *H. pylori* positive by histology, *H. pylori* was isolated from all 10 patients in the LGCR region and 7/10 patients in the HGCR region. Two patients had *H. pylori* growth only, six had *H. pylori* plus 1 additional bacterial species, and the remaining 12 had 2 to 9 non-*H. pylori* species, including 1 to 3 urease-positive species.

Of the 17 *H. pylori*-negative biopsy specimens, 10 had 1 to 2 urease-positive non-*H. pylori* species. Urease-positive bacteria commonly isolated included *Staphylococcus* spp. (*Staphylococcus condimenti*, *S. epidermidis*, *S. caprae*, and *S. warneri*) and *Streptococcus* spp. (*Streptococcus salivarius* and *S. mitis*), with fewer urease-positive *Gordonia* spp., *Neisseria* spp., *Citrobacter freundii*, and *Bacillus* spp.

Figure 1E depicts the urease-positive bacterial strains recovered from the biopsy specimens and their average urease activity. *H. pylori* SS1, used as the positive control, had the strongest urease activity (262 U/L). The urease activities of the *Staphylococcus* spp. and the *Streptococcus* spp. were markedly less than that of *H. pylori* (ranges, 0.46 to 15.1 U/L and 0.4 to 6.74 U/L, respectively). Given the high prevalence of both *S. salivarius* and *S. epidermidis* in these biopsy specimens, we elected to address whether these urease-positive bacteria had a demonstrable effect on *H. pylori* pathogenesis in the INS-GAS mouse model.

Effects of *S. salivarius* and *S. epidermidis* on *H. pylori* pathogenesis in GF INS-GAS mice. (i) Gastric colonization with *S. salivarius* and *S. epidermidis* does not produce gastritis in GF mice. GF INS-GAS mice were infected with *S. salivarius* MIT 14-1770 C1 from the HGCR region (S.sal-A) and *S. epidermidis* MIT 14-1777 C6 from the LGCR region (S.epi-A) to determine whether a mixed population of urease-positive bacteria would induce gastritis in the absence of *H. pylori* infection. Gastric cultures from mice euthanized at 5 months postinoculation (p.i.) confirmed persistent colonization with both bacteria. Infected mice had pathology scores similar to those of the control GF mice at this time point (data not shown).

(ii) Cocolonization with *S. salivarius* and *S. epidermidis* induced similar gastric pathology but decreased inflammatory markers in *H. pylori*-infected mice. To further assess the role of coinfection in gastritis and gastric cancer, mice were monoinfected with *H. pylori* or coinfected with *H. pylori*, *S. epidermidis*, and *S. salivarius* (Table 2, experiment 1). Control groups were uninfected or dosed with *S. epidermidis* and *S. salivarius* without *H. pylori* (Table 2). Mice dosed with *H. pylori* remained colonized at 5 months p.i. as indicated by *H. pylori* culture and PCR. The correlation of *H. pylori* CFU by culture method and DNA copy number in quantitative PCR (qPCR) assay was performed; the strong correlation ($r = 0.9927$) is presented (Fig. S1). We used the qPCR assay to represent *H. pylori* colonization levels in our study due to the higher sensitivity compared to the *H. pylori* culture method. *H. pylori* levels trended lower but were not significantly different

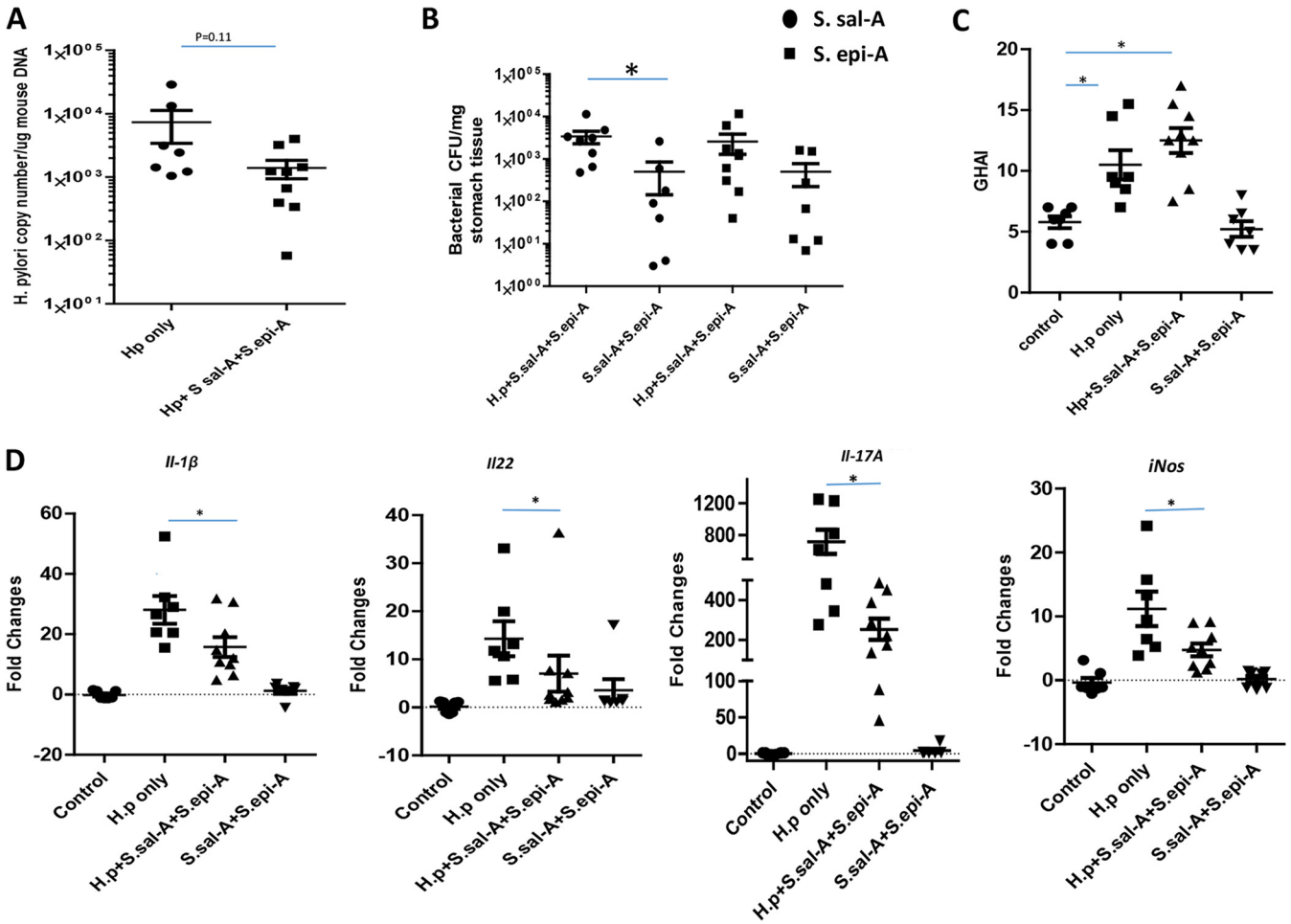

FIG 2 Experiment 1. Each point in the figure indicates an individual mouse. *, $P < 0.05$. (A) Quantitative PCR for *H. pylori* colonization. Cocolonization of *H. pylori*-infected mice with *S. salivarius* and *S. epidermidis* did not significantly affect *H. pylori* colonization levels. (B) *S. salivarius* (S.sal-A) and *S. epidermidis* (S.epi-A) persistently colonized the gastrointestinal tracts of GF mice coinfected with *H. pylori*. *S. salivarius* colonization levels in the stomachs of infected mice were significantly higher in the group coinfected with *H. pylori* than the *S. salivarius-S. epidermidis* group. (C) *H. pylori*-monoinfected mice had robust stomach pathology compared to control mice, but the difference was not significant compared to mice coinfected with *H. pylori* and *S. salivarius* (S.sal-A) and *S. epidermidis* (S.epi-A). (D) Compared to *H. pylori*-monoinfected mice, mice coinfected with all three bacteria had statistically lower *Il-1β*, *Il-17A*, and *Il-22* cytokine expression levels as well as lower *iNos* expression levels in the gastric tissues ($P < 0.05$). No changes in mRNA expression levels for *Ifn-γ*, *Tnf-α*, *Il-10*, *Il-11*, *Reg-3γ*, *Reg3-β*, and *Stat-3* were seen (Supplemental Fig. S2).

between monoinfected mice and mice coinfected with *H. pylori*, *S. salivarius*, and *S. epidermidis* (Fig. 2A). *S. salivarius* and *S. epidermidis* were isolated from the oral cavities, stomachs, and feces of infected mice (data not shown). The colonization levels of *S. epidermidis* in the stomachs of *S. epidermidis*- and *S. salivarius*-infected mice and mice coinfected with *H. pylori* were not significantly different. In contrast, *S. salivarius* colonization levels were significantly higher in the stomachs of mice coinfected with *H. pylori* than in *S. epidermidis*- and *S. salivarius*-infected mice (Fig. 2B).

As expected, *H. pylori*-infected mice developed significant gastric pathology compared to controls. *H. pylori*-infected mice coinfected with *S. salivarius* and *S. epidermidis* also developed significant pathology compared to controls, but the pathology did not statistically differ from that of mice monoinfected with *H. pylori*. *H. pylori* mice with and without *S. salivarius* and *S. epidermidis* coinfection had similar gastric lesions, including prominent mucosal and submucosal inflammation, lymphoid aggregates, mucosal atrophy, foveolar and glandular hyperplasia, epithelial defects, pseudopyloric hyperplasia, and moderate to severe dysplasia. Mice dually infected with *S. salivarius* and *S. epidermidis* mice had pathology scores similar to those of the control GF mice (Fig. 2C).

Interestingly, compared to *H. pylori*-monoinfected mice, mice coinfected with all

three bacteria (i.e., *H. pylori*, *S. salivarius*, and *S. epidermidis*) had statistically lower levels of expression of the cytokine genes *Il-1β*, *Il-22*, and *Il-17A*, as well as lower *iNos* expression levels, in gastric tissues ($P < 0.05$) (Fig. 2D). *Tnf-α* and *Ifn-γ* expression levels also trended lower, but gastric expression levels between the two groups for *Il-11*, *Reg-3γ*, *Reg3-β*, and *Stat-3* were not different (Fig. S2).

(iii) Coinfection with *H. pylori* and *S. salivarius* induced more severe gastric pathology than monoinfection with *H. pylori* or coinfection with *H. pylori* and *S. epidermidis*. The effects of coinfection with individual bacterial species on *H. pylori*-induced gastric disease were determined in monoinfected and dually infected mice, as indicated in Table 2, experiments 2 and 3. At 5 months postinfection, *H. pylori* was successfully recovered from stomach tissue by microaerobic culture (data not shown). There was no statistically significant difference in *H. pylori* colonization levels in the stomachs by qPCR analysis among the three groups infected with *H. pylori* (Fig. 3A). Mice monoinfected with *S. salivarius* had increased pathological changes compared with control mice; however, the changes were less severe than those in *H. pylori*-infected mice (Fig. 3B). All the mice infected with *H. pylori* had robust pathology compared to uninfected controls at 5 months p.i. Mice infected with *H. pylori* and *S. salivarius* had statistically higher overall pathology scores than monoinfected *H. pylori* mice or mice infected with *H. pylori* and *S. epidermidis*. Individual indices, including inflammation, hyperplasia, and dysplasia, were higher in *H. pylori*-*S. salivarius* coinfected mice than monoinfected *H. pylori* mice or mice coinfected with *H. pylori* and *S. epidermidis* (Fig. 3C and D). However, expression levels of tissue cytokines, including those encoded by *Il-1β*, *Il-22*, *Il-17A*, *Tnf-α*, *Ifn-γ*, and *iNos*, were comparable for *H. pylori*-monoinfected and *H. pylori*-*S. salivarius*-coinfected mice. Interestingly, *H. pylori*-*S. salivarius*-coinfected mice had statistically lower expression levels of *Il-10* than *H. pylori*-monoinfected mice (Fig. 3E).

(iv) *H. pylori* and *S. epidermidis* infected mice have statistically lower overall pathology and cytokine expression levels compared to *H. pylori* and *S. salivarius* coinfected mice. The INS-GAS mice coinfected with *H. pylori* and *S. epidermidis* had statistically lower pathology scores, including inflammation, hyperplasia, and dysplasia, than mice coinfected with *H. pylori* and *S. salivarius* (Fig. 3B to D). Similarly, selected proinflammatory tissue cytokine expression levels of *Il-1β*, *Il-22*, *Il-17A*, *Tnf-α*, and *Ifn-γ* were statistically lower in the coinfected *H. pylori* and *S. epidermidis* mice compared to coinfected *H. pylori* and *S. salivarius* mice. Although there was no statistical significance in pathology changes in *H. pylori* and *S. epidermidis* coinfected mice compared with monoinfected *H. pylori* mice, proinflammatory cytokines *Il-1β*, *Il-22*, *Tnf-α*, and *Ifn-γ* were significantly lower in mice coinfected with *H. pylori* and *S. epidermidis* (Fig. 3E).

In experiment 3 (Table 2), an additional *S. epidermidis* strain, MIT 14-1779 M5 (LGCR region), was used to investigate if the effects on coinfection with *H. pylori* were species or strain specific and to investigate the effect of monoinfection with *S. epidermidis*. Similar to the results in mice infected with *S. epidermidis* MIT 14-1777 C6, cocolonization of *H. pylori*-infected mice with *S. epidermidis* MIT 14-1779 M5 did not significantly affect *H. pylori* colonization levels (Fig. 4A), No significant differences were observed in gastric pathology in uninfected mice and mice monoinfected with *S. epidermidis* (Fig. 4B), nor were there significant differences in gastric pathological scores between coinfected mice and *H. pylori*-monoinfected mice (Fig. 4B). Expression levels of proinflammatory cytokines encoded by *Il-1β* and *Il-17A* as well as those of *iNos* in gastric mRNA were lower in *H. pylori*-*S. epidermidis*-coinfected mice (Fig. 4C).

(v) *S. epidermidis* reduced proinflammatory anti-*H. pylori* isotype IgG2a, while *S. salivarius* and *S. epidermidis* both reduced anti-inflammatory anti-*H. pylori* isotype IgG1. The observation of reduced gastric proinflammatory cytokines in mice coinfected with *H. pylori* and *S. epidermidis* compared to *H. pylori* alone was supported by reduced proinflammatory IgG2a responses to *H. pylori* in *H. pylori*-*S. epidermidis*-infected mice compared to mice infected with *H. pylori* alone ($P < 0.05$ in experiment 2 and $P < 0.01$ in experiment 3) (Fig. 5). In contrast, mice coinfected with *H. pylori* and *S. salivarius*

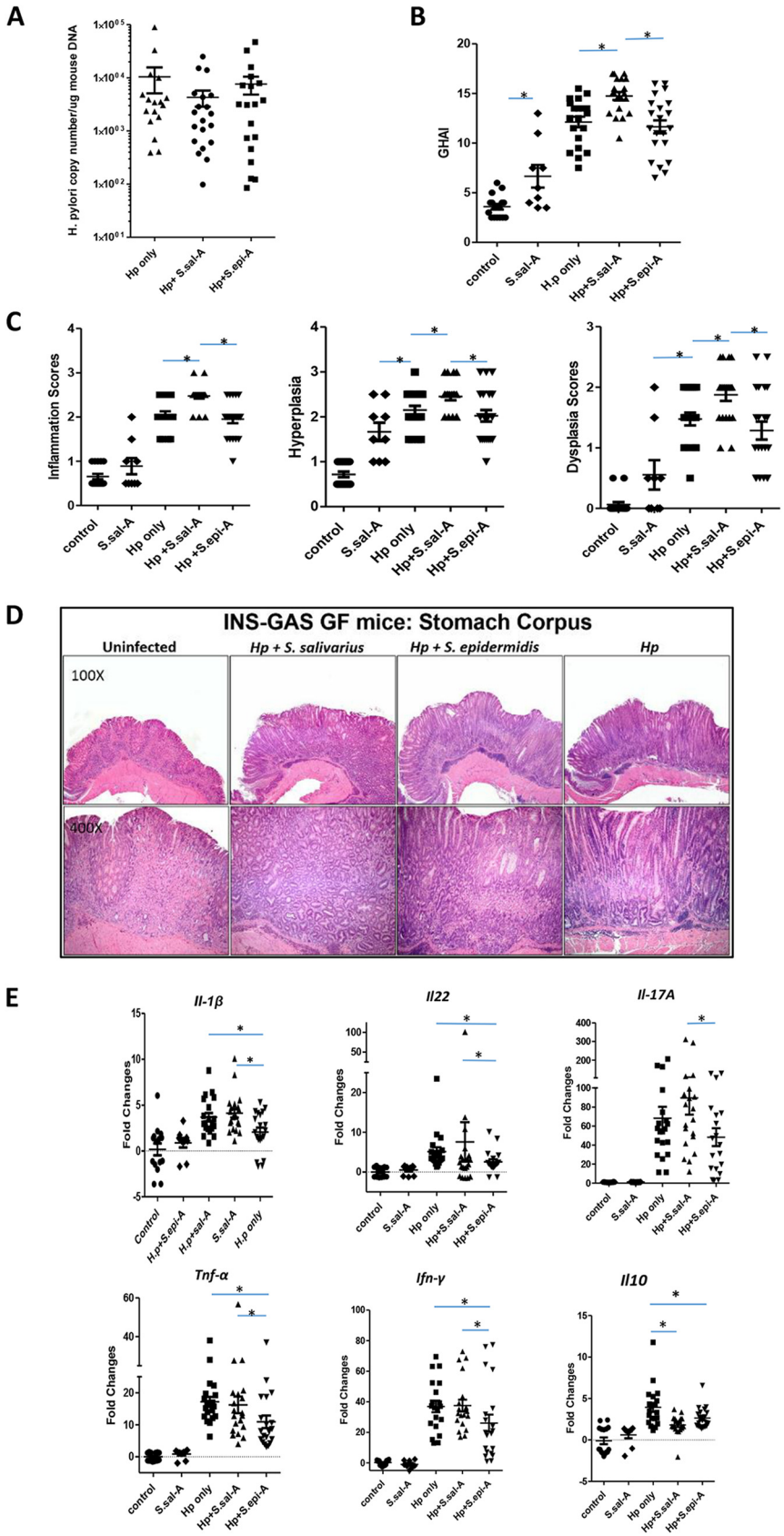

FIG 3 Experiment 2. (A) Quantitative PCR for *H. pylori* colonization. Cocolonization of *H. pylori*-infected mice with *S. salivarius* or *S. epidermidis* did not significantly affect *H. pylori* colonization levels. (B) Gastric

(Continued on next page)

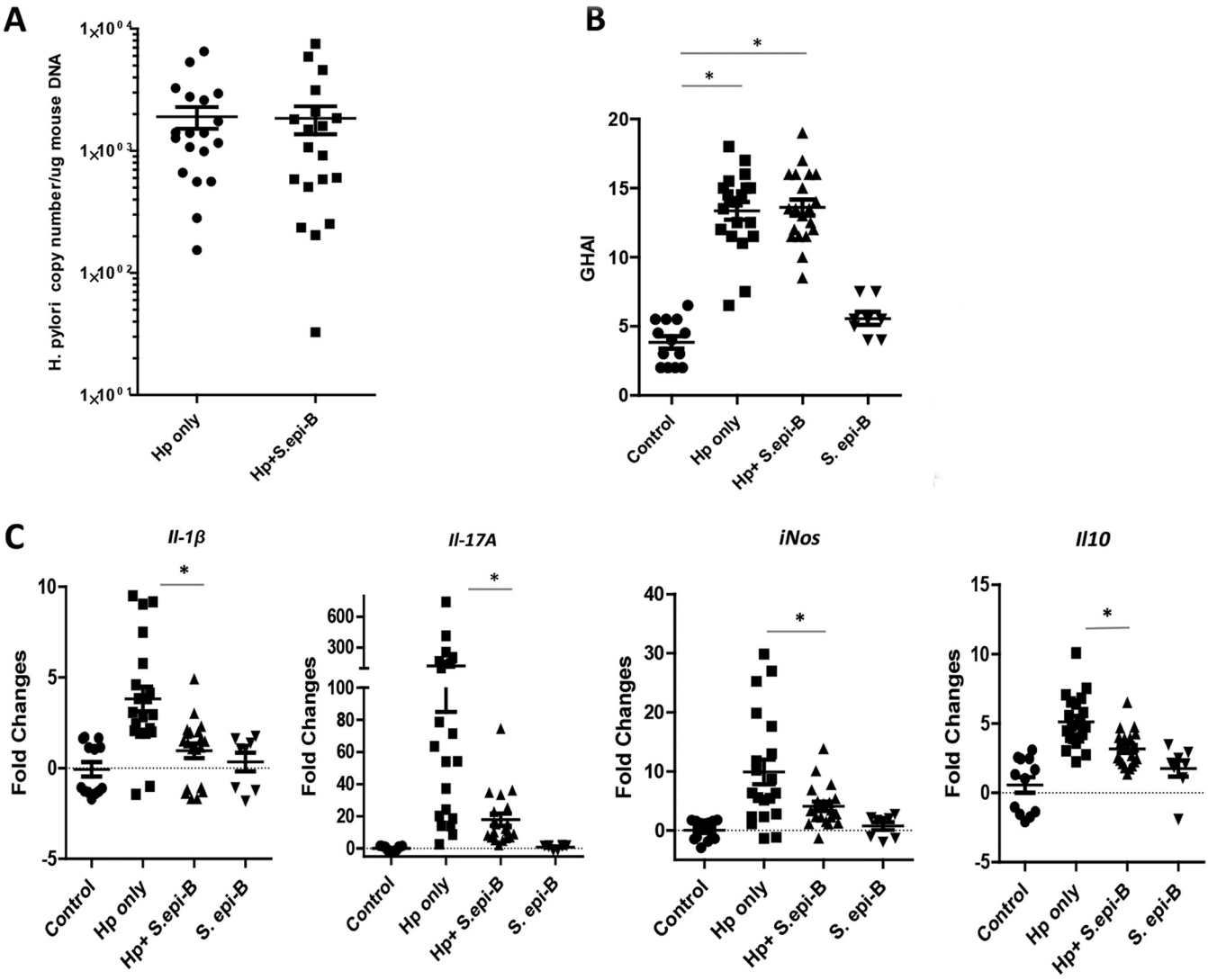

FIG 4 Experiment 3. Each point in the figure indicates an individual mouse. *, $P < 0.05$. (A) Quantitative PCR for *H. pylori* colonization; (B) gastric histologic activity index (GHAI). (C) Expression levels of proinflammatory cytokine genes *Il-1β* and *Il-17A* as well as *iNos* and Il-10 expression levels in gastric mRNA were lower in *H. pylori-S. epidermidis* M5-coinfected INS-GAS mice.

developed the lowest anti-inflammatory IgG1 response to *H. pylori* ($P < 0.001$). The IgG1 response to *H. pylori* in mice coinfected with *H. pylori* and *S. epidermidis* was lower than that in mice infected with *H. pylori* alone ($P < 0.01$ in experiment 2 and $P < 0.05$ in experiment 3) but was greater than the response to *H. pylori* and *S. salivarius* ($P < 0.01$) (Fig. 5, experiment 2).

FIG 3 Legend (Continued)
histologic activity index (GHAI) of GF INS-GAS mice. *H. pylori*-monoinfected mice were not significantly difference from *H. pylori-S. epidermidis* (S.epi-A)-coinfected mice. *H. pylori* coinfection with *S. salivarius* (S.sal-A) induced more severe gastric pathology. (C) INS-GAS mice coinfected with *H. pylori* and *S. salivarius* had significantly higher gastric inflammation, hyperplasia, and dysplasia scores than *H. pylori* only and *H. pylori-S. epidermidis* groups. *, $P < 0.05$. (D) *H. pylori* infection induced moderate gastric pathology compared to control INS-GAS mice. *H. pylori* coinfection with *S. salivarius* induced more severe pathology than *H. pylori* only and *H. pylori* with *S. epidermidis*. (E) mRNA levels of cytokines in stomachs of INS-GAS mice. Expression levels of the proinflammatory cytokines *Il-1β*, *Il-22*, *Il-17A*, *Ifn-γ*, and *Tnf-α* as well as *Il-10* were statistically lower in the *H. pylori-S. epidermidis*-coinfected mice than the *H. pylori-S. salivarius*-coinfected mice and *H. pylori*-monoinfected mice. Each point in the figure indicates an individual mouse. *, $P < 0.05$.

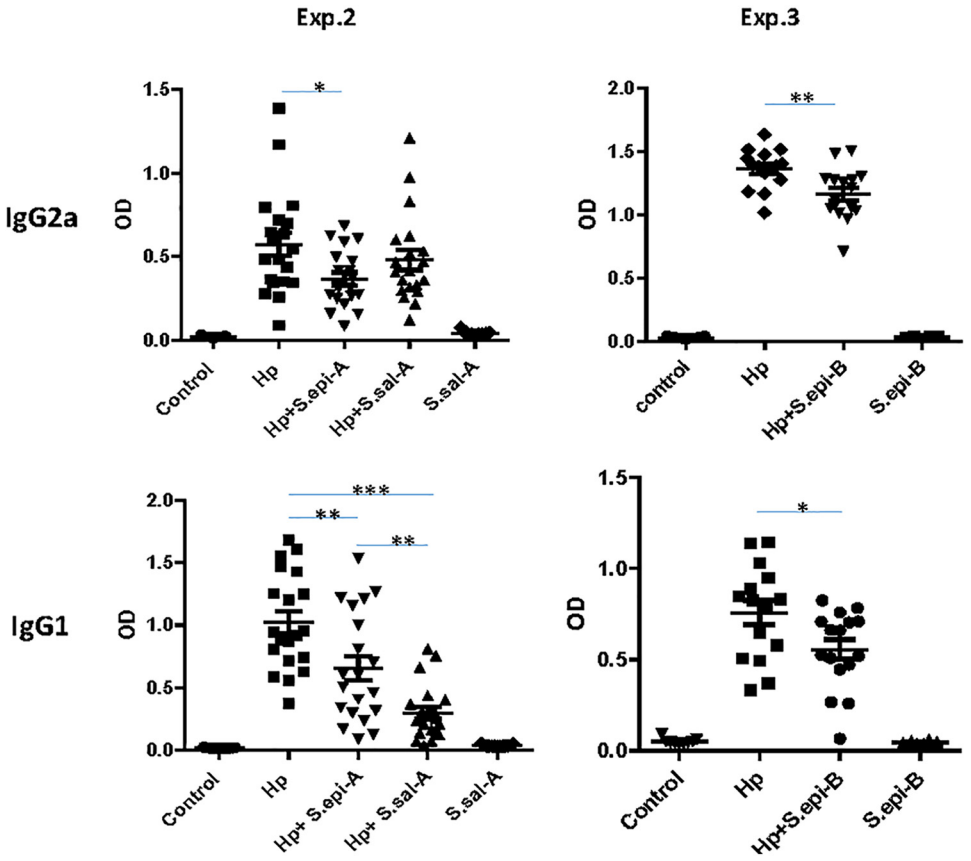

FIG 5 GF INS-GAS mouse serum antibody against *H. pylori* and *S. epidermidis* reduced proinflammatory anti-*H. pylori* isotype IgG2a. In experiments 1 and 2, *S. salivarius* and *S. epidermidis* both reduced anti-inflammatory anti-*H. pylori* isotype IgG1. Mice infected with *H. pylori* and *S. salivarius* developed the lowest anti-inflammatory IgG1 responses to *H. pylori*. *, $P < 0.05$; ** $P < 0.01$; ***, $P < 0.001$.

(vi) Mice coinfected with *H. pylori* and *S. salivarius* had significantly higher numbers of FoxP3- and Ki67-positive cells in gastric tissues. Immunohistochemistry (IHC) staining for FoxP3 and Ki67 was performed on paraffin-embedded gastric tissues. Increased numbers of FoxP3$^+$ cells were noticed in the mice coinfected with *H. pylori* and *S. salivarius*. Mice coinfected with *H. pylori* and *S. epidermidis* had significantly lower numbers of FoxP3$^+$ cells in their gastric mucosae than mice monocolonized with *H. pylori* ($P < 0.05$), as well as mice cocolonized with *H. pylori* and *S. salivarius* ($P < 0.001$) (Fig. 6A). Monoassociated *S. epidermidis* or *S. salivarius* mice had same levels of FoxP3$^+$ cells as control mice (Fig. 6A).

Proliferating epithelial cells were detected by Ki67 IHC staining. In control mice, Ki67-positive cells were observed in the isthmus regions of the corpus mucosa. In all mice infected with *H. pylori*, the Ki67 staining was significantly higher ($P < 0.05$) than in the control mice. Proliferating cells in the corpus expanded from the isthmus regions to hypertrophic foveolar regions. Mice coinfected with *H. pylori* and *S. salivarius* had significantly higher Ki67 staining in the corpus than *H. pylori*-monoinfected mice ($P < 0.05$) and mice coinfected with *H. pylori* and *S. epidermidis* ($P < 0.01$). There was no difference in Ki67 staining between *H. pylori*-monoinfected mice and mice coinfected with *H. pylori* and *S. epidermidis*. *S. epidermidis*- and *S. salivarius*-monoinfected mice had the same levels of Ki67-positive cells as control mice (Fig. 6B).

DISCUSSION

The human gastric microbiota composition and its changes after *H. pylori* colonization have been studied to investigate the mechanisms of *H. pylori*-induced gastric diseases (2).

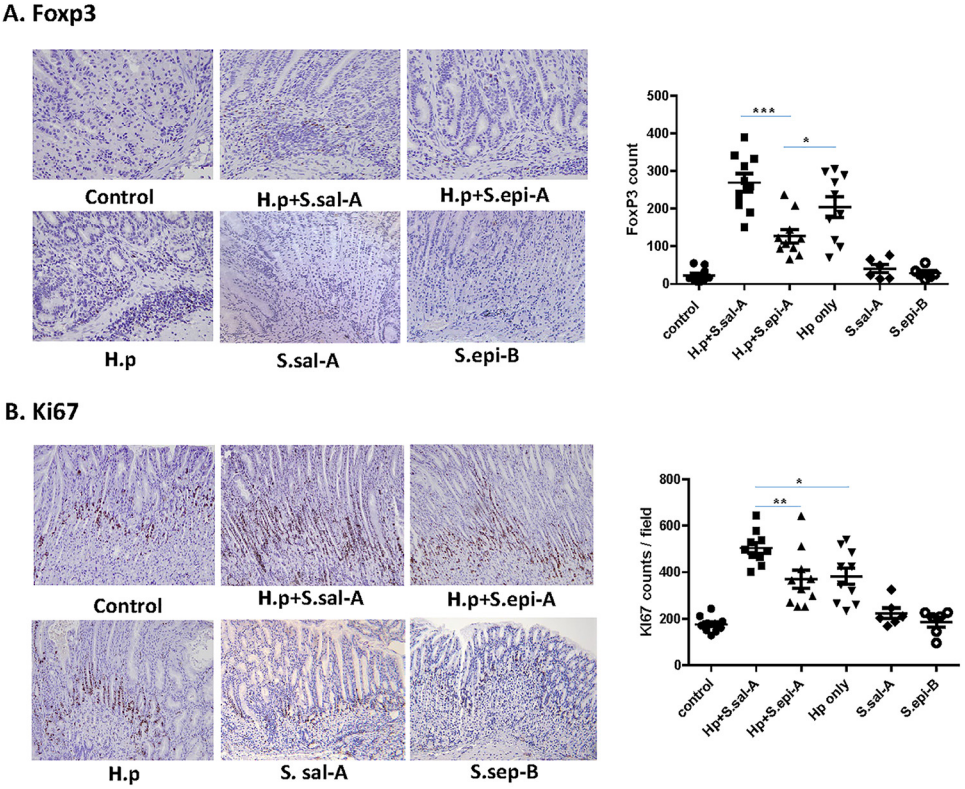

FIG 6 Quantifications of FoxP3 for Tregs (A) and the cell proliferation marker Ki67 (B) in the gastric tissue of INS-GAS mice were performed by IHC staining. The numbers of FoxP3- and Ki67-positive cells in the gastric mucosa and submucosa were counted for 10 fields (magnification, ×200) of mouse stomach corpus. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

Several studies in the recent literature have demonstrated by culture methods that multiple species of bacteria were present in stomachs colonized with *H. pylori*, as well as stomachs not colonized with *H. pylori*. Hu et al. cultured 103 biopsy samples from *H. pylori*-positive patients from China and reported that 65% of patients had non-*H. pylori* bacteria, of which the dominant genera were *Streptococcus*, *Neisseria*, *Rothia*, and *Staphylococcus* (9). Khosravi et al. reported the culturable gastric microbiota of 215 Malaysian patients, including both 131 *H. pylori*-positive and 84 *H. pylori*-negative patients. They found that the most abundant bacteria at the genus level were *Streptococcus*, *Neisseria*, *Klebsiella*, and *Lactobacillus*. In their study, *H. pylori* colonization did not significantly affect the diversity of the gastric microbiota. Interestingly, *Streptococcus* species were associated with peptic ulcer disease (10). Another study cultured the gastric mucosa from 50 chronic gastritis and 53 clinically normal patients and found that the abundance of *Streptococcus* and *Neisseria* were markedly higher in the gastritis group than those in the normal group, suggesting that certain bacterial species may play a role in the development of gastritis rather than being only transient microbes (11). Stomach samples from 346 children in China revealed that the most abundant and culturable bacteria were *Streptococcus*, *Neisseria*, *Rothia*, and *Staphylococcus* (12). In another report, stomach samples from healthy volunteers in Brazil were surveyed for culturable bacteria; *Veillonella* sp., *Lactobacillus* sp., and *Clostridium* sp. were the dominant species (13). Like other studies, the most abundant species in our study were bacteria known to colonize the oral cavity or upper respiratory tract, such as *Streptococcus*, *Veillonella*, and *Staphylococcus*. The prevalence of *Bacillus* isolation was much higher than in other studies (Table S1). The aerobic bacterium *B. pumilus* naturally resides in soils and colonizes the roots of plants. Its abundance in LGCR regions may be related to their geographic location and the dietary habits of LGCR region inhabitants (14). A significantly higher prevalence of *Staphylococcus* spp. in the LGCR region was

noted in our study. Our results in *H. pylori*-infected INS-GAS mice coinfected with *S. epidermidis* suggested that this bacterium may have a protective immunomodulatory effect on the progression of gastritis. The biological function of this organism involved in *H. pylori*-associated gastric cancer requires further investigation.

INS-GAS mice exhibit overexpression of human gastrin under the regulation of a rat insulin promoter. These mice have an age-dependent increase of parietal cell loss and gastric pathology. We previously reported that the normal intestinal and oral flora (IOF) in INS-GAS mice accelerated and promoted the progression of gastrointestinal intraepithelial neoplasia (GIN) (15), while antimicrobial treatment delayed onset of GIN in *H. pylori*-infected mice and, importantly, had the same effect in uninfected INS-GAS mice (16). Notably, *H. pylori*-free INS-GAS mice colonized with IOF developed GIN more quickly than GF INS-GAS mice, which remained free of GIN through 11 months of age (15). Similar results were observed in the *K19-Wnt1/C2mE* (Gan) mouse model of gastric adenocarcinoma (GAC). Specific-pathogen-free (SPF) Gan mice developed large gastric tumors, but gastric tumorigenesis was suppressed in GF Gan mice (17). These data suggest that non-*H. pylori* bacteria, including those considered potentially pathogenic or commensal microorganisms, can colonize the stomach and represent an important component when assessing GAC risk, particularly in *H. pylori*-infected, susceptible individuals (3, 5).

Although evidence supports a role for normal IOF in GAC, it is unknown if specific species of bacteria are required to modulate GAC progression or if the mechanism reflects gastric colonization with diverse microflora secondary to *H. pylori*-mediated gastric atrophy. Using the INS-GAS *H. pylori* mouse model, we evaluated if a restricted microbiota limited to three species of altered Schaedler's flora (rASF), including ASF356 *Clostridium* species, ASF361 *Lactobacillus murinus* and ASF519 *Bacteroides* species, was sufficient to contribute to GIN incidence after development of gastric atrophy secondary to *H. pylori* infection (18–20). We demonstrated that gastric colonization with three species of ASF was comparable to colonization with diverse gastrointestinal microbiota in promoting gastritis and *in situ* gastric carcinoma (20).

The literature cites the presence of non-*H. pylori* urease-positive bacteria as the cause of false positives in *H. pylori* urea breath tests, as ^{13}C urea is hydrolyzed by urease of these commensal bacteria (21–23). In our study, bacteria from multiple genera tested positive for urease activities. Although their urease activity levels were much lower ($<$15 U) than those of *H. pylori* (262 U), these bacteria might have a selective advantage in colonizing the stomach due to their ability to produce NH_3, which buffers the gastric acidity. We therefore selected two urease-positive bacteria, *S. salivarius*, present in both HGCR and LGCR patients, and *S. epidermidis*, predominantly present in the stomachs of LGCR patients, for further investigation.

In the current study, *S. epidermidis* was more commonly isolated from LGCR patients. In the INS-GAS mouse model, *S. epidermidis* efficiently colonized the stomach of *H. pylori*-infected INS-GAS mice. Importantly, even though *S. epidermidis* present in the mouse stomach with *H. pylori* did not change the severity of pathology compared with that in *H. pylori*-monoinfected mice, attendant reductions in the level of RNA expression of proinflammatory cytokine genes *Il-1β*, *Il-17A*, and *Ifn-γ*, as well as lower levels of *Il-22* and IgG2a expression, were noted. It has been reported that *S. epidermidis* strains, which can form biofilms, elicited production of lower levels of the proinflammatory cytokine genes *IL-1b*, *IFNr*, and *IL-12*, and increased levels of anti-inflammatory cytokines such as interleukin 13 (IL-13) in a primary human monocyte-derived macrophages model (24). Cogen et al. demonstrated that *S. epidermidis*-derived δ-toxin interacts with host-derived antimicrobial peptides, present in the innate immune system, to reduce the survival of group A *Streptococcus*, a human pathogen (25).

There were no statistically significant differences in *H. pylori* colonization levels in the stomachs of INS-GAS mice coinfected with either *S. epidermidis* or *S. salivarius*. This may be the result of *H. pylori* and non-*H. pylori* bacteria being present in different colonization niches of the stomach. Brandi et al. have reported that non-*H. pylori* bacteria

are grouped into clusters of different sizes localized mainly over the mucus layer of the stomach and only occasionally can be found embedded in the mucus and in direct contact with gastric microvilli in human biopsy samples (21). While *H. pylori* is spiral shaped with a bundle of unipolar flagella, it is highly motile and efficiently penetrates the mucus layers to reside in the internal mucus layer adjacent to epithelial cells (26, 27). *H. pylori* can also establish colonies deep in the gastric glands, and these populations can expand in size and colonize adjacent glands to provide protected niches during chronic infection (28).

S. salivarius is among the members of the healthy human oral microbiota which are regularly found in human stomach samples. It has been reported that an increased abundance of members of the genus *Streptococcus* can be found in antral gastritis and peptic ulcer diseases as well as in patients with multifocal atrophic gastritis with intestinal metaplasia (9, 10, 29). *S. salivarius* also persistently colonizes the stomachs of *H. pylori*-infected INS-GAS mice. Cocolonization by *H. pylori* and *S. salivarius* induced robust pathology and statistically higher pathology scores compared to *H. pylori* monoinfection of INS-GAS mice and coinfection with *H. pylori* and *S. epidermidis*. Tissue mRNA levels of the cytokines *Il-1*β, *Il-17A*, *Ifn-*γ, and *Tnf-*α and the antimicrobial peptides *Reg-3*β and *Reg-3*γ, as well as serum IgG2a, were comparable among the monoassociated *H. pylori* group and the *H. pylori-S. salivarius* group. Of interest, the expression levels of *Il-10* RNA in mice coinfected with *S. salivarius* and *H. pylori* were lower than in *H. pylori*-monoinfected mice. The presence of these chemokines during *H. pylori* infection leads to the recruitment of immune cells, including neutrophils, macrophages, dendritic cells (DCs), NK cells, and lymphocytes, to infection sites and causes persistent chronic inflammation and gastritis (30). In GF mice coinfected with *S. salivarius* and *H. pylori*, this non-*H. pylori* bacterium may serve as an additional proinflammatory stimulus augmenting the severity of *H. pylori*-induced pathology. *H. pylori* colonization levels were comparable between *H. pylori*-monoinfected mice and those coinfected with *H. pylori* and *S. salivarius*. The mechanism whereby *S. salivarius* increased pathology in *H. pylori*-infected mice is unknown. Certain strains of *S. salivarius* exhibit anti-inflammatory properties in the oral and digestive tract as well as affecting IL-8 secretion and innate immune responses in bronchial and pharyngeal epithelial cells (31–34). Nevertheless, studies involving the intrinsic immunomodulatory effect of *S. salivarius* on cells related to the immune system, including activated immune cells and overexpression of proinflammatory cytokines, have been reported. (35–37).

H. pylori infection induces gastric epithelial cells to produce the proinflammatory cytokines and chemokines such as *Il-8*, *Il-1*β, *Il-6*, *Il-17A*, *Il-22*, *Ifn-*γ, and *Tnf-*α to promote gastric inflammation by neutrophils and macrophages infiltrations and initiation of Th1 immune responses (38). Chronic gastritis can progress to gastric atrophy, intestinal metaplasia, and then dysplasia, leading to neoplastic transformation. Complex control of chronic inflammation in *H. pylori* infection is mediated by a balance of $T_H1/T_H2/T_H17$ and regulatory-T-cell (Treg) responses (39). *H. pylori* infection induces Th1 and Th17 responses, which cause chronic inflammation, but is insufficient to clear *H. pylori* infection. Persistent infection results in the expansion of Tregs to reduce inflammatory response to protect against exaggerated tissue damage. The transcription factor forkhead box protein 3 (FoxP3) is the hallmark of regulatory T cells. It has been frequently reported that Tregs are increased in gastric cancer patients and patients with severe gastritis (40, 41). Gastric $FoxP3^+$ cells were positively associated with the severity of *H. pylori*-induced gastric lesions in INS-GAS mice (42). $FoxP3^+$ Tregs were significantly more abundant in the gastric epithelial cells of mice coinfected with *S. salivarius* and *H. pylori* in our study. IL-17 stimulates gastric epithelial cells to release IL-8, which recruits neutrophils and enhances chronic inflammation (43). IL-17A and IL-22 are also associated with antimicrobial responses and the control of bacterial colonization (44). In our GF INS-GAS mouse model, the most significantly altered cytokine genes in coinfected mice were *Il-1*β, *Il-17A*, and *Il-22*, which were significantly upregulated in mice coinfected with *H. pylori* and *S. salivarius* but significantly downregulated in mice coinfected with *H. pylori* and *S. epidermidis*. Mice

coinfected with *H. pylori* and *S. salivarius* had significantly higher numbers of proliferating epithelial cells detected by Ki67 staining in the stomach than *H. pylori*-monoinfected mice ($P < 0.05$) and mice coinfected with *H. pylori* and *S. epidermidis* ($P < 0.01$).

This study confirms our earlier microbiome analysis of *H. pylori*-infected Colombian patients, demonstrating that both high- and low-gastric-cancer-risk patients have their stomachs colonized by a multitude of non-*H. pylori* bacteria (7). One of these bacteria, *Staphylococcus* sp. (OTU566), which had 99% sequence identity with *S. epidermidis*, was identified from the stomachs of LGCR patients at a much higher frequency than HGCR patients, which is consistent with our culture-based study on a similar patient population. Using a *H. pylori*-infected INS-GAS model, we demonstrated that *S. epidermidis* cocolonization with *H. pylori* attenuated proinflammatory responses. We attribute this finding to possible immunomodulating properties of *S. epidermidis* and its inhibitory effects of inflammation-associated *H. pylori* gastritis. Another possibility is that *S. epidermidis* also has antimicrobial effects on *H. pylori* and other commensal gastric colonizers, which could also afford protection in the gastric compartment of patients infected with *H. pylori* and other gastric compartment-colonizing microbes. It is of interest that in our study, *S. salivarius* and *H. pylori* induced statistically more severe gastritis than either *H. pylori* monoinfection or *H. pylori-S. epidermidis* coinfection. We hypothesize that the *S. salivarius* strain from the stomach of a Colombian patient elicited a stronger Th1- and Th17-mediated immune response, allowing the overexpression of proinflammatory cytokine genes *Il-1β*, *Il-17A*, and *Ifn-γ*, which promoted chronic gastric inflammation and more severe gastric hyperplasia and dysplasia in *H. pylori*-infected INS-GAS mice cocolonized with *S. salivarius*.

It is appreciated that GF mice do not recapitulate all facets of *H. pylori* disease in human. Unlike humans with an intact complex microbiota structure, the lack of microbes in germfree mice allows them to be challenged with defined populations of bacteria (i.e., *H. pylori* with *S. epidermidis/S. salivarius*) to evaluate how specific microbes affect disease states (i.e., gastric inflammation and cancer). In summary, this study reinforces the argument that non-*H. pylori* bacteria colonize the stomachs of humans and play a role in the severity of *H. pylori*-induced gastric cancer. Further, using GF INS-GAS mice, it was demonstrated that the urease-positive bacteria *S. epidermidis* and *S. salivarius* isolated from Colombian patients have the ability to promote or attenuate *H. pylori* associated gastritis.

MATERIALS AND METHODS

Study population, samples, and histopathology. As previously cited, subjects between 40 and 60 years of age with dyspeptic symptoms that warranted upper gastrointestinal tract endoscopy were recruited in Tumaco (LGCR) and Túquerres (HGCR) in 2010. Subjects that had received proton pump inhibitors, H2 receptor antagonists, or antimicrobials during the 30-day period previous to the endoscopic procedure were excluded from this study (7). Other exclusion criteria were major diseases or previous gastrectomy. Participation was voluntary, and informed consent was obtained from all participants. The ethics committees of the participating hospitals in Nariño and the Universidad del Valle in Cali, Colombia, and the Institutional Review Board of Vanderbilt University approved all study protocols, and all experiments were performed in accordance with the relevant guidelines and regulations. A sample population of 37 patients (19 from the HGCR region and 18 from the LGCR region) consisting of 17 males (average age, 50 years) and 20 females (average age, 49 years) were recruited for gastric endoscopy in this study. By histologic diagnosis, 32 patients had non-atrophic gastritis (NAG), 2 had multifocal atrophic gastritis (MAG), and 3 had MAG with intestinal metaplasia. Twenty of the patients were *H. pylori* positive by Warthin-Starry stain.

Bacterial culture methods for gastric biopsy specimens from patients. Antral biopsy specimens were frozen at −80°C in brain heart infusion (BHI) broth containing 20% glycerol. The biopsy specimens were thawed in an anaerobic atmosphere (10% CO_2, 10% H_2, 80% N_2) and were homogenized in BHI with 20% glycerol with tissue grinders. The homogenate was divided into aliquots to isolate bacteria under diverse culture conditions. For aerobic culture, the homogenates were plated onto chocolate agar, blood agar, MacConkey agar, and *Brucella* broth medium containing 10% fetal calf serum (FCS). The plates were incubated at 37°C in 5% CO_2 for 24 to 48 h. For anaerobic culture, the homogenates were plated onto prereduced *Brucella* blood agar plates (BBL) and inoculated into thioglycolate broth. The cultures were incubated at 37°C in an anaerobic chamber (Coy Lab Products) with mixed gas (10% CO_2, 10% H_2, 80% N_2) for 48 h. For microaerobic culture to detect the growth of *H. pylori*, the homogenates were plated onto *H. pylori* selective plates (45) and *Brucella* blood agar plates after passing through a 0.65-μm syringe filter. The plates were placed into a vented jar filled with mixed gas (10%

CO_2, 10% H_2, 80% N_2) and incubated at 37°C for up to 3 weeks. The plates were checked every 2 to 3 days for growth. All bacterial strains isolated from the different culture conditions were identified by 16S rRNA sequencing.

Urease activity assay. All bacterial isolates showing a distinct urease-positive reaction on urea agar slants were further evaluated for their urease activity. Briefly, the bacterial cells were grown in Trypticase soy broth (TSB) for 24 h with shaking at 150 rpm at 37°C. The cultures were washed and resuspended in phosphate-buffered saline (PBS) and adjusted to a 0.5 MacFarland turbidity standard. Urease activity of the bacterial isolates was measured using a urease activity assay kit (MAK120; Sigma-Aldrich) following the manufacturer's instructions. Urease activity was measured in duplicate or triplicate for each strain on separate occasions. One unit of urease is the amount of enzyme that catalyzes the formation of 1.0 μmol of ammonia per min at pH 7.0.

Germ-free-mouse experiments. The animal protocol was approved by the Massachusetts Institute of Technology Committee of Animal Care. GF INS-GAS mice on an FVB/N background [Tg(Ins1-GAS) 1Sbr] were maintained in a facility accredited by AAALAC International. GF mice were housed in sterile isolators on autoclaved hardwood bedding in solid-bottomed polycarbonate cages and fed autoclaved rodent diet (Prolab RMH 3000; PMI Nutrition International), and sterile water was provided *ad libitum*. Isolators for GF control mice were surveyed every other week and confirmed negative for microbial contaminants by culture, PCR using eubacterial primers, and Gram-stained fecal smears.

Seven- to 8-week-old male and female INS-GAS mice were infected by oral gavage with 200 μL of an *H. pylori* (SS1 strain) suspension at 1 unit of optical density at 600 nm (OD_{600})/mL ($\sim 2 \times 10^8$ CFU) on alternate days for a total of 3 doses (46). Coinfected mice were orally infected with *H. pylori* followed in 1 week by further dosing 200 μL of 1 OD_{600}/mL ($\sim 2 \times 10^8$ CFU) of either *S. salivarius*, *S. epidermidis*, or both organisms (Table 2). Mice were necropsied at 5 months postinfection.

(i) Necropsy. Immediately following CO_2 euthanasia of the mice, blood was collected via cardiac puncture; serum was separated and stored at −80°C. The stomach and proximal duodenum were aseptically removed and incised along the greater curvature. Four individual linear gastric strips from the lesser curvature were sectioned for culture, flash frozen for RNA analysis, and stored at −80°C for DNA extraction or preserved in 10% neutral buffered formalin for histopathologic evaluation (47). Gastric lesions were graded by a comparative pathologist who was blind to sample identity on an ascending scale from 0 to 4 for inflammation, epithelial defects, atrophy, hyperplasia, pseudopyloric metaplasia, dysplasia, and mucous metaplasia. A gastric histologic activity index (GHAI) was generated by combining scores for all criteria.

(ii) Quantification of *H. pylori*. A correlation assay between copy number in quantitative PCR and CFU in bacterial culture was performed. *H. pylori* SS1 strain was grown microaerobically on 5% sheep blood agar plates for 24 h. The bacterial suspension was adjusted to 1 OD_{600}/mL and serially diluted (10×) in *Brucella* broth. Bacterial suspensions were subjected in parallel to DNA extraction for qPCR and microanaerobic culture for bacterial CFU.

Colonization levels of gastric *H. pylori* SS1 were quantified using qPCR in the 7500 FAST real-time PCR system (Thermo Fisher Scientific, Waltham, MA) as previously described (48). Copy numbers of *H. pylori* were normalized to micrograms of mouse chromosomal DNA in the samples measured by qPCR using the 18S rRNA gene-based primers and probe mixture (Thermo Fisher Scientific, Waltham, MA).

(iii) CFU counts for *S. salivarius* or *S. epidermidis* in mouse stomach samples. Mouse stomach samples were weighed and homogenized with 1 mL of *Brucella* broth using a glass tissue grinder. The homogenate was diluted 10- and 100-fold in *Brucella* broth. Fifty microliters of each dilution was plated on CPS Elite plates (bioMérieux, Cambridge, MA) and incubated in a 5% CO_2 incubator at 37°C for 24 h. Blue colonies indicated growth of *S. salivarius*; white colonies indicated growth of *S. epidermidis*.

(iv) Cytokine mRNA expression profiles in stomach samples of GF INS-GAS mice. RNA was extracted from stomach tissue samples using the TRIzol reagent (Thermo Fisher Scientific, Waltham, MA). Total RNA (2 μg) was converted to cDNA using a high-capacity cDNA Archive kit following the manufacturer protocol (Thermo Fisher Scientific, Waltham, MA). cDNA levels for *Tnf-α*, *Ifn-γ*, *Il-1β*, *Il-22*, *Il-10*, *Il-17A*, and *iNos* mRNA were measured by quantitative PCR using commercial primers and probes for each cytokine. Briefly, duplicate 20-μL reaction mixtures contained 5 μL of cDNA, 1 μL of a commercial 20× primer-probe solution, 10 μL of 2× master mix (Thermo Fisher Scientific, Waltham, MA), and 4 μL of double-distilled H_2O. Relative expression of mRNA from infected and control samples was calculated using the comparative cycle threshold (C_T) method with RNA input standardized between samples by expression levels of the endogenous reference gene, *Gapdh*. Results from duplicate samples were plotted as fold changes between cells or tissues from infected and uninfected controls.

(v) Serology. Serum Th1-associated IgG2a and Th2-associated IgG1 responses to sonicated antigens of *H. pylori*, *S. epidermidis* and *S. salivarius* were measured by enzyme-linked immunosorbent assay (ELISA). To prepare antigens, *H. pylori* SS1 was grown on blood agar plates under microaerobic condition for 48 h, *S. salivarius* (MIT14-1770 C1) was grown in M17 medium, and *S. epidermidis* (MIT 14-1777 C6; MIT 14-1779 M5) was grown on *Brucella* broth overnight. Bacterial cells were pelleted and washed once with PBS by centrifugation at 12,000 rpm for 5 min. Pellets were resuspended in 5 mL of PBS and then sonicated on ice with an Ultrasonic liquid processor (Misonix, Newtown, CT) using the following program: amplitude, 35; power, 7 W at 30-s intervals for a total of 5 min with 1-min breaks between intervals. Sonicated samples were centrifuged at 12,000 rpm for 10 min at 4°C and filtered through a 0.2-μm syringe filter to remove cell debris.

For serum IgG measurement, 96-well Immulon II plates (Thermo Fischer Scientific, Waltham, MA) were coated with antigen at a concentration of 10 μg/mL and incubated overnight at 4°C. Wells were blocked with 200 μL per well of 2% bovine serum albumin (BSA) in PBS. Serum samples were diluted

1:100 in PBS supplemented with 1% FBS and plated at 100 μL/well. Biotinylated monoclonal anti-mouse antibodies produced by clones A85-1 (specific for mouse IgG1) and 5.7 (specific for mouse IgG2; BD Biosciences, San Jose, CA) were diluted 1:2,000 and used as secondary antibodies (100 μL/well). Incubation with ExtrAvidin-peroxidase (1:2,000, 100 μL/well) (Sigma, St. Louis, MO) was followed by 2,2′-azinobis (3-ethylbenzthiazoline-6-sulfonic acid) diammonium salt (ABTS) substrate (Kirkegaard and Perry Laboratories, Gaithersburg, MD) for color development at 405/590 nm.

(vi) Immunohistochemistry for Foxp3$^{\pm}$ and Ki67 cells. Foxp3 immunochemistry to detect regulatory T cells (Treg) was performed with Foxp3 antibody (1:50; FJK-16S; eBioscience, San Diego, CA). For assessment of epithelial cell proliferation, Ki67 (1:50, BD Biosciences, San Jose, CA) labeling was performed as described previously (49). Paraffin-embedded gastric tissues from 10 mice of each group were selected for these assays. The numbers of cells expressing nuclear staining for Foxp3 and for Ki67 were counted on 10 fields (×200) per stomach and are reported as the average number of positive cells per field.

Statistical analysis. Fisher's exact test was used for bacteria species isolated from human biopsy samples. Shannon index (H) was used to analyze bacterial diversities in different populations and was computed as follows:

$$H = -\sum_{i=i}^{s} p_i lnp_i$$

with the variance computed as

$$s_H^2 = \frac{\sum p(lnp)^2 - \left(\sum plnp\right)^2}{N} + \frac{S-1}{2N^2}$$

where p is the proportion of a species toward the total, S is the number of species, and N is the total abundance. The Hutcheson t test was used to compare the diversity between communities of interest (50).

GF mouse stomach lesion scores were analyzed using the Mann-Whitney U nonparametric test for ordinal data; *H. pylori* colonization, levels of cytokine mRNA expression, and serology results evaluated were compared by Student's t test. Statistical analysis was performed using R 3.6.3 (51) and GraphPad Prism 5.0 (GraphPad Software, Inc., La Jolla, CA). Results were considered significant at a P value of <0.05.

SUPPLEMENTAL MATERIAL

Supplemental material is available online only.

FIG S1, PDF file, 0.1 MB.

FIG S2, TIF file, 0.1 MB.

TABLE S1, DOCX file, 0.02 MB.

ACKNOWLEDGMENTS

We thank Damodaran Annamalai, Andrea Vargas for maintenance and care of germfree mice, Dylan A. Puglisi and Alejandra Aguilar for necropsy and sample collections, and Zhongming Ge for technique support and advice. We thank Ines Yang for analyses of a related next-generation sequencing data set that informed the experimental design of this study, and we thank Parisa Zarringhalam and Elaine Robbins for assistance with manuscript preparation.

This research is supported by NIH grants P30 ES02109 (to J.G.F.), P01 CA028842-29 (to J.G.F. and K.T.W.), and R35CA210088 (to T.C.W. and J.G.F.) and by German Research Foundation SPP 1656 (to S.S.) and funding in the framework of the German Center for Infection Research (DZIF; project number 04.802 to S.S. and projects number 06.804 and number 06.809 to C.J.). The granting agencies played no role in the study, preparation of article, or the decision to publish.

The authors declare that there are no conflicts of interest.

REFERENCES

1. Stearns JC, Lynch MDJ, Senadheera DB, Tenenbaum HC, Goldberg MB, Cvitkovitch DG, Croitoru K, Moreno-Hagelsieb G, Neufeld JD. 2011. Bacterial biogeography of the human digestive tract. Sci Rep 1:170. https://doi.org/10.1038/srep00170.
2. Maldonado-Contreras A, Goldfarb KC, Godoy-Vitorino F, Karaoz U, Contreras M, Blaser MJ, Brodie EL, Dominguez-Bello MG. 2011. Structure of the human gastric bacterial community in relation to *Helicobacter pylori* status. ISME J 5:574–579. https://doi.org/10.1038/ismej.2010.149.
3. Sanduleanu S, Jonkers D, De Bruine A, Hameeteman W, Stockbrügger RW. 2001. Non-*Helicobacter pylori* bacterial flora during acid-suppressive therapy: differential findings in gastric juice and gastric mucosa. Aliment Pharmacol Ther 15:379–388. https://doi.org/10.1046/j.1365-2036.2001.00888.x.

4. Mowat C, Williams C, Gillen D, Hossack M, Gilmour D, Carswell A, Wirz A, Preston T, McColl KEL. 2000. Omeprazole, *Helicobacter pylori* status, and alterations in the intragastric milieu facilitating bacterial N-nitrosation. Gastroenterology 119:339–347. https://doi.org/10.1053/gast.2000.9367.
5. Dicksved J, Lindberg M, Rosenquist M, Enroth H, Jansson JK, Engstrand L. 2009. Molecular characterization of the stomach microbiota in patients with gastric cancer and in controls. J Med Microbiol 58:509–516. https://doi.org/10.1099/jmm.0.007302-0.
6. Correa P, Cuello C, Duque E, Burbano LC, Garcia FT, Bolanos O, Brown C, Haenszel W. 1976. Gastric cancer in Colombia. III. Natural history of precursor lesions. J Natl Cancer Inst 57:1027–1035. https://doi.org/10.1093/jnci/57.5.1027.
7. Yang I, Woltemate S, Piazuelo MB, Bravo LE, Yepez MC, Romero-Gallo J, Delgado AG, Wilson KT, Peek RM, Correa P, Josenhans C, Fox JG, Suerbaum S. 2016. Different gastric microbiota compositions in two human populations with high and low gastric cancer risk in Colombia. Sci Rep 6:18594. https://doi.org/10.1038/srep18594.
8. López-Brea M, Alarcón T, Domingo D, Díaz-Regañón J. 2008. Inhibitory effect of Gram-negative and Gram-positive microorganisms against *Helicobacter pylori* clinical isolates. J Antimicrob Chemother 61:139–142. https://doi.org/10.1093/jac/dkm404.
9. Hu Y, He LH, Xiao D, Liu GD, Gu YX, Tao XX, Zhang JZ. 2012. Bacterial flora concurrent with *Helicobacter pylori* in the stomach of patients with upper gastrointestinal diseases. World J Gastroenterol 18:1257–1261. https://doi.org/10.3748/wjg.v18.i11.1257.
10. Khosravi Y, Dieye Y, Poh BH, Ng CG, Loke MF, Goh KL, Vadivelu J. 2014. Culturable bacterial microbiota of the stomach of *Helicobacter pylori* positive and negative gastric disease patients. ScientificWorldJournal 2014:610421. https://doi.org/10.1155/2014/610421.
11. Liu J, Xue Y, Zhou L. 2018. Detection of gastritis-associated pathogens by culturing of gastric juice and mucosa. Int J Clin Exp Pathol 11:2214–2220.
12. Guo C, Liu F, Zhu L, Wu F, Cui G, Xiong Y, Wang Q, Yin L, Wang C, Wang H, Wu X, Zhang Z, Chen Z. 2019. Analysis of culturable microbiota present in the stomach of children with gastric symptoms. Braz J Microbiol 50:107–115. https://doi.org/10.1007/s42770-018-0030-5.
13. Zilberstein B, Quintanilha AG, Santos MAA, Pajecki D, Moura EG, Alves PRA, Maluf Filho F, de Souza JAU, Gama-Rodrigues J. 2007. Digestive tract microbiota in healthy volunteers. Clinics (Sao Paulo) 62:47–54. https://doi.org/10.1590/s1807-59322007000100008.
14. Quintero-Lesmes DC, Herran OF. 2019. Food changes and geography: dietary transition in Colombia. Ann Global Health 85:28. https://doi.org/10.5334/aogh.1643.
15. Lofgren JL, Whary MT, Ge Z, Muthupalani S, Taylor NS, Mobley M, Potter A, Varro A, Eibach D, Suerbaum S, Wang TC, Fox JG. 2011. Lack of commensal flora in *Helicobacter pylori*-infected INS-GAS mice reduces gastritis and delays intraepithelial neoplasia. Gastroenterology 140:210–220. https://doi.org/10.1053/j.gastro.2010.09.048.
16. Lee C-W, Rickman B, Rogers AB, Muthupalani S, Takaishi S, Yang P, Wang TC, Fox JG. 2009. Combination of sulindac and antimicrobial eradication of *Helicobacter pylori* prevents progression of gastric cancer in hypergastrinemic INS-GAS mice. Cancer Res 69:8166–8174. https://doi.org/10.1158/0008-5472.CAN-08-3856.
17. Oshima H, Hioki K, Popivanova BK, Oguma K, Van Rooijen N, Ishikawa T-O, Oshima M. 2011. Prostaglandin E_2 signaling and bacterial infection recruit tumor-promoting macrophages to mouse gastric tumors. Gastroenterology 140:596–607.E7. https://doi.org/10.1053/j.gastro.2010.11.007.
18. Dewhirst FE, Chien CC, Paster BJ, Ericson RL, Orcutt RP, Schauer DB, Fox JG. 1999. Phylogeny of the defined murine microbiota: altered Schaedler flora. Appl Environ Microbiol 65:3287–3292. https://doi.org/10.1128/AEM.65.8.3287-3292.1999.
19. Sarma-Rupavtarm RB, Ge Z, Schauer DB, Fox JG, Polz MF. 2004. Spatial distribution and stability of the eight microbial species of the altered schaedler flora in the mouse gastrointestinal tract. Appl Environ Microbiol 70:2791–2800. https://doi.org/10.1128/AEM.70.5.2791-2800.2004.
20. Lertpiriyapong K, Whary MT, Muthupalani S, Lofgren JL, Gamazon ER, Feng Y, Ge Z, Wang TC, Fox JG. 2014. Gastric colonisation with a restricted commensal microbiota replicates the promotion of neoplastic lesions by diverse intestinal microbiota in the *Helicobacter pylori* INS-GAS mouse model of gastric carcinogenesis. Gut 63:54–63. https://doi.org/10.1136/gutjnl-2013-305178.
21. Brandi G, Biavati B, Calabrese C, Granata M, Nannetti A, Mattarelli P, Di Febo G, Saccoccio G, Biasco G. 2006. Urease-positive bacteria other than *Helicobacter pylori* in human gastric juice and mucosa. Am J Gastroenterol 101:1756–1761. https://doi.org/10.1111/j.1572-0241.2006.00698.x.
22. Michaud L, Gottrand F, Ganga-Zandzou PS, Wizla-Derambure N, Turck D, Vincent P. 1998. Gastric bacterial overgrowth is a cause of false positive diagnosis of *Helicobacter pylori* infection using 13C urea breath test. Gut 42:594–595. https://doi.org/10.1136/gut.42.4.594a.
23. Osaki T, Mabe K, Hanawa T, Kamiya S. 2008. Urease-positive bacteria in the stomach induce a false-positive reaction in a urea breath test for diagnosis of *Helicobacter pylori* infection. J Med Microbiol 57:814–819. https://doi.org/10.1099/jmm.0.47768-0.
24. Spiliopoulou AI, Kolonitsiou F, Krevvata MI, Leontsinidis M, Wilkinson TS, Mack D, Anastassiou ED. 2012. Bacterial adhesion, intracellular survival and cytokine induction upon stimulation of mononuclear cells with planktonic or biofilm phase Staphylococcus epidermidis. FEMS Microbiol Lett 330:56–65. https://doi.org/10.1111/j.1574-6968.2012.02533.x.
25. Cogen AL, Yamasaki K, Muto J, Sanchez KM, Crotty Alexander L, Tanios J, Lai Y, Kim JE, Nizet V, Gallo RL. 2010. *Staphylococcus epidermidis* antimicrobial delta-toxin (phenol-soluble modulin-gamma) cooperates with host antimicrobial peptides to kill group A Streptococcus. PLoS One 5:e8557. https://doi.org/10.1371/journal.pone.0008557.
26. Ottemann KM, Lowenthal AC. 2002. *Helicobacter pylori* uses motility for initial colonization and to attain robust infection. Infect Immun 70:1984–1990. https://doi.org/10.1128/IAI.70.4.1984-1990.2002.
27. Shimizu T, Akamatsu T, Sugiyama A, Ota H, Katsuyama T. 1996. *Helicobacter pylori* and the surface mucous gel layer of the human stomach. Helicobacter 1:207–218. https://doi.org/10.1111/j.1523-5378.1996.tb00041.x.
28. Fung C, Tan S, Nakajima M, Skoog EC, Camarillo-Guerrero LF, Klein JA, Lawley TD, Solnick JV, Fukami T, Amieva MR. 2019. High-resolution mapping reveals that microniches in the gastric glands control *Helicobacter pylori* colonization of the stomach. PLoS Biol 17:e3000231. https://doi.org/10.1371/journal.pbio.3000231.
29. Delgado S, Cabrera-Rubio R, Mira A, Suárez A, Mayo B. 2013. Microbiological survey of the human gastric ecosystem using culturing and pyrosequencing methods. Microb Ecol 65:763–772. https://doi.org/10.1007/s00248-013-0192-5.
30. Tran CT, Garcia M, Garnier M, Burucoa C, Bodet C. 2017. Inflammatory signaling pathways induced by *Helicobacter pylori* in primary human gastric epithelial cells. Innate Immun 23:165–174. https://doi.org/10.1177/1753425916681077.
31. Cosseau C, Devine DA, Dullaghan E, Gardy JL, Chikatamarla A, Gellatly S, Yu LL, Pistolic J, Falsafi R, Tagg J, Hancock REW. 2008. The commensal Streptococcus salivarius K12 downregulates the innate immune responses of human epithelial cells and promotes host-microbe homeostasis. Infect Immun 76:4163–4175. https://doi.org/10.1128/IAI.00188-08.
32. Guglielmetti S, Taverniti V, Minuzzo M, Arioli S, Stuknyte M, Karp M, Mora D. 2010. Oral bacteria as potential probiotics for the pharyngeal mucosa. Appl Environ Microbiol 76:3948–3958. https://doi.org/10.1128/AEM.00109-10.
33. Kaci G, Goudercourt D, Dennin V, Pot B, Doré J, Ehrlich SD, Renault P, Blottière HM, Daniel C, Delorme C. 2014. Anti-inflammatory properties of *Streptococcus salivarius*, a commensal bacterium of the oral cavity and digestive tract. Appl Environ Microbiol 80:928–934. https://doi.org/10.1128/AEM.03133-13.
34. Kaci G, Lakhdari O, Doré J, Ehrlich SD, Renault P, Blottière HM, Delorme C. 2011. Inhibition of the NF-kappaB pathway in human intestinal epithelial cells by commensal *Streptococcus salivarius*. Appl Environ Microbiol 77:4681–4684. https://doi.org/10.1128/AEM.03021-10.
35. van den Bogert B, Meijerink M, Zoetendal EG, Wells JM, Kleerebezem M. 2014. Immunomodulatory properties of Streptococcus and Veillonella isolates from the human small intestine microbiota. PLoS One 9:e114277. https://doi.org/10.1371/journal.pone.0114277.
36. Bouwer AL, Saunderson SC, Dunn AC, Lester KL, Crowley LR, Jack RW, McLellan AD. 2013. Rapid interferon-gamma release from natural killer cells induced by a streptococcal commensal. J Interferon Cytokine Res 33:459–466. https://doi.org/10.1089/jir.2012.0116.
37. Skovbjerg S, Martner A, Hynsjö L, Hessle C, Olsen I, Dewhirst FE, Tham W, Wold AE. 2010. Gram-positive and gram-negative bacteria induce different patterns of cytokine production in human mononuclear cells irrespective of taxonomic relatedness. J Interferon Cytokine Res 30:23–32. https://doi.org/10.1089/jir.2009.0033.
38. Algood HMS. 2020. T cell cytokines impact epithelial cell responses during *Helicobacter pylori* infection. J Immunol 204:1421–1428. https://doi.org/10.4049/jimmunol.1901307.
39. Jafarzadeh A, Larussa T, Nemati M, Jalapour S. 2018. T cell subsets play an important role in the determination of the clinical outcome of *Helicobacter pylori* infection. Microb Pathog 116:227–236. https://doi.org/10.1016/j.micpath.2018.01.040.

40. Lundgren A, Strömberg E, Sjöling A, Lindholm C, Enarsson K, Edebo A, Johnsson E, Suri-Payer E, Larsson P, Rudin A, Svennerholm A-M, Lundin BS. 2005. Mucosal FOXP3-expressing CD4+ CD25high regulatory T cells in *Helicobacter pylori*-infected patients. Infect Immun 73:523–531. https://doi.org/10.1128/IAI.73.1.523-531.2005.
41. Rezalotfi A, Ahmadian E, Aazami H, Solgi G, Ebrahimi M. 2019. Gastric cancer stem cells effect on Th17/Treg balance; a bench to bedside perspective. Front Oncol 9:226. https://doi.org/10.3389/fonc.2019.00226.
42. Ohtani M, Ge Z, Garcia A, Rogers AB, Muthupalani S, Taylor NS, Xu S, Watanabe K, Feng Y, Marini RP, Whary MT, Wang TC, Fox JG. 2011. 17-β-Estradiol suppresses *Helicobacter pylori*-induced gastric pathology in male hypergastrinemic INS-GAS mice. Carcinogenesis 32:1244–1250. https://doi.org/10.1093/carcin/bgr072.
43. Lina TT, Alzahrani S, Gonzalez J, Pinchuk IV, Beswick EJ, Reyes VE. 2014. Immune evasion strategies used by *Helicobacter pylori*. World J Gastroenterol 20:12753–12766. https://doi.org/10.3748/wjg.v20.i36.12753.
44. Algood HM, Allen SS, Washington MK, Peek RM, Jr, Miller GG, Cover TL. 2009. Regulation of gastric B cell recruitment is dependent on IL-17 receptor A signaling in a model of chronic bacterial infection. J Immunol 183:5837–5846. https://doi.org/10.4049/jimmunol.0901206.
45. Fox JG, Dangler CA, Taylor NS, King A, Koh TJ, Wang TC. 1999. High-salt diet induces gastric epithelial hyperplasia and parietal cell loss, and enhances *Helicobacter pylori* colonization in C57BL/6 mice. Cancer Res 59:4823–4828.
46. Lee C-W, Rao VP, Rogers AB, Ge Z, Erdman SE, Whary MT, Fox JG. 2007. Wild-type and interleukin-10-deficient regulatory T cells reduce effector T-cell-mediated gastroduodenitis in Rag2-/- mice, but only wild-type regulatory T cells suppress *Helicobacter pylori* gastritis. Infect Immun 75:2699–2707. https://doi.org/10.1128/IAI.01788-06.
47. Rogers AB, Taylor NS, Whary MT, Stefanich ED, Wang TC, Fox JG. 2005. *Helicobacter pylori* but not high salt induces gastric intraepithelial neoplasia in B6129 mice. Cancer Res 65:10709–10715. https://doi.org/10.1158/0008-5472.CAN-05-1846.
48. Fox JG, Wang TC, Rogers AB, Poutahidis T, Ge Z, Taylor N, Dangler CA, Israel DA, Krishna U, Gaus K, Peek RM. 2003. Host and microbial constituents influence *Helicobacter pylori*-induced cancer in a murine model of hypergastrinemia. Gastroenterology 124:1879–1890. https://doi.org/10.1016/S0016-5085(03)00406-2.
49. Lemke LB, Ge Z, Whary MT, Feng Y, Rogers AB, Muthupalani S, Fox JG. 2009. Concurrent *Helicobacter bilis* infection in C57BL/6 mice attenuates proinflammatory H. pylori-induced gastric pathology. Infect Immun 77:2147–2158. https://doi.org/10.1128/IAI.01395-08.
50. Hutcheson K. 1970. A test for comparing diversities based on the Shannon formula. J Theor Biol 29:151–154. https://doi.org/10.1016/0022-5193(70)90124-4.
51. R Core Team. 2020. R: a language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria. https://www.R-project.org/.