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Hydrogen sulfide-mediated resistance against water avoidance stress-induced gastritis by maintenance of gastric microbial homeostasis

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Abstract

Chronic persistent stress is an important cause of gastritis, but the underlying mechanism remains to be further researched, especially the role of the gastric microbiota in this process. Here, we used the water avoidance stress (WAS) test in mouse models for chronic stress-induced gastritis to investigate the underlying mechanisms of this disease. The effect of stress on the gastric microbiota was analyzed based on 16S rRNA sequencing; the changes in hydrogen sulfide (H_2S) and inflammatory cytokine levels in gastric tissues were detected by Western blotting, ELISA, immunofluorescence, and qRT-PCR. Hematoxylin and eosin staining was used as an indicator of the gastritis histological score. This finding is consistent with previous studies showing that gastric H_2S is negatively associated with the inflammatory index and might protect the gastrointestinal tract from inflammation. WAS-induced gastritis was associated with a reduction in H_2S release, which appeared to affect the homeostasis of the gastric microbiota of mice. Inflammation and microbial dysbiosis were partially reversed by sodium hydrosulfide (NaHS) and vitamin B6 (VB6) supplementation, suggesting the therapeutic potential of VB6 supplementation for the treatment of stress-induced gastritis. Gastritis has a serious impact on health and quality of life. An increasing number of people are suffering from chronic gastritis linked to a high-stress lifestyle, and our research provides clues for the prevention and treatment of stress-induced gastritis.

KEYWORDS
gastric microbiota, gastritis, H_2S, stress, VB6

1 | INTRODUCTION

With the development of DNA sequencing technology, it has been found that in addition to *Helicobacter pylori* (*H. pylori*), the stomach harbors a relatively stable flora (Bik et al., 2006; Nardone & Compare, 2015). Studies have shown that the gastric microbiota is associated with gastric cancer (Ferreira et al., 2018) and gastritis (Engstrand & Lindberg, 2013).

Gastritis is the inflammation of the lining of the stomach (Hunt et al., 2015), and approximately half of the world's population suffers from this condition (Magierowski, Magierowska, Kwiecien, & Brzozowski, 2015b). The main drivers of gastritis include *H. pylori* infection, use of nonsteroidal anti-inflammatory drugs (NSAIDs) (Hunt et al., 2015), drinking, smoking, and many other factors (Ko, Shin, Cho, Park, & Yoo, 2018), such as stress (Peters & Richardson, 1983). Previous research has shown that stressful life events can cause increased gastric acid secretion, which is a cause of ulceration (Peters & Richardson, 1983). Further research has found that skin sensation can cause an increase in vagal outflow and subsequently in gastric acid secretion, which may be an important factor underlying the gastric mucosal lesions caused by stress (Guo et al., 2012). Xie et al. have reported that parasympathetic overactivity is the predominant autonomic response to stress and is most likely the leading mechanism of stress-induced gastritis in rats (Xie, 2005). Other studies have shown that reactive oxygen species can damage the gastric mucosa and contribute to stress-induced gastritis in rats (Kwiecień, Brzozowski, Konturek, & Konturek, 2002a; Kwiecień, Brzozowski, & Konturek, 2002b), and epidermal growth factor, polyamines, and prostaglandins have therapeutic effects on stress-induced gastritis (Brzozowski, Konturek, Majka, Dembinski, & Drozdowicz, 1993; Magierowski et al., 2015a). Further research shows that hydrogen sulfide (H_2S) has a protective effect on gastric damage caused by stress (Bronowicka-Adamska et al., 2017; Lou, Geng, Du, & Tang, 2008; Magierowski et al., 2015b, 2016).

In these studies, the rats were subjected to brief and intense stress (water immersion restraint stress, WRS) for hours, and acute gastritis was induced. However, in modern society, people experience psychological stress for days or even months; it is necessary to use realistic animal models of long-term psychological stress to further understand the effects of long-term stress on the gastric microbiota and gastritis.

In our study, we used mice stimulated by water avoidance stress (WAS) for 10 days (Bradesi, 2005) to develop stress-induced gastritis. Our results showed that stress can induce gastric microbial dysbiosis and H_2S reduction, ultimately leading to gastritis. We also discovered the effectiveness of treatment with NaHS and vitamin B6 (VB6). VB6 is a cofactor of H_2S metabolism for stress-induced gastritis.

2 | MATERIALS AND METHODS

2.1 | Mice

Female C57BL/6 mice (ages 5–6 weeks) were purchased from Beijing Huafang Biotechnology Co., Ltd. and were housed in the Animal Breeding Center of Shandong University in the absence of specific pathogens.

2.2 | Mouse treatment

For the WAS test, the mice were placed on a small platform surrounded by shallow water at a temperature of 25°C for 1 hr per day for 10 days (Bradesi, 2005). During the daily hour of water exposure, the mice tried to avoid the water by carefully remaining on the platform, which caused stress. According to the experimental design, 20 mice were randomly divided into two groups (n = 10): one group was used as a control (nonstressed) and one group was subjected to WAS for 10 days to determine whether WAS could induce gastritis. A total of 50 mice were randomly divided into four groups (n = 10) and were subjected to the WAS test for 0 (control), 1, 4, 7, and 10 days to detect H_2S production. Thirty mice were randomly divided into three groups (n = 10): the nonstressed (control) group, WAS group, and WAS + NaHS group. In the nonstressed group, mice were raised in a standard environment and fed without any stress or treatment. In the WAS + NaHS group, 1 hr before being subjected to WAS, the mice were injected intraperitoneally (ip) with NaHS (100 μmol kg^{-1} day^{-1}; Sigma-Aldrich Co.); the mice were treated in this manner for 10 days. In the WAS + VB6 group, before being subjected to WAS, the mice were fed VB6 (4.5 mg/kg bw; Sigma-Aldrich Co.) daily via drinking water. In the PAG group, mice were injected intraperitoneally with PAG for 10 days (60 mg kg^{-1} day^{-1}, Sigma-Aldrich Co.).

2.3 | Release of H_2S in mouse gastric tissue

Based on a description in the literature (Xiao et al., 2015), we homogenized the gastric tissue in 50 mM ice-cold potassium phosphate buffer (pH 6.8). The reaction mixture included the following components: 10% (w/v) tissue homogenate (0.5 ml), 0.5 ml of potassium phosphate buffer (pH 7.4, 100 mM), 0.1 ml of pyridoxal 5′-phosphate (20 mM), and 0.1 ml of L-cysteine (L-Cys) (10 mM). We performed this experiment in a 25-ml flask containing the reaction mixture mentioned above. Before sealing, we rinsed the flask with N_2. The experiment was initiated by shifting the flask from ice to a shaking water bath at 37°C. After incubating at 37°C for 90 min, we added trichloroacetic acid (50%, 0.5 ml) to the reaction mixture to terminate the experiment and incubated the mixture for an additional 60 min at 37°C. The contents were then transferred to test tubes containing 3.5 ml of ultrapure water per tube. Then, we added 0.5 ml of a solution of *N*,*N*-dimethyl-*p*-phenylenediamine sulfate (20 mM) in HCl (7.2 M) and immediately added 0.4 ml of FeCl$_3$ (30 mM) to the mixture. We then incubated the mixture for an additional 20 min at the normal indoor temperature, and subsequently, we measured the optical absorbance of the final solution at 670 nm. A standard curve was generated with a known concentration of NaHS. The H_2S concentration was calculated from the calibration curve of the standard H_2S solution.

2.4 | H&E staining, histological evaluation, and IF

Gastric samples were embedded in paraffin and sliced into 5-μm-thick sections. Paraffin blocks were sectioned and stained with hematoxylin and eosin (H&E) for histopathological evaluation. The polymorphonuclear and mononuclear cells in the gastric antrum and in vivo were graded as follows (Ten Kate et al., 2009): 0 represents none; 1 represents some infiltration; 2 represents mild infiltration (indicating that there are very few aggregates in the submucosa and mucosa); 3 represents moderate infiltration

(indicating that there are several aggregates in the submucosa and mucosa); 4 represents marked infiltration (indicating that there are many large aggregates in the submucosa and mucosa); 5 represents dense infiltration of almost the entire mucosa; and 6 represents dense infiltration of the entire mucosa. Paraffin-embedded stomach tissue sections were immunostained with a cystathionine-γ-lyase (CSE) antibody (Proteintech Group, Inc.), followed by labeling with an Alexa Fluor-conjugated secondary antibody (ZSGB-BIO Ltd.). We used species-matched immunoglobulins as controls for every antibody. Finally, an Olympus FluoView scanning confocal microscope (Olympus) was used to generate the immunofluorescence (IF) images.

2.5 | RNA extraction and qRT-PCR

We used the TRIzol reagent (Invitrogen) according to the manufacturer's instructions to extract the total RNA. We then reverse transcribed the RNA to cDNA with the RevertAid First Strand DNA Synthesis (RT) Kit (Fermentas). Then, quantitative real-time PCR was performed with SYBR® Premix Ex Taq™ II (Takara) using an ABI Prism 7900 sequence detection system (Applied Biosystems) as follows: 1 cycle of 95°C for 30 s, followed by 40 cycles of 95°C for 5 s and 60°C for 31 s. Melt curve analysis was performed to confirm the specificity of the amplified product. β-Actin was used as an endogenous control. Relative expression of the target genes was determined using the $2^{-\Delta\Delta Ct}$ method. The primers are listed in Appendix Table A1.

2.6 | Western blotting and ELISA

Mouse stomach tissue was homogenized in radioimmunoprecipitation buffer (Solarbio) containing the proteinase inhibitor PMSF (Solarbio). After rotating at 4°C for 3 hr, the sample was centrifuged at 10,000 g to remove insoluble matter and was stored at −20°C. We determined the protein concentration using a BCA kit (Beyotime). Equal amounts of protein (60 μg) were separated by 10% SDS-PAGE and transferred to a PVDF membrane, which was blocked in 5% skim milk and then incubated with primary antibodies against cystathionine-β-synthase (CBS) (Proteintech Group, Inc.), CSE (Proteintech Group, Inc.), and β-actin (Abcam) overnight at 4°C. We then washed the membrane in TBS-T, incubated it with an anti-mouse or anti-rabbit horseradish peroxidase-conjugated secondary antibody, and developed it with an enhanced chemiluminescence reagent (ECL, Millipore). β-Actin was used as a loading control. ImageJ was used for Western blot image analysis. The IL-6, IL-1β, IL-18, and TNF-α expression levels in the gastric tissues of the mice were tested using ELISA kits (eBioscience) according to the manufacturer's instructions. The cytokine concentrations were calculated based on a standard curve.

2.7 | Microbial 16S rRNA gene sequencing and qRT-PCR

Microbial community samples were collected from the surfaces of the gastric samples according to a previously described method

(Osaki et al., 2006). Specifically, the whole gastric mucosal surface was gently scraped for at least 1 min using a sterile surgical blade that was premoistened with 1 ml of sterile phosphate-buffered saline in a 1.5-ml Eppendorf tube. DNA extraction was performed as described previously (Bik et al., 2006). Total genomic DNA was extracted using the QIAamp DNA Micro Kit (Qiagen) according to the manufacturer's protocol within 24 hr of obtaining the microbial samples. The concentration and integrity of bacterial DNA were assessed using a Nanodrop (Thermo Scientific) and by agarose gel electrophoresis, respectively.

We performed 16S rRNA gene amplicon sequencing on an Illumina MiSeq platform using the universal primers 515f (5′-GTGCCAGCMGCCGCGGTAA-3′) and 806r (5′-GGACTACHVGGGTWTCTAAT-3′), targeting the V4 hypervariable regions. One sample from the WAS + NaHS group could not be sequenced in the next step due to low DNA content. There was a 6-bp error-correcting barcode unique to each sample in the reverse primer. We performed sequencing at Novogene Bioinformatics Technology Co., Ltd., Beijing, China.

The reagents and instruments used in the microbial qRT-PCR assay were similar to those used to assay stomach tissue mRNA. Briefly, qRT-PCR was performed in a reaction mixture that contained nuclease-free water, forward and reverse primers (Appendix Table A1), microbiota-derived genomic DNA, and SYBR® Premix Ex Taq™ II (Takara) using an ABI Prism 7900 sequence detection system (Applied Biosystems). The thermal cycling conditions used were as follows: predenaturation at 94°C for 1 min; 45 amplification cycles of denaturation at 95°C for 20 s, annealing at 60°C for 20 s, and extension at 72°C for 40 s; and finally an additional extension at 72°C for 1 min. The relative abundance of the target bacteria was calculated according to the following principle (Kabeerdoss, Jayakanthan, Pugazhendhi, & Ramakrishna, 2015): The number of cycles required to detect the signal for the first time (the threshold cycle, Ct) is related to the original concentration of the DNA template. The DNA copy number is expressed as relative abundance, rather than the absolute number, of the designated microbial population. The Ct of the DNA in each target bacterial population can be detected during universal bacterial DNA amplification, and the formula to calculate the value is $2^{-\Delta Ct}$. In this formula, ΔCt is the difference between the Ct of the bacterium of interest and the Ct of universal bacteria. Thus, the relative abundance of the target bacterial population is the ratio of target bacterial rRNA to universal rRNA, which provides a quantitative comparison between different samples.

2.8 | Bioinformatics analysis

Bioinformatics analysis of the sequencing data was performed using quantitative insights into microbial ecology (QIIME; Caporaso et al., 2010). UCHIME software based on the UCHIME algorithm was used to remove low-quality reads, barcodes, primers, and chimeric sequences and finally obtain valid labels. The sequences were classified into operational taxonomic units (OTUs) with a 97% similarity threshold. Each OTU represents a selection sequence, and the classification

information for each representative sequence was annotated using the RDP classifier (version 2.2, http://sourceforge.net/projects/rdp-classifier/). OTUs were used for α-diversity analyses (including determination of the observed species, Chao1, Shannon, ACE, and Simpson indices), which were performed using QIIME (version 1.7.0) and visualized using R software (version 2.15.3). The jackknifed β-diversity containing unweighted Unifrac distances was calculated by QIIME software (version 1.7.0). These distances were visualized by principal coordinate analysis (PcoA) and unweighted pair group method with arithmetic mean (UPGMA). PcoA can help obtain and visualize master coordinates from complex multidimensional data. UPGMA clustering is a hierarchical clustering method that uses average links and was used to interpret distance matrices. Linear discriminant analysis combined with effect size (LEfSe) was implemented to identify the bacterial taxa that were differentially represented between groups at the genus or higher taxonomic levels.

To explore the metabolic activities of the gastric bacterial community, we identified KEGG (Kyoto Encyclopedia of Genes and Genomes) pathways using the bioinformatics tool PICRUSt (for phylogenetic analysis via reconstruction of unexplored communities; Langille et al., 2013). The functions were categorized at levels 2 and 3.

To identify the coassociation between mouse gastric microbiome samples and sets of taxonomic groups (OTUs), we visualized the sample OTU co-occurrence network in Cytoscape (version 3.1.0). The input files were obtained from the QIIME script make_otu_network.py performed on the groups of nonstressed and stressed mice, the groups of NaHS-treated and stressed mice, and the groups of nonstressed and NaHS-treated mice.

2.9 | Statistics

The biochemical data were analyzed and plotted using GraphPad Prism 6.0. The Western blot band intensities were analyzed by ImageJ, and the results are presented as the mean ± *SEM*. The differences in gene expression were analyzed using *t* tests for comparisons between two groups and ANOVA with the post hoc test if more than two groups were involved. Differences in the microbiome between two groups were analyzed using the Kruskal–Wallis rank sum test, and $p < .05$ was defined as a significant difference.

3 | RESULTS

3.1 | WAS-induced gastritis in C57BL/6 mice

To verify whether chronic persistent physiological stress can induce gastritis, the mice were subjected to WAS for 10 days, after which significant inflammation of the stomach tissue was observed (Figure 1a). The production of inflammatory cytokines, including IL-1β, IL-18, and IL-6, in the stomach tissues of the mice in the WAS group increased significantly (Figure 1b). Moreover, weight gain was slower in the WAS mice than in the nonstressed mice (Figure 1c).

3.2 | WAS reduces the production of H_2S in the mouse stomach by inhibiting the expression of CBS and CSE

Previous studies have reported that H_2S has a protective effect on gastric damage caused by stress (Magierowski et al., 2016). H_2S production in the mouse stomach was tested; a reduction in H_2S release was found in WAS mice (Figure 2a). H_2S is produced mainly by two enzymes, namely CBS and CSE, using L-Cys as the substrate (Łowicka & Bełtowski, 2007), and these two enzymes are expressed in the mouse stomach (Xiao et al., 2015). Therefore, we examined the expression of CSE and CBS in the gastric tissues of the nonstressed group and WAS mice by RT-PCR (Figure 2b), Western blot (Figure 2c), and IF (Figure 2d) analyses. The results showed that the expression of CBS and CSE was reduced in WAS mice, especially in those subjected to WAS for 10 days.

3.3 | Mice treated with the H_2S donor NaHS exhibited attenuation of WAS-induced gastritis

Considering that the reduction in H_2S production may be one of the causes of WAS-induced gastritis, 1 hr before the mice were subjected to WAS, they were ip injected with NaHS. The severity of gastritis was significantly alleviated (Figure 3a), as was the expression of pro-inflammatory factors (Figure 3b) and the production of H_2S (Figure 3c).

3.4 | NaHS protected the gastric microbiota from destruction in WAS-treated mice

Studies have shown that the composition of the stomach microbiota is related to stomach disease (Nardone & Compare, 2015). In our study, 16S rRNA gene sequencing of the microbiota on the surface of the gastric mucosa was carried out for the mice in the nonstressed, WAS, and NaHS-treated groups. We first compared the α-diversity of the samples as measured by the observed species, Chao1, ACE, and Fisher indices. The α-diversity scores tended to be higher for the WAS group than for the nonstressed group for all the indices, and after treatment with NaHS, the α-diversity scores were restored to normal levels (Figure 4a). Next, we plotted β-diversity to assess the extent of similarity of the microbial communities in each group. The WAS group was completely separated from the nonstressed group, while the NaHS-treated group was closely associated with the nonstressed group (Figure 4b). Furthermore, we investigated the co-occurrence network of OTUs between the nonstressed and WAS groups, the WAS and NaHS + WAS groups, and the nonstressed and NaHS + WAS groups. The nonstressed group deviated significantly from the WAS group, with fewer constituent OTUs and fairly little overlap; the NaHS-treated group was closely associated with the nonstressed group, with more constituent OTUs and relatively high overlap (Figure 4c).

Moreover, at the phylum level, the relative abundance of bacteria was quite different (Appendix Figure A1). The abundance of *Bacteroidetes* and *Proteobacteria* increased significantly in the

FIGURE 1 WAS-induced gastritis in mice. C57BL/6 mice (*n* = 10 mice per time point) subjected to WAS were analyzed at 1, 4, 7, and 10 days. (a) Representative gross photographs, micrographs (H&E, 200× s), and histological scores of the stomachs of the nonstressed and 10-day WAS groups are shown. (b) Average daily weight growth rate of mice during WAS. (c) Cytokine (IL-1β, IL-18, TNF-α, and IL-6) levels were measured in gastric tissues from the nonstressed and 10-day WAS groups by ELISA. The data representing the mean ± *SEM* were analyzed using *t* tests for two groups and ANOVA for three or more groups. **$p < .01$, ***$p < .001$
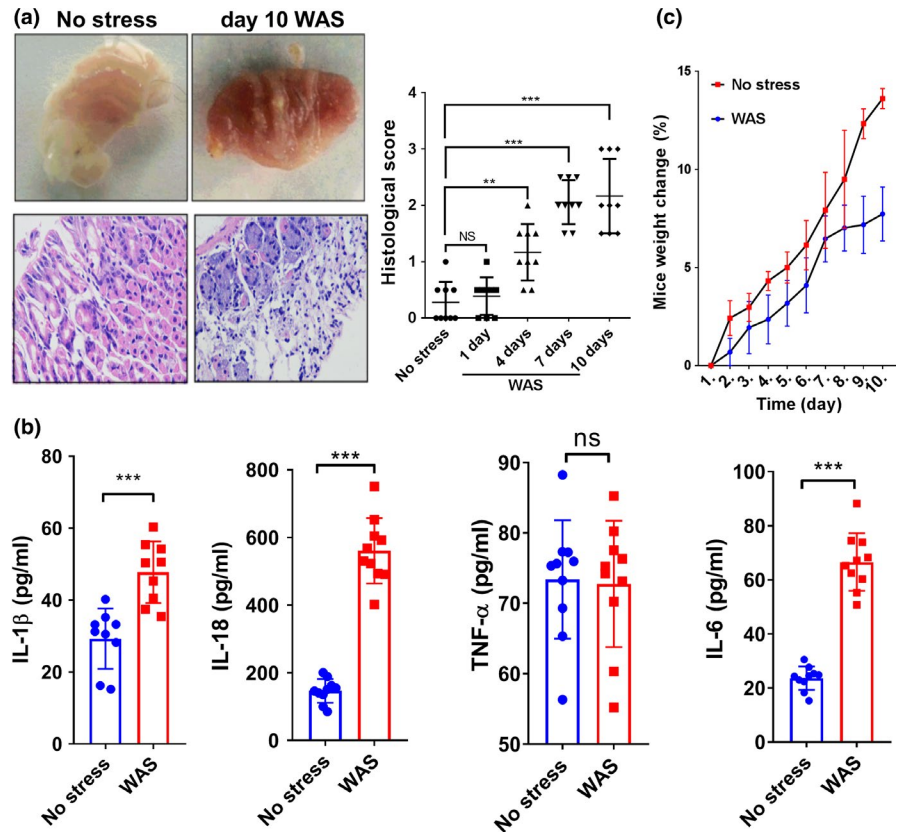
WAS group and was almost entirely restored in the NaHS-treated group (Appendix Figure A1).

To investigate altered phylotypes in the gastric microbiota that responded to WAS-induced gastritis and NaHS treatment, OTUs (relative abundance over 0.1%) between the nonstressed and WAS groups were analyzed by a volcano plot (Figure 5a). A total of 100 OTUs were screened, and these OTUs are presented as a heatmap that includes the nonstressed, WAS, and NaHS-treated groups (Figure 5b). For the WAS group, compared with the nonstressed group, 13 OTUs were enriched, including OTU_109, OTU_18, OTU_466, OTU_222, OTU_515, OTU_390, OTU_626, OTU_52, OTU_2, OTU_11, OTU_15, and OTU_20. Among these OTUs, the identifiable OTUs at the genus level included *Shewanella* (*n* = 1), *Turicibacter* (*n* = 1), *Lactobacillus* (*n* = 1), and *Kaistobacter* (*n* = 3). An additional 87 OTUs were eliminated or depleted by WAS, and the identifiable OTUs at the genus level included *Bilophila* (*n* = 1), *Sutterella* (*n* = 1), *Mesorhizobium* (*n* = 1), *Ochrobactrum* (*n* = 1), *Ruminococcus* (*n* = 6), *Oscillospira* (*n* = 9), *Butyricicoccus* (*n* = 1), *Anaerotruncus* (*n* = 2), *Coprococcus* (*n* = 3), [*Ruminococcus*] (*n* = 2), *Prevotella* (*n* = 2), *Parabacteroides* (*n* = 1), *Bacteroides* (*n* = 1), and [*Prevotella*] (*n* = 1). It is evident that the abundance of these OTUs after treatment with NaHS is very similar to that in the nonstressed group (Figure 5b).

Notably, at the class level, *Bacteroidales* and *Clostridiales* were the most abundant bacteria, and the abundance of these bacteria was significantly reduced in the WAS group compared with the nonstressed and WAS + NaHS groups (Figure 5b). Similarly, at the

phylum level, the relative abundance of *Firmicutes* and *Bacteriodetes* was markedly decreased in the WAS group (Figure 5b).

In addition, a taxon-based analysis at the genus level suggested that the relative abundances of *Shewanella* and *Turicibacter* were clearly increased after the mice were subjected to WAS, whereas the relative abundances of *Ruminococcus* and [*Prevotella*] were significantly decreased (Figure 6a–d).

3.5 | Analysis of the relationship between the gastric microbiota and gastritis

To study the relationship between bacterial taxa and gastritis more specifically, we generated a Spearman correlation heatmap and selected several inflammation markers related to gastritis (IL-18, IL-6, IL-1β, and inflammation score [IS]) and H_2S production (Figure 6e). *Shewanella* and *Turicibacter* showed a positive relationship with IL-18, IL-6, IL-1β, and IS and a negative relationship with H_2S production. In the case of *Ruminococcus* and [*Prevotella*], the opposite trend was observed.

3.6 | Functional metagenomic predictions

To gain a basic understanding of the alteration in the gastric microbiota induced by WAS, we used PICRUSt (Phylogenetic Investigation of Communities by Reconstruction of Unobserved States) analysis to predict the functional profiles of the microbiota. As shown in Figure 7, based on volcano plot analysis, 16
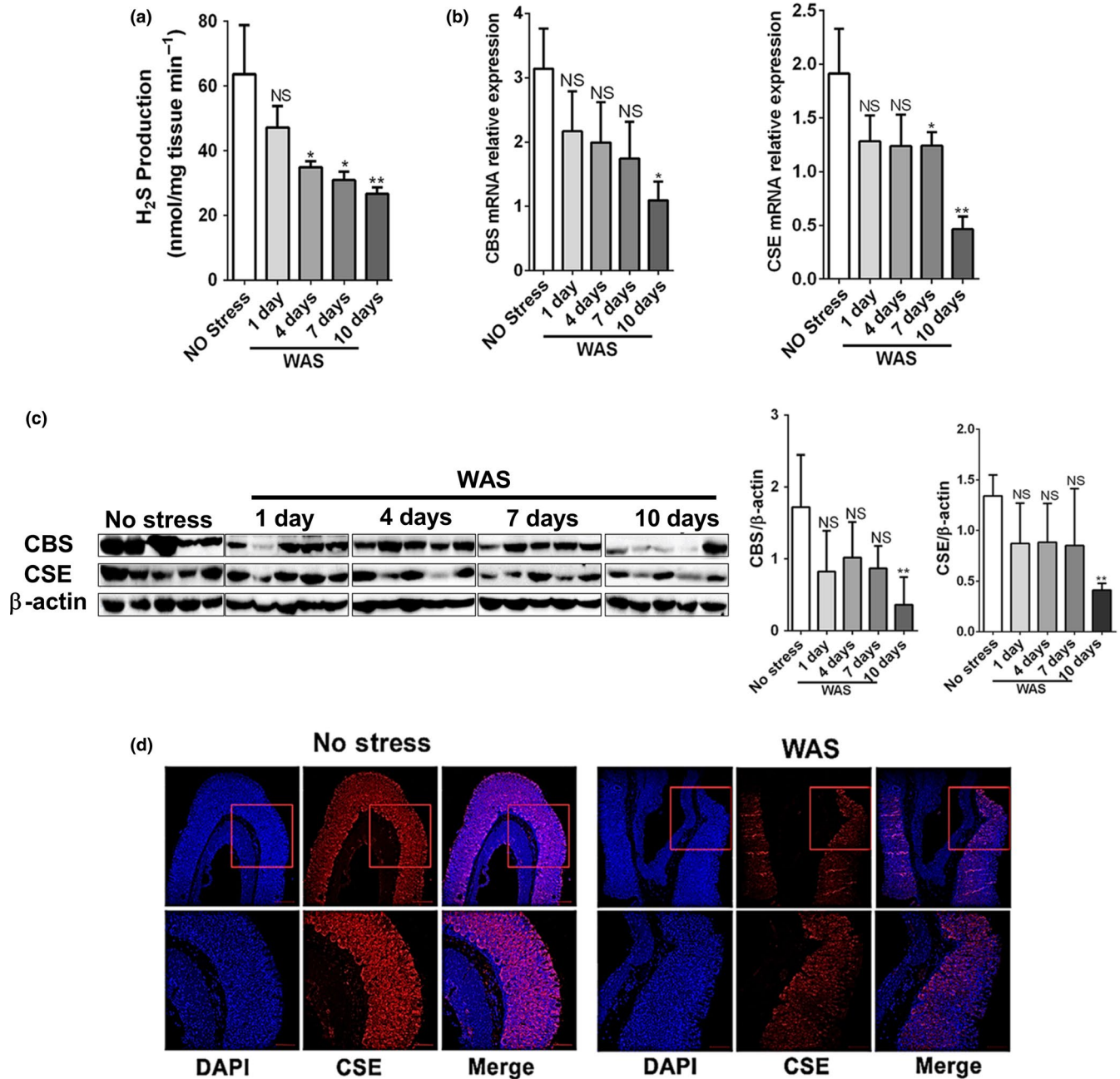
FIGURE 2 WAS reduced the release of H_2S by inhibiting CBS and CSE expression in mice. C57BL/6 mice (n = 5–10 mice per time point) were subjected to WAS for 10 days. (a) The production of H_2S released in gastric tissues was compared between the nonstressed mice (n = 10 per group) and WAS mice (n = 10) on days 1, 4, 7, and 10. qRT-PCR (b) and Western blotting (c) to evaluate CBS and CSE expression in gastric tissues from the nonstressed (n = 5) and 10-day WAS (n = 5) mice on days 1, 4, 7, and 10. The data are normalized to β-actin and represent the mean ± _SEM_. (d) The expression of CSE in stomach tissue was analyzed by an IF assay among the nonstressed mice and 10-day WAS mice. The data represent the mean ± _SEM_ and were analyzed using t tests for two groups and ANOVA for three or more groups. *p < .05, **p < .01

KEGG pathways of the gastric microbiota were predicted to be reduced in the WAS group. These pathways included amino acid metabolism, carbohydrate metabolism, energy metabolism, and lipid metabolism. Considering the stress-induced reduction in H_2S production in the stomachs of mice and its protective effect on gastritis (Bronowicka-Adamska et al., 2017; Lou et al., 2008; Magierowski et al., 2016, 2015b), we searched for H_2S metabolism-related pathways. The endogenous synthesis of H_2S involves three enzyme systems. Two of these systems require pyridoxal 5′-phosphate (P5P), the biologically active form of VB6, as a cofactor for their activity (Kimura, 2014). These two P5P-dependent enzymes for H_2S synthesis are CSE CBS. As shown in Figure 7, VB6 metabolism, which may be involved in the synthesis of H_2S, was decreased in the WAS group.
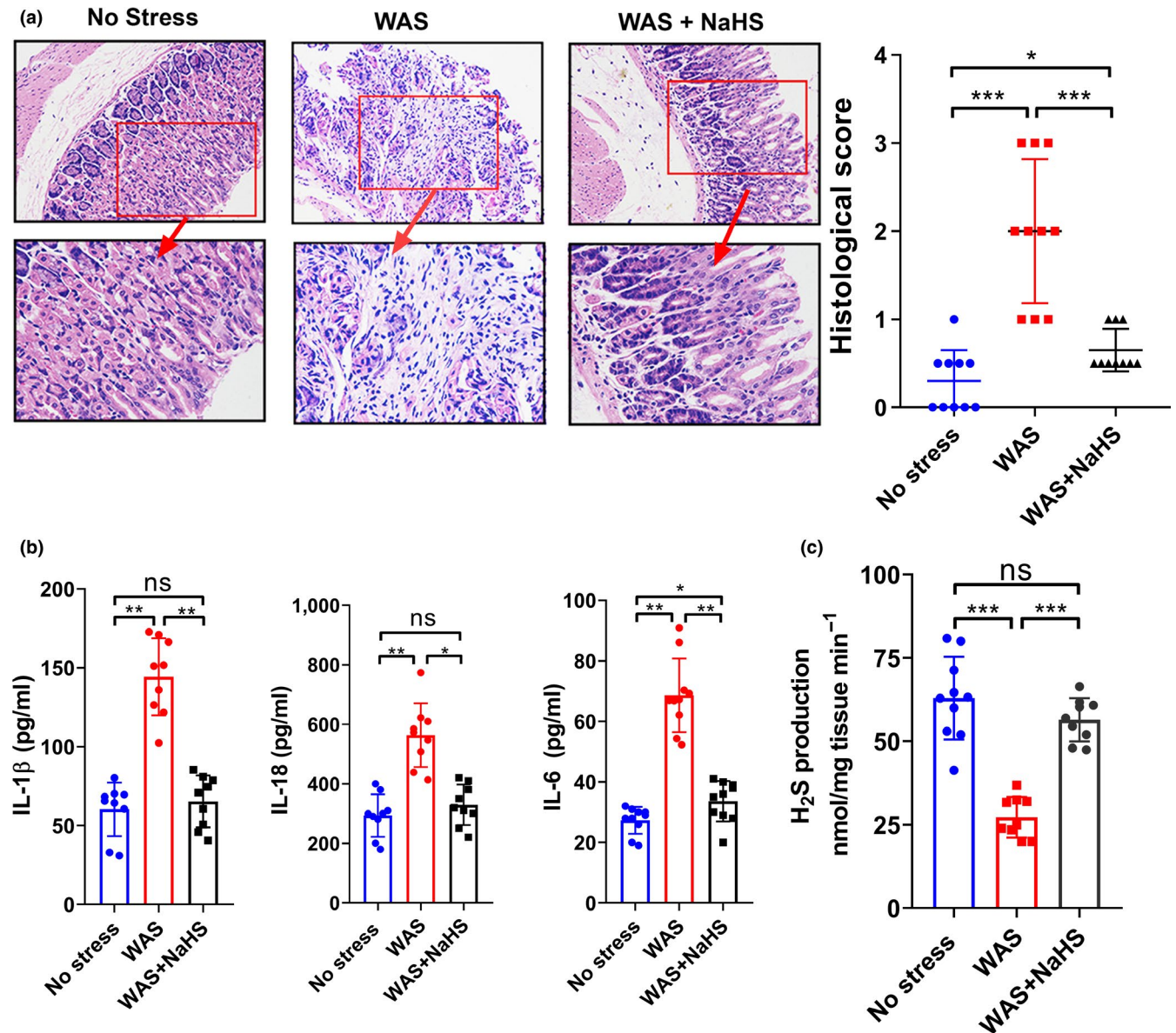
FIGURE 3 Intraperitoneal injection of NaHS in mice attenuated WAS-induced gastritis and increased H_2S production. (a) Histopathological examination (H&E, 200×) and histological scores of stomach tissues among the nonstressed mice (n = 10), 10-day WAS mice (n = 10), and NaHS-treated mice (n = 10). (b) Cytokine (IL-1β, IL-18, and IL-6) levels were measured by ELISA in gastric tissues from the nonstressed mice (n = 10), WAS mice (n = 10), and NaHS-treated mice (n = 10). (c) The production of H_2S in mouse gastric tissues was detected among the nonstressed (n = 10 per group), WAS (n = 10), and NaHS-treated (n = 10) mice. The data represent the mean ± *SEM* and were analyzed using t tests between two groups and the ANOVA test if more than two groups were involved. *p < .05, **p < .01, ***p < .001

3.7 | VB6 treatment led to WAS-induced gastritis and microbiota recovery

Based on the results of PICRUSt analysis and the fact that VB6 is involved in the synthesis of H_2S (Kimura, 2014), we fed the mice in the WAS group with VB6. Surprisingly, feeding with VB6 significantly reduced WAS-induced gastritis (Figure 8a) as well as the expression of inflammatory cytokines (Figure 8c). At the same time, VB6 treatment significantly restored H_2S production in mice in the WAS group to a level nearly equal to that in the nonstressed group (Figure 8b). Furthermore, feeding VB6 to mice prevents stress-induced gastric dysbiosis. At the class level, the relative abundance

of *Clostridia* and *Bacteroidia* in the WAS + VB6 group was similar to that in the nonstressed group (Figure 8d). Additionally, the abundance of *Shewanella* and *Turicibacter* decreased significantly in the WAS + VB6 group. However, feeding with VB6 did not increase the relative abundance of *Ruminococcus* and [*Prevotella*] (Figure 8e).

4 | DISCUSSION

In this study, we found that WAS can induce gastritis in mice, accompanied by a decrease in the H_2S production and gastric dysbiosis. Furthermore, H_2S was suggested to be essential for maintenance
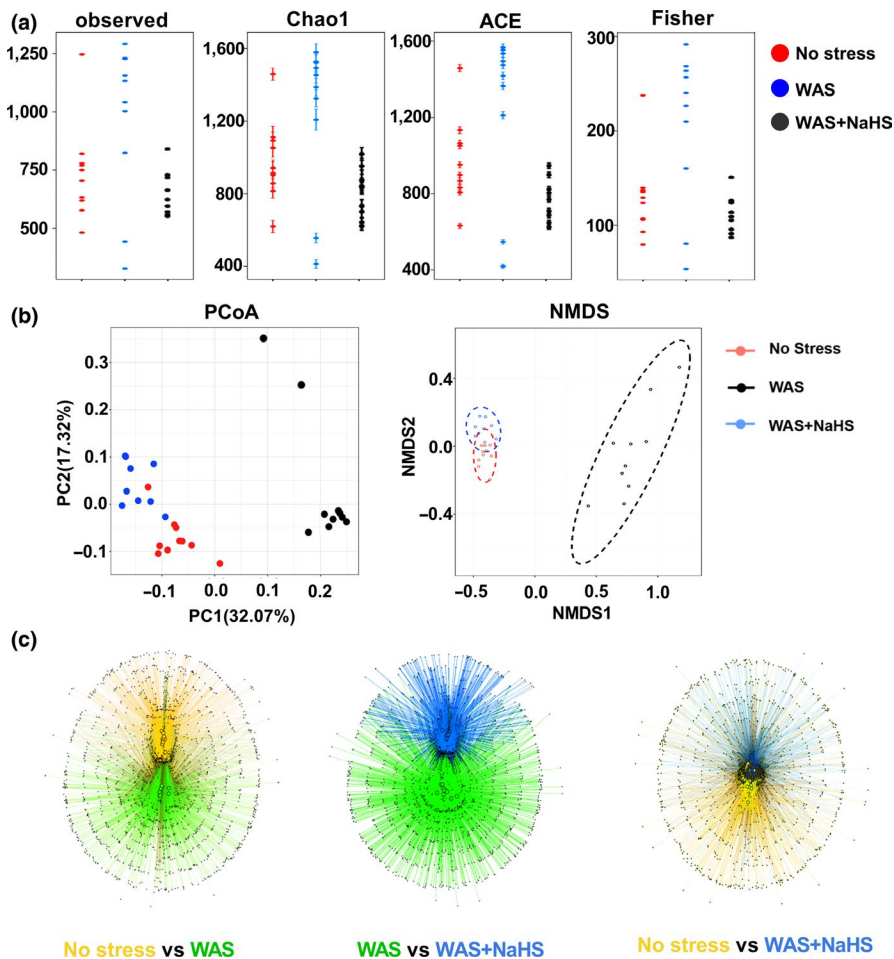
FIGURE 4 Diversity of the gastric microbiome in the nonstressed (n = 10), NaHS-treated (n = 9), and WAS (n = 10) groups. All figures are based on 16S rRNA sequencing data. For α-diversity (a), the bar plots show the observed Chao1, ACE, and Fisher indices. For β-diversity (b), principal coordinates analysis and nonmetric multidimensional scaling were used to analyze the different principal coordinates for OTUs from the nonstressed, NaHS-treated, and 10-day WAS samples. (c) OTU co-occurrence network. Cytoscape was used to visualize sample OTU co-occurrence patterns for the nonstressed, NaHS-treated, and 10-day WAS mice. In the network, samples are colored by treatment group (nonstressed samples, red; WAS samples, green; NaHS-treated samples, blue). Individual OTUs are represented by small black nodes. Lines connecting a sample to an OTU, colored by group, indicate that that a particular OTU was observed in that specific sample, forming the network

of homeostasis in the stomach. Based on the PICRUSt analysis, we found that VB6 can effectively prevent WAS-induced gastritis. Our results suggest that the reduction in H_2S production and gastric dysbiosis contribute to WAS-induced gastritis.

H_2S, an endogenous gaseous mediator, was recently considered the third "gasotransmitter," playing multiple physiological and pathophysiological roles in various body systems (Kimura, 2014; Li, Rose, & Moore, 2011; Wang, 2010), such as anti-oxidative (Liebert, Kimura, Dargusch, Schubert, & Kimura, 2006) and anti-inflammatory (Li, 2005; Zanardo et al., 2006) roles, and protecting cells from ischemia–reperfusion injury in different organs (Elrod et al., 2007; Johansen, Ytrehus, & Baxter, 2006; Wu et al., 2015). Previous studies have shown that inhibition of H_2S production contributes to stomach damage caused by NSAIDs (Fiorucci et al., 2005); H_2S can also protect the gastric mucosa during gastric ischemia–reperfusion injury in rats through antioxidant effects (Cui et al., 2013). Recent studies have found H_2S protected gastric epithelial cells from ischemia–reperfusion injuries (Guo, Liang, Shah Masood, & Yan, 2014), such as the attenuated gastric mucosal injury caused by WRS (Sun

et al., 2017), and protected cells against gastric mucosal injury caused by cold restraint stress in rats (Aboubakr, Taye, El-Moselhy, & Hassan, 2013). Xiao et al. showed that H_2S was involved in gastric accommodation (Xiao et al., 2015). Therefore, we wanted to explore the effect of WAS on the production of H_2S in the stomach. The present study shows that stress stimulation leads to reduced H_2S production in the mouse stomach, and injection of a CSE inhibitor (propargylglycine, PAG) into mice to inhibit H_2S production indeed induces gastritis (Appendix Figure A2a/b), which is consistent with previous research (Fiorucci et al., 2005). WAS-induced gastritis was effectively treated by injecting NaHS into the mice. Therefore, reduction in H_2S production in the stomachs of mice is an important cause of gastritis caused by stress, and H_2S provides gastroprotection against WAS-induced gastritis.

Garlic-derived H_2S donors have been observed to have antimicrobial effects against both planktonic gram-positive and gram-negative bacteria (Aston et al., 2011; Holly et al., 2013; Ross, O'Gara, Hill, & Sleightholme, 2001). Researchers have also reported that H_2S can prevent colitis and restore the intestinal microbiota biofilm (Motta et

FIGURE 5 Analysis of OTUs with relative abundance greater than 0.001 in each experimental group of mice (nonstressed [n = 10], NaHS-treated [n = 9], and WAS [n = 10] groups). (a) Volcano plot analysis of OTU changes in WAS mice compared with nonstressed mice. The p value is based on a t test. (b) Heatmap depicting the relative abundance of the filtered OTUs based on volcano map analysis across samples. A heatmap was constructed using the normalized log10 abundance of each OTU in each sample type. The taxonomies of the OTUs (genus, class, and phylum) are depicted on the right

(a)

(b)
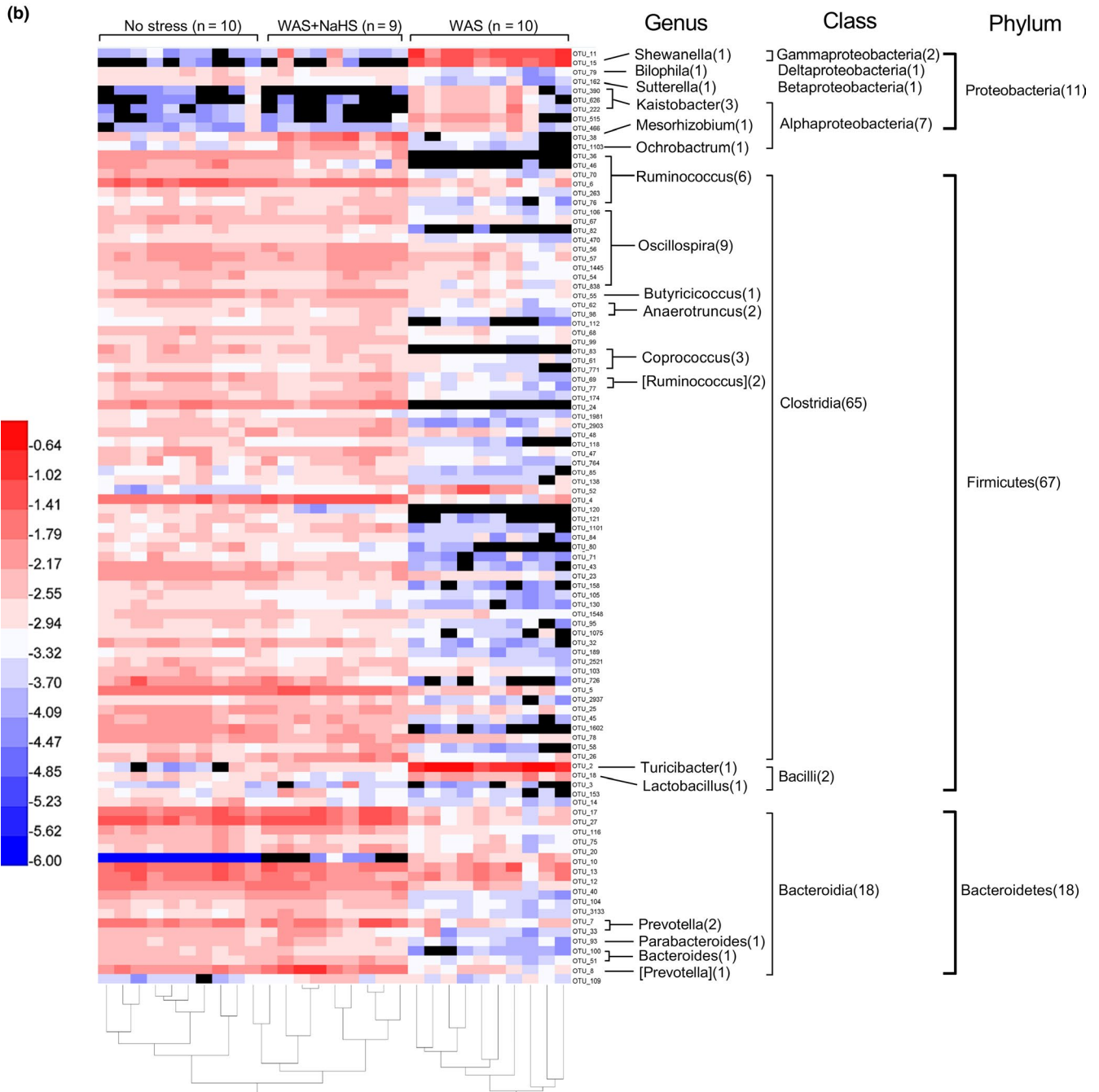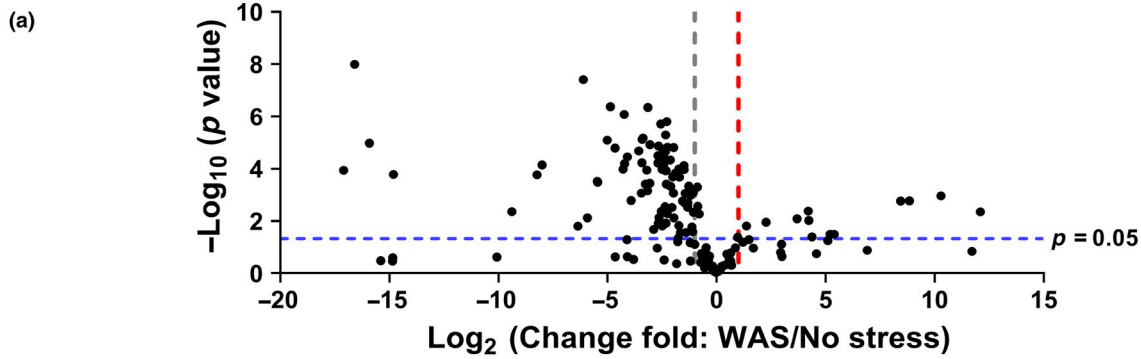
al., 2015). Therefore, H_2S can be seen as an stabilizing agent at the microbiome–mucosa interface (Wallace, Motta, & Buret, 2017). In this study, we found that simply inhibiting the production of H_2S can lead to dysbiosis (Appendix Figure 2c), and H_2S supplementation can reverse this effect. It is very difficult to explain how H_2S affects the changes in microorganisms in the stomach. Studies have reported that H_2S can reduce the cytotoxicity of bile acid (Buret et al., 2015),

and bile acid can regulate the composition of the gut microbiota (Islam et al., 2011; Wahlström, Sayin, Marschall, & Bäckhed, 2016). In addition, H_2S can regulate the differentiation of T cells and maintain immune homeostasis (Yang et al., 2015). The mammalian immune system plays an indispensable role in maintaining homeostasis with resident microbial communities (Hooper, Littman, & Macpherson, 2012). Considering that dysbiosis of the stomach is closely related
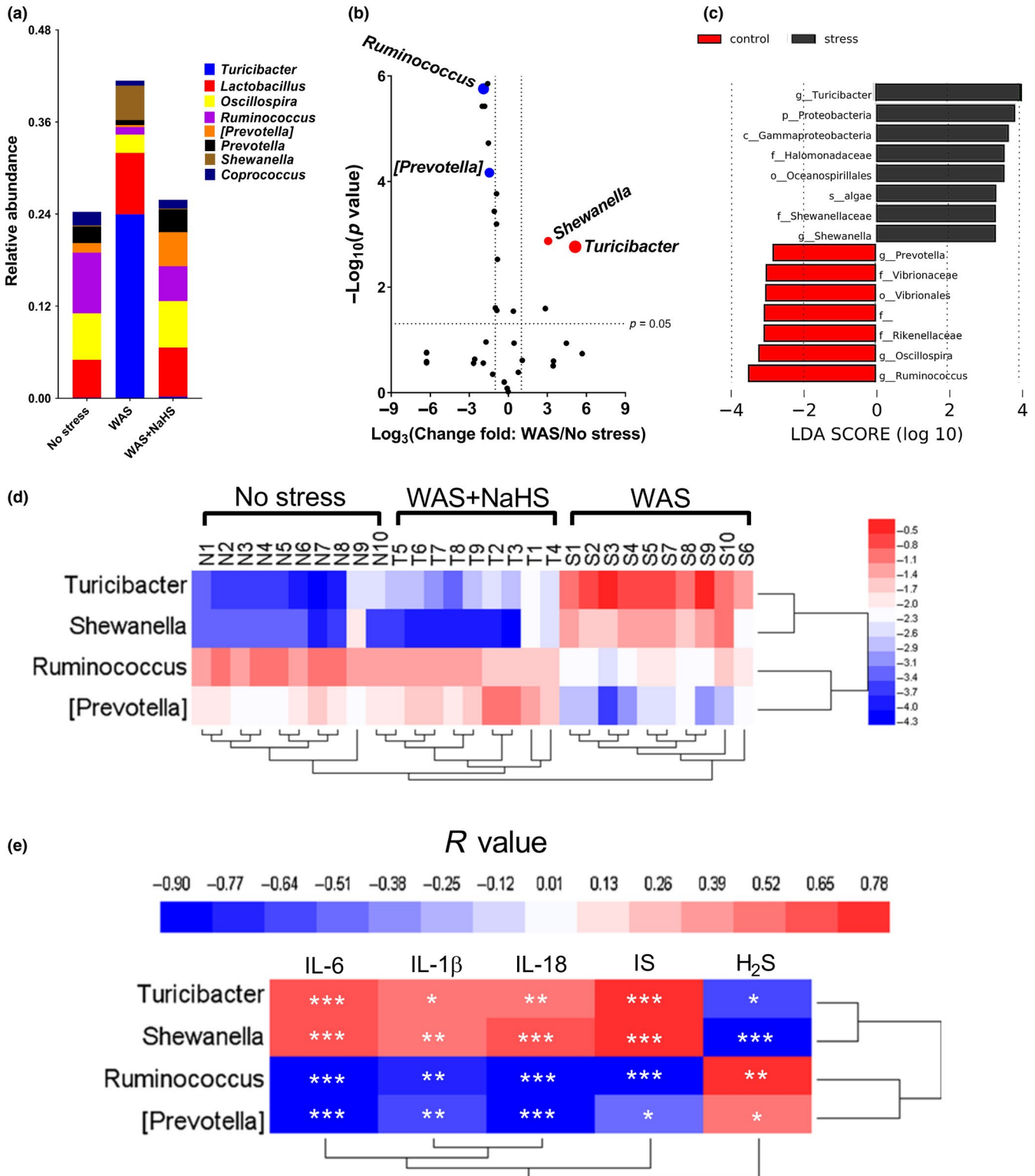
FIGURE 6 Comparison of the gastric microbiota in each group of mice (nonstressed [n = 10], NaHS-treated [n = 9], and WAS mice groups) at the genus level. (a) Distribution of the relative abundance of gastric bacteria in the community subgroups. The indicated bacteria were included when their 16S rDNA sequences were at least 1% of the total bacterial DNA content in at least one of the three groups of subjects. (b) Volcano plot analysis of gastric microbiota changes in WAS mice compared with nonstressed mice. The p value is based on a t test. According to Figure (a), bacteria with a relative abundance greater than 1% were selected and marked as key bacteria. (c) LEfSe analysis identified the most differentially abundant taxa between the WAS and nonstressed groups. Only taxa that met an LDA significance threshold of >2 and $p < .05$ are shown. (d) Heatmap showing the relative abundances of the key bacteria (genus level) for sampled individuals from the community subgroups. (e) Correlation between pro-inflammatory cytokines, IS, H_2S production, and relative abundance of intestinal bacteria at the genus level. The colors of the cells represent correlation R values from (−0.9)–0–0.8 (blue–white–red). Cells marked with an asterisk show significance after multiple comparisons of Spearman correlation. *$p < .05$, **$p < .01$, ***$p < .001$

to stomach diseases (Nardone & Compare, 2015), H_2S can prevent WAS-induced gastric dysbiosis, which may also contribute to protection against gastritis.

Studies have found that in addition to *H. pylori*, the stomach harbors a relatively stable flora (Bik et al., 2006; Nardone & Compare, 2015). The maintenance of bacterial homeostasis could be essential for stomach health. For example, nonatrophic gastritis can develop into intestinal metaplasia and then deteriorate to intestinal-type gastric cancer, and this gradual shift can be observed in the microbiota profile (Aviles-Jimenez, Vazquez-Jimenez, Medrano-Guzman, Mantilla, & Torres, 2014). Additionally, a positive correlation has been observed between *Streptococcus* abundance and peptic ulcer disease (Khosravi et al., 2014). Patients with antral gastritis show decreasing abundance of *Proteobacteria* and an increase in *Firmicutes* abundance at the phylum level compared with *H. pylori*-negative subjects (Li et al., 2009). The gastric microbiota composition differs

between paired benign and malignant tumor tissues in either Chinese or Mexican (Yu et al., 2017) samples.

During this study, WAS induced obvious changes in the gastric microbiota of mice; at the genus level, the abundances of *Shewanella* and *Turicibacter* were significantly increased in the stomachs of mice in the WAS group and showed positive correlations with gastritis (Figure 4). *Shewanella* species are very important bacteria that produce H_2S. An increase in the relative abundance of *Shewanella* may lead to an increase in the H_2S production in the stomachs of mice, in addition to consumption of substances involved in the synthesis of H_2S, such as VB6. The reduction in the H_2S synthesis substrate in the stomach leads to a decrease in the absorption rate of these substances in the stomach tissue, thereby reducing the production of H_2S in the stomach tissue. This effect may be one of the reasons for the decrease in H_2S production in the gastric tissue of mice in the WAS group. A recent study on pediatric Crohn's disease found

FIGURE 7 Comparison of the relative abundance of PICRUSt-generated functional profiles of the gastric microbiota among nonstressed mice (n = 10) and WAS mice (n = 10). (a) Volcano plot analysis of changed KEGG pathways in WAS mice compared with nonstressed mice. KEGG pathways with an average relative abundance greater than 0.1% in the two groups were included. The p value was based on a two-tailed and paired t test. (b) Based on volcano plot analysis, distinct gene categories were selected according to significant differences in gene categories at level 3 (t test, $p < .0001$; fold change >1.2). The bar plots on the left show the mean proportion of each KEGG pathway. The dot plots on the right show the differences in mean proportions between the two indicated groups using p-values. KEGG pathways involved in H_2S synthesis (VB6 metabolism) are marked
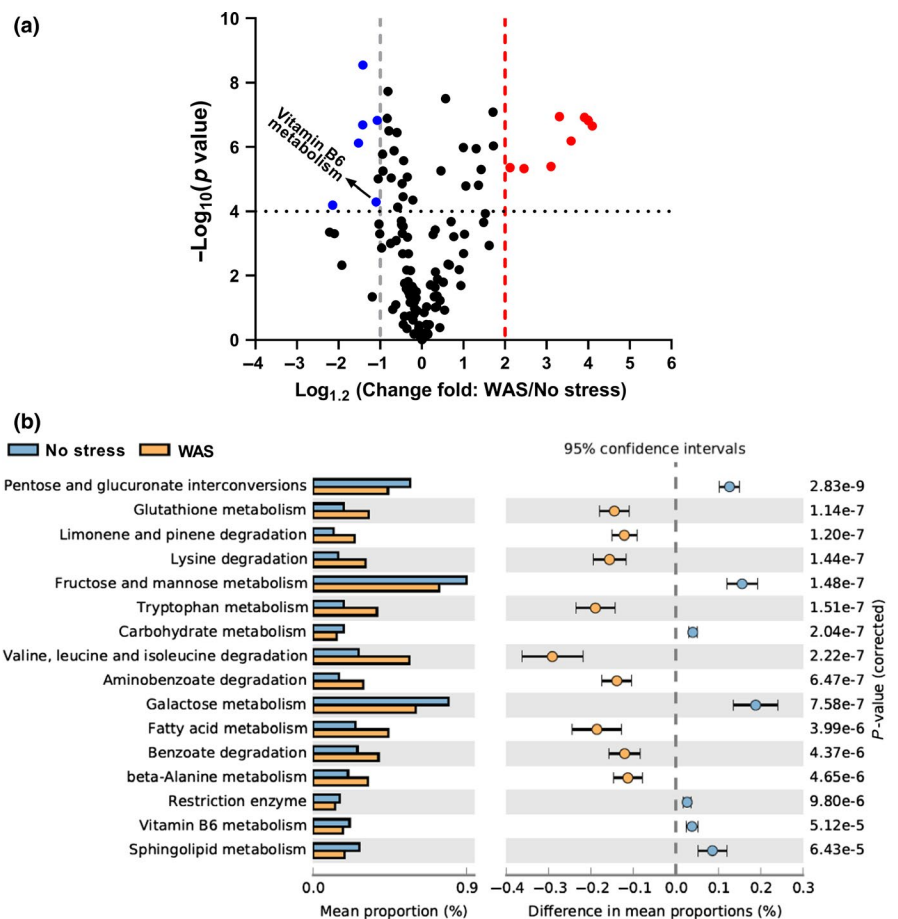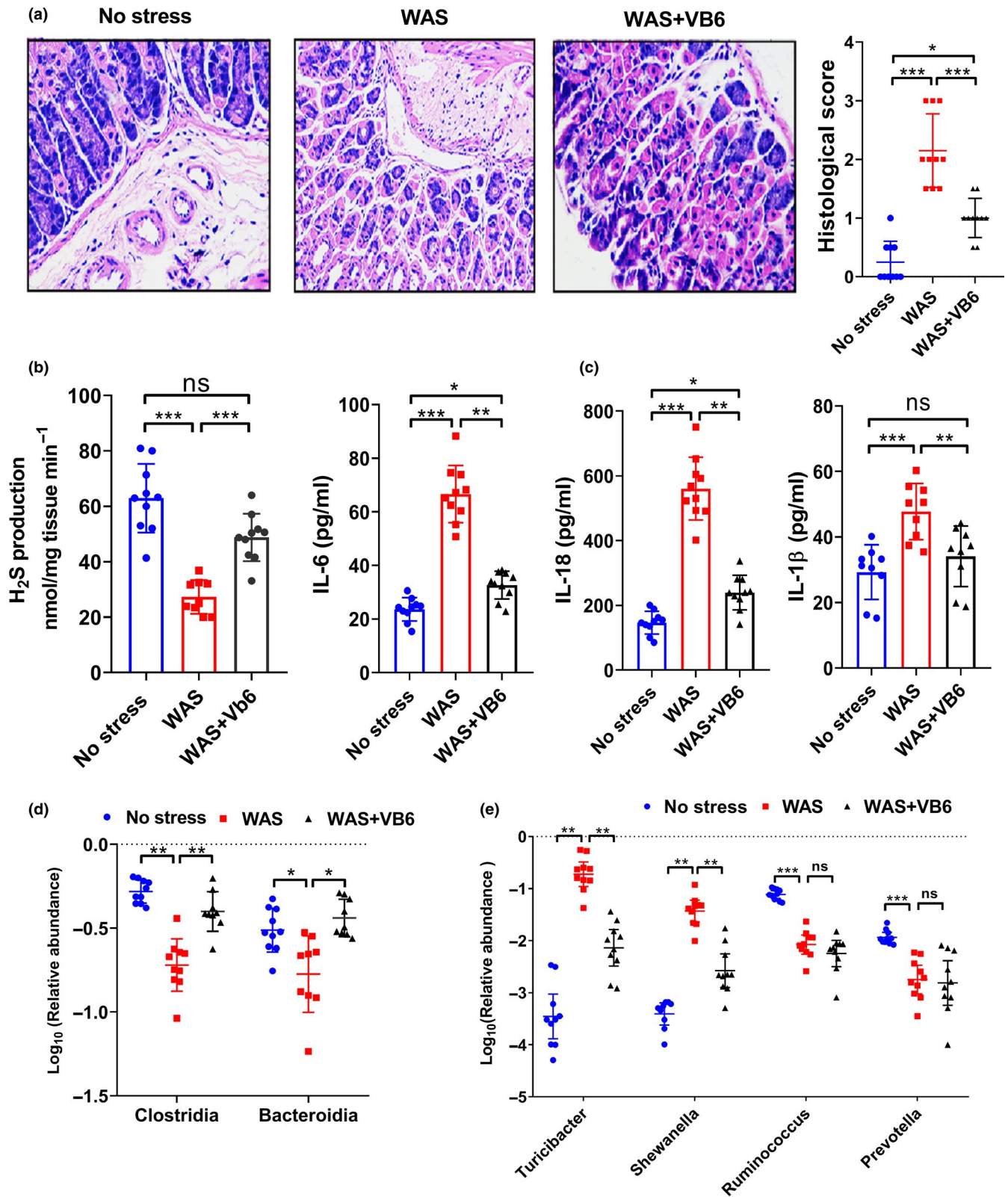
FIGURE 8 Gastritis and microbiota recovery in mice after VB6 treatment. (a) Histopathological examination (H&E, 200×) of gastric tissues from VB6-treated and WAS mouse sections. The right figure shows the histological scores based on histopathological examination. (b) Release of H_2S in the stomachs of the nonstressed, 10-day WAS, and VB6-treated mice; n = 10 per group. (c) ELISA showing IL-1β and IL-18 release in the nonstressed and VB6-treated mice. Comparison of the relative abundance of key bacteria at the class level (d) and genus level (e) among the nonstressed, 10-day WAS, and VB6-treated mice by qRT-PCR. The data represent the mean ± *SEM* and were analyzed using t tests between two groups. *p < .05, **p < .01, ***p < .001

that H$_2$S promotes damage in a small group of patients with genetic defects in mitochondrial oxidation of H$_2$S (Mottawea et al., 2016). Interestingly, the number of H$_2$S-producing bacteria in the enteric lumen of these patients also increased substantially (Mottawea et al., 2016). Another study observed a similar increase in the abundance of intestinal bacteria that produced H$_2$S in a rat model of the exacerbation of NSAID-related enteropathy through proton-pump inhibitors, which notably worsened NSAID-induced bowel disease (Bolla et al., 2011). Therefore, the increased relative abundance of *Shewanella* may be one of the factors that induced gastritis in the WAS group. Additionally, increased relative abundance of *Turicibacter* may be another cause of gastritis. A recent study reported an increase in the abundance of *Turicibacter* in fecal samples from rheumatoid arthritis (RA) patients (Chen et al., 2016). We have a relatively limited understanding of the roles that *Turicibacter* may play in gastritis. One study reported an increase in *Turicibacter* abundance in mice depleted of CD8 + T cells (Presley, Wei, Braun, & Borneman, 2010). Another study suggested that the presence of these bacteria may be associated with TNF expression (Jones-Hall, Kozik, & Nakatsu, 2015), and these bacteria were also isolated from the serum of patients with acute disease (Bosshard, Zbinden, & Altwegg, 1994). These findings suggest that the role of *Turicibacter* in the disease should be further rationally evaluated.

Our results suggest that WAS induces a decrease in the relative abundance of *Ruminococcus* and [*Prevotella*] in the stomachs of mice. *Ruminococcus* species play a major role in the digestion of resistant starch (Ze, Duncan, Louis, & Flint, 2012), which is associated with many health benefits, such as reversing infectious diarrhea and reducing the risk of diabetes and colon cancer (Armougom, Hugon, Lagier, Million, & Raoult, 2012). The study found that a *Ruminococcus* species is associated with an increased severity of irritable bowel syndrome, but most species are necessary for digestive function (Malinen et al., 2010). We hypothesized that the reduction in *Ruminococcus* abundance may attenuate the digestive capacity of mice. *Prevotella* spp. are members of the oral, vaginal, and gut microbiota. Previous studies showed that some *Prevotella* strains might be clinically important pathogens that can participate in human disease by promoting chronic inflammation (Larsen, 2017). However, recent studies have shown that the levels of some *Prevotella* strains are significantly reduced in stool samples from patients with depression (Jiang et al., 2015); stress is one of the important factors that induces depression (Praag, 2004). Therefore, WAS may cause reduced *Prevotella* abundance in the stomachs of mice.

It is known that VB6 is involved in the regulation of a healthy level of the amino acid homocysteine and plays a synergistic role in the maintenance of cardiovascular and neurological health. VB6 is also essential for energy metabolism (Cantuti-Castelvetri, Shukitt-Hale, & Joseph, 2000; Mariani, Polidori, Cherubini, & Mecocci, 2005). Studies have found that chronic stress leads to consumption of VB6 (Ford, Flicker, Singh, Hirani, & Almeida, 2013), and VB6 supplementation may be a therapeutic strategy to reduce stress (Stoney, 1999). Furthermore, previous studies have found that low levels of VB6

are associated with gastritis and gastric cancer (Sanderson & Davis, 1976). In our study, feeding mice with VB6 may have alleviated the effects of WAS, which may be one of the reasons why gastritis could be treated. In the context of gastritis, another important factor to consider is that VB6 is a substrate for H$_2$S synthesis (Kimura, 2014). In this study, we found that feeding VB6 to WAS mice increased the production of H$_2$S in the stomach tissue, and the abundance of bacteria (*Shewanella* and *Turicibacter*) associated with gastritis tended to be return to normal.

In summary, we found that decreased H$_2$S production was one of the factors associated with stress-induced gastritis; at the same time, H$_2$S can maintain homeostasis in the stomach, but the specific mechanism remains to be studied. We speculate that the occurrence of gastritis leads to microbial dysbiosis in the stomach and that dysbiosis aggravates gastritis. However, consumption of VB6 led to the maintenance of the relative stability of the stomach flora and increased the production of H$_2$S, which could be an alternative approach to prevent stress-induced gastritis.

CONFLICT OF INTEREST

None declared.

AUTHORS CONTRIBUTIONS

Yundong Sun: conceptualized, formally analyzed and supervised the study; Yundong Sun: involved in writing—original draft preparation; Wenjuan Li, Yundong Sun, Yan Li, and Yanbo Yu: acquired funding; Yingnan Han, Ya Li, Zhekai Hu, Xiao Wang, Xue Ren: investigated the study; Yundong Sun, Junze Liu: involved in writing—review and editing.

ETHICAL APPROVAL

All animal experiments were approved by the Animal Care Committee and Ethics Commission of Shandong University (LL-201602047).

DATA AVAILABILITY STATEMENT

The data supporting the findings are present within the article and have been uploaded to NCBI BioProject under accession number PRJNA391537. The other relevant files can be acquired from the authors upon request.

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APPENDIX A

TABLE A1 Primer sequences used in this study

qPCR primers	Sequence (5′–3′)	Target	References
ACTIN F	CGGTTCCGATGCCCTGAGGCTCT T	β-actin	Carmell et al. (2007)
ACTIN R	CGTCACACTTCATGATGGAATTGA		
CBS F	AGAAGTGCCCTGGCTGTAAA	Cystathionine beta synthase	This study
CBS R	CAGGACTGTCGGGATGAAGT		
CSE F	TCGTTTCCTGGAGACCAATC	Cystathionine gamma lyase	This study
CSE R	CTTAGCATGCTGCAGAGCAC		
Cloc F	ACTCCTACGGGAGGCAGC	Clostridia	Rivera-Chávez et al. (2016)
Cloc R	GCTTCTTTAGTCAGGTACCGTCAT		
Bac F	AGCAGCCGCGGTAAT	Bateroidia	Malo et al. (2010)
Bac R	GCATTTCACCGCTA		
TuriciF	CAGACGGGGACAACGATTGGA	Turicibacter (Genus)	Suchodolski et al. (2012)
TuricR	TACGCATCGTCGCCTTGGTA		
SheF	CGCGAT TGGATGAACCTAG	Shewanella (Genus)	Todorova and Costello (2006)
SheR	GGCTTTGC AACCCTCTGTA		
PreF	GGTTCTGAGAGGAAGGTCCCC	Prevotella (Genus)	Queipo-Ortuño et al. (2012)
PreR	TCCTGCACGCTACTTGGCTG		
RumF	CCTCTGACCGCTCTTTAATCGGAGCTTTCCTTC	Ruminococcus (Genus)	Rekha, Rizvi, and Jaishree (2006)
RumR	CCAGTTATCGGTCCCACCTTCGGCAGCT		
UniversalF	ACTCCTACGGGAGGCAGCAGT	All bacteria	Ley, Turnbaugh, Klein, and Gordon (2006)
Universal R	ATTACCGCGGCTGCTGGC		

FIGURE A1 Comparison of the mouse gastric microbiota in each group of mice (nonstressed (n = 10), NaHS-treated (n = 9), and WAS (n = 10) groups) at the phylum level. (a) Distribution of the relative abundance of gastric bacteria in the community subgroups. The indicated bacteria were included when their 16S rDNA sequences were at least 1% of the total bacterial DNA content in at least one of the three groups of subjects. (b) Analysis of the top three bacteria in terms of relative abundance. $*p < .05$, $**p < .01$, $***p < .001$
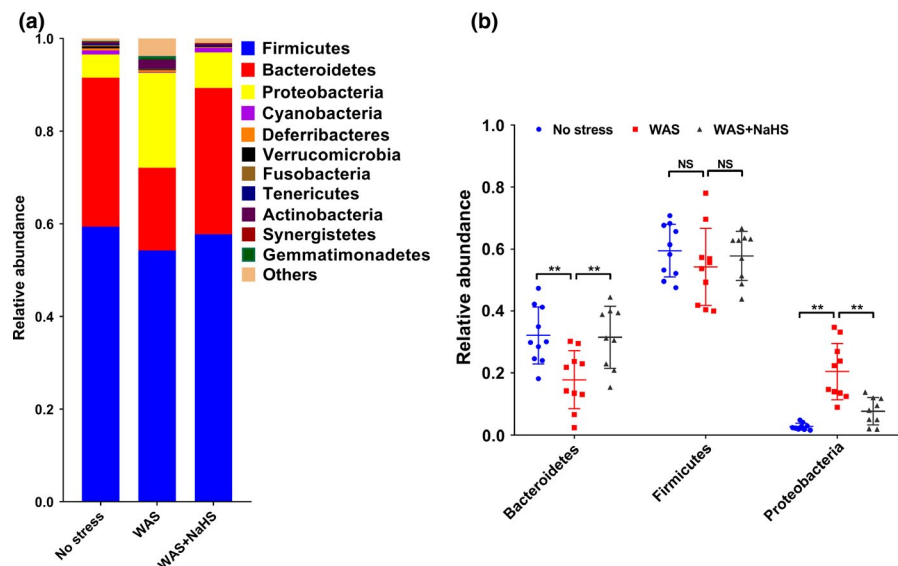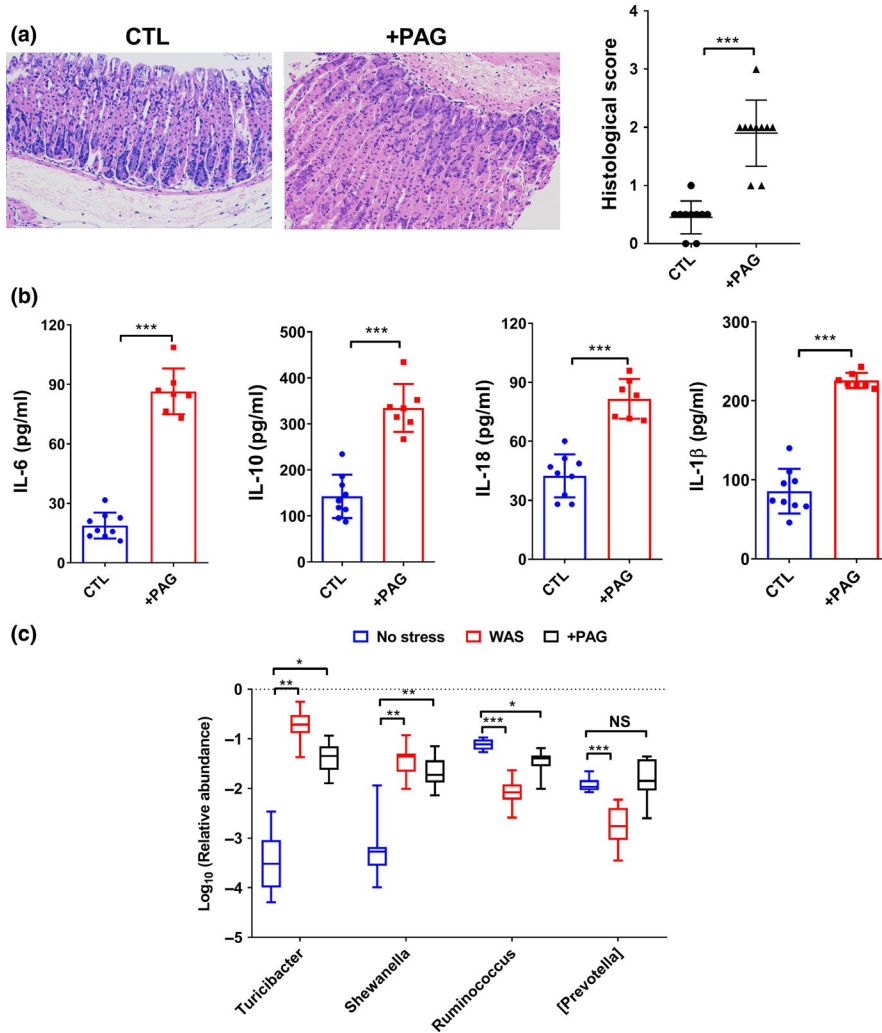
FIGURE A2 Injection of PAG induces gastritis and gastric dysbiosis in mice. (a) Gastric mucosal histopathological examination (H&E, 200×) between nontreated mice (control [CTL], $n = 10$) and mice injected with PAG for 10 days (propargylglycine, 60 mg/kg; $n = 10$). (b) Cytokines (IL-1β, IL-18, IL-10, and IL-6) were measured in gastric tissues from nontreated mice ($n = 10$) and mice injected with PAG ($n = 10$) by ELISA. (c) Relative abundances of *Turicibacter*, *Shewanella*, *Ruminococcus*, and *Prevotella* among the CTL mice ($n = 10$), WAS mice ($n = 10$), and mice injected with PAG for 10 days ($n = 10$). The data represent the mean ± *SEM* and were analyzed using *t* tests between two groups. *$p < .05$, **$p < .01$, ***$p < .001$