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Effects of choline supplementation on liver biology, gut microbiota, and inflammation in *Helicobacter pylori*-infected mice

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ABSTRACT

Aims: Diet is one of the factors affecting the pathogenicity of *Helicobacter pylori* (*H. pylori*) infection. Choline is a dietary component that is crucial for normal cellular function. However, choline intake imbalance can lead to liver injury, inflammation, and changes of the gut microbiota composition. The study aimed to explore the effects of choline supplementation on liver biology, gut microbiota, and inflammation in *H. pylori*-infected mice.
Main methods: Liver function was detected by biochemical and histopathological analysis. Serum inflammatory markers were measured using ELISA. Fecal microbial profiles were determined via 16S rRNA sequencing.
Key findings: The results showed that choline supplementation decreased serum LDL level, while increased the activities of serum AST and ALT in normal BALB/c mice. Besides, choline also reduced hepatic SOD and GSH-P*x* activities, and elevated hepatic MDA level of *H. pylori*-infected mice. Moreover, choline markedly enhanced the concentrations of inflammatory factors including LPS, CRP, IL-6, TNF-α, and CXCL1 in *H. pylori*-infected mice. Meanwhile, choline and *H. pylori* cotreatment altered the richness and diversity of the mice gut microbiota, and increased the relative abundance of *Escherichia_Shigella*, which had a significant positive correlation with the levels of LPS, CRP, IL-6, TNF-α and CXCL1.
Significance: Our data suggest, for the first time, that choline can aggravate *H. pylori*-induced inflammation, which may be associated with the alterations of gut microbiota. This study may provide novel insights into the possible effects of food-derived choline on *H. pylori* infection-related diseases.

1. Introduction

More than 50% of the world's population is infected with *Helicobacter pylori* (*H. pylori*), which is the main cause leading to gastric inflammation, peptic ulcer, and gastric cancer [1,2]. Besides that, *H. pylori* is also associated with some extragastric diseases, including non-alcoholic fatty liver disease (NAFLD), cardiovascular diseases, diabetes mellitus and neurologic disorders [3–5].

Clinic risk of *H. pylori* infection is closely related to the virulence of strains, host genetic polymorphisms and environmental factors such as diets [6]. Choline is an essential nutrient commonly found in milk, meat, and eggs [7]. It participates in several vital biological functions, which are vital for metabolism as well as the health of the brain, heart, skeletal muscles, and liver [8,9]. Nevertheless, insufficient or excessive intake of choline may result in liver injury and intestinal inflammation [10,11]. Animals obtain choline primarily from the diets described above or from the conversion of phosphatidylethanolamine (PE) to phosphatidylcholine (PC) followed by catabolism to choline [12]. PE

Abbreviations: H. pylori, *Helicobacter pylori*; CHO, cholestrol; TG, triglyceride; HDL, high-density lipoprotein; LDL, low-density lipoprotein; ALT, alanine aminotransferase; AST, aspartate aminotransferase; SOD, superoxide dismutase; GSH-P*x*, glutathione peroxidase; MDA, malonaldehyde; NEFA, non-esterified fatty acid; TMAO, trimethylamine *N*-oxide; LPS, lipopolysaccharide; CRP, C-reactive protein; IL-6, interleukin-6; TNF-α, tumor necrosis factor-α; CXCL1, chemokine (C-X-C motif) ligand 1; PCoA, principal coordinates analysis; UPGMA, unweighted pair group method with arithmetic mean; LEfSe, linear discriminant analysis (LDA) effect size

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and PC are the main phospholipids of human cell membranes that usually exist in food sources, such as egg yolks, soybeans, and beef [13,14]. In addition, PC is bactericidal against *H. pylori* due to its lytic activity [9,15]. Furthermore, choline and PC can be metabolized to trimethylamine (TMA) by the gut microbiota and subsequently oxidized to a NAFLD-related metabolite, trimethylamine-N-oxide (TMAO) by hepatic flavin-containing monooxygenases (FMOs) in the liver [9,16–18]. Taken with our previous study that TMAO may exacerbate *H. pylori*-induced inflammation in association with the gut microbiome [19], there appears to be an interaction among choline, *H. pylori*, gut microbiota, and inflammation. However, no relevant research has been conducted yet.

In this study, we assessed the levels of some liver function indicators and inflammatory markers in response to choline supplementation with or without *H. pylori* infection. We also investigated the effects of choline on the composition of the gut microbes in *H. pylori*-infected or uninfected mice. Our results indicate that choline supplementation and *H. pylori* infection may result in liver dysfunction, and choline may exacerbate the inflammation caused by *H. pylori* infection through gut microbiota modulation.

2. Materials and methods

2.1. H. pylori strain and culture

The strain used in this study was a clinical strain isolated from a patient with gastric ulcer and moderate gastritis at Sichuan Provincial People's Hospital. It was cultured on 3% (w/v) Columbia agar base (Oxoid, UK) supplemented with 1.2% (w/v) Brain heart infusion (Oxoid, UK), 7% (v/v) sheep blood, 10 μg/mL vancomycin, 10 μg/mL amphotericin, 2500 U/L polymyxin B sulfate salt, 5 μg/mL trimethoprim, and 10 μg/mL nalidixic acid (all purchased from Sigma, USA) and incubated at 37 °C in a microaerophilic environment (5%–6% O_2, 8%–10% CO_2, 85% N_2) for 5 days [20].

2.2. Animals and treatment

Female BALB/c mice (10 weeks old) were purchased from Chengdu Dashuo Experimental Animal Co., Ltd. (Chengdu, China). The mice were raised in a controlled environment (12-h light/dark cycle at 25 ± 2 °C) and allowed free access to food and water. After a one-week adaptation period, the mice were randomized into four groups (n = 12/group): Control group, Choline group, *H. pylori* group, and *H. pylori* + choline group. Two groups (the *H. pylori* + choline group and the *H. pylori* group) were infected with an oral gavage of 1×10^9 CFU of *H. pylori* once every two days and fed a normal chow diet and water with or without 1% (w/v) choline (Sigma, USA) supplementation [21]), whereas the other two (the control group and the choline group) received the same volume of saline and were provided with food and water accordingly. Body weight of each mouse was measured every week. All animal procedures were approved by the Ethics Committee of Sichuan University and conducted according to the guidelines for the care and use of laboratory animals.

2.3. Serum analysis

After 8 weeks feeding, overnight fasted mice were sacrificed for further experiments. Blood samples (n = 6–10/group) were allowed to clot for 30 min in microfuge tubes, after which serum samples were harvested for assessment by centrifugation at 3000 rpm for 20 min and were kept at −80 °C until analysis. Blood lipid levels (CHO, cholestrol; TG, triglyceride; HDL, high-density lipoprotein; LDL, low-density lipoprotein) and liver function (ALT, alanine aminotransferase; AST, aspartate aminotransferase) were detected by an automatic biochemistry analyzer (Hitachi, Japan). Serum lipopolysaccharide (LPS), C-reactive protein (CRP), interleukin-6 (IL-6), tumor necrosis factor-α (TNF-α) and chemokine (C-X-C motif) ligand 1 (CXCL1) concentrations were measured using commercially available ELISA kits obtained from Shanghai enzyme linked Biotechnology Co., Ltd. (Shanghai, China). Quantification of TMAO in serum samples (n = 3/group) were determined by LC/MS as previously described [19].

2.4. Hepatic SOD, GSH-Px, MDA and NEFA analysis

Hepatic tissues were homogenized in 9 volumes (w/v) of 0.9% ice-cold saline by an automatic homogenizer (Gering, China), and then centrifuged at 3000 r/min for 10 min. After that, the supernatant was collected for the measurement of superoxide dismutase (SOD), glutathione peroxidase (GSH-Px), malonaldehyde (MDA), non-esterified fatty acid (NEFA) and total protein concentrations using commercially available diagnostic kits (Nanjing Jiancheng Bioengineering Institute, China). And the results were expressed as U/mg protein, U/mg protein, nmoL/g protein, and μmoL/g protein, respectively.

2.5. Histopathological analysis

Mice liver specimens were fixed in 4% paraformaldehyde, embedded in paraffin, and cut into 5–7 μm sections. Hematoxylin and eosin (H&E) staining was performed on 6 samples from each group and the images were graded by a pathologist from Sichuan Provincial People's Hospital blinded to sample identity on an ascending scale from 0 to 4 (0 = absent; 1 = minimal (1–2 foci); 2 = mild (3–6 foci); 3 = moderate (7–12 foci); 4 = severe (> 12 foci)) according to lesions of the rat and mouse hepatobiliary system (with slight modification) [22]. Oil-red O staining (n = 6/group) was carried out on 8–10 μm frozen sections, and stained with Oil-red O for 8–10 min. The images were captured and analyzed using light microscopy (Leica, Germany). Liver lipid content was calculated as the percentage of the Oil-red O stained area over the total area using Image-pro Plus 6.0 software.

2.6. 16S rRNA gene sequencing and analysis

Fresh fecal samples (n = 5/group) were collected, frozen in liquid nitrogen immediately and then stored at −80 °C until use. Microbial genomic DNA was isolated from the frozen fecal samples using a QIAamp DNA Stool Mini Kit (Qiagen, Germany) following the manufacturer's protocols. The V3 and V4 regions of 16S rRNA gene were amplified and sequenced using the Illumina MiSeq platform at Biomarker Technologies Co., Ltd. (Beijing, China).

Procedures for data analysis were performed as previously described [19]. Firstly, raw tags were merged by FLASH (v1.2.7) [23], and then quality filtered by Trimmomatic (v0.33) [24]. Next, UCHIME (v4.2) was used to remove chimera sequences to obtain effective tags [25]. The tags were further clustered into operational taxonomic units (OTUs) at 97% identity utilizing UCLUST [26]. The OTUs were then aligned and classified into taxonomic groups according to the Silva database [27]. Finally, Microbial diversity analyses were conducted at BMKCloud (http://www.biocloud.net/). Chao1/ACE and Shannon/Simpson indexes were estimated as measures of bacterial abundance and diversity [28]. Principal coordinates analysis (PCoA) and unweighted pair group method with arithmetic mean (UPGMA) were used to distinguish differences in each group [29]. Linear discriminant analysis (LDA) effect size (LEfSe) was applied to identify the key bacterial contributors of different groups [30].

2.7. Statistical analysis

Data were expressed as mean ± standard deviation (SD). Statistical analysis was conducted with two-way ANOVA followed by the Turkey-Kramer test using GraphPad Prism 8.0 (GraphPad Software). *P* value less than 0.05 was considered statistically significant.

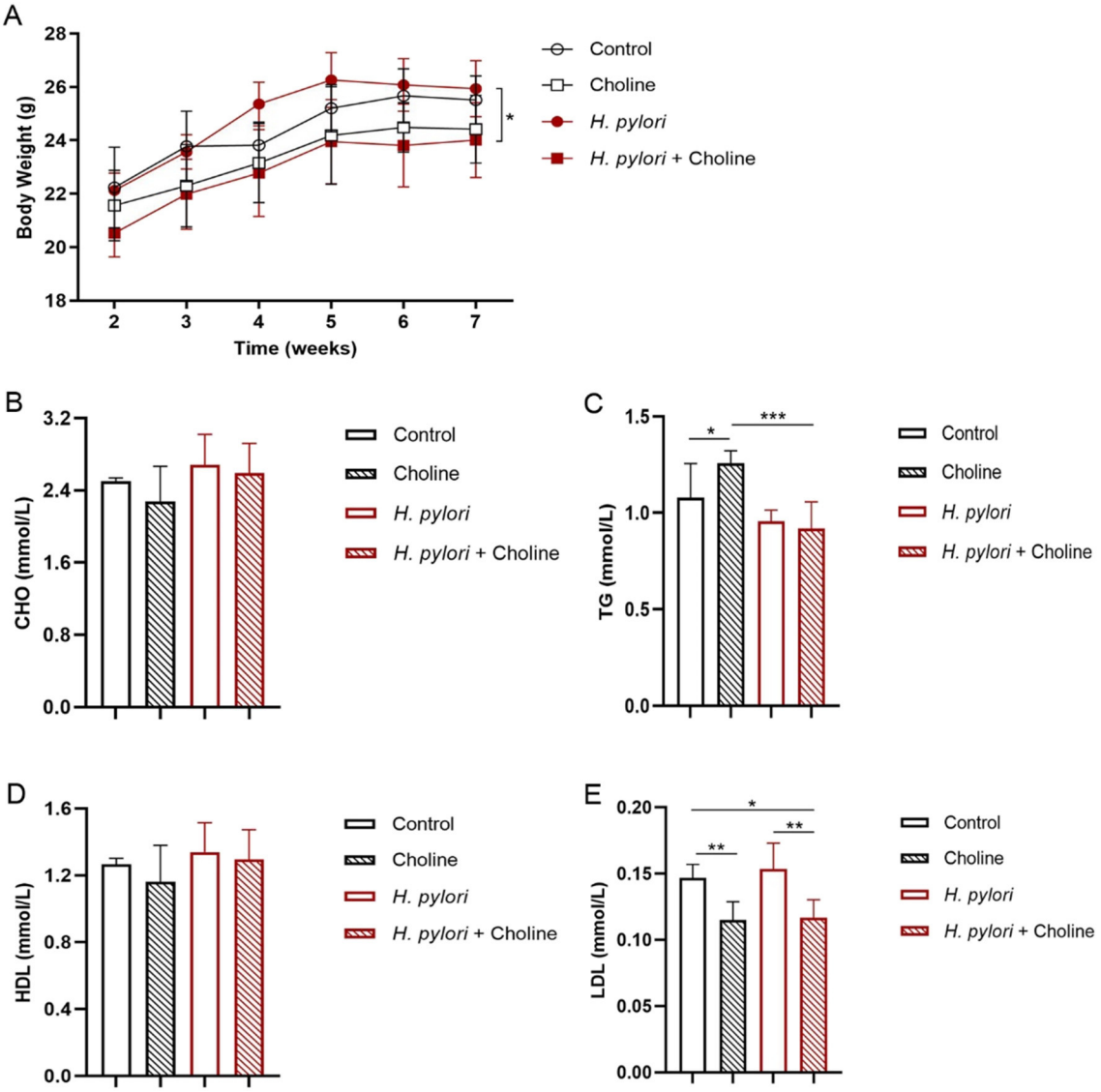

Fig. 1. Effects of choline supplementation on body weight (A) and serum levels of CHO (B), TG (C), HDL (D) and LDL (E) in the mice with or without *H. pylori* infection for 8 weeks. Values are expressed as means ± SD of 6–12 mice in each group. Statistical differences are denoted as follows: $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.

3. Results

3.1. Effects of choline supplementation on body weight and serum lipid profiles

The BALB/c mice were successfully infected with *H. pylori* after 8 weeks, which was verified by a rapid urease test and a positive *H. pylori* IgGb antibody test (Figs. S1 and S2). Changes in body weight of the mice are summarized in Fig. 1A. After treatment with choline for 8 weeks, the body weight of the tested mice was slightly decreased compared with the normal ones. In addition, there was a significant reduction in body weight of the *H. pylori* and choline cotreatment group as compared to the *H. pylori*-infected group. However, *H. pylori* infection did not alter the weight loss of the mice caused by choline.

As is known to all, the serum concentrations of CHO, TG, HDL and LDL are usually regarded as conventional indicators for clinical diagnosis of dyslipidemia, and the increased serum concentration of LDL is known to be one of the main risk factors for atherosclerosis [31,32]. Herein, we also examined if choline has a functional role in serum lipid profiles. As shown in Fig. 1B and D, no significant changes in serum CHO and HDL levels were observed among all groups. However, LDL levels were decreased in both *H. pylori*-infected and uninfected mice after choline feeding (Fig. 1E). In fact, choline can reduce the level of liver TG and facilitate lipid export from the liver [33]. As anticipated, liver lipid level had a decline and serum TG level had an increase in choline feeding mice (Figs. 1C, 2D and F). These results suggest that choline supplementation may exert a positive influence on lipid metabolism and help inhibit hepatic fat accumulation, and *H. pylori* infection does not seem to affect choline's role in these regards.

3.2. Effects of choline supplementation on liver biology

To determine the effects of choline supplementation with *H. pylori* infection on liver biology, we measured some hepatic indexes and histopathological changes in liver. Serum AST and ALT levels have been commonly employed as biochemical markers for early liver injury [34]. As can be seen in Fig. 2B and C, the activities of AST and ALT in the choline group were significantly higher than those in the control group. Moreover, the serum level of ALT increased dramatically after inoculation with *H. pylori*, while AST remained unchanged compared with the control. Unexpectedly, however, *H. pylori*-initiated ALT activity was suppressed by cotreatment with choline. Hepatic SOD and

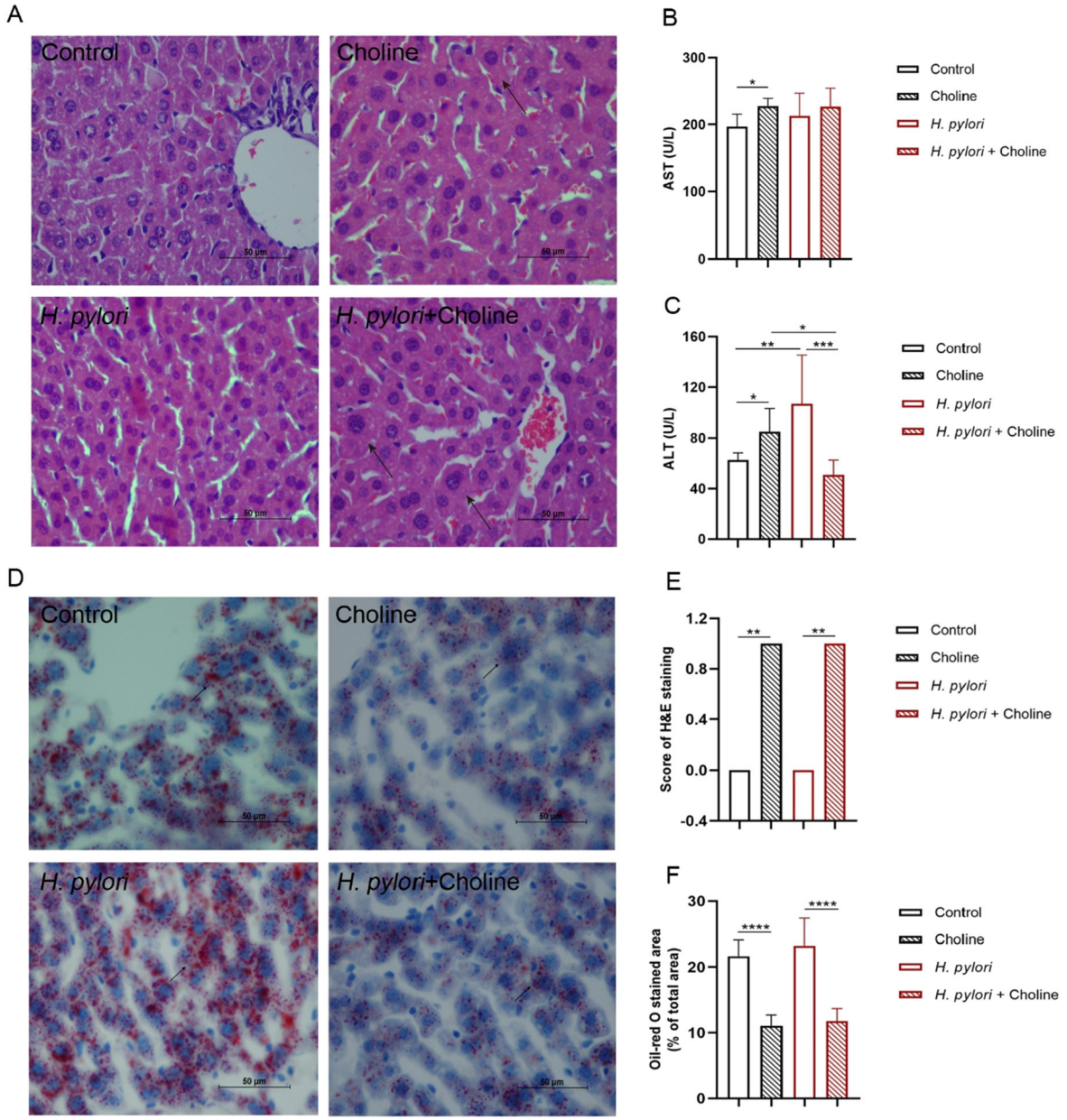

Fig. 2. Effects of choline supplementation on liver function of *H. pylori*-infected and non-infected mice. (A) H&E staining of the liver (original magnification of 400×, scale bar, 50 μm); (B and C) Serum enzymes activities of AST and ALT (n = 6); (D) Oil-red O staining of the liver (400×, scale bar, 50 μm); (E) Score of hepatic H&E staining (n = 6); (F) Quantification of liver Oil-red O staining (n = 6). Values are expressed as means ± SD. Significant differences are represented as follows: $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, $^{****}P < 0.0001$. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

GSH-P*x* activities are generally used as indicators of the antioxidant status, and MDA and NEFA levels are usually enlarged as a response to liver injury [34,35]. As illustrated in Fig. S3A and B, hepatic levels of MDA and NEFA were significantly elevated in choline feeding mice compared with the normal mice. Meanwhile, this choline-induced raising was strengthened by cotreatment with *H. pylori*. As expected, hepatic SOD and GSH-P*x* activities were obviously decreased in both choline-fed group and *H. pylori*-infected group as compare to the normal ones. A further decrease was observed in the choline and *H. pylori* cotreatment group (Fig. S3C and D). These results demonstrate that choline supplementation and *H. pylori* infection may lead to liver dysfunction in mice, and the combined effect of choline and *H. pylori* was more complex.

We subsequently examined the effects of choline supplementation on liver morphology. In comparison with the hepatic cellular architecture from the normal mice, HE staining showed hepatocyte hypertrophy in the portal vein conduit after choline feeding, and there was no evidence of pathological changes in the other two groups (Fig. 2A and E). The data together with the biochemical experiment illustrate that choline and *H. pylori* may have a negative impact on liver function in mice, but the damage to liver morphology may be mainly caused by choline.

3.3. Choline exacerbated the inflammation induced by H. pylori infection

Liver injury is generally associated with inflammation [36]. Therefore, we further examined some inflammation-related factors in the tested mice. CRP, an inflammatory marker synthesized in the liver, is considered to be an accurate and sensitive indicator of inflammatory activity [37]. LPS is mainly produced by gram-negative bacteria in the

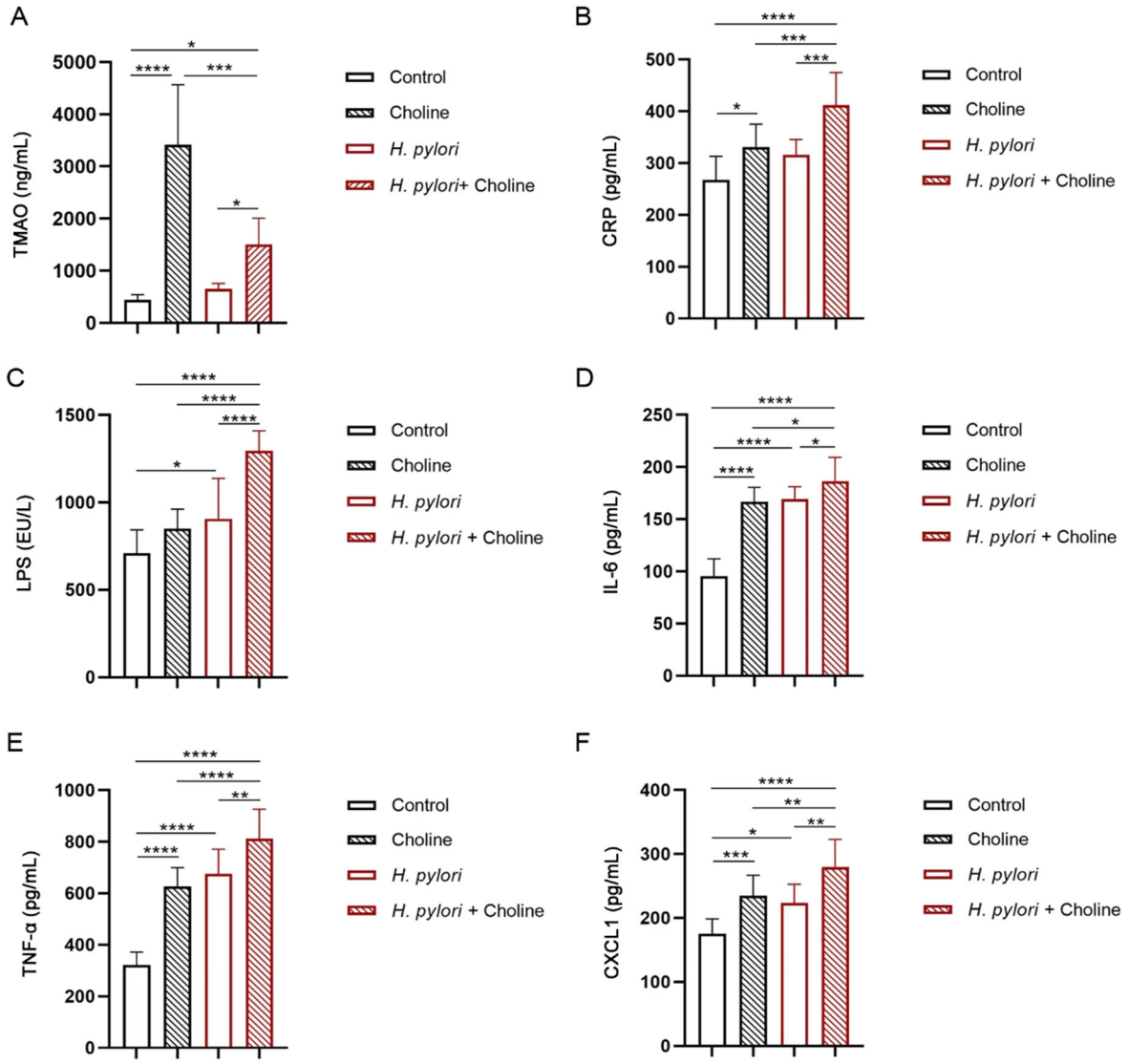

Fig. 3. Choline supplementation decreased serum TMAO level (A) and increased serum levels of inflammatory factors including CRP (B), LPS (C), IL-6 (D), TNF-α (E) and CXCL1 (F) in *H. pylori*-infected mice (n = 3–10). Values are expressed as means ± SD. Significant differences are represented as follows: $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, $^{****}P < 0.0001$.

intestinal ecosystem, which can induce inflammatory responses and regulate the expressions of inflammatory factors such as IL-6, TNF-α and CXCL1 [38]. As illustrated in Fig. 3B–F, serum levels of CRP, IL-6, TNF-α and CXCL1 were elevated in choline-fed mice compared with the control mice. A similar trend was observed in *H. pylori*-infected mice. Notably, an even higher rise in all the five factors (CRP, LPS, IL-6, TNF-α and CXCL1) were recorded in the group cotreated with *H. pylori* and choline. These findings demonstrate that choline aggravated *H. pylori*-induced inflammation.

3.4. Choline treatment changed the gut microbiome of H. pylori-infected mice

It has been widely shown in the literature that choline can be metabolized by the intestinal microbes to produce TMA, which is then oxidized to TMAO in liver [39]. We found that choline treatment strongly increased serum TMAO level, but the increase in TMAO induced by choline was markedly inhibited in *H. pylori*-infected mice (Fig. 3A). To explore whether *H. pylori* infection has any impact on choline intestinal metabolism, we investigated the composition of gut microbiota in mice by using 16S rRNA gene sequencing.

High-throughput sequencing generated 1,397,293 raw reads among all samples. After filtering out low-quantity sequences, 1,281,491 clean reads were obtained, and then the effective reads were clustered into OTUs based on 97% similarity level. As can be seen in Fig. S4, choline treatment reduced the number of OTUs in *H. pylori*-infected mice.

Alpha diversity of the intestinal flora calculated by Shannon, Simpson, Chao1 and ACE indexes are presented in Fig. 4A–D, showing that choline absorption significantly reduced the abundance and diversity of the gut microbiome in *H. pylori*-infected mice. Additionally, Beta diversity was used to analyze differences between samples (Fig. 5A). Similar results were seen when analyzed by PCoA and UPGMA (Fig. 5C, D and E, F). These observations implicate that four groups share different gut microbiota community structure.

We then assessed the taxonomic proportions of the mice enteric microorganisms. At the phylum level, gut microbiome of the mice was dominated by Bacteroidetes, Firmicutes and Proteobacteria in all groups (Fig. 4E). We found that the relative abundance of Proteobacteria in the control group (9.25%) was higher than that in the other three groups (choline group: 5.48%; *H. pylori* group: 5.94%; *H. pylori* + choline group: 4.90%; Fig. 4E), indicating that choline could inhibit the growth of corresponding bacteria in the intestine of *H. pylori*-

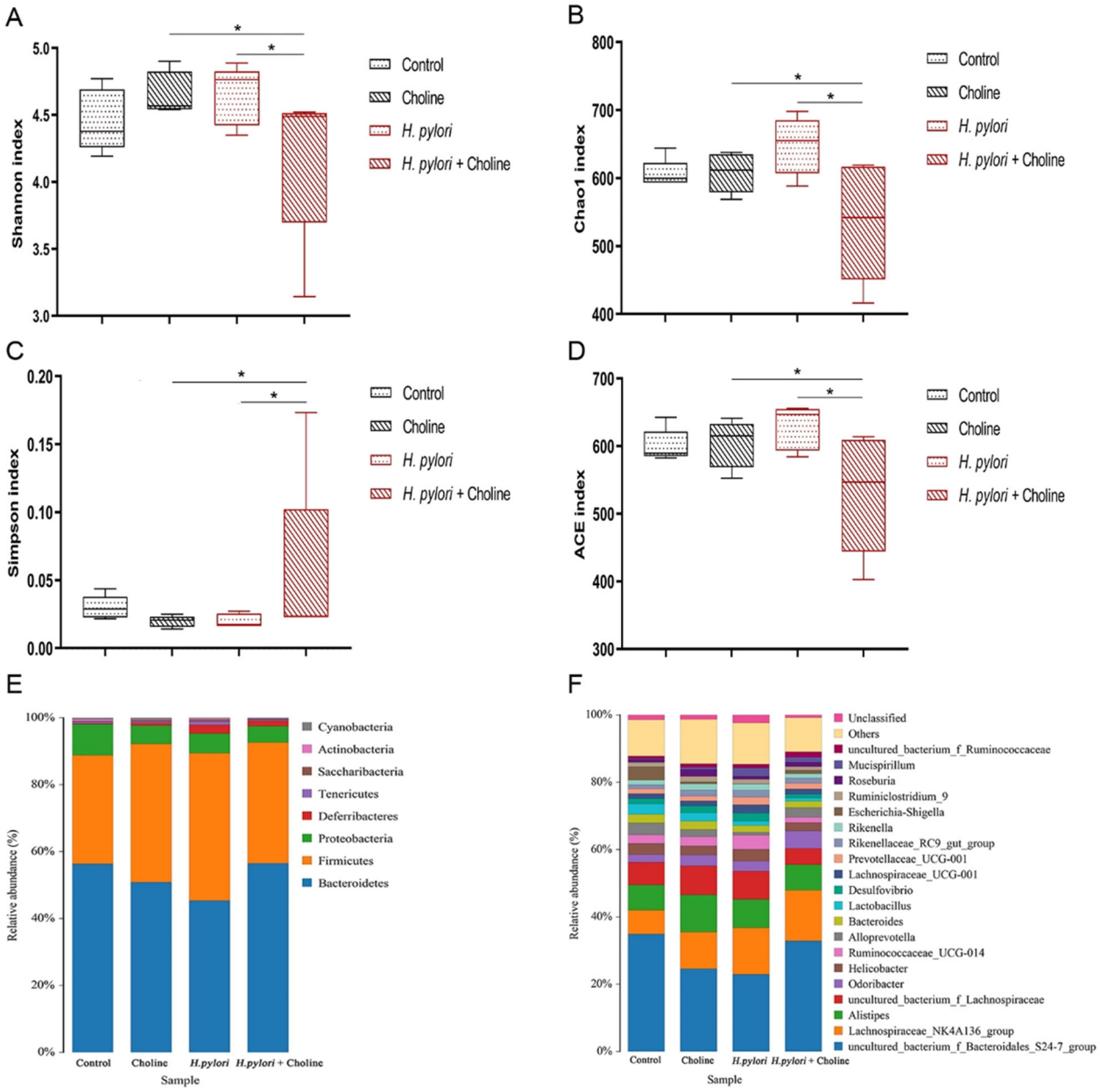

Fig. 4. Choline treatment altered the abundance and diversity of the gut microbiome in *H. pylori*-infected mice. Alpha diversity was calculated according to Shannon (A), Chao1(B), Simpson (C) and ACE (D) indexes; (E–F) The graphs showed relative abundances of the top 10 and top 20 bacterial species at the phylum (E) and genus (F) level, respectively. Data are expressed as means ± SD (n = 5). Statistical differences are denoted as follows: $^{*}P < 0.05$.

infected and uninfected mice. At the level of genus, we also found that choline reduced the relative abundance of *Helicobacter*, which belongs to the phylum Proteobacteria (Fig. 4F).

We further identified the key bacterial contributors in *H. pylori*-infected mice with or without choline treatment by LEfSe method (LDA score > 2, Fig. 5B). Remarkably, *Escherichia_Shigella* was more abundant in the group cotreated with *H. pylori* and choline compared to the *H. pylori* group, whereas *uncultured_bacterium_f_ Lachnospiraceae* was the most differentially abundant taxa in the *H. pylori* group.

3.5. Relationship between inflammation and alterations of gut microbes caused by choline and H. pylori infection

Gut microbiota is closely related to immune response [40]. In light of the above results and our previous findings that TMAO can also enhance *H. pylori*-triggered inflammation [19]. We next estimated the correlation between gut microbiome of top 20 abundance at genus level and some inflammatory factors (TNF-α, IL-6, CXCL1, LPS and CRP) as well as TMAO based on SparCC analysis. As can be seen in Fig. 6, *Escherichia_Shigella* showed a significant positive correlation with the levels of LPS, CRP, IL-6, TNF-α and CXCL1, while *Ruminococcaceae_UCG-014*, *Alistipes* and *Rikenellaceae_RC9_gut_group* were significantly negatively correlated with the levels of LPS, CRP, TNF-α and CXCL1. Ruminococcaceae_*UCG-014* was also inversely related to the TMAO level. These observations revealed that changes in intestinal flora are associated with inflammation in response to choline supplementation and *H. pylori* infection.

4. Discussion

In the present study, female BALB/c mice were used because female are likely to be more susceptible to choline than male. Heianza et al.

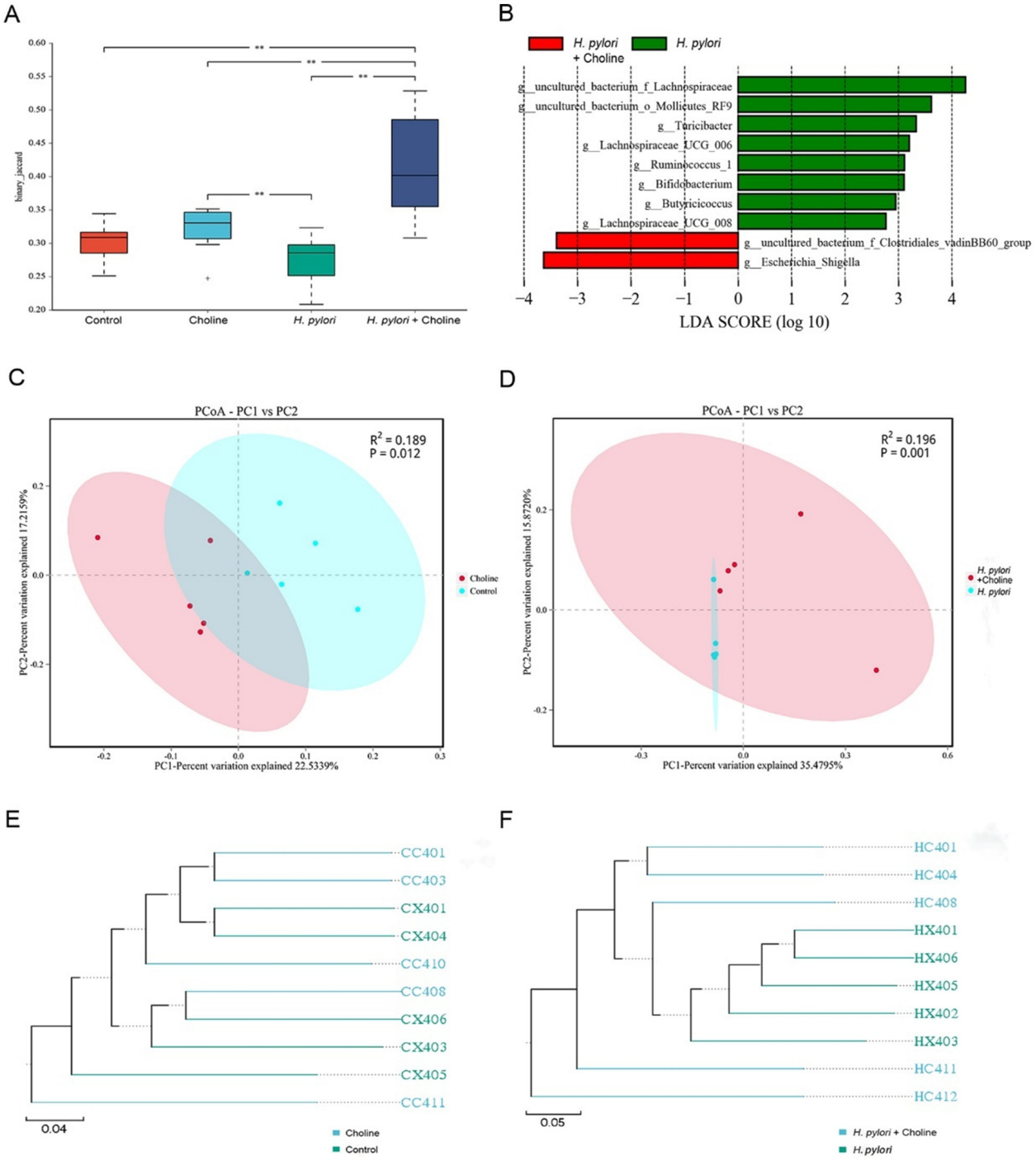

Fig. 5. Fecal microbial composition of the mice was significantly changed after cotreatment with choline and *H. pylori*. Beta diversity (A) measured the differences between samples. Similar trends were observed utilizing PCoA (C and D) and UPGMA (E and F); (B) The histogram of LEfSe analysis showed the biomarkers of the gut microbiota with statistics differences between the *H. pylori*-infected choline fed group and the *H. pylori*-infected group (LDA > 2). Data are expressed as means ± SD (n = 5). Statistical differences are denoted as follows: $^{**}P < 0.01$.

[41] suggested that choline, L-carnitine and TMAO are related to weight loss success, and these three may be biomarkers for successful reductions in body mass. Moreover, a study on female taekwondo and judo athletes demonstrated that choline supplementation can rapidly lose weight [42]. Additionally, another study monitored the effects of different concentrations of choline supplementation on rats, showing that the 15-fold choline group had a lower weight loss than the 10-fold and 5-fold groups [43]. The results of these studies indicate that choline can reduce body weight, but the extent of reduction depends on the amount of choline intake. Our data also observed that choline treatment slightly reduced the body weight of the normal mice, although the difference in body mass was not significant.

Choline supplementation markedly decreased hepatic activities of SOD and GSH-P*x*, while increased the levels of MDA and NEFA. Our findings indicate the liver injury in mice after choline administration, which are consistent with many previous studies [10,44,45]. Meanwhile, choline treatment reduced the serum LDL level, but elevated the activities of serum TG, AST, and ALT in the normal mice. Imajo et al. [46] showed that choline levels were positively related to the severity of liver steatosis, fibrosis, and nonalcoholic steatohepatitis (NASH) in

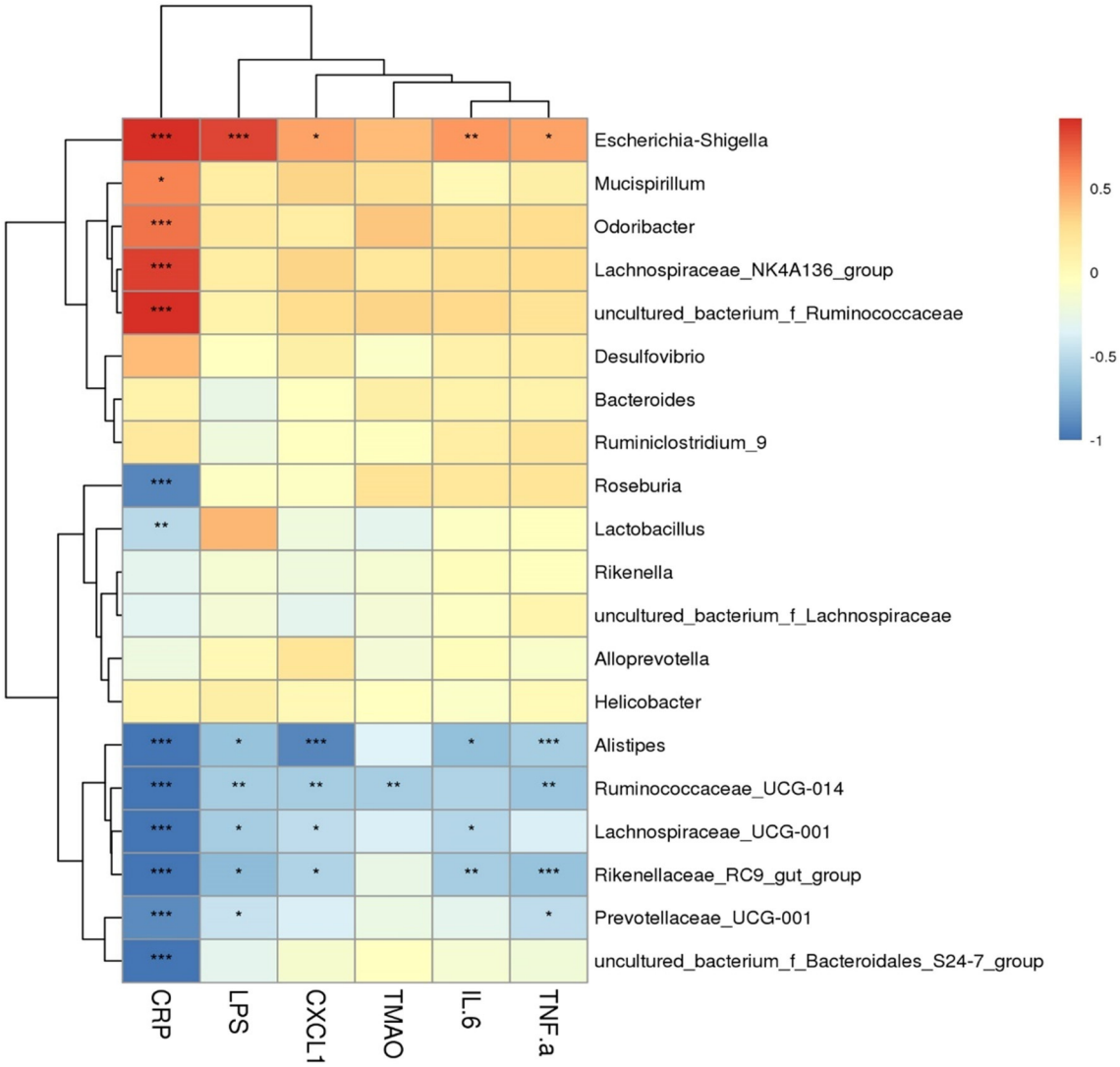

Fig. 6. The heatmap of the correlation between the gut microbiome of top 20 abundance at genus level and some inflammatory factors (TNF-α, IL-6, CXCL1, LPS and CRP) as well as TMAO based on SparCC analysis. Positive correlations are depicted in red, whereas negative correlations are in blue. Significant differences are represented as follows: $*P < 0.05$, $**P < 0.01$, $***P < 0.001$. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Japanese. Dietary intake of 3% choline could lead to vascular endothelial dysfunction and liver injury with the elevation of serum TG and LDL levels as well as ALT and AST activities [10,44]. Nevertheless, 1.2% choline intake alleviated a high fat diet-induced hepatic steatosis and weight gain in male mice in study of Brown et al. [47]. Additionally, Yu et al. [48] reported that choline supplementation was negatively associated with the risk of nonalcoholic fatty liver (NAFL) in Chinese women. However, Chen et al. [18] pointed out that there was no significant correlation between choline and NAFLD in community-based Chinese adults, but an adverse relationship in hospital-based patients, and this unfavorable association may be due to the TMAO levels. And apart from that, insufficient intake of choline may also result in signs of subclinical hepatic dysfunction [49]. Taken together, the effects of choline on liver biology probably depend on individual differences and the dosage of choline.

Some studies have demonstrated that *H. pylori* infection is linked to the increased risk of NAFLD [50,51], whereas others have revealed that *H. pylori* infection is not associated with NAFLD through cross-sectional studies in different countries [52,53]. Consistent with what we found in the present study, Ge et al. [54] also showed no significant differences in serum TG, HDL and AST levels between *H. pylori*-infected patients and normal control subjects. Interestingly, we detected that *H. pylori* infection obviously increased the level of ALT, which was attenuated by cotreatment with choline. *H. pylori* eradication has been shown to reduce serum levels of ALT and AST, indicating that *H. pylori* infection may be related to liver function [55]. We also observed the elevation of hepatic NEFA level and the reduction of SOD and GSH-P*x* activities after *H. pylori* infection. However, further studies are still needed to clarify this.

PC, an important dietary source of choline, is thought to have antibacterial effects against *H. pylori* [9,15]. Furthermore, Han et al. [56] suggested that methionine and choline-deficient diet increased the relative abundance of Helicobacteraceae in the mice gut microbiota. Helicobacteraceae is usually regarded as pathogenic species, among which *H. pylori* can cause a variety of inflammatory diseases by activating immune responses and gastrointestinal disorders. *H. pylori* has also been reported to interact with gut microbiota and its association with extragastric diseases [57,58]. Our 16S rRNA gene sequencing analysis showed that the relative abundance of *Helicobacter* in choline-fed groups were significantly lower in comparison with the other two groups, indicating that choline supplementation can reduce the relative abundance of *Helicobacter*. However, whether this is the case for *H.*

pylori remains to be seen.

It is well established that inadequate intake of choline or *H. pylori* infection can lead to inflammation. Rajaie et al. [59] and Jia et al. [44] illustrated that higher choline intake was related to the increased serum levels of CRP, IL-6 and TNF-α. In addition, our previous study showed that *H. pylori* stimulation altered the expression of immune-related genes and enhanced the secretion of iNOS, COX-2, IL-6 and CXCL1 in GES-1 cells [20,60,61]. In the current study, we observed that choline notably elevated serum concentrations of inflammatory makers including CRP, LPS, IL-6, TNF-α and CXCL1 in *H. pylori*-infected mice. Meanwhile, we found that choline supplementation dramatically increased serum TMAO level, which is consistent with the results from Anwar et al. [21]. TMAO, a metabolite derived from choline, can also aggravate the inflammation caused by *H. pylori* infection [19]. Wu et al. [19] reported that the expression of *H. pylori* virulent CagA and VacA genes were significantly increased in the presence of TMAO. Censini et al. [62] and Blaser et al. [63] demonstrated that gastric inflammation is highest with CagA strains of *H. pylori*. What is more, *H. pylori* CagA can promote the expressions of IL-6 and TNF-α via NF-κB p65 acetylation [64]. Thus, there is a possibility that choline may increase the virulence factors of *H. pylori* through TMAO, thereby exacerbating inflammation.

More strikingly, the elevated TMAO induced by choline was markedly suppressed in *H. pylori*-infected mice. Our previous study find that *H. pylori* has a TorA gene encoding TMAO reductase (Fig. S5), which can reduce TMAO to TMA. Moreover, there have been some literature reports about the correlation between TMAO and Ruminococcaceae [65,66]. Similarly, our heatmap analysis showed that the TMAO level was negatively correlated with *Ruminococcaceae_UCG-014*. Besides, the level of TMAO was also related to Desulfovibrionaceae and Lachnospiraceae [65,66], from which we found that the relative abundance of *Desulfovirobrio* and *uncultured_bacterium_f_ Lachnospiraceae* decreased after cotreatment with *H. pylori* and choline. These results indicate that changes in TMAO levels may be associated with gut microbes.

Further LEfSe and SparCC analysis observed that compared with the *H. pylori* group, *Escherichia_Shigella* was highly enriched in the group cotreated with *H. pylori* and choline. Meanwhile, *Escherichia_Shigella* was significantly positively correlated with the levels of LPS, CRP, IL-6, TNF-α and CXCL1. In contrast, *Ruminococcaceae_UCG-014*, *Alistipes* and *Rikenellaceae_RC9_gut_group* showed significant negative correlations with the levels of LPS, CRP, TNF-α and CXCL1. *Escherichia_Shigella* is a pro-inflammatory bacterium that can generate LPS [67], which is known to result in inflammation and stimulate the secretion of inflammatory factors such as iNOS, COX-2, IL-6 and TNF-a [38,68]. *Ruminococcaceae_UCG-014* and *Alistipes*, however, are considered to have anti-inflammatory activities, and the reduction in these bacteria is associated with inflammatory responses [69,70]. Additionally, the hydrogen-producing bacterium *Rikenellaceae_RC9_gut_group*, is thought to have the ability to suppress inflammatory cytokines, especially, IL-6, TNF-α and IL-1β [71,72]. All the information suggest that choline supplementation altered gut microbes in *H. pylori*-infected mice, which may contribute to inflammation.

5. Conclusions

In summary, we have shown for the first time that choline can exacerbate *H. pylori*-induced inflammation, which may be related to changes in gut microbiota. Hence, our present work may provide new insights into the effects of food-derived choline on *H. pylori* infection-related diseases.

Declaration of competing interest

The authors declare that there are no conflicts of interest.

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Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.lfs.2020.118200.

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