

# *Helicobacter pylori* infection does not promote hepatocellular cancer in a transgenic mouse model of hepatitis C virus pathogenesis

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**Keywords:** Hepatitis, helicobacter, cancer, infection, virus, bacteria, mice, transgenic

**Abbreviations:** hepatitis C virus, HCV; *Helicobacter pylori*, *H. pylori*; wild-type, WT; HCV transgenic, HT; gastric histologic activity index, GHAI; altered hepatocellular foci, AHF; large foci of cellular alteration, LFCA; hepatocellular adenoma, HCA; hepatocellular carcinoma, HCC; polymerase chain reaction, PCR; quantitative polymerase chain reaction, qPCR; hematoxylin and eosin, H&E; fluorescent in situ hybridization, FISH; enzyme-linked immunosorbent assay, ELISA; enterohepatic helicobacter species, EHS

*Helicobacter pylori* (*H. pylori*) and hepatitis C virus (HCV) infect millions of people and can induce cancer. We investigated if *H. pylori* infection promoted HCV-associated liver cancer. *Helicobacter*-free C3B6F1 wild-type (WT) and C3B6F1-Tg(Alb1-HCVN)35Sml (HT) male and female mice were orally inoculated with *H. pylori* SS1 or sterile media. Mice were euthanized at ~12 mo postinoculation and samples were collected for analyses. There were no significant differences in hepatocellular tumor promotion between WT and HT mice; however, HT female mice developed significantly larger livers with more hepatic steatosis than WT female mice. *H. pylori* did not colonize the liver nor promote hepatocellular tumors in WT or HT mice. In the stomach, *H. pylori* induced more corpus lesions in WT and HT female mice than in WT and HT male mice, respectively. The increased corpus pathology in WT and HT female mice was associated with decreased gastric *H. pylori* colonization, increased gastric and hepatic interferon gamma expression, and increased serum Th1 immune responses against *H. pylori*. HT male mice appeared to be protected from *H. pylori*-induced corpus lesions. Furthermore, during gastric *H. pylori* infection, HT male mice were protected from gastric antral lesions and hepatic steatosis relative to WT male mice and these effects were associated with increased serum TNF- $\alpha$ . Our findings indicate that *H. pylori* is a gastric pathogen that does not promote hepatocellular cancer and suggest that the HCV transgene is associated with amelioration of specific liver and gastric lesions observed during concurrent *H. pylori* infection in mice.

## Introduction

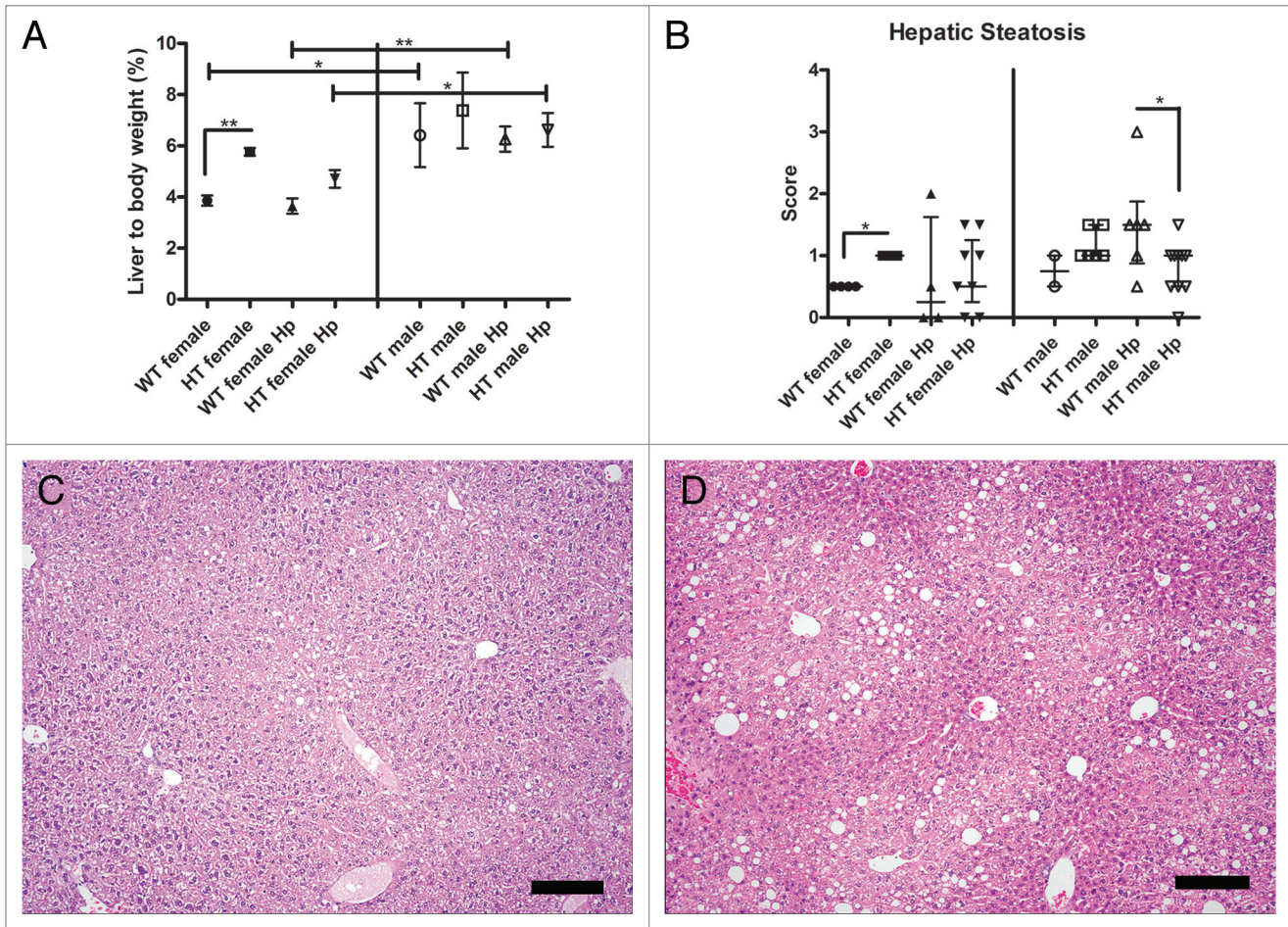
*Helicobacter pylori* (*H. pylori*) and hepatitis C virus (HCV) infect approximately 50% and 2% of the world's population, respectively.<sup>1,2</sup> The World Health Organization's International Agency for Research on Cancer considers *H. pylori* and HCV carcinogens.<sup>3</sup> *H. pylori* infection may induce gastritis leading to gastric adenocarcinoma whereas HCV can induce hepatitis and cirrhosis leading to hepatocellular carcinoma (HCC).<sup>2,4</sup> Gastric and liver cancers are ranked in the top ten most common cancers in both men and women worldwide and are two of the most common causes of cancer death.<sup>5</sup>

During HCV infection, environmental and/or host-associated factors are important and influence the progression to cirrhosis.<sup>6</sup> Some of these factors are age, gender, alcohol ingestion, obesity, diabetes, and co-infection with hepatitis B virus or human immunodeficiency virus.<sup>6</sup> Hepatic steatosis is also considered a

risk factor for HCC in humans with chronic HCV infection and may affect a patient's response to treatment.<sup>7,8</sup>

Studies suggest that *H. pylori* may impact HCV disease based on detection of *H. pylori* or "*H. pylori*-like" DNA in liver tissues from HCV patients.<sup>9-14</sup> *Helicobacter* spp. have been detected in bile and gallbladder tissues of chronic cholecystitis patients and the need for further research investigating a potential causal role of helicobacters in human liver disease has been proposed.<sup>15,16</sup> *H. pylori* was isolated from the liver of a human with Wilson's disease-associated liver cirrhosis.<sup>17</sup> In vitro studies using a human hepatoma cell line (Huh7) have also documented that *H. pylori* induces cell arrest and apoptosis of infected hepatocytes and also induces hepatocyte malfunction associated with formation of podosomes.<sup>18,19</sup> In addition, an enterohepatic helicobacter, *H. bilis*, can affect the modulation of various proteins including those involved with tumorigenesis in Huh7-derived cells transfected with HCV.<sup>20</sup> *H. hepaticus*, an enterohepatic helicobacter of

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**Figure 1.** (A) Liver to body weight ratio (%) in the different groups of mice. No liver or body weight was available for three mice in different groups including WT female Hp, HT female, and HT female Hp (\*,  $p < 0.05$ ; \*\*,  $p < 0.01$ ). (B) Hepatic steatosis scores in the different groups of mice (\*,  $p < 0.05$ ). (C) Normal liver in a hepatitis C virus transgenic male mouse infected with *H. pylori* (HT male Hp). (D) Mild fatty degeneration in the cytoplasm of centrilobular hepatocytes in a wild-type male mouse infected with *H. pylori* (WT male Hp). (C and D) bar size 100  $\mu\text{m}$ .

mice, can induce and promote HCC in A/JCr and HCV transgenic mice, respectively.<sup>21,22</sup>

The use of FL-N/35 (C57BL/6 background) mice has advanced the study of HCV pathogenesis. These mice are transgenic for FL-N which encodes the complete polyprotein of a genotype 1b strain of HCV. In humans, infection with the HCV genotype 1b strain appears to increase the risk of HCC development.<sup>23</sup> FL-N/35 mice usually develop hepatic steatosis and HCC after 10 and 13 mo of age, respectively, and these hepatic lesions appear to be more common in male than female mice.<sup>24,25</sup> These studies highlighted the role of structural and nonstructural viral proteins in HCV pathogenesis. Further studies using FL-N/35 mice have documented an attenuation of Fas-mediated apoptosis in transgenic hepatocytes, an increased risk of HCC by iron overload, and development of hepatocellular steatosis with decreased plasma triglycerides.<sup>26-28</sup> In the current study, we used a F1 hybrid of transgenic FL-N/35 and C3H/HeNTac mice as a mouse model of HCV to investigate the impact of *H. pylori* infection on liver cancer. The use of C3B6F1 mice in the current study was based on findings by the National Toxicology Program that B6C3F1 mice were susceptible to *H. hepaticus*-associated hepatitis and liver cancer.<sup>29</sup> Therefore, the F1 model was useful

to investigate whether another species of *Helicobacter* (*H. pylori*) could influence the pathogenesis of liver cancer in mice.

## Results

### HCV transgene increases liver weight in female mice

The liver to body weight ratio (%) was significantly higher in WT male mice relative to WT female mice ( $p < 0.05$ ) and in WT male Hp mice relative to WT female Hp mice ( $p < 0.01$ ). In addition, the liver to body weight ratio (%) was significantly higher in HT female mice relative to WT female mice ( $p < 0.01$ ) but not in HT male mice relative to WT male mice (Fig. 1A). Similar results were obtained when analyzing the absolute liver weight data. These findings suggest that male mice have a proportionately heavier liver than female mice and that the HCV transgene increases the liver weight in female mice. There were no significant differences in liver to body weight ratio (%) between HT male mice and HT male Hp mice, between HT female mice and HT female Hp mice, between WT male mice and WT male Hp mice, or between WT female mice and WT female Hp mice. Therefore, *H. pylori* infection did not increase liver weight in HT or WT mice.

**Table 1.** Percentage of mice with microscopic preneoplastic and/or neoplastic liver lesions in each group\*

Group (number of mice)	Mice with neoplasia			Total mice with hepatic preneoplasia (AHF and LFCA) and neoplasia
	Mice with HCA only	Mice with HCC only	Total mice with HCA and HCC	
WT female (n = 4)	0 (0%)	0 (0%)	0 (0%)	0 (0%)
HT female (n = 3)	1 (33.33%)	0 (0%)	1 (33.33%)	1 (33.33%)
WT female Hp (n = 4)	0 (0%)	0 (0%)	0 (0%)	0 (0%)
HT female Hp (n = 9)	0 (0%)	2 (22.22%)	2 (22.22%)	3 (33.33%)
WT male (n = 2)	0 (0%)	1 (50%)	1 (50%)	1 (50%)
HT male (n = 6)	2 (33.33%)	0 (0%)	2 (33.33%)	5 (83.33%)
WT male Hp (n = 6)	2 (33.33%)	1 (16.66%)	3 (50%)	4 (66.66%)
HT male Hp (n = 10)	0 (0%)	4 (40%)	4 (40%)	7 (70%)

\*No statistically significant differences were observed between the groups analyzed within each column. WT, wild-type; HT, hepatitis C virus transgenic. Preneoplasia includes altered hepatocellular foci (AHF) and large foci of cellular alteration (LFCA); Neoplasia includes hepatocellular adenoma (HCA) and hepatocellular carcinoma (HCC).

### HCV transgene increases hepatic steatosis in female mice

The hepatic steatosis score was significantly higher in HT female mice relative to WT female mice ( $p < 0.05$ ) and in WT male Hp mice relative to HT male Hp mice ( $p = 0.05$ ) (Fig. 1B, 1C, 1D). Histopathologic features of steatosis comprised hepatocellular cytoplasmic vacuolar change consistent with the formation of fat vacuoles. These findings suggest that the HCV transgene increases hepatic steatosis in HT female mice and decreases hepatic steatosis in HT male Hp mice. There were no significant differences in hepatic steatosis between HT male mice and HT male Hp mice, between HT female mice and HT female Hp mice, or between WT female mice and WT female Hp mice. The difference between WT male mice and WT male Hp mice could not be calculated due to the small sample size in the WT male group. Therefore, *H. pylori* did not modulate hepatic steatosis in HT mice or WT female mice.

### *H. pylori* infection does not promote hepatocellular tumors in HCV transgenic mice

The percentage of mice in each group with microscopic preneoplastic (altered hepatocellular foci (AHF) and large foci of cellular alteration [LFCA]) and/or neoplastic (hepatocellular adenoma [HCA] and hepatocellular carcinoma [HCC]) liver lesions is detailed in Table 1. Multiplicity values for microscopic preneoplastic and neoplastic liver lesions were recorded in *H. pylori*-infected and uninfected HT and WT mice; however, there were no significant differences in multiplicity of microscopic preneoplastic and neoplastic liver lesions between HT male mice and HT male Hp mice ( $p = 0.50$ ) and between HT female mice and HT female Hp mice ( $p = 0.80$ ) (Fig. 2A). There were no significant differences in the number of liver lobes with dysplasia and neoplasia between HT male mice and HT male Hp mice ( $p = 0.80$ ) and between HT female mice and HT female Hp mice ( $p = 0.50$ ) (data not shown). These results indicate that *H. pylori* infection does not promote liver tumors in C3B6F1-Tg(Alb1-HCVN)35Sml mice. HCCs were detected in livers of *H. pylori*-infected and uninfected HT and WT mice (Fig. 2B), and were characterized by poorly delineated proliferations of moderately-well differentiated, vacuolated, neoplastic hepatocytes, often organized in a trabecular pattern with

minimal intervening stroma (Fig. 2C). The multiplicity of preneoplasia and/or neoplasia and the number of mice with microscopic neoplastic liver lesions did not differ significantly between groups. None of the groups of mice developed a median hepatitis index score  $\geq 4$  and no significant differences in hepatitis index scores were observed in HT male Hp mice and HT female Hp mice relative to HT male mice and HT female mice, respectively (Supplementary Material).

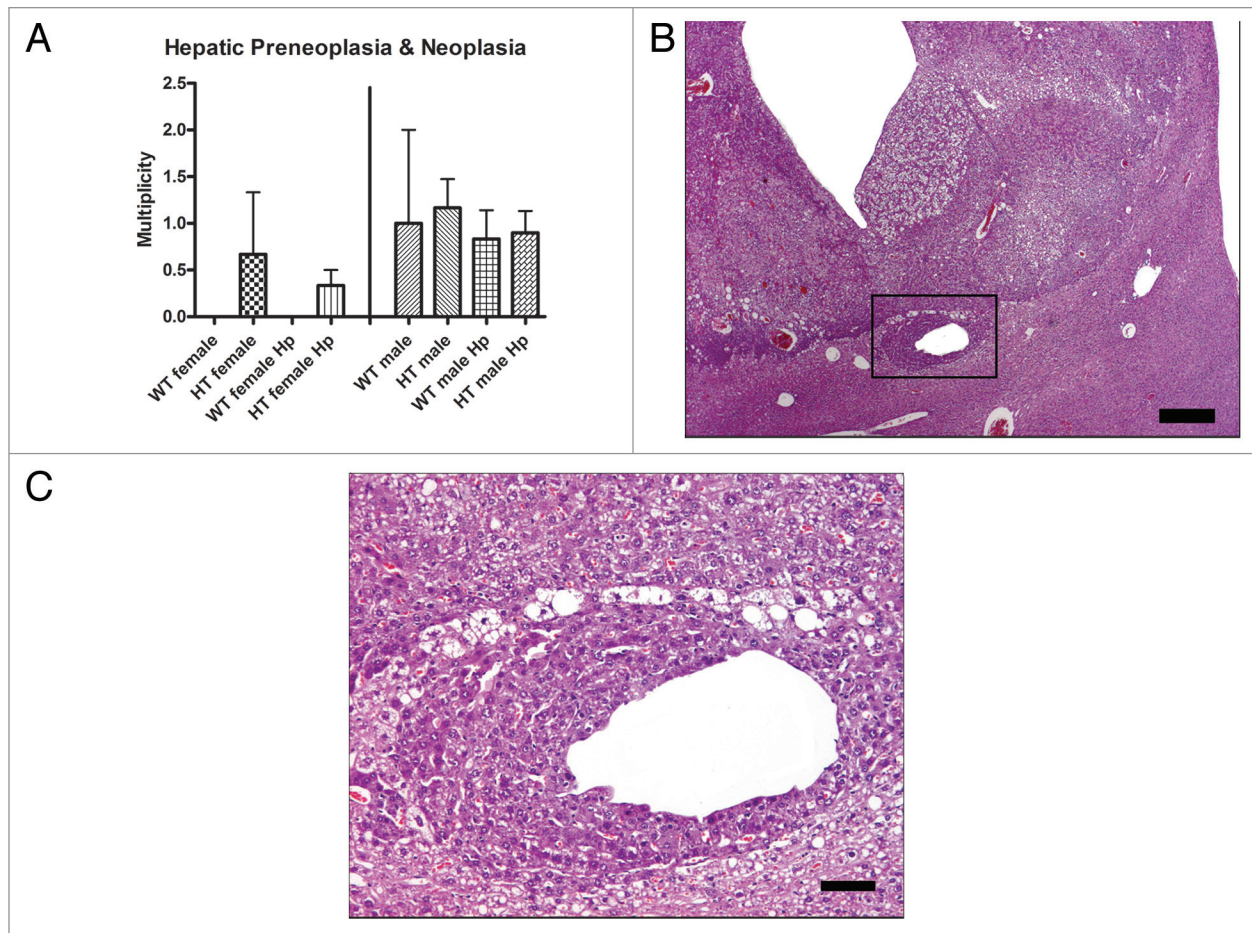
### *H. pylori* colonizes the stomach but not the liver

No *H. pylori* were detected in livers by quantitative PCR (qPCR). In the stomach, qPCR detected copies of *H. pylori* in all but three of the *H. pylori*-inoculated mice analyzed in this study (Table 2). In colonized mice, the values ranged from 28 to 165862 *H. pylori* copies per  $\mu\text{g}$  of mouse DNA. qPCR did not detect *H. pylori* in gastric tissues from 2 WT female Hp and 1 HT female Hp mice. No significant differences in *H. pylori* colonization were detected by qPCR between WT female Hp mice and WT male Hp mice; however, significantly fewer *H. pylori* were detected in the stomachs of HT female Hp mice than in the stomachs of HT male Hp mice ( $p < 0.05$ ) (Fig. 3A). This finding was consistent with the increased gastric corpus pathology observed in HT female Hp mice relative to HT male Hp mice. There were no significant differences in *H. pylori* colonization levels between WT female Hp mice and HT female Hp mice and between WT male Hp mice and HT male Hp mice.

*H. pylori* were detected by fluorescent in situ hybridization (FISH) in the gastric corpus and antrum of selected gastric sections from infected mice. In gastric sections with high numbers of *H. pylori*, the antrum appeared to be colonized at a higher level than the corpus. *H. pylori* were detected on the apical surface of epithelial cells at the luminal edge and within crypts (Fig. 3B and 3C).

### *H. pylori* infection causes the most severe gastric lesions in the corpus of WT and HT female mice

In the corpus, an increased gastric histologic activity index (GHAI) score was observed in WT female Hp mice relative to WT female ( $p < 0.05$ ) and WT male Hp mice ( $p = 0.07$ ) and in HT female Hp mice relative to HT female mice ( $p = 0.06$ ) and HT male Hp mice ( $p < 0.01$ ) (Fig. 4A-C). There were no



**Figure 2.** (A) Multiplicity of hepatic preneoplasia and neoplasia in the different groups of mice. (B) Hepatocellular carcinoma in a hepatitis C virus transgenic male mouse infected with *H. pylori* (HT male Hp). The tumor is poorly demarcated from the normal liver tissue and there is vacuolation of neoplastic cells and dilation of sinusoids. (C) Higher magnification (box in B) view of the junction between the poorly demarcated neoplasm and adjacent normal parenchyma. Neoplastic cells have cytoplasm with a deeper basophilic staining intensity and are often vacuolated. A focus of neoplastic cells also surrounds a distended hepatic sinusoid. (B) bar size 500  $\mu$ m. (C) bar size 100  $\mu$ m.

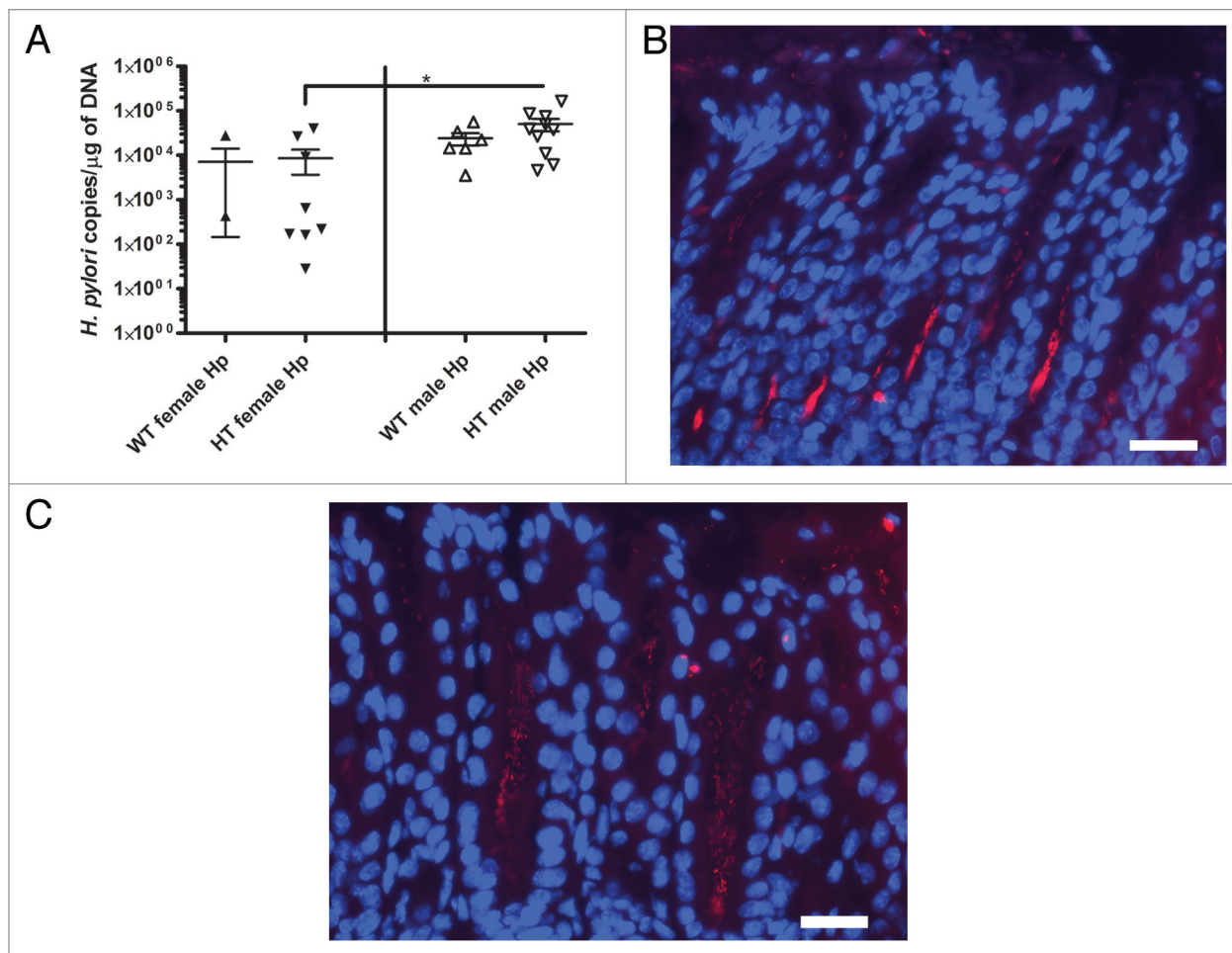
**Table 2.** Mouse strains, groups, and numbers in this study\*

	Sham		<i>H. pylori</i> (Hp)-infected	
	Female	Male	Female	Male
C3B6F1 <sup>†</sup>	4 (4)	2 (4)	4 (5)	6 (7)
C3B6F1-Tg(Alb1-HCVN)35Sml <sup>‡</sup>	3 (4)	6 (6)	9 (10)	10 (12)

\*Number in parentheses indicates number of mice at the initiation of the study. One WT male mouse was euthanized at ~6 mo postinoculation and another mouse in this group was diagnosed with osteosarcoma at the end of the study. One WT female Hp mouse was diagnosed with histiocytic sarcoma at the end of the study. One WT male Hp mouse was found dead at ~12 mo postinoculation. Two HT male Hp mice were euthanized at ~3 to 4 mo and ~4 mo postinoculation, respectively. The three mice euthanized before the end of the study did not exhibit preneoplastic and/or neoplastic liver lesions. One HT female and one HT female Hp mouse were diagnosed with lymphoma at the end of the study. The mice with other tumors (osteosarcoma, histiocytic sarcoma, and lymphoma) and the mice that were euthanized or died before the end of the study were not included in the final analyses. <sup>†</sup> Wild-type (WT); <sup>‡</sup>, HCV transgenic (HT).

significant differences in GHAI score between WT female Hp mice and HT female Hp mice and between WT male Hp mice and HT male Hp mice. The most common mucosal lesions were comprised of mild foveolar hyperplasia, oxyntic atrophy with mucous metaplasia, pseudopyloric intestinal metaplasia, epithelial dysplasia, and scattered inflammatory cell infiltrates; the latter of which also extended to involve the submucosa. The gastric GHAI scores were comparable between HT male and HT male Hp ( $p = 0.83$ ). The difference between WT male mice and WT male Hp mice could not be calculated due to the small sample size in the WT male group.

Increased gastric lesions in the antrum, characterized by mucosal hyperplasia, were observed in WT male Hp mice relative to WT male mice (no P value due to small sample size in the WT male group) and in WT female Hp mice relative to WT female mice ( $p < 0.05$ ). Gastric antral lesions were significantly greater in WT male Hp mice compared with HT male Hp mice ( $p < 0.05$ ) (Fig. 5A-C). The antral histopathology index scores were comparable between HT male mice and HT male Hp mice ( $p = 0.40$ ) and between HT female mice and HT female Hp mice ( $p = 0.19$ ).



**Figure 3.** (A) Colonization of *H. pylori* in the stomach analyzed with quantitative PCR (\*,  $p < 0.05$ ). Values are expressed as *H. pylori* copies per  $\mu\text{g}$  of mouse DNA. Values for three mice without *H. pylori* colonization are not shown in the graph. (B) Fluorescent in situ hybridization (FISH) of *H. pylori* in the gastric antrum of an infected hepatitis C virus transgenic male mouse (HT male Hp). (C) FISH of *H. pylori* in the gastric antrum of an infected hepatitis C virus transgenic female mouse (HT female Hp). (B and C) bar size  $\sim 20 \mu\text{m}$ .

In the antrum, exophytic, proliferative lesions were observed grossly in 2 of 6 (33%) WT male Hp mice. These exophytic antral lesions in WT male Hp mice were epithelial polyps, characterized by a proliferation of cells with antral epithelial differentiation (Fig. 5D). These lesions did not seem to be specific to WT male Hp mice as a microscopically similar antral polypoid lesion was diagnosed in a HT male Hp mouse that was euthanized at  $\sim 4$  mo postinoculation.

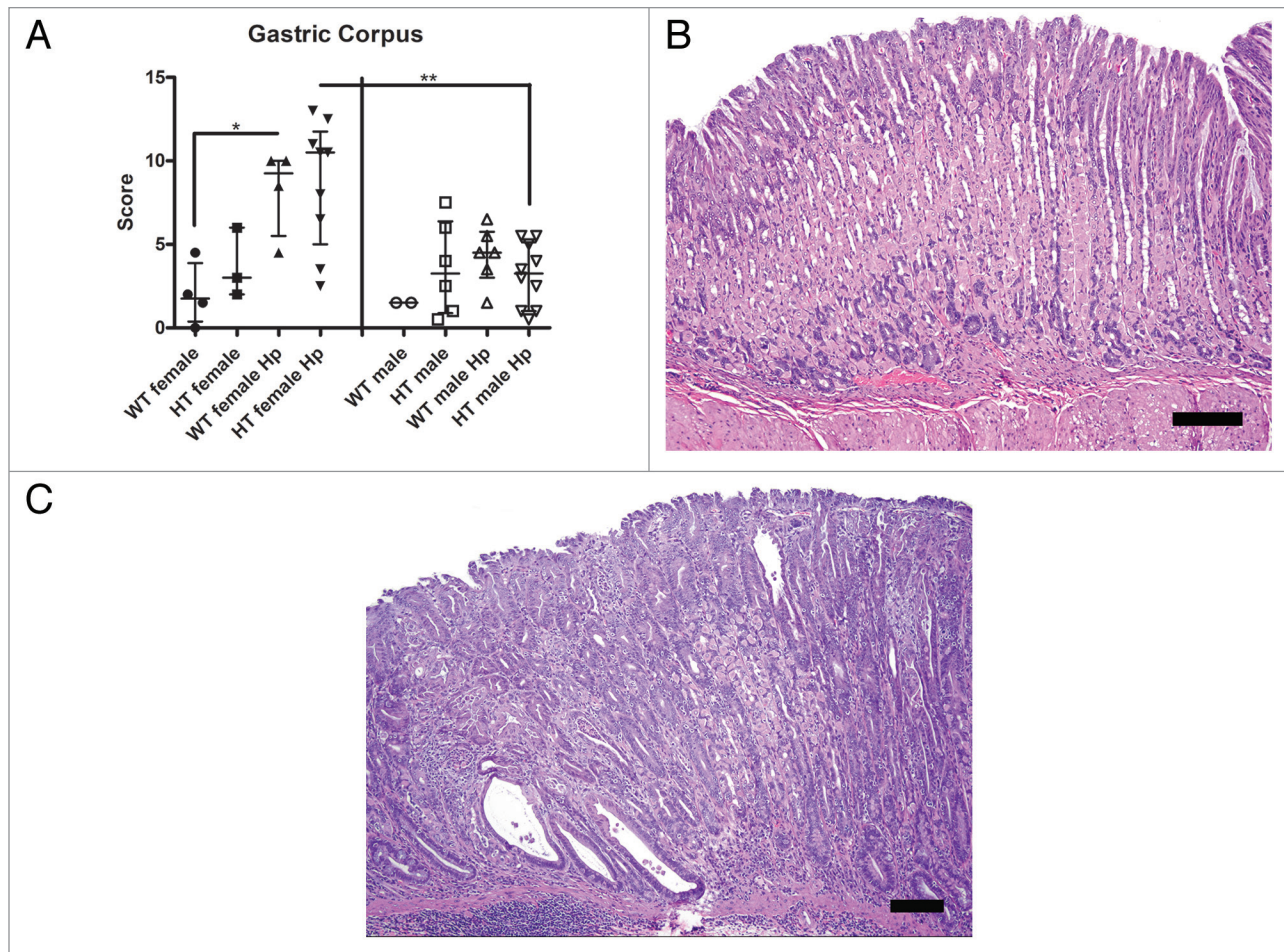
#### Female gender in combination with *H. pylori* infection increase the gastric and hepatic expression of interferon gamma

In the stomach, significant upregulation of *Ifng* was observed in HT male Hp mice relative to HT male mice and in HT female Hp mice relative to HT female mice (Fig. 6A). In addition, significant upregulation of *Ifng* was observed in WT female Hp mice relative to WT male Hp mice and in HT female Hp mice relative to HT male Hp mice (Fig. 6A). In the liver, significant differences in *Ifng* expression were observed between WT female Hp mice and WT male Hp mice and between HT female Hp mice and HT male Hp mice (Fig. 6B). There were no significant differences in hepatic *Ifng* expression between HT male

mice and HT male Hp mice and between HT female mice and HT female Hp mice. Gastric *Tnf* and *Il10* and hepatic *Il10* were significantly upregulated in *H. pylori*-infected female mice relative to *H. pylori*-infected male mice (Supplementary Material). Hepatic *Ifnb1* was significantly upregulated in HT female Hp mice relative to WT female Hp mice (Supplementary Material). The expression of gastric *Tnf* and *Il10* and hepatic *Il10* and *Ifnb1* did not differ significantly between HT male mice and HT male Hp mice and between HT female mice and HT female Hp mice.

#### *H. pylori* increases serum Th1 and Th2 immune responses in female mice

Compared with uninfected controls, all mice infected with *H. pylori* developed similar, robust serum IgG titers to *H. pylori* (Fig. 7A-C). A significantly greater Th1-associated IgG2c antibody response was measured in WT female Hp mice relative to WT male Hp mice and in HT female Hp mice relative to HT male Hp mice. Th2-associated IgG1 responses were also significantly higher in HT female Hp mice compared with HT male Hp mice.



**Figure 4.** (A) Gastric histologic activity index (GHA) score of the corpus in the different groups of mice (\*,  $p < 0.05$ ; \*\*,  $p < 0.01$ ). (B) Mild foveolar hyperplasia in the gastric corpus of a hepatitis C virus transgenic male mouse infected with *H. pylori* (HT male Hp). (C) Mucosal lesions in the gastric corpus of a hepatitis C virus transgenic female mouse infected with *H. pylori* (HT female Hp). Lesions consisted of pseudopyloric intestinal metaplasia, epithelial dysplasia, and multifocal inflammation in the mucosa and submucosa. (B and C) bar size 100  $\mu\text{m}$ .

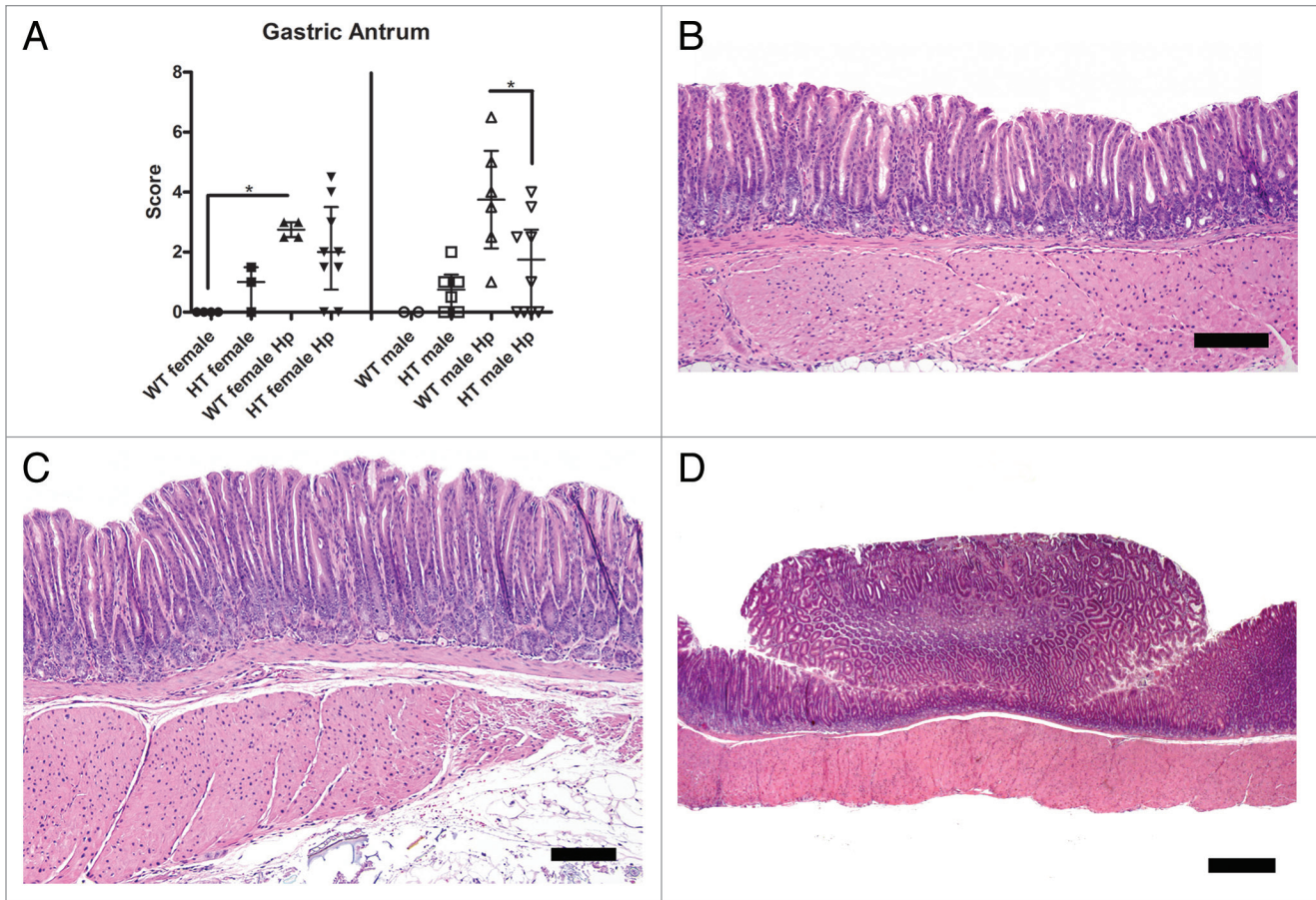
#### HCV transgene increases serum TNF- $\alpha$ during *H. pylori* infection in male mice

The serum concentration of TNF- $\alpha$  was significantly higher in HT male Hp mice relative to WT male Hp mice ( $185.5 \pm 11.6$  vs.  $138.6 \pm 17.1$ ,  $p < 0.05$ ) (Fig. 8). There were no significant differences in the serum concentration of TNF- $\alpha$  between HT male mice and HT male Hp mice and between HT female mice and HT female Hp mice. The serum concentration of IL1- $\beta$  was significantly lower in HT female Hp mice relative to HT male Hp mice and the serum concentration of MCP-1 was significantly lower in WT male Hp mice relative to WT male mice. The serum IL1- $\beta$  and MCP-1 findings were not consistent with the increased gastric corpus and antral pathology observed in HT female Hp and WT male Hp mice, respectively (Supplementary Material). No significant differences were observed in the serum concentration of IL-10 and IFN- $\gamma$  when comparing the different groups of interest; however, the serum concentration of IL-17A was significantly higher in HT male mice relative to HT female mice ( $p < 0.01$ ; data not shown) and the serum concentration of IL-6 was increased in WT female Hp mice relative to WT male

Hp mice ( $p = 0.06$ ; data not shown). There were no significant differences in the serum concentration of IL-1 $\beta$ , MCP-1, IL-10, IFN- $\gamma$ , IL-17A, and IL-6 between HT male and HT male Hp mice and between HT female and HT female Hp mice.

#### Discussion

Previous studies in humans infected with HCV suggested that *H. pylori* may play a role in the pathogenesis and progression of HCC. Direct and indirect mechanisms of helicobacter-induced liver damage involving toxin(s) and induction of pro-inflammatory cytokines, respectively, have been suggested.<sup>16</sup> The present study, utilizing C3B6F1-Tg(Alb1-HCVN)35Sml mice, characterized by hepatic steatosis in transgenic female mice, demonstrated that *H. pylori* colonized the stomach, but not the liver, and provides indirect evidence that *H. pylori* does not promote HCC during HCV infection in humans. However, we cannot exclude the possibility that *H. pylori* or "*H. pylori*-like" bacteria colonize the human liver during severe liver disease or cirrhosis as suggested by others.<sup>12,13,17,30-32</sup> Hepatic *H. pylori* colonization and pathology

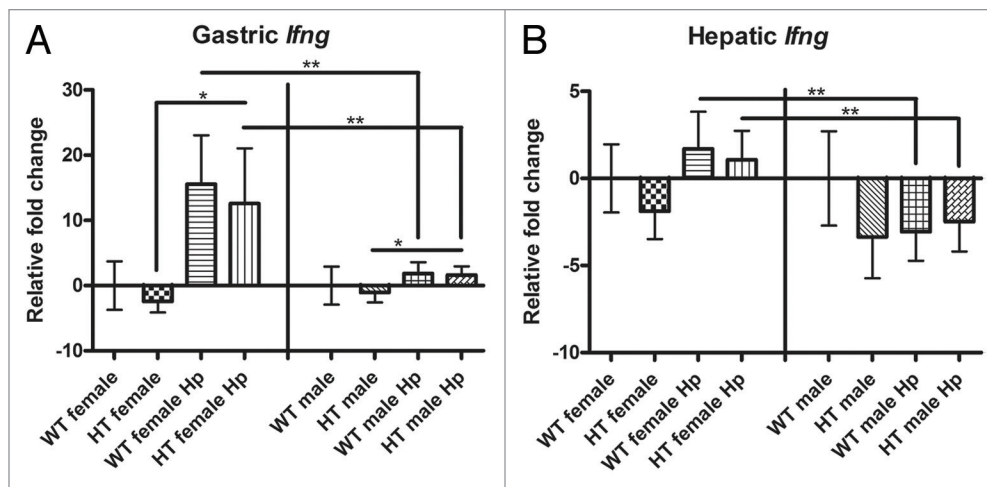


**Figure 5.** (A) Total histopathology index score of the antrum in the different groups of mice (\*,  $p < 0.05$ ). (B) Relatively normal gastric antrum of a hepatitis C virus transgenic male mouse infected with *H. pylori* (HT male Hp). (C) Mucosal hyperplasia with mild dysplasia in the gastric antrum of a wild-type male mouse infected with *H. pylori* (WT male Hp). (D) Polypoid lesion in the gastric antrum of a wild-type male mouse infected with *H. pylori* (WT male Hp). (B and C) bar size 100  $\mu\text{m}$ . (D) bar size 500  $\mu\text{m}$ .

may also depend on the immune response of the human host and/or *H. pylori* virulence factors such as CagA or VacA.<sup>10,33,34</sup> Since *H. pylori* SS1, used in the current study, appears to have a non-functional *cag* pathogenicity island, additional studies are needed in order to investigate the role of the *H. pylori* *cag* pathogenicity island in liver cancer.<sup>35</sup> It is important to note that enterohepatic *Helicobacter* spp. (EHS) may be involved in hepatobiliary diseases of humans.<sup>15,36-39</sup> Although *H. hepaticus* was not detected in the stools of HCV or hepatitis B virus patients with HCC, a previous study from our laboratory indicated that *H. hepaticus* promoted liver cancer in B6C3F1-Tg(Alb1-HCVN)35Sml mice and also promoted aflatoxin-induced liver cancer in C3H/HeNcr mice with persistent intestinal colonization of *H. hepaticus*, despite the lack of the organism in the liver.<sup>22,40</sup> The occurrence of HCCs in *H. pylori*-infected and uninfected HT and WT mice in our study is consistent with the natural susceptibility of aged B6C3F1 male mice to liver tumors.<sup>41</sup>

Hepatic steatosis is frequently observed in humans with chronic HCV infection and may increase the risk of HCC through increased oxidative stress or by promoting fibrosis.<sup>42</sup> The C3B6F1-Tg(Alb1-HCVN)35Sml mouse model used in the current study was characterized by increased hepatic steatosis

in female mice relative to WT female mice and no significant development of liver tumors. In contrast, its parental transgenic strain, the C57BL/6-Tg(Alb1-HCVN)35Sml (FL-N/35) mouse model, was characterized by hepatic steatosis and liver cancer in male mice.<sup>24,25</sup> This difference may be due in part to the mixed genetic background in our model since another HCV transgenic mouse model on B6C3F1 and B6C3F2 genetic backgrounds was characterized by more females than males developing hepatic steatosis.<sup>43</sup> In addition, subsequent generations of FL-N/35 mice no longer developed spontaneous liver tumors.<sup>27</sup> The tumor promoting effects of the HCV transgene depend on the host genetic background.<sup>44</sup> In our study, however, the HCV transgene protected from hepatic steatosis in *H. pylori*-infected male mice and this decrease in steatosis correlated with a significant increase in serum levels of TNF- $\alpha$ . Our findings are in contrast with human data in which chronic HCV patients with hepatic steatosis (and unknown *H. pylori* infection status) have increased serum levels of TNF- $\alpha$ .<sup>45</sup> However, TNF- $\alpha$ / $\beta$  deficient mice (C57BL/6J background) develop hepatomegaly and hepatic steatosis suggesting a role of TNF in control of lipid homeostasis.<sup>46</sup> The role of *H. pylori* in hepatic steatosis associated with HCV in humans has been reported.<sup>82</sup>



**Figure 6.** Gastric and hepatic gene expression in the different groups of mice: (A) Gastric interferon gamma (*Ifng*). (B) Hepatic *Ifng*. \*,  $p < 0.05$ ; \*\*,  $p < 0.01$ .

We observed that *H. pylori* infection induced increased gastric corpus pathology in C3B6F1 (WT and HT) female mice relative to male mice. This is consistent with previous studies utilizing C57BL/6J and C57BL/6 *gpt* delta mouse models which have documented that female mice develop more severe gastric lesions from *H. felis* and *H. pylori* infection, respectively.<sup>47,48</sup> *H. pylori*-infected female 129/Sv mice also showed increased gastric lesions compared with *H. pylori*-infected male 129/Sv mice.<sup>49</sup> Furthermore, HT male mice were protected from *H. pylori*-induced corpus lesions. In the gastric antrum, *H. pylori* induced gastric lesions in WT male and female mice. However, HT male and female mice were protected from *H. pylori*-induced gastric antral lesions and more antral lesions were observed in WT male Hp than in HT male Hp mice. These findings suggest that the HCV transgene protected the mice against gastric corpus and antral lesions during *H. pylori* infection. However, studies in humans with HCV-associated chronic hepatitis did not find a correlation between liver inflammation and gastric mucosal lesions.<sup>50</sup>

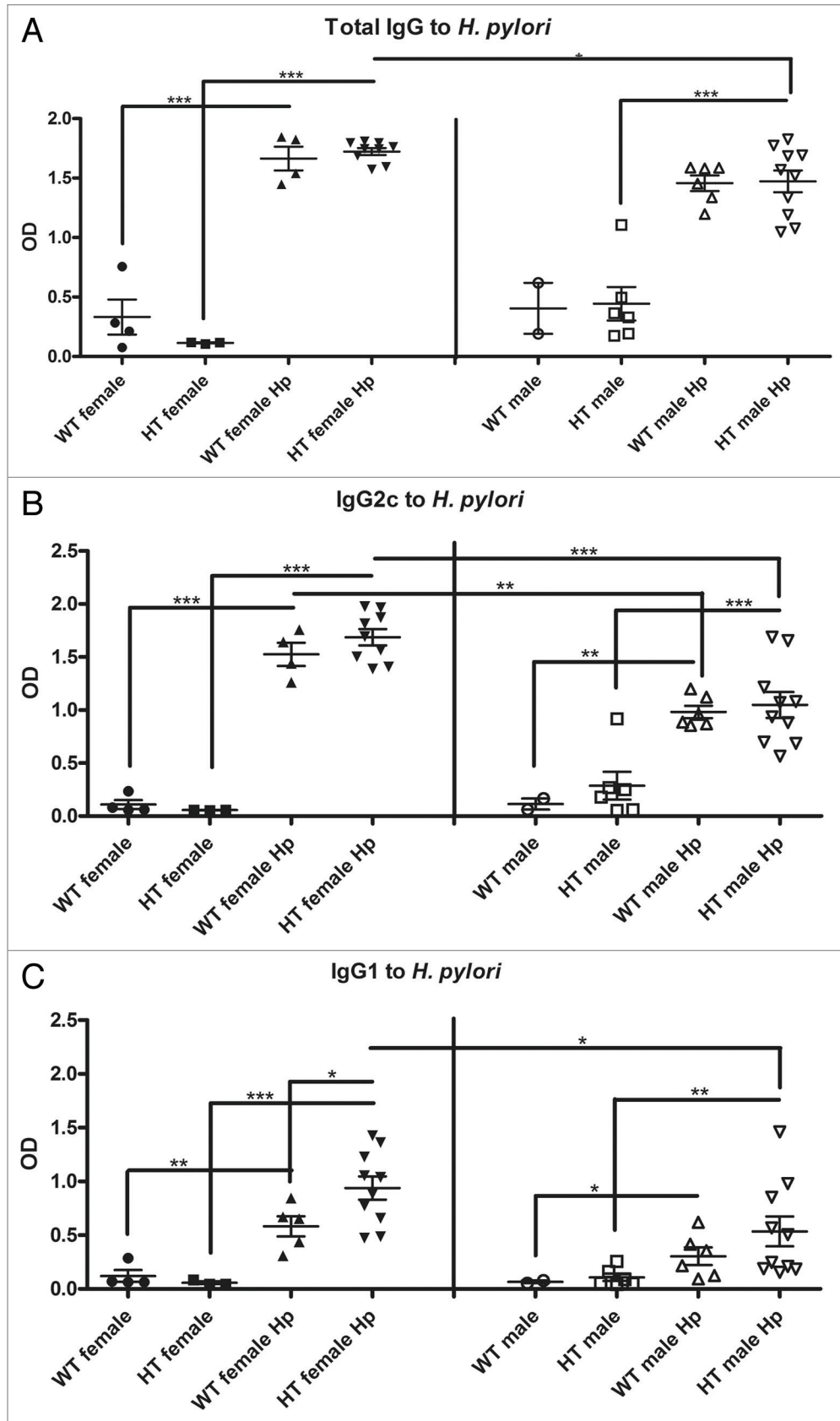
Dysplasia in the gastric antrum has been observed in trefoil factor family 2 knockout male and female mice (on a mixed B6129Sv background) infected with *H. pylori* and this finding correlated with upregulation of gastric *Ifng*.<sup>51</sup> During *H. pylori* infection the expression of *Ifng* was significantly higher in the stomach and liver of female mice relative to male mice. Similarly, in Mongolian gerbils infected with *H. pylori* SS1, the gastric *Ifng* expression was higher in females than in males at 36 weeks post-infection and females developed more gastric antral and corpus pathology than males at 36 weeks post-infection.<sup>52</sup> In our study, the upregulation of *Ifng* in the stomach was associated with a significant increase in Th1 immune response against *H. pylori*, an increase in gastric corpus pathology, and a decrease in *H. pylori* colonization levels. These findings are consistent with the absence of *H. pylori* in the stomach of three female mice originally inoculated with *H. pylori*. Interferon gamma mediates inflammation during *H. pylori* infection with a subsequent reduction in *H. pylori* colonization.<sup>53</sup> Increased gastric pathology in female C57BL/6 *gpt* delta mice at 6 mo postinfection has been associated with decreased

*H. pylori* colonization levels.<sup>48</sup> *H. pylori* also modulates the hepatic expression of innate immunity-, inflammation-, and fibrogenesis-associated genes in female C57BL/6 mice.<sup>54</sup> Increased hepatic expression of *Ifng* in female mice in our study may contribute to the activation of NF- $\kappa$ B with a resulting pro-inflammatory response.<sup>54,55</sup>

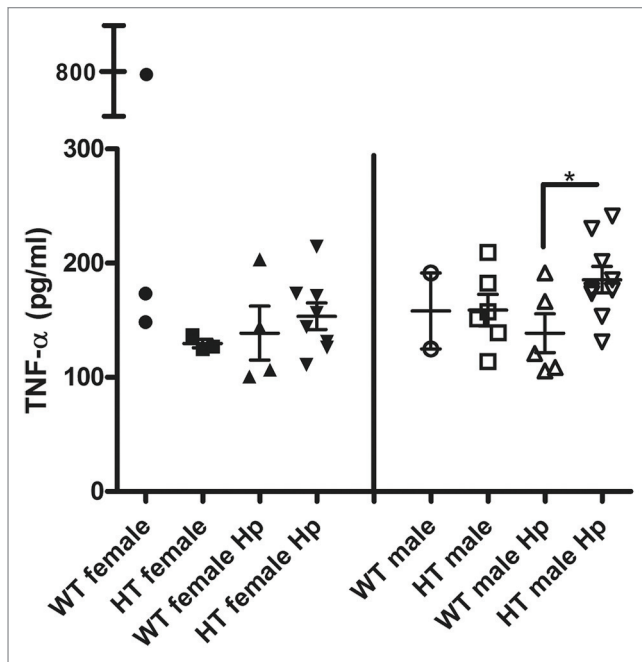
A potential beneficial effect of *H. pylori* infection has been documented in humans with HCV. Increased levels of iron in the body may contribute to HCC development.<sup>56</sup> *H. pylori*-positive HCV patients have lower levels of hepatic iron deposits than *H. pylori*-negative HCV patients.<sup>57</sup> This finding may be consistent with the association of *H. pylori* infection and iron deficiency anemia.<sup>58</sup> Interestingly, an excess-iron diet increased the risk of HCC in FL-N/35 mice whereas an iron-depleted diet increased the risk of gastric cancer in a gerbil model of *H. pylori* infection.<sup>27,59</sup> In addition, chronic *H. felis* infection induced iron deficiency in hypergastrinemic INS-GAS mice.<sup>60</sup> Whether *H. pylori* infection can decrease the risk of HCC in FL-N/35 mice fed a high iron diet requires additional studies.

*H. pylori* infection may prolong viral infection by decreasing the cytotoxic T cell response.<sup>61</sup> Recent studies also suggest that the DNA of *H. pylori* includes a high ratio of immunoregulatory to immunostimulatory sequences that are associated with decreased type I interferon (IFN- $\alpha$ ) and amelioration of colitis.<sup>62,63</sup> However, in the context of HCV infection, decreased IFN- $\alpha$  signaling could promote chronic infection by decreasing the host immune response against the virus.<sup>64,65</sup> *Ifnb1*, the type I interferon gene encoding IFN- $\beta$ , induces degradation of HCV proteins through autophagy.<sup>66</sup> In addition, through nucleotide-binding oligomerization domain 1, *Ifnb1* also partially protects the infected host by decreasing *H. pylori* gastric colonization.<sup>67</sup> We observed significant upregulation of hepatic *Ifnb1* in HT female Hp mice relative to WT female Hp mice suggesting that the HCV transgene contributed to a type I interferon response during *H. pylori* infection. Since the transgenic HCV mouse model used in the current study does not involve viral infection, it is not possible to determine the effect of *H. pylori* infection on HCV replication. The high prevalence of





**Figure 7.** Serological immune response against *H. pylori* in the different groups of mice: (A) Total IgG. (B) Th1 (IgG2c). (C) Th2 (IgG1). OD, optical density. \*,  $p \leq 0.05$ ; \*\*,  $p \leq 0.01$ ; \*\*\*,  $p \leq 0.001$ .



**Figure 8.** Serum concentration of tumor necrosis factor  $\alpha$  (TNF- $\alpha$ ) in the different groups of mice. \*,  $p < 0.05$ .

*H. pylori* in humans justifies the need for future studies designed to investigate if *H. pylori* and/or its DNA affect HCV replication in animal models. Mouse models with “humanized” livers and HCV infection may provide novel opportunities for delineating the effect of *H. pylori* on liver disease and cancer in humans.<sup>68</sup>

In summary, utilizing a transgenic mouse model of HCV pathogenesis and hepatic steatosis, we ascertained that *H. pylori* was not able to colonize the liver nor did the organism promote liver cancer. In contrast, previous studies using B6AF1 and AB6F1, B6C3F1-Tg(Alb1-HCVN)35Sml, and C3;B6 mice infected with the prototype EHS, *H. hepaticus*, demonstrated that this bacterium induced persistent hepatitis and/or liver tumors.<sup>22,69,70</sup> Our results therefore would argue that EHS rather than *H. pylori*, are more likely to be associated with human hepatobiliary diseases including cancer. Our experimental evidence is supported by studies in which EHS have been identified in humans with inflammatory and neoplastic conditions including cholecystitis, bile duct and gallbladder cancer as well as by studies suggesting the presence of non-*H. pylori* helicobacters in patients with hepatobiliary diseases including primary biliary cirrhosis and primary sclerosing cholangitis.<sup>15,36-39</sup> Future studies in humans with HCV and other hepatobiliary diseases should attempt to culture helicobacters not only in liver, but also the gastrointestinal tract, and compare isolates by detailed molecular techniques to ascertain their taxonomic classifications.<sup>15,17</sup>

## Materials and Methods

### Mice and experimental design

FL-N/35 mice [C57BL/6-Tg(Alb1-HCVN)35Sml; Mouse Genome Informatics ID: MGI:3513779] were obtained from

Stanley M. Lemon (University of North Carolina School of Medicine). Male C57BL/6-Tg(Alb1-HCVN)35Sml mice were bred to C3H/HeNTac female mice and the C3B6F1-Tg(Alb1-HCVN)35Sml [HCV transgenic (HT)] and C3B6F1 wild-type (WT) progeny were used for the current study. C3B6F1 mice were genotyped for the transgene using primers HCV-F (5'-CAACCCTACGTACAGCTG-3') and HCV-R (5'-GGTAGTCAACCATGCACC-3') and the following polymerase chain reaction (PCR) conditions: 1 cycle of 94 °C for 5 min, 35 cycles of 94 °C for 75 s, 52 °C for 75 s and 72 °C for 75 s, and a final cycle of 72 °C for 10 min. Prior to experimental inoculation, C3B6F1 mice were determined to be free of *Helicobacter* spp. by PCR of their fecal DNA using primers that detect *Helicobacter* spp.<sup>15,71</sup> Specific pathogen-free WT and HT mice were assigned to groups based on genotype and gender and were orally-inoculated every day for three days with 0.2 mL of media only (sham) or media with *H. pylori* SS1 ( $\sim 2 \times 10^8$  organisms per inoculum) at  $\sim 2$  mo of age. The sham inoculated groups are designated: WT female, HT female, WT male, and HT male. The *H. pylori*-inoculated groups are designated: WT female Hp, HT female Hp, WT male Hp, and HT male Hp (Table 2). Mice were housed in Association for Assessment and Accreditation of Laboratory Animal Care International-accredited facilities and fed ProLab® RMH 3000 (LabDiet®, 5P00) and provided reverse osmosis water ad libitum. Mice were euthanized at  $\sim 12$  mo postinoculation. This study was approved by the Massachusetts Institute of Technology Committee on Animal Care.

### Histopathological evaluation

Immediately following euthanasia by CO<sub>2</sub> inhalation, mouse body weight was recorded and blood was collected by cardiac puncture. A complete necropsy was performed and liver weight was measured. The stomach was collected and incised along the line of the greater gastric curvature, luminal contents were removed, and the mucosa was rinsed with sterile PBS. For histopathologic evaluation, linear strips extending from the gastric squamocolumnar junction to the proximal duodenum were taken along the lesser curvature. The liver was separated into left, right, median, and caudate lobes, and a section from each was collected. Stomach and liver sections were fixed in 10% neutral-buffered formalin and were routinely processed for histology. Sections of stomach and liver were also frozen in liquid nitrogen and stored at  $\sim -80^{\circ}\text{C}$  for RNA extraction and at  $\leq -20^{\circ}\text{C}$  for DNA extraction. Sera were also stored at  $\leq -20^{\circ}\text{C}$ .

Five-micrometer-thick sections were stained with hematoxylin and eosin (H&E) for histopathological evaluation by a board-certified veterinary pathologist (N.M.A.P.) blinded to treatment groups. H&E-stained stomach sections were scored for corpus and antral pathology. Lesions in the corpus were scored according to a previously described scoring system, on an ascending scale of 0 to 4 for inflammation, epithelial defects, hyperplasia, dysplasia, atrophy and intestinal metaplasia; a GHAI was calculated as the sum of the scores for all parameters listed.<sup>72</sup> Lesions in the antrum were scored based on a modification of the system used for lower intestinal lesions. The degree and frequency of epithelial defects, inflammation, and hyperplasia were scored on a scale of 0 to 4 with ascending severity (0, none; 1, minimal;

2, mild; 3, moderate; and 4, severe); epithelial dysplasia was also graded using a scale of 0 to 4 (0, normal; 1, mild dysplastic changes; 2, adenoma or low-grade dysplasia; 3, high grade dysplasia or carcinoma in situ; and 4, invasive carcinoma).<sup>51,73</sup> A total histopathology index for the antrum was generated by adding the scores for inflammation, epithelial defects, hyperplasia, and dysplasia. For the liver, a hepatitis index was calculated by combining individual scores for lobular, portal and interface hepatitis, as well as the number of lobes (out of a total of 4) that contained 5 or more inflammatory lesions.<sup>74</sup> Hepatitis was defined by a hepatitis index equal to, or greater than, 4.<sup>74</sup> Liver sections were also scored for hepatic steatosis, preneoplastic (AHF and LFCA) and neoplastic (HCA and HCC) lesions.<sup>75,76</sup> Images were obtained using an Olympus DP25 (Olympus Corporation, Tokyo, Japan) digital camera. Adobe® Photoshop® CS4 and CS6 were used for image processing. Automatic contrast and automatic color correction were performed for each image. Additionally, the eraser tool was used to remove extraneous, artifactually sloughed material in the intestinal lumen.

#### FISH for *H. pylori*

Formalin-fixed paraffin-embedded stomach sections were deparaffinized and rehydrated. After drying, pre-heated (74.5°C for 10 min) hybridization buffer consisting of 0.9 M NaCl, 20 mM Tris-HCL (pH 7.2), 0.1% SDS, 30% formamide, and including 10 ng/μl of the Hpy-1-Cy3 probe<sup>77</sup> was applied on the tissue sections and these were then covered with parafilm. The tissue sections were incubated in a chamber overnight at 48°C. The next day, sections were rinsed with double-distilled water and then washed 15 min (covered to prevent light exposure) with a pre-heated washing buffer consisting of 0.9 M NaCl, 20 mM Tris-HCL (pH 7.2), and 0.01% SDS. A second 15 min (covered) wash was then performed using another pre-heated washing buffer consisting of 0.9 M NaCl and 20 mM Tris-HCL (pH 7.2). Finally, the sections were rinsed with double-distilled water and dried. Vectashield® Mounting Medium with DAPI (Vector Laboratories, H-1500) was then applied to the sections followed by placement of coverslips. Sections were examined with a Zeiss Axioskop 2 Plus microscope with filters for DAPI, rhodamine, and FITC and photos were obtained with a QIClick™ digital CCD camera (QImaging, Surrey, BC Canada). Two slides per *H. pylori*-infected group and one slide from an uninfected sham group (WT female) were analyzed for fluorescent organisms in a blinded fashion by a board-certified veterinary pathologist (N.M.A.P.).

#### *H. pylori* colonization

DNA was extracted from a longitudinal section of stomach incorporating antrum and corpus and from a section of the left liver lobe using High Pure PCR Template Preparation Kit (Roche). qPCR for *H. pylori* was utilized to assess *H. pylori* colonization as previously described.<sup>78</sup> The following primers were used: 5'-CAAATCGCTGGCATTGGT-3' (forward) and 5'-CTTACCAGGCTAAGGCTTCA-3' (reverse).<sup>78</sup> The internal probe was: 5'-AACAAAGACATGCAAGATGGCGTTAAAAACA-3'.<sup>78</sup>

#### Gene expression analyses

RNA was extracted from a longitudinal section of stomach incorporating antrum and corpus and from the left liver lobe

using TRIzol® Reagent (Invitrogen-Life Technologies). Five micrograms of RNA were converted to cDNA using High Capacity cDNA Reverse Transcription Kit (Applied Biosystems). Gastric and hepatic gene expression of Tumor necrosis factor (*Tnf*) (Assay ID: Mm99999068\_m1), Interferon gamma (*Ifng*) (Assay ID: Mm01168134\_m1), Interferon β 1, fibroblast (*Ifnb1*)<sup>66</sup> (Assay ID: Mm00439546\_s1), Interleukin 10 (*Il10*) (Assay ID: Mm01288386\_m1) were measured using commercial TaqMan® primer-probe sets (Applied Biosystems-Life Technologies). The volume of each reaction consisted of 5 μl of sample cDNA, 4 μl of UltraPure™ Distilled Water (Gibco-Life Technologies, 10977), 1 μl of primer-probe, and 10 μl of TaqMan® Fast Universal PCR Master Mix (2×) (Applied Biosystems, 4352042). Duplicate reactions were performed for each sample. Gene expression was determined using the comparative C<sub>T</sub> method relative to Glyceraldehyde-3-phosphate dehydrogenase (*Gapdh*) (Applied Biosystems, 4352661), per PE Applied Biosystems' User Bulletin # 2 ABI PRISM 7700 Sequence Detection System and using uninfected WT male and WT female mice as controls for male and female mice, respectively.

#### Enzyme-linked immunosorbent assay (ELISA) for serum antibody to *H. pylori* antigens

Serum IgG, Th1-associated IgG2c, and Th2-associated IgG1 responses to outer membrane antigens of *H. pylori* were measured by ELISA as previously described.<sup>79,80</sup> Antigen was coated on Immulon II plates (Thermo Fisher Scientific, 3455) at a concentration of 1 μg/ml (IgG) or 10 μg/ml (isotypes) with sera diluted 1:100. Biotinylated secondary antibodies included goat anti-mouse IgG (Southern Biotech, 1030-03) and monoclonal anti-mouse antibodies produced by clones A85-1 and 5.7 (BD Biosciences, 553441 and 553504, respectively) for detecting IgG1 and IgG2c, respectively. Incubation with ExtrAvidin®-Peroxidase (Sigma, E2886) was followed by 2, 2'-azinobis (3-ethylbenzthiazoline-6-sulfonic acid) diammonium salt (ABTS®) substrate (Kirkegaard and Perry Laboratories, 50-65-02 and 50-64-02) for color development. Absorbance (optical density) development at 405/590 nm was recorded by an ELISA plate reader (Dynatech MR7000, Dynatech Laboratories, Inc.).

#### Serum cytokine assay (xMAP® Technology-Luminex)

Serum samples were analyzed using the Bio-Plex Pro™ Mouse Cytokine Th17 Panel A 6-Plex Group I (BIO-RAD, M60-00007NY) which included IL-1β, IL-10, IL-6, IL-17A, IFN-γ, and TNF-α. In addition, MCP-1 (MCAF; BIO-RAD, 171-G5019M) was added to the assay following the manufacturer's recommendations. Most samples were analyzed in duplicate. Four serum samples were not analyzed (samples were hemolyzed).

#### Statistical analyses

Statistical analyses were performed using GraphPad Prism version 5.03 for Windows (GraphPad Software). The combined multiplicity of hepatic preneoplasia and neoplasia was defined as the number of preneoplastic (AHF and LFCA) and neoplastic (HCA and HCC) microscopic liver lesions in each group divided by the total number of mice in the group.<sup>76,81</sup> The Mann Whitney test (2 tailed) was used for analyses of corpus' GHAI and antrum's

total histopathology index, hepatic steatosis, multiplicity of microscopic preneoplastic and neoplastic liver lesions, and hepatitis index scores. The Fisher's exact test was used to compare the multiplicity of microscopic preneoplastic and/or neoplastic liver lesions and the numbers of mice with microscopic preneoplastic and/or neoplastic liver lesions between the groups of mice. The unpaired T test (2 tailed) was used for analyses of liver weight, liver to body weight ratio (%), number of liver lobes with dysplasia and neoplasia, multiplicity of microscopic preneoplastic and neoplastic liver lesions, *H. pylori* colonization, and gene expression. The unpaired T test (2 tailed) with Welch correction was used to analyze the serological immune response against *H. pylori*. Results are shown as median with interquartile range (for hepatic steatosis, corpus' GHAI, antrum's total histopathology index, and hepatitis index), mean  $\pm$  standard error of the mean [for liver to body weight ratio (%), multiplicity, colonization, serological immune response against *H. pylori*, and serum cytokine concentrations], or

as mean  $\pm$  standard deviation (for gene expression). Results were considered significant if  $p \leq 0.05$ .

#### Disclosure of Potential Conflicts of Interest

No potential conflict of interest was disclosed.

#### Supplemental Materials

All supplemental materials may be found here: [www.landesbioscience.com/journals/gutmicrobes/article/26042](http://www.landesbioscience.com/journals/gutmicrobes/article/26042)

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