



Helicobacter pylori Induced Gastric Immunopathology Is Associated with Distinct Microbiota Changes in the Large Intestines of Long-Term Infected Mongolian Gerbils

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Abstract

Background: Gastrointestinal (GI) inflammation in mice and men are frequently accompanied by distinct changes of the GI microbiota composition at sites of inflammation. *Helicobacter (H.) pylori* infection results in gastric immunopathology accompanied by colonization of stomachs with bacterial species, which are usually restricted to the lower intestine. Potential microbiota shifts distal to the inflammatory process following long-term *H. pylori* infection, however, have not been studied so far.

Methodology/Principal Findings: For the first time, we investigated microbiota changes along the entire GI tract of Mongolian gerbils after 14 months of infection with *H. pylori* B8 wildtype (WT) or its isogenic $\Delta cagY$ mutant (MUT) strain which is defective in the type IV secretion system and thus unable to modulate specific host pathways. Comprehensive cultural analyses revealed that severe gastric diseases such as atrophic pangastritis and precancerous transformations were accompanied by elevated luminal loads of *E. coli* and enterococci in the caecum and together with *Bacteroides/Prevotella* spp. in the colon of *H. pylori* WT, but not MUT infected gerbils as compared to naïve animals. Strikingly, molecular analyses revealed that *Akkermansia*, an uncultivable species involved in mucus degradation, was exclusively abundant in large intestines of *H. pylori* WT, but not MUT infected nor naïve gerbils.

Conclusion/Significance: Taken together, long-term infection of Mongolian gerbils with a *H. pylori* WT strain displaying an intact type IV secretion system leads to distinct shifts of the microbiota composition in the distal uninflamed, but not proximal inflamed GI tract. Hence, *H. pylori* induced immunopathogenesis of the stomach, including hypochlorhydria and hypergastrinemia, might trigger large intestinal microbiota changes whereas the exact underlying mechanisms need to be further unraveled.

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Introduction

Chronic *Helicobacter (H.) pylori* infection represents a significant health burden affecting approximately half of the world's population [1]. Even though the vast majority of infected individuals remains asymptomatic or displays rather minor unspecific symptoms, the pathogen can cause chronic gastritis, peptic ulcer, gastric adenocarcinoma or mucosa-associated lymphoid tissue lymphoma [2,3]. As a consequence of epidemiological studies, in 1994 the WHO has declared *H. pylori* a class I carcinogen [4]. *H. pylori* produce a plethora of virulence factors inducing gastric immunopathology. Among these, the vacuolating

cytotoxin A (VacA) and cytotoxin-associated antigen A (CagA) have been studied intensively [5,6,7,8,9].

Strains carrying a *cag* pathogenicity island (*cagPAI*) are able to translocate the effector protein CagA via the type IV secretion system (T4SS) into the host cell. CagA gets tyrosine phosphorylated by host kinases and interferes with the signal transduction pathways regulating cell polarity, inflammation, proliferation, and apoptosis [5,10]. The *H. pylori* mutant $\Delta cagY$ is defective in the T4SS, thus representing a less virulent strain, which lacks the ability to induce a corpus dominant atrophic gastritis as confirmed in our previous Mongolian gerbil study [9]. As compared to mice, the Mongolian gerbil infection model has been proven to better

mimic key features of pathophysiology and long-term sequelae in *H. pylori* infected humans (reviewed in [11,12,13,14]). Following *H. pylori* infection of gerbils, like in humans the gastric inflammatory process starts in the antrum mucosa and further expands to the corpus whereas in infected mice the antrum is rarely involved. Importantly, *H. pylori* type I strains expressing CagA, VacA and a functional type IV secretion system only persist in a Mongolian gerbil, but not murine stomach in a genetically stable fashion. In addition, infected gerbils develop severe gastric lesions such as peptic ulcer and gastric adenocarcinoma [11,12,13,14].

Recent reports have highlighted that *H. pylori* infection is associated with changes in the gastric microenvironment, which in turn affects the gastric microbiota composition. In our own study, for instance, we have shown that following *H. pylori* infection bacterial species usually restricted to the lower intestinal tract (such as *Bacteroides/Prevotella* spp. and clostridia, among others) were present in stomach samples of *H. pylori* infected mice, whereas *Lactobacillus* spp. were predominant in control animals [15]. Furthermore, in a previous report Mongolian gerbils with gastritis and duodenitis harbored significantly higher *Bacteroides* spp. numbers at sites of inflammation 12 weeks following *H. pylori* infection [16].

So far, microbiota changes in *H. pylori* infected Mongolian gerbils have only been investigated at sites of inflammation itself [16,17,18]. Therefore, for the first time we investigated potential changes of the microbiota composition distal to the inflammatory process, particularly in long-term *H. pylori* infection. Results represent a comprehensive survey of the microbiota composition within the entire gastrointestinal (GI) tract of Mongolian gerbils 14 months following *H. pylori* wildtype infection as compared to its isogenic Δ cagY mutant strain applying cultural and molecular methods.

Results

Inflammatory Responses in the Gastrointestinal Tract of Mongolian Gerbils Following Long-term *H. pylori* Infection

Acute and chronic GI inflammation is accompanied by shifts within the intestinal microbiota composition at sites of inflammation. In the presented study, we were interested whether long-term *H. pylori* infection results in luminal microbiota changes at sites of gastric inflammation or beyond in the distal GI tract. To address this question Mongolian gerbils were orally infected with *H. pylori* B8 wildtype (WT) or its isogenic B8 Δ cagY mutant (MUT) strain. Fourteen months thereafter, either *H. pylori* WT and MUT strain could be re-isolated at comparable loads from the stomach in 66.7% and 100% of infected Mongolian gerbils, respectively. In *H. pylori* WT and MUT infected animals, gastric pathology was characterized by lymphoid aggregates in the antrum (100% with either strain) and corpus (100% and 33.3%, respectively), erosions (100% and 83.3%, respectively), ulcers (75.0% and 0%, respectively), parietal cell atrophy (100% and 33.3%, respectively), and metaplastic changes (90.9% and 33.3%, respectively), gastritis cystica profunda (75.0% and 33.3%, respectively), and focal dysplasia (25.0% and 0%, respectively) (**Fig. 1, Table 1**). After the establishment of a severe pangastritis in *H. pylori* WT strain infected animals, gerbils developed significant increases in pH values from 1.5 to 4 (hypochlorhydria) and plasma gastrin concentrations (hypergastrinemia) (refer to [9]).

Of note, significant histopathological signs of inflammation could not be observed in hematoxylin and eosin (H&E) stained paraffin sections derived from the small and large intestinal tract of infected animals (not shown). To further assess quantitative pro-

inflammatory immune cell responses in the colonic mucosa of infected Mongolian gerbils, we performed *in situ* immunohistochemical stainings of colonic paraffin sections with an antibody against CD3 to visualize T lymphocytes. *H. pylori* WT infected gerbils displayed significantly higher numbers of T lymphocytes in their colonic mucosa as compared to MUT infected or naïve animals 14 months post infection (p.i.) (**Fig. 2**).

Distinct Microbiota Changes in the Large, but not small Intestinal Tract of Mongolian Gerbils Following Long-term *H. pylori* Infection

We next performed a comprehensive cultural survey of the microbiota composition in the entire GI tract of long-term *H. pylori* infected Mongolian gerbils. Fourteen months following *H. pylori* WT or MUT infection, the total bacterial load increased by almost two orders of magnitude in the stomach lumen due to increased numbers of *Lactobacillus* spp. as compared to uninfected controls, but only reached statistical significance in the *H. pylori* WT group ($p < 0.01$) (**Fig. 3**). Interestingly, only in 30–40% of naïve gerbils Gram-negative species such as *E. coli*, *Proteus* sp. and *Bacteroides/Prevotella* spp. could be cultured from the gastric lumen (with a median of $< 10^6$ CFU per g for either bacterial species). During 14 months of long-term infection with the *H. pylori* WT or MUT strain, however, a trend towards higher gastric Gram-negative commensal loads was observed (approximately four and two orders of magnitude, respectively; not significant, n.s.) when compared to uninfected gerbils (**Fig. 3**).

In proximal parts of the small intestine such as duodenum (**Fig. 4**) and jejunum (**Fig. 5**) no differences in the luminal microbiota composition of infected versus non-infected gerbils could be observed. In the ileal lumen of *H. pylori* WT infected animals, however, lower *Lactobacillus* spp. numbers (approximately 1.5 orders of magnitude; $p < 0.05$) were detected as compared to naïve or with the *H. pylori* MUT strain infected gerbils at 14 months p.i. (**Fig. 6**).

Strikingly, in the distal compartment of the GI tract, such as the caecum (**Fig. 7**) and colon (**Fig. 8**), *H. pylori* WT, but not MUT strain infected gerbils harbored significantly higher *E. coli* and *Enterococcus* spp. loads (1.0–1.5 orders of magnitude; $p < 0.01$) 14 months following infection when compared to naïve animals. In addition, numbers of *Bacteroides/Prevotella* spp. were up to 100 times higher in colonic lumen of *H. pylori* WT infected animals as compared to naïve controls (**Fig. 8E**). Notably, no significant differences in microbiota composition could be observed in Mongolian gerbils suffering from distinct gastric immunopathology 14 months following infection, irrespective whether *H. pylori* WT strain could be re-isolated or not at necropsy (not shown).

We finally applied molecular methods for detection of fastidious or uncultivable bacterial species in the entire GI tract. Genetic fingerprints generated by PCR-based denaturing gradient gel electrophoresis (DGGE) and subsequent sequence analyses revealed that most prominent bacterial DNA bands occurring in caecal and colonic specimens 14 months following *H. pylori* WT, but not MUT strain infection referred to enterobacteria (**Fig. 9**). Thereby the increased abundance of commensal *E. coli* upon long-term *H. pylori* WT infection as detected by culture could be independently confirmed. Strikingly, *Akkermansia*, an uncultivable species involved in mucus degradation, could exclusively be detected in the caecal and colonic lumen of long-term *H. pylori* WT, but neither MUT infected (not shown) nor naïve gerbils (**Fig. 9**). Interestingly, genetic fingerprints from luminal samples taken from either stomach, duodenum, jejunum or ileum were comparable in *H. pylori* WT, MUT strain infected and naïve gerbils, respectively (not shown), further supporting results derived

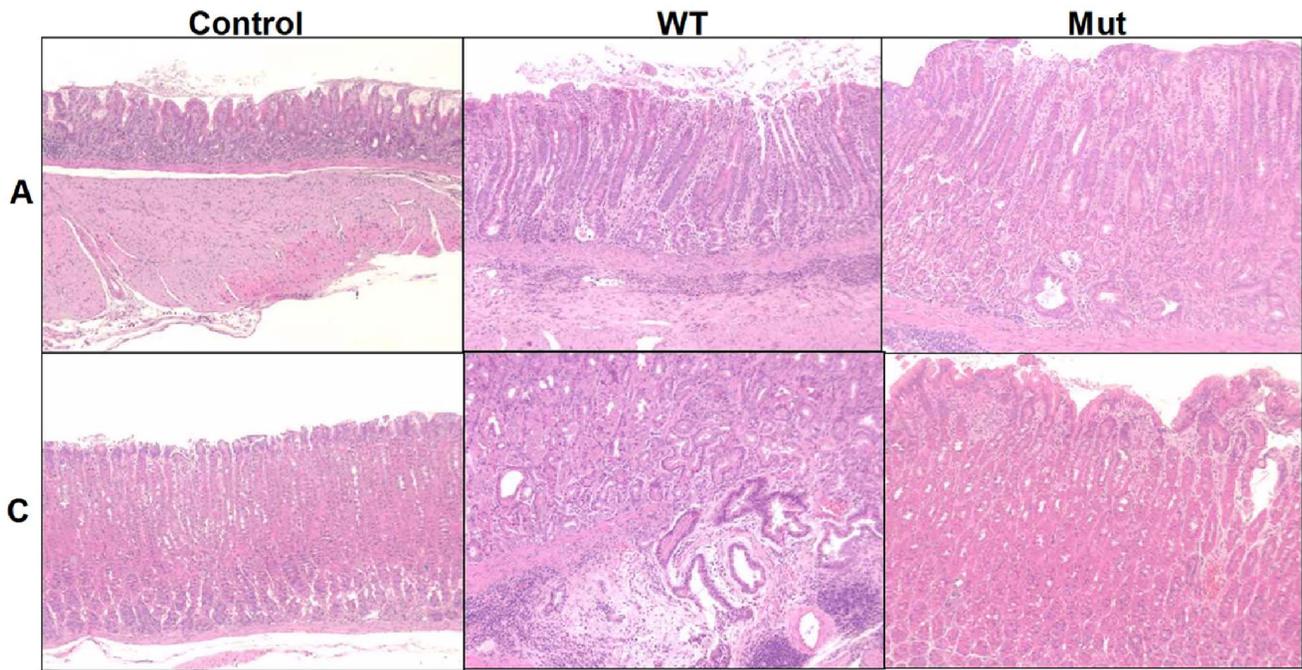


Figure 1. Gastric histopathology in Mongolian gerbils 14 months following *H. pylori* infection. Mongolian gerbils were infected with *H. pylori* wildtype strain B8 (WT; middle panels) or *H. pylori* mutant strain lacking *cagY* (Mut; right panels) as described in Methods. Fourteen months following infection, stomach biopsies separated in antrum (A) and corpus (C) mucosa were taken and paraffin sections stained with H&E. Uninfected age-matched animals served as negative controls (Control; left panels). Representative photomicrographs are shown (100 x magnification). doi:10.1371/journal.pone.0100362.g001

from culture. Taken together, long-term *H. pylori* WT infection of Mongolian gerbils leads to distinct quantitative as well as qualitative changes of the microbiota composition within the uninfamed distal, but not proximal inflamed GI tract.

Discussion

In the study presented here we performed a comprehensive survey of microbiota changes along the entire GI tract in Mongolian gerbils following long-term infection with *H. pylori* for 14 months comparing B8 wildtype with its isogenic *cagY* mutant strain defective in the type IV secretion system. Most notably, we for the first time extended the microbiota analyses to locations distal “classical” sites of inflammation (such as stomach and

duodenum) to the lower small and large intestines. Our presented data are well in line with our studies in other rodent inflammation models revealing that acute and chronic inflammation of the small as well as large intestinal tract in mice is associated with distinct shifts of the microbiota composition towards overgrowth with commensal *E. coli*, *Bacteroides/Prevotella* spp. and enterococci at sites of inflammation [19,20,21,22,23]. Given that in the presented study the significant microbiota shifts were exclusively detected following infection with the *H. pylori* WT strain able to translocate the effector protein CagA into the host cells, it is somewhat surprising, though, that at site of gastric inflammation no statistical significant differences in microbial colonization densities could be observed. As we could show in previous studies [9,10], *H. pylori* induced gastritis in antrum mucosa of Mongolian gerbils is *cagPAI-*

Table 1. Gastric pathology in Mongolian gerbils.

Animal group	Number of animals	Duration of experiment/ time of infection (months)	Gastric pathology
Uninfected control	12	14	None
B8 infected (WT)	11	14	Severe inflammation in antrum and corpus mucosa; Lymphoid aggregates in antrum and corpus mucosa; Hyperplasia in antrum and corpus mucosa; Erosions and gastric ulcer; Parietal cell atrophy in corpus mucosa; Mucous gland metaplasia; Gastritis cystica profunda; Focal dysplasia; Gastric pH: 4
B8-Δ <i>cagY</i> infected (Mut)	6	14	Moderate and mild inflammation in antrum and corpus mucosa, respectively; Lymphoid aggregates mainly in antrum mucosa; Hyperplasia in antrum mucosa only; Erosions but no gastric ulcer; Rarely observed atrophy, metaplasia, or gastritis cystic profunda; No dysplasia; Gastric pH: 1–2

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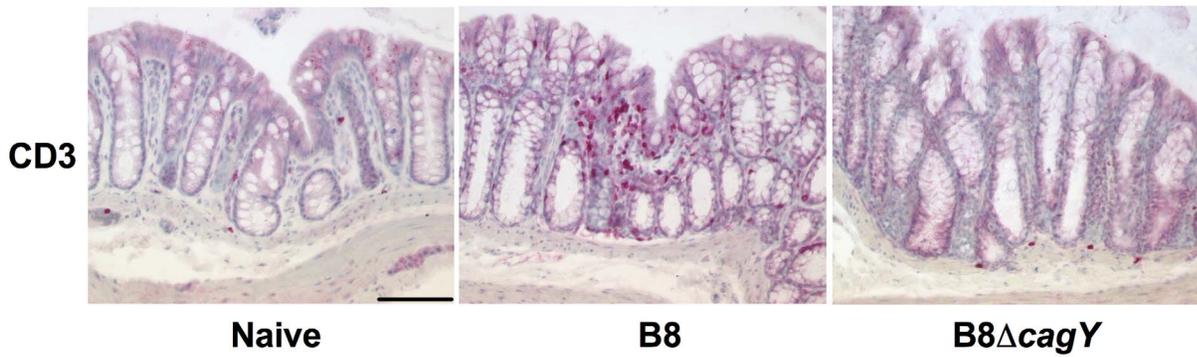


Figure 2. *In situ* T lymphocyte responses in colonic sections of Mongolian gerbils 14 months following *H. pylori* infection. Mongolian gerbils were infected with *H. pylori* wildtype strain B8 (B8; middle) or *H. pylori* mutant strain lacking *cagY* (B8Δ*cagY*; right). Fourteen months following infection, colonic biopsies were taken and paraffin sections stained for CD3 by immunohistochemistry to visualize T lymphocytes *in situ*. Uninfected age-matched animals served as negative controls (Naive; left). Representative photomicrographs of positively stained cells are shown (200 x magnification, scale bar 50 μm).
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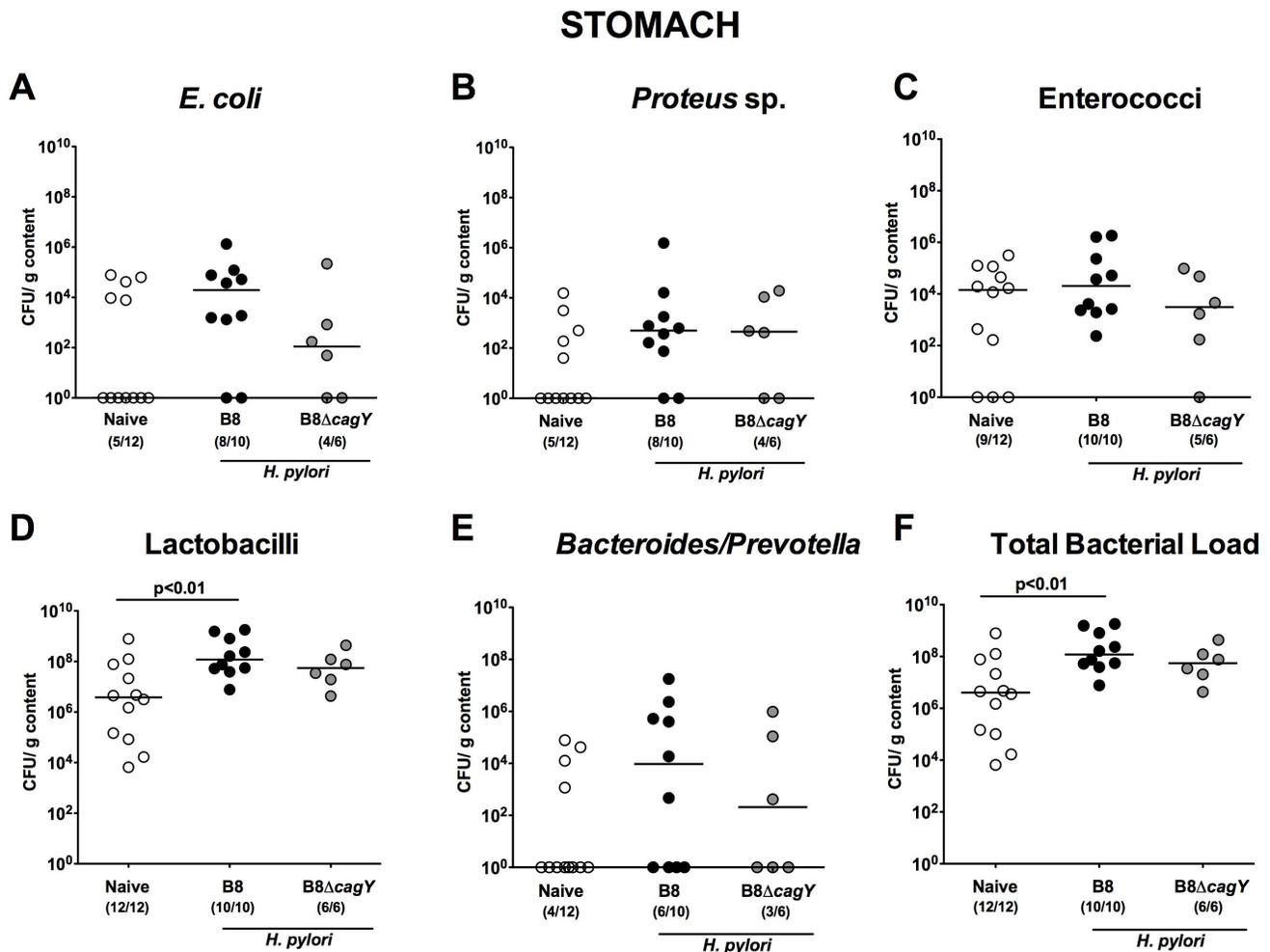


Figure 3. Stomach microbiota composition in Mongolian gerbils 14 months following *H. pylori* infection. Fourteen months following oral infection of Mongolian gerbils with *H. pylori* wildtype strain B8 (B8; black circles) or *H. pylori* mutant strain lacking *cagY* (B8Δ*cagY*; grey circles), the microbiota composition of luminal stomach contents were quantitatively analyzed by culture as described in Methods. Uninfected age-matched animals served as negative controls (Naive; white circles). Numbers of live (A) *E. coli*, (B) *Proteus sp.*, (C) Enterococci, (D) Lactobacilli, (E) *Bacteroides/Prevotella* spp. as well as the (F) total bacterial load are indicated as colony forming units (CFU) per g luminal content. Numbers of animals harboring the respective bacterial species are given in parentheses. Medians and significance levels (p-values) determined by Mann-Whitney-U test are indicated. Data shown were pooled from three independent experiments.
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DUODENUM

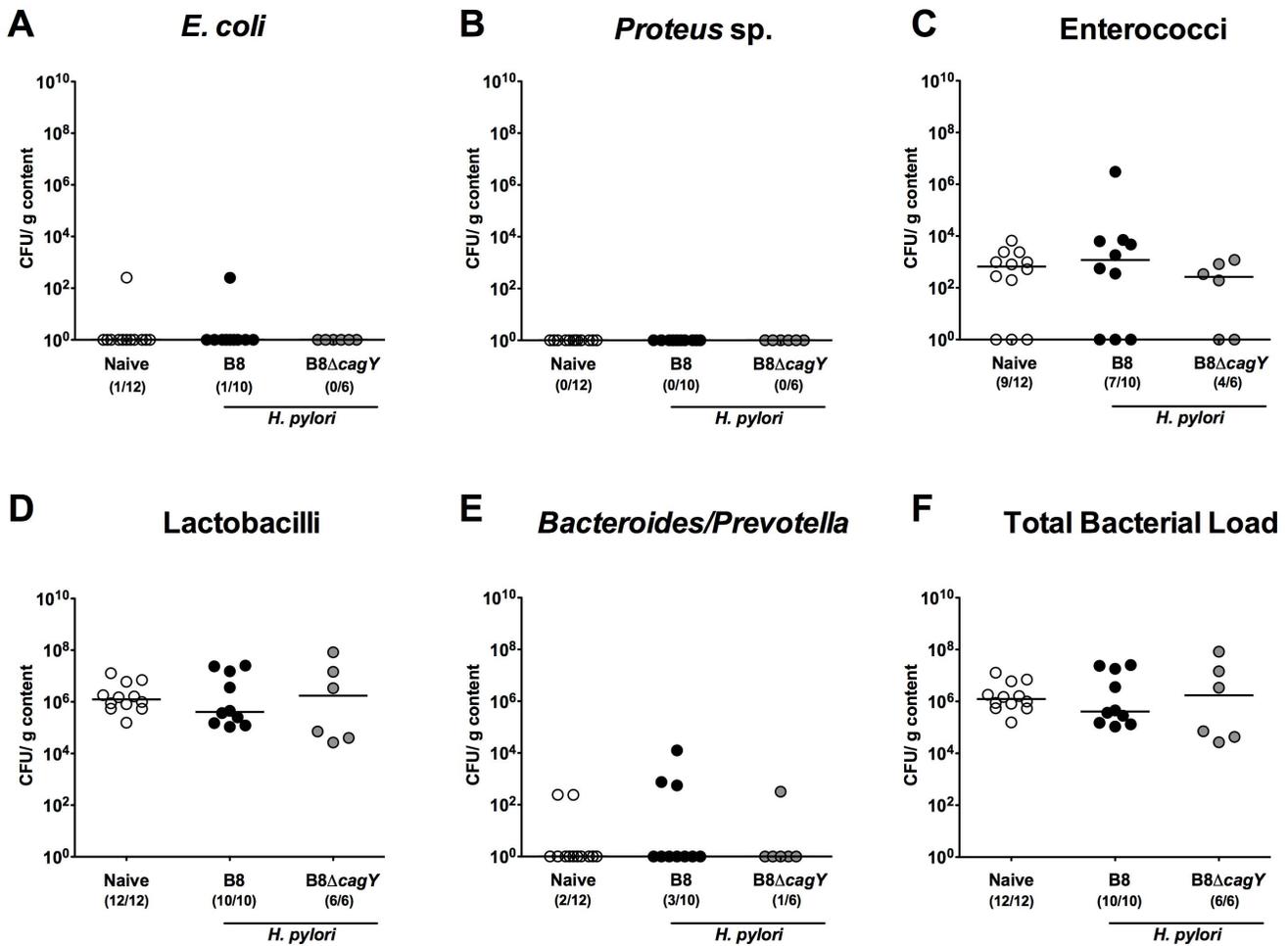


Figure 4. Duodenal microbiota composition in Mongolian gerbils 14 months following *H. pylori* infection. Fourteen months following oral infection of Mongolian gerbils with *H. pylori* wildtype strain B8 (B8; black circles) or *H. pylori* mutant strain lacking *cagY* (B8Δ*cagY*; grey circles), the microbiota composition of luminal **duodenum** contents were quantitatively analyzed by culture as described in Methods. Uninfected age-matched animals served as negative controls (Naïve; white circles). Numbers of live (A) *E. coli*, (B) *Proteus sp.*, (C) Enterococci, (D) Lactobacilli, (E) *Bacteroides/Prevotella* spp. as well as the (F) total bacterial load are indicated as colony forming units (CFU) per g luminal content. Numbers of animals harboring the respective bacterial species are given in parentheses and medians indicated. Data shown were pooled from three independent experiments.
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independent, whereas a severe inflammation of the corpus mucosa is induced via a *cagPAI*-dependent mechanism. Only *H. pylori* WT infected gerbils developed a pangastritis with a complete atrophy of the parietal cells resulting in a significant up-rise of the intragastric pH value (hypochlorhydria) followed by hypergastrinemia. Moreover, CagA translocation and hypochlorhydria have been shown to be pivotal prerequisites for *H. pylori* induced long-term sequelae in humans such as atrophic gastritis, dysplasia or gastric adenocarcinoma [24,25,26,27]. Furthermore, most of these animals also developed a mucous gland metaplasia producing atypical mucus. This is in sharp contrast to *H. pylori* MUT strain infected gerbils that do not develop such sequelae. One must take into account, however, that the observed lack of statistical significant differences in gastric bacterial colonization densities might be due to the relatively high standard deviations in either group since there was a trend towards four orders of magnitude higher (median) *E. coli* and *Bacteroides/Prevotella* spp. loads in *H.*

pylori WT, but not MUT strain infected gerbils at 14 months p.i. when compared to naïve animals.

In a previous molecular study of the gastric microbiota composition we could demonstrate that *E. coli* and *Bacteroides/Prevotella* spp. were exclusively abundant in stomachs of mice 8 weeks following *H. pylori* infection, but absent in uninfected controls [15]. A possible explanation for the gastric abundance of commensals that are usually restricted to the intestines could be that following chronic *H. pylori* infection the “acidic barrier” breaks (and thus the pH within the stomach lumen increases). Subsequently Gram-negative species such as *E. coli* and *Bacteroides/Prevotella* spp. perorally ingested by the coprophagous rodents via their own feces have an advantage to grow in the formerly hostile environment and win the intraluminal competition with other commensal bacteria for niches and nutrients in the stomach. In contrast to mice, however, Mongolian gerbils tend not to coprophage their own feces if supplied with excess food. Therefore, gerbils kept under SPF conditions will not ingest

JEJUNUM

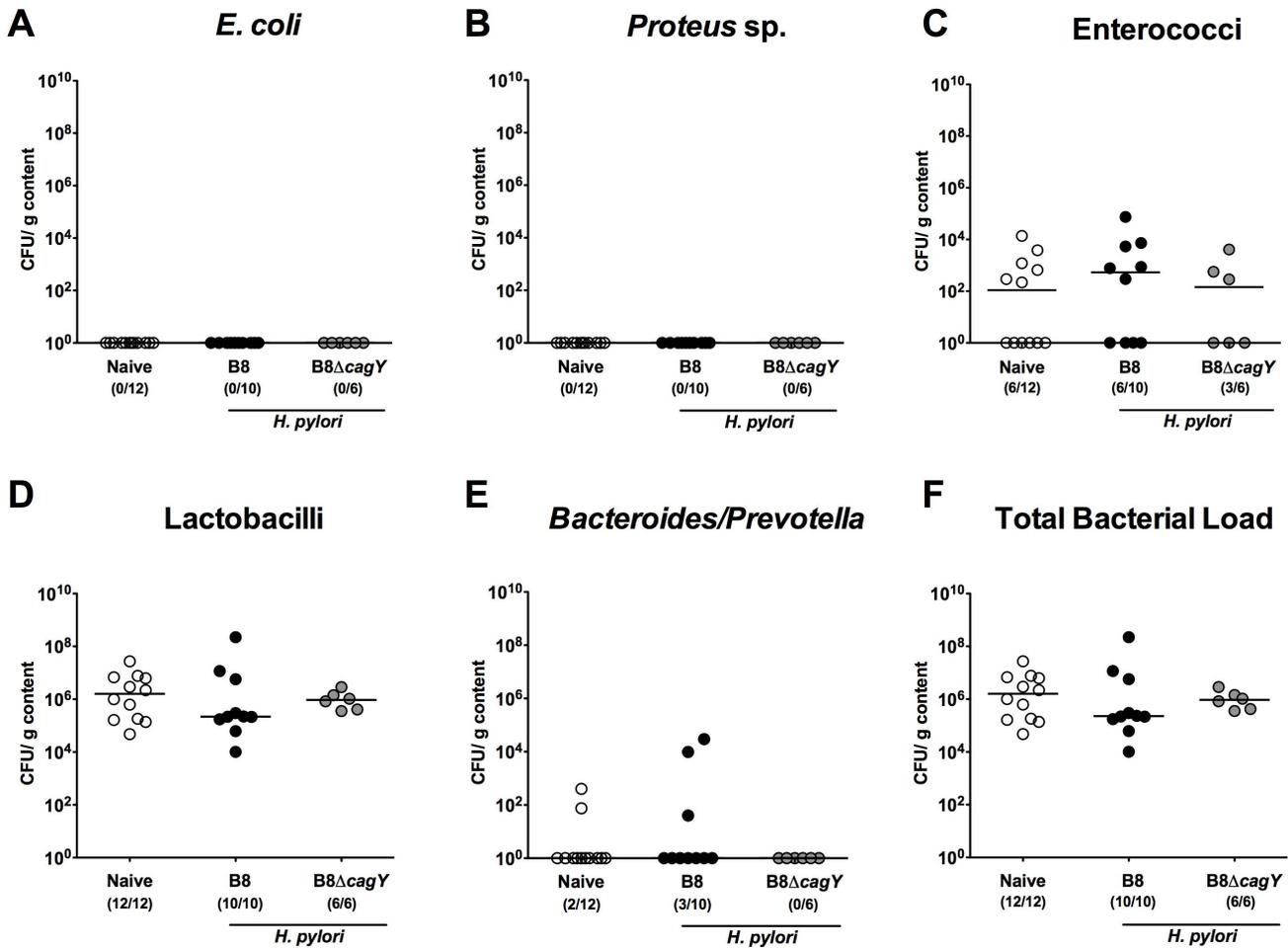


Figure 5. Jejunal microbiota composition in Mongolian gerbils 14 months following *H. pylori* infection. Fourteen months following oral infection of Mongolian gerbils with *H. pylori* wildtype strain B8 (B8; black circles) or *H. pylori* mutant strain lacking *cagY* (B8Δ*cagY*; grey circles), the microbiota composition of luminal jejunum contents were quantitatively analyzed by culture as described in Methods. Uninfected age-matched animals served as negative controls (Naive; white circles). Numbers of live (A) *E. coli*, (B) *Proteus sp.*, (C) Enterococci, (D) Lactobacilli, (E) *Bacteroides/Prevotella* spp. as well as the (F) total bacterial load are indicated as colony forming units (CFU) per g luminal content. Numbers of animals harboring the respective bacterial species are given in parentheses and medians indicated. Data shown were pooled from three independent experiments. doi:10.1371/journal.pone.0100362.g005

intestinal commensals. This would explain the lack of significant abundance of Gram-negative intestinal commensals in the stomach of gerbils here.

In the past some studies attempted to profile the microbiota of *H. pylori*-infected Mongolian gerbils at sites of inflammation [16,17,18,28]. Yin et al. previously showed that gerbils in which the pathogen could still be re-isolated 12 weeks following *H. pylori* infection harbored higher *Bacteroides*, *Enterococcus* and *Staphylococcus* spp., but tended to harbor lower lactobacilli loads in their inflamed stomach and duodenum as compared to animals having expelled the pathogen in the meantime [16]. Furthermore, the gastric and duodenal microbiota composition in *H. pylori*-negative gerbils following infection appeared to be similar to naïve, uninfected animals. The authors concluded that the abundant species had adapted to the *H. pylori* induced changes of the intraluminal milieu at site of infection and inflammation, whereas the lactobacilli population had not [16]. Whether the lower lactobacilli densities was a consequence of *H. pylori* infection (due to milieu changes and lack of adaptation) or had initially rather facilitated the pathogen's

establishment in the stomach cannot be answered. In contrast, in our study 14 months following *H. pylori* infection, gerbils exhibited even significantly (two orders of magnitude) higher lactobacilli and total bacterial loads in the stomach as compared to uninfected animals most likely as a consequence of the hypochlorhydria and, hence, increased pH within the gastric lumen. In addition, a different repertoire of expressed β -defensins [29] and/or abundance of B lymphocyte subsets [30] in lymphoid aggregates of *H. pylori*-infected as compared to naïve gerbils might have contributed to the observed differences in gastric lactobacilli loads. Whereas in our study gerbils had been infected for over one year, Yin et al. had performed their analyses 12 weeks p.i. Of note, the microbiota changes in the interplay with pathogen and host responses are a dynamic system and substantially change over time. Given that here we have not assessed the commensal microbiota composition to an earlier time point, results derived following short-term *H. pylori* infection might differ for several (patho-) physiological reasons.

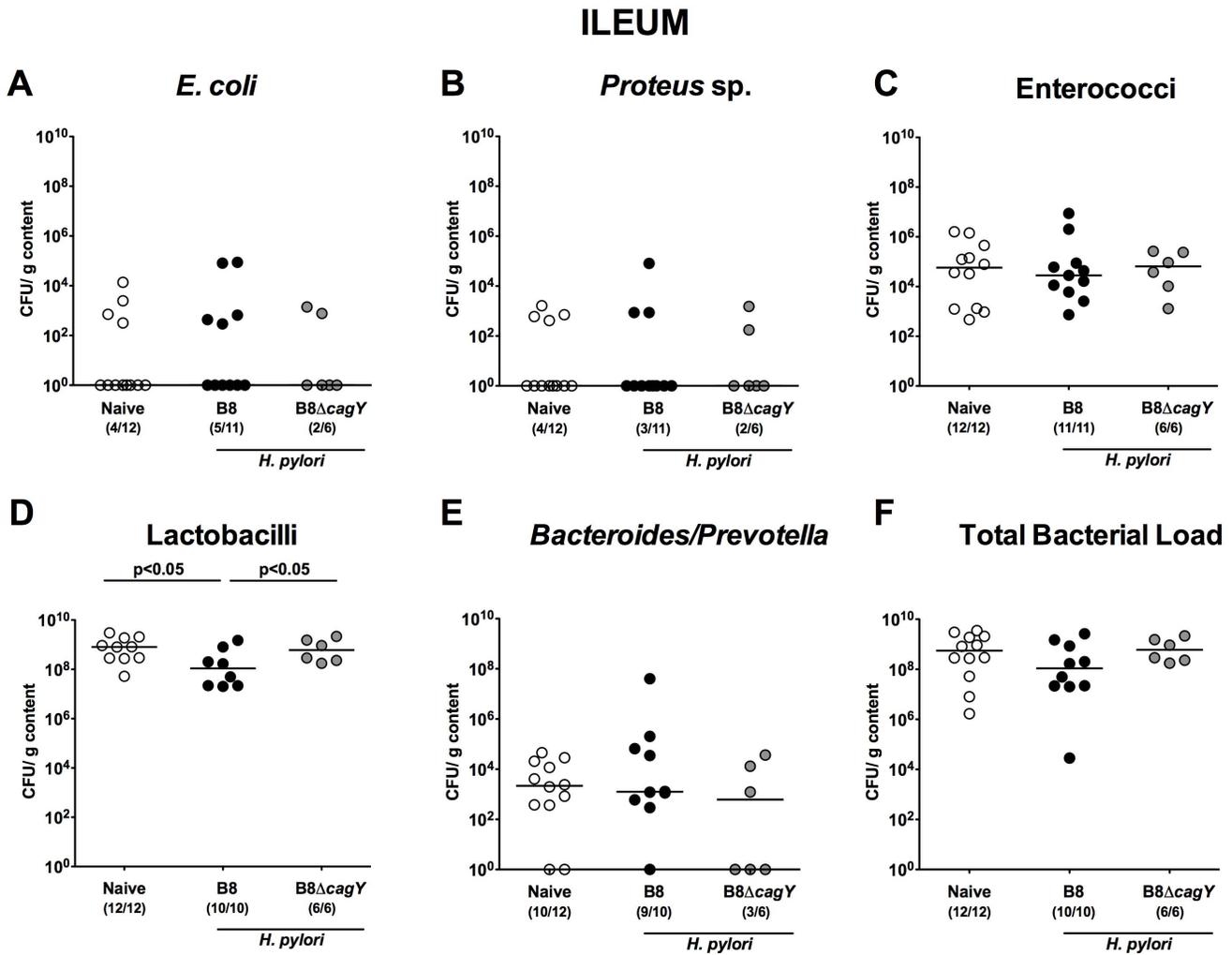


Figure 6. Ileal microbiota composition in Mongolian gerbils 14 months following *H. pylori* infection. Fourteen months following oral infection of Mongolian gerbils with *H. pylori* wildtype strain B8 (B8; black circles) or *H. pylori* mutant strain lacking *cagY* (B8Δ*cagY*; grey circles), the microbiota composition of luminal ileum contents were quantitatively analyzed by culture as described in Methods. Uninfected age-matched animals served as negative controls (Naive; white circles). Numbers of live (A) *E. coli*, (B) *Proteus sp.*, (C) Enterococci, (D) Lactobacilli, (E) *Bacteroides/Prevotella* spp. as well as the (F) total bacterial load are indicated as colony forming units (CFU) per g luminal content. Numbers of animals harboring the respective bacterial species are given in parentheses. Medians and significance levels (p-values) determined by Mann-Whitney-U test are indicated. Data shown were pooled from three independent experiments.
 doi:10.1371/journal.pone.0100362.g006

In our study, applied molecular methods (such as PCR-based DGGE analysis with subsequent sequencing of DNA bands of interest) not only confirmed culture-based results (e.g. enterobacterial such as *E. coli* overgrowth). Strikingly, *Akkermansia*, an uncultivated species involved in mucus degradation [31], could be detected in the caecum and colon of *H. pylori* WT, but not MUT strain infected or naive gerbils. This suggests that the accumulation of *Akkermansia* is driven by *H. pylori* induced inflammation and, hence, depends on a functional Cag pathogenicity island. Thus, intestinal abundance of bacterial species further compromising host barrier function by mucus degradation, might be one important puzzle piece in the complex pathogenesis of *H. pylori*-induced sequelae, also in humans.

Overall, it is most surprising, however, that in our study even though the *H. pylori* induced immunopathology was prominent within the stomach, the microbiota shifts became exclusively overt in the large and not small intestinal tract where no mucosal histopathological changes of the intestinal mucosa could be

observed. Interestingly, we were able to detect increased numbers of CD3+ cells in the large (but not small) intestinal mucosa and lamina propria in long-term *H. pylori*-WT, but not MUT strain infected Mongolian gerbils. It is tempting to speculate that recruited T lymphocytes might subsequently induce the release of cytokines and chemokines, which in turn promote further inflammatory responses leading to a compromised epithelial barrier function. As a consequence, a broken epithelial barrier might lead to changes within the intraluminal milieu subsequently promoting the luminal growth of intestinal commensals such as Gram-negative species as we could show in murine acute and chronic inflammation of the small as well as large intestinal tract [19,20,21,22,23]. In addition, one might speculate that via the neuroendocrine route, for instance, mediators (e.g. gastrin, somatostatin, cytokines, low proton concentration, among other factors) released in the stomach during *H. pylori* infection and induced immunopathology [32,33] exert their effect in the lower intestinal tract. Of note, patients suffering from *H. pylori* infection

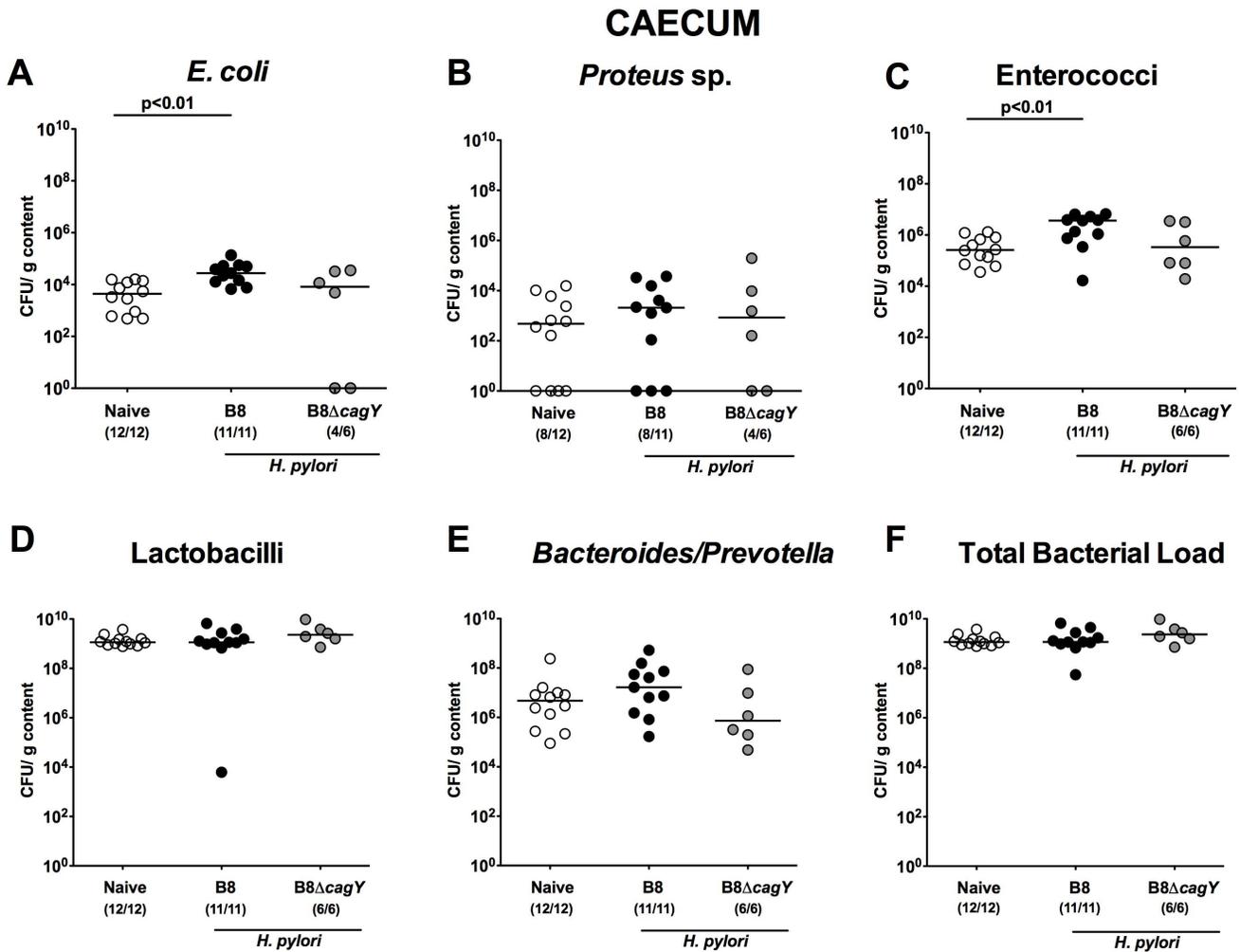


Figure 7. Caecal microbiota composition in Mongolian gerbils 14 months following *H. pylori* infection. Fourteen months following oral infection of Mongolian Gerbils with *H. pylori* wildtype strain B8 (B8; black circles) or *H. pylori* mutant strain lacking *cagY* (B8Δ*cagY*; grey circles), the microbiota composition of luminal caecum contents were quantitatively analyzed by culture as described in Methods. Uninfected age-matched animals served as negative controls (Naive; white circles). Numbers of live (A) *E. coli*, (B) *Proteus sp.*, (C) *Enterococci*, (D) *Lactobacilli*, (E) *Bacteroides/Prevotella* spp. as well as the (F) total bacterial load are indicated as colony forming units (CFU) per g luminal content. Numbers of animals harboring the respective bacterial species are given in parentheses. Medians and significance levels (p-values) determined by Mann-Whitney-U test are indicated. Data shown were pooled from three independent experiments. doi:10.1371/journal.pone.0100362.g007

and hypochlorhydria occasionally complain about rather unspecific intestinal symptoms such as irregular bowel movements, flatulence or abdominal pain of otherwise unknown origin [34,35], which might be attributed to bacterial microbiota changes (e.g. overgrowth of some commensals and disappearance of others from the complex bacterial community) in the lower intestine during the disease process.

Taken together, long-term infection of Mongolian gerbils with an *H. pylori* WT strain displaying an intact type IV secretion system leads to distinct shifts of the microbiota composition in the distal uninflamed, but not proximal inflamed GI tract. Hence, *H. pylori* induced immunopathogenesis of the stomach, including hypochlorhydria and hypergastrinemia, might trigger large intestinal microbiota changes whereas the exact underlying mechanisms need to be further unraveled.

Materials and Methods

Ethics Statement

All animal experiments were conducted according to the European Guidelines for animal welfare (2010/63/EU) with approval of the commission for animal experiments headed by the “Regierung von Oberbayern” (AZ 55.2-1-54-2531-41/04 and 55.2-1-54-2531-78/05). Animal welfare was monitored twice daily by assessment of clinical conditions.

Bacterial Strains

Helicobacter pylori B8 (WT), a recently sequenced ([36]; B128 parental strain), Mongolian gerbil-adapted type I-strain (CagA, VacA: s1m2) and its isogenic mutant B8Δ*cagY* (MUT) were used in this study as previously described [10]. Both strains carried a chromosomal streptomycin resistance cassette allowing quantitative recovery of *H. pylori* from the gerbil stomach by antibiotic selection (streptomycin 250 mg/L; [37]) as described earlier [9,10].

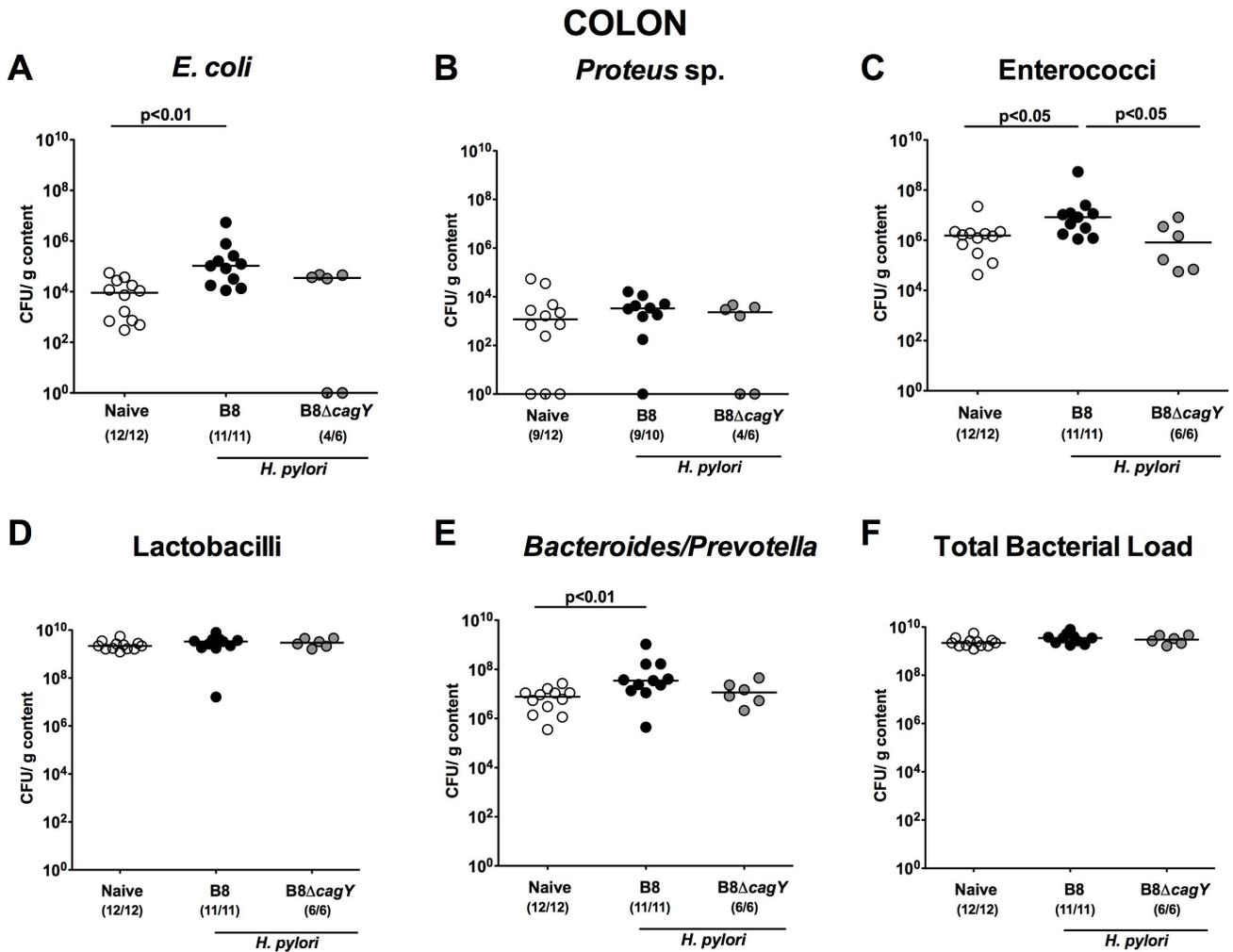


Figure 8. Colonic microbiota composition in Mongolian gerbils 14 months following *H. pylori* infection by culture. Fourteen months following oral infection of Mongolian gerbils with *H. pylori* wildtype strain B8 (B8; black circles) or *H. pylori* mutant strain lacking *cagY* (B8 Δ cagY; grey circles), the microbiota composition of luminal **colon** contents were quantitatively analyzed by culture as described in Methods. Uninfected age-matched animals served as negative controls (Naïve; white circles). Numbers of live (A) *E. coli*, (B) *Proteus* sp., (C) Enterococci, (D) Lactobacilli, (E) *Bacteroides/Prevotella* spp. as well as the (F) total bacterial load are indicated as colony forming units (CFU) per g luminal content. Numbers of animals harboring the respective bacterial species are given in parentheses. Medians and significance levels (p-values) determined by Mann-Whitney-U test are indicated. Data shown were pooled from three independent experiments.
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Animals and Infection Experiments

Outbred Mongolian gerbils from our own breeding colony (Max von Pettenkofer-Institute, LMU Munich, Germany) were bred and maintained under specific pathogen free (SPF) conditions and housed in SEALS SAFE IVC cages (H-Temp, Tecniplast, Hohenpeissenberg, Germany) in an air-conditioned biohazard room (room temperature, $23 \pm 5\%$; 12/12 hours light/dark cycle) with free access to a commercial gerbil diet (ssniff Gerbil, SSMIFF, Soest, Germany) and sterile tap water. Female animals at the age of 8–12 weeks were challenged orogastrically three times over five consecutive days with approximately 10^9 viable *H. pylori* B8 wildtype or *H. pylori* B8 Δ cagY mutant strain. Age-matched control animals were incubated with identical volumes of sterile Brucella broth alone. Animals were sacrificed after defined time of infection (14 months) by isofluran treatment (Abbott, Germany). Gastrointestinal samples from each Mongolian gerbil were collected in parallel for immunohistological, microbiological, and molecular analyses. The stomach was opened along the great curvature, and

the gastric tissue conserved separately in antrum and corpus as previously described [10]. In addition luminal contents of stomach, duodenum, jejunum, ileum, caecum, and colon were collected under sterile conditions in sterile PBS and immediately stored on ice.

Histopathology and Immunohistochemistry of Gastrointestinal Tissues

Histopathological changes were determined in GI tissue samples immediately fixed in 5% formalin and embedded in paraffin. Sections (5 μ m) were stained with H&E and examined by light microscopy (100 x and 400 x magnification). *In situ* immunohistochemical analysis of paraffin sections taken from the colon was performed as described previously [38]. A primary antibody against CD3 (#N1580, Dako, Denmark, dilution 1:10) was used to visualize T lymphocytes in the colonic mucosa *in situ* (200 x magnification).

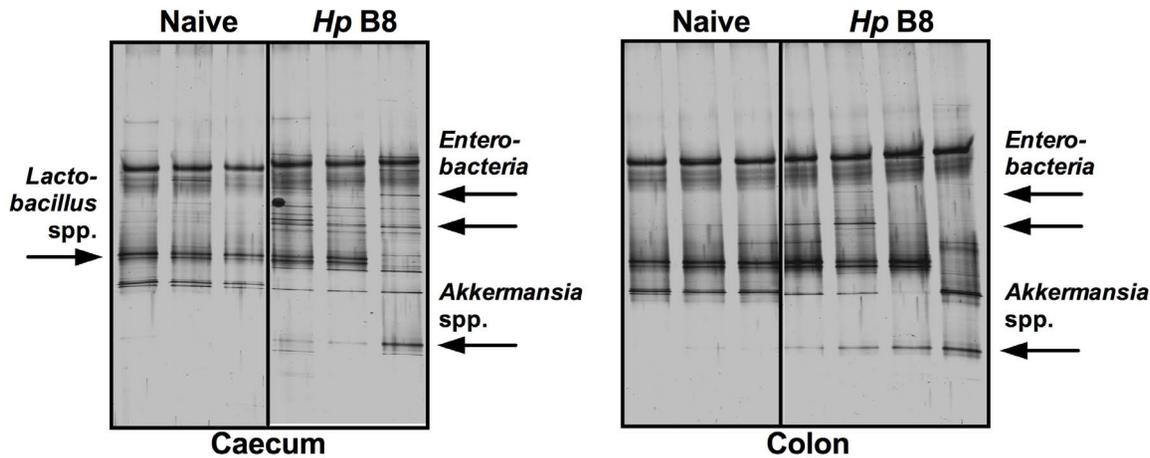


Figure 9. Molecular caecum and colon microbiota analysis in Mongolian gerbils 14 months following *H. pylori* infection. Fourteen months following oral infection of Mongolian gerbils with *H. pylori* wildtype strain B8 (*Hp* B8; right lanes within each panel), the microbiota composition of luminal **caecum** (left panel) and **colon** (right panel) contents were subjected to PCR-DGGE analysis of PCR-amplified total bacterial 16S rRNA gene fragments as described in Methods, and compared to uninfected age-matched controls (Naive; left lanes within each panel). Sequence analysis revealed that indicated DNA bands appearing 14 months following *H. pylori* B8 infection refer to enterobacteria and *Akkermansia* spp. Shown DGGE profiles of the bacterial microbiota are representative for three independent experiments. doi:10.1371/journal.pone.0100362.g009

Cultural Analysis of the Luminal Gastrointestinal Microbiota

Cultural analysis of the intestinal microbiota along the entire GI tract was performed as described previously [20]. Luminal contents taken from stomach, duodenum, jejunum, ileum, caecum, and distal colon were resuspended in PBS, weighted, and 100 µl aliquots of serial dilutions plated onto solid media (Oxoid). Bacteria were grown at 37°C for 2 days under aerobic or for 4 days under anaerobic conditions, and total numbers determined by colony counting on Columbia blood agar. Bile esculin, McConkey, anaerobe 5% sheep blood agar supplemented with kanamycin and vancomycin, and Rogosa (Merck) media were used for quantitative identification of enterococci, enterobacteria (such as *E. coli* and *Proteus* spp.), *Bacteroides/Prevotella* spp., and lactic acid bacteria (mainly lactobacilli), respectively. Bacteria were subcultivated and further investigated by Gram-staining and by biochemical analysis with the API20E, API50 CH, and API Rapid ID 32A systems (Biomérieux) and confirmed by sequence analyses in cases of inconclusive identification. Results were expressed as colony forming units (CFU) per g of luminal content. The detection limit of viable pathogens was ≈25 CFU per g.

Molecular Microbiota Analysis

Molecular detection of fastidious or uncultivable bacterial communities in stomach, duodenum, jejunum, ileum, caecum, and distal colon was performed as previously described [20]. Respective luminal contents were removed, resuspended in PBS, and centrifuged (16,000 g/10 min/4°C). The sediment was resuspended in 0.5 ml of lysis buffer (500 mM Tris (pH 9.0), 20 mM EDTA, 10 mM NaCl, 1% SDS) and incubated with proteinase K (2 mg/ml; Sigma-Aldrich) for 1 h at 56°C. After bead beating with zirconium-silica beads (0.2 mm), the total DNA isolated by phenol-chloroform extraction served as template for PCR amplification.

Genetic fingerprints were generated by PCR denaturing gradient gel electrophoresis (PCR-DGGE; [39]). Variable regions

6–8 in bacterial 16S rRNA genes were amplified (denaturation at 95°C/5 min, then 25 cycles of 93°C/45 s, 64°C/1 min, 72°C/1 min, final elongation at 72°C/7 min) from total gut content DNA with GC clamp (underlined) primer GC968F (5'-CGCCCCGGGGCGCGCCCCGGGCGGGGCGGGGGCAG-GGGGGAACGCGAAGAACCCTTAC-3', nt 968-84 in *E. coli* 16S rRNA) and primer R1378 (5'-CGGTGTGTACAAGGCCCGG-GAACG-3', nt 1401-1378 in *E. coli* 16S rRNA). Amplicons (300 ng) were electrophoresed on a DCode System (Bio-Rad) at 80 V/60°C for 16 h in a polyacrylamide gel containing 35–60% urea/formamide. DNA band profiles were visualized by silver staining. For sequence analysis, DGGE bands were stained with SYBR green I (Fluka), visualized under UV light, and cut off the gel matrix. DNA was eluted by shaking in double-distilled water (ddH₂O) overnight at 37°C. After reamplification by PCR, the amplicons were cloned (pCR2.1 TOPO-TA, Invitrogen life technologies) and sequenced for phylogenetic identification.

Statistical Analysis

Medians and levels of significance were determined using Mann-Whitney-U Test. Two-sided probability (*P*) values ≤0.05 were considered significant. Experiments were repeated twice.

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Author Contributions

Conceived and designed the experiments: MMH AF RP TW UBG SB GR. Performed the experiments: MMH AF RP TW SB GR. Analyzed the data: MMH AF RP TW CL SB GR. Contributed reagents/materials/analysis tools: CL UBG. Wrote the paper: MMH AF SB GR.

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