Effect of the proton pump inhibitor omeprazole on the gastrointestinal bacterial microbiota of healthy dogs

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Abstract

The effect of a proton pump inhibitor on gastrointestinal (GI) microbiota was evaluated. Eight healthy 9-month-old dogs (four males and four females) received omeprazole (1.1 mg kg⁻¹) orally twice a day for 15 days. Fecal samples and endoscopic biopsies from the stomach and duodenum were obtained on days 30 and 15 before omeprazole administration, on day 15 (last day of administration), and 15 days after administration. The microbiota was evaluated using 16S rRNA gene 454-pyrosequencing, fluorescence in situ hybridization, and qPCR. In the stomach, pyrosequencing revealed a decrease in Helicobacter spp. during omeprazole (median 92% of sequences during administration compared to >98% before and after administration; \( P = 0.0336 \)), which was accompanied by higher proportions of Firmicutes and Fusobacteria. FISH confirmed this decrease in gastric Helicobacter \(( P < 0.0001)\) and showed an increase in total bacteria in the duodenum \(( P = 0.0033)\) during omeprazole. However, Unifrac analysis showed that omeprazole administration did not significantly alter the overall phylogenetic composition of the gastric and duodenal microbiota. In feces, qPCR showed an increase in Lactobacillus spp. during omeprazole \(( P < 0.0001)\), which was accompanied by a lower abundance of Faecalibacterium spp. and Bacteroides-Prevotella-Porphyromonas in the male dogs. This study suggests that omeprazole administration leads to quantitative changes in GI microbiota of healthy dogs.

Introduction

The secretion of gastric acid is one of the first defense mechanisms in the body to avoid the introduction of potentially harmful infectious agents into the intestinal tract. Gastric acid is secreted by the parietal cells and is regulated by complex paracrine, endocrine, and neural pathways (Yao & Forte, 2003).

Proton pump inhibitors (PPIs) are drugs of widespread therapeutic use in human and veterinary medicine. PPIs inhibit the secretion of gastric acid by blocking the H⁺/K⁺-ATPase in gastric parietal cells (Sachs et al., 1995). In humans, a recent retrospective study of 125 patients showed that advanced age, low serum albumin concentrations, and concomitant use of PPIs were significant risk factors for Clostridium difficile-associated diarrhea (Kim et al., 2010), an important disease with increasing rates of mortality (Dawson et al., 2009). Likewise, a recent large study involving 5387 elderly subjects, and a systematic review of 2948 patients, have linked the use of PPIs with an increased risk of diarrhea (Pilotto et al., 2008) and a higher risk of enteric infections (Leonard et al., 2007), respectively.

The mechanisms by which the suppression of gastric acid secretion predisposes patients to an increased risk of gastrointestinal (GI) disease are not well understood. For example, while there is mounting evidence suggesting an association between the use of PPIs and C. difficile-associated disease (Dial, 2009), gastric acid does not kill C. difficile spores (Rao et al., 2006), which are believed to be crucial for the transfer of the microorganism (Dawson et al., 2009). Also, a large case-control cohort study of more than 170 000 ever-users of acid-suppressing drugs, including PPIs, showed no association of antacid use with...
bacterial gastroenteritis (Garcia Rodriguez & Ruigomez, 1997) and a recent review of the literature indicates that bacterial overgrowth during PPIs administration rarely leads to clinical disease (Williams & McColl, 2006). These observations illustrate the possibility that the development of GI disorders in patients that are treated with gastric acid inhibitors is a multi-factorial phenomenon rather than an isolated association (Canani & Terrin, 2010).

The GI tract of mammals is home to a vast number of different microbial groups, all acting in close symbiosis with one another and with their host (Neish, 2009). Despite the widespread medical use of PPIs and its potential involvement in intestinal dysbiosis (Vesper et al., 2009), only a few studies have explored the effect of these compounds on GI microbial communities, mainly using culture techniques for specific microorganisms (e.g. Helicobacter pylori) (Sharma et al., 1984; Fried et al., 1994; Saltzman et al., 1994; Verdu et al., 1994; Logan et al., 1995; Thorens et al., 1996). However, culture techniques are by definition restricted to cultivable microorganisms, a group representing a small proportion of all GI microbiota (Rajilic-Stojanovic et al., 2007). Culture independent, 16S rRNA gene-based techniques have greatly enhanced our knowledge of intestinal microbial inhabitants, but these techniques have rarely been used to evaluate the effect of gastric acid inhibition on the overall composition of the GI bacterial microbiota (Williams & McColl, 2006; Vesper et al., 2009).

As in humans, PPIs and other inhibitors of gastric acid secretion are frequently used in dogs with disorders of the upper GI tract. However, the effect of omeprazole or any other suppressor of gastric acid secretion on the GI bacterial microbiota of dogs has not been investigated in detail and was the primary objective of this study.

Materials and methods

Study design

Eight intact clinically healthy mixed-breed dogs, four male and four female, were entered into this study. All dogs were 9 months old, of similar weight (18.6 ± 2.0 kg), and were fed once a day with a commercial diet (8755 Teklad: 21% protein, 4% fiber, Harlan). Omeprazole capsules (Zegerid, Santarus) were administered orally (8755 Teklad: 21% protein, 4% fiber, Harlan). Omeprazole (1.1 mg kg\(^{-1}\)) and were fed once a day with a commercial diet containing (8755 Teklad: 21% protein, 4% fiber, Harlan). Omeprazole (1.1 mg kg\(^{-1}\)) and were fed once a day with a commercial diet containing 20 mL of water orally. Multiple mucosal biopsy specimens from the gastric body and the proximal duodenum (12–15 from each site) were obtained from all dogs on Days 30 (Day −30) and 15 (Day −15) before omeprazole administration, on the last day of omeprazole administration (Day 15), and 15 days after the end of omeprazole administration (Day 30). Biopsies were collected by endoscopy under general anesthesia (sedation with butorphanol 0.2 mg kg\(^{-1}\) IM 15 min before induction with thiopental IV 15 mg kg\(^{-1}\) followed by endotracheal intubation and maintenance of anesthesia with sevoflurane in 100% oxygen via a circle system). For both stomach and duodenum, three biopsies were flash frozen in liquid nitrogen for DNA extraction and 6–7 biopsies were harvested and placed into 10% formalin for FISH analysis and histological assessment according to the guidelines of the World Small Animal Veterinary Association (Day et al., 2008). Gastric juice (~ 5 mL) was obtained before each endoscopic procedure via an endoscopic catheter, and the pH measured immediately with a pH paper (EMD Chemicals) and a pH meter. Fecal samples were collected by rectal palpation on Days −30, −15, 15, and 30, and stored at −80 °C until analysis. The study protocol was approved by the Institutional Animal Care and Use Committee of the University of Illinois (approval number: 08261).

DNA extraction

Genomic DNA was extracted from the biopsies and feces using a bead-beating phenol–chloroform method as described elsewhere (Suchodolski et al., 2010).

Massive parallel 454-pyrosequencing

The gastric and duodenal mucosa-adherent microbiota were evaluated using pyrosequencing of biopsy samples collected on Days −30 and −15 (before omeprazole administration), on Day 15 of omeprazole administration, and on Day 30 (after discontinuation of omeprazole administration) using a bacterial tag-encoded FLX-Titanium 16S rRNA gene amplicon pyrosequencing (bTE-FAP), as described previously for canine intestinal samples (Handl et al., 2011). To estimate total bacterial diversity, sequences were depleted of barcode primers, chimeras, plastid, mitochondrial, and any non-16S bacterial reads (< 70% identity to any known high-quality 16S sequence). Chimeras were depleted using Black Box Chimera Check (B2C2) as described previously (Gontcharova et al., 2010). Because sequence number may impact diversity estimates, an equal number of high-quality sequences were used for each sample. In order to use all available samples, a total of 1000 sequences were selected randomly from each sample, sequences < 350 were removed and the rest of the sequences trimmed to 350 bp and aligned with MUSCLE (Edgar, 2004). The alignment was inspected visually and deemed accurate. A distance matrix was calculated from the alignment with PHYLIP (Felsenstein, 2005;
gaps considered). Operational Taxonomic Units (OTUs) then were assigned by mothur using the read.otu command (Schloss et al., 2009).

To evaluate bacterial taxonomic community structure, Phred20 quality reads were trimmed to remove tags and primer sequences, depleted of chimera, plastid, mitochondrial, non-16S reads, and sequences < 250 bp. The final sequences were evaluated using blastn against a continuously curated high-quality 16S rRNA gene database derived from the National Center for Biotechnology Information (NCBI) as described previously (Cephas et al., 2011). blast outputs, based upon top-hit designations, were compiled to generate percentage files at each taxonomic level as described previously. Sequences with identity scores to known or well-characterized 16S rRNA gene sequences greater than 97% identity (<3% divergence) were resolved at the species level, between 95% and 97% at the genus level, between 90% and 95% at the family level, between 85% and 90% at the order level, between 80% and 85% at the class level, and 77–80% at the phylum level. Sequence information is available through GenBank within a short read archive (SRA) under accession SRA0456271.

**Quantitative real-time PCR (qPCR)**

In an effort to support the main findings of pyrosequencing (see below), the abundance of total bacteria, Helicobacter, Lactobacillus, and Enterococcus spp. was estimated by qPCR in the obtained DNA samples from the gastric and duodenal biopsies using published oligonucleotides (Supporting Information, Table S1). TaqMan® reaction mixtures (total 10 μL) contained 5 μL of TaqMan® Fast Universal PCR master mix (2×), No AmpErase® UNG (Applied Biosystems), 1 μL of water, 0.4 μL of each primer (final concentration: 400 nM), 0.2 μL of the probe (final concentration: 200 nM), 1 μL of 1% bovine serum albumin (BSA, final concentration: 0.1%), and 2 μL of DNA (1:10 or 1:100 dilution), and the PCR conditions were: 95 °C for 20 s, and 40 cycles at 95 °C for 5 s, and 10 s at the optimized annealing temperature (Table S1). SYBR-based reaction mixtures (total 10 μL) contained 5 μL of SsoFast® EvaGreen® supermix (Biorad Laboratories), 1.6 μL of water, 0.4 μL of each primer (final concentration: 400 nM), 1 μL of 1% BSA (final concentration: 0.1%), and 2 μL of DNA (1:10 or 1:100 dilution). PCR conditions were 95 °C for 2 min, and 40 cycles at 95 °C 5 s and 10 s at the optimized annealing temperature (Table S1). A melt curve analysis was performed for SYBR-based qPCR assays under the following conditions: 1 min at 95 °C, 1 min at 55°C, and 80 cycles of 0.5 °C increments (10 s each). Amplicons were also visualized in an agarose gel (1%) to confirm the presence of one band of the expected molecular size.

The qPCR data for Helicobacter, Lactobacillus, and Enterococcus spp. were normalized to the qPCR data for total bacteria, and all samples were run in duplicate.

The abundance of total bacteria, Bifidobacterium, Lactobacillus, the Bacteroides-Prevotella-Porphyromonas group, gamma-Proteobacteria (Class), Firmicutes (Phylum), Clostridium perfringens, as well as C. difficile and the C. difficile gene-encoding toxin B, was evaluated in feces using published oligonucleotides (Table S1). The abundance of Ruminococaceae (Family) and the genus Faecalibacterium was also evaluated using family and genus-specific oligonucleotides (as assessed by 16S rRNA gene clone libraries) recently developed at our laboratory. The decision to target only a subset of fecal bacterial groups was based on previous reports suggesting that these groups are highly abundant phyotypes in feces of dogs (e.g. Ruminococaceae) and because of purported beneficial properties (e.g. Faecalibacterium and Bifidobacterium spp.). SYBR-based qPCR assays were performed as described above (without BSA) at the optimized annealing temperature (Table S1). A commercial real-time PCR thermal cycler (CFX96TM; Biorad Laboratories) was used for all qPCR assays. The DNA concentration of all fecal samples was adjusted to 5 ng μL⁻¹.

**Fluorescence in situ hybridization (FISH)**

An average of six biopsies (range: 4–7 per organ evaluated) were obtained at each time point and from each dog, fixed in neutral-buffered 10% formalin for less than 48 h, and embedded in paraffin. Histological sections (4 μm) were evaluated using FISH with oligonucleotide probes 5’-labeled with 6-FAM or Cy-5 targeting the 16S rRNA gene of total bacteria and Helicobacter spp. (Table S1) as described previously (Jergens et al., 2009), with minor modifications (no formamide in the hybridization buffer in an effort to reduce toxic waste and because the addition of formamide did not significantly increase the fluorescent signal, and 48 and 50 °C for hybridization and washing, respectively). Gastric and duodenal bacteria were quantified every 3–5 microscopic fields throughout the mucosal perimeter of each biopsy, depending on the unique morphology of each specimen, using a Zeiss Stemi digital confocal microscope (Carl Zeiss Microimaging). To facilitate the quantification of bacteria at different levels of the glass slide, at least three consecutive pictures were taken sequentially throughout the vertical z-axis (each picture separated from one another by 0.5 μm) from each microscopic field. A C-aphochromat (63× water correction) objective lens was used for all FISH analyses. Total bacteria were quantified using FISH in both gastric (universal probe labeled with 6-FAM) and
duodenal (universal probe labeled with Cy5) biopsies, while the genus *Helicobacter* (FISH probes labeled with Cy5) was only quantified in the gastric biopsies.

**Statistical analysis**

To assess the diversity of the GI microbiota, the Shannon–Weaver (Shannon & Weaver, 1963) and Chao1 (Chao, 1987) diversity indices were calculated using MOTHUR. Alterations of microbial communities before, during, and after omeprazole administration were investigated using an unweighted UniFrac distance matrix in the QIIME software (Caporaso et al., 2010). In short, the high-quality sequences as described above were aligned using MUSCLE, and an optimized tree was generated (also using MUSCLE). This tree served as the input tree for the unweighted UniFrac distance metric.

Parametric analyses. A general linear mixed model
using the MIXED procedure of SAS® 9.2 (SAS® Institute, Inc.) was used to analyze the qPCR data with time, gender, and time*gender interaction as fixed effects. The inclusion of the interaction between time and gender is justified by the fact that all dogs were the same age, had a very similar body weight, and were subjected to the same diet and environmental conditions. In addition, time was also used in the REPEATED statement to model the repeated measures (before, during, and after omeprazole administration), and dog was included as a random effect. The log_{10} gastric *Helicobacter* FISH counts were analyzed using a general linear mixed model in *SAS*® 9.2 and the same approach described for qPCR data. Post hoc multiple comparisons were performed using the Tukey–Kramer method. All model residuals showed a distribution very close to normal, thus indicating valid models.

Nonparametric analyses. The Friedman’s test in Prism5 (GraphPad Software, CA) was used to compare the pyrosequencing data (percentage of sequences) for each bacterial group separately, gastric non-*Helicobacter* total FISH counts, and the indexes of bacterial richness and diversity. Post hoc multiple comparisons were performed using the Dunn’s post-test. The NPAR1WAY procedure in *SAS*® 9.2 was used to compare intragastric pH and duodenal bacterial FISH counts. A P-value of < 0.05 was considered to be statistically significant for all analyses.

**Results**

**Side effects of omeprazole administration and intragastric pH**

All dogs remained clinically healthy throughout the study. Intragastric pH was significantly increased during omeprazole administration (median pH: 7.4, interquartile range: 7.2–7.9) when compared with intragastric pH on Days 1 (1.7, 1.5–1.9) and 15 (1.8, 1.5–2.1) before administration, and Day 30 after omeprazole administration (1.5, 1.4–6.8) (P = 0.0037). The pH measurements did not correlate linearly or quadratically with gastric or duodenal bacterial FISH counts, pyrosequencing, or qPCR data (results not shown).

**Number of sequences obtained by bTEFAP**

A total of 142 026 (stomach) and 133 449 (duodenum) sequences (~ 4000 per sample evaluated) were analyzed. The number of sequences ranged from 1199 to 7734 sequences in gastric samples and 1028–9383 sequences in duodenal samples. On average, the number of sequences per dog varied from 3176 to 5476 in gastric samples and from 1955 to 6590 in duodenal samples. With the exception of the gastric microbiota of two male dogs at only one different time point each, the gastric and duodenal microbiota formed completely separated phylogenetic clusters (Fig. S1), suggesting a distinctive microbiota in each of the evaluated segments of the GI tract.

**bTEFAP in gastric biopsies**

In the stomach, a median of 36 OTUs (> 97% sequence identity, range: 18–119) was detected per dog per time point. There was a higher bacterial richness in the stomach during omeprazole administration (median: 56, range: 26–70; median before and after: 35, range: 18–119) but this difference did not reach significance (Table 1). Bacterial diversity was not significantly modified. While we observed significant changes in specific bacterial groups in response to omeprazole administration (see below), the constructed dendrograms based on the UniFrac distance metric did not reveal an obvious clustering of animals according to treatment period (Fig. S2). The great majority (> 90% on average at baseline) of the obtained sequences from the stomach were classified as *Proteobacteria*, a phylum that decreased in its relative abundance during omeprazole administration (P = 0.0427, Table S2). This effect was more evident on the genus *Helicobacter* (P = 0.0336). The median percentage of *Helicobacter* spp. sequences during omeprazole administration was 92%, while the median percentage before and after omeprazole was > 98%. This decrease in the relative abundance of *Helicobacter* spp. during omeprazole administration was accompanied by an increase in the relative abundance of other genera of the phyla *Proteobacteria* (especially *Actinobacillus*), *Firmicutes* (especially *Streptococcus*), and *Fusobacteria* (Table S2).
FISH in gastric biopsies

Gastric *Helicobacter* spp. and non-*Helicobacter* bacteria were counted throughout the mucosal side of a total of 155 gastric biopsies from a similar number of microscopic fields (Table S3 and Fig. S3). There was a significant effect of omeprazole on the abundance of gastric *Helicobacter* (*P* < 0.0001), and there was no difference in abundance of gastric *Helicobacter* between the male and female dogs (*P* = 0.3161) (Fig. 1). Also, there was a significant interaction between time and gender (*P* = 0.0323), suggesting that the change in gastric *Helicobacter* organisms over time was different between the male and female dogs (Fig. 1). Also, in the stomach, non-*Helicobacter* bacteria were observed more frequently during omeprazole administration (median: 3, range: 0–20) than on Day −30 (median: 0, range: 0–3) and Day −15 (median: 1, range: 0–6) before omeprazole administration, and 15 days after omeprazole administration on Day 30 (median: 0, range: 0–2) (*P* = 0.0300).

Quantitative real-time PCR in gastric biopsies

There was no significant effect of omeprazole administration on gastric total bacteria (*P* = 0.0687), there was no difference in bacterial abundance between the male and female dogs (*P* = 0.7566), but there was a significant interaction between time and gender (*P* < 0.0001) (Fig. 2). In the male dogs, there was a higher bacterial abundance during omeprazole administration on Day 15 (*P* = 0.0093) and on Day 30 after discontinuation of omeprazole administration (*P* = 0.0007) when compared with that on Day −30 before omeprazole administration (Fig. 2). There was no significant effect of omeprazole administration on total gastric bacteria in the female dogs, and there was no significant effect of omeprazole administration on the abundance of gastric *Helicobacter* and *Lactobacillus* spp. (Fig. 2).

Table 1. Median (interquartile range) indices of bacterial diversity (Shannon–Weaver and Chao1 3%) and richness (OTU 3%) on Days −30 and −15 before omeprazole administration, during omeprazole administration (Day 15), and after discontinuation of omeprazole administration (Day 30). *P*-values were obtained by nonparametric Friedman’s tests.

<table>
<thead>
<tr>
<th></th>
<th>Day −30</th>
<th>Day −15</th>
<th>Day 15</th>
<th>Day 30</th>
<th><em>P</em>-values</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Stomach</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Shannon</td>
<td>1.2 (0.9/1.6)</td>
<td>1.4 (1.0/1.9)</td>
<td>1.4 (0.9/2.4)</td>
<td>0.9 (0.8/1.3)</td>
<td>0.3476</td>
</tr>
<tr>
<td>Chao1</td>
<td>59 (38/81)</td>
<td>77 (48/83)</td>
<td>98 (56/194)</td>
<td>47 (38/74)</td>
<td>0.0917</td>
</tr>
<tr>
<td>OTU</td>
<td>35 (29/40)</td>
<td>37 (31/45)</td>
<td>56 (38/92)</td>
<td>30 (24/49)</td>
<td>0.2468</td>
</tr>
<tr>
<td><strong>Duodenum</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>Shannon</td>
<td>4.1 (3.4/4.6)</td>
<td>3.9 (3.6/4.3)</td>
<td>2.9 (2.4/4.2)</td>
<td>3.3 (2.7/4.0)</td>
<td>0.1447</td>
</tr>
<tr>
<td>Chao1</td>
<td>324 (274/470)</td>
<td>387 (229/453)</td>
<td>236 (131/395)</td>
<td>239 (142/471)</td>
<td>0.1116</td>
</tr>
<tr>
<td>OTU</td>
<td>184 (140/246)</td>
<td>191 (129/228)</td>
<td>112 (85/218)</td>
<td>131 (103/220)</td>
<td>0.2898</td>
</tr>
</tbody>
</table>

These estimates are based on 1000-sequence subsamples. Chao1 and OTU estimates were rounded up to better fit in the table.

bTEFAP in duodenal biopsies

In the duodenum, a median of 173 OTUs (> 97% sequence identity, range: 52–285) was detected per dog per time point. Omeprazole administration was not associated with significant differences in the indexes of bacterial richness and/or diversity (Table 1). While we observed significant changes in specific bacterial groups in response to omeprazole administration (see below), the constructed dendrograms based on the Unifrac distance metric did not reveal any obvious clustering of animals according to treatment period (Fig. S4). Bacterial
representatives of at least seven different phyla were identified in the duodenum (Table S4). The majority of the obtained sequences from the proximal duodenum were classified as *Firmicutes*, followed by *Proteobacteria* and *Bacteroidetes*. On average, these three bacterial phyla comprised more than 80% of all sequences at all time points. Omeprazole administration was associated with a higher relative abundance of *Enterococcus* (*P* = 0.0137) and a lower relative abundance of *Helicobacter* (*P* = 0.0287) and *Porphyromonas* (*P* = 0.0316), but there was no statistically significant difference in all the rest of the bacterial groups analyzed (Table S4).

Interestingly, all four male dogs had an increase in the Class *Bacilli* (Phylum *Firmicutes*) during omeprazole administration (all had > 70% during omeprazole administration, while only two had more than 10% at either baseline evaluation) (Fig. S5). This effect was also evident at the order Lactobacillales and the genera *Enterococcus* and *Lactobacillus* in three of the four male dogs. This consistent increase in *Bacilli* during omeprazole administration in the male dogs was associated with a lower abundance of other bacterial phyla (especially *Proteobacteria* and *Bacteroidetes*) during omeprazole administration. In the female dogs, no such consistent changes in the proportions of duodenal bacteria were observed.

**FISH in duodenal biopsies**

Duodenal total bacteria were counted in a total of 132 biopsies from a similar number of microscopic fields (Table S3). While the median number of bacteria per microscopic field was zero for all time points (range: 0–3), nonparametric analyses revealed higher numbers of bacteria during omeprazole administration (*P* = 0.0033). The sum of all counted bacteria during omeprazole administration was 40 bacteria (male dogs only: 34), while the median sum of all other time points was eight bacteria. All the observed bacteria were morphologically similar.
(i.e. rod-shaped, 2–3 μm long). It is possible that we underestimated bacterial populations using FISH owing to issues with probe penetration and/or over-fixation with formalin.

**Quantitative real-time PCR in duodenal biopsies**

There was a significant effect of omeprazole administration on the abundance of total duodenal bacteria \((P = 0.0003)\), but there was no difference between genders and there was no significant interaction between omeprazole administration and gender. Regardless of gender, there was a higher bacterial abundance on Day 15 during omeprazole administration when compared to Day \(-15\) before omeprazole administration \((P = 0.0295)\). Also, there was a higher bacterial abundance in the duodenum on Day 30 after omeprazole administration when compared to that on Day \(-30\) \((P = 0.0040)\) and Day \(-15\) \((P = 0.0009)\) before omeprazole administration (Fig. 3). In contrast to the pyrosequencing results that showed a decrease of *Helicobacter* spp. in the duodenum during omeprazole administration, the genus *Helicobacter* was detected only at six isolated time points in the duodenum of five dogs (three male and two female dogs). *Enterococcus* spp. was detected only in two male dogs during omeprazole administration on Day 15. There was a significant effect of omeprazole on duodenal *Lactobacillus* spp. \((P < 0.0001)\) with male dogs having a higher abundance of duodenal *Lactobacillus* when compared with female dogs \((P = 0.0168)\). Also, there was a significant interaction between omeprazole administration and gender \((P < 0.0001)\) (Fig. 3). The male dogs had a significantly higher abundance of *Lactobacillus* spp. in the duodenum during omeprazole administration when compared to all time points before and after omeprazole administration \((P < 0.0001\) for all multiple comparisons) (Fig. 3).

**Quantitative real-time PCR for the analysis of fecal microbiota**

One fecal DNA sample (from one female dog, Day \(-15\) before omeprazole administration) was not available and was treated as a missing value. All time points in all dogs were PCR negative for *C. difficile* and the *C. difficile* gene-encoding toxin B. *C. perfringens* was detected in all female dogs on Day \(-30\) before omeprazole administration only. Regardless of gender, there was a significant increase in fecal *Lactobacillus* during omeprazole administration when compared with all other time points \((P < 0.0001, \text{Fig. 4})\). This increase in *Lactobacillus* was accompanied, in the male dogs only, by a decrease of *Faecalibacterium* and the *Bacteroides-Prevotella-Porphyromonas* group (Fig. 4).

![Fig. 3. Quantitative real-time PCR results for total duodenal bacteria (a) and *Lactobacillus* spp. (b) on Day 30 (D \(-30\)) and Day 15 (D \(-15\)) before omeprazole administration, the last day of omeprazole administration (D 15), and 15 days after the completion of omeprazole administration (D 30). Error bars represent the mean and the standard error. Horizontal brackets represent statistical significance \((P < 0.0001)\). *Significantly different \((P = 0.0295)\) than Day 15 before omeprazole administration (D \(-15\)), regardless of gender. †Significantly different than Day \(-15\) \((P = 0.0040)\) and Day \(-30\) \((P = 0.0009)\) before omeprazole administration, regardless of gender. qPCR data for *Lactobacillus* spp. were normalized to qPCR data for total bacteria.](image-url)
Discussion

PPIs and other suppressors of gastric acid secretion are used extensively in both human and veterinary patients with suspected disorders of the upper GI tract. Despite the widespread use of these compounds in dogs and the cumulative evidence suggesting an association between PPI use and GI infections in human patients, there are no studies to date that have evaluated the effect of PPIs or any other gastric acid suppressor on the composition of the canine GI microbiota. The results of this study suggest that orally administered omeprazole at a dose of 1.1 mg kg$^{-1}$ twice a day for 15 days can alter the quantitative composition of the gastric, duodenal, and fecal bacterial microbiota of healthy dogs.

In this study, omeprazole administration led to a decrease in gastric Helicobacter spp., an effect which was more evident on the quantitative FISH analysis. While a growing number of investigations suggest that PPIs can also lead to a decrease in the abundance of gastric H. pylori in humans, most studies have evaluated the effect of PPIs on this bacterium only in combination with other pharmaceuticals such as antibiotics (Graham & Fischbach, 2010; Luther et al., 2010; Wu et al., 2010). Also, the histological density of H. pylori in the gastric body and antrum of humans was reduced after 4 weeks of omeprazole treatment, while it was increased in the fundus (Logan et al.,...
1995). Other studies have confirmed this phenomenon (Ishihara et al., 2001). In the current study, we collected biopsies only from the gastric body and antrum, and therefore, we cannot confirm an overall decrease in gastric Helicobacter in all regions of the stomach. Moreover, the quantitative real-time PCR assay used in this study did not confirm the decrease in gastric Helicobacter spp. abundance during omeprazole administration, an effect suggested by both bTEFAP and FISH. It is possible that the qPCR assay used here does not detect all canine gastric species and strains of Helicobacter. For instance, while both the reverse primer and the oligo probe detect all Helicobacter spp. that have been isolated from the stomach of dogs (Neiger & Simpson, 2000), the forward primer may not detect H. bilis and Flexispira rappini (see Table S1). The latter may be especially relevant as it includes multiple Helicobacter taxa (Dewhirst et al., 2000). These observations raise the interesting question of whether the effect of omeprazole is different among different species and/or strains of gastric Helicobacter, a hypothesis that is indirectly supported by a recent study showing that the effect of pantoprazole (another PPI) on growth and morphology of bacteria was different among several strains of oral Lactobacillus spp. (Altman et al., 2008).

The mechanism by which omeprazole leads to a decrease in gastric Helicobacter is unclear and controversial (Canani & Terrin, 2010). Omeprazole could have an indirect effect by means of raising intragastric pH, which in turn could allow other non-Helicobacter bacteria to thrive. Alternatively, omeprazole may act directly by means of a direct bactericidal effect. For instance, it has been shown that omeprazole inhibits the growth of gram-positive and gram-negative bacteria in vitro, including H. pylori (Jonkers et al., 1996). More recent studies also support a direct effect of PPIs on H. pylori (Suzuki et al., 2003; Nakamura et al., 2007). This effect may be due to a direct effect on the proton pumps of the bacteria, as these enzymes have been identified at least in H. pylori (Melchers et al., 1998) and Streptococcus pneumoniae (Hoskins et al., 2001). Thus, it has been hypothesized that these enzymes of bacterial origin may serve as extrinsic sites of action for PPI therapy (Vesper et al., 2009). However, while much research has focused on H. pylori, dogs are not known to harbor this species in the stomach but other Helicobacter spp. such as H. felis and H. heilmannii (Neiger & Simpson, 2000; Shinozaki et al., 2002). To date, the effect of PPIs on other non-H. pylori gastric Helicobacter spp. has not been investigated.

The decrease in gastric Helicobacter abundance during omeprazole administration was accompanied by a higher relative abundance of other bacteria, especially Streptococcus, Lactobacillus, Fusobacterium, and Actinobacillus, as suggested by pyrosequencing. It is likely that other, non-Helicobacter bacteria were able to thrive in the stomach during the temporary reduction in intragastric acidity. It is also possible that some of these bacteria possess a direct antagonist effect against Helicobacter spp., as suggested by a recent study of the effect of two strains of Lactobacillus on H. pylori (Cui et al., 2010). However, it is not clear whether the bacteria that were found more abundantly during omeprazole administration were native to the stomach or foreign, for example, from the mouth and esophagus. One study suggested that the human stomach could contain its own distinct microbial ecosystem (Bik et al., 2006), but the authors warned that this observation was based on a comparison of gastric, oral, and esophageal bacterial communities from different subjects with different clinical syndromes.

In the duodenum, omeprazole led to an increased relative abundance in Lactobacillus and Enterococcus in the male dogs, which likely caused the observed higher abundance of all bacteria suggested by FISH analysis. In the past, an abnormal accumulation of bacteria in the small bowel of dogs was termed as small intestinal bacterial overgrowth (SIBO; Johnston, 1999), but the understanding of this phenomenon has undergone several advances (Hall, 2011), in part because of the complex microbial composition discovered in the canine small intestine (Mentula et al., 2005; Suchodolski et al., 2008a, b; Xenoulis et al., 2008; Suchodolski et al., 2009, 2010). In small animal veterinary medicine, small intestinal dysbiosis is a currently used term to define a clinical syndrome caused by an alteration, either qualitative, quantitative, or both, of one or more groups of the small intestinal microbiota. Although the observed changes in the composition of the duodenal microbiota during omeprazole administration may be considered a dysbiosis (from its baseline composition), its clinical significance remains to be determined.

In addition to the changes in the stomach and duodenum, our results also suggest that omeprazole can alter the composition of the fecal microbiota. Similarly, one recent study showed that orally administered omeprazole can lead to changes in fecal microbial communities of mice in a dose-dependent manner (Kanno et al., 2009). However, unlike the current study that showed a higher abundance of some fecal bacteria (e.g. Lactobacillus) accompanied by a lower abundance of other bacteria (e.g. Faecalibacterium and Bacteroides) during omeprazole administration, Kanno et al. showed that all groups of fecal bacteria (with the exception of Bifidobacterium) increased during omeprazole administration in mice (Kanno et al., 2009). Because omeprazole is metabolized by the hepatic cytochrome P450 system after absorption from the small intestine and about 80% of the metabolites are excreted in urine (Petersen, 1995), it is unlikely...
that omeprazole reaches the large intestine, at least in its native form. Thus, our results and the results reported by Kanno et al. suggest that it is the increase in the bacterial load entering the large intestine that is responsible for the changes observed in the fecal microbiota. Another factor affecting the fecal microbiota during inhibition of gastric acid could be the change in the composition of dietary protein reaching the large intestine (Zentek et al., 2003), as gastric acid plays a key role in the initial stages of protein digestion. It seems likely that both mechanisms contribute to the changes observed in the fecal microbiota. The decrease in Faecalibacterium during omeprazole administration in the male dogs is especially interesting, as these bacteria possess anti-inflammatory properties (Sokol et al., 2008) and have been found to be depleted during episodes of colitis in humans (Sokol et al., 2009).

Finally, the interaction between the effect of omeprazole on the GI bacterial microbiota and gender suggested in this study may deserve scrutiny in future studies. Interestingly, Zhang et al. (2006) showed that higher endogenous progesterone concentrations in women could have a stimulatory effect on the P450 3A (CYP3A) activity, which plays an essential role in the metabolism of omeprazole in the liver (Andersson et al., 1993, 1994). However, all the females in the current study did not show signs of their first heat season until weeks after the last sample collection, and it has been shown that bitches have undetectable serum concentrations of progesterone during anestrous (Hase et al., 1999).

In summary, this study suggests that orally administered omeprazole can alter the quantitative abundance of several bacterial communities throughout the GI tract of healthy dogs. Particularly, in this study, omeprazole administration was associated with a decrease in Helicobacter spp. and an increase of other bacteria in the stomach. Also, omeprazole administration was associated with higher numbers of total bacteria and an increase in Lactobacillus in the duodenum of the male dogs. Lastly, omeprazole led to an increase in fecal Lactobacillus, which was accompanied by a decrease in Faecalibacterium and the Bacteroides-Prevotella-Porphyromonas group in the male dogs. However, omeprazole administration was not associated with major qualitative changes in the phylogenetic composition of the stomach and the duodenum, as evaluated by Unifrac analysis of pyrosequencing results. Further studies are warranted to investigate the clinical significance of these findings.

Acknowledgements
Part of this investigation was presented in the form of an abstract in the Forum 2010 and 2011 of the American College of Veterinary Internal Medicine. The study was funded by the College of Veterinary Medicine at the University of Illinois and the Gastrointestinal Laboratory at Texas A & M University. The authors acknowledge Angie Otto and Megan Miller for their help during the animal phase of the study.

References


Supporting Information

Additional Supporting Information may be found in the online version of this article:

**Fig. S1.** Dendrogram illustrating the phylogenetic clustering of the gastric (GB: gastric biopsy) and duodenal bacterial microbiota.

**Fig. S2.** Dendrogram illustrating the phylogenetic clustering of the gastric microbiota.

**Fig. S3.** Serial images of gastric *Helicobacter* spp. throughout the vertical z-axis in one microscopic field from a gastric biopsy.

**Fig. S4.** Dendrogram illustrating the phylogenetic clustering of the duodenal microbiota.

**Fig. S5.** Percentage of sequences in the male (left) and the female (right) dogs at the class *Bacilli* (a), order *Lactobacillales* (b), and *Lactobacillus* on Day 30 (Day −30) and Day 15 (Day −15) before omeprazole administration, the last day of omeprazole administration (Day 15), and on Day 30 after discontinuation of omeprazole administration.

**Table S1.** Oligonucleotides used in this study for quantitative real-time PCR (qPCR) assays and fluorescent *in situ* hybridization (FISH).

**Table S2.** Median (interquartile range) proportions of pyrosequencing tags in the stomach on Day 30 (Day −30) and Day 15 (Day −15) before omeprazole administration, the last day of omeprazole treatment (Day 15), and 15 days after omeprazole treatment (Day 30).

**Table S3.** Number of microscopic fields analyzed for each gender for FISH analyzes in the gastric (stomach) and the duodenal (duodenum) biopsies.
Table S4. Median (interquartile range) proportions of pyrosequencing tags in the duodenum on Day 30 (Day $-30$) and Day 15 (Day $-15$) before omeprazole administration, the last day of omeprazole treatment (Day 15), and 15 days after omeprazole treatment (Day 30).

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