Localization of Helicobacter spp. in the fundic mucosa of laboratory Beagle dogs: an ultrastructural study

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Abstract
In dogs Helicobacter spp. are found in all gastric regions usually localized in the surface mucus, gastric glands and parietal cells. The aim of this study was to detail the distribution of Helicobacter spp. in the fundic mucosa of asymptomatic Beagle dogs and their intracellular localization within parietal cells, in order to evaluate species-specific pathogenetic effects on gastric cells. The presence of Helicobacter spp. was investigated by immunohistochemistry, TEM, and PCR in the fundic mucosa of six Beagle dogs. Helicobacter spp. were found in all dogs examined, and H. bizzozeronii and H. felis were identified by PCR and confirmed by TEM. In the lumen of the fundic glands, co-localization was common. H. bizzozeronii was present in larger numbers than H. felis in both intraluminal and intraparietal localization. The amounts of H. bizzozeronii were similar in superficial and basal portions of the glands. H. felis was predominantly localized in the superficial portions of gastric glands but almost absent from the base. Within parietal cells, most Helicobacter organisms were intracanalicular, but intact and degenerate Helicobacter organisms were also visualized free in the cytoplasm or in secondary lysosomes. No specific degenerative lesions were found in infected parietal cells. Helicobacter organisms were also observed within macrophages in the lamina propria. In conclusion, there is a differential distribution of H. bizzozeronii and H. felis in the fundic mucosa of Beagle dogs, and their intracellular localization in parietal cells and macrophages suggests novel pathogenic scenarios for the development of immune response and maintenance of chronic gastritis in dogs.

Introduction
Gastric colonization by spiral-shaped bacteria of the genus Helicobacter is common in dogs and to date several species of Helicobacter have been identified in the canine stomach, including Helicobacter felis, Helicobacter bizzozeronii, Helicobacter salomonis, “Candidatus Helicobacter heilmannii”, Helicobacter bilis, Helicobacter (Flexispira) rappini, and Helicobacter cynogastricus [1-6]. Dogs may be infected with more than one species of Helicobacter that are indistinguishable on light microscopy, appearing as large tightly coiled spiral-shaped bacteria [7]. Different species of canine gastric Helicobacter can be categorized by their morphological features (i.e. size, number and degree of tightness of the spirals, presence of periplasmic fibrils, number and position of flagella) using transmission electron microscopy (TEM). In addition, in the early work of Lockard and Boler (1970) morphologically different organisms were classified as Lockard types 1 to 3 [8,9].

The regional distribution of Helicobacter spp. throughout the canine stomach (i.e. cardia, fundus, and pylorus) is well studied [7], but knowledge of the distribution of different Helicobacter spp. within these regions is incomplete. Helicobacter organisms in dogs reside in the surface mucus, gastric pits, and gastric glands, colonizing both the lumen and the gastric cells. In particular, canine and feline Helicobacter spp. (as “Candidatus H. heilmannii” in humans) have great predilection for parietal cells of the fundic mucosa, where they are usually described to colonize the intracellular canaliculi and sporadically the

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cytoplasm [7,10-14]. Since the virulence of many pathogenic bacteria is directly related to invasion it is possible that the development of Helicobacter associated gastritis in dogs is related to their intracellular localization (intracanalicular or intracytoplasmic) or possibly invasion of the lamina propria through the basal lamina, as reported for H. pylori, which is now considered as an invasive and facultative intracellular organism [15-17]. The ability of Helicobacter spp. infection in dogs to establish an intracellular niche may also vary by species or even strain, and this may impact on their pathogenicity and ability to evade antibiotics typically employed to treat Helicobacter gastritis in dogs [18].

The aim of this study was to gain insight into the distribution and localization of distinct Helicobacter spp. in the lumen of gastric glands and within parietal cells in the fundic mucosa, using a limited number of asymptomatic Beagle dogs. This was done by applying a combination of morphological (immunohistochemical and ultrastructural) and molecular methods (species-specific PCR) and allowed to evaluate the presence of distinct colonization niches and possible species-specific pathogenetic effects on gastric cells.

Materials and methods

Animals and sampling

Three male (No. 1, 2, 3) and three female (No. 4, 5, 6) Beagle dogs (Marshall Beagles), purchased from Greenhill Italy (Montichiari, Brescia, Italy) were investigated. The dogs were 8-9 months old, and were part of a general toxicology study performed in GlaxoSmithKline S.p.A., where they served as the control group. They were housed singly or in pairs, in standard husbandry and environmental conditions. All the work involving animals was carried out in accordance with Italian regulation governing animal welfare and protection (Legislative Decree n. 116/1992), the European Directive 86/609/EEC, and according to the internal GlaxoSmithKline Committee on Animal Research & Ethics (CARE) review, with related codes of practice.

Dogs were euthanized by intravenous injection of barbiturate. Prior to necropsy, the animals were deprived of food overnight. The stomach was opened along the inner curvature and macroscopically examined. Three samples were taken from the body (greater curvature) region from each dog: one was snap frozen in liquid nitrogen and stored at -80°C for PCR analysis; one was fixed in 10% buffered formalin for histopathology; one was fixed in 2.5% glutaraldehyde in 0.1 mol/L cacodylate buffer pH 7.2, containing 0.01 mol/L CaCl₂ for at least 12 h at 4°C for ultrastructural examination.

DNA Extraction and PCR amplification

DNA was extracted using DNeasy Tissue kit (QIAGEN, GmbH D-40724, Hilden, Germany) according to the manufacturer's instructions. Primers used in this study for the amplification of Helicobacteraceae, canine gastric Helicobacter spp. (H. bizzozeronii, H. salomonis and H. felis), canine enterohepatic Helicobacter spp., H. pylori, “Candidatus H. heilmannii”, H. felis, and H. bizzozeronii are listed in Table 1.

All PCR reactions were performed in an Eppendorf thermocycler (Mastercycler gradient, Eppendorf GA, Hamburg, Germany) in a final volume of 25 μL containing 1 μL of extracted DNA, 12.5 μL of PCR Master Mix (Promega, Madison, Wisconsin, USA), and 0.5 μM of each primer (Integrated DNA Technologies, Coralville, IA, USA). Ten microliters of each amplification product were analyzed by gel electrophoresis in 1% agarose gels in Tris-acetate EDTA buffer (TAE). The gel was stained with ethidium bromide (0.5 mg/L) and examined under UV transilluminator for the presence of amplified DNA; the size of the expected fragment was compared to a 100 bp reference marker.

Table 1 Oligonucleotide primers used in this study

| Primer | Sequence | Target sequence | Reference |
|--------|----------|-----------------|-----------|
| C97    | 5’-GCT ATG ACG GGT ATC C-3’ | 16S rRNA gene Helicobacteraceae | [19] |
| C05    | 5’-ACT TCA CCC CAG TGC CTG-3’ | 16S rRNA gene Helicobacteraceae | [19] |
| CAR557f| 5’-TGC GTA GGC GGG GGT GTA AG-3’ | 16S rRNA gene H. felis, H. bizzozeronii, H. salomonis | [20] |
| CAR636r| 5’-CAG AGT TGT AGT TTC AAA TGC-3’ | 16S rRNA gene H. felis, H. bizzozeronii, H. salomonis | [20] |
| H. pylori 1 | 5’-GGA ATT CCA GAT CTA TGA AAA AGA TTA GCA GAA AAG-3’ | Urease B gene H. pylori | [21] |
| H. pylori 2 | 5’-GGA ATT CGT CCA CCT AGA AAA TGC TAA AAG GGT GTT G-3’ | Urease B gene H. pylori | [21] |
| Heil 1F | 5’-GGG CCA TAA AGT GGC CTT AGG-3’ | Urease B “Candidatus H. heilmannii” | [21] |
| Heil 2R | 5’-CTG GTC GTA GAT GAC AGG-3’ | Urease B “Candidatus H. heilmannii” | [21] |
| H. felis 1 | 5’-ATG AAA CTA ACG CCT AAA GAA CTA G-3’ | Urease B gene H. felis | [21] |
| H. felis 2 | 5’-GGA GAG ATA AAG TCA ATA TGC GT-3’ | Urease B gene H. felis | [21] |
| H. bizz 1 | 5’-GAA GTC GAA CAT GAC TGC AC-3’ | Urease B gene H. bizzozeronii | [5] |
| H. bizz 2 | 5’-GTT CAC ATT AGC CCC ATC AG-3’ | Urease B gene H. bizzozeronii | [5] |
| HelINTF1 | 5’-TGA ATG CTA GTT GTC GCC CTG CTT G-3’ | 16S rRNA gene enterohepatic Helicobacter spp. | [22] |
| HelINTR1 | 5’-CTC CCT TAG ATG GCT CAG CCG AAC T-3’ | 16S rRNA gene enterohepatic Helicobacter spp. | [22] |
(Fermentas Inc, Maryland, USA). Negative controls in which the DNA extract was omitted were included with each reaction.

**Helicobacteraceae PCR**

The 16S rRNA gene of members of the *Helicobacteraceae* family was amplified by PCR using primers C97/C05 [19] generating a 1200 bp amplicon. For C97/C05 amplification samples were heated to 94°C for 10 min, followed by 30 amplification cycles of denaturation at 94°C for 1 min, primer annealing at 58°C for 1 min and 30 s, and extension at 72°C for 2 min, with a final extension at 72°C for 10 min.

**Gastric Helicobacter spp. PCR**

Primers CAR557f and CAR636r were used to amplify a 78 bp fragment of the 16S rRNA gene of the canine gastric *Helicobacter* species (*H. bizzozeronii, H. salomonis* and *H. felis*) [20]. For CAR557f/CAR636r amplification samples were heated to 94°C for 9 min, followed by 35 amplification cycles of denaturation at 94°C for 30 s, primer annealing at 60°C for 30 s, and extension at 72°C for 45 s, with a final extension at 72°C for 5 min. Additionally, all gastric samples were investigated using species-specific primers for *H. pylori*, “Candidatus H. heilmannii”, *H. felis*, and *H. bizzozeronii* urease B gene amplification, as previously reported [5,21].

**Enterohpetic Helicobacter spp. PCR**

Primers HelINTF1 and HelINTR1 were used to amplify a 345 bp fragment of the 16S rRNA gene of the canine *Helicobacter* species [22]. For HelINTF1/HelINTR1 amplification samples were heated to 94°C for 2 min, followed by 27 amplification cycles of denaturation at 94°C for 30 s, primer annealing at 60°C for 30 s, and extension at 72°C for 30 s, with a final extension at 72°C for 7 min.

**Histopathology**

For light microscopy examination, formalin-fixed samples were embedded in paraffin wax, sectioned at 4 μm thickness, and stained with hematoxylin and eosin (HE).

For immunohistochemistry (IHC), formalin-fixed, paraffin-embedded 4 μm sections were deparaffinized, rehydrated and treated with 3% hydrogen peroxide in distilled water for 20 min. The sections were labelled by the avidin-biotin-peroxidase (ABC) procedure [23] with a commercial immunoperoxidase kit (Vectastain Standard Elite; Vector Laboratories, Burlingame, CA, USA). A polyclonal rabbit anti-*H. pylori* antibody (Dako, Glostrup, Denmark) was applied at a working dilution of 1:1000 at 4°C overnight. The immunohistochemical reaction was developed with 3,3’ dianinobenzidine (DAB) for 1 min and sections were counterstained for 1 min with Mayer’s haematoxylin. Negative controls for each sample were prepared by replacing the primary antibody with PBS containing 10% normal goat serum.

Known positive control sections were included in each immunolabeling assay.

Samples were examined for the presence of inflammation (infiltrates of lymphocytes, plasma cells, and macrophages) using a semiquantitative scoring system as follows: 0 (absent), +1 (mild), +2 (moderate), +3 (marked).

Immunostained sections were scored for the presence of *Helicobacter* antigen within the parietal cells (P) and in the lumen (L) of gastric glands using a semiquantitative scoring system as follows: 0 (absence of *Helicobacter* antigen in the parietal cell/lumen of the gastric glands); +1 (presence of *Helicobacter* antigen in the parietal cell/lumen of < 5% of the gastric glands); +2 (presence of *Helicobacter* antigen in the parietal cell/lumen of 5-50% of the gastric glands); +3 (presence of *Helicobacter* antigen in the parietal cell/lumen of > 50% of the gastric glands) and extending to the base of the glands).

**Transmission electron microscopy (TEM)**

Glutaraldehyde fixed samples were transferred into phosphate buffer after 72 h, postfixed for 90 min in 1% osmium tetroxide (wt/vol), and then stained with 2% (wt/vol) uranyl acetate in barbiturate buffer pH 7.3 for 60 min. After dehydration and embedding in Epon (glycride ether 100, Merck, Darmstadt, Germany), 1 μm-thick sections were stained with toluidine blue and the areas of interest were selected. Thin sections were stained with uranyl acetate and lead citrate. Electron microscopy was carried out using a Philips Morgagni™ 268D transmission electron microscope (FEI Company, Eindhoven, The Netherlands) operating at 90 kV.

*Helicobacter* spp. were identified according to their morphological features (size, number of spirals, presence of periplasmic fibrils, number and position of flagella) [2,3,24]. The distribution and number (bacterial load) of *Helicobacter* spp. was evaluated in 10-20 gastric glands and in 4-8 parietal cells of the fundic mucosa from 3 dogs (No. 1-3). The superficial (pit/isthmus/neck) and basal portions of the gastric glands were investigated as distinct colonization sites and the number of distinct morphological types of *Helicobacter* spp. was evaluated within parietal cells (including intracanicular and intracytoplasmic compartments).

**Statistical analysis**

Differences in regional distribution of intraluminal *Helicobacter* load between superficial (pit/isthmus/neck) and basal portions of the gastric glands were evaluated using the Mann-Whitney test. Differences in *Helicobacter* species (*H. bizzozeronii*-like or *H. felis*-like organisms) load in the same location (superficial and basal portions of the gastric glands, and intraparietal localization) were
evaluated using the Wilcoxon signed-rank test. Statistical significance was set at $P = 0.001$.

**Results**

**PCR**

*Helicobacteraceae* and canine gastric *Helicobacter* spp. DNA was amplified in all fundic samples from all six dogs. Species-specific *Helicobacter* PCR detected *H. bizzozeronii* and *H. felis* in all six dogs. PCR for “*Candidatus* *H. heilmannii*”, *H. pylori* and enterohepatic *Helicobacter* spp. was negative for all samples.

**Gross pathology and histopathology**

No gross lesions were observed in the stomach of the dogs. Histologically, mild chronic inflammation was present in the fundic mucosa of 5 of 6 dogs (Table 2). Focal to multifocal infiltrates of small numbers of lymphocytes, plasma cells, and macrophages were found in the lamina propria, mainly localized in the deepest portion of the mucosa, close to the muscularis mucosae. In HE-stained sections, ill-defined elongated bacteria consistent with *Helicobacter* spp. were visible at high power magnification (400×) in the mucus covering the surface epithelium, the gastric pits, lumen of gastric glands, and occasionally in parietal cells. In all dogs, most parietal cells were morphologically normal; however, small numbers of parietal cells with a large cytoplasmic vacuole containing bacterial organisms were found, localized mainly in the upper third of the gastric gland, around the isthmus region.

The degree of *Helicobacter* spp. colonization of the stomach assessed by IHC is summarized in Table 2. Positive immunostaining of *Helicobacter* antigen was characterized by a coarse granular brown precipitate easily detectable against the light blue background. Moderate to large numbers of *Helicobacter* spp. were visible in the fundic mucosa, in particular the *Helicobacter* antigen was found in the mucus covering the surface epithelium, the gastric pits, lumen of gastric glands, and intracellularly within parietal cells, multifocally contained in large vacuoles. There was no difference in *Helicobacter* colonization density between the lumen and parietal cells. Within parietal cells, the *Helicobacter* antigen was visible as 1-2 μm in diameter, well demarcated, brown dots or less frequently as a fine brown granular staining diffuse to the whole cytoplasm. Occasionally, well defined spiral shaped organisms were visible in parietal cells (Figure 1).

**Transmission electron microscopy (TEM)**

Bacteria were identified by TEM in the fundic mucosa from all the dogs examined. They appeared as numerous, 4-8 μm in length × 0.6-0.8 μm in width, tightly to loosely coiled spiral-shaped bacteria with or without periplasmic fibrils, and with bipolar flagellar tufts (composed of up to six flagella) consistent with two types of large canine gastric *Helicobacter* spp. According to the results of PCR and aforementioned ultrastructural features they were identified as *H. bizzozeronii*-like and *H. felis*-like (Figures 2A and 2B).

In the superficial mucus layer, low numbers of free-floating *Helicobacter* spp. were seen, occasionally admixed with bacteria with disrupted walls and variably condensed or vacuolated cytoplasm (degenerated bacteria). In the lumen of the fundic glands, co-localization of the two morphologically distinct *Helicobacter* spp. was common. *Helicobacter* spp. load in the lumen of gastric glands and parietal cells is summarized in Table 3. Intraluminal load of total *Helicobacter*-like organisms and *H. bizzozeronii*-like organisms was similar in superficial and basal portions of the glands ($P = 0.4146$ and $P = 0.5817$), while the load of *H. felis*-like was significantly higher in the superficial portions compared to the base ($P = 0.0007$). Both in the superficial and basal portions, there were significantly more *H. bizzozeronii*-like organisms than *H. felis*-like organisms ($P < 0.0001$). In parietal cells, there were significantly more *H. bizzozeronii*-like organisms than *H. felis*-like organisms ($P = 0.0004$).

Intraluminal bacteria were localized close to the luminal surface of gastric epithelial cells and the luminal pole of the intracanalicular system of parietal cells. Although *H. felis*-like organisms were often in contact with parietal cell membranes and microvilli, no adhesion to cellular membranes, pedestal formation, actin condensation or disruption of intercellular junctions were found. Within parietal cells, electrondense structures morphologically consistent with bacterial organisms were frequently identified. In most cases, bacteria were found in the intracellular portion of the canalicular system (Figure 2C). Both well preserved spiral-shaped forms, with or without periplasmic fibrils, and rounded bodies (transverse sections of bacteria) were observed (Figure 2C). Bacteria were also present within the

![Table 2](http://www.veterinaryresearch.org/content/42/1/42)

**Table 2 Gastric inflammation and *Helicobacter* spp. colonization of fundic mucosa in 6 laboratory Beagle dogs infected by *Helicobacter* spp.**

| Dog no. | Inflammation (0-3) | *Helicobacter* spp. colonization (IHC) (0-4) |
|---------|-------------------|--------------------------------------------|
|         |                   | **L** | **P** |
| 1       | 0                 | 3     | 3     |
| 2       | 1                 | 3     | 3     |
| 3       | 1                 | 4     | 4     |
| 4       | 1                 | 3     | 3     |
| 5       | 1                 | 4     | 4     |
| 6       | 1                 | 3     | 3     |

$L$ = lumen of gastric glands.  
$P$ = parietal cells.
cytoplasm of parietal cells enveloped by a closely apposed membrane, occasionally focally disrupted, or free in the cytoplasm or within secondary lysosomes (Figure 2D). Condensation or lysis of bacterial structures consistent with bacterial degeneration was occasionally visualized within the cytoplasm of parietal cells (Figure 2E). In both colonized and uncolonized parietal cells, secondary lysosomes were often present and degenerating swollen mitochondria were occasionally observed. Macrophages in the lamina propria of fundic mucosa had numerous secondary lysosomes similar to those detected in parietal cells, and, rarely, intracellular *Helicobacter* organisms were found in their cytoplasm. In a single case, *Helicobacter* organisms were in association with remnants of an entire phagocytized cell (Figure 2F).

**Discussion**

*Helicobacter* spp. were identified by histology, IHC, TEM, and PCR in the fundic mucosa of all the dogs examined in this study, confirming the high prevalence (100%) of the infection in healthy laboratory Beagle dogs [1,12,25]. *Helicobacter*-like organisms were visualized in the surface mucus, lumen of gastric glands (extending from the gastric pit to the base), and within parietal cells, but not within mucous epithelial cells and chief cells, in agreement with previous studies [12,13,26]. On the basis of species-specific PCR, all of the dogs were found to be coinfected with *H. bizzozeronii* and *H. felis* and this finding was supported by TEM, which showed two morphologically distinct *Helicobacter* types, *H. bizzozeronii*-like and *H. felis*-like, in the fundic mucosa. Coinfection with *H. bizzozeronii*-like and *H. felis*-like organisms, has been previously observed in laboratory Beagles [12,25].

In depth analysis of TEM images suggests that these *Helicobacter* species are adapted for colonization of distinct niches in the fundic mucosa. In the lumen of fundic glands, co-localization of the two distinct morphologic types was common. *H. bizzozeronii*-like organisms colonized the glands from the pit to the base, while *H. felis*-like organisms were localized in the more superficial portions of the gastric glands (pit, isthmus and neck regions), but virtually absent from the base. In all dogs and portions of gastric glands evaluated, *H. bizzozeronii*-like organisms were significantly more abundant than *H. felis*-like organisms. Within parietal cells, *H. bizzozeronii* was also the most common species, and this can be simply the result of the lower abundance of *H. felis* in the glands of these dogs or a consequence of the lower affinity of *H. felis* towards the parietal cells compared to *H. bizzozeronii*. The latter hypothesis is supported by the presence of higher proportion of *H. felis*-like organisms in the glands versus parietal cells (*H. felis* in glands/parietal cells = 4.05) compared to *H. bizzozeronii*-like organisms (*H. bizzozeronii* in glands/parietal cells = 1.49). Altogether, these findings may indicate that *H. bizzozeronii* could be more host-adapted than *H. felis* in dogs.

Some previous studies have suggested that *H. felis* is more pathogenic than *H. bizzozeronii* in dogs and also experimentally in mice and gerbils, resulting in necrosis and/or apoptosis of parietal cells with subsequent parietal cell loss, whereas *H. bizzozeronii* appears non pathogenic even in cases of heavy bacterial load [27-30]. In the present study, however, there were no specific and relevant ultrastructural parietal cell degenerative lesions with either of *Helicobacter* species, but the lack of cell damage could also be related to the limited sampling of the fundic mucosa that was performed in this study. Additionally, no evidence of tight attachment to gastric epithelial cells and no intercellular invasion by canine gastric *Helicobacter* spp. were found and this contrasts...
with *H. pylori* which is commonly found to attach to the cell membrane of epithelial cells by different forms of adhesive structures (i.e. pedestals or membrane fusion), and invade intercellular space with disruption of intercellular junctions [15,17,31]. Although there was no attachment to the cell membrane, *H. felis*-like organisms were often in direct but loose contact with the plasma cell membranes and microvilli of parietal cells, confirming previous ultrastructural observations performed in dogs and gerbils [27,30].

Canine and feline *Helicobacter* spp., as well as "*Candidatus* H. heilmannii" in humans, have greater affinity to
parietal cells than does *H. pylori* in man, and are usually found in their intracellular canaliculi [7,10-13,27,32,33]. Also in the present study most of the intracellular organisms were intracanalicular, but cross-sections of *Helicobacter* organisms or degenerating bacterial fragments were found directly free in the cytoplasm of parietal cells, in association with secondary lysosome-like structures, or occasionally enveloped by a closely apposed membrane which could be focally interrupted resulting in the partial penetration of the organism into the cytoplasm. These ultrastructural findings are consistent with a true intracytoplasmic localization, often associated with bacterial degradation. It is not clear how *Helicobacter* organisms penetrate into the cytoplasm of parietal cells, and whether the intracytoplasmic organisms are associated with parietal cell degeneration, since only sporadic degenerative alterations of organelles were found in both infected and uninfected parietal cells.

By IHC, intracellular *Helicobacter* antigen within parietal cells appeared as well demarcated dots or diffuse cytoplasmic staining of parietal cells. Based on the ultrastructural findings, the immunohistochemical dots are likely the cross-sections of intracellular bacteria or their degrading forms, while the diffuse cytoplasmic staining could be the result of cytoplasmic bacterial degradation and dispersion of bacterial contents (including antigens) throughout the cytoplasm.

Sporadically, *Helicobacter* spp. and their fragments were found by TEM, but not by IHC, in the cytoplasm of macrophages in the lamina propria of the fundic mucosa. The presence of *Helicobacter* organisms within macrophages can be the result of phagocytosis of bacteria released in the extracellular space after parietal cell necrosis or phagocytosis of degenerated parietal cells containing intracellular bacteria, as suggested by the presence of intracytoplasmic remnants of an entire phagocytized cell in association with *Helicobacter* organisms within a macrophage found in the lamina propria. Direct invasion of lamina propria with phagocytosis by macrophages seems less likely the mechanism since no evidence of transepithelial invasion was found. To our knowledge, canine *Helicobacter* spp. have not been so far described within macrophages, in contrast to *H. pylori* which has been described to invade the lamina propria and translocate via macrophages to gastric lymph nodes, leading to the chronic stimulation of the immune system and thus contributing to the maintenance of chronic gastritis in people [15-17,34].

In the fundic mucosa of this small group of Beagle dogs, we observed high levels of mucosal *Helicobacter* colonization but clinical signs of gastritis were absent and only mild chronic lymphoplasmacytic gastritis was detected. The absence of a correlation between *Helicobacter* colonization density, severity of inflammation, and clinical signs has been reported by several studies [7,13,14,26,35].

Previous studies have demonstrated that *Helicobacter* infection in dogs induces a specific systemic humoral response with reported seroconversion after infection and a Th1-biased local immune response [14;27,35], but the mechanisms of activation of the immune response in association with canine *Helicobacter* spp. infection have been so far not investigated. The intracytoplasmic localization of canine *Helicobacter* spp. in parietal cells and macrophages in the lamina propria disclose novel pathogenic scenarios for the development of the cell-mediated
...and humoral immune response and maintenance of chronic gastritis in dogs. Activation of the immune response might be related to an antigenic stimulation of CD4+ T cells either by direct epithelial cell presentation of Helicobacter antigens in combination with induced expression of class II MHC and B7 costimulatory molecules, or by lamina propia macrophages after phagocytosis of Helicobacter spp. secondary to parietal cell necrosis, with degradation and antigen presentation directly to immunocompetent cells recruited in the lamina propria or after nodal migration, similarly to what was recently suggested for the pathogenesis of chronic gastritis elicited by H. pylori in humans [17,34,36].

In conclusion, we found that morphologically different Helicobacter species tend to differ in their abundance and regional distribution in the fundic mucosa. Free mixing of Helicobacter spp. was observed all along the length of gastric glands. The amounts of H. bizzozeronii were similar in superficial and basal portions of the glands. Overall H. bizzozeronii was more abundant than H. felis in both intraluminal and intraparietal location. H. felis was predominantly localized in the superficial portions of the gastric glands and was almost absent from the base. We also showed a true intracytoplasmic localization of both viable and degenerate Helicobacter organisms in parietal cells (within lysosomes and free in the cytoplasm), and rarely in macrophages in the lamina propria. The intraparietal and intramacrophagic presence of Helicobacter spp. are of interest in elucidating the mechanisms involved in the modulation of the immune response against gastric Helicobacter spp. in affected dogs. However, despite the high levels of colonization and ultrastructural findings, no clinical signs and only mild histological gastritis were observed, suggesting that immune tolerance might be involved in canine Helicobacter spp. infection.

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Authors’ contributions
AL carried out the ultrastructural studies, and drafted the manuscript. IF performed part of the molecular genetic studies. KS performed part of the molecular genetic studies and the statistical analysis. ES participated in the design of the study and critically revised the manuscript. CR participated in the design of the study, carried out the histopathological studies and co-drafted the manuscript. All authors read and approved the final manuscript.

Competing interests
The authors declare that they have no competing interests.

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