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Presence of *Helicobacter* spp. and *Fusobacterium gastrosuis* in the stomach of domestic and wild animals: an epidemiological and molecular study

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Presence of *Helicobacter* spp. and *Fusobacterium gastrosuis* in the stomach of domestic and wild animals: an epidemiological and molecular study

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Declaration:

In accordance to the disposed in “nº 2, alínea a, do Art.º 31º do Decreto-Lei nº 230/2009”, the author of this thesis declares to have actively participated in the elaboration and execution of experimental work that led to the results presented, as well as in its interpretation and in the writing of the respective manuscripts.

List of Publications:

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Flores AR, Lobo J, **Nunes F**, Rêma A, Lopes P, Carvalho L, Bartosch C, Amorim I, Gärtner F. Canine ovarian gonadoblastoma with dysgerminoma overgrowth: a case study and literature review. J Ovarian Res. 2019 Sep 23;12(1):89. doi: 10.1186/s13048-019-0561-x. PMID: 31547830; PMCID: PMC6757443.

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“Para ser grande, sê inteiro: nada
Teu exagera ou exclui.
Sê todo em cada coisa. Põe quanto és
No mínimo que fazes.
Assim em cada lago a lua toda
Brilha, porque alta vive.”

In Odes de Ricardo Reis. Fernando Pessoa.

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Abstract

The *Helicobacter* genus is a group of spiral-shaped motile Gram-negative bacteria, composed of at least 75 described species that can colonize the gastrointestinal tract of humans and animals. *Helicobacter pylori* (*H. pylori*) was the first species described in 1983. Nowadays, it is well known that *H. pylori* infects over half the global population causing several and potentially severe gastric pathologies in humans. Nevertheless, within this genus, there are other important bacteria, namely non-*Helicobacter pylori* helicobacters (NHPHs), such as *H. suis*, *H. felis*, *H. salomonis*, *H. bizzozeronnii, H. ailurogastricus*, *H. heilmannii* also associated with the development of gastrointestinal disease in humans and animals (dogs, cats, pigs and wild animals). In fact, in swine, *H. suis* in association with *Fusobacterium gastrosuis* (*F. gastrosuis*) may have a synergetic role in gastric ulcer pathophysiology, seriously affecting animal production and welfare and causing significant economic losses.

The primary source of NHPH infections in humans is most likely direct or indirect contact with infected animals. Therefore, animal contact remains a risk factor.

The present study aimed to investigate the presence of *Helicobacter* spp. and *F. gastrosuis* in the stomach of domestic (pigs) and few wild animals' species (wild boars, wild rabbits and quails) and, whenever possible, the presence of these bacteria and associated gastric histopathological changes in these hosts was assessed.

The occurrence of NHPH and *F. gastrosuis* in pigs and wild boars and its association with gastric histopathological alterations was confirmed through microscopic and molecular analysis. Gastritis was found in 95.8% of the pig samples and 71.4% of the wild boar samples. *Helicobacter* spp. DNA was detected in 78.9% and 64.3% of the gastric pig and wild boar samples, respectively. The presence of *F. gastrosuis* DNA in the stomach of pigs and wild boars was 35.2% and 42.9%, respectively. It is also reported the presence of NHPH in 91.7% of the wild rabbits and the presence of NHPH in 100% in wild quails with one positive sample for *H. pylori*.

Considering the zoonotic potential of some of the described bacteria and the increasing public health concern regarding antimicrobial resistance, it was described that antimicrobial resistance (AMR) to metronidazole and tetracyclines may occur in *H. pylori*-like organisms from pigs, where one sample was positive for *frxA* gene and two samples were positive for *16S rRNA* mutation gene.

Lastly, there may be limited knowledge and awareness of Portuguese veterinarians regarding *Helicobacter* spp., namely *H. suis* and *H. pylori* as a result of the analysis done to the questionnaire applied to Portuguese veterinarians.

Overall, a high prevalence of *Helicobacter* spp. in the analyzed pig gastric samples collected from Portuguese abattoirs was found. Also wild boars may be affected by *F. gastrosuis*. In addition, since some of the *H. pylori*-like bacteria detected in pig gastric samples may carry antimicrobial resistance genes, the need to improve awareness among Portuguese veterinarians regarding these bacteria is emphasized since these professionals play a unique and important role as public health promoters.

Resumo

O género *Helicobacter* é um grupo de bactérias helicoidais Gram-negativas, composto por pelo menos 75 espécies diferentes que podem colonizar o trato gastrointestinal de humanos e animais. *Helicobacter pylori* (*H. pylori*) foi descrita pela primeira vez em 1983. Sabe-se que *H. pylori* infecta mais de metade da população global e causa várias patologias gástricas graves em humanos. Contudo, existem outras helicobactérias gástricas importantes, nomeadamente não-*Helicobacter pylori* helicobacters (NHPHs), tais como *H. suis*, *H. felis*, *H. salomonis, H. bizzozeronnii*, *H. ailurogastricus* e *H. heilmannii*. As NHPHs também estão associadas ao desenvolvimento de doenças gastrointestinais em humanos e animais (como cães, gatos, porcos e animais selvagens). De facto, em suídeos, *H. suis* esta relacionada a patologias gástrica e quando associada com *Fusobacterium gastrosuis* (*F. gastrosuis*) pode ter um papel sinérgico no desenvolvimento de úlceras gástricas com repercussões serias na produção e bem-estar animal que se refletem em perdas económicas significativas.

A principal via de infeção por NHPH em humanos é, provavelmente, o contato direto ou indireto com animais infetados, sendo por isso a convivência e o contato com animais vistos como fatores de risco significativos.

O objetivo deste trabalho foi determinar a presença de *Helicobacter* spp. e *F. gastrosuis* no estomago de animais domésticos (porcos) e algumas espécies de animais selvagens (javalis, coelhos e codornizes) e quando possível, investigar a presença destas bactérias e a sua associação com alterações gástricas histopatológicas

A ocorrência de NHPHs e *F. gastrosuis* em porcos e javalis, bem como a sua associação com patologia gástrica nestas espécies utilizando técnicas de diagnóstico molecular e histológicas. Sendo que 95,8% e 71,4% das amostras gástricas de porco e javali, respetivamente, apresentavam gastrite. Foi detetado DNA de espécies de *Helicobacter* em 78,9% das amostras gástricas de porco e 64,3% das amostras gástricas de javali. Também foi detetado DNA de *F. gastrosuis* em 35,2% e 42,9% das amostras gástricas de porcos e javalis, respetivamente. Já nos coelhos e codornizes selvagens, 91,7% das amostras gástricas dos coelhos selvagens continham DNA de NHPH enquanto 100% das amostras de codornizes selvagens continham DNA de NHPH e uma das amostra foi positiva para DNA de *H. pylori*.

Considerando o potencial zoonótico de algumas das bactérias descritas e a crescente preocupação relativamente à resistência aos antibióticos, foi ainda possível descrever que a resistência antimicrobiana ao metronidazol e à tetraciclina pode ocorrer em *H. pylori*-like detetada em suínos pela deteção de uma amostra positiva para a presença do gene *frxA* e duas amostras positivas para a presença de mutação do gene *16s rRNA*.

Este estudo permitiu ainda evidenciar através de um inquérito por questionário aplicado aos médicos veterinários portugueses que estes poderão estar pouco sensibilizados relativamente a infeções de *Helicobacter* spp. em animais, nomeadamente *H. suis* e *H. pylori.*

No geral, este estudo teve como objetivo realizar uma abordagem integrativa concluindo que existe uma elevada prevalência de *Helicobacter* spp. nas amostras gástricas de suínos domésticos analisadas recolhidas em matadouros portugueses e que os javalis podem ser infetados por *F. gastrosuis*. Já os coelhos selvagens e codornizes selvagens podem atuar como reservatórios para as espécies de *Helicobacter* estudadas. Além disso, algumas das bactérias *H. pylori*-like de amostras de suínos domésticos podem ser portadoras de genes de resistência antimicrobiana, o que pode reforçar a importância de melhorar a sensibilização dos médicos veterinários portugueses para esta temática, uma vez que desempenham um papel importante como promotores de saúde pública, apesar do seu conhecimento limitado sobre infeções animais por *Helicobacter* spp..

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List of Abbreviations

AMR – Antimicrobial resistance

ARG – Antimicrobial resistance gene

***babA* –** Blood group antigen binding adhesion

BLBLI – Beta-lactam and beta-lactamase inhibitor

***cagA* –** Cytotoxin associated gene A

DNA – Deoxyribonucleic acid

***dupA* –** Duodenal ulcer promoting gene A

ECDC – European Center for Disease Prevention and Control

fiapA – Immunosuppressive protein A

fldA - *Flavadoxin*

FVE – Federation of Veterinarians of Europe

GGT – Gama glutamyl transpeptidase

glmM – *Phosphoglucosamine mutase*

gyrA – *Gyrase subunit A*

gyrB – *Gyrase subunit B*

hefC – *Multidrug exporter*

IARC – International Agency for Research on Cancer

MALT – Mucosa-associated lymphoid tissue

mdaB - Quinine redutase

mRNA – Messenger ribonucleic acid

mutS – Mismatch repair ATPase

NADPH – Nicotinamide adenine dinucleotide phosphate

napA – neutrophil-activating protein

neuA – Gene involved in sialic acid biosynthesis

neuB - Gene involved in sialic acid biosynthesis

NHPH – Non-*Helicobacter pylori Helicobacter*

oipA – Outer inflammatory protein A

OMPs – Outer membrane proteins

OMV – Ordem dos Médicos Veterinários

OMV – Outer membrane vesicles

PCR – Polymerase chain reaction

PgbA – Plasminogen binding protein A

PgbB– Plasminogen binding protein B

PMNs – Polymorphonuclears

porA – pyruvate-oxidoreductase A

PPI – Proton pump inhibitors

qPCR – Quantitative polymerase chain reaction

rpoB – RNA polymerase subunit B

rRNA – Ribosomal ribonucleic acid

RUT – Rapid urease test

SNPs – Single nucleotide polymorphism

sodB – Superoxide dismutase B

UBT – Urease breath test

ureA – Urease subunit A

ureAB – Urease subunit AB

ureB – Urease subunit B

VacA – Vacuolating cytotoxin A

wecB - Gene involved in sialic acid biosynthesis

WHO – World Health Organization

WOAH – World Organization for Animal Health

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Chapter I

General Introduction

Scientific Aims

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General Introduction

1 - Gastric Helicobacters in humans and animals

The *Helicobacter* genus is a group of Gram-negative bacteria, composed of at least 75 different species which can colonize the gastrointestinal tract of humans and animals [1-3]. The number of *Helicobacter* (*H.*) species has been increasing since the first description of the human pathogen *Helicobacter pylori* (*H. pylori*) in 1983 [3].

H. pylori infects over half the global population causing gastritis, gastroduodenal ulcers, gastric adenocarcinoma and mucosa associated lymphoid tissue (MALT) lymphoma [4,5].

H. pylori is the most known and studied bacterium of this genus but there are other gastric helicobacters, non-*Helicobacter pylori* helicobacters (NHPH), that also are associated with gastrointestinal diseases in humans and animals [3,6,7].

The most common gastric NHPH in humans are *H. suis*, *H. heilmannii*, *H. felis*, *H. salomonis* and *H. bizzozeronii* and all of these with zoonotic potential [3,8]. These NHPHs have an extremely fastidious nature and are commonly found in the stomach of dogs, cats and pigs and other animals and are associated with gastrointestinal disease [3,7,9-11].

NHPH infections in humans are less frequent when compared with *H. pylori* infections and within these, *H. suis* infection is the most common [3,12], although in a recent study the canine/feline gastric NHPHs *H. bizzozeronii* and *H. felis* were the most prevalent species in human gastric patients and not *H. suis* [13]. Interestingly NHPH infections are often associated with less active or less severe gastritis but on other hand, in humans, are linked with a higher risk for the development of low-grade MALT lymphoma compared to *H. pylori* [3,9,14].

The primary source of NHPH infections in humans is most likely direct or indirect contact with infected animals. Living around animals is consequently viewed as a significant risk factor [10,15-18]. A higher prevalence of these diseases has been observed in farmers, slaughterhouse employees, pet owners and children, who have frequent contact with animals [15,19-21]. Consuming contaminated water or, in the case of *H. suis*, eating contaminated pork, are potential additional routes of infection [14,19,20,22,23] .

1.1 - *Helicobacter pylori*

1.1.1 – Epidemiology

H. pylori was discovered by Marshall and Warren to infect the epithelium of the human stomach [24,25]. *H. pylori* is the most common and best-studied species that infects half of the world's human population. Since 1994 *H. pylori* has been categorized as a class-

I carcinogenic agent by the International Agency for Research on Cancer (IARC) of the World Health Organization (WHO), because it is the strongest known risk factor for severe gastric complication development [25,26]. It is associated with chronic gastritis, gastric and/or duodenal ulcers, gastric carcinoma, and MALT, accounting for approximately 75% of all human gastric cancer cases [5]. For this reason, the eradication of *H. pylori* infection remains a public health concern worldwide [5]. Most of the infections with *H. pylori* occur during childhood and persist for a lifetime [5,27]. It is also speculated that soiled drinking water can be a source of contamination, and this can be influenced and vary according to the frequency and distribution of *H. pylori* among the different socioeconomic populations as well as the standards of public hygiene in different countries [5,22,28,29]. But the exact mechanism, pathways and behavioral patterns that lead to the widespread of the infection are still unknown [5,30].

The transmission routes of *H. pylori* are not completely understood, and the human stomach is supposedly the only known reservoir [31]. New infections are believed to occur due to direct human-human transmission or environmental contamination, but lately, there were some reports of identification of these bacteria in animals, food, and the environment [4,22,23,30]. The human-human transmission can be divided in vertical transmission and horizontal transmission [5]. It is hypothesized that the most common transmission may occur via fecal-oral routes, oral-oral transmission is also important despite lack of universal support evidence and gastric-oral transmission occurs primarily in children [5,32]. Strong environmental and occupational restrictions are linked to animal-to-human and occupational exposure, whereas person-to-person and foodborne infections constitute the primary transmission modes of *H. pylori* [5,32].

1.1.2 - Pathophysiology

An infection with *H. pylori* results in acute gastritis with an initial invasion of the gastric mucosa and its epithelial layer by polymorphonuclear leukocytes (PMNs). The chronicity of this acute process leads to chronic gastritis characterized by infiltration of the gastric mucosa with plasma cells and lymphocytes associated to the PMNs of the acute phase [30]. Chronic infections can further result in intestinal metaplasia or atrophy of the gastric mucosa. The chronic active inflammation of the gastric and intestinal mucosa can subsequently result in gastroduodenal ulcers [30,33]. The death of immune and epithelial cells at the site of the immune response provides nutrients to the gastric pathogen, allowing it to continue colonizing the stomach over the lifetime of the host. *H. pylori* can neutralize surrounding acid through the secretion of urease, an enzyme that converts urea into bicarbonate and ammonia [25]. This increases the risks for the development of gastric

disease with the prominence of gastric cancer and peptic ulcer [30,33]. Therefore, antibiotic eradication of *H. pylori* is the cure for most cases of peptic ulcer disease [14,30,33].

1.1.3 - Pathogenesis mechanisms

H. pylori uses several virulence factors for colonization of the human gastric mucosa and gastric pathogenicity (Table 1). It also encodes many outer membrane proteins (OMPs) vital for the bacterial life cycle and that play an important role in the colonization [34-36].

Table 1 – *H. pylori* virulence factors and their function.

Virulence genes		Action	References
Bacterial colonization	*BabA*	Adhesion;	[34-38]
	SabA	Adhesion;	
	OipA	Proinflammatory; Promotes colonization; Inhibits the apoptosis of gastric cells;	
	HopQ	Facilitate the transfer of crucial pathogenic factor CagA to host cells;	
	homB	Improve the adhesion of *H. pylori* to the host; Promote the secretion of inflammatory factors;	
Gastric pathogenicity	*CagA*	Functions as a eukaryotic signaling hub; Induction of proinflammatory responses; Induction of carcinogenesis; Modulation of apoptosis; Promotion of genetic instability;	
	VacA	Promotion of immune tolerance and chronic infection; Modulation of autophagy; Induces apoptosis; Powerful immunosuppressant;	
	HtrA	Protects the pathogen; Promotes infection;	
	OMV	Protects the pathogen; Promotes infection;	

Table 1 (continuation) – *H. pylori* virulence factors and their function.

Bacterial colonization Other functions	*OMV*	Impair cellular function; Modulate host immune defenses;	[34-38]
	GGT	Pathogens growth promotion; Autophagy regulation; Bacterial internalization in human gastric cells; Protection of bacteria to commence a persistent infection; Induces apoptosis; Inhibits gastric cell proliferation; Arrests the cell cycle; Generates reactive oxygen species;	
	dupA	Promote ulcer formation; Functions not yet fully understood;	

The ability to adhere to the gastric epithelium is critical for colonization success because it protects the bacteria from clearance mechanisms such as liquid flow, peristaltic movement, washing, and mucus shedding. Attachment provides bacteria with a source of nutrients and facilitates the transmission of bacterial toxins or other virulence factors to host cells, resulting in the formation of a persistent infection [34-38].

H. pylori adapts to the acidic environment of the lumen by using motility, chemotaxis, urease synthesis, and other processes to populate a narrow, protected niche near the epithelial cell surface [34-38].

Most of the bacteria are free-swimming in the mucus gel, while some adhere straight to the epithelium's surface through different virulence factors [34-38].

Several of these virulence factors such as *CagA, VacA* are linked to an elevated risk of cancer and peptic ulcer [37].

1.1.4 - Diagnostic tests

There are different diagnostic techniques that can be used to diagnose *H. pylori* infections (Table 2). There are several factors that dictated the method used, and one of them is regarding resources available and the type of technique, non-invasive *vs*. invasive [39-44]. The same diagnostic techniques have been used in humans and animals [45].

Table 2 – Advantages and disadvantages of the different *H. pylori* diagnostic methods.

Technique		Advantages	Disadvantages	References
Non-invasive				
	Urea Breath Test (UBT)	Simple; Non-invasive; Safe; High sensitivity, specificity and accuracy; Detection of eradication of infection.		[39-44]
	Faecal Antigen Test	Fast, simple, and inexpensive.	False-negative results occur in the low bacterial load, recent use of the antibiotics, bismuth and proton-pump inhibitors; The unwillingness of patients and doctors to do this test; Problems of keeping and carrying samples.	[39-44]
	Serology	Cost-effectiveness cheapest; Widely available; Applicable to diagnosis for patients treated with antibiotics and Proton Pump Inhibitors.	Failure to distinguish between acute infection and previous contact; Unusable in confirming cure after therapy.	[39-44]
Invasive				
	Endoscopy	Acquisition of gastric biopsies and leads to a definitive diagnosis of infection.	Time-consuming and requires so much skill;	[39-44]
	Rapid Urease Test (RUT)	Fast, inexpensive and simple.	Sensitivity and specificity are lower in gastric ulcer bleeding and intestinal metaplasia.	[39-44]
	Histology	The gold standard in the direct diagnosis of *H. pylori* in mucosa and	Observer dependency; time-consuming; Needing a lot of skill, and high cost.	[39-44]

Table 2 (continuation) – Advantages and disadvantages of the different *H. pylori* diagnostic methods.

		investigation of the eradication condition.		[39-44]
	Culture	Determination of patterns of antimicrobial resistance and sensitivity.	Expensive, complicated, and time-consuming test.	[39-44]
	Molecular techniques (Polymerase Chain Reaction - PCR, qPCR)	High sensitivity, specificity; No need for specific transportation.	Expensive; Need special skills; False-positive results due to detect DNA pieces of dead bacteria.	[39-44]

1.2 - *Helicobacter suis*

The presence of *Helicobacter*-like organisms in the stomach of pigs was reported for the first time in the 90's by Queiroz et al. 1990 [46] and the name *Gastrospirillum suis* was proposed by Mendes et al. 1990 [47]. Later on, after De Groote et al. 1999 study, was classified as *Candidatus* Helicobacter suis [48] and now *Helicobacter suis* [49]. *H. suis* is a spiral-shaped, non-sporulating Gram-negative bacteria that is associated with ulceration of the non-glandular stomach and gastritis in pigs [49,50].

1.2.1 – Epidemiology

The presence of *H. suis* has been described in domestic and wild animals as well as in humans [9,49,51-54].

H. suis in pigs originated from non-human primates via a possible host jump from macaques to pigs, after which domestication has had a significant impact on the spread of this pathogen among pigs [55,56].

H. suis colonizes the gastric mucosa of 60-95% of pigs at slaughter age [12,52].

Prevalence rates of *H. suis* colonizing the gastric mucosa of pigs, vary from 10-60%, depending on geographical area, diagnostic tools and management and hygiene standards applied. The prevalence is found to be increasing with age being 2% in suckling piglets and 90% in adult pigs [7].

There are strong indications that pigs may serve as a source of infection for humans [57].

NHPH have been associated with human gastric disease with prevalence ranging from 0.2-6%. *H. suis* is the most prevalent gastric NHPH in humans [2,58,59]. *H. suis* can be associated with gastric diseases such as gastritis, gastric ulceration, MALT lymphoma and

occasionally gastric adenocarcinoma [51,55,57,59,60], as well as there is some association with extra-gastrointestinal diseases [61-63].

H. suis has been detected in saliva but not in feces of pigs. Therefore, transmission is suggested to occur via the oral-oral route or the gastric-oral route [7]. However despite *H. suis* DNA isolation from pork meat and pork carcasses [19,20] and the previous report of a pig veterinarian infected with *H. suis* [15], the route and stage of life of *H. suis* infection in humans are unknown [64] and it may persist for life in the host in the absence of proper treatment [65].

1.2.2 - Pathophysiology

In pigs, *H. suis* colonizes the fundic and pyloric gland zone of the stomach and is often observed near or even inside the canaliculi of parietal cells and that can show signs of degeneration [7,51,65,66]. *H. suis* infection leads to an increased number of gastrin-producing cells and a decreased number of somatostatin-producing cells resulting in excessive acid production since gastrin stimulates and somatostatin inhibits the secretion of hydrochloric acid by parietal cells [67].

It was also shown that *H. suis* infection triggers abnormal mRNA expression levels of *Atp4a*, responsible for acid production and regulation, and induces a marked up-regulation of transcriptional levels of *Shh*, a critical factor involved in gastric organogenesis, glandular differentiation and gastric homeostasis [51].

H. suis infection in pigs has been associated with gastritis, ulceration of the *pars oesophagea* and decreased daily weight gain [7,52,68].

During the *H. suis* acute phase of the infection, there is a decreased gastric acid secretion in the distal glandular part of the stomach [51,52]. During the chronic phase, there is a secretion of excessive amounts of gastric acid in the distal part of the stomach which may lead to increased contact of the *pars oesophagea* with hydrochloric acid. Since this part of the stomach is not protected by mucous it is more prone to hyperkeratosis, erosion and ulcer formation [51,52].

1.2.3 - Pathogenesis mechanisms

H. suis has homolog genes to *H. pylori* involved in acid acclimation, chemotaxis, adhesion, oxidative stress resistance and motility (Table 3) that help with colonization and pathogenesis to the host [57]. It was hypothesized that primate-associated *H. suis* strains might be more virulent than porcine strains, at least in mouse models of human gastric disease [66].

Table 3 – *H. suis* virulence factors and their function.

Virulence factors	Function	Reference
HorB Surface lipoprotein HpaA	Adhesion.	[57]
fibrinonectin/fibrinogen-binding protein	Enhance its adherence to injured gastric tissue.	
neuA, *neuB*, and *wecB*	Contributes to evasion of the host complement defense system.	
napA, *sodB*, *katA*, *mutS*, *mdaB*, and peroxiredoxin coding sequence	Oxidative-stress resistance; May harbour a defense mechanism against the host inflammatory response; Contributes to chronic gastric colonization by this bacterium.	
cag23/E *cagX*	Proinflammatory; Higher risk for gastric adenocarcinoma.	
vacA (non-funtional)	Cytotoxicity; Immunomodulation.	
HP-NapA protein	Proinflammatory and immunodominant protein.	
PgbA PgbB	Contribute to obstructing the natural healing process of gastric ulcers.	
fldA	Colonization of *H. suis* and MALT lymphoma development in *H. suis* infected people.	
porA to *D*	Colonization of *H. suis* and MALT lymphoma development in *H. suis* infected people.	
ureA ureB	Neutralizing the gastric acid creating neutral pH surrounding the bacterium.	
GGT	Alterations in gastric acid secretion by parietal cells.	[69]

1.2.4 - Diagnostic tests

Different diagnostic techniques can be used for the detection of *H. suis* from microscopic examination and special staining (Giemsa), immunohistochemistry, urease activity test, and molecular analysis of biopsies samples by PCR and sequencing [55,70].

1.3 – Antimicrobial resistance

Antimicrobial resistance (AMR) is one of the most concerning One Health issues worldwide [71], in fact, the World Health Organization (WHO) has classified the most important resistant bacteria at a global level for which there is an urgent need for new treatments [72]. This classification was done according to the species and the type of resistance resulting in three priority tiers: critical, high and medium. In this list, *H. pylori* is classified as high risk, specifically for clarithromycin-resistance [72].

Based on the antimicrobial importance to treat human infections and the antibiotics used in veterinary medicine, WHO published a priority list of antimicrobials grouped into three categories: Critically important (1) subdivided into highest priority and high priority; Highly important (2) and Important (3) [73]. Likewise, the World Organization for Animals Health (WOAH) developed a list of antimicrobials of veterinary importance further classified as: veterinary critically important, veterinary highly important, and veterinary important antimicrobials [74].

Several genetic changes (e.g. single-nucleotide polymorphism - SNPs), physiological changes of the bacterial cells (e.g. upregulation of the efflux pumps) and cellular adaptation properties (e.g. biofilm formation) have arisen and contributed to *H. pylori* antimicrobial resistance. With *H. pylori* different antimicrobial resistance patterns can be considered: single-drug resistance, multi-drug resistance, and heteroresistance, depending on how many drugs they are resistant to [75].

H. pylori shows acquired resistance to several drugs: tetracyclines, beta-lactams, nitroimidazoles, fluoroquinolones, macrolides and rifampicin. Regarding tetracyclines *H. pylori* resistance is mainly associated with single, double, or triple mutations in the *16S rRNA* gene. The main factor of amoxicillin resistance in *H. pylori* are mutations in *pbp1A* gene as well as other possible mechanisms as mutations in genes encoding efflux pumps and porins such as *hofH*, *hefC*, and *hopC*. *H. pylori* metronidazole resistance is due to mutations involving the genes *rdxA* and *frxA* and other mutations are suspected to play a role in the resistance mechanism as mutation of ferric uptake regulator. Resistance to clarithromycin is due to point mutations in the peptidyl transferase region encoded in domain V of 23S rRNA and three mutations in this domain, A2141G/C and A2143G respectively, are responsible for more than 90% of clarithromycin-resistant *H. pylori* strains. Another

mechanism involved is associated with multidrug efflux pump transporters, mainly the resistance-nodulation-cell-division [76]. *H. pylori* resistance to levofloxacin usually results from a point mutation in the quinolone resistance-determining regions of the *gyrA* gene. A mutation at position 463 in *gyrB* may also be responsible for some cases of fluoroquinolone-resistant *H. pylori,* Rifabutin resistance is due to a point mutation in the rifampicin resistance determined region of the *rpoB* gene [75,76].

Some antimicrobial resistance mechanisms have also been described in NHPH. Acquired resistance to azithromycin, enrofloxacin, lincomycin, and spectinomycin seems to occasionally occur in feline *H. heilmannii* isolates as reported by Matos et al. 2020 [77]. Berlamont et al. 2019 showed that acquired resistance occasionally occurs in *H. suis* isolates. Porcine isolates are intrinsically less susceptible to β-lactam antibiotics and tetracyclines than primate isolates [78]. The presence of a mutation in the 23s rRNA gene conferring clarithromycin resistance in *H. pylori*, has recently been described in *H. suis* [79].

2 - *Fusobacterium* spp.

Fusobacteria are gram-negative non-sporing bacilli and strict anaerobes [80]. The members of this genus produce butyric acid as their major metabolic end-product. The cell wall contains diaminopimelic acid and/or lanthionine as the dibasic amino acid in their peptidoglycan [80,81]. They tend to form long filamentous rods, described as fusiform or spindle shaped, several appear short with rounded ends [80]. There are 34 recognized species, which includes the novel *Fusobacterium gastrosuis* (*F. gastrosuis*) identified and described in the study published by De Witte et al. (2017) [1,81].

Fusobacteria are found in mixed infections. They may colonize the mucous membranes of humans and animals as commensal bacteria of the upper respiratory and gastrointestinal tracts and can be involved in a wide variety of infections, usually of the pyonecrotic type [80,81]. Depending on the host species Fusobacteria can play different roles: in humans, they can be associated with gingivitis, dental plaque formation, dental and periodontal abscesses, and liver abscesses; in horses, Fusobacteria can play a role in oral and lower respiratory tract disease, intra-abdominal abscesses; in cattle, they can cause necrotic laryngitis and footrot; in pigs, Fusobacteria have been associated with lameness and facial skin necrosis and gastric ulceration [81].

2.1 - *Fusobacterium gastrosuis*

A novel species, *F. gastrosuis*, was identified after a metagenomic analysis of the gastric microbiota of pigs by De Witte et al. (2017) [81].

F. gastrosuis takes part of the gastric microbiota of pigs, colonizing the *pars*

oesophagea. It is characterized as gram-negative rods with rounded ends, non-capsulated with the presence of swelling and globular forms and occasionally long filaments [81,82]. The strains are indole producers and show proline arylamidase and glutamic acid decarboxylase activity [81]. It shows high sequence similarity with *Fusobacterium* spp., such as *F. mortiferum*, *F. russi*, *F. ulcerans*, *F. varium* and *F. necrogens* [81].

It was shown that *F. gastrosuis* is abundantly present in the non-glandular *pars oesophagea* of the porcine stomach [81]. This anatomical region is usually affected by ulceration, very common in pigs, with a prevalence up to 93% [82]. Gastric ulceration can lead to discomfort, a decrease in daily weight gain, and sudden death [81]. It is known that gastric ulceration is multifactorial and multiple agents can be involved such as *H. suis*, *Lactobacillus* spp. and *Bacillus* spp. [81,82].

2.1.1 – Epidemiology

F. gastrosuis is part of the gastric microbiota and colonizes the *pars oesophagea* of pigs [82]. There are reports of *F. gastrosuis* in the nasal and oral microbiota of dogs [68] and in the feces of humans which may indicate that *F. gastrosuis* might be able to colonize a wide range of hosts [68,83].

2.1.2 - Pathophysiology

Fusobacterium spp. can aggravate necrosis when tissue damage is initiated by other microorganisms or environmental factors [82]. For example, an altered gastric environment secondary to *H. suis* infection can lead to colonization and invasion of the *pars oesophagea* by *F. gastrosuis* producing epithelial cell death induced by its metabolites [68]. *F. gastrosuis* can induce cell death in oesophageal and gastric epithelial cell lines and possibly play a role in gastric ulceration [68].

F. gastrosuis was found at high colonizing rates in the oral cavity of 2–3-month-old pigs indicating that the oropharynx, and potentially its tonsils, may function as a natural reservoir for this bacterium [68]. *F. gastrosuis* may enter the stomach through ingestion of food, water, or saliva, after which it may opportunistically colonize the upper, non-glandular *pars oesophagea*, but continues unclear if the *F. gastrosuis* belongs to the oral microbiota or if it may induce oropharyngeal pathologies [68].

2.1.3 - Pathogenesis Mechanisms

A variety of virulence-associated genes were shown by genome sequence analysis of *Fusobacterium* spp. [68]. *Fusobacterium* spp. are able to adhere to and actively invade

host cells without the aid of other factors, due to the presence of genes encoding adhesins and membrane-related proteins. *Fusobacterium* spp. can aggravate necrosis when tissue damage is initiated by other microorganisms or environmental factors [68].

De Witte et al. (2019) concluded that *F. gastrosuis* has homologous sequences in the proteome that have been reported to be involved in structural organization, nutrient uptake, pathogenesis, antibiotic resistance, virulence, immune evasion and other processes essential for the survival of *F. gastrosuis* [68].

F. gastrosuis has virulent factors that have been reported to be involved in adhesion, invasion, immune-evasion, capsule formation, resistance to antibiotics and uptake of magnesium, zinc and iron (Table 4). This species has genes associated with active invasion of host cells [68].

Table 4 – *F. gastrosuis* virulence factors and their function [68].

Virulence factors	Function
Leukotoxin	Cell death.
Immunosuppressive protein A (fiapA)	Immune-evasion; Inhibits T-cell activation.
Haemagglutinin	Attachment to epithelial cells.
Outer membrane proteins	Mediate adherence to other pathogenic bacteria and host cells; Suppress host immune system; Induce cell death in lymphocytes.
Serine protease	Degrades the extracellular matrix proteins fibrinogen and fibronectin as well as collagen I and collagen IV; Contribute to damage of periodontal tissues; Helps the evasion of the immune system.
Hemolysin	Lyses erythrocytes and creates anaerobic environment at the site of infection; Stimulates the production of IL-1β and TNFα.
ABC transporters	Facilitates iron uptake; Related with antibiotic resistance.
Cytolysin	Forms pores in cell membrane; Induces apoptosis, promotes cellular invasion; Triggers inducible Nitric oxide synthase (iNOS) and cytokine release.
Lipooligosaccharides synthesis	Involved in immune system evasion; Attachment to epithelial tissue; Mediator of the proinflammatory response.

2.1.4 - Diagnostic tests

F. gastrosuis diagnosis is based on microbiologic culture and molecular biology techniques such as PCR [68,81].

2.1.5 - Antimicrobial Resistance

Data regarding antimicrobial susceptibility testing for *Fusobacterium* spp. are limited [84]. About 4-15% of *Fusobacterium* species can display resistance to penicillins that is generally associated to the production of β-lactamases [85,86]. Some *Fusobacterium* species are resistant to penicillin G, ceftriaxone, clindamycin, or moxifloxacin [84]. Typically, *Fusobacterium* species are susceptible to metronidazole, Beta-lactamase inhibitors (BLBLI)s, cephalosporins, carbapenems, and clindamycin [85]. There are no studies to date, regarding *F. gastrosuis* AMR.

3 - Synergetic role of *H. suis* and *F. gastrosuis*

It is known that *H. suis* has an active role in pig's gastric ulceration [51-53]. Gastric ulceration is of multifactorial origin and changes in gastric microbiota composition and infectious agents seem to play a role in the development of ulcers of the *pars oesophagea* [68,82]. *H. suis* induced impaired acid secretion that may favor changes in the gastric microbiota promoting the development and infection with *F. gastrosuis* [68].

F. gastrosuis can play a role in gastric ulceration by aggravating pre-existent tissue damage and necrosis [81,82].

De Witte et al. (2017) reported that *H. suis* positive pigs had highly abundant *F. gastrosuis* in the gastric microbiota [81].

It is hypothesized that *H. suis* colonizes and invades the *pars oesophagea* altering the gastric environment that potentiates *F. gastrosuis* infection and pathogenesis [52,68,82].

During the acute phase of *H. suis* infection, the gastric acid secretion is downregulated resulting in higher numbers of *F. gastrosuis* in the *pars oesophagea* which may facilitate the initiation of the lesion process. In a chronic phase of the infection, gastric acid secretion is stimulated which can lead to aggravation of pre-established lesions of the *pars oesophagea* that is not protected due to lack of mucus [52,68,82].

4 - Zoonotic potential: Impact on One Health

The WHO and the European Center for Disease Prevention and Control (ECDC) define zoonoses as infectious diseases that can jump from animals to humans via direct or indirect contact through food, water or the environment [87,88]. The zoonotic agents can be bacteria, viruses, parasites, or fungi. They represent a worldwide public health problem due to humans' close relationship with domestic, production, and wild animals [88].

Several NHPHs are known and described to be zoonotic, these bacteria which

naturally colonize the stomach of mammals and birds have been detected in human clinical samples [2,6,8,11,14,18,89].

Routes of transmission are not completely known but the close contact with animals and the possible water, food, and environmental contamination must be considered [14,90,91] since there are reports of isolation of NHPH from gastric samples of different animals as well as from saliva and in feces [92-95].

Particularly, *H. suis* has been reported, in several studies, to be associated with gastritis, gastric ulcer and duodenal ulcer in children and adults [6,9,12,14,59,60,89,96,97] as well as MALT lymphoma [6,8,59]. There are some studies reporting its linkage to neurological diseases [61-63].

Besides the direct identification of *H. suis* in human clinical samples it was also isolated from pig meat and pork carcasses [19,20], which suggests that human exposure to *H. suis* may occur through the food chain.

There are also reports of *H. pylori* in non-human animals which raises questions regarding the possible potential for these animals to act as reservoirs for *H. pylori* and perpetuate its spread in the environment [21,54,98].

Regarding *F. gastrosuis* there were only two reports on the identification of these bacteria in other species than pigs, one in dogs and one in humans but not directly related with development of pathology associated [68,83].

It is also debatable the role of non-human animals in the transmission of *H. pylori* or its role as reservoirs and spread of this bacterium since there are reports of identification of *H. pylori* DNA in wild boars, wild quails, dogs [21,54,98], and in other animals as well as in the food chain and in the environment [29,99-101].

Considering all of these, it would be important to raise awareness of the general population for this issue, since there continues to exist a lack of awareness regarding these topics [102,103]. Although it would have some expenses, implementing awareness programs on zoonotic illnesses within communities could help lessen the public health effects of zoonotic infections in human-domestic animal-wildlife interface areas [104,105].

Detecting and responding to emerging zoonotic pathogens, prioritizing the diseases of greatest public health concern, and successfully launching appropriate prevention, detection, and response strategies all require collaborations across sectors relevant to zoonotic diseases, particularly among human and animal (domestic and wildlife) health disciplines [106]. It is essential to build efficient systems for coordination and collaboration between the animal, human, and environmental health sectors. This can be done by bringing the various sectors together to address the most pressing endemic zoonotic illnesses.

To inform those who care for both humans and animals, as well as the general

public, on zoonotic disease transmission and prevention and the importance of the use of personal preventative measures to avoid or decrease exposure to vector-borne and other zoonotic diseases, safe farming methods, knowing the dangers associated with animal interaction and exposure, and biosecurity measures may all be covered in community education programs [106].

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Scientific Aims

General aim

This research project aimed to: determine the presence of gastric *Helicobacter* species and *F. gastrosuis* DNA in pigs and wild boars and its association with gastritis; determine the presence of gastric *Helicobacter* species in game and the possible role of these animals as reservoirs for gastric helicobacter species; assess the presence of AMR genes in *H. pylori*-like positive samples; assess the knowledge and awareness of Portuguese veterinarians regarding gastric *Helicobacter* species infections.

Specific aims:

- *Article I*

The aim of this paper was to determine the presence of gastric *Helicobacter* species and *F. gastrosuis* and their association with gastritis in pigs and wild boars from Portugal. Gastric samples were collected, and histological and molecular biology technics were applied in order to determine: first, the presence of *Helicobacter* spp. and *F. gastrosuis* DNA in each sample; second, to analyze histologically each sample in order to determine the presence of gastritis; third, to associate gastritis with the presence of *Helicobacter* spp. and/or *F. gastrosuis* DNA.

- *Article II*

The aim of this paper was to assess wild boar gastric samples for the presence of eight gastric *Helicobacter* species DNA: *H. pylori*, *H. suis*, *H. felis*, *H. ailurogastricus*, *H. salomonis*, *H. bizzozeronnii* and *H. heilmannii* through a conventional PCR with species-specific primers for each species of interest.

- *Article III*

Considering the close contact between different wild animal species, especially game species, and domestic animals, the aim of this paper was to investigate the presence of gastric *Helicobacter* species in gastric samples of wild rabbits and wild quails.

- *Manuscript I*

The aim of this paper was to determine if the *H. pylori*-like DNA-positive samples obtained among all the animal samples (pigs, wild boars and wild quails) were positive for AMR gene mutations that may potentially confer resistance for antimicrobials.

- *Article IV*

Regarding the veterinarian exposure to gastric *Helicobacter* species and the role of veterinarians as public health and One health promoters, the aim of this paper was to assess the perception and knowledge of Portuguese veterinarians regarding the zoonotic transmission of gastric Helicobacters.

Chapter II

Molecular detection of *Helicobacter* spp. and *Fusobacterium gastrosuis* in pigs and wild boars and its association with gastric histopathological alterations

(Article I)

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VETERINARY RESEARCH

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Molecular detection of *Helicobacter* spp. and *Fusobacterium gastrosuis* in pigs and wild boars and its association with gastric histopathological alterations

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Abstract

Besides *Helicobacter pylori*, a Gram-negative bacterium that may cause gastric disorders in humans, non-*Helicobacter pylori* helicobacters (NHPH) may also colonize the stomach of humans and animals. In pigs, *H. suis* can induce gastritis and may play a role in gastric ulcer disease, possibly in association with *Fusobacterium gastrosuis*. In the present study, gastric samples from 71 slaughtered pigs and 14 hunted free range wild boars were tested for the presence of DNA of *F. gastrosuis* and gastric *Helicobacter* species associated with pigs, dogs cats and humans, using species-specific PCR assays, followed by sequencing of the amplicon. These gastric samples were also histopathologically evaluated. Almost all the pigs presented gastritis (95.8%). *Helicobacter* spp. were detected in 78.9% and *F. gastrosuis* in 35.2% of the animals. *H. suis* was the most frequently identified *Helicobacter* species (57.7% of the animals), followed by a *H. pylori*-like species (50.7%) and less often *H. salomonis* and *H. felis* (each in 2.8% of the animals). *H. suis* was most often detected in the glandular (distal) part of the stomach (*pars oesophagea* 9.9%, oxyntic mucosa 35.2%, antral mucosa 40.8%), while the *H. pylori*-like species was mainly found in the non-glandular (proximal) part of the stomach (*pars oesophagea* 39.4%, oxyntic mucosa 14.1%, antral mucosa 4.2%). The great majority of wild boars were also affected with gastritis (71.4%) and *Helicobacter* spp. and *F. gastrosuis* were detected in 64.3% and 42.9% of the animals, respectively. *H. bizzozeronii* and *H. salomonis* were the most frequently detected *Helicobacter* species, while a *H. pylori*-like species and *H. suis* were only occasionally identified. These findings suggest that these microorganisms can colonize the stomach of both porcine species and may be associated with gastric pathology. This should, however, be confirmed through bacterial isolation. This is the first description of the presence of *F. gastrosuis* DNA in the stomach of wild boars and a *H. pylori*-like species in the *pars oesophagea* of the porcine stomach.

Keywords: *Sus scrofa*, stomach, gastritis, gastric pathology, One Health

Introduction

Helicobacter species are Gram-negative, spiral-shaped motile bacteria that colonize the gastrointestinal tract of humans and a wide range of animals [1–4]. Studies have been done over the years to investigate their association with gastrointestinal and extra-gastrointestinal diseases [5]. In humans, *Helicobacter pylori* (*H. pylori*) is the most common gastric pathogen, with an estimated prevalence

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of 44.3% worldwide [6]. Other spiral-shaped non-*Helicobacter pylori* helicobacters (NHPH) have also been associated with gastric diseases in humans. These gastric helicobacters can be responsible for development of gastritis, gastroduodenal ulcers, gastric adenocarcinoma and mucosa-associated lymphoid tissue (MALT) lymphoma [2, 7, 8].

Zoonotic NHPH such as *H. suis*, *H. felis*, *H. bizzozeronii*, *H. salomonis*, and *H. heilmannii*, with an estimated prevalence ranging from 0.2 to 6% in symptomatic humans, naturally colonize the stomach of dogs, cats and pigs [6, 9].

Helicobacter suis is found mainly in the fundic and pyloric gland zone of the pig stomach [10]. This bacterium presents tropism for the gastric acid-producing parietal cells [11]. Its prevalence appears to be very low prior to weaning, but increases rapidly thereafter, being very high at slaughter age (77%) and in adults (>90%) [10–12]. *H. suis* infection causes gastritis, decreased daily weight gain, and may play a role in induction of gastric ulcers possibly in association with *Fusobacterium gastrosuis* (*F. gastrosuis*), further affecting animal production and welfare [13–15].

Fusobacteria are Gram-negative, non-sporing, strict anaerobic bacilli. They tend to form long filamentous rods, described as fusiform, spindle-shaped short with rounded ends [16]. There are 27 recognized species, including *F. gastrosuis* [17]. Fusobacteria can be involved in a wide range of mixed infections. Depending on the host species they can play different roles: in humans they can be associated with gingivitis, dental plaque formation, dental and periodontal abscesses and liver abscesses; while in pigs, they have been associated with lameness, facial skin necrosis and gastric ulceration [17].

Fusobacterium gastrosuis was primarily identified in the *pars oesophagea* of the pig stomach [17]. This anatomical region may be affected by ulceration, with a prevalence of up to 93% [18]. Gastric ulceration can lead to discomfort, decrease in daily weight gain, and sudden dead [17]. It is known that gastric ulceration is multifactorial and multiple agents can be involved such as *H. suis* and *F. gastrosuis* [17].

Several *Helicobacter* spp. have been described to have a zoonotic potential and therefore close contact between humans, domestic animals, and wild animals deserves more attention [19–23]. Although reservoirs of wild and domestic animals can be considered important sources of emerging infectious diseases, it is the human impact on ecological systems that determines the level of risk at the human/animal interface upon the occurrence of emerging zoonotic diseases [22, 23]. From an eco-epidemiological perspective, wild boars have an important role in spreading several pathogens [24–26].

The aim of this study is to determine the presence of gastric helicobacters and *F. gastrosuis* in a group of pigs and wild boars and describe the most relevant gastric histopathological alterations associated.

Materials and methods

Sample collection

Pig gastric tissues samples were collected from a total of 71 domestic pigs slaughtered at two slaughterhouses in the center of Portugal: one in Coimbra ($n=46$, from 3 different herds) and another in Santarém ($n=25$, from 2 different herds). All sampled animals were older than 6 months according to the slaughter animal registries.

Wild boar gastric tissue samples were collected from 14 hunted animals during two national campaigns, one in the north and other in the center of Portugal (Vila Real and Coimbra districts, respectively). Based on tooth evaluation [26], all sampled animals were older than 9 months.

From each animal, two samples were collected from the three different anatomical gastric regions: *pars oesophagea*, *antrum* and *fundus*, using a sterile disposable Kruuse® biopsy punch of 8 mm per site. After collection, from each region one sample was fixed in 10% phosphate-buffered formalin for histopathology and the other sample was stored at −20 °C for further DNA extraction and molecular analysis.

Gastric tissue sampling was performed within one hour and 4 h after slaughter, in pigs and wild boars respectively. The animals were not slaughtered, euthanized, or hunted to carry out this study, and the fresh gastric tissue specimens were purchased and obtained as sub-products derived from the normal meat inspection activity occurring in the slaughterhouses or during the national campaigns. Additionally, any of these actions were performed solely for research purposes and the researchers had no influence in the selection and execution of the slaughters nor in the national hunting campaign's inspection procedures. Ethical approval was obtained from the i3S Animal Welfare and Ethics Review Body (ref. 2021-4).

Sample evaluation

Histological examination

After fixation, gastric tissues were routinely processed, paraffin-embedded and stained with hematoxylin and eosin (HE) for histopathology. The severity of gastritis was scored according to the human Updated Sydney System [14, 27] classification, with some modifications [13]. The same criteria were applied to the *pars oesophagea*, as described by Yamasaki et al., although this gastric region is not included within the human Updated Sydney System [28, 29]. Diffuse infiltration with inflammatory cells and the presence of lymphoid aggregates and lymphoid

follicles in the mucosa and submucosa were also evaluated [13, 14, 27, 28] (Table 1).

A semi quantitative estimation of the overall inflammatory degree for each gastric area (*pars oesophagea*, oxyntic and antral mucosa) was calculated using a composite score which consisted of adding the partial values previously obtained [13, 27]. Additionally, each gastric section was also microscopically evaluated for the presence of hyperplasia, erosion, ulceration, and fibrosis.

Finally, the overall inflammatory score was correlated with the presence of *H. pylori*, *H. suis*, *H. felis*, *H. salomonis*, *H. bizzozeronii*, *H. heilmannii*, *H. ailurogastricus* and *F. gastrosuis* using Pearson Correlation coefficient.

DNA extraction, PCR conditions, and sequencing

DNA was extracted from the frozen tissue samples, using EXTRACTME® DNA tissue Kit (BLIRT, Poland) according to the manufacturer's instructions.

All the samples were tested for the presence of *H. pylori*, *H. suis*, *H. salomonis*, *H. bizzozeronii*, *H. felis*, *H. heilmannii*, *H. ailurogastricus* and *F. gastrosuis* DNA through conventional PCR, according to previously described protocols (Additional file 1) [2, 18, 30, 31]. As can be seen in Additional file 1: Table S1, for detection of *H. pylori* DNA, two different PCR tests were used, one targeting the *ureAB* gene and the other one targeting the *glmM* gene.

Aliquots of each PCR product were electrophoresed on 1.5% agarose gel stained with Xpert Green Safe DNA gel stain (GRISP, Porto, Portugal) and examined for the presence of specific fragment under UV light. DNA fragment size was compared with the standard molecular weight, 100 bp DNA ladder (GRISP, Porto, Portugal) and the molecular weight of the positive controls (Additional file 1). For negative control, distilled water was used.

To exclude false-positive samples, the amplicons from each positive sample were sequenced. Bidirectional sequencing was performed using Sanger method at the genomics core facility of the Institute of Molecular Pathology and Immunology of the University of Porto. Sequence editing and multiple alignments were performed using MegaX Molecular Evolutionary Genetic Analysis version 10.1.8. The sequences obtained were subject to the basic local alignment search tool (BLAST) using the non-redundant nucleotide database.

Statistical analysis

Statistical analysis was performed using SAS®9.4 (SAS Institute Inc., 2019. Copyright® 2019 SAS Institute Inc., Cary, NC, USA). Correlations between different variables (erosion, ulceration, hyperplasia, fibrosis and presence of *Helicobacter* spp. and or *F. gastrosuis*) were investigated using Pearson correlation coefficient, r results of 0 meaning no correlation, $r<0.3$ low degree of correlation, $r\geq 0.3$ to $r<0.5$ moderate degree of correlation, and $r\geq 0.5$ to 1 high degree of correlation. Differences were considered statistically significant at $p\leq 0.05$.

Table 1 Histological parameters used to establish a scoring system based on the Updated Sydney System and De Witte et al. and Gastritis overall scoring system [13]

Parameter		Scoring
Inflammatory cells (lymphocytes, plasm cells, neutrophils, eosinophils)	Absent	0
	Mild infiltration (<5 cells per HPF)	1
	Moderate infiltration (≥5–20 cells per HPF)	2
	Severe infiltration (≥20 cells per HPF)	3
Lymphoid follicles in the superficial and deep mucosa	No aggregates of lymphoid cells	0
	Presence of one aggregate of lymphoid cells with minimal organization into a follicular structure	1
	Presence of at least one large follicle measuring at least 300 µm in diameter and/or more than one small aggregate of lymphoid cells per histological section	2
	At least two large lymphoid follicles measuring at least 300 µm in diameter per histological section and/or deformation of the mucosal caused by large lymphoid follicles	3
Gastritis overall scoring system		
Score		
0	Normal	
1	Mild gastritis	
2	Moderate gastritis	
≥3	Severe gastritis	

HPF: high power field, i.e., 400× total magnification.

Differences between the different variables (erosion, ulceration, hyperplasia, fibrosis and presence of *Helicobacter* spp. and or *F. gastrosuis*) of the *pars oesophagea*, oxyntic mucosa, and antral mucosa were investigated using non-parametric Kruskal–Wallis, Chi-Square test. A p-value ≤ 0.05 was considered to be significant.

Results

Pigs

A total of 426 gastric samples were collected from 71 animals: 213 for histopathology and 213 for molecular evaluation.

Histopathology

Only 192 of the 213 available samples for histopathology were microscopically evaluated and classified, since 21 samples were in poor preservation conditions impairing histological examination. Thus 57 samples from *pars oesophagea*, 69 from the oxyntic mucosa, and 66 from the antral mucosa were analyzed.

Normal histological features were only observed in 3/71 (4.2%) pigs. Signs of inflammation were diagnosed in 95.8% of pigs. Indeed, 52 out of 57 samples (91.2%) of *pars oesophagea*, 60 out of 69 samples (86.9%) of oxyntic mucosa and all the samples of antral mucosa analyzed (100%) exhibited gastritis (Figure 1). Among samples with gastritis, hyperplasia was present in 28/52 (53.8%) of *pars oesophagea* and fibrosis in 49/60 (81.6%) of oxyntic and in 44/66 (66.6%) of antral mucosa (Table 2).

Remarkably, almost all samples of antral mucosa presented severe inflammation (61/66 or 92.4%) and amongst these, fibrosis was also a common finding (40/61 or 65.6%) (Table 2). When comparing the three different gastric zones, there were statistically significant differences for the presence of erosion, ulceration, hyperplasia, and fibrosis ($p \leq 0.05$) (Additional file 2).

Presence of Helicobacter spp. and F. gastrosuis DNA through PCR analysis

In ten pigs, no DNA amplification was achieved, neither for *Helicobacter* spp. nor for *F. gastrosuis*.

Out of all animals ($n=71$), *Helicobacter* spp. were detected in 56 stomachs (78.9%) with *H. suis* being the most frequently identified species (41/71 or 57.7%), followed by a *H. pylori*-like species (36/71 or 50.7%) and less often *H. salomonis* and *H. felis* (2/71 or 2.8% each).

Regarding the *H. pylori* PCR results, 36/71 samples amplified *H. pylori* DNA with homologies ranging from 96.3 to 100% (Additional file 3) using the *ureAB* gene primers but no amplification was achieved when using the *glmM* gene primers. Therefore, these cases were reclassified as *H. pylori*-like.

Positive results for *F. gastrosuis* were obtained in 25 out of the 71 pigs (35.2%), although *F. gastrosuis* was mainly found in association with helicobacters (20/71 or 28.2%) rather than alone (5/71 or 7.0%). Indeed, in 27/71 of the positive samples (38.0%), only one species was identified, while mixed infections with two or more distinct bacteria were detected in 34/71 (47.9%) (Table 3). The most frequent bacterial combination was *H. pylori*-like + *H. suis* (12/71 or 16.9%) and *H. pylori*-like + *H. suis* + *F. gastrosuis* (10/71 or 14.1%) (Table 3).

Bacteria were differently distributed throughout the porcine gastric compartments (Table 4). *H. pylori*-like DNA was mostly detected in the *pars oesophagea* (28/71, 39.4%), while *H. suis* was most frequently identified in the oxyntic (25/71, 35.2%) and antral mucosa (29/71, 40.8%). There was also a statistically highly significant moderate degree of correlation regarding the presence of *H. pylori*-like + *H. suis* DNA in the *pars oesophagea*, as well as a statistically significant low degree of correlation between the presence of *H. pylori*-like and *H. suis* in the *pars oesophagea* ($r=0.29$ $p \leq 0.05$) and oxyntic mucosa ($r=0.25$, $p \leq 0.05$) (Table 4).

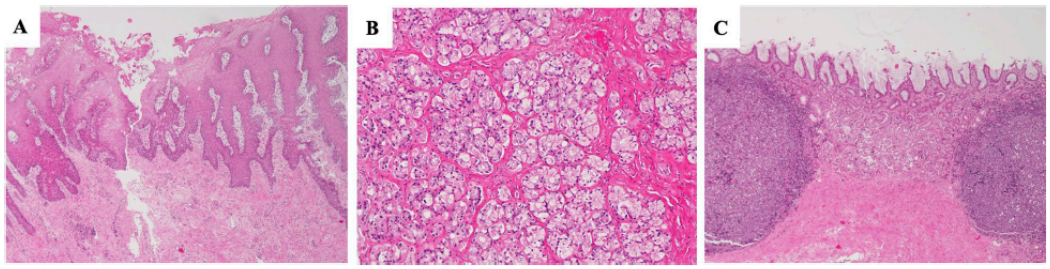

Figure 1 Main histopathological features observed in the different regions of pigs' stomach. A Erosion and irregular and papillary hyperplasia of the lining epithelium of *pars oesophagea*. HE, ×40; **B** Mild inflammation of oxyntic mucosa. HE, ×100. **C** Severe inflammation and deformation of the antral mucosal caused by large lymphoid follicles and fibrosis. HE, ×40.

Table 2 Results of the histopathology of the different gastric regions of pigs' stomach

Number of samples per gastric zone	Pars oesophagea		Oxyntic mucosa		Antral mucosa	
	N = 57		*N* = 69		*N* = 66	
	n/N	%	*n/N*	%	*n/N*	%
Normal mucosa	5/57	8.8	9/69	13.0	0/66	0.0
Score 0						
Fibrosis	0/5	0.0	6/9	66.7	0/0	0.0
Erosion	1/5	20.0	0/9	0.0	0/0	0.0
Ulceration	0/5	0.0	0/9	0.0	0/0	0.0
Hyperplasia	3/5	60.0	0/9	0.0	0/0	0.0
Mild gastritis	16/57	28.1	28/69	40.6	2/66	3.0
Score 1						
Fibrosis	2/16	12.5	23/28	82.1	1/2	50.0
Erosion	4/16	25.0	1/28	3.6	1/2	50.0
Ulceration	2/16	12.5	0/28	0.0	0/2	0.0
Hyperplasia	8/16	50.0	0/28	0.0	0/2	0.0
Moderate gastritis	9/57	15.8	13/69	18.8	3/66	4.5
Score 2						
Fibrosis	0/9	0.0	12/13	92.3	3/3	100.0
Erosion	0/9	0.0	2/13	15.4	0/3	0.0
Ulceration	0/9	0.0	0/13	0.0	0/3	0.0
Hyperplasia	6/9	66.7	0/13	0.0	0/3	0.0
Severe gastritis	27/57	47.4	19/69	27.5	61/66	92.4
Score ≥ 3						
Fibrosis	0/27	0.0	14/19	73.7	40/61	65.6
Erosion	5/27	18.5	1/19	5.3	12/61	19.7
Ulceration	1/27	3.7	0/19	0.0	21/61	34.4
Hyperplasia	14/27	51.9	0/19	0	2/61	3.3

Regarding the correlation between the presence of both bacteria and the respective histological findings, a statistically highly significant moderate correlation was observed between the presence of *H. felis* and erosion in the oxyntic mucosa ($r = 0.33$, $p \leq 0.01$); a statistically significant low degree of correlation between the presence of *H. pylori*-like and erosion ($r = 0.23$, $p \leq 0.05$) in the antral mucosa as well as a statistically significant correlation between the presence of *F. gastrosuis* and ulceration of the antral mucosa ($r = 0.31$, $p \leq 0.05$) (Table 5).

Wild boars

A total of 84 samples were collected from 14 animals: 42 samples for histopathological examination and 42 samples for molecular evaluation.

Histopathology

Only 30 of the 42 available samples for histopathology were microscopically evaluated and classified, since 12 were in poor preservation conditions impairing histological examination (10 of the *pars oesophagea*, 10 of the oxyntic mucosa and 10 of the antral mucosa).

Normal histological features were not identified in wild boars. Microscopically, gastritis was diagnosed in 8 out of 10 samples of *pars oesophagea* (80.0%), 4 out of 10 samples of oxyntic mucosa (40.0%), and 9 out of 10 samples of antral mucosa (90.0%). In most representative cases of *pars oesophagea* the inflammation was mild (4/10 or 40.0%), whereas in the antral mucosa the inflammation was severe (7/10 or 70.0%) (Figure 2).

Regarding the cases diagnosed with gastritis of *pars oesophagea* (8/10), 3 out of 8 were associated with erosion (37.5%) and 2 out of 8 with hyperplasia (25.0%) (Table 6).

Helicobacter spp. and F. gastrosuis identification through PCR analysis

In 3 out of 14 wild boars, no DNA amplification was achieved, neither for *Helicobacter* spp. nor for *F. gastrosuis*. Out of all animals, *Helicobacter* spp. were detected in 9 sampled stomachs (78.6%) with both *H. bizzozeronii*

Table 3 ***Helicobacter*** **spp. and** ***F. gastrosuis*** **DNA PCR positive samples per pigs' stomach**

Specific PCR-positive results	*n/N*	%
Overall identification		
Helicobacter spp. and/or *F. gastrosuis*	61/71	85.9
Helicobacter spp.	56/71	78.9
H. suis	41/71	57.7
H. pylori-like	36/71	50.7
H. felis	2/71	2.8
H. salomonis	2/71	2.8
F. gastrosuis	25/71	35.3
Single bacteria		
H. suis	14/71	19.7
H. pylori-like	7/71	9.9
F. gastrosuis	5/71	7.0
H. felis	1/71	1.4
Multiple bacteria		
H. pylori-like + *H. suis*	12/71	16.9
H. pylori-like + *F. gastrosuis*	5/71	7.0
H. suis + *F. gastrosuis*	4/71	5.6
H. pylori-like + *H. salomonis*	1/71	1.4
H. suis + *H. felis*	1/71	1.4
H. pylori-like + *H. suis* + *H. salomonis* + *F. gastrosuis*	1/71	1.4
H. pylori-like + *H. suis* + *F. gastrosuis*	10/71	14.1

and *H. salomonis* being the most frequently identified species (4/11 or 36.4%), while a *H. pylori*-like species (2/11 or 18.2%) and *H. suis* (1/11 or 9.1%) were less often detected (Table 7).

Regarding the *H. pylori* PCR results, 2/14 samples amplified *H. pylori* DNA with homologies of 100% (Additional file 2: Table S2) using the *ureAB* gene primers but no amplification was achieved when using the *glmM* gene primers. Therefore, these cases were reclassified as *H. pylori*-like.

Positive results for *F. gastrosuis* were obtained in 6 out of the 14 wild boars (42.9%), although it was mainly found in association with helicobacters (4/11 or 36.4%) rather than alone (2/11 or 18.2%). Indeed, in 6/14 of the positive sampled stomachs (42.9.0%), only one species was identified while mixed infections with two or more distinct bacteria were detected in 5/14 (35.7%) (Table 7). The most frequent bacterial combination was *H. salomonis* + *F. gastrosuis* (2/14 or 14.3%) (Table 7).

Bacteria were differently distributed throughout the different regions of wild boars' stomach (Table 8).

Regarding the identification of DNA of only one bacteria per sample site, the *pars oesophagea* samples presented 2/14 (14.3%) DNA for *F. gastrosuis*, 2/14 (14.3%) DNA for *H. salomonis* and 1/14 (7.1%) DNA for *H. bizzozeronii*. The oxyntic mucosa had 2/14 (13.3%) of the samples with *F. gastrosuis* DNA, 1/14 (7.1%) with *H. pylori*-like DNA, 1/14 (7.1%) with *H. suis* DNA and 1/14 (7.1%) with *H. bizzozeronii* DNA. The antral mucosa had 4/14 (28.5%) of the samples with *F. gastrosuis* DNA followed by *H. salomonis* DNA in 3/14 (21.4%) of the samples (Table 8).

Regarding the antral mucosa, there was a statistically significant high degree of correlation between the

Table 4 ***Helicobacter*** **spp. and** ***F. gastrosuis*** **detected through PCR in the different regions of pigs' stomach**

Number of samples	Pars oesophagea		*r*	*p*	Oxyntic mucosa		*r*	*p*	Antral mucosa		*r*	*p*
	N = 71				*N* = 71				*N* = 71			
	n/N	%			*n/N*	%			*n/N*	%		
Single bacteria												
H. pylori-like	28/71	39.4			10/71	14.1			3/71	4.2		
H. suis	7/71	9.9			25/71	35.2			29/71	40.8		
H. felis	0/71	0.0			2/71	2.8			0/71	0.0		
H. salomonis	0/71	0.0			0/71	0.0			2/71	2.8		
F. gastrosuis	12/71	16.9			4/71	5.6			10/71	7.6		
Multiple bacteria												
F. gastrosuis + *H. pylori*-like	8/71	11.3	0.29	0.03	1/71	1.4	0.25	0.04	1/71	1.4	0.11	0.38
H. pylori-like + *H. suis*	6/71	8.5	0.36	< 0.01	1/71	1.4	−0.07	0.56	2/71	2.8	−0.05	0.71
F. gastrosuis + *H. suis*	0/71	0.0	−0.04	0.79	2/71	2.8	0.20	0.09	4/71	5.6	−0.03	0.79
H. suis + *H. felis*	0/71	0.0	–	–	1/71	1.4	0.05	0.69	0/71	0.0	–	–
F. gastrosuis + *H. pylori*-like + *H. suis*	1/71	1.4	–	–	1/71	1.4	–	–	0/71	0.0	–	–

r: Pearson correlation coefficient.

p: *p*-value ≤ 0.05 was considered to be significant.

Table 5 Correlation between the PCR-positive results obtained in the different stomach regions and the gastric histological findings in pigs

		Erosion			Ulceration			Hyperplasia			Fibrosis		
	n/N	**10/57**	***r***	***p***	**3/57**	***r***	***p***	**31/57**	***r***	***p***	**2/57**	***r***	***p***
Pars oesophagea													
H. pylori-like	28/57	1/10	−0.11	0.40	1/3	−0.03	0.81	14/31	0.04	0.64	2/2	0.20	0.13
H. suis	7/57	1/10	−0.03	0.83	0	−0.08	0.57	4/31	0.04	0.77	0	−0.07	0.63
H. felis	0/57	0	–	–	0	–	–	0	–	–	0	–	–
F. gastrosuis	12/57	2/10	−0.04	0.74	0	−0.12	0.39	7/31	−0.01	0.93	0	−0.10	0.46
	n/N	**4/69**	***r***	***p***	**0**	***r***	***p***	**0**	***r***	***p***	**4**	***r***	***p***
Oxyntic mucosa													
H. pylori-like	10/69	0	−0.10	0.40	0	–	–	0	–	–	0	−0.21	0.08
H. suis	25/69	2/4	0.07	0.56	0	–	–	0	–	–	2/4	0.0	0.16
H. felis	2/69	1/4	0.33	< 0.01	0	–	–	0	–	–	1/4	0.15	0.19
F. gastrosuis	4/69	1/4	0.20	0.09	0	–	–	0	–	–	1/4	0.08	0.51
	n/N	**13/66**	***r***	***p***	**21/66**	***r***	***p***	**2/66**	***r***	***p***	**44/66**	***r***	***p***
Antral mucosa													
H. suis	29/66	5/13	−0.02	0.85	10/21	0.13	0.31	1/2	−0.04	0.78	16/44	0.02	0.86
H. pylori-like	3/66	2/13	0.23	0.02	0	−0.01	0.31	0	−0.04	0.77	1/44	−0.02	0.86
H. felis	0/66	0	–	–	0	–	–	0	–	–	0	–	–
F. gastrosuis	10/66	2/13	−0.11	0.36	4/21	0.31	0.011	1/2	0.08	0.50	3/44	−0.20	0.11

r: Pearson correlation coefficient.

p: *p*-value ≤ 0.05 was considered to be significant.

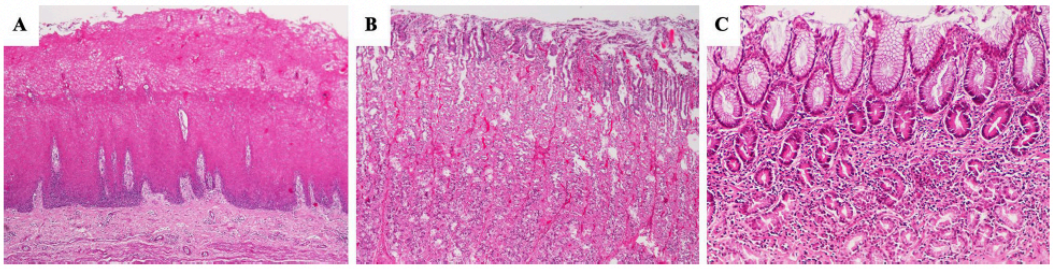

Figure 2 Main histopathological features observed in the different regions of wild boars' stomach. A Hyperkeratosis and hyperplasia of the lining epithelium of *pars oesophagea*. HE, ×40; **B** Mild inflammation of oxyntic mucosa. HE, ×40 and **C** Moderate and diffuse inflammatory infiltration of antral mucosa. HE, ×100.

presence of *H. salomonis* and *H. bizzozeronii* ($r = 0.67$, $p \leq 0.05$) (Table 8).

Concerning the correlation between the presence of *Helicobacter* spp., *F. gastrosuis* DNA and different histological findings, there was a highly statistically significant correlation between the presence of *H. bizzozeronii* and hyperplasia in the *pars oesophagea* (Table 9).

Discussion

It is known that the presence of helicobacters may be associated with gastric disease in humans and animals. *F. gastrosuis* has been described to possibly play a role in gastric ulceration in pigs [17, 18].

This study reports a high occurrence of gastritis in pigs and wild boars, 95.8% and 100% respectively, as

Table 6 Results of the histopathology of the different gastric regions of wild boars' stomach

Number of samples per gastric zone	Pars oesophagea		Oxyntic mucosa		Antral mucosa	
	N = 10		*N* = 10		*N* = 10	
	n/N	%	*n/N*	%	*n/N*	%
Normal mucosa	2/10	20.0	6/10	60.0	1/10	10.0
Score 0						
Fibrosis	0/2	0.0	0/6	0.0	0/1	0.0
Erosion	1/2	50.0	0/6	0.0	0/1	0.0
Ulceration	0/2	0.0	0/6	0.0	0/1	0.0
Hyperplasia	0/2	0.0	0/6	0.0	0/1	0.0
Mild gastritis	4/10	40.0	1/10	10.0	1/10	10.0
Score 1						
Fibrosis	0/4	0.0	0/1	0.0	0/1	0.0
Erosion	2/4	50.0	0/1	0.0	0/1	0.0
Ulceration	0/4	0.0	0/1	0.0	0/1	0.0
Hyperplasia	1/4	25.0	0/1	0.0	0/1	0.0
Moderate gastritis	2/10	20.0	2/10	20.0	1/10	10.0
Score 2						
Fibrosis	0/2	0.0	0/2	0.0	0/1	0.0
Erosion	0/2	0.0	0/2	0.0	0/1	0.0
Ulceration	0/2	0.0	0/2	0.0	0/1	0.0
Hyperplasia	0/2	0.0	1/2	50.0	0/1	0.0
Severe gastritis	2/10	20.0	1/10	10.0	7/10	70.0
Score ≥ 3						
Fibrosis	0/2	0.0	0/1	0.0	0/7	0.0
Erosion	1/2	25.0	0/1	0.0	0/7	0.0
Ulceration	0/2	0.0	0/1	0.0	0/7	0.0
Hyperplasia	1/2	25.0	0/1	0.0	0/7	0.0

was previously described by Hessing et al., Robertson et al., De Witte et al. [18, 32, 33].

Regarding the pig samples that presented gastritis: 51.9% of the *pars oesophagea* tissues were positive for one or more *Helicobacter* spp. and *F. gastrosuis* and, specifically 23.1% were positive for a *H. pylori*-like species and 11.5% for a *H. pylori*-like species in combination with *F. gastrosuis* (Additional file 4). In the oxyntic mucosa samples, 50.0% were positive for *Helicobacter* spp. and *F. gastrosuis* but, in contrast, the species most often detected was *H. suis*. In addition, positive cases for *F. gastrosuis* were always accompanied by a *Helicobacter* species (Additional file 4). Among the porcine antral gastritis cases, 57.6% were positive for *Helicobacter* spp. and *F. gastrosuis,* while 36.4% were *H. suis* positive, 7.6% *F. gastrosuis* positive and 6.1% *F. gastrosuis* plus *H. suis* positive (Additional file 4).

In both pigs and wild boars, *F. gastrosuis* was detected in association with *Helicobacter* species which corroborates their potential synergy to induce gastric pathology (Additional files 4 and 5).

Particularly in pigs, *F. gastrosuis* may have a synergetic role with *H. suis* in gastric ulceration as described by De Witte et al. [18]. De Bruyne et al. described a correlation between *H. suis* infection and the development of gastritis [14]. *H. suis* can affect the acid gastric secretion by altering the number and/or function of parietal D and G cells, as well as interfere with the sonic hedgehog (Shh) signaling pathway that regulates the gastric acid secretion and is involved in the gastric organogenesis, glandular differentiation and gastric homeostasis. This may lead to gastroesophageal ulceration as well as affect the gastric microbiota since the presence of *H. suis* alters the gastric environment which may promote the proliferation of other microorganisms such as *F. gastrosuis* in the non-glandular zone leading to gastritis and ulceration [10, 11, 34].

In pig samples, gastric ulceration and erosion were higher in the antral mucosa samples positive for *H. suis* and *F. gastrosuis* and a statistically significant correlation for the presence of *F. gastrosuis* with ulceration ($r = 0.31$, $p \leq 0.05$), and *H. pylori*-like with erosion ($r = 0.27$,

Table 7 ***Helicobacter*** **spp. and** ***F. gastrosuis*** **DNA PCR positive samples per wild boars' stomach**

Specific PCR-positive results	*n/N*	%
Overall identification		
Helicobacter spp. + *F. gastrosuis*	11/14	78.6
Helicobacter spp.	9/14	64.3
H. bizzozeronii	4/14	28.6
H. salomonis	4/14	28.6
H. pylori-like	2/14	14.3
H. suis	1/14	7.1
F. gastrosuis	6/14	42.9
Single bacteria		
F. gastrosuis	2/14	14.3
H. salomonis	2/14	14.3
H. pylori-like	0/14	0.0
H. suis	1/14	7.1
H. bizzozeronii	1/14	7.1
Multiple bacteria		
H. bizzozeronii + *F. gastrosuis*	2/14	14.3
H. pylori + *H. salomonis*	1/14	7.1
H. pylori-like + *F. gastrosuis*	1/14	7.1
H. bizzozeronii + *H. salomonis* + *F. gastrosuis*	1/14	7.1

$p \leq 0.05$) was demonstrated. The presence of *H. suis* and *F. gastrosuis* in pigs was previously reported and it was hypothesized that bacterial interaction can lead to gastric lesions and ulceration of the *pars oesopahea* [10, 18].

Compared to pigs, wild boars had a lower percentage of a *H. pylori*-like species and *H. suis* in the analyzed samples. However, both had close percentages of samples positive for *F. gastrosuis* (35.2% in pigs vs 42.9% in wild boars). Previous studies have reported the presence of *Helicobacter* spp. in wild boars [26, 35, 36], but this remains the first description of *F. gastrosuis* DNA detection and its possible relation with gastric erosion and ulceration in this species, as it has been described in pigs [10, 17, 18].

DNA of zoonotic *Helicobacter* spp. were detected in the pigs' and wild boars' stomach samples screened in this study, so the close contact between wildlife, domestic animals, and humans should be a concern for the transmission of bacteria with zoonotic potential that raises awareness in a One health perspective [4, 5, 19, 21, 24–26].

In the present study, DNA from *H. felis*, *H. bizzozeronii* and *H. salomonis*, which naturally colonize the stomach of dogs and cats, was detected in the stomach of pigs and/or wild boars. It is not clear whether these species are really able to colonize the stomach of these animals or whether our results are due to, for instance, environmental contamination.

Two PCR assays were used for detection of *H. pylori* DNA, one targeting the *ureAB* gene and the other one targeting the *glmM* gene. Although a number of studies have been published diagnosing *H. pylori* infection based on a *ureAB* gene PCR only [19, 37, 38], some reports indeed claim that *H. pylori* identification should include these two different target genes amplification [39–41]. Lu et al. compared five PCR methods for detection of *H. pylori* DNA of 24 culture-positive samples obtained from 50 human gastric samples, including PCRs targeting the *ureA* and *glmM* gene. These authors considered the *glmM* gene PCR to be the most appropriate method for detection of *H. pylori* organisms in clinical samples [42]. After

Table 8 Specific ***Helicobacter*** **spp. and** ***F. gastrosuis*** **detected through PCR in the different regions of wild boars' stomach**

Number of samples	Pars oesophagea		*r*	*p*	Oxyntic mucosa		*r*	*p*	Antral mucosa		*r*	*p*
	N = 14				*N* = 14				*N* = 14			
	n/N	%			*n/N*	%			*n/N*	%		
Single bacteria												
H. pylori-like	0/14	0.0			1/14	7.1			0/14	0.0		
H. suis	0/14	0.0			1/14	7.1			0/14	0.0		
H. bizzozeronii	1/14	7.1			1/14	7.1			0/14	0.0		
H. salomonis	2/14	14.3			0/14	0.0			3/14	21.4		
F. gastrosuis	2/14	14.3			4/14	28.6			5/14	28.5		
Multiple bacteria												
H. pylori-like + *F. gastrosuis*	0/14	0.0	–	–	1/14	7.1	–	–	0/14	0.0	–	–
H. bizzozeronii + *F. gastrosuis*	1/14	7.1	0.22	0.55	1/14	7.1	–	–	0/14	0.0	–	–
H. bizzozeronii + *H. salomonis* + *F. gastrosuis*	0	0	–	–	0	0.0	–	–	1/14	7.1	0.67	0.04

r: Pearson correlation coefficient.

p: *p*-value ≤ 0.05 was considered to be significant.

Table 9 Correlation between the PCR-positive results obtained in the different stomach regions and the gastric histological findings in wild boars

		Erosion			Ulceration			Hyperplasia			Fibrosis		
	n/N	**4/10**	***r***	***p***	**0/10**	***r***	***p***	**2/10**	***r***	***p***	**0/10**	***r***	***p***
Pars oesophagea													
H. pylori-like	0/10	0	–	–	0	–	–	0	–	–	0	–	–
H. suis	0/10	0	–	–	0	–	–	0	–	–	0	–	–
H. bizzozeronii	1/10	0	–	–	0	–	–	2/2	0.94	< 0.01	0	–	–
H. salomonis	0/10	0	–	–	0	–	–	0	–	–	0	–	–
F. gastrosuis	4/10	2/4	0.36	0.31	0	–	–	1/2	0.03	0.93	0	–	–
	n/N	**0/10**	***r***	***p***	**0/10**	***r***	***p***	**1/10**	***r***	***p***	**0**	***r***	***p***
Oxyntic mucosa													
H. pylori-like	2/10	0	–	–	0	–	–	0	–	–	0	–	–
H. suis	1/10	0	–	–	0	–	–	0	–	–	0	–	–
H. bizzozeronii	0/10	0	–	–	0	–	–	0	–	–	0	–	–
H. salomonis	0/10	0	–	–	0	–	–	0	–	–	0	–	–
F. gastrosuis	5/10	0	–	–	0	–	–	0	–	–	0	–	–
	n/N	**0/10**	***r***	***p***	**0/10**	***r***	***p***	**0/10**	***r***	***p***	**0**	***r***	***p***
Antral mucosa													
H. pylori-like	0/10	0	–	–	0	–	–	0	–	–	0	–	–
H. suis	0/10	0	–	–	0	–	–	0	–	–	0	–	–
H. bizzozeronii	2/10	0	–	–	0	–	–	0	–	–		–	–
H. salomonis	5/10	0	–	–	0	–	–	0	–	–	0	–	–
F. gastrosuis	6/10	0	–	–	0	–	–	0	–	–	0	–	–

r: Pearson correlation coefficient.

p: ***p*-value** ≤ **0.05 was considered to be significant.**

analyzing 290 human gastric samples, Elnosh et al. concluded that a *glmM* gene based PCR showed higher sensitivity and specificity than a *ureA* gene based PCR [43]. In another investigation, only 10 out of the 50 human gastric samples analyzed turned out positive using the *glmM* gene whereas 25 were PCR-positive using the *ureA* gene [44].

It is remarkable that more than 50% of the pigs were positive in the *ureAB* PCR, while none of the samples were positive in the *glmM* PCR. Sequencing of the amplicons obtained in the *ureAB* PCR, revealed high homologies with *H. pylori ureAB* ranging from 96.3 to 100%. Natural colonization of the stomach of pigs with *H. pylori* has not been described before. We hypothesize that a *H. pylori*-like species, with a similar *ureAB* gene as *H. pylori*, was detected. This organism seems to preferably colonize the proximal, non-glandular part of the stomach whereas *H. suis* mainly colonizes the distal part. In any case, our results should be confirmed by isolation and identification of this *H. pylori*-like species from the stomach of pigs.

Krakowka et al. described a *Helicobacter* species present in the stomach of pigs, which had a curve-shaped morphology similar to that of *H. pylori* [45]. Unfortunately, no taxonomic studies or genome sequences have been published for this *Helicobacter* species and its exact identity is unclear [10]. Comparison with our results is therefore not possible.

This study reports the presence of a *H. pylori*-like species, gastric NHPH, and *F. gastrosuis* DNA in the stomach of pigs and wild boars and the putative gastric histopathological alterations associated. The results suggest that pigs and wild boars may act as reservoirs for these bacteria.

Further research should be carried out, including studies with a larger sampled population of wild boars, to assess the real prevalence of these bacteria in this animal species and to better understand their possible role in the development of gastric pathology.

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s13567-022-01101-5.

Additional file 1. Primer sequences used for detection of *Helicobacter* spp. and *F. gastrosuis* and thermocycling conditions.

Additional file 2. Nonparametric Kruskal–Wallis, Chi Square test applied to the three gastric zones comparing the different variables in pigs.

Additional file 3. BLAST identity percentage interval of the different *Helicobacter* spp. and *F. gastrosuis* sequences obtained.

Additional file 4. Number of *Helicobacter* spp. and *F. gastrosuis* DNA positive samples associated with gastritis score per pig gastric zone.

Additional file 5. Number of *Helicobacter* spp. and *F. gastrosuis* DNA positive samples associated with gastritis score per wild boar's gastric zone.

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Authors' contributions
Conceptualization: FCN, TLM, FH and IA; Methodology: FCN, TLM, FH and IA; formal analysis: FCN, NC, TLM, ET, FH and IA; investigation: FCN, ST, ET, SB, AR; resources: FCN, TLM and IA; data curation: FCN, TLM, FH and IA; writing—original draft preparation: FCN, TLM and IA; writing—review and editing: FCN, ST, TLM, AR, FG, ET, FH and IA; supervision: TLM, IA, FH. All authors read and approved the final manuscript.

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Declarations

Ethics approval and consent to participate
This study was approved by i3S Animal Welfare and Ethics Review Body (ref. 2021-4).

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

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Additional file 1 Primer sequences used for detection of *Helicobacter* spp. and *F. gastrosuis* and thermocycling conditions

Helicobacter species	Primer	Sequence	Target gene	Amplicon size	Thermal cycle conditions			*Helicobacter* strain used as positive control
					Nr. Cycles	Temp. (°C)	Time	
H. suis	BFHsuis_F1	AAA ACA MAg gCg ATC gCC CTg TA	*ureA*	150bp	40	95 60 72	20 sec 30 sec 30 sec	HS1
	BFHsuis_R1	TTT CTT CgC CAg gTT CAA AgC g	*ureA*					
H. heilmannii	Hh-IceA-FWQ	gTT TCC AAC CAA AAg ACT CA	*iceA*	135 bp	30	94 55 72	30 sec 30 sec 30 sec	ASB1.4
	Hh-IceA-RVQ	ATT gCC TAg Agg TTg TgT Tg	*iceA*					
H. ailurogastricus	Ha-LpsA-FWQ	CTT gAg TAC ggC gAT gTC AAT	*lpsA*	136 bp	30	94 55 72	30 sec 30 sec 30 sec	ASB7.1
	Ha-LpsA-RVQ	ggg gAA AAA TgT gCT TgA AgT	*lpsA*					
H. salomonis	Hsal_FQ_PAR	CTC TTA TgA gTT ggA CTT ggT gCT CAC CAA T	*ureAB*	91 bp	45	94 61 72	30 sec 30 sec 1 min	R1051
	Hsal_RQ_PAR	TTT gCC ATC TTT AAT TCC AAT gTC ggC	*ureAB*					
H. felis	BFHfel_F2	gCT ggT ggC ATC gAT ACg CAT	*ureAB*	154 bp	45	94 60 72	30 sec 30 sec 1 min	CS1
	BFHfel_R2	TTT TTA gAT TAg CgC gTC Cgg gA	*ureAB*					
H. bizzozeronii	Hbizz_FQ_PAR	CCA ACA AAT CCC CAC AgC ATT TgC CAg	*ureAB*	91 bp	45	94 58 72	1 min 1 min 1 min	R1053
	Hbizz_RQ_PAR	AgT CCC ATC AgC Wgg WCC TgT TCC CCC AC	*ureAB*					
H. pylori	BFHpyl_F1	AAA gAg CgT ggT TTT CAT ggC g	*ureAB*	217 bp	45	94 59 72	30 sec 30 sec 1 min	26695
	BFHpyl_R1	ggg TTT TAC CgC CAC CgA ATT TAA	*ureAB*					
	Hpy3F	TTATCGGTAAAGACACCA GAAA	*glmM*	144	45	94 54 72	30 sec 30 sec 30 sec	SS1
	Hpy3R	ATCACAGCGCATGTCTTC	*glmM*					
F. gastrosuis	GB_F	GCA GCT CAA AGA GCA AGA GAA GCA	*gyrB*	158bp	35	95 63 72	20 sec 30 sec 30 sec	CDW1T
	GB2_R	CTT CCC TGC TTT GCA GAA CCT CC	*gyrB*	158bp				

Additional file 2 Nonparametric Kruskal-Wallis, Chi Square test applied to the three gastric zones comparing the different variables in pigs.

	finsco	fib	eros	ulc	hyperp	hpyl	hsuis	hfelis	hsal	fuso
Kruskal-Wallis Test										
Chi-Square	9.12	69.18	6.24	33.39	77.95	36.44	17.05	3.58	3.75	6.385
DF	2	2	2	2	2	2	2	2	2	2
Pr > Chi-Square	**0.0105**	**<.0001**	**0.0442**	**<.0001**	**<.0001**	**<.0001**	**0.0002**	0.1666	0.1533	**0.0411**

finsco – final score of gastritis; neut – neutrophils; eosin – eosinophils; fib – fibrosis; eros – erosion; hpyl – H. *pylori*; hsuis – *H. suis*; hfelis – *H. felis*; hsal – *H. salomonis;* fuso – *F. gastrosuis*; - $p < 0.05$ – statistically significant differences between gastric zones (*pars esopahea*, *antrum* and oxyntic mucosa)

Additional file 3 BLAST identity percentage interval of the different *Helicobacter* spp. and *F. gastrosuis* sequences obtained.

Animals	Bacteria	Identity	Reference strain Accession Number
Pigs	*H. pylori*	96.3-100%	CP048599
		99.2-100%	CP024946
		99.3-100%	CP032027
		98.6%	CP048600
		100%	CP011330
		98.1-100%	CP032037
	H. suis	95.6-100%	AP023036
		96.8-100%	AP023046
	H. felis	95.9%	JQ736360
		97.1%	FQ670179
	H. salomonis	100%	AJ130882
	F. gastrosuis	96.0-100%	LN906798
Wild Boars	*H. bizzozeronii*	95.3-99.2%	FR871757
	H. felis	99.1%	FQ670179
	H. suis	98.9%	AP023046
	H. pylori	100%	AF507994
		100%	AY368264
	H. salomonis	96.4%	AF508005
		100%	AY368266
	F. gastrosuis	97.0-100%	LN906798

Additional file 4 Number of *Helicobacter* spp. and *F. gastrosuis* DNA positive samples associated with gastritis score per pig gastric zone.

	Pars oesophagea					Oxyntic Mucosa					Antral mucosa				
	Normal	Mild gastritis	Moderate gastritis	Severe gastritis	Total	Normal	Mild gastritis	Moderate gastritis	Severe gastritis	Total	Normal	Mild gastritis	Moderate gastritis	Severe gastritis	Total
	n = 5	*n* = 16	*n* = 9	*n* = 27	*n* = 57	*n* = 9	*n* = 28	*n* = 13	*n* = 19	*n* = 69	*n* = 0	*n* = 2	*n* = 3	*n* = 61	*n* = 66
H. pylori-like	1	6	0	6	13	1	3	2	1	7	0	0	0	1	1
H. suis	0	0	0	0	0	3	10	2	5	20	0	0	2	22	24
H. felis	0	0	0	0	0	0	1	0	0	1	0	0	0	0	0
H. salomonis	0	0	0	0	0	0	0	0	0	0	0	0	0	2	2
F. gastrosuis	0	3	0	0	3	0	0	0	0	0	0	0	1	4	5
H. pylori-like + *H.suis*	0	1	1	3	5	0	0	1	0	1	0	0	0	1	1
H.suis + *H. felis*	0	0	0	0	0	0	0	0	1	1	0	0	0	0	0
H. pylori-like + *F. gastrosuis*	2	2	0	4	8	0	0	0	1	1	0	0	0	1	1
H. suis + *F. gastrosuis*	0	0	0	0	0	0	1	1	0	2	0	0	0	4	4
H. pylori-like + *H.suis* + *F. gastrosuis*	0	0	1	0	1	0	1	0	0	1	0	0	0	0	0

Additional file 5 Number of *Helicobacter* spp. and *F. gastrosuis* DNA positive samples associated with gastritis score per wild boar's gastric zone

	Pars oesophagea					Oxyntic mucosa					Antral mucosa				
	Normal	Mild gastritis	Moderate gastritis	Severe gastritis	Total	Normal	Mild gastritis	Moderate gastritis	Severe gastritis	Total	Normal	Mild gastritis	Moderate Gastritis	Severe gastritis	Total
	$n = 2$	$n = 4$	$n = 2$	$n = 2$	$n = 10$	$n = 6$	$n = 1$	$n = 2$	$n = 1$	$n = 10$	$n = 1$	$n = 1$	$n = 1$	$n = 7$	$n = 10$
H. pylori-like	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
H. suis	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
H. bizzozeronii	0	0	0	1	1	1	0	0	0	1	0	0	0	0	0
H. salomonis	0	0	1	0	1	0	0	0	0	0	0	0	0	1	1
F. gastrosuis	0	1	0	1	2	1	1	0	0	1	1	0	0	2	3
F.gastrosuis + H. bizzozeronii	0	1	0	0	1	0	0	1	0	1	0	0	0	0	0
H.bizzozeronii + H. salomonis + F. gastrosuis	0	0	0	0	0	0	0	0	0	0	0	0	0	1	1

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Chapter III

Presence of *Helicobacter pylori* and *H. suis* DNA in Free-Range Wild Boars

(Article II)

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animals

MDPI

Brief Report

Presence of *Helicobacter pylori* and *H. suis* DNA in Free-Range Wild Boars

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Simple Summary: *Helicobacter pylori* and *H. suis* are associated with gastric pathologies in humans. To obtain better insights into the potential role of wild boars as reservoirs of these pathogens, gastric samples of 14 animals were tested for the presence of *H. pylori* and *H. suis* DNA. Two wild boars were found PCR-positive for *H. pylori* and one for *H. suis*. This indicates that these microorganisms may colonize the stomach of wild boars.

Abstract: *Helicobacter pylori* (*H. pylori*) is a Gram-negative bacterium that infects half of the human population worldwide, causing gastric disorders, such as chronic gastritis, gastric or duodenal ulcers, and gastric malignancies. *Helicobacter suis (H. suis)* is mainly associated with pigs, but can also colonize the stomach of humans, resulting in gastric pathologies. In pigs, *H. suis* can induce gastritis and seems to play a role in gastric ulcer disease, seriously affecting animal production and welfare. Since close interactions between domestic animals, wildlife, and humans can increase bacterial transmission risk between species, samples of gastric tissue of 14 free range wild boars (*Sus scrofa*) were evaluated for the presence of *H. pylori* and *H. suis* using PCR. Samples from the antral gastric mucosa from two animals were PCR-positive for *H. pylori* and another one for *H. suis*. These findings indicate that these microorganisms were able to colonize the stomach of wild boars and raise awareness for their putative intervention in *Helicobacter* spp. transmission cycle.

Keywords: one health; wildlife; zoonosis; *Helicobacter* spp.; PCR; *Sus scrofa*

1. Introduction

The number of infectious diseases has been increasing in humans, with about 60% of those being zoonotic [1]. Of these emerging zoonoses, about 72% are transmitted from wildlife animals [2].

Wild boars are known to be reservoirs of a considerable number of zoonotic bacteria, viruses, and parasites [3], and these infections can be bi-directional (wild/domestic). The

exposure to wild boars' pathogens can happen through different pathways: Direct contact, meat consumption, or indirect intake of contaminated water, food, or through the environment [4]. Changes in human living habits, increased hunting activities, and consumption of wild boar meat play a role in the risk of human exposure to infectious agents [3].

Helicobacter species are Gram-negative, spiral-shaped motile bacteria that colonize the gastrointestinal tract of both humans and animals [5–7], and have been studied over the years for their association with gastrointestinal diseases [8]. In humans, *Helicobacter pylori* (*H. pylori*) is the most common gastric pathogen, affecting more than half of the world's population, being responsible for development of gastritis, gastroduodenal ulcers, gastric adenocarcinoma, mucosa-associated lymphoid tissue (MALT) lymphoma, and extra digestive diseases [5,9,10]. In addition, *Helicobacter suis* (*H. suis*) is the most prevalent human gastric non-*Helicobacter pylori Helicobacter* (NHPH) and has been associated with a range of gastric pathologies, including MALT lymphoma, and possibly also extra digestive diseases. Recent reports reinforce that these infections most likely originate from pigs, emphasizing their zoonotic potential [5,11–14].

In pigs, *H. suis* mainly colonizes the fundic and pyloric gland zone of the stomach [15]. The bacterium presents tropism for the gastric acid-producing parietal cells [16]. Its prevalence appears to be very low prior to weaning, but increases rapidly thereafter, being very high at slaughter age (77%) and in adults (>90%) [15–17]. *H. suis* infection causes gastritis, decreased daily weight gain, and plays a role in induction of gastric ulcers, clearly affecting animal production and welfare [18–20].

In pigs, there is a report of a natural infection by a *H. pylori*-like bacterium, described as attached to the mucosa of the cardiac and antral portions of the stomach of two out of four healthy young pigs, without gross lesions associated [6,21]. This agent seemed to be morphologically similar to, but antigenically different from *H. pylori*, and its exact identity is not clear [18].

Until now, studies trying to assess the presence of *H. suis* in wild boars (*Sus scrofa*) have been mainly unsuccessful, as described by Flahou et al. [14] and others [22,23].

Helicobacter spp. have been described to have zoonotic potential and the close contact between humans, domestic animals, and wild animals deserves more consideration [14,24]. Although reservoirs of wild and domestic animals can be considered as important sources of emerging infectious diseases, it is the human impact on ecological systems that determines the level of risk at the human/animal interface upon the occurrence of emerging zoonotic diseases [14,24].

From an eco-epidemiological perspective, wild boars have an important role in spread of several pathogens [3,4]; thus, the aim of this study was to screen different regions of the stomach (*Pars oesophagea*, fundic, and pyloric gland zone) collected from wild boars for the presence of *H. pylori* and *H. suis*.

2. Materials and Methods

2.1. Samples Collection

Samples were collected using convenience sampling from fourteen hunted animals during two national campaigns, one in the north and other in the center of Portugal (Vila Real and Coimbra districts, respectively) (Figure 1). All the sampled animals were older than 9 months according to teeth assessment [25,26]. From each animal, gastric samples were collected from three different gastric regions: *Pars oesophagea*, fundic gland zone, and pyloric gland zone (gastric *antrum)* using a Kruuse® Biopsy punch 8 mm. After collection, samples were stored at −20 °C until DNA extraction.

The animals were not slaughtered or euthanized in order to carry out this study, and the fresh gastric tissue specimens were obtained as sub-products derived from the normal activity associated with the meat inspection procedures occurring during these conventional campaigns. None of the actions was performed solely for research purposes and the researchers had no influence on the campaign organization, nor in the meat inspection actions.

Figure 1. Illustration of the geographical origin of the samples collected in Portugal and evaluated in this study. Samples were collected from 10 wild boars during a national campaign in Vila Real (VR10) and 4 wild boars during a national campaign in Coimbra (C4).

2.2. DNA Extraction, PCR Conditions and Sequencing

DNA was extracted from 8-mm gastric frozen tissue samples, using EXTRACTME® DNA tissue kit (BLIRT, Poland), according to the instructions provided by the supplier.

All the samples were tested for *H. pylori* and *H. suis* by conventional PCR, according to previously described protocols (Table 1).

Table 1. Primer sequences used for detection of *H. pylori* and *H. suis* and thermo cycling conditions.

Primer	Sequence	Region	Amplicon Size	Cycle			Ref.
				Nr. Cycles	Temp. (°C)	Time	
BFHsuis_F1	5′-AAA ACA MAG GCG ATC GCC CTG TA-3′	*ureA* gene	150bp	40	95 60 72	20 s 30 s 30 s	[5]
BFHsuis_R1	5′-TTT CTT CGC CAG GTT CAA AGC G-3′	*ureA* gene					
BFHpyl_F1	5′-AAA GAG CGT GGT TTT CAT GGC G-3′	*ureAB gene*	217bp	45	94 59 72	30 s 30 s 1 min	[27]
BFHpyl_ R1	5′-GGG TTT TAC CGC CAC CGA ATT TAA-3′	*ureAB gene*					

Aliquots of each PCR product were electrophoresed on 1.5% agarose gel, stained with Xpert Green Safe DNA gel stain (GRISP, Porto, Portugal) and examined for the presence of a specific fragment under UV light. DNA fragment size was compared with the standard molecular weight, 100bp DNA ladder (GRISP, Porto, Portugal), and the molecular weight of the positive controls (*H. pylori* with 217 and *H. suis* with 150 bp) (Figure S1). As a negative control, distilled water was used. As positive controls, DNA was extracted from pure cultures of *H. pylori* strain 26695 and *H. suis* strain HS1.

To exclude false positive samples, the amplicons from each positive sample were sequenced. Bidirectional sequencing was performed with Sanger method at the genomics core facility of the Institute of Molecular Pathology and Immunology of the University of Porto, Portugal. Sequence editing and multiple alignments were performed with the MegaX Molecular Evolutionary Genetic Analysis version 10.1.8. The sequences obtained were subject to the basic local alignment search tool (BLAST) using the non-redundant nucleotide database (http://blast.ncbi.nlm.nih.gov/Blast.cgi, accessed on 6 January 2021) [28,29].

3. Results and Discussion

A total of 42 samples, collected from 14 wild boars, were analyzed.

Based on PCR results, two samples corresponding to two distinct animals were *H. pylori* PCR-positive and another, also corresponding to a different animal, was *H. suis* PCR-positive. These positive samples all originated from the pyloric gland zone (gastric *antrum)* of these animals.

The bidirectional sequencing and BLAST analysis of consensus sequences of partial *ureA* and *ureB* genes showed a homology of 100% with *H. pylori* (GenBank® accession no. AF507994 and AY368264) for two of them and the other positive sample showed a homology of 95.39% with *H. suis* (GenBank® accession no. EF204592).

In the current study, *H. pylori* DNA was detected in the pyloric gland zone of the stomach (gastric *antrum)* from two animals and *H. suis* DNA in the same gastric region of another animal. This region is also a preferential colonization site of *H. suis* in domesticated pigs [17]. These findings might indicate that wild boars are occasionally colonized by *H. pylori* and *H. suis.* It can, however, not be excluded that presence of DNA of these microorganisms might be a consequence of recent contamination; for instance, through contact with domesticated pigs in the case of *H. suis*, or contaminated water or environments for both species. Both *H. pylori* PCR-positive cases did not present any macroscopic gastric alterations. The wild boar stomach where *H. suis* DNA was detected showed signs of mild antral inflammation consisting of mild erythema and congestion at gross examination. Unfortunately, the tissue preservation conditions prevented their detailed microscopic evaluation and the presence of microorganisms with *Helicobacter*-like morphology could not be examined. Further studies using a larger sample and including immunohistochemical and histopathological analysis of gastric tissue from wild boars are absolutely relevant to confirm our findings.

To the authors' knowledge, this is the first report of *H. pylori* DNA detected in gastric samples of free-ranging wild boars and the first report of *H. pylori* and *H. suis* DNA detection in free-range wild boars in Portugal.

Previously, gastric *antrum* specimens of 17 free-ranging wild boars from Poland were tested, using a *Helicobacter* genus-specific 16S rRNA PCR and sequence analysis of positive samples [23]. In one sample, DNA was detected of a microorganism related to the group of NHPH mainly associated with dogs and cats (previously referred to as *Helicobacter heilmannii* type 2) [23]. More recently, a novel *Helicobacter* species, *H. apri*, was described in wild boars, but this is an enterohepatic *Helicobacter* species [22]. In another survey, very low numbers of *H. suis* were detected in the cardiac gland zone and *Pars oesophagea* from 2 out of 9 wild boars from Belgium. These gastric regions are not the preferential *Helicobacter* colonization sites in the porcine stomach, possibly indicating recent contamination for instance through contact with domesticated pigs or their excretes [14].

Wild boars are known to be reservoirs for several agents for important infectious diseases transmissible to other wild animals, domestic animals, and humans [3,4]. Depending on the pathogen properties and on wild boar density and management, the eco-epidemiological role of these animals can vary from a dead-end over spill over, up to maintenance host [3,4]. Contacts between wild boars and outdoor domestic pigs should be considered a risk for transmission of these pathogens that can directly affect the swine production and animal welfare, since wild boars and domestic pigs belong to the same species (*Sus scrofa*) [3,4,17,19,30].

Hunting dogs and humans can be exposed to wild boars' pathogens either from fresh carcass contact, handling or consumption of raw, undercooked meat, or indirect contact from contaminated water or environment, and therefore, high risk exposure would include game wardens, hunters, butchers, and other wildlife professional duties [3,4]. Another aspect to consider is the increasing wild boar population and its adaptability to urban areas that can prompt wild boar contact with humans in these areas, making humans more vulnerable to be exposed to wild boars' pathogens [3,4,31]. The current findings raise *One health* concerns regarding the impact of *H. suis* and *H. pylori* in wild boar welfare, its role

and impact on bacterial spread and transmission to the environment, and also to wild and domestic animals, and, ultimately, to humans [3,13,24,31].

4. Conclusions

H. pylori and *H. suis* DNA was detected in the stomach of free-range wild boars, which might indicate that these animals were colonized by these microorganisms. It can be hypothesized that wild boars might act as reservoirs and contribute to the spread of the *H. pylori* and *H. suis* in the environment, raising a public health concern.

Supplementary Materials: The following are available online at https://www.mdpi.com/article/10.3390/ani11051269/s1, Figure S1: Example of agarose gel electrophoresis of PCR products of *H. pylori* gene fragments. M, molecular marker; +C, DNA extracted from pure culture of 26695 strain was used as a positive control; WB, wild boar DNA; -C, negative control consisting solely of mix solution (NC).

Author Contributions: Conceptualization, F.C.N., T.L.M. and I.A.; methodology, F.C.N., T.L.M., S.T. and I.A.; formal analysis, F.C.N., P.B., T.L.M. and I.A.; investigation, F.C.N.; resources, F.C.N., T.L.M. and I.A.; data curation, F.C.N., T.L.M. and I.A.; writing—original draft preparation, F.C.N., T.L.M. and I.A.; writing—review and editing, S.T., T.L.M., I.A., P.B., C.d.W., F.H. and F.G.; supervision, T.L.M., I.A., C.d.W., F.H. and F.G. All authors have read and agreed to the published version of the manuscript.

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Supplementary data

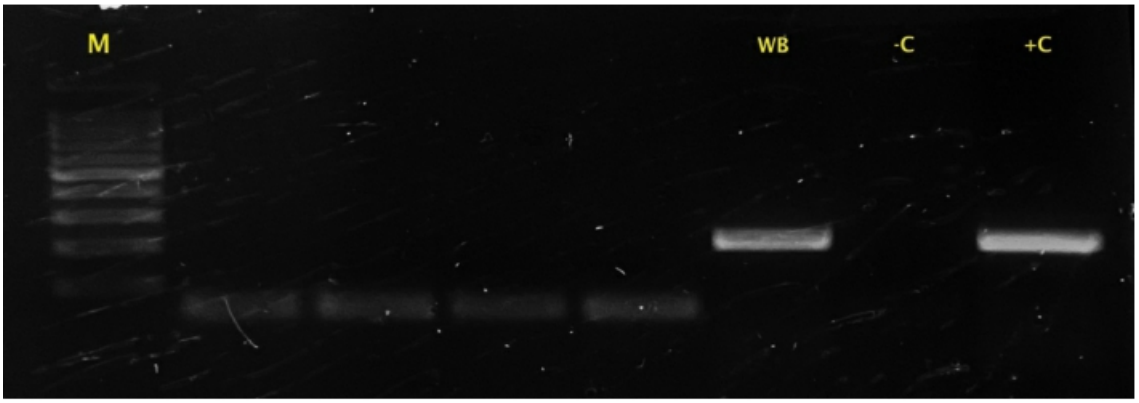

Figure S1. Example of agarose gel electrophoresis of PCR products of *H. pylori* gene fragments**.** M, molecular marker; +C, DNA extracted from pure culture of 26695 strain was used as a positive control; WB, wild boar DNA; -C, negative control consisted solely of mix solution (NC)..

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Chapter IV

Molecular detection of human pathogenic gastric *Helicobacter* species in wild rabbits (*Oryctolagus cuniculus*) and wild quails (*Coturnix coturnix*)

(Article III)

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zoonotic diseases

MDPI

Brief Report

Molecular Detection of Human Pathogenic Gastric *Helicobacter* Species in Wild Rabbits (*Oryctolagus cuniculus*) and Wild Quails (*Coturnix coturnix*)

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Abstract: Wildlife plays a major role in the maintenance and transmission of multihost pathogens. Several *Helicobacter* spp. have been described to have zoonotic potential; thus, human, domestic and wild animal interactions deserve more attention. In this study, the presence of the DNA of human pathogenic gastric *Helicobacter* species was determined in gastric samples collected from wild rabbits and wild quails during the national hunting campaigns in Portugal. Eleven out of the 12 wild rabbits (91.7%) and all six wild quails tested (100%) were PCR positive for one or more gastric *Helicobacter* species. In both animal species, *H. felis*, *H. bizzozeronii* and *H. salomonis* DNA were detected. In addition to these non-*Helicobacter pylori Helicobacter* spp. (NHPH), *H. pylori* DNA was also identified in gizzard samples of wild quails. These findings might indicate that wild rabbits and wild quails may act as reservoirs and contribute to the *H. pylori* and NHPH environment dissemination, causing both Public Health and One health concerns to arise.

Keywords: one health; PCR; wildlife; gastric *Helicobacter*; zoonoses

1. Introduction

Wildlife plays a major role in the maintenance and transmission of multihost pathogens and the understanding of the role of host species in the epidemiological cycle is essential to prevent diseases caused by zoonotic pathogens [1]. Although both wild and domestic animal reservoirs can be considered important sources of emerging infectious diseases, it is the human impact in the ecological systems that commands the level of risk at the humans/animals interface upon zoonotic disease emergences episodes [2]. In addition, the number of infectious diseases of zoonotic origin has been increasing, and approximately 72% are transmitted from wildlife [3,4].

Helicobacter species are Gram-negative, motile bacteria with a helical form that colonize the gastrointestinal tract of humans and a wide range of animal species [5–8]. Gastric

Helicobacter species have been studied over the years for their association with gastrointestinal diseases and zoonotic potential [9]. These bacteria have been widely described in humans, dogs and cats [8,10,11].

Helicobacter pylori (*H. pylori*) is the most prevalent gastric pathogen in humans, infecting over half the global population and causing gastritis, gastroduodenal ulcers, gastric adenocarcinoma and mucosa associated lymphoid tissue (MALT) lymphoma [5,12,13]. Gastric non-*Helicobacter pylori Helicobacter* species (NHPH) include a range of *Helicobacter* species previously described as *Helicobacter heilmannii* type 1 (*H. suis*) and *Helicobacter heilmannii* type 2 (*H. felis*, *H. bizzozeronii*, *H. salomonis*, *H. heilmannii* and *H. ailurogastricus*) [8,14].

In addition, gastric NHPH have been observed in 0.2–6% of human gastric biopsies and have been associated with a range of gastric pathologies, especially MALT lymphoma, as well as extra digestive diseases [8,15–17]. *H. suis* is the most common NHPH in humans suffering from gastric disorders, followed by *H. salomonis*, *H. felis*, *H. heilmannii* and *H. bizzozeronii* [5,8,15]. Humans may acquire these infections through contact with animals since most of NHPH are animal associated bacteria, but only some of them are recognized as potentially zoonotic [9,11,18].

There are two reports of *Helicobacter* species in rabbits. In these studies, gastric NHPH (*H. felis* and *H. salomonis*) DNA was detected in rabbit stomach samples [19,20]. Regarding birds, enterohepatic but not gastric *Helicobacter* species are commonly detected and mainly in intestinal or faecal samples [18,21–24].

Despite humans being considered the natural reservoir for *H. pylori,* this *Helicobacter* species has been detected in other domestic and wild animals [8,11,20,24–27]. Nevertheless, to the authors' knowledge, there is a lack of studies regarding the presence of gastric *Helicobacter* species in both wild rabbits and wild birds.

From an eco-epidemiological perspective, wild animals may have an important role in the spread of several pathogens [28–31].

Hunting dogs and humans can become infected with game pathogens through direct contact with fresh carcasses and the handling or consumption of raw or undercooked meat; thus, as a result, game wardens, hunters, butchers and other wildlife professionals are at high risk. Therefore, the aim of this study was to screen different regions of wild rabbit stomachs (fundus and corpus) and wild quail gizzards for the presence of DNA from *H. pylori* and zoonotically important NHPH, mainly associated with pigs, dogs and cats.

2. Materials and Methods

2.1. Animals and Samples Collection

Wild rabbits (*Oryctolagus cuniculus*) and wild quails (*Coturnix coturnix*), shot during three national hunting campaigns held in the centre of Portugal (Coimbra district), were subjected to convenience sampling. All the sampled animals were adults. Dissection of the stomach was performed using disposable scalpels and disposable tweezers. Representative gastric tissues from the fundus and corpus of wild rabbits and from the gizzard of wild quails were collected using a sterile disposable Kruuse® (Langeskov, Denmark) Biopsy punch 8 mm per sample, rendering cross-contamination between samples unlikely, and then they were stored at −20 °C for further DNA extraction.

The animals were not slaughtered or euthanized in for this investigation and the stomach specimens were obtained as sub-products of the usual meat inspection activity that takes place throughout these campaigns. None of the acts were taken exclusively for the purpose of research, and the researchers had no influence over the campaign's planning or meat inspection methods.

2.2. DNA Extraction and PCR Conditions and Sequencing

DNA was extracted from 8 mm of gastric frozen tissue samples, using the EXTRACTME® DNA tissue Kit (BLIRT S.A., Gdansk, Poland) and according to instructions provided by the supplier.

All the samples were tested for the presence *H. pylori*, *H. felis*, *H. salomonis*, *H. bizzozeronii*, *H. heilmannii*, *H. suis* and *H. ailurogastricus* DNA through conventional PCR analysis, according to previously described protocols (Table 1).

Table 1. Primer sequences used for detection of *H. pylori* and NHPH and thermo cycling conditions.

Helicobacter Species	Primer	Sequence	Target Gene	Amplicon Size	Thermal Cycle Conditions			*Helicobacter* Species Culture Used as Positive Control
					Nr. Cycles	Temp. (C)	Time	
H. suis	BFHsuis_F1	AAA ACA MAg gCg ATC gCC CTg TA	ureA	150 bp	40	95 60 72	20 s 30 s 30 s	HS1
	BFHsuis_R1	TTT CTT CgC CAg gTT CAA AgC g	ureA					
H. heilmannii	Hh-IceA-FWQ	gTT TCC AAC CAA AAg ACT CA	iceA	135 bp	30	94 55 72	30 s 30 s 30 s	ASB1.4
	Hh-IceA-RVQ	ATT gCC TAg Agg TTg TgT Tg	iceA					
H. ailurogastricus	Ha-LpsA-FWQ	CTT gAg TAC ggC gAT gTC AAT	lpsA	136 bp	30	94 55 72	30 s 30 s 30 s	ASB7.1
	Ha-LpsA-RVQ	ggg gAA AAA TgT gCT TgA AgT	lpsA					
H. salomonis	Hsal_FQ_PAR	CTC TTA TgA gTT ggA CTT ggT gCT CAC CAA T	ureAB	91 bp	45	94 61 72	30 s 30 s 1 min	R1051
	Hsal_RQ_PAR	TTT gCC ATC TTT AAT TCC AAT gTC ggC	ureAB					
H. felis	BFHfel_F2	gCT ggT ggC ATC gAT ACg CAT	ureAB	154 bp	45	94 60 72	30 s 30 s 1 min	CS1
	BFHfel_R2	TTT TTA gAT TAg CgC gTC Cgg gA	ureAB					
H. bizzozeronii	Hbizz_FQ_PAR	CCA ACA AAT CCC CAC AgC ATT TgC CAg	ureAB	91 bp	45	94 58 72	1 min 1 min 1 min	R1053
	Hbizz_RQ_PAR	AgT CCC ATC AgC Wgg WCC TgT TCC CCC AC	ureAB					
H. pylori	BFHpyl_F1	AAA gAg CgT ggT TTT CAT ggC g	ureAB	217 bp	45	94 59 72	30 s 30 s 1 min	26695
	BFHpyl_R1	ggg TTT TAC CgC CAC CgA ATT TAA	ureAB					

Aliquots of each PCR product were electrophoresed on a 1.5% agarose gel stained with Xpert Green Safe DNA gel stain (GRISP, Porto, Portugal) and examined under UV light for the presence of specific fragments. The size of the DNA fragment was compared to the standard molecular weight, the 100 bp DNA ladder (GRISP, Porto, Portugal) and the molecular weight of the positive controls (Table 1). For the negative control, distilled water was used. For the positive controls, DNA was extracted from pure cultures of each *Helicobacter* species tested (Table 1).

To exclude false positive samples, the amplicons of each positive sample were sequenced. Bidirectional sequencing was carried out using the Sanger method at the Genomics core facility of the University of Porto's Institute of Molecular Pathology and

Immunology. MegaX Molecular Evolutionary Genetic Analysis version 10.1.8 was used for sequence editing and multiple alignments. The sequences obtained were subject to the basic local alignment search tool (BLAST) using the non-redundant nucleotide database (http://blast.ncbi.nlm.nih.gov/Blast.cgi, accessed on 7 December 2021).

3. Results and Discussion

A total of 30 gastric samples were evaluated: 24 were collected from 12 wild rabbits and consisted of equal amounts of fundus and corpus mucosa, and six were collected from six wild quails.

Eleven out of the 12 wild rabbits (91.7%) and the six (100%) wild quails were PCR positive for one or more gastric *Helicobacter* species.

3.1. Wild Rabbits

Among the 11 PCR positive animals, the most prevalent *Helicobacter* was *H. salomonis* (9/11), followed by *H. bizzozeronii* (5/11), and then *H. felis* (2/11) (Table 2).

Table 2. Number and percentage of positive PCR results for gastric *Helicobacter* species in wild rabbits.

	Wild Rabbits (*n* = 12)	**Fundus (*n* = 12)**	**Corpus (*n* = 12)**
H. salomonis	9 (75.0%)	8 (66.7%)	8 (66.7%)
H. bizzozeronii	5 (41.7%)	1 (8.3%)	5 (41.7%)
H. felis	2 (16.7%)	1 (8.3%)	1 (8.3%)

Concerning the wild rabbit specimens, and regardless of the stomach counterpart: four animals were *H. salomonis*-positive, one was *H. felis*-positive, another was *H. bizzozeroni*-positive, four were *H. bizzozeronii* plus *H. salomonis*-positive and one was *H. felis* plus *H. salomonis*-positive (Table A1).

According to the gastric location, nine samples of each fundus and corpus location were PCR positive for at least one *Helicobacter* species (Table 2).

The bidirectional sequencing and BLAST analysis of consensus sequences obtained showed a homology of 97% to 100% with the respective species (Table A1).

Co-infections, i.e., the presence of more than one *Helicobacter* species, were detected in 41.7% of wild rabbits; the most frequent bacterial association was *H. salomonis* plus *H. bizzozeronii* (33.3%) and *H. salomonis* plus *H. felis* (8.3%). Regarding the *Helicobacter* species identified, these results are in agreement with those previously described in pets, as well as industrial and laboratory rabbits, which reported *H. felis*, *H. salomonis* and *H. bizzozeronii* DNA in the gastric *corpus* [19,20], but are here reported for the first time in free range wild rabbits.

3.2. Wild Quails

Of the six PCR positive animals, the most frequent species was *H. salomonis* (6/6), followed by *H. bizzozeronii* (2/6), and then *H. felis* (1/6) and *H. pylori* (1/6) (Table 3).

Concerning the wild quails: three were *H. salomonis*-positive, one was *H. salomonis* plus *H. bizzozeronii*-positive, one was *H. salomonis* plus *H. pylori*-positive and another was *H. salomonis* plus *H. felis* plus *H. bizzozeronii*-positive (Table A1).

The bidirectional sequencing and BLAST analysis of the consensus sequences obtained showed a homology of 97% to 100% with the respective species (Table A1).

Table 3. Number and percentage of positive PCR results for gastric *Helicobacter* species in wild quails.

	Wild Quails (n = 6) Gizzard
H. salomonis	6 (100%)
H. bizzozeronii	2 (33.3%)
H. felis	1 (16.7%)
H. pylori	1 (16.7%)

Co-infections were detected in 50.0% of the animals and different bacteria associations were found: *H. salomonis* plus *H. bizzozeronii* (16.7%), *H. salomonis* plus *H. pylori* (16.7%) and *H. salomonis plus H. felis* plus *H. bizzozeronii* (16.7%). These are novel results and constitute new findings that report the presence of different gastric *Helicobacter* species DNA in birds.

Previous studies reported the detection of enterohepatic *Helicobacter* in wild birds' faecal samples using PCR [21–23].

To the authors knowledge, this is the first time that gastric NHPH is detected in the fundus/corpus of free range wild rabbits and NHPH and *H. pylori* DNA in the gizzard of wild quails. This might indicate that wild rabbits and wild quails may act as reservoirs and contribute to the *H. pylori* and NHPH environment dissemination, causing both Public Health and *One health* concerns to arise. DNA extraction of the wild rabbit samples and the wild quail samples was performed on different days. Disposable sterile scalpels and tweezers were used to open the stomachs and, for each sample collection, a sterile disposable Kruuse® Biopsy punch was used, rendering cross-contamination between samples unlikely. It can, however, not be excluded that the presence of *Helicobacter* DNA in the stomach from some of the animals tested is a consequence of recent contamination from the environment, as described by [32]. Future studies using a larger sample size and including histopathological analysis of gastric tissues is, therefore, necessary to confirm these findings and to associate the presence of *Helicobacter* spp. with possible gastric alterations.

Wild animals, including leporids and birds, are known to be reservoirs for several infectious and transmissible diseases [30,33], raising concerns regarding zoonotic pathogens cross-species spillover [34]. The close contact of wild animals with domestic animals and humans is continuously increasing due to different factors, which can drive the zoonotic spillovers to become more common [29,34] and directly affect animal and human health [30,31,33,34].

Hunting dogs and humans can be exposed to game pathogens through direct contact with fresh carcasses, the handling or consumption of raw or undercooked meat or indirect contact through contaminated water or the environment; thus, game wardens, hunters, butchers and other wildlife professional duties are at high risk.

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Appendix A

Table A1. Wild rabbits and wild quails PCR positive results collection and sequencing data according to sample anatomical collection.

Animals (n = 18) Samples (n = 30)		*H. bizzozeronii*	*Accession nr./ Homology* *	*H. felis*	*Accession nr./ Homology*	*H. salomonis*	*Accession nr./ Homology*	*H. pylori*	*Accession nr./ Homology*	*H.suis*	*Accession nr./ Homology*	*H. ailuro-gastricus*	*Accession nr./ Homology*	*H. heil-mannii*	*Accession nr./ Homology*
WR1	Fundus	-			-		-		-		-		-		-
	Corpus	-			-		-		-		-		-		-
WR2	Fundus	-			+		FQ670179 99.35%		-		-		-		-
	Corpus	-			-		-		-		-		-		-
WR3	Fundus	-			-		-		-		-		-		-
	Corpus	+		FR871757 99.42%	-		-		-		-		-		-
WR4	Fundus	-			-		+		AJ130882 100%		-		-		-
	Corpus	-			-		+		AJ130882 100%		-		-		-
WR5	Fundus	-			-		+		AJ130882 100%		-		-		-
	Corpus	+		FR871757 100%			+		AJ130882 100%		-		-		-
WR6	Fundus	-			-		-				-		-		-
	Corpus	+		FR871757 95.08%	-		+		AJ130882 100%		-		-		-
WR7	Fundus	-			-		+		AJ130882100%		-		-		-
	Corpus	-			-		+		AJ130882 100%		-		-		-
WR8	Fundus	+		AF508003 97.11%	-		+		AJ130882 100%		-		-		-
	Corpus	+		FR871757 100%	-		+		AJ130882 100%		-		-		-
WR9	Fundus	-			-		+		AJ130882 100%		-		-		-
	Corpus	-			-		+		AJ130882 100%		-		-		-
WR10	Fundus	-			-		+		AJ130882 100%		-		-		-
	Corpus	-			+		JQ736360 100%	+	AJ130882 100%		-		-		-
WR11	Fundus	-			-		+		AJ130882 100%		-		-		-
	Corpus	+		AF508003 100%	-		+		AJ130882 100%		-		-		-

Table A1. *Cont.*

Animals (*n* = 18) Samples (*n* = 30)	***H. bizzozeronii***	***Accession nr./ Homology ****	***H. felis***	***Accession nr./ Homology***	***H. salomonis***	***Accession nr./ Homology***	***H. pylori***	***Accession nr./ Homology***	***H.suis***	***Accession nr./ Homology***	***H. ailuro-gastricus***	***Accession nr./ Homology***	***H. heil-mannii***	***Accession nr./ Homology***
WR12	Fundus	-		-		+	AJ130882 100%	-		-		-		-
	Corpus	-		-		-		-		-		-		-
Q1	Gizzard	-		-		+	AJ130882 100%	-		-		-		
Q2	Gizzard	-		-		+	AJ130882 100%	-		-		-		
Q3	Gizzard	-		-		+	AJ130882 100%	-		-		-		
Q4	Gizzard	+	FR871757 100%	-		+	AJ130882 100%	-		-		-		
Q5	Gizzard	+	FR871757 100%	+	AF116580 97.12%	+	AJ130882 100%	-		-		-		
Q6	Gizzard	-		-		+	AJ130882 100%	+	CP024947 100%	-		-		

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Chapter V

Molecular detection of Metronidazole and Tetracycline resistance genes in *Helicobacter pylori*-like positive gastric samples from pigs

(Manuscript I – submitted)

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antibiotics

MDPI

Communication

Molecular detection of Metronidazole and Tetracycline resistance genes in *Helicobacter pylori*-like positive gastric samples from pigs

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Abstract: Antimicrobial resistance is a major public health concern. The aim of this study was to assess the presence of antibiotic resistance genes, previously reported in *Helicobacter pylori*, in gastric samples of 36 pigs, in which DNA of *H. pylori*-like organisms had been detected. Based on PCR and sequencing analysis, two samples were positive for the *16S rRNA* mutation gene, conferring tetracycline resistance, and one sample was positive for the *frxA* gene with a single nucleotide polymorphism, conferring metronidazole resistance. All three amplicons showed highest homology with *H. pylori* associated antibiotic resistance gene sequences. These findings indicate that acquired antimicrobial resistance may occur in *H. pylori*-like organisms associated with pigs.

Keywords: antimicrobial resistance, antimicrobial resistance gene, gastric helicobacters, PCR, Sus scrofa, One Health

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1. Introduction

Over 50% of the world population is infected with the Gram-negative bacterium *Helicobacter pylori* (*H. pylori*), one of the main causes of acute and chronic gastritis, peptic ulcer disease, gastric adenocarcinoma and gastric mucosa-associated lymphoid tissue (MALT) lymphoma, which can also cause extra-gastrointestinal diseases [1-4]. Humans can also be infected with gastric non-*Helicobacter pylori Helicobacter* (NHPH) species, with an estimated global prevalence of 0.2-6% in patients undergoing a gastroscopy [5-7] and a prevalence of 20.8-29.1% in selected *H. pylori*-negative gastric patient cohorts [8,9]. Besides a pathophysiological involvement in gastric disease, gastric NHPHs have also been associated with extra-digestive diseases [3].

In animals, there are several studies reporting infections with helicobacters and their association with gastric alterations [5,10-13]. Among these, there are sporadic reports of infections with *H. pylori*-like organisms in pigs [14-19]. These *H. pylori*-like organisms appear to carry genes similar to *H. pylori*, including the *ureAB* gene. However, no amplification of the *glmM* (*ureC*) gene was achieved in these pig samples positive for a *H. pylori*-specific, *ureAB* based PCR assay. Therefore, the term *H. pylori*-like organisms is used [19].

In humans, there are different therapeutic approaches to *H. pylori* infections, although standard treatment consists of triple therapy that includes a proton-pump inhibitor and two antibiotics. Among the antibiotic choices, amoxicillin, clarithromycin and metronidazole are the most commonly used, being considered the first line of treatment [1,20-25].

Antimicrobial resistance (AMR) is one of the most concerning One Health issues worldwide [26]. In fact, the World Health Organization (WHO) has classified the most important resistant bacteria at a global level for which there is an urgent need for new treatments [27]. This classification was done according to the species and the type of resistance resulting in three priority tiers: critical, high and medium, where *H. pylori* is classified as high risk, specifically for clarithromycin-resistance [27].

Based on the importance of antimicrobials for treating human infections and the antibiotics used in veterinary medicine, the WHO published a priority list of antimicrobials grouped into three categories: (1) Critically important, subdivided into highest priority and high priority, (2) Highly important and (3) Important [28]. Likewise, the World Organization for Animal Health (WOAH) developed a list of antimicrobials of veterinary importance further classified as: veterinary critically important, veterinary highly important, and veterinary important antimicrobials [29]. Within these lists, amoxicillin is classified as critically important (WHO, WOAH), clarithromycin as critically important (WHO), tetracycline as highly important (WHO, WOAH), and metronidazole as important (WHO) [28,29].

Penicillins and tetracyclines are among the most commonly used antimicrobial classes in pigs [30,31]. The use of antimicrobials has been associated with direct and indirect impacts on the gastrointestinal microbiota and its antimicrobial resistome [30]. This has raised public health concerns due to selective pressure on opportunistic pathogens.

The main mechanism that contributes to *Helicobacter* resistance development is the acquisition of point mutations in the DNA. In the specific case of *H. pylori*, it acquires resistance via chromosomal mutations and horizontal transfer of resistance genes [32,33].

Regarding metronidazole, a nitroimidazole that acts as a bactericidal agent by interacting with a nitroreductase homolog, *rdxA*, resistance to it in *H. pylori* has been linked to mutations in the gene *rdxA*, while changes in the gene *frxA*, which encodes for NADH:flavin oxidoreductase, have also been implicated [32].

Tetracyclines bind to the ribosomal 30S subunit, inhibiting protein synthesis. Tetracycline resistance can be acquired in the majority of bacterial species through efflux systems or through ribosomal protection proteins. A reduction in membrane permeability, changes in ribosome binding, enzymatic antibiotic degradation, active efflux, and changes in membrane permeability all appear to play a role in tetracycline resistance. In *H. pylori*, resistance to tetracyclines seems to be conferred by mutations in the *16S rRNA* gene [34].

The aim of this study was to assess the presence of gene mutations associated with resistance to amoxicillin, metronidazole, clarithromycin, and tetracycline in porcine gastric samples that were shown to be positive for *H. pylori*-like DNA in a previous study [19].

2. Results

2.1. PCR results

Out of the 36 *H. pylori*-like positive tested samples, three *pars oesophagea* samples (8.3%) were PCR-positive for genes conferring resistance to antimicrobials. Two were found PCR-positive for the *16S rRNA* mutation gene conferring tetracycline resistance and one was found PCR-positive for the *frxA* gene that can be associated with metronidazole resistance in the presence of a single nucleotide polymorphism (SNP) (Table 1).

Table 1. PCR results regarding AMR genes per gastric region.

***H. pylori*-like positive samples**	***frxA* gene PCR positive (n/N) (%)**	***rdxA* gene PCR positive (n/N) (%)**	***16S rRNA* mutation gene PCR positive (n/N) (%)**	***23S rRNA* gene PCR positive (n/N) (%)**	***Pbp1A* gene PCR positive (n/N) (%)**
Pars oesophagea (N=29)	1/29 (3.4%)	0/29 (0.0%)	2/29 (6.9%)	0/29 (0.0%)	0/29 (0.0%)
Oxyntic mucosa (N=7)	0/7 (0.0%)	0/7 (0.0%)	0/7 (0.0%)	0/7 (0.0%)	0/7 (0.0%)

2.2. Sequencing and Sequence analysis of Positive PCR products

The bidirectional sequencing and basic local alignment search tool (BLAST) analysis of consensus sequences of partial *16S rRNA* mutation gene amplicons showed an identity ranging from 98.6-100% with *H. pylori* (accession nr. OP389222) *16S rRNA* mutation conferring resistance to tetracycline (ARO:3003510) for both positive samples.

The other positive sample was also subject to bidirectional sequencing and BLAST analysis of the consensus sequence. The amplicon showed an identity of 99.85% to *H. pylori* (accession nr. CP026515). The obtained sequence was also analyzed using the Resistance Gene Identifier (RGI) to predict resistomes from nucleotides on homology and SNP models. The RGI criteria fully corresponded with *H. pylori frxA* mutation conferring resistance to metronidazole, with SNP Y62D, with an identity of the matching region of 99.07% (ARO:3007056) [35-37].

3. Discussion

Our results showed that AMR associated mutation genes presenting highest homology with *H. pylori* associated genes occurred in three samples from the stomach of pigs, that were previously reported as *H. pylori*-like positive.

To achieve these results, we partly relied on the methodology described by Diab et al. (2018) [38] and Lee et al. (2018) [39] who also aimed to detect antibiotic resistance genes (ARGs) of *H. pylori* conferring resistance to clarithromycin, metronidazole, amoxicillin and tetracycline, however in human patients. The former examined gastric biopsy specimens from patients found positive based on rapid urease testing and the presence of *H. pylori 16S rRNA*. The latter research was performed using *H. pylori* isolates obtained from patients. AMR gene PCR positive amplicons were in both studies also sequenced and subjected to BLAST for sequence and mutation analysis, but not analyzed using the Comprehensive Antibiotic Resistance Database (CARD). Although AMR in *H. pylori*-like positive samples had not been studied yet, there are other studies that investigated the same genes in other animal samples positive for other *Helicobacter* species [40-42].

In our study, two of the analyzed porcine gastric tissue samples from the *pars oesophagea* were positive for the *16S rRNA* mutation gene conferring resistance to tetracycline. Another sample of the *pars oesophagea* was positive for the *frxA* gene with SNP Y62D mutation conferring resistance to metronidazole. The three samples were subject to BLAST with homologies with *H. pylori* ranging from 98.6 to 100%. In humans, metronidazole is regarded as one of the drugs of choice in triple therapy against *H. pylori*

infections. The overall prevalence of *H. pylori* resistance to metronidazole was found to be 47.2%, which is highest in Africa (75.0%), followed by South America (52.9%), Asia (46.6%), Europe (31.2%) and North America (30.5%) [43], implicating reduced treatment efficacy in humans [21]. In addition, tetracyclines are also commonly used in rescue eradication regimens against *H. pylori*, displaying a worldwide resistance rate of 11.7% [43]. Of note, none of the included samples were positive for *H. pylori 23S rRNA* potentially associated with clarithromycin conferring point mutations. Since this is a *H. pylori* species-specific PCR assay (not cross-reacting with *23S rRNA* gene sequences of other gastric NHPHs according to *in silico* analysis), this may further confirm our previous findings regarding the presence of *H. pylori*-like DNA in porcine gastric samples rather than DNA of *H. pylori* itself.

Although the use of antibiotics for growth promotion is prohibited in several countries, including European Union member states, tetracyclines are still used as a growth promoter in many countries. In pigs, tetracyclines are generally the most commonly used antibiotics [31,42,44][45]. Tetracycline resistance genes are some of the most abundant ARGs in the pig microbiome [31,46]. A study conducted by Liu et al. (2019) detected tetracycline ARGs in all evaluated (22/22) pork samples [42]. Ricker et al. (2020) isolated and extracted DNA from porcine feces for retrospective ARG analysis. They reported that the use of tetracyclines in pigs promotes co-selection for resistance genes for aminoglycosides and tetracyclines [47], although this has not been described in *Helicobacter* species.

Apart from our study, acquired resistance to tetracyclines has also been reported to occasionally occur in *H. suis* isolates obtained from the stomach of pigs [40].

During the processing of pig carcasses, the surface of the carcass and the parts destined for retail sale can get contaminated with organisms coming from the hide/skin of the animals, gut content, worker's hands, and the slaughter environment [48]. This can predispose humans to contact with AMR pathogens and ARGs as stated by Liu et al. (2019). This suggests that ARGs, could be potentially transmitted to humans via the meat industry chain/feed supply, pig feeding and pork production [42].

Furthermore, antibiotic use eliminates susceptible pathogens allowing resistant strains to continue to evolve and multiply. Selective pressure from antimicrobial exposure thus provides resistant pathogens with an evolutionary advantage and favors their spread [30].

Although our results point towards the presence of ARGs in *H. pylori*-like organisms conferring resistance to tetracycline and metronidazole, the interpretation of these findings should be done with caution. The significance for human and animal health is not yet completely clear, since there are no reports of *H. pylori*-like infections in humans and the relevance of *H. pylori*-like organisms in both animals and humans requires further investigation. Studies should be conducted with a larger sample size and ideally, isolation of the *H. pylori*-like bacteria should be performed in order to characterize the organisms and test for antimicrobial susceptibility in depth.

4. Materials and Methods

4.1. Sample selection

DNA extracts from 36 gastric samples from pigs (29 samples of the *pars oesophagea* and 7 samples of oxyntic mucosa), containing *H. pylori*-like DNA as shown by PCR and sequencing analysis, were analyzed for the presence of *H. pylori*-specific ARGs [17-19].

4.2. PCR conditions and sequencing

The *H. pylori*-like positive samples were subjected to conventional PCR assays to test for the presence of genes related to AMR in *H. pylori*, including *Pbp1A* (amoxicillin), *rdxA* and *frxA* (metronidazole), *16S rRNA* mutation gene (tetracycline) and *23S rRNA* (clarithromycin) in order to identify point mutations (Table 2).

Aliquots of each PCR product were electrophoresed on 1.5% agarose gel stained with Xpert Green Safe DNA gel stain (GRISP, Porto, Portugal) and examined for the presence of specific fragments under UV light. DNA fragment size was compared with the standard molecular weight, 100bp DNA ladder (GRISP, Porto, Portugal). For the negative control, distilled water was used. No positive control was used on PCR to test resistance genes.

The amplicons of each positive sample were sequenced. Bidirectional sequencing was performed using the Sanger method at the Genomics core facility of the Institute of Molecular Pathology and Immunology of the University of Porto, Portugal. Sequence editing and multiple alignments were performed with the MegaX Molecular Evolutionary Genetic Analysis version 10.1.8 [49]. The sequences obtained were subject to BLAST analysis using the non-redundant nucleotide database (http://blast.ncbi.nlm.nih.gov/Blast.cgi) [36,37]. Sequences were also analyzed through the CARD to identify additional antibiotic resistance gene mutations [35].

Table 2. Primer sequences and thermocycling conditions for detection of genes and mutation genes conferring resistance to antimicrobials.

Antimicrobials		Sequence	Target gene	Thermo cycle conditions			Reference
				Temp. (°C)	Time	Nr. cycles	
Amoxicillin	Forward	GCG ACA ATA AGA GTG GCA	*Pbp1A*	95	3′	35	[38,39]
				95	1′		
	Reverse	TGC GAA CAC CCT TTT AAA T		56	1′		
				72	5′		
				72	10′		
Metronidazole	Forward	AAT TTG AGC ATG GGG CAG A	*rdxA*	95	5′	35	[38,39]
				94	30″		
	Reverse	GAA ACG CTT GAA AAC ACC CCT		60	30″		
				72	1′		
				72	10′		
	Forward	TGG ATA TGG CAG CCG TTT A	*frxA*	95	5′	35	[38,39]
				95	30″		
	Reverse	GGT TAT CAA AAA GCT AAC AGC G		58	30″		
				72	1′		
				72	10′		
Tetracycline	Forward	CGG TCG CAA GAT TAA AAC	*16S rRNA mutation*	95	10′	45	[38]
				95	5″		
	Reverse	GCG GAT TCT CTC AAT GTC		55	2″		
				72	30″		
				72	10′		
Clarithromycin	Forward	TCA GTG AAA TTG TAG TGG AGG TGA AAA	*23S rRNA*	95	10′	40	[38]
				92	15″		
	Reverse	CAG TGC TAA GTT GTA GTA AAG GTC CA		60	1′		
				72	1′		
				72	10′		

5. Conclusions

AMR is a One Health concern mainly related to the animal and human use of antibiotics. Our results demonstrate that AMR may occur in *H. pylori*-like organisms from pigs. The significance of these findings for public and animal health requires further

investigation, including attempts to isolate and in depth characterize these organisms and to determine their possible pathogenic significance.

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Chapter VI

Perception and knowledge of Portuguese veterinarians about zoonotic transmission of *Helicobacter pylori* and *Helicobacter suis*: The need for One Health Intervention

(Article IV)

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Article

Perception and Knowledge of Portuguese Veterinarians about the Zoonotic Transmission of *Helicobacter pylori* and *Helicobacter suis*: The Need for One Health Intervention

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Abstract: *Helicobacter* species can colonize the gastrointestinal tract of both humans and animals, and are associated with gastrointestinal and extra-gastrointestinal diseases. Some studies indicate that animals, health professionals, and people in close contact with animals might be at higher risk for infection with gastric *Helicobacter* spp. Considering that veterinarians are professionals at risk for infection with zoonotic gastric Helicobacters and are also seen by many as health communicators concerning zoonoses, the aim of this study was to evaluate the Portuguese veterinarians' perception and knowledge of *Helicobacter* spp. infection and its zoonotic risk/potential. Therefore, a structured questionnaire composed of 34 dichotomic, multiple-choice, rating scale, matrix, drop-down, and open-ended questions was developed and given to Portuguese veterinarians via an online platform from May 2021 to July 2021, and statistical analysis was used to obtain results. There was a total of 149 respondents, most of them (73.8%) being females. Evidently, Portuguese veterinarians have a limited perception regarding *Helicobacter* spp. infections. Of the respondents that *"have heard of Helicobacter"*, 17.6% do not know which animal species can be affected by it. Most of the companion animal veterinarians (76.2%) do not consider *Helicobacter* spp. infection a differential diagnosis when evaluating animals with gastritis. A significant percentage (37.2%) of the respondents that have "heard of *H. suis*" do not consider it a zoonotic bacterium. There is a need for education and sensitization of veterinarians regarding the potential zoonotic risk of *Helicobacter* spp. in order to elucidate these professionals to this One Health issue, as the number of reports of non-*Helicobacter pylori Helicobacter* in livestock, companion, and wild animals is increasing.

Keywords: awareness; NHPH; One Health; public health; occupational health; risk communication; zoonosis

1. Introduction

Helicobacter species can colonize the gastrointestinal tract of both humans and animals and are characterized as Gram-negative, spiral-shaped motile bacteria associated with several diseases [1–4].

In 1994, the World Health Organization (WHO) classified *Helicobacter pylori* (*H. pylori*) in the first group of carcinogenic agents, and its eradication remains a public health concern worldwide [5].

In humans, *H. pylori* is the most common gastric pathogen, affecting more than half of the world's population, playing a major role in the development of gastritis, gastroduodenal ulcers, gastric adenocarcinoma, mucosa-associated lymphoid tissue (MALT) lymphoma, and extra-digestive diseases [1,6–8].

Gastric non-*Helicobacter pylori Helicobacter* (NHPH) have been associated with a wide range of pathologies, from MALT lymphoma to extra-digestive diseases, and have been diagnosed in 0.2–6% of human gastric biopsies [2,4,9].

In humans, *Helicobacter suis* (*H. suis*), naturally hosted by pigs, is the most prevalent NHPH and has also been associated with a range of gastric and extra-digestive pathologies [1,4,10–13]. Recent reports reinforce that these infections most likely originate from pigs, emphasizing their zoonotic potential [12–15].

H. pylori infection is acquired predominantly during childhood and persists throughout life without treatment [5]. The route of transmission is not 100% known and the human stomach is considered the only known reservoir, despite having been identified in other species' stomachs [5,14,16,17]. Human-to-human transmission may occur through different pathways (such as gastro-oral, oral-oral, and fecal-oral), but there are other routes also hypothesized to be foodborne transmissions as the organism has been identified in food and water [5,17,18]. Indeed, *H. pylori* has been detected in drinking water, seawater, vegetables, and foods of animal origin. *H. pylori* survives in complex foodstuffs such as milk, vegetables, and ready-to-eat foods [19] and it has been demonstrated to transmit between humans and animals [20]. These types of contaminations are hypothesized to be the cause of new infections [5]. *H. suis* DNA was detected on pork carcasses in slaughterhouses, and the bacterium can persist for up to 48 h in experimentally contaminated pork. Therefore, pork consumption may constitute a new route of *H. suis* transmission in humans [21]. *H. suis* has also been associated with gastric disease in animal health professionals, emphasizing its zoonotic importance [12]. Thus, as some studies indicate, animals, health professionals, and people in close contact with animals might be at a higher risk for infection [22].

Considering that veterinarians are professionals at risk for infection with zoonotic species of gastric Helicobacters, and are also seen by many as health communicators concerning zoonoses, the aim of this study was to evaluate the Portuguese veterinarians' perception and knowledge of *Helicobacter* spp. infection and its zoonotic risk/potential.

2. Materials and Methods

2.1. Data Collection

Data collection was performed using a structured questionnaire, specifically developed for this study in Portuguese. The draft questionnaire was designed based on the bibliographic review and three focus groups with veterinarians. Then, it was tested before being released. The questionnaire includes information related to general demographic (date of birth, gender, region), contact with animals (specifically pigs and wild boars), and the context and duration of the contact. Information regarding food consumption, consumption of pig and wild boar meat or meat products, as well as the frequency of weekly consumption and food safety, was also assessed. Moreover, data regarding perception and knowledge of *Helicobacter* spp., *H. suis*, *H. pylori*, infection, and zoonotic risk was assessed. Data regarding the possible gastric disease of each participant presumably associated or not with *Helicobacter* spp. infection was also addressed. Finally, these data were collected regarding the participants' interest in receiving further information about

Helicobacter spp. infection in animals and humans and how they would like to receive any further information.

The following variables were considered for the present study: general demographic characteristics, contact with animals, nutritional habits, food safety habits, veterinarians' knowledge of *Helicobacter* spp., perception of each individual gastric pathology, and receptiveness to obtain more information regarding the survey topic.

This process resulted in the final survey, composed of 34 dichotomic, multiple-choice, rating scale, matrix, drop-down, and open-ended questions.

2.2. Study Design and Participants

A cross-sectional study using data from an online survey based on the snowball sampling method was performed [23]. A virtual snowball sampling survey was disseminated, firstly, through closed social networking channels (namely, Facebook®, Menlo Park, CA, USA) and, secondly, through the researcher's mailing lists to work colleagues and veterinarian friends. The inclusion criteria consisted of age $\geq$23, being a certified veterinarian, and being based in Portugal. Everyone who followed the link accepted to participate. Since the survey did not have information on where the participants were recruited (e.g., Facebook or mailing list), due to confidentiality, we cannot calculate the proportion of participants that come from each networking channel. For the present study, data was gathered from May 2021 to July 2021, corresponding to a convenience sample of 149 individuals.

2.3. Ethics Approval and Consent to Participate

Ethical approval was obtained from the i3S Animal Welfare and Ethics Review Body (ref.2021-4). According to the Ethical Principles for Medical Research involving human subjects expressed in the Declaration of Helsinki and the current national legislation, all participants were asked to give their informed consent. Since this was an online survey, participants had to select the option: "accept to participate in the study to proceed with the survey". The questionnaire was confidential, and no data that allowed the identification of the participants was collected.

2.4. Statistical Analysis

These data were analyzed using IBM SPSS® Statistics v27 (IBM, Armonk, NY, USA). Descriptive statistics were generated to verify the variables described above. Cross-tables and chi-square analyses were used to explore possible associations between nominal variables and Cramer's V test, with an oscillation between 0 and 1, to assess the intensity of the relationship between variables. Statistical significance was set at $p < 0.05$.

3. Results

3.1. Sociodemographic Distribution

Of the 149 questionnaires were completed, 73.8% (110/149) of the respondents were female veterinarians and 26.2% (39/149) were male veterinarians. The mean age was 37.2 years-old and 71.1% (106/149) were companion animal veterinarians.

There were respondents from 17 of the 18 districts of Portugal with the greater percentages of respondents from Lisbon (20.8%), Coimbra (14.1%), and Porto (13.4%) in concordance with the most populated regions in Portugal (Figure 1).

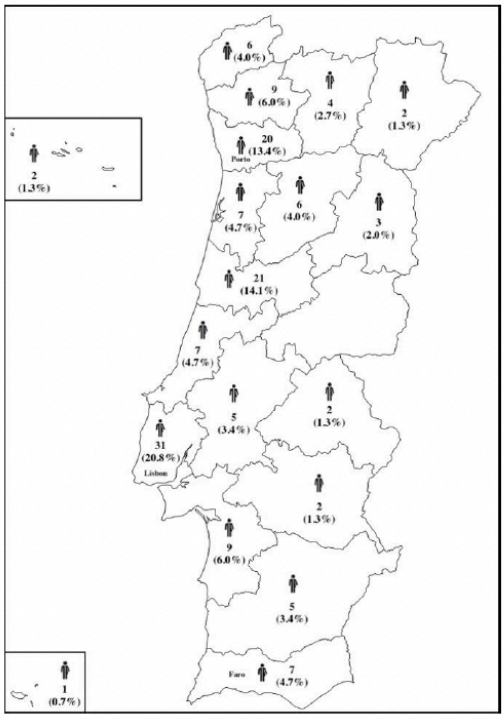

Figure 1. Distributions of the respondents enrolled in the questionnaire.

3.2. Knowledge about Helicobacter spp. Infection

When the questionnaire asked "*have you heard of Helicobacter?*", only 0.7% (1/149) chose "*no*" and 99.3% (148/149) chose "*yes*". Of the respondents that chose "*yes*", 97.3% (144/148) answered "*yes, I know what it is*" and 2.7% (4/148) "*yes, but I don't know what it is*".

Of the 148 respondents that "*have heard of Helicobacter*", when asked the question "*have you heard of H. suis?*", 47.0% replied "*no*", 38.3% replied "*yes, and I know what it is*", 13.4% replied "*yes, but I don't know what it is*", and 0.7% preferred not to answer. Regarding the question "*Have you heard of H. pylori?*", 2.0% selected "*no*", 92.6% selected "*yes, and I know what it is*", and 4.7% selected "*yes, but I don't know what it is*".

From the 148 respondents that "*have heard of Helicobacter,*" 17.6% did not know which animal species could be affected by *Helicobacter*, and only 4.0% stated that they "*do not know*" clinical signs associated with *Helicobacter* spp. infection in animals or humans.

Of the 106 respondent veterinarians who practice in companion animal clinics, the majority never (22.6%) or rarely (50.0%) consider *Helicobacter* spp. infection as a differential diagnosis of gastritis in companion animals; on the other hand, 21.6% frequently and 3.8% always consider it.

Of those 106 companion animal veterinarians, 22.6% have already diagnosed gastritis associated with *Helicobacter* spp. infection while 54.7% never have. Within the 24 companion animal veterinarians that have diagnosed gastritis associated with *Helicobacter* spp. infection, 22.6% diagnosed it in dogs, 8.3% in cats, and 12.5% in other species.

Of the 149 respondents, 47.7% consider *Helicobacter* spp. a zoonotic bacteria, 13.4% do not consider it a zoonotic bacteria, and 38.9% "*do not know*".

Although 71 veterinarians consider the zoonotic potential of *Helicobacter* spp., most (45.1%, 32/71) referred to an ambiguous answer when asked how the transmission between

animals and humans occurs. Some indicated "*food contamination*" (21.1%, 15/71), "*feco-oral route*" (16.9%, 12/71), or "*oral-oral route*" (8.5%, 6/71), while 11.2% (8/71) stated that they "*do not know*".

3.3. Contact with Pigs and Wild Boars and Consumption of Their Meat or Meat Products

Of the total respondents, 79.2% (118/149) had contact with pigs and/or wild boars: 31.4% (37/118) as livestock animal veterinarians, 23.7% (28/118) as companion animal veterinarians, 11.9% (14/118) as meat inspectors, 10.2% (12/118) when they were students, 1.7% (2/118) as hunters, 1.7% (2/118) as livestock farmers, and 19.5% (23/118) in other circumstances.

Of the 118 veterinarians who had contact with pigs and or wild boars, 52.5% have been in contact with pigs for less than 5 years and 47.5% for more than 5 years.

Regarding consumption of pig and wild boar meat or meat products: 89.9% (134/149) consumed pork at least once per week and 90.6% (135/149) consumed pork products, 38.9% (58/149) consumed wild boar meat at least once per week, and 18.1% (27/149) consumed wild boar meat products at least once per week.

3.4. Food Safety and Hygiene

More than one-fourth of the veterinarians (28.8%, 34/149) refer to the use of borehole water during food preparation or for ingestion.

Regarding the preparation of food at home, 98.7% (147/149) prepare food at home at least once per week.

Of the 147 respondents that prepare food at home, 97.9% (144/147) state that they "*have hygiene and safety measures during food preparation*", 0.7% (1/147) state that do not have hygiene and safety measures during food preparation, and 1.4% (2/147) state they "*do not know if they have hygiene and safety measures during food preparation*". When questioned about which type of hygiene and safety measures they practice: 81.3% (117/144) mentioned "*food washing*", 45.1% (65/144) "*cooking*", 27.8% (40/144) "*food separation to avoid cross-contamination*", 10.4% (15/144) "*refrigeration*", and 3.5% (5/144) "*hand washing*".

3.5. Gastric Pathology among Veterinarians

Of the respondent veterinarians, 83.9% (125/149) admitted to having "*gastric pain*", 69.1% (103/149) "*had gastritis*", 77.2% (115/149) "*suffered from gastric reflux/heartburn*", and 30.9% (46/149) did some type of gastric diagnostic test (Table 1). Finally, 21.5% (32/149) of the respondents are medicated for gastric disease.

A total of 22 (14.7%) of the 149 participants responded that they had gastric disease associated with *Helicobacter*. Of this number, 100% (22/22) claim to have been treated for *Helicobacter pylori* infection, with 50.0% (11/22) cured or eradicated, 18.2% (4/22) not cured or eradicated, and 31.8% (7/22) did not know if they got cured or if eradication was achieved.

3.6. Interest in Receiving Information on Helicobacter spp. Infections

Lastly, the respondents were questioned if they would like to receive more information about *Helicobacter* spp. infection in livestock animals, companion animals, or humans. Most (85.9%, 128/149) said "*yes*", and 32.0% (41/149) responded that they would like to get it through the Portuguese Order of Veterinarians (*Ordem dos Médicos Veterinários*, OMV).

3.7. Statistical Associations between Variables

When applying statistical analysis to determine if there was any association between them, it was possible to conclude that there are some statistically significant associations (with green background in Table 2) whose strengths vary from weak (<0.1) to moderate (<0.3) considering the Cramer's V and the degree of freedom =1.

Table 1. Assessment of veterinarians' gastric pathology: questions and answers (numbers and percentages).

Question	N	Answer	%
Did you use to have gastric pain?	149	Never	16.1
		Rarely	61.7
		Frequently	20.8
		Always	1.3
Have you had gastritis?	149	Never	30.9
		Rarely	55.7
		Frequently	13.4
Do you suffer from gastric reflux/heartburn?	149	Never	22.8
		Rarely	54.4
		Frequently	20.8
		Always	2.0
Have you done any gastric diagnostic test?	149	Yes	69.1
		No	30.9
If yes, what diagnostic test was performed?	103	Endoscopy only	56.5
		Endoscopy with biopsy	19.6
		Abdominal echography	8.7
		Rapid urease test	6.5
		Abdominal radiographs	2.2
		Ambiguous answer	6.5
Do you take any medication for gastric problems?	149	No	78.5
		Yes	21.5
If yes, which?	32	Omeprazole	50.0
		Pantoprazole	31.3
		Sucralfate	31.3
		Esmoprazole	21.9
		Antibiotics	6.3
		Ranitidine	3.1

Table 2. Statistical association between variables.

Question		Gender	Age	Years of Contact with Pigs	Do You Eat Pig Meat?	Do You Use Borehole Water to Prepare Food?	Do You Prepare Food at Home?	Do You Consider *Helicobacter* spp. Infection a Zoonosis?	Would You Like to Receive More Information about *Helicobacter* spp. Infection in Production Animals, Companion Animals, or Humans?
	Variables	Male/Female	[25–35]/[36–68]	[0–5]/[>5]	Yes/No	Yes/No	Yes/No	Yes/No	Yes/No
Have heard of *Helicobacter* spp.?	Yes/No	$p > 0.05$	$p > 0.05$	$p > 0.05$	$p > 0.05$	$p > 0.05$	$p > 0.05$	$p > 0.05$	$p = 0.013$; cv = 0.203
Have heard of *H. suis*?	Yes/No	$p > 0.05$	$p > 0.05$	$p > 0.05$	$p > 0.05$	$p > 0.05$	$p > 0.05$	$p < 0.001$; cv = 0.322	$p = 0.045$; cv = 0.204
Have heard of *H. pylori*?	Yes/No	$p > 0.05$	$p > 0.05$	$p > 0.05$	$p > 0.05$	$p > 0.05$	$p > 0.05$	$p > 0.05$	$p = 0.001$; cv = 0.299
Do you consider *Helicobacter* spp. infection a zoonosis?	Yes/No	$p > 0.05$	$p = 0.021$; cv = 0.189	$p > 0.05$	$p > 0.05$	$p > 0.05$	$p > 0.05$	$p > 0.05$	$p > 0.05$
Did you use to have gastric pain?	Yes/No	$p > 0.05$	$p > 0.05$	$p > 0.05$	$p = 0.047$; cv = 0.163	$p > 0.05$	$p > 0.05$	$p > 0.05$	$p > 0.05$
Do you take any medications for gastric problems?	Yes/No	$p = 0.036$; cv = 0.172	$p > 0.05$	$p > 0.05$	$p > 0.05$	$p > 0.05$	$p > 0.05$	$p > 0.05$	$p = 0.010$; cv = 0.212
Have you had gastric disease associated with Helicobacter infection?	Yes/No	p > 0.05	$p > 0.05$	$p > 0.05$	$p > 0.05$	$p = 0.029$; cv = 0.179	$p > 0.05$	$p = 0.037$; cv = 0.171	$p = 0.040$; cv = 0.169
Have you had treatment for *Helicobacter* spp. infection?	Yes/No	$p = 0.003$; cv = 0.277	$p > 0.05$	$p = 0.020$; cv = 0.268	$p > 0.05$	$p > 0.05$	$p > 0.05$	$p = 0.029$; cv = 0.219	$p = 0.23$; cv = 0.226
Do you consider hygiene and safety measures during food preparation?	Yes/No	$p > 0.05$	$p = 0.036$; cv = 0.172	$p > 0.05$	$p > 0.05$	$p > 0.05$	$p < 0.01$; cv = 0.398	$p > 0.05$	$p > 0.05$

cv = Cramer's V.

4. Discussion

There are several species of gastric Helicobacters, with *H. pylori* being one of the most studied since humans remain the natural host. *H. pylori* is associated with gastric disease as it may cause gastritis, peptic ulcer disease, gastric carcinoma, and mucosa-associated lymphoid tissue (MALT) lymphoma. Besides this species, other NHPHs with zoonotic potential, such as *H. suis*, have been associated with gastric and neurologic pathologies in humans [2,4,10,24,25]. Considering the close contact between humans and animals and the late reports of *Helicobacter* spp. in humans [26], the late reports of *H. pylori* human-animal transmission and infection [20], or the detection of *H. pylori* DNA in other animal species [14,16], and also the description of human *H. suis* infection [1,27], including a report in a veterinarian [12], our goal was to investigate the awareness of veterinarians regarding *Helicobacter* species, such as *H. pylori* and *H. suis*.

However, there are two limitations that should be pointed out: one is the small sample size and the other is that the majority of the respondents were female veterinarians, so the generalizability of our findings is limited.

Most of the 149 questionnaire respondents were females (73.8%), as expected according to the 2018 European veterinary survey applied and published by the Federation of Veterinarians of Europe (FVE) [28]. Additionally, according to the OMV, on the 12th February 2020, there were 4071 (61%) female veterinarians and 2551 (39%) male veterinarians registered in Portugal. Therefore, when associating different variables with gender, there is expected to be a deviation towards the female gender.

Despite some of the NHPHs being considered zoonotic or with zoonotic potential [25,29], in this study 37.2% of the respondents that "*heard of H. suis*" do not consider it a zoonosis ($p < 0.001$; cv = 0.322). However, people directly affected by *H. pylori* infection seem to be aware of this hypothesis since 68.2% of the respondents that "*had gastric disease associated with Helicobacter*" ($p = 0.037$; cv = 0.171) and 68.2% that "*had treatment for Helicobacter infection*" ($p = 0.029$; cv = 0.219) consider it a zoonosis.

Interestingly, 59.0% of the respondents between the ages of 25 and 35 years-old consider *Helicobacter* spp. infections a zoonosis, while only 39.8% of the respondents between the ages of 36 and 68 years-old do ($p = 0.021$; cv = 0.189). We hypothesize that the recent emergence of zoonoses globally [30] may have made younger professionals more aware of these diseases.

Some studies report the presence of *H. suis* on pig carcasses and retail meat which can pose a transmission route [21,31]. Intriguingly, 42.4% of the respondents to the questionnaire that claim "*to eat pork*" regularly also "*have gastric pain*" ($p = 0.047$; cv = 0.163). Although no direct association can be made, this result should be explored in further studies.

Regarding food safety and hygiene, despite 97.9% of the respondents that prepare food at home stating that they "*have hygiene and safety measures during food preparation*" ($p < 0.01$; cv = 0.398), only 3.5% mention "*hand washing*" as a food safety practice. Similar findings were reported in a study by Stratev et al. (2017) concluded that, despite the high level of awareness of food safety, these practices were low among veterinary medicine students in Bulgaria [32].

With our study we were able to assess that most of the respondents have limited perception and knowledge about the zoonotic/anthropozoonotic risks of *H. suis* and *H. pylori* infections. When questioned "*have you heard of Helicobacter*", 99.3% chose"*yes*" with the majority of the respondents (97.3%) knowing *H. pylori* but only 51.7% acknowledging *H. suis*, and interestingly, 17.6% of respondents that "*have heard of Helicobacter*" did not know which animals' species could be affected. Furthermore, 13.4% do not consider *Helicobacter* spp. a zoonotic bacteria and 38.9% "*do not know*", even though 96.6% claimed that they "*have heard of Helicobacter and know what it is*" and 38.3% "*have heard of H. suis and know what it is*".

It can also be hypothesized that *Helicobacter* spp. infections among animals could be underdiagnosed due to a lack of knowledge/awareness of veterinarians regarding this

bacterial infection. In fact, 17.6% did not know the animal species that could be affected, and of the 106 veterinarians that practice at companion animals' clinics, 72.6% never (22.6%) or rarely (50.0%) consider *Helicobacter* spp. infection as a differential diagnosis of gastritis in companion animals.

When assessing gastric pathology among the respondents, 59.4% of females responded positively to "*did you use to have gastric pain*" and 68.2% of females "*have had treatment for Helicobacter*" ($p = 0.003$; cv = 0.277), which follows the study published by Khoder et al. (2021) that reports a higher occurrence of *H. pylori* infections in females [33].

We also concluded that veterinarians with higher contact time (>5 years) with pigs (52.9%) also "*had had treatment for Helicobacter infection*" ($p = 0.020$; cv = 0.268), which again may indicate the occupational risk of these professionals as reported by Joosten et al. (2013) [12].

Remarkably, 86.5% of the respondents that "*have heard of Helicobacter spp.*" ($p = 0.013$; cv = 0.203), 85.7% that "*have not heard of H. suis*" ($p = 0.045$; cv = 0.204), 87.2% that "*have heard of H. pylori*" ($p = 0.001$; cv = 0.299), 100% that "*take medication for gastric pain*" ($p = 0.010$; cv = 0.212), 100% that "*have gastric problems associated with Helicobacter infection*" ($p = 0.040$; cv = 0.169), and 100% that "*had treatment for H. pylori infection*" ($p = 0.023$; cv = 0.226) would like to receive more information about this bacterial infection in livestock, companion animals, and humans. This may suggest that despite having some insights about the subject, they would like to get further information and enrich their knowledge.

On the other side, we should not neglect that a surprising percentage of veterinarians (14.1%) do not wish to receive additional information.

To the authors' best knowledge there are no studies that assess the perception, awareness, and knowledge of veterinarians regarding *Helicobacter* spp. infections. This makes our results the first to be reported and challenging to compare since there are no similar studies among veterinarians that are up to date.

It is known that zoonotic diseases are a growing concern, amounting to approximately 60% of the existing human pathogens, and over 75% of those can be tracked to animals [34]. Sometimes, zoonotic diseases manifest in animals before they infect humans [35], so veterinarians possess specialty training in zoonoses and, thereafter, should be a great resource of information on this subject [36]. In addition, m according to Spear et al. (2015), people would be willing to consult with a veterinarian on the advice of their physician if they had a zoonotic disease [36]. Some studies also show that veterinarians should be active not only in controlling zoonotic diseases in animals but also in providing information for patients and physicians, and that physicians assign them the duty of educating populations about zoonoses [36–38]. This happens because physicians are uncomfortable with their knowledge about zoonoses [36,38]. Therefore, physicians consign veterinarians the duty of educating the public about zoonoses, yet neither of these experts communicate with the other [35].

Despite the results of our study being suggestive of limited knowledge and awareness of veterinarians regarding *Helicobacter* spp. infections, they continue to play an important role in the promotion of public health [35,39]. Pet owners are more likely to contact their veterinarian than their physician regarding information about zoonoses [40], along with the fact that they consider the role of veterinarians important as public health promoters [39]. So further training programs addressing vet communication skills should take into account the particular issues of emerging zoonoses, and veterinarians need to have proper knowledge so they can assess and explain the risks to their clients [41].

The medical and veterinary communities should collaborate closely in clinical, public health, and research contexts since zoonoses can infect both animals and humans [42].

Veterinarians are in the perfect position to give animal owners trustworthy information since they are more aware of the possible hazards of zoonotic diseases and how to minimize them.

There is a need for collaboration between animal, human, and environmental professionals in an objective One Health perspective due to the increase in emerging zoonotic

diseases. Veterinarians seem to show greater awareness of the importance of cooperation activities and continuous cross-sectional formation than physicians [34].

Nonetheless, communication between veterinarians and physicians seems to be insufficient [37], so it is important to create awareness of this need and promote cooperation and communication since the One Health initiative aims to reduce this professional gap between veterinarians and physicians [43], and both parts can and should play a role on public health education.

5. Conclusions

There is a need for education and sensitization of veterinarians regarding the zoonotic risk of *Helicobacter* spp. in order to elucidate these professionals to this One Health issue since the number of reports of NHPH in livestock, companion, and wild animals are increasing.

The results of this study allowed us to conclude that communication and sensibilization regarding *Helicobacter* spp. infections in animals and its zoonotic potential should be done by veterinarians since there are 37.2% of them that heard of *H. suis* but do not consider it a zoonosis. A special focus should be implemented regarding veterinarians older than 35 years since only 39.8% of these consider *Helicobacter* spp. infections as potential zoonoses.

The veterinarian respondents demonstrated interest in receiving more information regarding *Helicobacter* spp. infections in production animals, companion animals, or humans which could, in fact, increase their awareness on this topic, and improve their role as clinicians and public health agents.

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Institutional Review Board Statement: Ethical approval was obtained from the i3S Animal Welfare and Ethics Review Body (ref.2021-4) in March 2021. According to the Ethical Principles for Medical Research involving human subjects expressed in the Declaration of Helsinki and the current national legislation, all participants were asked to give their informed consent. Since this was an online survey, participants must select the option: to accept to participate in the study to proceed with the survey. The questionnaire was confidential, and no data that allow the identification of the participants was collected.

Informed Consent Statement: Informed consent was obtained from all subjects involved in the study.

Data Availability Statement: Not applicable.

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Chapter VII

General discussion

Conclusions and future perspectives

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General Discussion

It is known that the presence of helicobacters may be associated with gastric disease in humans and animals and *F. gastrosuis* has been described to possibly play a role in gastric ulceration in pigs [1,2]. In this sense, in the study of pathologies associated with these bacteria, the interaction between domestic animals-wild animals-humans should be taken into account from a One Health perspective.

Therefore, the aims of this study were: to assess the presence of *Helicobacter* spp. and *F. gastrosuis* DNA in swine samples and its possible association with gastric disease (Chapter II, Article I); to assess the role of wild animals as possible reservoirs for gastric helicobacters (Chapters III and IV, Articles II and III respectively); to evaluate the presence of AMR genes within the *H. pylori*-like PCR positive samples (Chapter V, Manuscript I) and lastly to assess the knowledge, attitudes and practice of veterinarians regarding gastric helicobacters with focus on *H. pylori* and *H. suis* (Chapter VI, Article IV).

The overall results report the presence of *Helicobacter* spp. DNA in the stomach of 78.9% (56/71) of the pigs, 64.3% (9/11) of the wild boars, 91.7% (11/12) of the wild rabbits and 100% (6/6) of the wild quails tested. Regarding the presence of *F. gastrosuis* DNA, it was present in 35.2% (25/71) of the pigs and 42.9% (6/14) of the wild boars. Wild rabbits and wild quails were not tested for this bacterium. Regarding the most prevalent *Helicobacter* species DNA amplified in the different animal species tested: in pigs this was *H. suis* (57.7% or 41/71) followed by *H. pylori*-like (50.0% or 36/71); in wild boars was *H. salomonis* and *H. bizzozeronii* (28.6% or 4/14 each) followed by *H. pylori*-like (14.3% or 2/14); in wild rabbits this was *H. salomonis* (75.0% or 9/12) and in wild quails was *H. salomonis* (100% or 6/6) (Chapters II to IV, papers I to III).

The high percentage of *H. suis* in pig samples was not surprising as similar results were already described by Hellemans *et al.* (2007) and Baele *et al.* (2008) [3,4]. Compared to pigs, the occurrence of both *H. pylori*-like species and *H. suis* was lower in wild boars but both animal species displayed similar percentages regarding *F. gastrosuis*. Although previous studies reported the presence of *Helicobacter* spp. [5-7], to the best of our knowledge this remains the first description of *F. gastrosuis* DNA detection in wild boars. The present investigation included, for the first time, free-range wild rabbits. Nevertheless, the results achieved go towards those previously described in pets and industrial and laboratory rabbits, which identified *H. felis*, *H. salomonis* and *H. bizzozeronii* DNA in the gastric corpus [8,9]. Regarding wild quails, the absence of publications prevents comparisons or critical interpretations of the results herein obtained. Indeed, to the best of our knowledge, this was the first time that gastric *Helicobacter* DNA was detected in wild

quails [10-12].

Due to the discrepant and relatively small sample size tested, these results must be interpreted with caution, and cannot be extrapolated to the general population of the different animal species evaluated. For instance, we analyzed the results from 71 pigs, 14 wild boars, 12 wild rabbits, and 6 wild quails. The difference between sample sizes was related with limitations in obtaining wild animal samples due to the restrictions applied to the activities that could take place during the pandemic and lockdown, reflected in a limited number of national hunting campaigns.

Regarding the histopathological analysis of pig and wild boar samples, in general, the results report a high occurrence of gastritis in these animal species (95.8% and 100% respectively), as previously described by Hessing et al. (1992), Robertson et al. (2002) and De Witte et al. (2019) [1,13,14].

It was found that 50.0% to 57.6% of the pig samples with gastritis, depending on the gastric portion analyzed (*pars oesophagea*, oxyntic mucosa or *antrum*) were associated with the presence of one or more *Helicobacter* sp. and *F. gastrosuis*, with highlight to *H. pylori*-like and *H. suis*. In both pigs and wild boars, *F. gastrosuis* was detected in association with *Helicobacter* species corroborating their potential synergy to induce gastric pathology [1]. In our study, in pig samples, gastric ulceration and erosion were higher in the antral mucosa samples positive for *H. suis* and *F. gastrosuis.* A statistically significant correlation was achieved for the presence of *F. gastrosuis* and the existence of ulceration and with the presence of *H. pylori*-like and erosion. These evidences are partially supported by other studies, where it was hypothesized that *H. suis* and *F. gastrosuis* bacterial interaction can lead to gastric lesions and ulceration of the *pars oesopahea* [1,15] in the porcine stomach. These lesions could be caused by *H. suis* that alters the gastric environment and may promote the proliferation of other microorganisms such as *F. gastrosuis* in the non-glandular zone leading to pH alterations and ultimately to gastritis and ulceration [15-17].

In wild boars, a statistically significant correlation between the presence of *H. bizzozeronii* and hyperplasia of the *pars oesophagea* was found. Regarding the presence of *F. gastrosuis* and histological alterations, no statistically significant results were obtained. However *F. gastrosuis* DNA was detected in association with *Helicobacter* species DNA which may corroborate their potential synergy to induce gastric pathology, as it has been described in pigs [1,2,15]. To better study this hypothesis, a study including a larger number of samples, as well as *in vitro* studies should be carried out in order to identify the molecular mechanisms underlying the pathogenesis of these bacteria in wild boars' gastric tissue.

Concerning the samples of wild rabbits and wild quails', the time elapsed from the animals' slaughter until sample collection, led to tissue autolysis that impaired the histological evaluation to assess gastritis and its possible correlation with the presence of *Helicobacter* spp. DNA in these animal species. Nevertheless, to ensure that these tissue alterations would not display further implications, DNA concentration and purity were measured for all specimens by spectrophotometry (NanoDrop®) and only those samples whose values fell within the accepted ranges were subjected to molecular analysis and submitted to PCR evaluation and sequencing analysis.

When analyzing our data, co-infections with two or more bacteria were detected in 47.9% (34/71) of the pigs and the most frequent bacterial combination was *H. suis* + *H. pylori*-like (16.9% or 12/71) followed by *H. suis* + *H. pylori*-like + *F. gastrosuis* (14.1% or 10/71). With respect to wild boars, 35.7% (5/14) presented co-infections and the most frequent combination was *H. salomonis* + *F. gastrosuis* (14.3% or 2/14). Co-infections were detected in 41.7% (5/12) of wild rabbits; mainly with *H. salomonis* + *H. bizzozeronii* (33.3% or 4/12) and *H. salomonis*+ *H. felis* (8.3% or 1/12). In wild quails, co-infections were detected in 50.0% (3/6) of the animals and different bacteria associations were found: *H. salomonis*+ *H. bizzozeronii* (16.7% or 1/6), *H. salomonis* + *H. pylori* (16.7% or 1/6) and *H. salomonis*+ *H. felis*+ *H. bizzozeronii* (16.7% or 1/6).

In the present study, DNA from *H. felis, H. bizzozeronii* and *H. salomonis,* which are recognized as natural colonizers of canine and feline stomachs, was also detected in the stomach of pigs and/or wild boars, wild rabbits and wild quails. However, it was not possible to determine whether these species are able to colonize the stomach of these animals species or whether our results are due to, for instance, environmental contamination. To exclude this last hypothesis and to determine if these bacterial species are indeed capable of colonizing the stomach of these animal species, thorough *in vitro* adherence and infection assays and *in vivo* infection studies should be performed.

Following the recommendations of other authors and as detailed in Chapter II (Article I), those samples initially considered to be *H. pylori* DNA-positive were subjected to a second set of *H. pylori*-specific primers targeting the *glmM* gene, [18-21]. The amplicons obtained from *H. pylori* DNA-positive PCR samples using species-specific primers targeting *ureAB* gene showed identities higher than 96% with *H. pylori,* when subjected to BLAST analysis. However, these were negative when subjected to a second set of primers targeting the *glmM* gene. Since natural colonization of swine stomach with *H. pylori* has not been described before, we hypothesize that *H. pylori*-like species was detected. Ideally, an appropriate approach would be the *in vitro* isolation and whole genome sequencing of these *H. pylori*-like organisms.

Additionally, all gastric samples from pigs, wild boars and wild quails in which the molecular tools employed culminated in a positive *H. pylori*-like case were then submitted to analysis of antimicrobial resistance genes (ARGs). To determine the presence of ARGs, species-specific primers targeting genes or mutated genes conferring resistance to different antimicrobials were used: *Pbp1A* gene (amoxicillin), *rdxA* and *frxA* genes (metronidazole), *16S rRNA* mutation gene (tetracycline) and *23S rRNA* gene (clarithromycin). As described in Chapter V, our results showed AMR mutation genes in three pig *H. pylori*-like positive samples. Two out of the three analyzed gastric tissue samples were also positive for *16S rRNA* mutation gene conferring resistance to tetracycline. One sample was positive for *frxA* gene encoding NADPH nitroreductase with SNP Y62D mutation, conferring resistance to metronidazole.

Considering that all three samples were PCR positive using *H. pylori* species-specific primers for *ureAB* gene (with a homology higher than 96.3 to 100%), and they were also PCR-positive when using primers specific for *H. pylori* ARGs (with homology with *H. pylori* ranging from 98.6-100%), it may be assumed that these samples may actually come from a bacterium very similar to *H. pylori* and as such, they also carry similar ARGs. The same *frxA* gene has been used in other studies to search for a mutation that would confer metronidazole resistance. Marques et al. (2019) concluded that mutations in genes encoding NADPH nitroreductase (*rdxA* and *frxA* genes) were associated with *H. pylori* resistance to metronidazole [22]. In their study, they reported resistance to metronidazole through amplification of *rdxA* and *frxA* genes by PCR followed by sequence analysis and comparison [22]. Diab et al. (2018) detected *Pbp1A*, *rdxA*, *23S rRNA* genes and *16S rRNA* mutation gene conferring resistance to amoxicillin, metronidazole, clarithromycin and tetracycline respectively, by PCR and sequencing in human *H. pylori* PCR positive samples [23].

In another study with animal samples conducted by Liu et al. (2019), tetracycline ARG was detected in all the evaluated (22/22) pig meat samples [24] and *H. pylori* AMR to tetracyclines has been identified in other animals' species such as in poultry samples, as described by Hamada et al. (2018) [25]. In this investigation, *H. pylori* was detected through PCR in seven out of 90 broiler samples and five of the *H. pylori-positive* samples were resistant to oxytetracycline [25]. Apart from our study, acquired resistance to tetracyclines and beta-lactams can occasionally occur in other *Helicobacter* species, such as gastric *H. suis* isolates from pigs, as reported by Berlamont et al. (2019) [26].

In summary, although requiring further exploration, our data reveal the presence of DNA from *Helicobacter* spp. in the stomach of pigs, wild boars, wild rabbits and wild quails, raising some questions and concerns regarding the contact between wild/domestic animals

and humans in the dissemination of agents with a putative impact on public and environmental health [5, 27-32]. Therefore, considering the position of veterinarians as professionals exposed to animals and therefore to most bacteria belonging to the genus *Helicobacter*, as well as their important role as public health promoters, the objective of the investigation presented in Chapter VI (Article IV) was to evaluate the knowledge, attitudes and practice of veterinarians on this subject. Taking into account the popularity of *H. pylori* as a reference species and its confirmed role as a carcinogen agent in humans, and the high prevalence and recognized pathogenicity of *H. suis* in pigs, these were the main *Helicobacter* species focused in the questionnaire addressed to Portuguese veterinarians.

Considering the current number of veterinarians registered and practicing in Portugal (6622), few answers were received (2.3%, 149/6622) which, probably, is not entirely representative of the general perception of the professionals but provided interesting results and tendencies. Most of the 149 questionnaire respondents were females (73.8%), as expected according to the 2018 European veterinary survey applied and published by the Federation of Veterinarion of Europe (FVE) and the Portuguese Order of Veterinarians (OMV) [27].

Despite some of the NHPHs being considered zoonotic or with zoonotic potential [28-30], in this study 37.2% of the respondents that "heard of *H. suis*" do not consider it a zoonosis. However, people directly affected by *H. pylori* infection seem to be aware of this hypothesis, since 68.2% of the respondents that "had gastric disease associated with *Helicobacter*" and 68.2% that "had treatment for *Helicobacter* infection" consider it a zoonosis. Interestingly, 59.0% of the respondents between the ages of 25 and 35 years-old consider gastric *Helicobacter* spp. infections a zoonosis. This may raise the hypothesis that the recent emergence of zoonoses globally may have made younger professionals more aware of these diseases [31].

It was also possible to infer that, regarding food safety and hygiene, only 3.5% mention "hand washing" as a food safety practice despite 97.9% of the respondents that prepare food at home stating that they "have hygiene and safety measures during food preparation". Similar findings were reported by Stratev et al. (2017) who concluded that, despite the high level of awareness of food safety, these practices were low among veterinary medicine students in Bulgaria [32].

Overall, the data analysis suggests that most of the respondents have limited perception and knowledge about the putative zoonotic/anthropozoonotic risks of *H. suis* and *H. pylori* infections. When questioned "have you heard of *Helicobacter*", 99.3% chose "yes" with most of the respondents (97.3%) knowing *H. pylori* but only 51.7% acknowledging *H.*

suis. Interestingly, 17.6% of respondents that "have heard of *Helicobacter*" did not know which animals' species could be affected. Furthermore, 13.4% do not consider *Helicobacter* spp. zoonotic bacteria and 38.9% "do not know", even though 96.6% claimed that they "have heard of *Helicobacter* and know what it is" and 38.3% "have heard of *H. suis* and know what it is".

It can also be hypothesized that *Helicobacter* spp. infections or its association with gastrointestinal diseases among animals could be underdiagnosed due to a lack of knowledge/awareness of veterinarians regarding this bacterial infection. In fact, the data reveals that 17.6% did not know the animal species that could be affected, and of the 106 veterinarians that practice at companion animals' clinics, 72.6% never or rarely consider *Helicobacter* spp. infection as a differential diagnosis of gastritis in companion animals despite studies suggesting the possible association between *Helicobacter* spp. infections and gastritis in companion animals [33-36].

When assessing gastric pathology among the respondents, 59.4% of females report to have gastric pain and 68.2% had treatment for *H. pylori* infection which goes along with results found in a study published by Khoder et al. (2021) that reports a higher occurrence of *H. pylori* infections in females [37]. The data also suggests that veterinarians with higher contact time (more than 5 years) with pigs (52.9%) also "had had treatment for *Helicobacter* infection", which again may support the theory of the occupational risk of these professionals as reported by Joosten et al. (2013) [38].

Lastly, veterinarians who had some knowledge/experience with *Helicobacter* infections seem to be more open to receiving further information on this topic as detailed in the article presented in Chapter VI.

There are no studies that assess the perception, awareness, and knowledge of veterinarians regarding *Helicobacter* spp. infections which make this one the first to be reported. On the other hand, it also turns it challenging to compare. It is known that zoonotic diseases are a growing concern, accounting for approximately 60% of the existing human pathogens, and over 75% of those can be linked to animals [39]. Sometimes, zoonotic diseases manifest in animals before they infect humans [40], so veterinarians possess specialty training and knowledge in zoonoses, constituting an important resource of information and advice on this topic [41]. Despite the results of this study being suggestive of limited knowledge and awareness of veterinarians regarding gastric *Helicobacter* spp. infections, they continue to play a unique, referential and extremely important role in multidisciplinary public health teams in preventing and combating diseases [40,42]. The medical and veterinary communities should collaborate closely, not only in the context of

zoonoses but in a wide range of intervention areas. This collaboration will enhance fundamental, clinical and translational research, and the conservation of species using translational medicine and environmental research and under the umbrella of the “One Health” concept which, among others, aims to reduce the professional gap between veterinarians and physicians, encouraging both to take part in public health and environmental education [43].

Conclusions and future perspectives

There is a high prevalence of *Helicobacter* in the analyzed gastric samples collected in Portuguese abattoirs. Wild boars may be affected by *F. gastrosuis* and this bacterium may play a role in the development of gastric pathology in this species, which has also been suggested for pigs. It may be hypothesized that wild rabbits and wild quails may act as reservoirs for the studied *Helicobacter* species. Additionally, some of the gastric *H. pylori*-like bacteria identified in these animal species may carry ARG. As a last conclusion, there may be limited knowledge and awareness of Portuguese veterinarians regarding *Helicobacter* spp., namely *H. suis* and *H. pylori.*

After these conclusions, it would be interesting to continue some of the studies with a larger sample and further techniques. Ideally, a larger sample of wild boars, wild rabbits, and wild quails; a gastric swab for bacterial culture, isolation, and antibiotic susceptibility test from each gastric sample should be performed to rule out contamination of the gastric counterparts or if indeed the bacteria were able to colonize these different hosts as well as a histopathologic evaluation of all the samples collected. Besides this, *in vitro* adherence and infection studies could be relevant to determine if these bacteria can colonize and infect these animal species and the respective pathways of adhesion and infection. It would be also interesting to prepare a questionnaire to be applied to the general population in order to assess their knowledge and awareness regarding *Helicobacter* infections and possible risks of exposure.

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DOUTORAMENTO

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