Experimental studies on *Helicobacter suis* virulence and control

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List of abbreviations

8-OHdG	8-hydroxydeoxyguanosine
Ac	acivicin
ADP	adenosine diphosphate
AGS	human gastric adenocarcinoma cell line
AP-1	activator protein-1
Apaf-1	apoptotic peptidase-activating factor 1
ASK1	Apoptosis signal-regulating kinase 1
ATCC	American type culture collection
ATP	adenosine triphosphate
ATPase	adenosine triphosphatase
BabA	blood group antigen-binding adhesin
Bak	BCL2-antagonist/killer
Bax	BCL2-associated X protein
bp	base pair
Bcl	B-cell leukemia/lymphoma
BHI	brain heart infusion
BLAST	basic local alignment search tool
C.	Clostridium
c3	caspase-3
CagA	cytotoxin-associated protein
cag PAI	cytotoxin-associated gene pathogenicity island
caspase	cysteine-dependent aspartate-directed protease
CD	cluster of differentiation
CFU	colony-forming unit
COX	cyclooxygenase
CT	cholera toxin
CXCL	CXC ligand
cyt *c*	cytochrome *c*
D	day
DISC	death-inducing signalling complex
DNA	deoxyribonucleic acid
$\Delta\Psi m$	mitochondrial membrane potential
E.	*Escherichia*
EGC	expanding germinal center
ELISA	enzyme-linked immunosorbent assay
EPIYA	Glu-Pro-Ile-Tyr-Ala
Erk	extracellular signal-regulated kinase
FADD	Fas-associated death domain

List of abbreviations

FasL	Fas ligand
FCS	fetal calf serum
FISH	fluorescent *in situ* hybridization
FSC	forward scatter
fur	ferric uptake regulator
g	gram
GGT	γ-glutamyl transpeptidase
GSH	reduced glutathione
GSSG	oxidized glutathione
GST	Glutathione S-transferase
gyrB	gyrase subunit B
GPI-AP	glycosylphosphatidylinositol-anchored proteins
h	hour
H.	*Helicobacter*
H&E	haematoxylin and eosin
HBSS	Hank's balanced salt solution
His	histidine
HpaA	*H. pylori* adhesin A
HP-NAP	*H. pylori* neutrophil activating protein
HRP	horseradish peroxidase
HspA	heat-shock protein A
hsp60	heat-shock protein 60 gene
IFN	interferon
Ig	immunoglobulin
IKK*β*	I*κ*B-kinase *β*
IL-8	interleukin-8
iNOS	inducible nitric oxide synthase
INS-GAS	insulin-gastrin
ITS	internally transcribed rRNA spacer
JNK	c-Jun N-terminal kinase
K.	*Kazachstania*
kb	kilobase
kDa	kilodalton
kV	kilovolt
l	liter
L.	*Lactobacillus*
LEL	lymphoepithelial lesion
LF	lymphoid follicle
LPS	lipopolysaccharide
LT	heat-labile toxin of enterotoxigenic *Escherichia coli*

M	molar
MALT	mucosa-associated lymphoid tissue
MAP	mitogen-activated protein
MCL1	myeloid cell leukemia sequence 1
Mek	mitogen-activated protein kinase kinase
MHC	major histocompatibility complex
min	minute
ml	milliliter
mm	millimeter
mM	millimolar
MOI	multiplicity of infection
MWCO	molecular weight cut-off
MyD88	myeloid differentiation primary response gene 88
µg	microgram
µM	micromolar
NADPH	nicotinamide adenine dinucleotide phosphate
NCBI	National Center for Biotechnology Information
NF-κB	nuclear factor-κB
NHPH	non-*H. pylori Helicobacter*
NLR	NOD-like receptor
nm	nanometer
NOD	nucleotide-binding oligomerization domain
NSAID	non-steroidal anti-inflammatory drug
NTPase	nucleoside triphosphatase
OD	optical density
PAMP	pathogen-associated molecular patterns
PARP	poly (ADP-ribose) polymerase
PAS	periodic acid-Schiff
PBS	phosphate-buffered saline
PCR	polymerase chain reaction
pH	measure of acidity or basicity
PHYLIP	PHYLogeny Interference Package
pi	post infection
PI	propidium iodide
PKC	protein kinase c
PRM	pathogen-recognition molecules
rHSGGT	recombinant *H. suis* GGT
ROS	reactive oxygen species
RPTP	receptor protein tyrosine phosphatases
rRNA	ribosomal RNA

List of abbreviations

RT	room temperature
SAB	Sabouraud
SabA	sialic acid-binding adhesin
SD	standard deviation
SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis
SH2	Src homology 2
SHP-2	SH2-containing tyrosine phosphatase
SPF	specific pathogen free
SSC	side scatter
TCA	tricarboxylic acid
TEM	transmission electron microscopy
TGF	transforming growth factor
Th	T helper
TLR	Toll-like receptor
TNF	tumor necrosis factor
TNFR	tumor necrosis factor receptor
TRADD	TNFR associated death domain
Treg	regulatory T cell
TREM	triggering receptors expressed on myeloid cells
U	unit
ureA/B	urease subunit A/B
VacA	vacuolating cytotoxin
vol	volume
w	weeks
WT	wild-type

General Introduction

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1 Gastric helicobacters in humans and pigs

It was first reported in 1984 that gastritis and gastric ulcer disease in humans can be caused by a curved bacterium (Marshall and Warren, 1984), which was named *Helicobacter pylori* in 1989 (Goodwin et al., 1989). Nowadays, it is clear that this bacterium plays an important role in the development of gastritis, peptic ulcer disease, gastric adenocarcinoma and mucosa-associated lymphoid tissue (MALT) lymphoma (Parsonnet et al., 1991; Stolte et al., 1993; Kusters et al., 1996). This bacterium is very successful in the way that it colonizes the human stomach, since in developing countries, more than 80% of the population is infected with *H. pylori,* even at young age. In more developed countries, the prevalence rate of *H. pylori* generally remains under 40% and is considerably lower in children and adolescents than in adults and elderly people (Kusters et al., 2006; Pounder and Ng, 1995).

On histology, *H. pylori* bacteria are identified on the basis of their typical localization in gastric mucus or closely associated with mucus-secreting gastric epithelial cells and their characteristic, slightly curve-shaped morphology (Testerman et al., 2001). *H. pylori*, however, is not the only bacterial pathogen capable of colonizing the human gastric mucosa. Indeed, non-*H. pylori* helicobacters with a typical spiral-shaped morphology have been found in 0.2 to 6% of gastric biopsies (Heilmann and Borchard, 1991; Stolte et al., 1994; Svec et al., 2000; Solnick et al., 2003). These spiral-shaped non-*H. pylori* helicobacters were first described in 1987 (Dent et al., 1987) and were originally referred to as "*Gastrospirillum hominis*" (McNulty et al., 1989). Analysis of the 16S rRNA gene of these uncultivated organisms resulted in their classification in the genus *Helicobacter*. They were provisionally named "*H. heilmannii*" after the German pathologist Konrad Heilmann, who first studied the pathology associated with these microorganisms (Heilmann and Borchard, 1991). Humans infected with these large spiral-shaped organisms have been reported to suffer from gastritis (Stolte et al., 1997) sometimes accompanied by gastric ulcer, gastric MALT lymphoma and even gastric adenocarcinoma (Stolte et al., 1997; Debongnie et al., 1998; Morgner et al., 1995; Morgner et al., 2000). Further research on "*H. heilmannii"* has been seriously hampered by the very fastidious nature of these microorganisms. Even today, to our knowledge, only two "*H. heilmannii"* strains have been cultured from human tissue (Andersen et al., 1996; Kivistö et al., 2010). Recent investigations, however, have shown that these gastric non-*H. pylori* helicobacters comprise different species which have been detected in the stomach of different animal species (De Groote et al., 2005; Van den Bulck et al., 2005).

1.1 Gastric non-*H. pylori Helicobacter* nomenclature

Since the description of *H. pylori,* the number of species in the genus *Helicobacter* has rapidly expanded. Today, a large number of non-*H. pylori Helicobacter* species have been described in a wide variety of animals and humans and the genus *Helicobacter* contains at least 32 species with validly published names (http://www.bacterio.cict.fr/h/helicobacter.html). The frequent changes in nomenclature of non-*H. pylori* helicobacters colonizing the stomach of humans have caused much confusion, not only among clinicians, but also among bacteriologists. Today, there is a serious problem in trying to reach international agreement on the nomenclature of this complex and expanding group of micro-organisms, all of which have in common their tightly coiled morphology and their difficulty to culture *in vitro*.

Subsequent to the renaming of "*Gastrospirillum hominis*" as "*H. heilmannii*", further genetic analysis of the 16S rRNA gene revealed two types that differed by more than 3% in their nucleotide sequence, which prompted the subclassification of the non-*H. pylori* helicobacters into "*H. heilmannii"* type 1 and "*H. heilmannii"* type 2.

It is now accepted that "*H. heilmannii"* type 1 is identical to the recently cultured and described *Helicobacter suis*, formerly *"Candidatus* Helicobacter suis*"* (De Groote et al., 1999; O'Rourke et al., 2004b; Baele et al., 2008b), a spiral-shaped bacterium that colonizes the stomach of more than 60 % of slaughter pigs (Mendes et al., 1991; Grasso et al., 1996; Park et al., 2004; Hellemans et al., 2007b). This micro-organism was first designated "*Gastrospirillum suis*" (Mendes et al., 1990, Queiroz et al., 1990). Almost ten years later, sequencing of the 16S rRNA gene, fluorescent in-situ hybridization (FISH) and electron microscopy showed that this organism belongs to the genus *Helicobacter* and is sufficiently different from all existing species to constitute a new taxon. Because at that time this species could not be thoroughly characterized due to the lack of pure *in vitro* isolates, the organism was described as "*Candidatus* Helicobacter suis*"* (De Groote et al., 1999). Only a few years ago, a successful *in vitro* cultivation method has been developed, resulting in the valid description of *H. suis* as a species (Baele et al., 2008b).

The situation with regard to "*H. heilmannii*" type 2 is even more complex. This type does not represent a single *Helicobacter* species but rather a group of species, including *H. felis, H. bizzozeronii* and *H. salomonis*, which all three have been isolated from the stomachs of cats and dogs. To add to the confusion, one uncultivable species detected in the stomachs of humans, wild felids, dogs and cats was named "*Candidatus* Helicobacter heilmannii"

(O'Rourke et al., 2004b). Very recently, however, we succeeded in isolating this bacterium from the gastric mucosa of cats and proposed the name *Helicobacter heilmannii* (Smet et al., 2011). Two other closely related species, one of which was isolated from a dog and the other from a cat, have been described as *H. cynogastricus* and *H. baculiformis,* respectively (Van den Bulck et al., 2006; Baele et al., 2008a). For the latter two species, however, no information is available about their presence in humans.

Besides the fact that all non-*H. pylori Helicobacter* species are spiral-shaped and possess variable numbers of bipolar flagella, differences in morphology between different non-*H. pylori Helicobacter* species have been described. *H. felis* is characterized by the presence of periplasmic fibrils, which can be lost during *in vitro* cultures (Eaton et al., 1996). Similarly, *H. cynogastricus* and *H. baculiformis* possess periplasmic fibrils (Van den Bulck et al., 2006; Baele et al., 2008) while *H. bizzozeronii, H. salomonis, H. heilmannii* and *H. suis* do not have periplasmic fibrils (Hänninen et al., 1996; Jalava et al., 1997; Baele et al., 2008; Smet et al., 2011). *H. felis* and *H. bizzozeronii* are large and tightly coiled organisms (Lee et al.,1988; Hänninen et al., 1996), whereas *H. salomonis* is shorter and less tightly coiled (Jalava et al., 1997). *H. cynogastricus* generally is the largest non-*H. pylori Helicobacter* species and upon repeated subculture the bacteria become less tightly coiled. *H. baculiformis* is a large, slender to slightly spiral rod with periplasmic fibrils (Baele et al., 2008a). The recently isolated *H. heilmannii* is morphologically very similar to *H. suis* and both species possess tight coils (De Groote et al., 1999; Baele et al., 2008b; Smet et al., 2011). Despite these differences and because, based on our observations and those of others (Eaton et al., 1996), morphology can slightly alter when cultured *in vitro* or after repeated subculture, microscopic investigation of biopsy samples is not an accurate method for species identification.

Sequencing of the 16S and 23S ribosomal RNA encoding genes allows differentiation of *H. suis* from the other gastric non-*H. pylori Helicobacter* species mentioned above, but it can not distinguish between *H. felis*, *H. bizzozeronii*, *H. salomonis, H. cynogastricus, H. baculiformis* and "*Candidatus* H. heilmannii" (Dewhirst et al., 2005; Van den Bulck et al., 2006; Baele et al., 2008). For differentiation between these species sequencing of the *hsp60* gene (Mikkonen et al., 2004), the urease A and B genes (Neiger et al., 1998; O'Rourke et al., 2004b) and *gyrB* gene (Hannula and Hänninen, 2007) is useful, as well as whole cell protein profiling (Vandamme et al., 2000) if pure *in vitro* cultures are available.

Phylogenetic trees for the gastric helicobacters are shown in Figure 1 (16S rRNA gene sequence) and Figure 2 (partial *ureA* and *ureB* gene sequences).

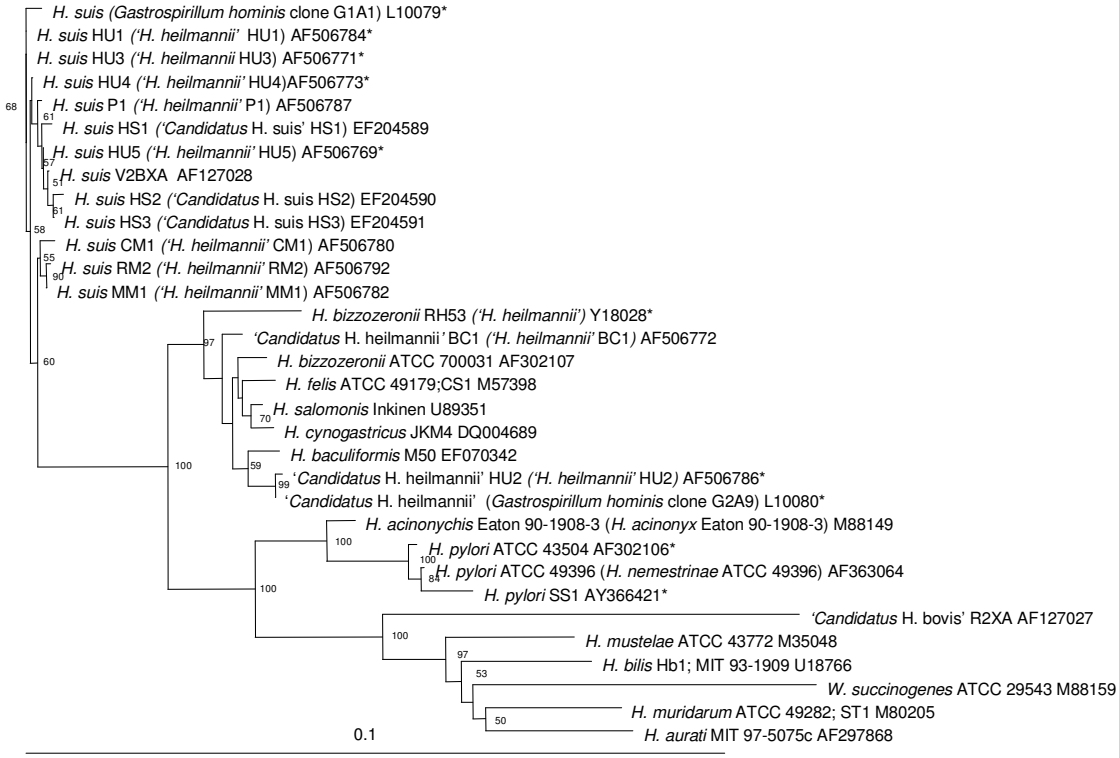

Figure 1: Phylogenetic tree, based on the near-complete 16S rRNA gene sequences from gastric *Helicobacter* species and other closely related bacteria. Sequences were aligned by using the CLUSTAL W program (Thompson et al., 1994) and a phylogenetic tree was constructed using the neighbour-joining method (Saitou & Nei, 1987) via the PHYLIP package (Felsenstein, 1989), using DNADIST for distance analysis (Kimura, 1980). Bootstrap values (for branches present in more than 50 out of 100 resamplings of the data) are indicated at the nodes. Original names found at the Entrez Nucleotide database (NCBI) are shown between brackets. Sequences marked with an * are derived from bacteria present in the stomach of humans. Bar, 0.1 substitution per nucleotide position.

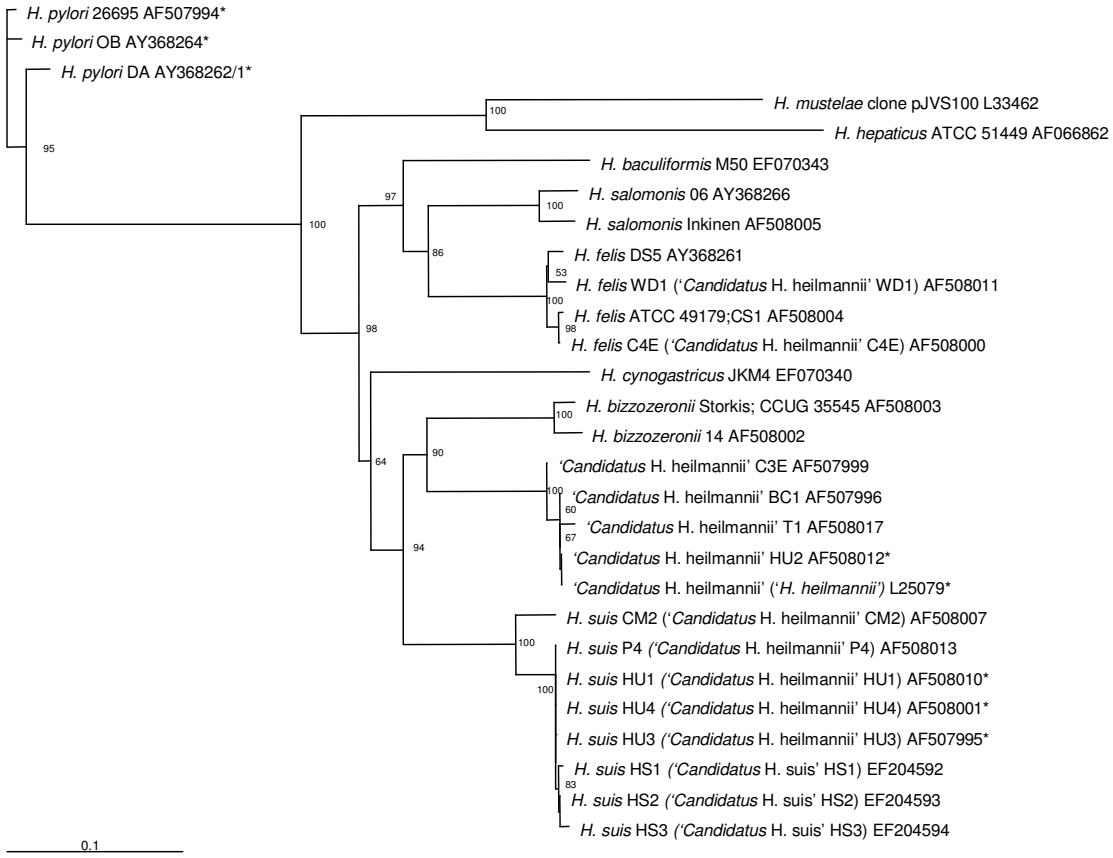

Figure 2: Phylogenetic tree, based on the partial *ureA* and *ureB* gene sequences from gastric *Helicobacter* species and other closely related bacteria. Sequences were aligned by using the CLUSTAL W program (Thompson et al., 1994) and a phylogenetic tree was constructed using the neighbor-joining method (Saitou and Nei, 1987) via the PHYLIP package (Felsenstein, 1989), using DNADIST for distance analysis (Kimura, 1980). Bootstrap values (for branches present in more than 50 out of 100 resamplings of the data) are indicated at the nodes. Original names found at the Entrez Nucleotide database (NCBI) are shown between brackets. Sequences marked with an * are derived from bacteria present in the stomach of humans. Bar, 0.1 substitution per nucleotide position.

In literature, gastric infections with spiral-shaped bacteria in humans are often referred to as "*H. heilmanni"* or "*H. heilmannii"*-like organism infections. However, at present, the name "*H. heilmannii*" should be reserved to the species as described by Smet et al. (2011) and should no longer be used as a covering name, according to taxonomical rules. To avoid confusion, we propose to use the term "gastric non-*H. pylori* helicobacters" to designate these spiral-shaped bacteria when only results of histopathology or crude taxonomic data are available and to reserve true species designations for those situations in which the species is defined.

1.2 Gastric non-*H. pylori* helicobacters in humans

1.2.1 Origin of infection

In 1999, Andersen and coworkers succeeded for the first time in the *in vitro* isolation of a non-*H. pylori Helicobacter* strain from human gastric mucosa. Later, phenotypic analysis, sequencing of the 16S rRNA gene, DNA-DNA hybridization analysis and whole-cell protein profiling revealed that this isolate belongs to the species *H. bizzozeronii* (Jalava et al., 2001). Recently, another *in vitro* isolate was obtained from a human gastric non-*H. pylori Helicobacter* infection. Despite the low prevalence of *H. bizzozeronii* in human biopsies, polyphasic identification analysis revealed that this second isolate also belonged to this species (Kivistö et al., 2010). O'Rourke et al. (2004b) demonstrated that 3 human and 4 porcine non-*H. pylori Helicobacter* strains isolated *in vivo* in mice showed a very high degree of homology, based on 16S rRNA and partial *ureA* and *ureB* gene sequences. This led to the conclusion that they represent the same species, later described as *H. suis* (O'Rourke et al., 2004b; Baele et al., 2008). Another human *in vivo* isolate in this study was shown to cluster with helicobacters from domestic and wild feline species and was designated '*Candidatus* Helicobacter heilmannii' (O'Rourke et al., 2004b).

The prevalence of non-*H. pylori* helicobacters in humans suffering from gastric disease varies between 0.1 and 6% (Stolte et al., 1994; Boyanova et al., 2003; Yang et al., 1998; Yali et al., 1998; Ierardi et al., 2001). Although the exact mechanisms of transfer remain largely unknown, human infections with non-*H. pylori Helicobacter* organisms most likely originate from animals. Several case reports suggest the transmission of these large spiral-shaped bacteria from dogs to humans (Thomson et al., 1994; Jalava et al., 2001; De Bock et al., 2007) or from cats to humans (Lavelle et al., 1994; Stolte et al., 1994; Dieterich et al., 1998; van Loon et al., 2003; Hiroshi et al., 2008). Additionally, it has been shown that living in close proximity to dogs, cats and especially pigs is a significant risk factor for contracting these infections (Meining et al., 1998). The intensity of contact with animals is thought to be important as well, since a higher incidence of these infections has been noted in pig farmers and people having intensive contact with pet animals (Stolte et al., 1994; Yoshimura et al., 2002; van Loon et al., 2003). It is remarkable that *H. suis* is the most prevalent gastric non-*H. pylori Helicobacter* species in humans (Trebesius et al., 2001; Van den Bulck et al., 2005). This might indicate that the infectivity in humans of cat- or dog-related strains is less than that of *H. suis*, or else that consumption of contaminated pig meat might also be an, albeit sporadic, source of infection.

1.2.2 Clinical significance of gastric non-*H. pylori Helicobacter* infections in humans

Reports in literature about human non-*H. pylori* helicobacter infections often lack a detailed description of the exact causative *Helicobacter* species and should therefore be interpreted with caution. Clinical symptoms associated with non-*H. pylori* helicobacters in man can be characterized by atypical complaints such as acute or chronic epigastric pain and nausea. Other aspecific symptoms include dyspepsia, heartburn, vomiting, hematemesis, abdominal pain, irregular defecation frequency and consistency, and dysphagia, often accompanied by a decreased appetite (Morgner et al., 1995; Goddard et al., 1997; Dieterich et al., 1998; Yang et al., 1998; Mention et al., 1999; Yoshimura et al., 2002; Seo et al., 2003; Sykora et al., 2003; Van Loon et al., 2003). However, some people infected with non-*H. pylori* helicobacters do not present obvious clinical signs (Mazzucchelli et al., 1993).

Very often, inspection of the gastric mucosa through endoscopy is performed in humans suffering from gastric disease. In persons infected with long spiral bacteria, a variety of lesions is observed, ranging from a normal to slightly hyperemic mucosa, mucosal edema, nodular inflammation, and the presence of multiple erosions and ulcerations in the antrum or in the duodenum (Goddard et al., 1997; Debongnie et al, 1998; Dieterich et al., 1998; Mention et al., 1999; Yoshimura et al., 2002; Seo et al., 2003; Sykora et al., 2003; Van Loon et al., 2003). In contrast to a *H. pylori* infection, non-*H. pylori Helicobacter* colonization is predominantly focal and is found predominantly in the antrum (Stolte et al., 1997). Microscopic examination of gastric biopsies of non-*H. pylori Helicobacter*-infected patients often reveals a chronic gastritis, characterized by infiltration with lymphocytes and plasma cells, sometimes organized into lymphoid aggregates (Morgner et al., 1995; Stolte et al., 1997; Ierardi et al., 2001; Sykora et al., 2003; Van Loon et al., 2003). Compared to a *H. pylori*-associated gastritis, gastritis associated with non-*H. pylori* helicobacters is mostly less active and less severe (Stolte et al., 1997), although a predominant acute neutrophilic inflammation can also be observed (Yoshimura *et al.,* 2002). Other lesions, such as atrophic gastritis and intestinal metaplasia, are rare and observed less frequently in humans infected with non-*H. pylori* helicobacters compared to *H. pylori*-infected patients (Debongnie et al., 1995; Goddard et al., 1997; Stolte et al., 1997).

Interestingly, the risk of developing MALT lymphoma is higher with non-*H. pylori* helicobacters than with *H. pylori* (Stolte et al., 1997 and 2002; Morgner et al., 2000). Both gastritis and MALT lymphoma have been reported to resolve after clearance of the non-*H. pylori Helicobacter* infections, further underlining the causal relationship (Morgner et al.,

2000). Again, however, no description of the exact *Helicobacter* species was included. Only occasionally, the presence of non-*H. pylori Helicobacter* bacteria has been associated with gastric adenocarcinoma, mostly accompanied by necrosis and erosive or ulcerative lesions (Yang et al., 1995; Morgner et al., 1995). This is accompanied by a mild non-active gastritis in the corpus tissue surrounding the tumor and a mild, moderately active gastritis in the antrum.

1.3 *Helicobacter suis* in pigs

In 1990, the first description of large, spiral-shaped bacteria in the mucus layer of lumen and antral pits of pig stomachs was made (Mendes et al., 1990; Queiroz et al., 1990). Initially, "*Gastrospirillum suis"* was proposed as a name, but subsequent characterization showed that this organism in fact belonged to the genus *Helicobacter* (De Groote et al., 1999). A new name, '*Candidatus* Helicobacter suis' was then proposed and despite numerous attempts worldwide, the first successful *in vitro* isolate was only obtained in 2007, by using a new biphasic culture method, which finally led to the description of *H. suis* as a new species (Baele et al., 2008b). Sequence analysis of 16S rRNA, 23S rRNA, partial *hsp60* and partial *ureAB* gene sequences confirmed that *H. suis* is identical to the previously called "*H. heilmannii"* type 1, colonizing the stomach of humans and other primates (O'Rourke et al., 2004b).

1.3.1 Prevalence of *Helicobacter suis* in pigs

The pig stomach is often colonized by *Helicobacter suis.* Its reported prevalence depends on the study. Mostly, however, this bacterium is detected in more than 60% of the pigs at slaughter age (Barbosa et al., 1995; Grasso et al., 1996; Cantet et al., 1999; Park et al., 2004; Hellemans et al., 2007b). Recently, similar prevalences have been described in the US, with varying prevalence rates depending on the geographic region (Kopta et al., 2010). Despite a very high prevalence in adult pigs, much lower degrees of infection have been shown in younger animals. Hellemans et al. (2007b) detected a prevalence of only 2% in suckling piglets, which however increased rapidly after weaning. This might be due to the protective effect of sow milk, which has been shown to inhibit *H. pylori* colonization in mice through the presence of *H. pylori*-binding epitopes on milk proteins (Gustafsson et al., 2006).

1.3.2 Possible role of *Helicobacter suis* in porcine gastric pathology

Gastric *H. suis* infection in pigs has been shown to cause gastritis in experimentally and naturally infected pigs, mainly in the antrum (Mendes et al., 1991; Grasso et al., 1996; Queiroz et al., 1996; Park et al., 2000; Hellemans et al., 2007a; Kumar et al., unpublished results). This gastritis shows a spatial association with the main site of colonization, as shown in experimentally infected pigs (Hellemans et al., 2007a; Kumar et al., unpublished results). Sapierzynski et al. (2007) demonstrated that an *H. suis* infection in pigs results in an increased number of gastrin producing cells and a decreased number of somatostatin producing cells. Since gastrin stimulates and somatostatin inhibits the secretion of hydrochloric acid by parietal cells, this may result in excessive acid production.

The non-glandular part of the pig stomach is a small rectangular area around the esophageal opening, covered by a stratified squamous epithelium and also called the pars esophagea of the stomach. Besides the strong association with gastritis, *H. suis* infection has also been associated with ulcers of this non-glandular part of the stomach, although *H. suis* bacteria probably do not colonize this specific stomach region (Barbosa et al., 1995; Queiroz et al., 1996; Choi et al., 2001; Roosendaal et al., 2000; Hellemans et al., 2007b). Other research groups did not find this association (Grasso et al., 1996; Cantet et al., 1999; Melnichouk et al., 1999; Park et al., 2000; Szeredi et al., 2005), so the exact role of *H. suis* in the development of these lesions remains to be elucidated. The discrepancies found in literature may be due to differences in laboratory techniques for demonstration of *Helicobacter,* different sampling practices or differences in virulence between different *H. suis* strains. In any case, hyperkeratosis and ulceration of the non-glandular part of the stomach have been reported in many countries (Friendship et al., 2006). Up to 80% of the market pigs in Australia (Robertson et al., 2002) and 60% of the sows (Hessing et al., 1992) in the Netherlands have been described to present gastric lesions.

The development of lesions in the non-glandular pars esophagea is most likely a process involving different factors, including stress, transport, the presence in the stomach of short chain fatty acids and pelleting and fine grinding of the feed (Potkins et al., 1989; Hessing et al., 1992; Elbers et al., 1994; Argenzio et al., 1995). The latter factor could have an influence on the fluidity of gastric contents (Elbers et al., 1994), leading to an increased contact of the stratified squamous epithelium of the non-glandular region with the luminal content of the distal part with acid, bile (refluxed from the duodenum) and pepsin. Chronic insult of the non-glandular region will eventually lead to ulceration.

Ulceration of the porcine gastric non-glandular mucosa may result in decreased feed intake, a decrease in daily weight gain and even sudden death due to fatal hemorrhage (Ayles et al., 1996; Friendship et al., 2006), thus leading to significant economic losses. There is also little doubt that this disease can cause pain and discomfort. Interestingly, a decrease in daily weight gain has also been observed in pigs experimentally infected with *H. suis,* however without a clear association with the development of lesions of the non-glandular part of the stomach (Kumar et al., 2010).

Besides *H. suis*, other *Helicobacter* species have been described in the stomach of pigs, including a *H. pylori*-like bacterium, responsible for the development of ulcers of the pars esophagea in gnotobiotic piglets (Krakowka et al., 2006), *H. bilis* and *H. trogontum* (Hänninen et al., 2003 and 2005). The main site of colonization of the latter two is most probably the lower intestinal tract and it remains to be determined whether these bacteria are able to colonize the porcine stomach and cause gastric pathology.

2 The host response to *Helicobacter pylori* infection

Throughout the years, *H. pylori* has adapted to its host in a remarkable way. Infection with this bacterium persists for life, despite the development of an often distinct host response (Wilson and Crabtree, 2007). Because the evoked inflammation plays a central role in *H. pylori*-induced gastric pathology, a brief overview (summarized in Figure 3) is distilled from the abundance of available literature.

2.1 Innate immune recognition of *H. pylori*

H. pylori is mainly considered an extracellular pathogen, implying that the interactions with epithelial cells are important for the induction of the host response. Toll-like receptors (TLR) are important pathogen-recognition molecules (PRM) expressed by epithelial cells and cells of the innate immunity system, including dendritic cells and macrophages (Kaparakis et al., 2010), and TLRs are involved in the recognition of pathogen-associated molecular patterns (PAMP). TLR2, -4, -5 and -9 are expressed by gastric epithelial cells in the antrum and corpus of both patients suffering from *H. pylori* gastritis and those with a non-inflamed gastric mucosa (Schmausser et al., 2004). *H. pylori* LPS has been shown to activate NF-κB through TLR2 (Smith et al., 2003), while most authors agree that *H. pylori* can not be

recognized by TLR4 (Backhëd et al., 2003; Smith et al., 2003). Bacterial flagellin is typically recognized by TLR5, but conflicting reports have been made about a possible recognition of *H. pylori* flagellin by this specific receptor (Smith et al., 2003; Andersen-Nissen et al., 2005). Besides LPS, also the *H. pylori* neutrophil-activating protein (HP-NAP) has been described as a TLR2 agonist (Amedei et al., 2006). Eventually, the recruitment of myeloid differentiation primary response gene 88 (MyD88) to the formed TLR complex leads to activation of transcription factors NF-κB or activator protein-1 (AP-1) and production of pro-inflammatory cytokines (Allison et al., 2009; Kaparakis et al., 2010).

In addition to surface-expressed TLR's, cytosolic PRM's also exist, including the nucleotide-binding oligomerization domain (NOD)-like receptor (NLR) family. Nod1 has been shown to be responsible for the recognition of *H. pylori* peptidoglycan, delivered by the type IV secretion system (Viala et al., 2004). This results in the induction of NK-κB activation and subsequent production of pro-inflammatory cytokines, including CXCL-8 (IL-8) and a murine IL-8 homologue, CXCL-2 (Viala et al., 2004), although other mechanisms are also involved here, as shown by the fact that direct contact between epithelial cells and metabolically inactive bacteria is sufficient to induce IL-8 expression (Rieder et al., 1996). Interestingly, transformation of *H. pylori* to coccoid forms, which retain their infectious ability (Wang et al., 1997), promotes their escape from Nod1 detection, which probably involves modifications of cell wall peptidoglycan (Chaput et al., 2006). A variety of other receptor proteins have been shown to play a role in innate *H. pylori* immunity, including triggering receptors expressed on myeloid cells (TREM) (Kaparakis et al., 2010).

2.2 Inflammatory cells involved in the *H. pylori*-induced innate and acquired immune response

After production of pro-inflammatory cytokines by epithelial cells exposed to *H. pylori*, innate immune cells are recruited, including neutrophils, macrophages and dendritic cells (Kaparakis et al., 2010). An important *H. pylori* factor involved in enhancement of early immune responses is the *H. pylori* neutrophil activating protein (HP-NAP). Interestingly, a homologue of the coding gene is found in the *H. suis* genome (Vermoote et al., 2011). HP-NAP promotes the adherence of neutrophils to endothelium, transendothelial migration of neutrophils, chemotaxis of leukocytes and the release of ROS by neutrophils and monocytes (Evans et al., 1995; Satin et al., 2000 ; Montecucco and de Bernard, 2003; Brisslert et al. 2005; Polenghi et al., 2007). Macrophages and dendritic cells are important for the

recruitment of $CD4^+$ T helper (Th) cells to the gastric mucosa, which orchestrate the adaptive immune response. After stimulation of peripheral blood mononuclear cells with whole *H. pylori* bacteria or bacterial products, expression of cytokines as IFN-γ and IL-12 is upregulated, eliciting a predominant Th1 response (Haeberle et al., 1997; Meyer et al., 2000; Kaparakis et al., 2010). In *H. pylori*-infected patients, most infiltrating $CD4^+$ T cells indeed show a Th1 phenotype (Sommer et al., 1998; Bamford et al., 1998), although cells showing a Th1 and a Th2 phenotype have both been observed in the *H. pylori*-infected gastric mucosa (Robinson and Atherton, 2010). This was confirmed in numerous experimental mouse infection studies. For instance, Smythies et al. (2000) have shown that, in C57BL/6 mice, *H. pylori*-elicited inflammation is regulated by an increase of IFN-γ expression and a downregulation of IL-4 expression, which is a distinct Th2 cytokine. In this same study, gene-deficient IL-$4^{-/-}$ animals were shown to have more severe gastritis compared to wild-type mice. A *H. pylori* virulence factor involved in this polarization again is HP-NAP, which has been shown to strongly induce a shift from a Th2 to a Th1 response (Amedei et al., 2006).

More recently, IL-17, a Th17 cytokine, has been shown to be involved in neutrophil infiltration during *H. pylori* infection and different IL-17 expression patterns have been described in different mouse strains (Scott Algood et al., 2007; Shiomi et al., 2008). Mice infected with *H. pylori* have been shown to develop a mixed Th17/Th1 response, with the Th17/IL-17 pathway preceding and modulating the Th1 response (Shi et al., 2010). In IL-$17^{-/-}$ mice, a decreased *H. pylori* colonization and inflammation is observed. In another study, *H. pylori*-infected mice lacking the IL-17A receptor were shown to contain fewer neutrophils in their gastric mucosa, compared to wild-type mice. Interestingly, this was accompanied by an increased presence of gastric B cells and lymphoid follicles, highlighting an important role of IL-17 signaling in chronic *H. pylori*-induced inflammation (Scott Algood et al., 2009).

Regulatory T cells (Tregs) are mainly $CD4^+$ T cells expressing high levels of CD25 (IL-2Rα) and actively controlling or suppressing the function of other cells (Robinson and Atherton, 2010). The most important cytokine secreted by these Tregs is considered IL-10. Infection of IL-$10^{-/-}$ mice, lacking IL-10 expression, with *H. pylori* or *H. felis* elicits a more severe chronic gastritis compared to that seen in wild-type mice (Ismail et al., 2003), which can lead to spontaneous eradication of helicobacters in these animals (Blanchard et al., 2004; Matsumoto et al., 2005). These results are supported by others. For instance, studying the role of mucosal dendritic cells, which are more abundant during *H. pylori* infection, show that these cells induce a Treg-biased response suppressing the induction of a Th17 response. This could in part be responsible for the chronicity of the infection (Kao et al., 2010). Seen from a

clinical perspective, Robinson et al. (2008) have suggested that low numbers of Tregs result in an increased Th1 response, leading to more severe gastritis and the development of gastric ulcers. In addition to regulatory T cells, other mechanisms are involved in a decreased immune response towards a *H. pylori* infection. The bacterium has been shown to cause apoptosis of macrophages, possibly resulting in a decreased immune response (Gobert et al., 2002; Menaker et al., 2004) and certain *H. pylori* virulence factors, including VacA and *H. pylori* GGT, have been shown to impair the host immune response by inhibiting the activation and proliferation of T cells (Gebert et al., 2003; Schmees et al., 2007).

In infected hosts, *H. pylori* stimulates the production of antibodies, as shown by the presence of anti-*H. pylori* IgG and IgM in serum and IgG and IgA in gastric contents, respectively (Ferrero et al., 1998). As discussed in section 6, specific antibodies are not required for protection against *H. pylori* infection, which is confirmed by several studies on the immune response elicited by *H. pylori* infection. In B cell-deficient mice, early *H. pylori* colonization has been shown to follow the normal pattern, described in wild-type mice (Akhiani et al., 2004). This, however, finally resulted in the development of a severe gastritis and clearance of the bacteria, which lead the authors to conclude that specific anti-*H. pylori* antibodies can even facilitate bacterial colonization and counteract the host resistance against *H. pylori* infection.

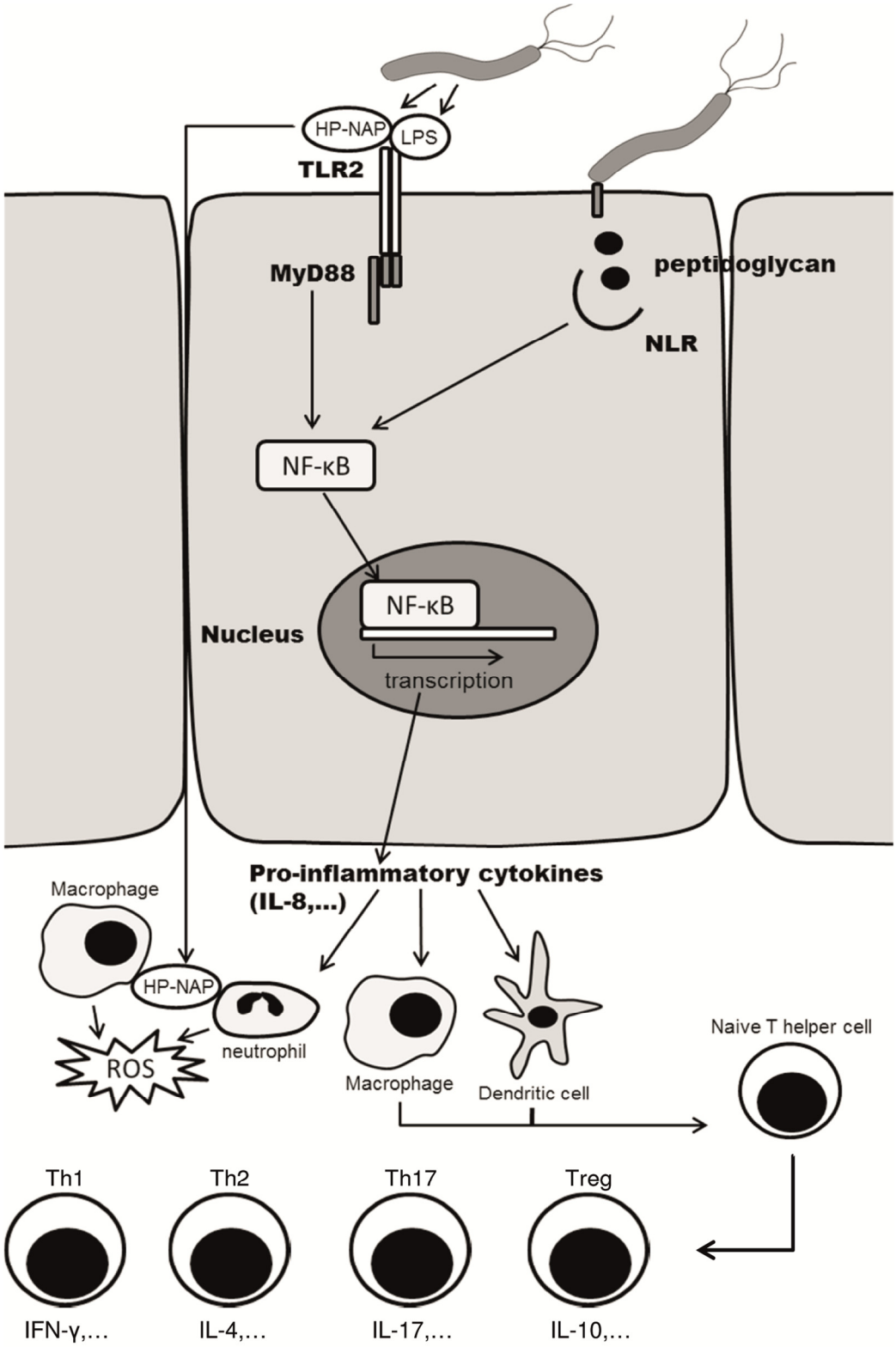

Figure 3: Summarized overview of the host response to *Helicobacter pylori* infection. After innate immune recognition of *Helicobacter pylori* pathogen-associated molecular patterns (PAMP) by pathogen-recognition molecules (PRM), a signalling cascade is initiated, leading to activation and translocation of various transcription factors. This in turn leads to production of pro-inflammatory cytokines, promoting the recruitment of innate immune cells, including neutrophils, macrophages and dendritic cells. Subsequent expression of cytokines by these cells leads to the recruitment of various $CD4^+$ T helper cells. A more detailed description can be found in the text of section 2.

3 Virulence factors of gastric helicobacters involved in gastric epithelial cell death

Gastric epithelial, mainly apoptotic, cell death is considered an important feature contributing to *H. pylori*-related gastric disease (Shirin and Moss, 1998; Blaser and Atherton, 2004). Most of the research concerning *Helicobacter* virulence factors contributing to gastric epithelial cell death has been done with *H. pylori* and is summarized in this section and Figure 4. Far less information is available about the virulence mechanisms of non-*H. pylori* helicobacters. Although some virulence factors of non-*H. pylori* helicobacters may indeed be similar to those described for *H. pylori,* there are also important differences.

Urease

For colonization of the host stomach, all gastric *Helicobacter* species require a family of genes involved in the production of urease (Andrutis et al., 1995; Eaton and Krakowka, 1994). This enzyme consists of two subunits, UreA and UreB. Urease is mainly localised in the cytoplasm but also becomes associated with the surface of the viable bacteria after autolysis of surrounding bacteria (Phadnis et al., 1996; Krishnamurthy et al., 1998; Marcus and Scott, 2001). *In vitro*, this autolysis occurs at the onset of the stationary culture phase (Fujita et al., 2005). Urease hydrolizes urea to ammonia and carbon dioxide and is an important mechanism of survival required to colonize the stomach. The production of ammonia, most likely occurring at or near the inner bacterial membrane, neutralizes the hydrochloric acid of the stomach, creating a neutral microenvironment around the bacterium (Hong et al., 2003).

The urease-mediated production of ammonia has been shown to reduce the viability of gastric epithelial cells (Smoot et al., 1990). Others have suggested that ammonia accelerates cytokine-induced apoptosis in gastric epithelial cells, while the urease or ammonia molecules alone are unable to induce apoptosis (Igarashi et al., 2001). Either process, however, results in damage of the mucosal barrier, thereby releasing nutrients for the bacterium and sustaining the inflammation process (Smoot et al., 1990).

VacA

Already more than 20 years ago, Leunk et al. (1988) found that broth culture supernatants of the majority of *H. pylori* isolates induced cytoplasmic vacuolation of epithelial cells *in vitro*. The factor involved was shown to be heat-labile and protease-sensitive, strongly suggesting a protein was responsible for the observed effects. A few years

later, a soluble protein was purified from *H. pylori* broth culture supernatant (Cover and Blaser, 1992). The coding gene, *vacA,* is present in nearly all *H. pylori* strains, although not all strains induce vacuolation of epithelial cells *in vitro* (Leunk et al., 1988; Cover et al., 1990; Cover et al., 1994). The *vacA* gene possesses several polymorphic sites, namely the signal (s) region, the midregion (m) and the intermediate region. Type s1/m1 strains have been shown to produce higher levels of cytotoxin activity *in vitro* compared to s1/m2 strains, whereas s2 genotypes are inactive in vacuolating cytotoxicity assays (Atherton et al., 1995). From a clinical perspective, *H. pylori* strains with vacA type s1/m1 have been shown to be associated with duodenal and gastric ulceration and gastric adenocarcinoma, whereas the intermediate region is even a more important marker for *H. pylori* strains associated with gastric adenocarcinoma (Rhead et al., 2007). Interestingly, recombinant strains with altered toxicity have been described to emerge during human infection (Aviles-Jimenez et al., 2004).

The *vacA* gene encodes a pro-toxin of approximately 140 kDa, although VacA proteins of this size have never been reported. During secretion, the amino-terminal signal sequence and a carboxy-terminal domain are cleaved to yield a mature toxin of around 88 kDa, which is secreted through an autotransporter mechanism (Cover and Blanke, 2005). This mature, secreted protein can undergo further limited cleavage into what are believed to be 2 subunits, called p33 and p55, although other names have been used quite frequently (Blaser and Atherton, 2004; Backert et al., 2010). Once secreted, VacA has been described to interact with various receptors on the eukaryotic plasma membrane, including Receptor Protein Tyrosine Phosphatases (RPTP) α and β (Yahiro et al., 1999; Yahiro et al., 2003; Fujikawa et al., 2003; Rieder et al., 2005a). Binding to the latter influences signalling rather than vacuolation (Fujikawa et al., 2003) and an increase of this specific interaction has been described after acidic treatment of VacA (Yahiro et al., 2003). Several authors have described an important interaction between VacA and membrane lipid rafts (Ricci et al., 2000; Schraw et al., 2000; Kuo et al., 2003), which are dynamic assemblies of cholesterol, sphingolipids and proteins, including constitutive glycosylphosphatidylinositol-anchored proteins (GPI-AP). The sensitivity of epithelial cells to VacA has been described to depend on the presence of one or more GPI-AP (Ricci et al., 2000), although Schraw et al. (2000) found that these were no prerequisite for internalization of VacA. More recently, it was shown that sphingomyelin is important for VacA binding to cells and its association with membrane lipid rafts (Gupta et al., 2008).

After binding to the plasma membrane, VacA oligomers form low-pH-triggered anion-selective channels in the plasma membrane (Czajkowsky et al., 1999; Szabo et al., 1999). The

toxin is internalized through an actin-dependent endocytic pathway (Ricci et al., 2000), which is, again, only possible after acidic activation of VacA (McClain et al., 2000). Once inside the host cell, VacA causes accumulation of large vacuoles, which are hybrid compartments presenting both late endosomal and lysosomal features (Molinari et al., 1997). Important driving forces in this process include v-ATPase, which is colocalized on the vacuolar membranes with the late endosomal marker Rab7, a GTPase (Papini et al., 1996; Papini et al., 1997. Most likely, the presence of VacA channels stimulates v-ATPase proton pump activity, causing acidification of the endosomal compartments with a subsequent increase of the osmotic pressure (Genisset et al., 2007).

Besides vacuolation, which is mainly observed *in vitro*, VacA has also been shown to be an important cause of epithelial cell apoptosis, which is often detected in *H. pylori*-infected humans (Kuck et al., 2001; Blaser and Atherton, 2004). Cover et al. (2003) have shown that mainly a type s1/m1 VacA toxin causes apoptosis in a dose-dependent manner. No apoptosis could be detected when cells were incubated with a chimaeric s2/m1 toxin or a VacA mutant protein. Early studies showed that purified VacA causes a decrease of the mitochondrial membrane potential and a subsequent dramatic decrease of intracellular levels of ATP, however both independent from vacuolation (Kimura et al., 1999). These mitochondrial changes cause an impairment of the glutathione metabolism, requiring ATP, most likely increasing the chance of developing oxidative stress-induced cell damage (Kimura et al., 2001). Additionally, a release of cytochrome *c* in the cytosol has been observed in VacA-treated cells (Galmiche et al., 2000). Interestingly, Yamasaki et al. (2006) showed that VacA reduced the membrane potential of isolated mitochondria, without the release of cytochrome *c*. In intact cells, however, VacA-induced cytochrome *c* release could indeed be observed and involved the activation of pro-apoptotic regulators, including Bax. Additionally, a specific inhibitor of v-ATPase, bafilomycin A1, inhibited VacA-induced vacuolation, but not VacA-induced apoptosis, confirming the theory of Kimura et al. (1999) that different mechanisms underlie both effects. Very recently, it was shown that the small VacA subunit acts as a small pore-forming toxin in the inner mitochondrial membrane (Domańska et al., 2010), but future investigations should clarify possible pathological consequences of this observation.

As to the presence of VacA in non-*Helicobacter pylori* helicobacters, Xiang *et al.* (1995) did not detect the gene nor the protein in *H. felis*. Very recently, the whole-genome sequence of two *H. suis* strains has been determined, revealing the absence of a *vacA* homolog in *H. suis* strain 5, but also the presence of a *vacA* paralog in *H. suis* strain 1. Sequence analysis, however, suggests that no functional toxin is produced (Vermoote et al., 2011).

Further research is needed to confirm or enfeeble this hypothesis. To the author's knowledge, no information is available on the presence or absence of VacA in other gastric non-*H. pylori Helicobacter* species.

Cag PAI

The most widely investigated virulence factor of *H. pylori* is the Cytotoxin-associated genes Pathogenicity Island or *cag* PAI, encoding a type IV secretion system, which has, until now not been reported to be present in any other *Helicobacter* species. It represents a DNA insertion element of approximately 40 kb, carrying about 30 genes (Akopyants et al., 1998; Backert et al., 2010). In *H. pylori* strains carrying a functional *cag*PAI, a syringe-like structure is formed, capable of penetrating gastric epithelial cells and delivering the CagA protein into the host cells (Odenbreit et al., 2000; Backert et al., 2008). Some other proteins encoded by the *cag*PAI are VirB proteins, including channel or core components, NTPases supplying energy for secretion, and the components forming the pilus, of which VirB2 (CagC) is the major component (Andrzejewska et al., 2006; Backert et al., 2008). Once delivered inside the eukaryotic cell, CagA becomes phosphorylated on tyrosine residues by Src, a tyrosine kinase (Stein et al., 2002). Another *cag*PAI-encoded protein, CagL interacts with integrins at the cell surface, in this way triggering CagA delivery in the target cell and activation of Src (Kwok et al, 2007). The CagA phosphorylation sites are so-called EPIYA motifs (Glu-Pro-Ile-Tyr-Ala), which are present in variable numbers at the carboxy-terminal half of CagA (Backert et al., 2001; Stein et al., 2002). Phosphorylated CagA is recruited to the plasma membrane where it forms a complex with Src homology 2 (SH2) domains of the protein tyrosine phosphatase SHP-2 (Higashi et al., 2002a; Rieder et al., 2005a). Interestingly, differences in CagA phosphorylation and subsequent interaction with SHP-2 have an influence on the biological activity of CagA (Higashi et al., 2002b). These diversities are caused by variations in both the number and sequence of phosphorylation sites. The CagA-specific sequence from East Asian *H. pylori* strains virtually always induces a stronger binding to SHP-2, resulting in an increased ability of causing the hummingbird phenotype (see below) *in vitro*. In CagA proteins from Western *H. pylori* strains, SHP-2 binding is determined by the number of EPIYA motifs (Higashi et al., 2002b; Rieder et al., 2005a). The importance of this finding is confirmed by the observation that *H. pylori* strains that deliver CagA with more phosphorylation motifs are most often associated with gastric cancer (Azuma et al., 2002; Argent et al., 2004).

A variety of effects have been attributed to the presence of a functional *cag* PAI in *H. pylori*. Some of these effects depend on the phosphorylation of CagA. The elongation and scattering of *H. pylori*-infected host cells, also known as the 'hummingbird' phenotype, is probably the best known (Segal et al., 1999). The interaction between phosphorylated CagA and SHP-2 induces cytoskeletal rearrangements, in part through activation of the Rap1->B-Raf->Erk signalling pathway (Higashi et al., 2004). Eventually, deregulation of SHP-2, caused by CagA-SHP-2 complex formation results in apoptosis of gastric epithelial cells *in vitro* (Tsutsumi et al., 2003). Besides interaction with SHP-2, phosphorylated CagA is known to also interact with other host cell proteins, including the tyrosine kinase Csk. This interaction downregulates CagA-SHP-2 signaling, underlining the complexity of cellular signalling after CagA translocation (Tsutsumi et al., 2003).

Other effects are dependent on the translocation of CagA, but not phosphorylation. Examples are the activation of the pro-inflammatory transcription factor NF-κB through the Ras->Raf->Mek->Erk pathway (Brandt et al., 2005) and the CagA-mediated upregulation of the expression of the anti-apoptotic myeloid cell leukemia sequence 1 (MCL1) protein (Mimuro et al., 2007) *in vivo,* which contrasts with the CagA-induced increase of apoptosis described by Tsutsumi et al. (2003) *in vitro*. Finally, effects can also depend on the type IV secretion system encoded by the genes in the *cag* PAI, however without the need for injection of CagA in the host cell. Viala et al. (2004) described that peptidoglycan delivery through the same pilus is responsible for Nod1 activation. This results in activation of NF-κB and the upregulated translation of the pro-inflammatory cytokine interleukin-8.

Le'Negrate et al. (2001) showed that intestinal epithelial cell apoptosis *in vitro* was mainly dependent on the presence of a *cag* PAI, rather than VacA. Peek et al. (1999), however, showed that the enhanced apoptosis induced in AGS cells by CagA-positive strains, was in part dependent of the simultaneous action of VacA. The results of yet another study showed that *H. pylori* exerts pro-apoptotic effects, which are apparently more dependent on a functional *cag* PAI than on an intact vacuolating cytotoxin. Simultaneously, however, *H. pylori*-mediated anti-apoptotic effects were described to be mediated by activation of NF-κB (Maeda et al., 2002). Also for *H. pylori*-infected humans, conflicting reports have been made on the effect of CagA on apoptosis. Overexpression of both pro-apoptotic and anti-apoptotic proteins has been detected in gastric biopsies of *H. pylori*-infected humans, with the higher apoptosis rates associated with CagA-positive strains (Cabral et al., 2006). Peek et al. (1997), however, found that the number of apoptotic cells is lower in the gastric epithelium of humans infected with a CagA-positive strain compared to individuals infected with a CagA-negative

strain. Additionally, these CagA-positive strains were associated with an increase of epithelial cell proliferation. In contrast, Moss et al. (2001) found an increased gastric epithelial cell proliferation in *H. pylori*-infected humans, unrelated to the CagA status but associated with the presence of gastritis. In this study, increased epithelial cell apoptosis was only observed in humans infected with a CagA-positive *H. pylori* strain.

The available literature on the *cag* PAI and the vacuolating cytotoxin reveal very complex mechanisms used by *H. pylori* to control both cell death and proliferation. These two genes are thought to downregulate each other's effects on epithelial cells, raising the possibility of avoiding excessive cellular damage (Argent et al., 2008). Recently, this was confirmed by showing that CagA inhibits VacA-induced apoptosis by two complementary mechanisms, one dependent on the prevention of pinocytosis of VacA by tyrosine-phosphorylated CagA and one dependent on blocking of VacA-induced apoptosis at the mitochondrial level, which requires no phosphorylation of CagA (Oldani et al., 2009).

GGT

By focusing on the genetic and molecular characterization of genes that are known to be expressed by all *H. pylori* clinical isolates and only a restricted number of bacteria belonging to other genera, Chevalier et al. (1999) identified a catalytically active enzyme, γ-glutamyl transpeptidase (GGT), as essential for colonization of mice. In contrast, the results of another study suggest that this enzyme is not essential for colonization of both mice and piglets, although the presence of this enzyme provides a clear advantage for *H. pylori* colonization (McGovern et al., 2001). A *H. pylori* membrane preparation fraction has been shown to induce apoptosis of a gastric adenocarcinoma cell line (AGS) (Shibayama et al., 2003). This fraction contains the *H. pylori* GGT, inducing apoptosis in a dose-dependent manner. Additionally, the apoptosis-inducing capacity is partly inhibited when cells are treated with a *H. pylori* GGT mutant, lacking enzymatic GGT activity. The authors hypothesize that the bacteria could gain essential nutrients from the apoptotic cells, which was confirmed by showing that this enzyme very efficiently hydrolyzes both glutamine and glutathione, causing a depletion of both substrates (Shibayama et al., 2007). Kim et al. (2007) confirmed that *H. pylori* GGT causes apoptosis of AGS cells. The analysis of various factors revealed cytochrome *c* release by mitochondria, activation of caspase-3 and -9, upregulation of Bax and downregulation of Bcl-2 and Bcl-Xl, suggesting apoptosis mediated through mitochondrial damage. More recent work of these same authors with recombinant *H. pylori* GGT showed that the enzyme inhibits growth of AGS cells by inducing cell cycle arrest in the

G1-S transition phase, associated with the downregulation of several members of the cyclin and cyclin-dependent kinase families (Kim et al., 2010). In addition to its role in the induction of cell death, *H. pylori* GGT has also been shown to inhibit T-cell proliferation (Schmees et al., 2007). This effect depends on the catalytic activity of the enzyme, disrupting Ras-dependent signalling and finally inducing G1 cell cycle arrest. Apoptosis of T cells was not observed. Very recently, *H. pylori* GGT was shown to be an important factor in the development of peptic ulcer disease. In this study, NF-κB activation was shown to upregulate the expression of pro-inflammatory IL-8 (Gong et al., 2010).

Interestingly, GGT activity is detected in all gastric non-*H. pylori Helicobacter* species (Haesebrouck et al., 2009). As part of this thesis, the role of *H. suis* GGT in the induction of gastric epithelial cell death was thorougly investigated.

LPS

Experimental application of *H. pylori* lipopolysaccharide (LPS) to the gastric surface epithelium of rats causes a marked increase of epithelial cell apoptosis (Piotrowski et al., 1997). This was confirmed in primary guinea pig gastric mucosal cells by Kawahara et al. (2001). However, the fact that *H. pylori* LPS induces apoptosis only at much higher concentrations compared to *E. coli* LPS, suggests that it has a lower toxicity compared to the latter (Durkin et al., 2006). Interestingly, this study showed that *H. pylori* LPS and ibuprofen, a NSAID, interact to enhance gastric cell loss.

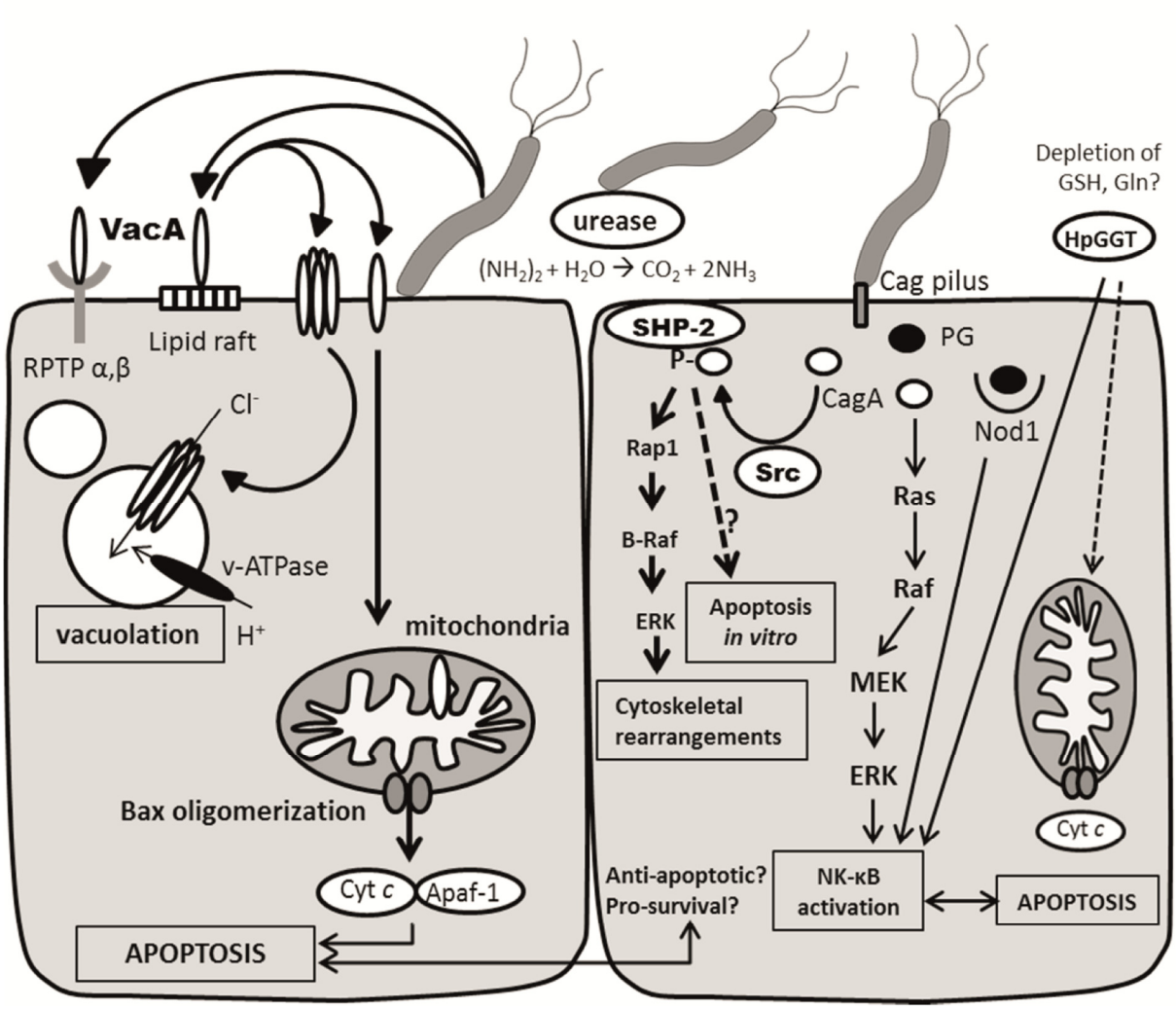

Figure 4: Summarized overview of important *Helicobacter pylori* virulence factors involved in the promotion or inhibition of gastric epithelial cell death. The **urease** enzyme leads to the production of ammonia (NH_3), which reduces the viability of gastric epithelial cells. The secreted vacuolating cytotoxin, **VacA**, interacts with various plasma membrane receptors and is internalized. VacA oligomerization is involved in the formation of large cytoplasmic vacuoles. Besides vacuolation, VacA is an important cause of epithelial cell apoptosis. The ***cag* PAI**, a type IV secretion system, injects CagA and peptidoglycan (PG) into epithelial cells. After injection into the host cell, CagA can become phosphorylated (P-o) on tyrosine residues. Phosphorylated and unphosphorylated CagA, as well as injected bacterial peptidoglycan, cause a variety of changes inside the host cell, including cytoskeletal rearrangements and activation of NF-κB. Finally, the *H. pylori* **GGT** enzyme is involved in the induction of mitochondria-mediated apoptosis. More detailed descriptions can be found in the text of section 3.

4 Oxidative stress and cell death in gastric *Helicobacter* infections

4.1 Oxidative stress, apoptosis and necrosis

It is well-known that reactive oxygen species (ROS) can cause molecular changes to lipids, proteins and DNA, finally leading to apoptosis or necrosis (Fiers et al., 1999; Duprez et al., 2009) (Figure 5). Typically, activation of caspases is observed during apoptosis, and these caspases (including effector caspases such as caspase-3) are activated mainly through the intrinsic or extrinsic apoptotic pathway. Briefly, the intrinsic pathway is activated through different stimuli, including DNA damage, and acts through the mitochondria. The apoptotic stimulus promotes Bax/Bak oligomerization which leads to the formation of a channel in the outer mitochondrial membrane, through which cytochrome *c* is released into the cytosol. The subsequent association between cyt *c* and Apaf-1 leads to activation of the caspase cascade (Riedl and Salvesen, 2007; Duprez et al., 2009; Circu and Aw, 2010). The extrinsic pathway involves binding of death ligands such as, but not limited to, FasL and TNF-α, to their receptors on the cell surface, including Fas and TNFR-1 (TNF receptor 1). This can lead to recruitment of specific death domains, including FADD (Fas-associated death domain) and TRADD (TNFR associated death domain), the subsequent formation of a DISC (death-inducing signalling complex) and activation of the caspase cascade through the initiator caspase-8 (Peter and Krammer, 2003; Duprez et al., 2009; Circu and Aw, 2010). ROS, for instance, can induce apoptosis through different pathways, including ROS-induced ASK1/JNK signalling, either leading to upregulated expression of proapoptotic factors such as TNFα and FasL or to mitochondrial signalling resulting in mitochondrial cytochrome *c* release (Circu and Aw, 2010).

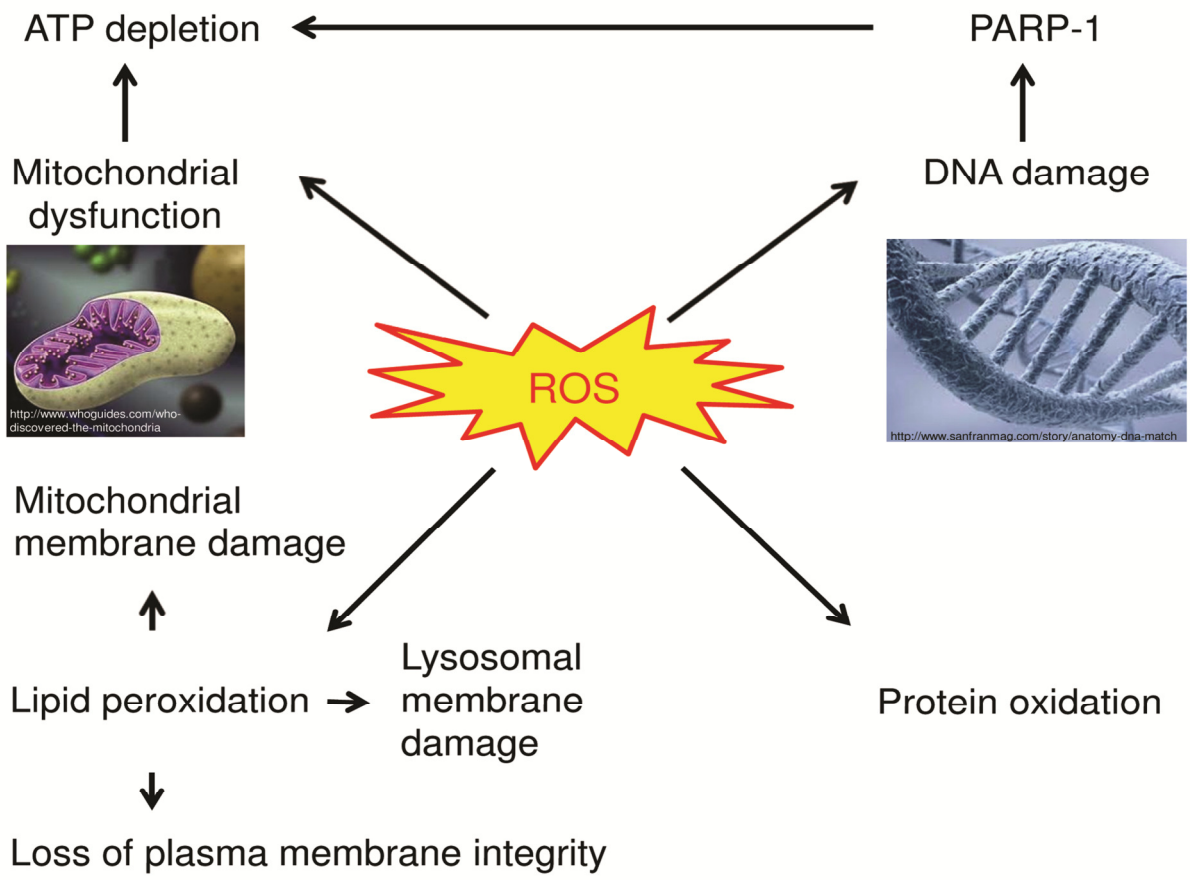

Figure 5: ROS can induce damage to lipids, proteins and DNA. Upon DNA damage, poly(ADP-ribose) polymerase 1 is hyperactivated, leading to ATP depletion. ROS-induced lipid peroxidation can lead to membrane damage of various organelles and loss of plasma membrane integrity (Adapted from Vanlangenakker et al., 2008).

As opposed to apoptosis, primary necrosis or oncosis is characterized by cytoplasmic and organelle swelling and loss of plasma membrane integrity (Duprez et al., 2009; Kroemer et al., 2009). Irrespective of the stimulus, ROS are often involved in the induction of necrosis. They can cause oxidative DNA damage, triggering the overactivation of PARP-1 (Figure 3), a poly(ADP-ribose) polymerase involved in DNA repair, which can lead to depletion of cytosolic ATP (Los et al., 2002; Vandenabeele et al., 2010). ROS can also cause lipid peroxidation, both of organelle membranes and the plasma mebrane, which can result in loss of plasma membrane integrity (Vanlangenakker et al., 2008). Lipid peroxidation of mitochondrial membranes impairs oxidative phosphorylation, maintenance of the mitochondrial membrane potential and the mitochondrial Ca^{++} buffering capacity (Orrenius et al., 2007). Lyosomal membranes are also a possible target, resulting in the leakage of protease or efflux of Ca^{++}. Interestingly, large amounts of redox-active iron are present in lysosomes, enabling the possible auto-amplification of ROS through the Fenton reaction, generating the highly reactive hydroxyl radical: $\mathbf{H_2O_2 + Fe^{2+} \rightarrow OH^- + \underline{HO\cdot} + Fe^{3+}}$

Proof of the possible threat of redox-active iron is also provided by the finding that TNF-α-induced ROS formation is enhanced by JNK1-dependent ferritin degradation (Antosiewicz et al. 2007). The degradation of this iron-binding protein causes an increase of the labile iron pool (Vandenabeele et al., 2010).

4.2 *H.* pylori-induced oxidative stress

Only very little is known about a possible relation between non-*H. pylori Helicobacter* infection and oxidative stress, although it has been shown that a chronic *H. felis* infection causes oxidative damage through the recruitment of neutrophils (Kawasaki et al., 2005). Impairment of IKK*β*/NF-κB signaling also results in an increased level of oxidative stress and subsequent apoptosis or necrosis in gastric epithelial cells of mice chronically infected with *H. felis* (Shibata et al., 2010).

In contrast to the limited data available for non-*H. pylori* helicobacters, gastric infection with *H. pylori* has regularly been shown to cause an increase of the oxidative stress burden. Increased levels of lipid peroxidation have been observed in *H. pylori*-positive patients (Farinati et al., 1996). Also, higher levels of oxidized and nitrated proteins have been described in these patients, indicating cells are exposed to higher levels of oxidative and nitrative stress. These alterations at the protein level were clearly associated with the presence of chronic gastritis and duodenal ulcers (Li et al., 2001). At the DNA level, the formation of 8-hydroxydeoxyguanosine (8-OHdG) is considered the main change induced by reactive oxygen species. Significantly higher concentrations of 8-OHdG have been detected in the mucosa of humans suffering from chronic atrophic gastritis and intestinal metaplasia. But the most important factor involved in the induction of DNA oxidative damage in the gastric mucosa is mere infection with *H. pylori* (Farinati et al., 1998; Papa et al., 2002). Interestingly, the increased local levels of ROS are also reflected in the peripheral blood of *H. pylori*-infected humans (Mashimo et al., 2006).

Various factors contribute to these detrimental changes. First, *H. pylori* bacteria generate substantial amounts of superoxide radicals themselves, an effect which is negligible in, for instance, *E. coli* (Nagata et al., 1998; Ding et al., 2007). Additionally, several reports have been made about the *H. pylori*-induced production of reactive oxygen species (ROS) in epithelial cells. Compared to non-infected humans, gastric mucosa from *H. pylori*-infected humans shows an altered expression of proteins that have been reported to be affected by oxidative stress, including members of the aldehyde dehydrogenase family (Baek et al. 2004).

In vitro, *H. pylori* causes an increase of spermine oxidase expression and activity, resulting in the generation of H_2O_2 (hydrogen peroxide) and subsequent oxidative DNA damage (Xu et al., 2004). Finally, development of gastritis is often seen in *H. pylori*-infected humans (Kusters et al., 2006). Among other inflammatory cells, neutrophils are recruited to the site of inflammation, generating reactive oxygen and nitrogen species (Yoshikawa and Naito, 2000). *H. pylori* has been shown to be capable of triggering the oxidative burst of both monocytes and neutrophils *in vitro* (Nielsen and Andersen, 1992). This was confirmed in human gastric biopsies, showing higher levels of ROS released by neutrophils when patients were infected with *H. pylori* (Jung et al., 2001). Eradication of *H. pylori* has been shown to cause a decrease in the presence of inducible nitric oxide synthase (iNOS), resulting in lower levels of nitric oxide (NO) production (Felley et al., 2002). NO can combine with superoxide to form peroxynitrite, capable of inducing host cell damage.

Although conflicting results have been published, several virulence factors have been shown to be important for the increased generation of ROS during *H. pylori* infection. The *H. pylori* neutrophil activating protein (HP-NAP) is important for the development of gastritis. Interestingly, a homologue of the coding gene is found in the *H. suis* genome (Vermoote et al., 2011). HP-NAP promotes the adherence of neutrophils to endothelium, transendothelial migration of neutrophils, chemotaxis of leukocytes and the release of ROS by neutrophils and monocytes (Evans et al., 1995; Satin et al., 2000 ; Montecucco and de Bernard, 2003; Brisslert et al. 2005; Polenghi et al., 2007). Unemo et al. (2005), however, found that not HP-NAP but instead the sialic acid-binding adhesin (SabA) is important for the induction of the oxidative burst in neutrophils. In gastric epithelial cells *in vitro*, *H. pylori* strains with a functional *cag* PAI have been shown to induce higher levels of intracellular ROS compared to isogenic *cag* PAI-deficient mutant strains (Ding et al., 2007). Also in *H. pylori*-infected humans, CagA-positive strains have been associated with the highest production of ROS and subsequent oxidative DNA damage (Papa et al., 2002), although Farinati et al. (2008) did not find a relation between the CagA status and the increase of oxidative DNA damage in patients suffering from gastric cancer. Two other *H. pylori* virulence factors, GGT and VacA, have been described to cause a higher production of ROS by gastric epithelial cells (Bagchi et al., 2002; Gong et al., 2010). For both proteins, an impaired metabolism of glutathione, an important free thiol involved in protection against oxidative stress, has been suggested to underlie these changes (Kimura et al., 2001; Gong et al. 2010).

As a consequence of the increased oxidative stress burden in humans infected with *H. pylori*, the bacteria are also confronted with these hostile agents. *H. pylori* combats this

oxidative stress using a number of enzymes, such as superoxide dismutase, catalase and a family of peroxiredoxins, including alkylhydroperoxide reductase and a thiol-reductase (Wang et al., 2006). Interestingly, the oxidative stress-inducing HP-NAP has been shown to protect *H. pylori* bacteria from oxidative stress, through ferric uptake regulator (*fur*)-involved regulation of *napA* expression and intracellular colocalization of HP-NAP with DNA, protecting it from damaging by free radicals (Cooksley et al., 2003).

4.3 The protective role of glutathione against oxidative stress

The tripeptide reduced glutathione (L-γ-glutamyl-L-cysteinylglycine) is by far the most abundant free thiol in eukaryotic cells (Meister and Anderson, 1983). It has an important role in metabolisation and detoxification of exogenous compounds (e.g. drugs) involving the activity of various Glutathione S-transferases (GST's), conjugation reactions with endogenous metabolites and radiation protection (Meister and Anderson, 1983; Sau et al., 2010). Above all, it is an important antioxidant which degrades reactive oxygen species (ROS), including O_2–derived free radicals, as well as O_2–derived nonradical species such as hydrogen peroxide (H_2O_2) (Circu and Aw, 2010). The antioxidant activity involves the conversion of reduced glutathione (GSH) to its oxidized form, glutathione disulfide (GSSG) (Meister and Anderson, 1983; Pompella et al., 2003). This can take place through numerous reactions, including both nonenzymatic transhydrogenation, often mediated by metal ions, and enzymatic pathways, involving the activity of selenium-containing GSH peroxidases and transhydrogenases (Meister and Anderson, 1983; Valencia et al., 2001; Pompella et al. 2003). In turn, GSSG can be reduced to GSH via the NADPH-dependent GSSG reductase (Meister and Anderson, 1983; Pompella et al., 2003).

The vast majority of glutathione can be found intracellularly, compartmentalized as distinct redox pools in cytosol, mitochondria, endoplasmatic reticulum and nucleus, with concentrations in the mM range (Meister and Anderson, 1983; Body et al., 1979; Circu and Aw, 2010). The set of reactions involved in GSH synthesis is often referred to as the γ-glutamyl cycle (Meister and Anderson, 1983). Briefly, intracellular synthesis involves the activity of different ATP-dependent enzymes such as γ-glutamylcysteine synthetase and GSH synthetase (Orlowski and Meister, 1970; Meister and Anderson, 1983). However, extracellular GSH is also found in tissues and plasma in the micromolar range (20-30 μM; Anderson and Meister, 1980; Yang et al., 1997; Inoue, 2001). Indeed, during GSH metabolism a significant amount is translocated outside the cells, often mediated by GSH

transporters (Griffith and Meister, 1979a; Meister et al., 1979; Griffith and Meister, 1979b; Dethmers and Meister, 1981; Lu et al., 1996; Shibayama et al., 2007). Outside the cell, it can serve as a substrate for membrane-bound γ-glutamyl transpeptidase (GGT), which catalyzes transfer of the γ-glutamyl moiety to various acceptors, including amino acids, dipeptides and GSH (Meister and Anderson, 1983; Del Bello et al., 1999; Pompella et al., 2003). After further cleavage by dipeptidases the constituent amino acids (or dipeptides) are taken up by the cells, where they can be utilized in *de novo* glutathione synthesis (Griffith and Meister, 1979b; Meister and Anderson, 1983; Zhang et al., 2005). Interestingly, plasma membrane-bound GGT of mammalian cells has been shown to initiate pro-oxidant reactions through the catabolism of GSH (Dominici et al., 1999; Maellaro et al., 2000). Transpeptidation of the γ-glutamyl group generates the more reactive thiol cysteinyl-glycine, leading to the production of H_2O_2 through the reduction of Fe^{3+} and subsequent production of thiyl radicals and the superoxide anion (O_2•—) (Dominici et al., 1999).

Cells depleted of reduced glutathione are more sensitive to oxidative stress (Ueda et al., 1998). However, various studies show that the ratio between GSH and GSSG is a better indicator for determining the redox state of a cell (Schafer et al., 2001). Despite elevated levels of GSH *per se*, low cellular ratios of GSH/GSSG have been linked with decreased cell survival (Pias et al., 2003). *In vitro* GSSG treatment of cells results in increased production of ROS leading to a dose-dependent induction of cell death through the p38 MAP kinase pathway (Filomeni et al., 2003). This can be counteracted by treatment of cells with GSH ethyl ester, which is known to increase the intracellular GSH content, underlining the importance of the GSH/GSSG balance. Similarly, depletion of GSH through impaired *de novo* synthesis results in an overproduction of ROS, leading to apoptosis through the activation of protein kinase c (PKC)-delta (Marengo et al., 2008). This can, however, be prevented by supplementation with other antioxidants, showing that the observed effect depends on the formation of ROS. In contrast, GSH depletion through transporter-mediated efflux has been shown to induce apoptosis independent of ROS formation (Franco et al., 2007). During oxidative stress, an optimal mitochondrial GSH/GSSG ratio has been shown to be crucial for further cell survival (Circu and Aw, 2010). Indeed, a decrease of mitochondrial GSH has been shown to result in enhanced production of mitochondrial ROS, loss of mitochondrial membrane potential and release of cytochrome *c*, which is known to initiate apoptosis (Lluis et al., 2007).

In the stomach, intracellular glutathione concentrations are high (up to 10 mM), compared to most other tissues (Body et al., 1979; Meister and Anderson, 1983; Mårtensson

et al., 1990). Farinati et al. (1996) have described an increase of both the reduced and oxidized glutathione content of gastric mucosa of *H. pylori*-infected humans suffering from chronic nonatrophic gastritis. Similar results have been described for total (sum of reduced and oxidized) glutathione contents in the stomach of *H. pylori*-infected Mongolian gerbils (Suzuki et al., 1999). However, these findings are in contrast with the results from other studies, describing lower levels of reduced glutathione in *H. pylori*-infected human patients compared to *H. pylori*-negative individuals (Jung et al., 2001; Shirin et al., 2001).

5 *In vivo* modelling of non-*H. pylori Helicobacter*-related gastric disease in rodents

Most of the current knowledge on the complex interplay between gastric helicobacters and their hosts derives from experimental infection studies in small rodents, mainly because of the small size, low price and easy manipulation of these animals. This overview will mainly focus on popular *in vivo* models of non-*H. pylori Helicobacter* associated gastric disease.

Mouse

The vast majority of experimental *in vivo* research on the pathogenesis of *H. pylori* has been conducted in mice. In the early days, *in vivo H. pylori* research in mice was often hampered by a poor and inconsistent colonization ability of the *H. pylori* strains used. After long-term adaptation in mice, Lee et al. (1997) introduced the Sydney strain (SS1), which showed an excellent chronic colonization ability and became one of the standard strains, although it lacks a functional *cag*PAI (Crabtree et al., 2002). Both in C57BL/6 and BALB/c mice, this strain elicited an active chronic gastritis, evolving to severe atrophy. In the years to follow, various severe gastric pathologies associated with a *H. pylori* infection in humans were reproduced in experimentally infected mice. These included MALT lymphoma, which also developed with CagA- and VacA-negative strains (Wang et al., 2003), and adenocarcinoma following an increased mucosal expression of IL-1β, although these lesions were only generated in hypergastrinemic INS-GAS mice (Fox et al., 2003).

In the meantime, however, *H. felis* became more popular to model *H. pylori*-induced gastric pathologies *in vivo*, mainly because of its high and reproducible colonization ability in mice. *H. felis* infection was shown to cause gastritis in different mouse strains, including BALB/c and C57BL/6 mice, which are considered to be mainly Th2 and Th1 responders, respectively. A moderate to severe chronic inflammation could be observed in the

corpus/fundus region of C57BL/6 mice, along with increased numbers of neutrophils and the development of other lesions, including mucosal erosions, mucous cell hyperplasia and atrophy (Mohammadi et al., 1996; Sakagami et al., 1996). In contrast, no corpus atrophy and a milder inflammation in fundus or antrum were seen in BALB/c mice. In a comparative *H. felis*/*H. pylori* infection study in C57BL/6 mice, *H. felis* was shown to induce more severe inflammation, both in fundus and antrum, compared to *H. pylori* strain SS1 and only *H. felis* infection was associated with an increased epithelial cell proliferation in the fundus (Court et al., 2002). Interestingly, the onset of chronic inflammation and loss of parietal and chief cells was shown to be earlier in female mice, compared to males, further underlining the importance of host factors in the development of gastric pathologies (Court et al., 2003).

Besides severe inflammation, more severe pathologies have been observed in *H. felis*-infected mice. Long-term infection of BALB/c mice with this bacterium has been shown to induce lesions identical to those seen in patients suffering from gastric MALT lymphoma, including poor definition of B-cell germinal centers in lymphoid aggregates and lympho-epithelial lesions (Enno et al., 1995). In contrast, long-term *H. felis* infection of wild-type C57BL/6 mice has been shown to lead to the development of chronic gastritis, atrophy, metaplasia, dysplasia and finally adenocarcinoma, which is similar to the sequence of pathologies leading to the formation of gastric cancer in humans (Cai et al., 2005). Interestingly, bacterial eradication in mice with severe lesions significantly prevented gastric cancer-related deaths in these animals. Takaishi et al. (2009) have revealed an important role for gastrin in *H. felis*-induced gastric corpus carcinogenesis in C57BL/6 mice, whereas this same hormone seems to inhibit the development of similar lesions in the antrum. Finally, in outbred Swiss mice, which are expected to show a more balanced Th response, both lymphoid tissue and glandular lesions have been shown to develop in response to a *H. felis* infection (Ferrero et al., 2000).

Besides their extensive use in research on *H. felis*-induced gastric pathology, mouse models have also been used with other non-*H. pylori Helicobacter* species. Until now, no successful mouse colonization by *H. salomonis* has been described, whereas *H. bizzozeronii* induces less striking pathological changes in mice, when compared to *H. felis* (De Bock et al., 2005). In literature, several studies describe experimental infection of mice with *"H. heilmannii*" or "tightly coiled spiral bacteria". Often, however, this name is misleading, as several of these studies probably have in fact used *H. suis* bacteria, obtained from mucus or homogenized gastric tissue of infected mice, pigs or non-human primates, because no *in vitro* isolate was available. Infection of mice with these inocula induced inflammation, already 7

days after inoculation (Moura et al., 1993). During long-term infection studies of up to 2 years, infiltration of gastric mucosa with lymphocytes and plasma cells with subsequent development of lymphoid follicles has been observed (Park et al., 2003; Park et al., 2008). Cinque et al. (2006) have described this inflammation to be mainly driven by a Th1 response, in C57BL/6 and even in BALB/c mice, although others have described a mixed Th1/Th2 response in C57BL/6 mice (Park et al., 2008). In BALB/c mice infected experimentally with different *"H. heilmannii"* bacteria, "isolated" *in vivo* in mice and originating both from humans and animals, gastric MALT lymphoma has been shown to develop starting from 18 months post infection (O'Rourke et al., 2004a). In this study, the most severe pathological changes were seen in mice infected with *in vivo* "isolates" from humans and different non-human primates and in fact belonging to 2 different species, *H. suis* and "*Candidatus* H. heilmannii". Similar lesions were observed in C57BL/6 mice infected for at least 6 months with an *in vivo* "isolate" that was erroneously designated "*Candidatus* H. heilmannii" (Nakamura et al., 2007), but that in fact belonged to the species *H. suis* (Baele et al., 2009) and the development of MALT lymphoma-like lesions was accompanied by an increased detection of several vascular endothelial growth factors (Nishikawa et al., 2007). In contrast to what has been described for *H. pylori*, the development of gastritis, caused by this same "isolate" has been shown not to depend upon the presence of Peyer's patches in the small intestine (Nagai et al., 2007; Nobutani et al., 2010). In conclusion, until before the start of the present PhD research, no experimental studies in rodent models have been carried out with pure cultures of *H. suis*, due to the lack of an *in vitro* isolation protocol.

Mongolian gerbil

The first experimental evidence that *H. pylori* alone can result in the development of gastric adenocarcinoma was provided by a long-term experimental infection study for up to 62 weeks in Mongolian gerbils (Watanabe et al., 1998). Moreover, chronic *H. pylori* infection in this animal model brings about other severe lesions, including intestinal metaplasia and development of gastric ulcers (Watanabe et al., 1998; Ikeno et al., 1999). The rapid development of these lesions in this animal model urged other research groups to start using the Mongolian gerbil model for research on *H. pylori*-induced gastric pathology.

When gerbils are infected experimentally with *H. pylori* for 4 weeks, inflammation can mainly be observed in the antrum. This gastritis, however, has been shown to extend to the corpus in the majority of animals infected for over 30 weeks (Crabtree et al., 2004) and this corpus inflammation shows a strong correlation with the presence of an intact *cag*PAI

(Rieder et al., 2005b). Interestingly, the cytokine response in female animals shows a strong Th1 profile whereas in male animals, a much more moderate Th1-polarized response was observed (Crabtree et al., 2004). In animals of both genders, no increased expression of Th1-downregulating cytokines TGF-β and IL-10 was found. As for the balance between cell death and proliferation, experimental *H. pylori* infection has been shown to induce apoptosis in the antrum of gerbils 2-4 weeks after challenge (Peek et al., 2000). This increased apoptotic ratio dropped back to baseline levels at later timepoints and was succeeded by increased epithelial cell proliferation, peaking at 16-20 weeks post inoculation.

Only few studies describe experimental infection of gerbils with non-*H. pylori Helicobacter* species. Court et al. (2002) have shown that *H. felis* infection for 4 weeks mainly induces antral gastritis and an increased epithelial cell proliferation in this same region. These bacteria have, however, also been shown to induce severe loss of parietal cells, starting from the limiting ridge at the forestomach/stomach transition zone and extending into the fundus (De Bock et al., 2006a and 2006b) (Figure 6). This was associated with increased apoptosis and proliferation at the transition zone between normal and affected tissue, occasional pyloric gland metaplasia and complete loss of the parietal cell population in some animals after 70 days of infection. Interestingly, *H. felis* could be found in close association with parietal cells, in contrast to *H. bizzozeronii*, which induced similar but less severe lesions compared to *H. felis* (De Bock et al., 2006b).

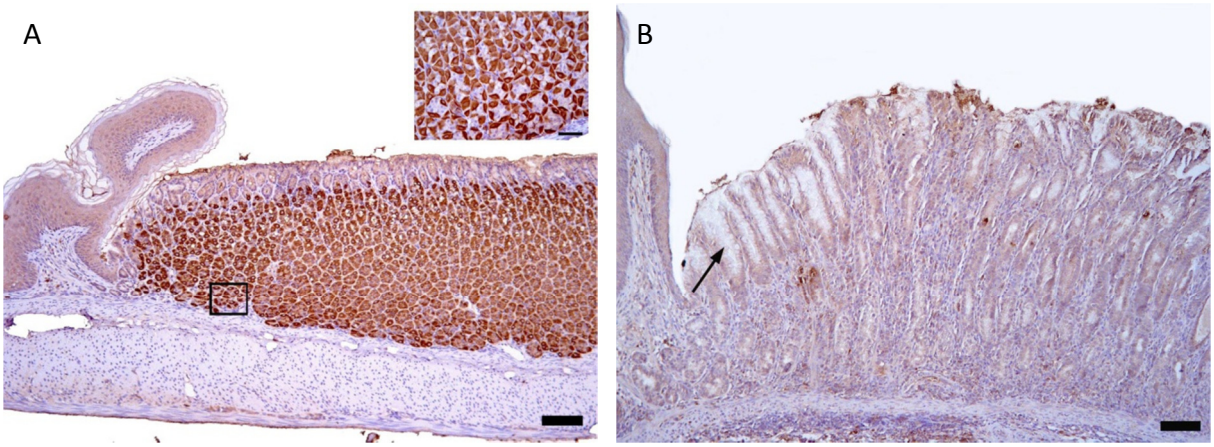

Figure 6: H^+-K^+-ATPase staining of (A) an uninfected control gerbil, indicating differential staining between purple chief cells and brown stained parietal cells (Bar insert = 50 µm). (B) Gerbil infected with *H. felis* for 3 weeks, showing severe parietal cell loss and "antralisation" (arrow). (De Bock et al., 2006a).

6 Immunization against gastric helicobacters

Gastric infection in humans with *H. pylori* or a non-*H. pylori Helicobacter* species is so widespread that eradication therapies with antimicrobial agents are limited to patients suffering from gastric disease. Similarly, the prevalence of *H. suis* in pigs is very high (Hellemans et al., 2007b), which makes eradication of this zoonotic agent with antimicrobials virtually impossible. Moreover, as for other bacteria, clinicians face an increasing problem of antimicrobial resistance in the *H. pylori* population (Malfertheiner et al., 2007). For these reasons, vaccination is considered a valuable approach for controlling gastric *Helicobacter* infections and disease (Blanchard and Nedrud, 2010). Similar to experimental *Helicobacter* infection studies, the mouse has by far been the most widely used animal model. The present overview will thus focus on vaccination studies in the mouse model, complemented with some information on vaccination studies in other animals and humans.

6.1 Candidate antigens for *Helicobacter* vaccination

To determine the efficacy of different *Helicobacter* vaccination strategies, the mouse model has been by far the most widely used (Nedrud, 2001). Especially in the nineties and mainly because a stable chronic *H. pylori* infection mouse model was not available before the mid-nineties (Nedrud, 2001), challenge of mice was often done with *H. felis,* both after immunization with *H. pylori* and *H. felis* antigens.

In the past, but also more recently, whole bacterial cell preparations have often been used for vaccination (Goto et al., 1999; Eisenberg et al., 2003; Garhart et al., 2003; Nyström et al., 2006; Sutton et al., 2007). In several of these studies and both after prophylactic and therapeutic immunization, a strong reduction of *Helicobacter* colonization was observed, sometimes even leading to complete protection. However, in these cases, culture and histology were used for assessment of protection, which might not be the most sensitive techniques. In addition to whole bacterial cell lysates, purified or recombinant proteins have also been used. Although the IgG antibody response to *H. pylori* urease is poor in naturally infected humans (Leal-Herrera et al., 1999), this enzyme or its subunits have often been used in mouse vaccination studies, both in prophylactic (Michetti et al., 1994; Pappo et al., 1995; Ermak et al., 1998; Morihara et al., 2007) and therapeutic (Corthésy-Theulaz et al., 1995; Dieterich et al., 1999; Guy et al., 1999) vaccination studies. For both strategies, a complete protection was suggested in some animals in certain studies, although culture, histology,

urease activity or a combination of these techniques were used for assessing the level of protection. Michetti et al. (1994) found that quantification of urease activity in the stomach was the most sensitive of these three techniques.

The important *H. pylori* virulence factors CagA and VacA have also been used as antigens for immunization (Ghiara et al., 1997), although VacA has been shown to inhibit T-cell activation and proliferation (Gebert et al., 2003; Rieder et al., 2005a) and CagA is not secreted by all *H. pylori* strains, which could make these proteins less suitable for vaccination (Blanchard and Nedrud, 2010). HP-NAP, which is a key modulator of *H. pylori*-induced gastritis has also been used for prophylactic vaccination purposes and has been shown to induce similar partial protection rates, compared to vaccination with CagA and *H. pylori* lysate (Satin et al., 2000). Other antigens used in *H. pylori* mouse vaccination studies include catalase (Radcliff, 1997), the blood group antigen-binding adhesin BabA (Hultberg et al., 2005) and the lipoprotein HpaA. The latter has been shown to confer protection against subsequent *H. pylori* challenge in some mouse strains (inbred BALB/c and outbred QS mice), but not in others (inbred C57BL/6 mice), underlining the importance of the host genetic background (Sutton et al., 2007). In this same study, however, therapeutic immunization was efficient in all three mice strains and *H. pylori* lysate was shown to be more effective than HpaA. The authors concluded that HpaA could be a candidate for vaccination against *H. pylori,* however most likely as a component of a multiantigenic vaccine. The fact that multicomponent vaccines could provide better protection compared to one single antigen was also shown by Ferrero and coworkers, already in 1995. They found that dual antigen vaccination with a combination of Heat-shock protein A (HspA) from *H. pylori* in combination with the *H. pylori* ureB subunit conferred protection against *H. felis* challenge in all animals, whereas some animals in groups vaccinated with only one of both antigens became infected after *H. felis* challenge. Again, protection was assessed by histology and urease activity, which might not be the most sensitive techniques.

6.2 Possible immunization routes

Besides the use of the appropriate antigen (combinations), the choice of the immunization route with the appropriate adjuvant has also been shown to play an important role in *Helicobacter* vaccination studies. In general, vaccination strategies were designed to generate an optimal immune response at the mucosal surface, in line with strategies designed for other mucosal bacterial infections (Blanchard and Nedrud, 2010). This includes oral,

gastric, intranasal and rectal administration, combined either with mucosal adjuvants or delivery vehicles. Some studies indicate that intranasal administration yields better results, compared to the intragastric route (Garhart et al., 2002; Sutton et al., 2007). As adjuvants for mucosal immunization, cholera toxin (CT) and the heat-labile toxin of enterotoxigenic *Escherichia coli* (LT) have been the most widely used in mice, although they are known to cause diarrhoea in humans, even at low doses (Corthésy-Theulaz et al., 1995; Guy et al., 1998; Guy et al., 1999; Banerjee et al., 2002; Garhart et al., 2002; Nyström et al., 2006; Sutton et al., 2007; Svennerholm and Lundgren, 2007). As a consequence, several mouse vaccination studies have been perfomed with success using CT- or LT-based adjuvants with reduced toxicity. Examples are vaccination using the recombinant non-toxic cholera toxin B subunit (Kubota et al., 2005), detoxified mutant LT toxins (Ghiara et al., 1997; Satin et al., 2000) and an adjuvant containing the ADP-ribosylating ability of the cholera toxin A subunit combined with a dimer of an immunoglobulin-binding fragment of *Staphylococcus aureus* protein A (Akhiani et al., 2006). Several other adjuvants have also been used with success in mucosal *H. pylori* mouse immunization studies. These include linear polysaccharides such as chitosan (Xie et al., 2007) and immunostimulatory CpG oligonucleotides, both alone or in combination with antigens (Raghavan et al., 2003; Jiang et al., 2003). Interestingly, when using a combination of *H. felis* lysate, CT and CpG, a sterilizing immunity was assessed by means of quantitative PCR, revealing a possible synergy between both adjuvants (Jiang et al., 2003). The use of *H. pylori* ghosts, which are bacterial cell envelopes devoid of cytoplasmic contents, has shown a certain degree of efficacy (Panthel et al., 2003; Hoffelner et al., 2008; Talebkhan et al., 2010), as did the use of vectors expressing *Helicobacter* urease subunits, including *Lactobacillus* spp. and attenuated *Salmonella* strains (Corthésy-Theulaz et al., 1998; Corthésy et al., 2005).

Since *H. pylori* infection also recruits inflammatory cells from the circulation (Blanchard and Nedrud, 2010), protective immunity may also be achieved through parenteral vaccination. Indeed, a certain degree of protection has been described using prophylactic and therapeutic vaccination via several possible parenteral administration routes, mostly accompanied by the use of established adjuvants, such as complete and incomplete Freund's adjuvant (Eisenberg et al., 2003; Morihara et al., 2007) and aluminium hydroxide (alum) (Ermak et al., 1998; Gottwein et al, 2001; Nyström et al., 2006). Rarely, complete protection was assessed, and again, histology and detection of urease activity were the applied techniques. Interestingly, systemic unadjuvanted vaccination with bacterial lysate or formalin-fixed *Helicobacter pylori* has also been shown to confer partial protection against challenge

with a heterologous *H. pylori* strain, as determined by bacterial culture (Harbour et al., 2008). Conflicting results have been reported when comparing the efficacy of mucosal vs. systemic immunization routes, but most studies suggest that mucosal immunization is the better of two options (Ermak et al., 1998; Guy et al., 1999; Nyström et al., 2006).

6.3 Mechanisms of vaccine-mediated protection against *H. pylori* infection

In prophylactic *H. pylori* mouse vaccination studies, an enhanced gastritis is often observed after challenge of immunized mice (Blanchard and Nedrud, 2010). This post-immunization gastritis is often associated with the degree of protection against colonization, as shown by a clear correlation between the severity of post-immunization gastritis and a reduction of the bacterial load (Goto et al., 1999; Garhart et al., 2002; Morihara et al., 2007). Eventually, however, the disparity in gastritis between infected animals and immunized/challenged animals disappears, showing that this is a transient event, both in the *H. pylori* and *H. felis* mouse immunization model (Sutton et al., 2001; Garhart et al., 2002). Identification of cell populations has shown that protection mainly depends on the presence of a MHC class II-restricted $CD4^+$ T cell population (Ermak et al., 1998; Ruggiero et al., 2003), which was confirmed by protection induced in mature B and T cell-deficient *rag*$1^{1/1}$ mice, by transfer of purified $CD4^+$ T cells from vaccinated C57BL/6 mice (Gottwein et al., 2001). As for the polarization of the $CD4^+$ T-helper cells, some conflicting reports can be found in literature. Although initially, an elicited Th2 response (with prophylactic systemic alum-adjuvanted and therapeutic mucosal CT-adjuvanted immunization) or a balanced Th2/Th1 response were shown to confer protection (Ermak et al., 1998; Saldinger et al., 1998; Gottwein et al., 2001), it is now accepted that Th1 responses are probably most important for *Helicobacter* immunity (Taylor et al., 2008; Blanchard and Nedrud, 2010). Garhart et al. (2003) found that IL-4 is not essential for vaccine-induced protection and Nyström and Svennerholm (2007) described a protection after therapeutic oral vaccination which was strongly related to specific mucosal $CD4^+$ T cells with a Th1 profile. As for the role of IFN-γ in this protective response, the debate is still ongoing. The same goes for other cytokines, including IL-12p40 and the IL-23/IL-17 pathway (Blanchard and Nedrud, 2010). The latter has been described as being important for neutrophil-dependent protection (DeLyria et al., 2009), which was again questioned by the same research group (Blanchard and Nedrud, 2010). Very recently, it has been shown that *H. pylori* infection causes an imbalance between interleukin-17^+ helper T cell (Th17) and regulatory T cells (Tregs) in favour of the Tregs and

resulting in a suboptimal Th17 response and failure to eliminate the bacterium (Kao et al., 2010). Possibly, T cell activation at lymph nodes due to immunization could circumvent this Treg-mediated suppression of immunity (Blanchard et al., 2004).

Generally, antibodies are considered not to be essential for vaccine-mediated immunity to *H. pylori*. Several research groups have been able to obtain a similar level of protection in antibody-deficient mice, when compared to wild-type animals (Ermak et al., 1998; Gottwein et al., 2001; Garhart et al., 2003), although others have described a strong relation between protection and mainly the presence of antigen-specific IgA antibodies, both local and circulating (Czinn et al., 1993; Nyström and Svennerholm, 2007; Morihara et al., 2007).

6.4 Immunization against gastric helicobacters: other animal models and non-*H. pylori* helicobacters

Only few studies have described vaccination in the Mongolian gerbil model. Jeremy et al. (2006) achieved a successful protection, assessed by culture, histology and urease, against *H. pylori* challenge in gerbils vaccinated orogastrically with *H. pylori* whole bacterial cell sonicate along with CT. Interestingly, protection in this animal model was not accompanied by post-immunization gastritis, 6 weeks after challenge of immunized animals. Intragastric and, to a lesser extent, intramuscular immunization with a combination of UreB, HspA, HpaA and low-toxic $LT_{R72DITH}$ or $Al(OH)_3$, respectively, also have been shown to induce a good protection against *H. pylori* challenge (Wu et al., 2008). As a consequence, the gerbil model may be very useful for *H. pylori* vaccination studies, especially since highly virulent type I strains, with a functional CagA type IV secretion system and a functional VacA are incompatible with a stable colonization in the mouse model (Hoffelner et al., 2008). In Mongolian gerbils, however, a stable chronic infection with type I *H. pylori* strains is possible (Rieder et al., 2005b) and VacA has even been suggested to be important for chronic persistence in gerbils (Hoffelner et al., 2008). A clear disadvantage of this rodent model, however, is the relative lack of tools, including antibodies and options of genetic manipulation (Hoffelner et al., 2008).

Some *Helicobacter* vaccination studies have been done in animals other than rodents. Two studies by the same research group describe both prophylactic and therapeutic vaccination against *H. pylori* infection in gnotobiotic piglets (Eaton and Krakowka, 1992; Eaton et al., 1998). Prophylactic vaccination did not prevent infection by subsequent

challenge but reduced bacterial colonization, whereas therapeutic vaccination was unsuccessful. In cats and dogs, a prophylactic oral immunization with recombinant urease and LT and a therapeutic intramuscular vaccination with a multicomponent vaccine and aluminium hydroxide, respectively, have been shown to elicit a reduced *H. pylori* colonization (Batchelder et al., 1996; Rossi et al., 2004). In non-human primates, only some studies describe a partial protection by immunizing with urease against subsequent *H. pylori* challenge or natural *H. pylori* infection (Dubois et al., 1998; Lee et al., 1999b; Solnick et al., 2000). This partial protection was achieved with oral vaccination or oral priming and subsequent parenteral vaccination, whereas parenteral vaccination alone was unsuccessful. Therapeutic vaccination protocols have been described to confer limited protection when using parenteral administration or a combination of parenteral and oronasal vaccination (Guy et al., 1998; Lee et al., 1999a).

For gastric non-*H. pylori* helicobacters, vaccination studies are mostly limited to *H. felis*, as discussed in the previous sections. Additionally, Dieterich et al. (1999) have described a prophylactic and therapeutic vaccination regimen against a so-called "*H. heilmannii*" challenge. Similar results were obtained for prophylactic intranasal vaccination using recombinant "*H. heilmannii*" ureB or *H. pylori* urease, both adjuvanted with CT. The results of therapeutic immunization with *H. pylori* urease suggested that successful treatment is more difficult to achieve for "*H. heilmannii*" infection when compared to *H. felis*. However, caution is needed, since gastric tissue of "*H. heilmannii*"-infected mice was used for challenge and the exact species status of the "*H. heilmannii*" strain remains unknown. Finally, a single mouse study describes both intranasal (+ CT) and subcutaneous (+ saponin formulation) immunization with heterologous *H. pylori* and *H. felis* whole bacterial cell antigens against challenge with "*Candidatus* H. suis", which was unculturable at the time of publication (Hellemans et al., 2006). Although urease activity in the stomach of vaccinated/challenged mice was often reduced to basal levels, all immunization protocols conferred only partial protection, since all animals remained positive in a *H. suis*-specific PCR.

6.5 Immunization against gastric helicobacters in humans

In early human *H. pylori* vaccination trials, mucosal administration of recombinant *H. pylori* urease and LT was used, often leading to diarrhoea in volunteers (Michetti et al., 1999; Banerjee et al., 2002; Sougioultzis et al., 2002). Often, an immune response was elicited,

although LT was shown to be more immunogenic than urease. Only in the study by Michetti et al. (1999), the effect on *H. pylori* colonization was assessed, after therapeutic vaccination, however no eradication could be achieved. Similarly, successful eradication could not be achieved after therapeutic immunization using a formalin-inactivated whole-cell preparation (Kotloff et al., 2001).

To avoid the undesirable side-effects of toxic adjuvants such as LT, several research groups have performed vaccination with *H. pylori* urease, expressed by an attenuated *Salmonella enterica* vector. Also in the most recent study, this did not induce satisfactory protection against *H. pylori* challenge, although higher percentages of *H. pylori*-responsive T cells were detected in patients with reduced *H. pylori* colonization (Aebischer et al., 2008). Finally, intramuscular administration of a multicomponent vaccine, composed of CagA, VacA, HP-NAP and aluminium hydroxide, elicited no adverse reactions and generated a humoral and antigen-driven T-cell proliferation and cytokine production (Malfertheiner et al., 2008). It remains, however, to be elucidated whether this vaccination protocol can really protect against colonization with *H. pylori* and the subsequent development of severe gastric pathologies.

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Scientific Aims

Worldwide, infection with *Helicobacter (H.) pylori* is considered to be the major cause of gastritis, peptic ulcer disease, gastric adenocarcinoma and mucosa-associated lymphoid tissue (MALT) lymphoma in humans. Besides *H. pylori,* large, spiral-shaped non-*H. pylori* helicobacters have also been associated with these gastric disorders and comprise different species naturally occurring in the stomach of various animal species. *H. suis* colonizes the stomach of the vast majority of pig populations and is the most prevalent non-*H. pylori Helicobacter* species detected in humans suffering from gastric disease.

For all non-*H. pylori Helicobacter* species in general and *H. suis* in particular, very little is known about their exact role in human gastric pathology. In part, the very fastidious nature of these bacteria has hampered the progress of non-*H. pylori Helicobacter*-related research. Our research group was the first to successfully isolate *H. suis in vitro* in 2008, opening new doors to investigate the exact role of this bacterium in gastric pathology and to develop possible strategies to control this infection.

To obtain better insights into the pathogenesis of human gastric diseases associated with a *H. suis* infection, experimental infections with pure cultures of the bacterium are essential. In the past, various animal models of human gastric disease, including the Mongolian gerbil model, have been used to investigate *H. pylori*-induced gastric pathology. Therefore, the **first aim** of this thesis was to investigate the possibility of using the Mongolian gerbil model in *H. suis* research.

In the **second study**, experimental *H. suis* infections in mouse and Mongolian gerbil models were performed in order to examine in detail the interactions between the bacterium and the gastric mucosa of the host.

Prevention and eradication of gastric *Helicobacter* infection are two very important steps to drastically reduce the number of people suffering from gastric disease. Therefore, in the **third study**, *in vitro* grown *H. suis* was used to vaccinate mice, in a first attempt to evaluate the protective effect of the *in vitro* cultured organisms.

Loss of gastric epithelial cells has been shown to be a major factor contributing to *H. pylori*-induced gastric pathology. For *H. suis*, however, nothing is known about its possible role in human gastric epithelial cell death. Therefore, it was the **fourth aim** of this thesis to

investigate *H. suis*-induced cell death and to characterize *H. suis* virulence factors involved in this process.

Experimental Studies

Chapter 1

Experimental *Helicobacter suis* infection in Mongolian gerbils: interference of a concomitant *Kazachstania heterogenica* infection

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Abstract

Background: The Mongolian gerbil model is often used to investigate the interactions between different gastric *Helicobacter* species and the gastric tissue. A preliminary screening of a gerbil population intended for use in *Helicobacter suis* infection studies revealed a natural yeast infection in the stomach of these animals. After identification, we have investigated the effect of the gastric yeast infection on the outcome of an experimental *H. suis* infection in Mongolian gerbils.

Materials and methods: Yeast cells were isolated from the stomachs of Mongolian gerbils. Identification was done by Internally Transcribed rRNA Spacer 2 Region (ITS2) PCR fragment length analysis. To investigate a possible pathologic role of this yeast, Mongolian gerbils were infected experimentally with this yeast. Co-infection with the newly isolated *Helicobacter suis* was performed to investigate possible interactions between both micro-organisms.

Results: *Kazachstania heterogenica* was found colonizing the stomach of Mongolian gerbils, mainly in the antrum. Few pathologic changes were seen in the stomachs of infected animals. Experimental co-infection of gerbils with this yeast and the newly isolated *Helicobacter suis* showed a significant increase in inflammation in animals infected with both micro-organisms compared to animals infected only with *Helicobacter suis*.

Conclusions: *Kazachstania heterogenica* colonizes the stomach of Mongolian gerbils in exactly the same regions as gastric *Helicobacter* species. The uncontrolled presence of this yeast in the gerbil stomach can lead to an overestimation of the inflammation caused by *Helicobacter* in this animal model.

Introduction

Helicobacter (H.) suis is a newly isolated *Helicobacter* species (Baele et al., 2008) colonizing the gastric mucosa of the vast majority of the pig population (Park et al., 2004; Hellemans et al., 2007). This large spiral-shaped bacterium has been suggested to play a role in the development of hyperkeratosis and ulcers of the pars oesophagea in the pig stomach (Haesebrouck et al., 2009). In addition, this *Helicobacter* species is identical to *'H. heilmannii"* type 1 (Haesebrouck et al., 2009; O'Rourke et al., 2004). Bacteria belonging to the group comprising *"H. heilmanni"* type 1 and 2 have been associated with gastritis, gastric ulcers and even gastric MALT lymphoma in a minority of the human population (Debongnie et al., 1995; Debongnie et al., 1998; Morgner et al., 2000; Stolte et al., 2002). To date, very little is known about the pathogenesis of an *H. suis* infection.

Rodent models are commonly used to study the pathogenesis of gastric *Helicobacter* infections. In *H. pylori* research, the mouse and the Mongolian gerbil (*Meriones unguiculatus*) model are the two rodent species most commonly used to investigate the interactions between this bacterium and the gastric tissue (O'Rourke and Lee, 2003; Rogers and Fox, 2004). Also, vaccination studies against *H. pylori* infection are performed in the Mongolian gerbil model (Jeremy et al., 2006; Wu et al., 2008). Besides extensive use in *H. pylori* research, the Mongolian gerbil has also been used to investigate the pathogenic effects of other gastric helicobacters, such as *H. felis* and *H. bizzozeronii* (De Bock et al., 2006a and 2006b). During a preliminary screening of a gerbil population prior to their use for the study of *H. suis* infection, yeast-like organisms were found colonizing the antral region of their stomachs. The purpose of the present study was to identify the yeasts and to study the effect of a gastric yeast infection on the outcome of an experimental *H. suis* infection in Mongolian gerbils.

Materials and Methods

Natural yeast infection in the stomach of Mongolian gerbils: animals, sampling and processing of samples

Seventeen specific-pathogen-free (SPF) female gerbils of six weeks old were purchased from an authorized laboratory animal supplier (supplier A). The animals were barrier-maintained in a room with controlled environment and housed in filtertop cages. They were fed an autoclaved diet containing 18% protein (Teklad Global Rodent Diet, Harlan NL, Horst, The Netherlands) and received autoclaved tap water ad libitum. Two animals were sacrificed by cervical dislocation after isoflurane (5% vol/vol) anaesthesia at eight weeks of age and seven animals at thirteen weeks of age. The eight remaining animals were sacrificed at eightteen weeks of age. The stomachs were opened along the greater curvature and rinsed with sterile HBSS (Gibco, Carlsbad, USA). For histological analysis, a longitudinal strip of tissue along the greater gastric curvature from the oesophagus to the duodenum, was taken from all animals. After fixation in 4 % phosphate buffered formalin, tissues were processed by standard methods and embedded in paraffin. Serial 5 µm sections were cut and stained with haematoxylin and eosin (H&E), periodic acid-Schiff (PAS) and Gomori silver to visualize the yeasts.

To isolate the yeast cells, a swab was taken from the antrum pyloricum of ten animals (two animals of eight weeks old and eight animals of eightteen weeks old) and plated onto Sabouraud (SAB) dextrose agar (Oxoid, Basingstoke, UK) containing penicillin and streptomycin (Sigma-Aldrich, St. Louis, USA). Plates were incubated aerobically at 37°C for at least 48 hours. A faecal sample was collected from the cage housing the two animals euthanized at eight weeks of age, homogenized in sterile HBSS and plated out as described above.

Identification of the yeast strains

Two yeast colonies obtained from the antral swabs of five animals (n= 10) as well as two colonies obtained from the faecal sample were used for subcultivation and subsequent identification to species level. DNA-extraction of single colonies was done by a boiling-freezing method. Next, identification was done by Internally Transcribed rRNA Spacer 2 Region (ITS2) PCR fragment length analysis (De Baere et al., 2002). Further discrimination between the members of the *Kazachstania (K.) telluris* complex was carried out by sequence

analysis of the ITS2-region followed by comparison of the obtained sequence to all known sequences in Genbank using the BLAST software at NCBI. An isolate from each animal was passed onto new SAB agar plates and frozen at -80°C in a medium consisting of 7.5 g glucose, 25 ml Brain Heart Infusion broth and 75 ml sterile inactivated horse serum (isolates 001-005).

Bacterial and yeast strains for experimental infection

H. suis strain HS5, isolated as described previously from the gastric mucosa of a sow (Baele et al., 2008), was grown on Brucella agar (Oxoid, Basingstoke, UK) supplemented with 20% fetal calf serum, 5 mg/l amphotericin B (Fungizone; Bristol-Myers Squibb, Epernon, France), *Campylobacter* selective supplement (Skirrow, Oxoid; containing 10 mg/l vancomycin, 5 mg/l trimethoprim lactate and 2500 U/l polymyxin B) and Vitox supplement (Oxoid). In addition, the pH of the agar was adjusted to 5 by adding HCl to a final concentration of approximately 0.05%. One ml of Brucella broth (Oxoid) with a pH of 5 was added on top of the agar to obtain biphasic culture conditions, essential for cultivation of this bacterium. After three days of incubation at 37°C in microaerobic conditions, the broth, containing the bacteria, was harvested. The final concentration was adjusted to an optical density of 1 at 660 nm, corresponding to approximately 2 x 10^8 viable bacteria/ml, as determined by counting bacteria in an improved Neubauer counting chamber.

K. heterogenica (isolate 002) was first grown on SAB plates, as described above. After 48 hours, one colony was suspended in 50 ml of SAB broth (containing 10g special peptone (Oxoid), 20 g glucose and 1000 ml distilled water at a pH of 5,5) and grown with shaking overnight (16 hours) at 37°C to obtain a liquid culture containing 2.5 x 10^7 CFU/ml.

Experimental infection of Mongolian gerbils with *Kazachstania heterogenica*

The experimental protocol was approved by the Ethical Committee of the Faculty of Veterinary Medicine, Ghent University, Belgium. Thirty-four female SPF outbred gerbils (Crl:MON) of five weeks old were obtained from another authorized laboratory animal supplier (supplier B) and housed as described above. Preliminary screening of four extra animals showed gerbils of this supplier to be free of gastric *K. heterogenica* infection by direct culture from both stomach and faeces (as described above). Twenty-three animals were inoculated two times at 48 hours intervals (Day 0 and Day 2) with 10^7 CFU of *K. heterogenica*, grown overnight in SAB broth. Eleven animals served as uninfected controls and were inoculated with SAB broth. Inoculation was performed intragastrically using a ball-

tipped gavage needle, under isoflurane (IsoFlo; Abbott, Illinois, USA) anaesthesia. Five infected animals were euthanized at Day (D) 4, as well as two uninfected controls. At D8, D15 and D22 of the experiment, six infected and three uninfected animals were euthanized. This was done by cervical dislocation following isoflurane anaesthesia. The stomach of each animal was resected, opened along the greater curvature and rinsed with sterile HBSS (Gibco). Yeast isolation and histological analysis were performed as described above. H&E-stained sections were used for blind scoring of the intensity of gastric inflammation (infiltration of mononuclear cells and neutrophils), according to the Updated Sydney System (Dixon et al., 1996). PAS-stained sections were used for blind scoring of the colonization with *K. heterogenica*, with a scale from 0 (no yeasts visible) to 3 (extensive colonization).

Experimental co-infection of Mongolian gerbils with *Kazachstania heterogenica* and *Helicobacter suis*

The experimental protocol was approved by the Ethical Committee of the Faculty of Veterinary Medicine, Ghent University, Belgium. Fourty female SPF outbred gerbils (Crl:MON) of five weeks old were obtained from laboratory animal supplier B (see previous section) and housed as described above. Preliminary screening of four extra animals with an *H. suis*-specific PCR showed gerbils of this authorized laboratory animal supplier to be free of *H. suis.* As described above, these animals were also shown to be free of *K. heterogenica.* The animals were divided into 5 groups (Table 2). The animals from the control group (group 1) were inoculated with SAB broth at D0 and D2, with Brucella broth at D14 and D16, and again with SAB broth at D21 and D23. Gerbils in group 2 were inoculated with 10^7 CFU of *K. heterogenica* at D0 and D2. At D14 and D16 they were administered Brucella broth, followed by SAB broth administration at D21 and D23. Animals in the third group were given SAB broth at D0 and D2, inoculated with 10^8 CFU of *H. suis* at D14 and D16, and given SAB broth again at D21 and D23. Animals from group 4 were given SAB broth at D0 and D2. Inoculation of *H. suis* (10^8 CFU) was performed at D14 and D16, followed by administration of *K. heterogenica* (10^7 CFU) at D21 and D23. Animals in group 5 received inoculation with *K. heterogenica* (10^7 CFU) at D0 and D2 and with *H. suis* (10^8 CFU) at D14 and D16. At D21 and D23 the gerbils were given SAB broth. At D35, all animals were sacrificed. All inoculations were performed intragastrically as described above. Euthanasia was performed as described in the previous section. At euthanasia, the stomach of each animal was resected, opened along the greater curvature and rinsed with sterile HBSS (Gibco). The procedure for yeast isolation was performed as described above. Samples for PCR analysis and urease

testing were taken in both antrum and fundus using disposable sterile biopsy punches (∅ 3mm). DNA extraction was done with the DNeasy Tissue Kit (Qiagen, Hilden, Germany) according to the manufacturer's protocol. All samples were screened for the presence of *H. suis* DNA using an *H. suis* specific PCR (De Groote et al., 2000). For urease testing, samples were immersed in 500 µl of rapid urease test (CUTest; Temmler Pharma, Marburg, Germany) and incubated at 37°C for 3 h. After centrifugation (5 min., 1000 x g), the level of urease activity was measured by spectrophotometric analysis at 550 nm. Basal values were determined by measuring the optical density of the rapid urease test incubated for 3h without addition of a tissue sample (negative control). All urease values were subtracted with the mean value of the negative control samples. Histological evaluation was performed as described above. An additional 5 µm section was used for immunohistochemical staining of *Helicobacter* bacteria, by using a polyclonal genus-specific rabbit anti-*H. pylori* antibody (1/320; DakoCytomation, Glostrup, Denmark) (De Groote et al., 2000). The *H. suis* colonization density was scored blindly according to the Updated Sydney System (Dixon et al., 1996).

Statistical analysis

All statistical analyses were performed using SPSS, version 17 (SPSS Inc., Chicago, Illinois, USA). For comparison of urease values between different groups, a Student's t-test was performed. For comparison of inflammation and both colonization scores, a Wilcoxon rank-sum test was used. Tests were performed at the 5% global significance level.

Results

Kazachstania heterogenica occurs naturally in the stomach of Mongolian gerbils

In twelve out of seventeen animals, on histological examination a mild to severe colonization with yeast cells was seen in the antrum, ranging from a few yeast cells to massive numbers forming a thick layer on the mucosa. The organisms were intensely positive by periodic acid-Schiff and Gomori Silver staining. Most of the yeast cells were seen in the surface mucus layer, although a substantial number of yeast cells also were seen in close association with the apical surface of the epithelial cells. In some animals, yeast cells were seen deep in the crypts.

In eight out of seventeen animals, a limited number of yeast cells were also observed in the fore stomach-stomach transition zone (i.e. limiting ridge). This colonization, however, was always substantially milder than that seen in the antrum. Colonization in the fundus was limited to a small region in only one animal.

Most animals had a well-developed gastric mucosa with a normal foveolate surface and no inflammatory cell infiltration. In three out of seventeen animals, however, a mild inflammation was observed, mainly consisting of small lymphocytic infiltrates in the mucosa and submucosa of the antrum. This region was always colonized by yeast cells.

Yeast cells were isolated from seven out of ten antral swabs and from the stool sample. In all seven of these animals, yeasts were also detected on histological sections. No organisms were isolated from the remaining three animals which were also histologically negative. Macroscopically, separate colonies shared the same phenotype and were visible after twenty four hours, ranging from 1mm to 3mm in diameter. Two colonies per plate obtained from the antral swabs of five animals and the faecal sample were selected for subcultivation and further analysis.

ITS2-PCR fragment length analysis of all selected colonies resulted in a fragment length of 329 bp. This fragment length was already present in the Basehopper database as *Kazachstania telluris* complex. Previously we obtained different fragment lengths for strains of this complex, which was divided into five separate species four years ago (Kurtzman et al., 2005). The obtained fragment length corresponded to *K. heterogenica*, one of those new species. Final confirmation was done by ITS2 sequence analysis, resulting in a 100% sequence similarity to strains CBS 2675^{T} and CBS 2778, which were used for the description of the species *K. heterogenica* (Kurtzman et al., 2005). No other yeast species were detected in this study.

Experimental infection of Mongolian gerbils with *Kazachstania heterogenica* leads to a predominant antral colonization without an appreciable inflammatory response

As opposed to control animals, in which no yeasts could be isolated from the antral region of the stomach, all *Kazachstania*-infected animals were positive for *K. heterogenica* culture in the antral stomach region at all four timepoints after experimental infection.

At four days post infection (D4), colonization by *K. heterogenica* was already established, since all animals had a colonization score of at least 1 in the antrum (Table 1). In contrast, only 2 out of 5 animals showed PAS-positive organisms colonizing the fundus at the forestomach-stomach transition zone. At 8 days post infection, 4 out of 6 animals showed a

maximum colonization score of 3 in the antrum, while only one animal showed no visible colonization at the forestomach-stomach transition zone. Colonization scores did not increase further in course of time.

When comparing active and chronic inflammation between *K. heterogenica* infected and uninfected groups at all timepoints, no differences were observed ($p>0.05$) (data not shown). Only five infected animals, spread out over all timepoints, showed a very mild neutrophilic infiltrate (score 1), mainly in the antrum. Moreover, when using time as stratification factor, no differences in active ($p = 0.147$) and chronic ($p = 1$) antral inflammation were observed between uninfected and infected animals.

Table 1. Colonization results of gerbils infected experimentally with *K. heterogenica*

Timepoint of euthanasia	n	Number of animals with respective colonization score in fundus at forestomach-stomach transition zone				Number of animals with respective colonization score in antrum			
		0*	1	2	3	0	1	2	3
D4	5	3	2	0	0	0	2	2	1
D8	6	1	5	0	0	0	0	2	4
D15	6	2	4	0	0	0	0	4	2
D22	6	0	6	0	0	0	0	3	3

* colonization scores: 0, 1, 2, 3

n: total number of *K. heterogenica*-infected animals euthanized at each time point

Kazachstania heterogenica exacerbates a *Helicobacter suis* infection in Mongolian gerbils

K. heterogenica was isolated from the antral stomach region of all animals inoculated with this yeast, whereas all animals not inoculated with *K. heterogenica* were negative for this yeast. Animals infected only with *K. heterogenica* (group 2), showed higher *Kazachstania* colonization scores on PAS-stained sections in the antrum compared to the animals infected both with *K. heterogenica* and *H. suis* (groups 4 and 5) (Table 2) [$P(2\text{-}4) = 0.032$; $P(2\text{-}5) = 0.003$]. Pooling the data from groups 4 and 5 confirmed these findings [$P(2\text{-}4/5) = 0.004$].

Table 2. Experimental infection study with *H. suis* and *K. heterogenica*: colonization scores and urease values

Group	Number of animals/ group	Inoculation D0/D2	Inoculation D14/D16	Inoculation D21/D23	*H. suis* colonization score antrum: mean (min-max)	*K. heterogenica* colonization score antrum: mean (min-max)	Urease value antrum : mean ± SD
1	5	SAB broth	Brucella broth	SAB broth	0 (0-0)	0 (0-0)	0 ± 0
2	5	*K. heterogenica*	Brucella broth	SAB broth	0 (0-0)	3 (2-3)	0 ± 0
3	10	SAB broth	*H. suis*	SAB broth	2 (1-2)	0 (0-0)	1.601 ± 0.110
4	10	SAB broth	*H. suis*	*K. heterogenica*	2 (2-3)	2 (1-3)	1.533 ± 0.178
5	10	*K. heterogenica*	*H. suis*	SAB broth	2 (1-3)	2 (1-2)	1.524 ± 0.063

D: day of the experiment; Min-max: minimum-maximum individual score per group; SD: standard deviation; SAB: Sabouraud

All animals infected with *H. suis* (groups 3, 4 and 5) were positive in the *H. suis* specific PCR, in contrast to the animals in both the negative and *K. heterogenica* control group (data not shown). The results of the urease testing showed positive values, mainly in the antrum, for all *H. suis*-infected animals (Table 2). Although slightly lower values for urease activity were detected in the antrum of groups 4 and 5 compared to animals which received *H. suis* inoculation only (group 3), these differences were not significant [P(3-4) = 0.323; P(3-5) = 0.075; P(3-4/5) = 0.125]. This was confirmed by the results of the *Helicobacter* colonization scores, showing no difference in antral colonization density between animals infected with *H. suis* alone and animals infected with both *H. suis* and *K. heterogenica* (Table 2). Often, both organisms colonized the exact same places in the antrum (Figure 1A) and were seen in close proximity of each other (Figure 1B).

Results of the inflammation scores in the antrum are shown in figure 2. All animals in the negative control group (group 1) showed a normal gastric mucosa with no inflammatory cell infiltration (Figure 3). Four out of five animals infected with *K. heterogenica* alone showed no inflammation, while one animal showed a very mild lymphocytic infltrate. All *H. suis*-inoculated animals had an inflamed gastric mucosa, mainly visible as lymphocytic infiltrates in the antrum. Animals infected experimentally with both *H. suis* and *K. heterogenica* (groups 4 and 5, respectively) (Figures 4A and 4B) showed higher inflammation scores than animals infected with *H. suis* only (group 3) (Figures 5A and 5B) [P(3-4) = 0.104; P(3-5) = 0.042]. This difference was confirmed by pooling the data from groups 4 and 5 [P(3-

4/5) = 0.039]. This was done because no differences in antral inflammation were seen between groups 4 and 5 [P(4-5) = 0.706].

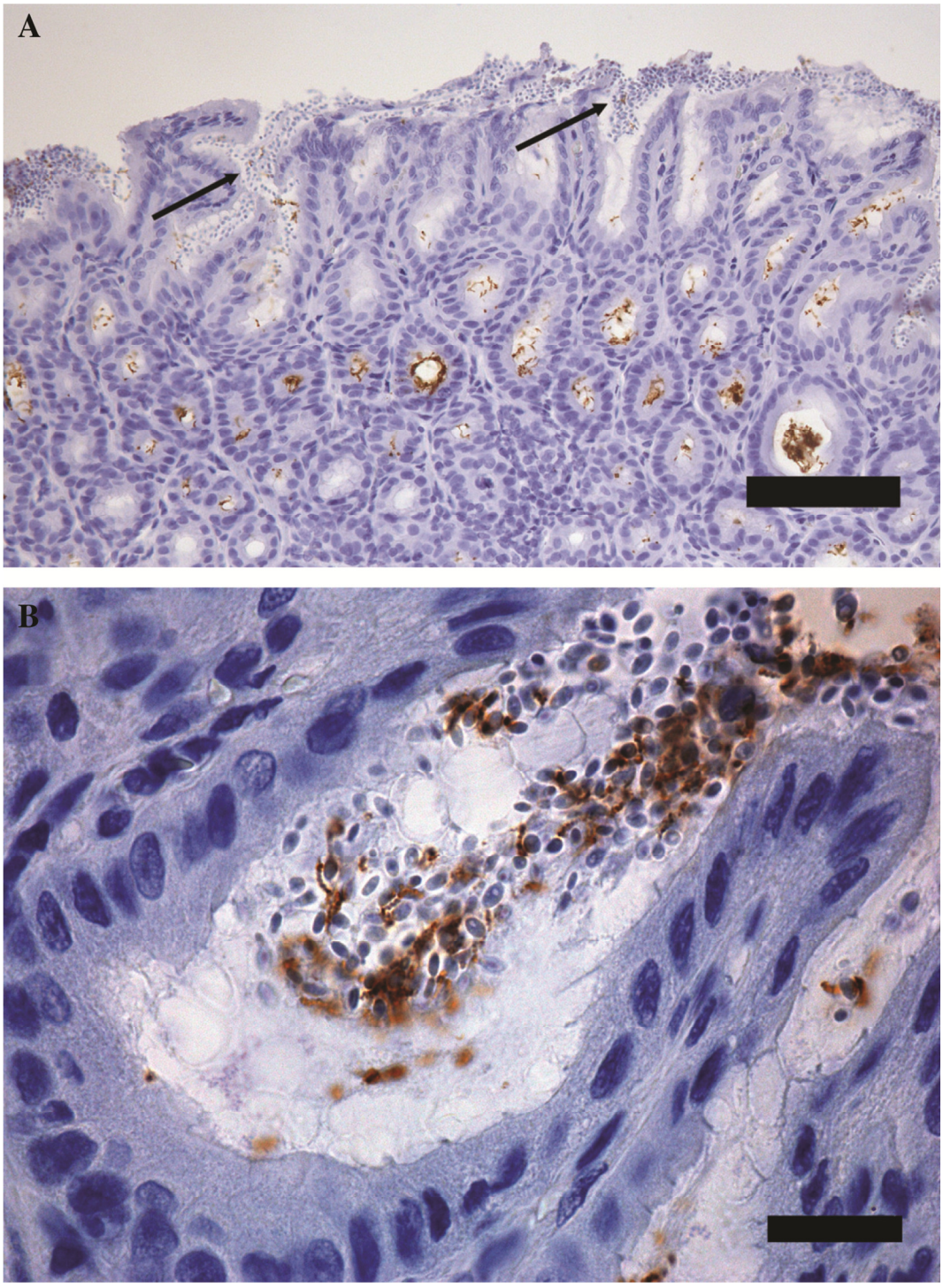

Figure 1 (A) Immunohistochemical *Helicobacter* staining showing antral colonization with *H. suis* (brown; score 2 on a scale from 0-3) and *K. heterogenica* (arrows; score 2 on a scale from 0-3). Original magnification: 200x; bar: 100 µm. **(B)** Immunohistochemical *Helicobacter* staining of a stomach gland showing antral colonization both with *H. suis* (brown) and *K. heterogenica*. Original magnification: 1000x; bar: 20 µm.

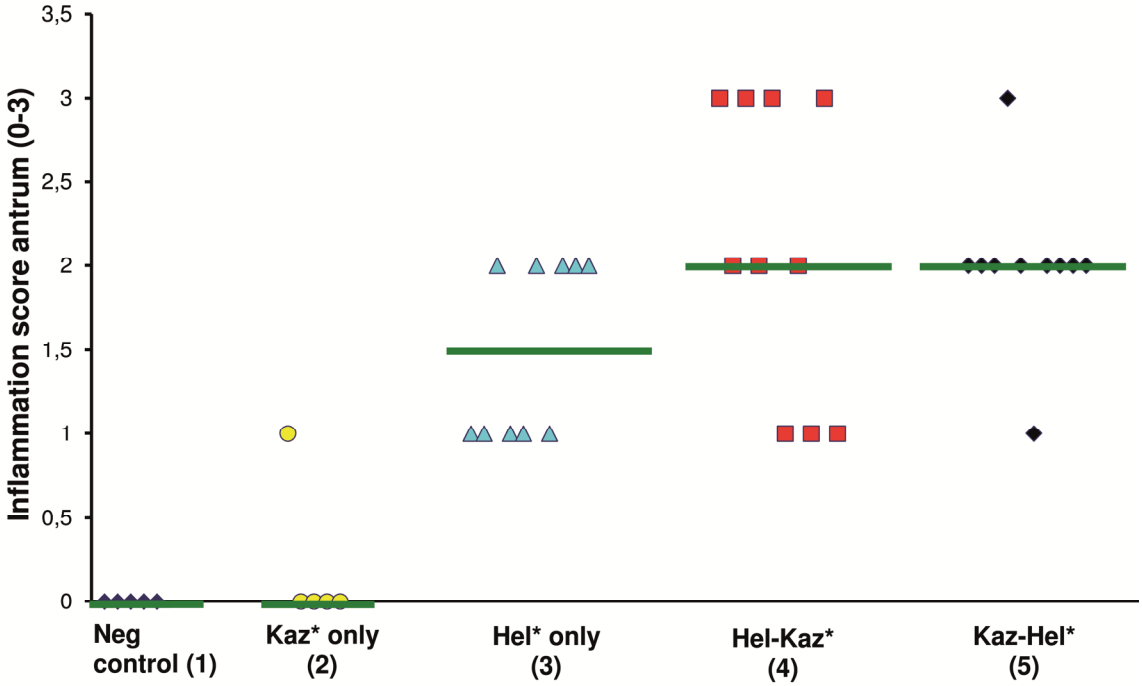

Figure 2 Antral inflammation scores (0-3) per group (between brackets). Shown are the individual scores of each animal. Bars represent the median antral inflammation score of each group. * Kaz: experimental infection with *Kazachstania heterogenica.* Hel: experimental infection with *Helicobacter suis*.

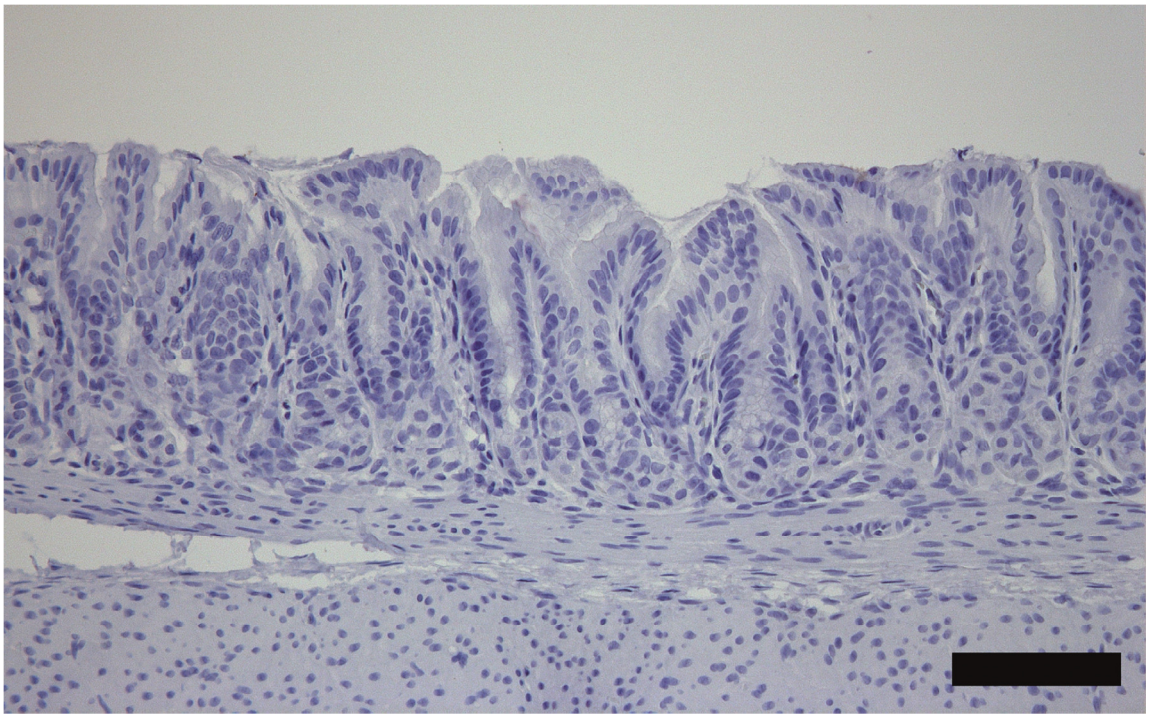

Figure 3 Immunohistochemical *Helicobacter* staining of a negative control animal showing no antral colonization with *H. suis* nor *K. heterogenica*. No inflammation can be seen. Original magnification 200x; bar: 100 µm.

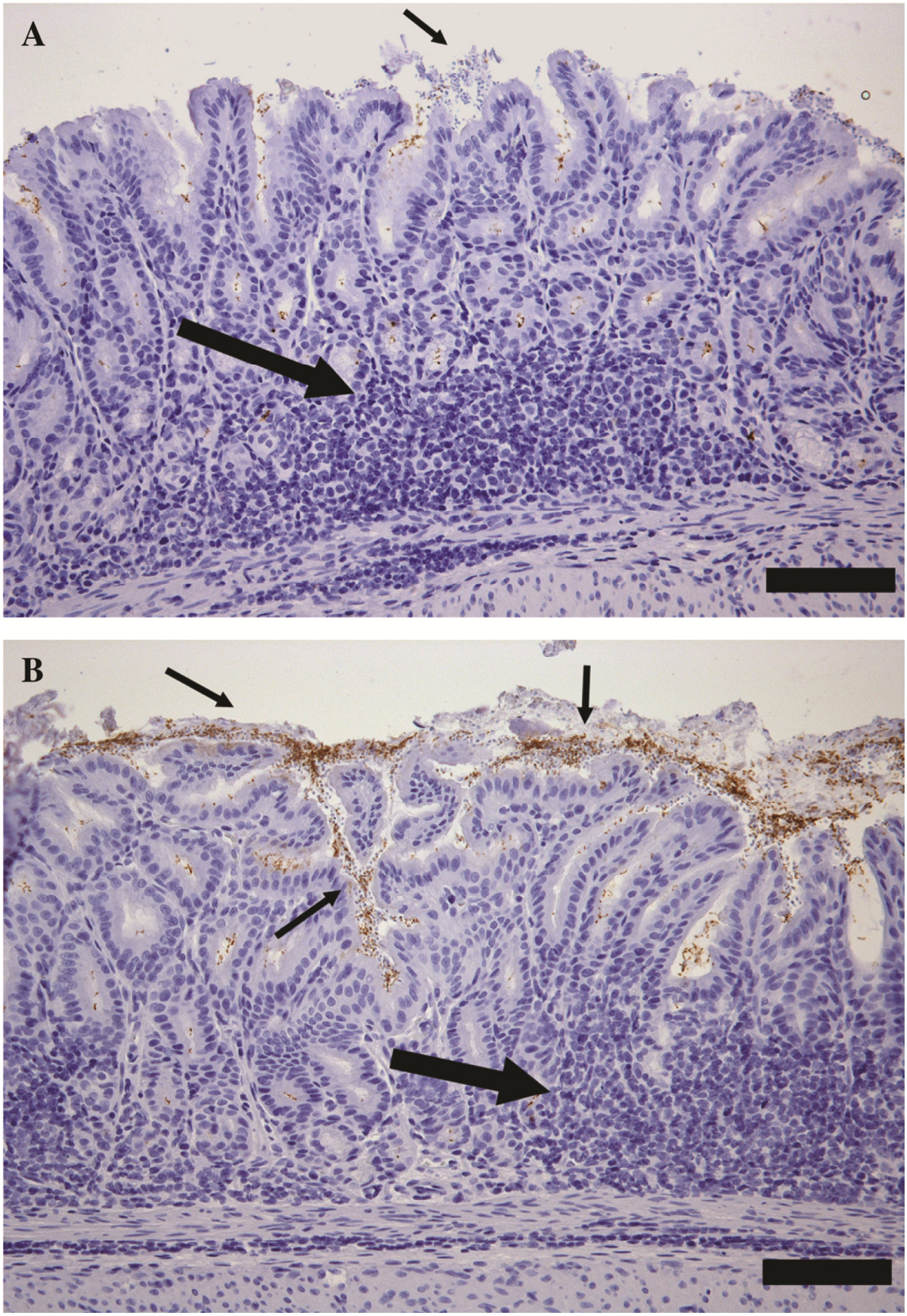

Figure 4 (A) and (B) Immunohistochemical *Helicobacter* staining of the antrum of an animal infected with both *H. suis* and *K. heterogenica* showing antral colonization with *H. suis* (brown) and *K. heterogenica* (small arrows) and a more severe inflammation (large arrow) compared to animals infected with *H. suis* alone. Original magnification 200x; bar: 100 µm.

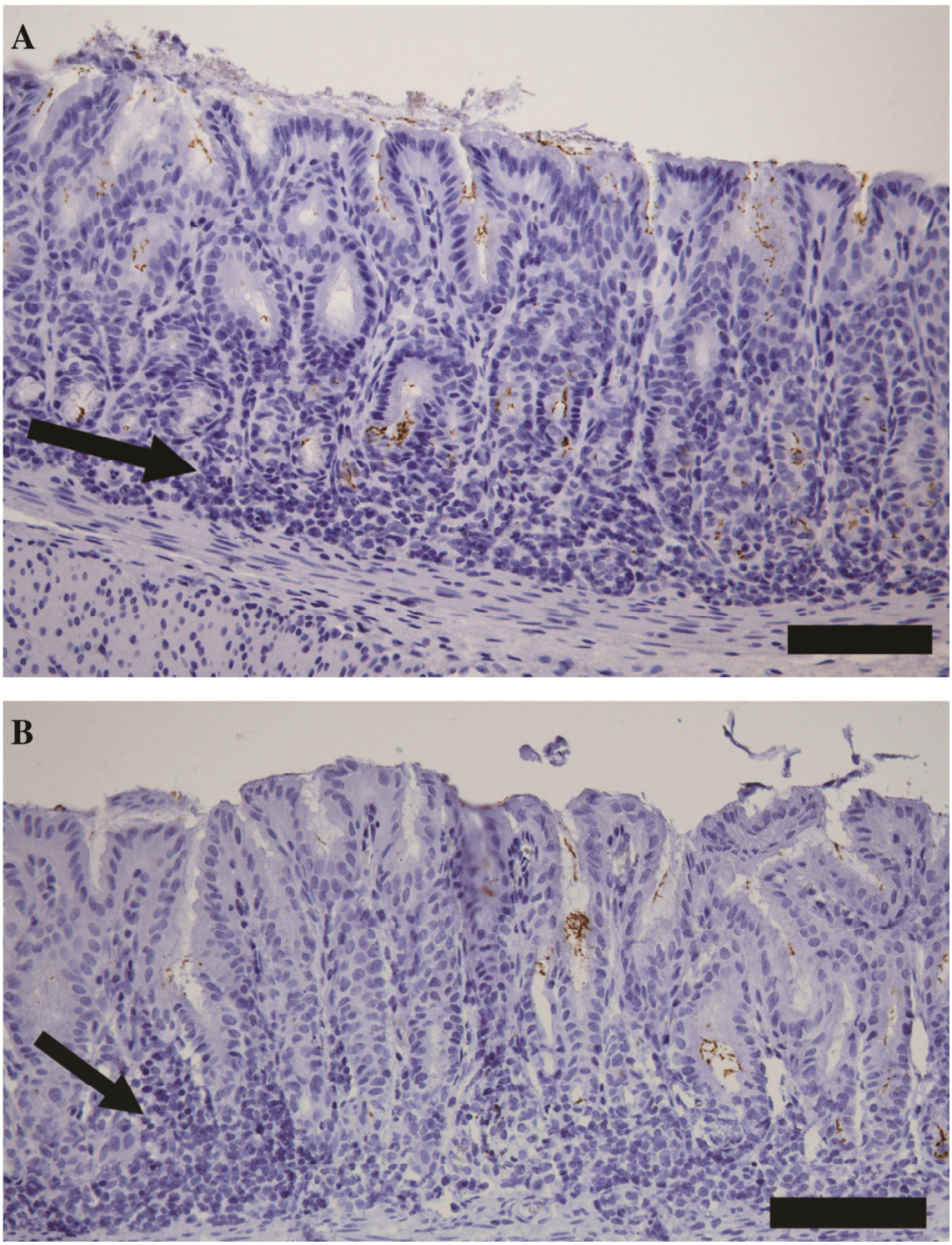

Figure 5 (A) and (B) Immunohistochemical *Helicobacter* staining of the antrum of an animal infected with *H. suis* alone showing *H. suis* bacteria (brown) and a mild to moderate inflammatory cell infiltrate (arrow). Original magnification 200x; bar: 100 µm.

Discussion

Over the past fifteen years, the mouse model has been widely used to investigate *Helicobacter* pathology. Until now, only a few reports have been published about gastric yeast infections in these animals (Savage and Dubos, 1967; Artwohl et al., 1988; Kurtzman et al., 2005). During the past decade, the Mongolian gerbil model has increasingly been used to study the pathogenesis of *Helicobacter* infections. The data presented here describe the spontaneous colonization of the gerbil stomach with yeasts. Sequence analysis of a 198 bp fragment from Internally Transcribed Spacer 2 allowed identification as *Kazachstania heterogenica,* a yeast belonging to the *Kazachstania* (*Arxiozyma*) *telluris* complex.

All of the yeast-infected animals in the first study (naturally infected) and all animals experimentally infected with *K. heterogenica* were colonized mainly in the antrum, showing striking similarities between the colonization patterns of *K. heterogenica* and gastric helicobacters in the Mongolian gerbil. Namely, this pattern of colonization is similar to what Court et al. (2002) reported in Mongolian gerbils experimentally infected for 4 weeks with *H. pylori*. Similar results were obtained for Mongolian gerbils infected with *H. felis* and *H. bizzozeronii* (De Bock et al., 2006a and 2006b) and the results of the present study reveal that also *H. suis* shows the same colonization pattern. In the studies of De Bock and coworkers, as well as in the present study, antral biopsies were more often PCR- positive than fundus tissues and immunohistochemical staining for *Helicobacter* bacteria showed bacteria mainly in the antrum. Nevertheless, bacteria could also be found near the forestomach-stomach transition zone. Remarkably, *K. heterogenica* was also found near the forestomach-stomach transition zone in some animals in the present study.

When infected with gastric *Helicobacter* species, Mongolian gerbils develop lesions similar to those seen in humans, such as chronic active gastritis, intestinal metaplasia, gastric ulcer and even gastric cancer (Watanabe et al., 1998; Ikeno et al., 1999)**.** Court et al. (2002) have found that *H. pylori*-infected Mongolian gerbils show an inflammatory response in their antral mucosa at four weeks after infection. In contrast, little or no inflammatory changes were observed in the corpus mucosa. In the present study, similar changes were observed in Mongolian gerbils infected for 3 weeks with *H. suis* alone.

Three out of seventeen animals naturally infected with *K. heterogenica* suffered from mild lymphocytic infiltrates in association with *K. heterogenica*, indicating that infection with these yeast cells may independently cause mild pathologic changes in the gastric mucosa of Mongolian gerbils. In total, twelve separate colonies were tested in the present study. All of

them were shown to be *K. heterogenica.* However, it can not be excluded that other yeast species were also present in the gerbil stomachs in lower numbers. Possibly, other yeast species can also have an effect on inflammation. For instance, Kurtzman et al. (2005) have found a chronic inflammation with lymphocytes in a minority of mice infected with *Candida pintolopesii,* a closely related yeast species. Moreover, Schofield et al. (2005) have described an immune response elicited by gastric candidiasis in germ-free immunocompetent C57BL/6 and BALB/c mice. In contrast to the naturally infected animals in the present study, none of the animals infected experimentally with *K. heterogenica* showed a gastric lymphocytic infiltrate, except one *Kazachstania*-infected animal of group 2 in the *Helicobacter/Kazachstania* co-infection study. All experimentally infected animals in the *Kazachstania* infection study were euthanized within twenty two days after the first inoculation. So possibly, the naturally infected animals were colonized for a longer time, although this can not be stated with certainty.

To our knowledge, this is the first report on co-infection of *Helicobacter* and yeasts colonizing the stomach. Several interactions between *H. pylori* and other micro-organisms have already been described. Ansorg and Schmid (1998) described the possibility of *H. pylori* to adhere to different *Candida* species. Moreover, Rokka et al. (2006) found that culture supernatants of different lactobacilli inhibit the *in vitro* growth of *H. pylori*. The third experiment in this paper has shown that gerbil stomachs can be colonized simultaneously with *H. suis* and *K. heterogenica*. Colonization with *K. heterogenica* did not seem to influence the degree of colonization with *H. suis.* However, a significant increase in inflammation was observed in animals infected with both micro-organisms, already three weeks after experimental *H. suis* infection. The underlying mechanism remains to be investigated. Nevertheless, these results show that the uncontrolled presence of *K. heterogenica* can lead to an overestimation of the inflammation and thus pathology caused by *Helicobacter* organisms in Mongolian gerbils. Therefore, Mongolian gerbils having a gastric infection with *K. heterogenica* or related yeasts should be considered unsuitable for experimental research on gastric helicobacters.

The stomach mucosa by mere secretion of acid constitutes an antimicrobial barrier, and thus a very harsh environment for microbes. The fact that two totally different classes of micro-organisms colonize the same stomach regions suggests that these sites constitute special niches where these organisms find environmental factors suiting some very specific needs. Both antrum pyloricum and limiting ridge constitute transition zones between an acid-secreting mucosa and a non-acid-secreting mucosa. De Bock et al. (2006b) showed that *H.*

felis induced loss of parietal cells from the limiting ridge onwards and progressing in time. Simultaneously, bacteria moved their residence in parallel with their preferred niche. Further *in vivo* and *in vitro* studies are needed not only to investigate the unique features of *K. heterogenica* but also to further elucidate its possible interactions with gastric helicobacters.

Acknowledgements

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Chapter 2

Helicobacter suis Causes Severe Gastric Pathology in Mouse and Mongolian Gerbil Models of Human Gastric Disease

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Abstract

Background: "*Helicobacter (H.) heilmannii"* type 1 is the most prevalent gastric non-*H. pylori Helicobacter* species in humans suffering from gastric disease. It has been shown to be identical to *H. suis*, a bacterium which is mainly associated with pigs. To obtain better insights into the long-term pathogenesis of infections with this micro-organism, experimental infections were carried out in different rodent models.

Methodology/Principal findings: Mongolian gerbils and mice of two strains (BALB/c and C57BL/6) were infected with *H. suis* and sacrificed at 3 weeks, 9 weeks and 8 months after infection. Gastric tissue samples were collected for PCR analysis, histological and ultrastructural examination. In gerbils, bacteria mainly colonized the antrum and a narrow zone in the fundus near the forestomach/stomach transition zone. In both mice strains, bacteria colonized the entire glandular stomach. Colonization with *H. suis* was associated with necrosis of parietal cells in all three animal strains. From 9 weeks after infection onwards, an increased proliferation rate of mucosal epithelial cells was detected in the stomach regions colonized with *H. suis*. Most gerbils showed a marked lymphocytic infiltration in the antrum and in the forestomach/stomach transition zone, becoming more pronounced in the course of time. At 8 months post infection, severe destruction of the normal antral architecture at the inflamed sites and development of mucosa-associated lymphoid tissue (MALT) lymphoma-like lesions were observed in some gerbils. In mice, the inflammatory response was less pronounced than in gerbils, consisting mainly of mononuclear cell infiltration and being most severe in the fundus.

Conclusions/Significance: *H. suis* causes death of parietal cells, epithelial cell hyperproliferation and severe inflammation in mice and Mongolian gerbil models of human gastric disease. Moreover, MALT lymphoma-like lesions were induced in *H. suis*-infected Mongolian gerbils. Therefore, the possible involvement of this micro-organism in human gastric disease should not be neglected.

Introduction

Although infection with *Helicobacter (H.) pylori* is considered to be the major cause of gastritis, peptic ulcer disease (Kusters et al., 2006; Marshall and Warren, 1984), gastric adenocarcinoma (Parsonnet et al., 1991) and mucosa-associated lymphoid tissue (MALT) lymphoma (Parsonnet et al., 1994) in humans, these gastric diseases have also been associated with other helicobacters, nowadays referred to as gastric non-*H. pylori Helicobacter* (NHPH) species or "*H. heilmannii"* (Morgner et al., 1995; Stolte et al., 1997; Debongnie et al., 1998; Mrgner et al., 2000). The latter, however, has never been a validated species name, since "*H. heilmannii"* represents a group of closely related, but distinct bacterial species, mainly present in different animal species and including *H. felis, H. bizzozeronii, H. salomonis, "Candidatus* H. heilmannii" and *H. suis* (Haesebrouck et al., 2009). A common feature of these bacteria is their very fastidious nature, which seriously hampers the progress of gastric NHPH-related research. *H. suis* has only recently been cultured *in vitro* (Baele et al., 2008) and is in fact identical to *"H. heilmannii"* type 1 (O'Rourke et al., 2004b). It is the most prevalent gastric NHPH species in humans (Van den Bulck et al., 2005; De Groote et al., 2005). Moreover, its prevalence is probably underestimated since histological examination of a gastric biopsy, which is commonly performed in humans suffering from gastric disease, is considered not to be the best diagnostic test for infections with *H. suis* and other NHPH species (Debongnie et al., 1995).

Numerous studies have boosted the knowledge regarding the pathogenesis of *H. pylori* infections in humans. In contrast, only very few data are available dealing with the pathogenesis of *H. suis* infections in humans (Haesebrouck et al., 2009). In the past, several infection studies have been performed in mice with *"H. heilmannii"* or "tightly coiled spiral bacteria", however often without a clear identification of these bacteria to the species level. Moreover, mucus or homogenized gastric tissue of infected mice, pigs or non-human primates was always used as inoculum (Moura et al., 1993; Peterson et al., 2001; Park et al., 2003; O'Rourke et al., 2004a; Cinque et al., 2006; Nakamura et al., 2007; Nishikawa et al., 2007; Park et al., 2008). This implies that other micro-organisms were also inoculated along with the helicobacters, which might influence the results, as has been shown for gastric yeasts interfering with a gastric *H. suis* infection in Mongolian gerbils (Flahou et al., 2010).

To obtain better insights into the pathogenesis of human gastric diseases associated with *H. suis*, experimental infections with pure cultures of the bacterium are essential. In *H.*

pylori research, different rodent models have been shown to be very useful to obtain significant insights into the pathogenesis of this infection (O'Rourke and Lee, 2003; Rogers and Fox, 2004). Therefore, in the present study, C57BL/6 mice, BALB/c mice and Mongolian gerbils were used to explore the interactions between *H. suis* and the gastric mucosa. Mainly in Mongolian gerbils, long-term infection with *H. suis* was associated with severe gastric pathology, including necrosis of gastric epithelial cells and the development of gastric MALT lymphoma-like lesions.

Materials and Methods

Ethics statement

The *in vivo* experimental protocol was approved by the Ethical Committee of the Faculty of Veterinary Medicine, Ghent University, Belgium (EC 2007/022; May 21, 2007).

Animal and bacterial strains

Twenty-seven specific-pathogen-free (SPF) female six-week-old mice of each of two strains (BALB/c and C57BL/6) were purchased from Harlan NL (Horst, The Netherlands). Twenty-seven female SPF outbred gerbils (Crl:MON) of six weeks old were obtained from Charles River Laboratories (Brussels, Belgium). Preliminary screening showed these animals to be free of *Kazachstania heterogenica* (Flahou et al., 2010). All animals were fed and housed as described previously (De Bock et al., 2006).

H. suis strain HS5 was isolated from the gastric mucosa of a sow as described previously [10]. Bacteria were grown under microaerobic conditions (85% N_2, 10% CO_2, 5% O_2; 37°C; 72 h) on biphasic Brucella (Oxoid, Basingstoke, UK) culture plates supplemented with 20% fetal calf serum (HyClone, Logan, UT, USA) and Vitox supplement (Oxoid) (Flahou et al., 2010). The bacteria were harvested and the final concentration was adjusted to 2 x 10^8 viable bacteria/ml, as determined by counting in an improved Neubauer counting chamber.

Experimental procedure

Both for the mice strains and gerbils, 18 animals were inoculated three times at 48 hours intervals with 0.4 ml of the bacterial suspension. Nine animals of each strain (BALB/c, C57BL/6 and Mongolian gerbil) were inoculated with Brucella broth (Oxoid) with a pH of 5

and served as negative controls. Inoculation was performed intragastrically under isoflurane anaesthesia, using a ball-tipped gavage needle. At 3 weeks, 9 weeks and 8 months after the first inoculation, six *H. suis* infected and three control animals of each group were euthanized by cervical dislocation under isoflurane anaesthesia. The stomach of each animal was resected and samples were taken for PCR analysis, histopathological and ultrastructural examination.

PCR analysis.

From each animal, one sample of approximately 4 mm^2 was taken both in fundus and antrum. The DNeasy Tissue Kit (Qiagen, Hilden, Germany) was used for DNA extraction according to the manufacturer's protocol. All samples were screened for the presence of *H. suis* DNA using an *H. suis* specific PCR (De Groote et al., 2000).

Histological and ultrastructural examination

Two longitudinal strips of gastric tissue were cut from the oesophagus to the duodenum along the greater curvature. One strip was fixed in 4% phosphate buffered formaldehyde, processed by standard methods, and embedded in paraffin for light microscopy. Nine consecutive sections of 5 µm were cut. After deparaffinization and hydration, heat-induced antigen retrieval was performed in citrate buffer (pH 6.0) using a microwave oven. Slides were incubated with 3% H_2O_2 in methanol (5 min) and 30% goat serum (30 min) to block endogenous peroxidase activity and non-specific reactions, respectively.

On the first section, the *H. suis* colonization density was scored, as shown in Table 1, according to the Updated Sydney System (Dixon et al., 1996) after immunohistochemical staining using a polyclonal genus-specific rabbit anti-*H. pylori* antibody (1/320; DakoCytomation, Glostrup, Denmark) (De Groote et al., 2000). The second section was stained with haematoxylin and eosin (H&E) to score the intensity of the overall gastritis (infiltration with mononuclear and polymorphonuclear cells), also using a visual analog scale similar to the Updated Sydney System (Dixon et al., 1996) but with some modifications, as shown in Table 2. Both diffuse infiltration and the presence of lymphoid aggregates and lymphoid follicles were taken into consideration. Moreover, these H&E stained sections were used for counting the numbers of neutrophils. For differentiation between T and B lymphocytes, staining of CD3 and CD20 antigens, respectively, was performed on sections three and four, using a polyclonal rabbit anti-CD3 antibody (1/100; DakoCytomation) and a polyclonal rabbit anti-CD20 antibody (1/100; Thermo Scientific, Fremont, USA),

respectively. Incubation with primary antibodies directed against *Helicobacter,* CD3 and CD20 was followed by incubation with a biotinylated goat anti-rabbit IgG antibody (1/500; DakoCytomation). After rinsing, the sections were incubated with a streptavidin-biotin-HRP complex (DakoCytomation) and the colour was developed with diaminobenzidine tetrahydrochloride (DAB) and H_2O_2. A primary antibody directed against the F4/80 surface marker (1/50; Santa Cruz Biotechnology, Inc., Santa Cruz, USA) was used for highlighting mature macrophages. Detection was done using a rat ABC staining system (Santa Cruz Biotechnology, Inc.) Apoptotic cells were identified by immunohistochemical staining on section six using a rabbit polyclonal antibody directed against active caspase-3 and an anti-rabbit HRP-AEC cell and tissue staining kit (R&D Systems, Minneapolis, USA). Replicating cells were identified on section seven using a mouse monoclonal anti-Ki67 antibody (1/25; Novocastra Laboratories Ltd, Newcastle upon Tyne, UK) and a biotinylated goat anti-mouse IgG antibody (1/200; DakoCytomation). Subsequent visualization was done as described for *Helicobacter*, CD3 and CD20 staining. Parietal cells were identified by immunohistochemical staining for the hydrogen potassium ATPase using a mouse monoclonal antibody (1/200; Abcam Ltd, Cambridge, UK) and a biotinylated goat anti-mouse IgG antibody (1/200; DakoCytomation). Subsequent visualization was done as described for *Helicobacter*, CD3 and CD20 staining. Finally, a monoclonal mouse anti-cytokeratin antibody (1/50; DakoCytomation) was used to highlight lymphoepithelial lesions (LEL's). These sections were further processed using an EnVision^{TM}+ system for use with mouse primary antibodies (DakoCytomation).

During each immunohistochemical staining protocol, appropriate washing steps were included. After counterstaining of nuclei with haematoxylin, slides were dehydrated and mounted.

To determine the numbers of cells belonging to defined immune cell populations (T cells, B cells, macrophages and neutrophils) *in situ*, positive cells were counted in five randomly chosen High Power Fields (magnification: x400), both in fundus and antrum. For each animal, an average of the positive cell count was determined for both stomach regions. For inflammatory aggregates and lymphoid follicles, the average area percentage of T and B cell populations was determined by using Optimas 6.51 image analysis software (Media Cybernetics, Inc., Bethesda, USA). Finally, for each individual animal, these data were translated into the respective percentages of both lymphocytic populations in aggregates and follicles.

The rates of apoptosis and epithelial cell proliferation were also determined by counting the number of active caspase-3 positive and Ki67 positive epithelial cells, respectively, in five randomly chosen High Power Fields at the level of the gastric pits, both in antrum and fundus. For each animal, an average of the positive cell count was determined for both stomach regions.

The second longitudinal strip of tissue was processed for Transmission Electron Microscopy (TEM) as described previously (De Bock et al., 2006). Semithin sections (2µm) were stained with toluidine blue to select the most appropriate regions for ultrathin sectioning. Ultrathin sections were stained with uranyl acetate and lead citrate solutions before examining under a Jeol EX II transmission electron microscope at 80 kV.

Statistical analysis

Colonization and inflammation scores of infected groups of different animal strains were compared pairwise using the Wilcoxon rank sum test, using time as a stratification factor and a Bonferroni adjusted significance level of 0.05/3=0.0167. For analysis of defined immune cell populations, data from the three time points post infection were analyzed separately as appropriate, by analysis of variance with a Bonferroni post hoc test or the Wilcoxon rank sum test with Bonferroni adjustment of significance levels for multiple comparisons. The numbers of Ki67 positive and active caspase-3 positive cells were analyzed by analysis of variance with a Bonferroni post hoc test assuming normally distributed error terms.

Results

Throughout the experiment, the animals were checked on a daily basis. Even by the end of the experiment, no animals showed overt clinical signs of discomfort or pain.

Bacterial colonization

Throughout the experiment, all control animals were negative for *H. suis* in PCR and immunohistochemical staining. All infected animals were PCR-positive in both antrum and fundus. The colonization levels of the infected animals are shown in Table 1. In general, colonization scores were higher in C57BL/6 mice compared to BALB/c mice (P = 0.0016 for fundus; P = 0.0035 for antrum). For both mice strains and for all timepoints, bacteria were

seen throughout the entire glandular stomach, both in antrum and fundus. For the gerbils, however, colonization in the antrum was moderate to marked at all timepoints (Table 1; Figure 1A), whereas colonization in the fundus remained limited to a very narrow zone at the limiting ridge (Figure 1B). Moreover, in some gerbils, no bacteria could be visualized in the fundic region. Although not statistically significant, colonization scores in infected BALB/c mice tended to drop in the course of time (P = 0.18 for fundus; P = 0.08 for antrum), whereas a tendency to increase was observed in the antrum of Mongolian gerbils (P = 0.15).

Table 1: Gastric *H. suis* colonization in experimentally infected mice and gerbils

Time pi [a]	Group	Colonization score fundus [b]				Colonization score antrum [b]			
		0 [c]	1 [c]	2 [c]	3 [c]	0 [c]	1 [c]	2 [c]	3 [c]
3 weeks	**BALB/c**	0	3	3	0	0	1	3	2
	C57BL/6	0	0	6	0	0	0	3	3
	Gerbil	1	5	0	0	0	2	2	2
9 weeks	**BALB/c**	0	4	2	0	1	2	3	0
	C57BL/6	0	2	4	0	0	1	4	1
	Gerbil	3	3	0	0	0	3	1	2
8 months	**BALB/c**	0	6	0	0	2	2	2	0
	C57BL/6	0	1	5	0	0	0	3	3
	Gerbil	2	4	0	0	0	0	1	5

[a] pi: post infection
[b] Displayed are the numbers of animals out of six animals in each infected group with a specific colonization score.
[c] 0, no bacteria; 1, mild colonization; 2, moderate colonization; 3, marked colonization

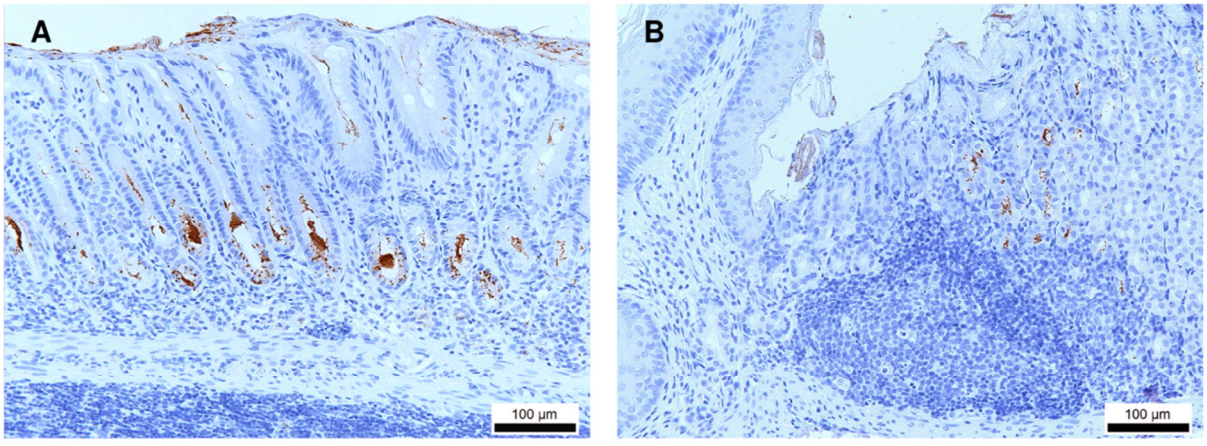

Figure 1: Immunohistochemical *Helicobacter* staining of a gerbil stomach, showing *H. suis* colonization. *H. suis* bacteria (brown) are seen (A) in the glands of the antrum and (B) at the forestomach/stomach transition zone, accompanied by a focal infiltrate of mononuclear cells. Original magnification: 200x.

Inflammation

At all timepoints, all uninfected control animals showed normal histomorphology, with little inflammatory cell infiltration in the gastric mucosa. For both mice strains and for the gerbils, inflammation in infected animals was characterized by mononuclear and polymorphonuclear cell infiltration in the lamina propria mucosae or the tunica submucosa or both, depending on the individual animal. The individual overall inflammation scores of the *H. suis*-infected animals are shown in Table 2. For BALB/c mice at all three timepoints and C57BL/6 mice at 8 months post infection, inflammation was observed mainly in the fundus. In general, BALB/c mice showed higher inflammation scores in the fundic region, compared to C57BL/6 mice (P=0.004). In Mongolian gerbils, however, inflammation in the fundic region was always limited to a very narrow zone near the limiting ridge, i.e. the forestomach-stomach transition zone (Figure 1B). Generally, a more severe inflammation was observed in the antrum of infected gerbils, compared to BALB/c and C57BL/6 mice (P<0.0001).

Table 2. Overall gastric inflammation in experimentally infected mice and gerbils

Time pi [a]	Group	Inflammation score fundus [b]					Inflammation score antrum [b]				
		0 [c]	1 [c]	2 [c]	3 [c]	4 [c]	0 [c]	1 [c]	2 [c]	3 [c]	4 [c]
3 weeks	**BALB/c**	4	2	0	0	0	4	2	0	0	0
	C57BL/6	5	1	0	0	0	5	1	0	0	0
	Gerbil	5	1	0	0	0	1	1	2	2	0
9 weeks	**BALB/c**	0	4	2	0	0	6	0	0	0	0
	C57BL/6	5	1	0	0	0	3	3	0	0	0
	Gerbil	1	3	2	0	0	0	0	1	4	1
8 months	**BALB/c**	0	0	4	2	0	5	1	0	0	0
	C57BL/6	1	1	4	0	0	5	1	0	0	0
	Gerbil	0	4	2	0	0	0	0	2	1	3

[a] pi: post infection
[b] Displayed are the numbers of animals out of six animals in each infected group with a specific overall inflammation score.
[c] 0, no infiltration with mononuclear and/or polymorphonuclear cells; 1, mild diffuse infiltration with mononuclear and/or polymorphonuclear cells or the presence of one small (50-200 cells) aggregate of inflammatory cells; 2, moderate diffuse infiltration with mononuclear and/or polymorphonuclear cells and/or the presence of 2-4 inflammatory aggregates; 3, marked diffuse infiltration with mononuclear and/or polymorphonuclear cells and/or the presence of at least five inflammatory aggregates; 4, diffuse infiltration of large regions with large aggregates of mononuclear and/or polymorphonuclear cells

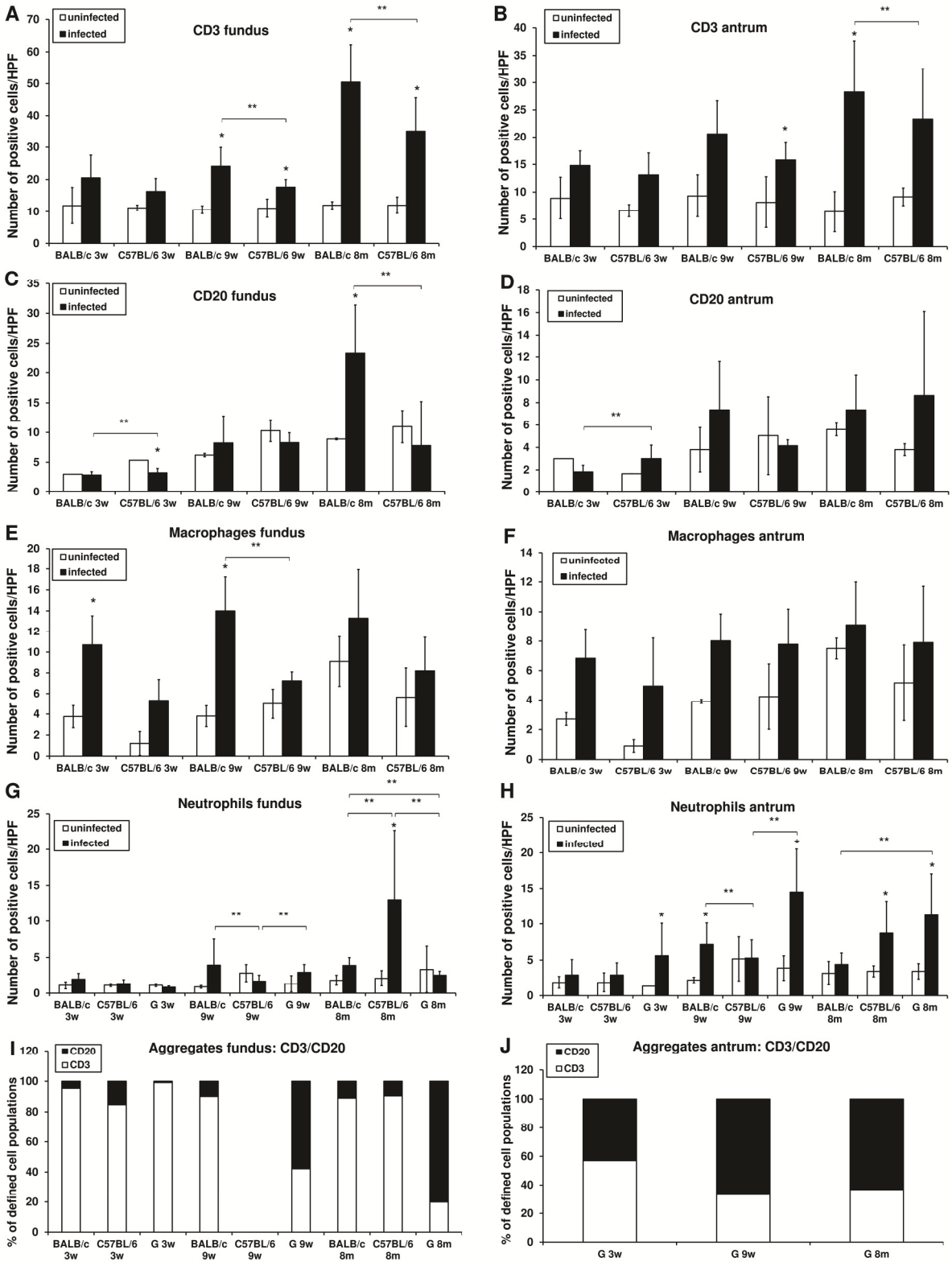

Figure 2: Counting of defined immune cell populations and image analysis of lymphocytic aggregates and lymphoid follicles. (A-H) Shown are the average (±SD) numbers of cells/High Power Field (HPF) belonging to a defined immune cell population, including T cells (CD3-positive), B cells (CD20-positive), macrophages (F4/80-positive) and neutrophils. * depict statistically significant differences between corresponding *H. suis*-infected and uninfected control animals. ** depict statistically significant differences between infected groups of different animal strains at the same time point post infection. (I-J) Shown are the average percentages of T (CD3-positive) and B (CD20-positive) lymphocytes in lymphocytic aggregates and lymphoid follicles in *H. suis*-infected animals. BALB/c and C57BL/6, mice strains; G, gerbil; 3w, 3 weeks post infection; 9w, 9 weeks post infection; 8m, 8 months post infection.

Counting of defined immune cell poulations is shown in Figure 2. At 9 weeks and 8 months post infection, an increase of the number of T cells (CD3-positive) was observed in the fundus of infected animals of both mice strains (Figure 2A). This increase was always higher for BALB/c mice, compared to C57BL/6 mice. Similar results were seen in the antrum, however not always statistically significant (Figure 2B). At 3 and 9 weeks post infection, no statistically significant increase could be observed for diffuse infiltration of the fundic mucosa with B cells (CD20 positive) in infected animals as compared to control animals. However, at 8 months post infection, a higher number of B cells was detected only in the fundus of infected BALB/c mice (Figure 2C). In the antrum of both mice strains, no significant differences were found for B cell infiltration between uninfected and infected animals (Figure 2D). For BALB/c mice infected for 3 and 9 weeks, a significantly higher number of macrophages could be detected in the fundus when compared to the control animals (Figure 2E). For C57BL/6 mice, similar results could be observed, however not statistically significant. Additionally, for the antrum of both mice strains, this increase was never statistically significant (Figure 2F). In both mice strains, neutrophils were always vastly outnumbered by mononuclear cells. At 8 months post infection, infected C57BL/6 mice showed higher numbers of neutrophils, compared to control animals, both in fundus and antrum (Figure 2G and Figure 2H). An increase of the number of neutrophils could be seen in BALB/c mice at 9 weeks post infection, an observation which was absent at the last time point of euthanasia.

The presence of inflammatory cell aggregates and lymphoid follicles is shown in Table 3. Both the number of *H. suis*-infected animals with inflammatory cell aggregates and the mean number of aggregates/animal were always equal (3 weeks post infection) or higher (9 weeks and 8 months post infection) for BALB/c mice compared to C57BL/6 mice. At 8 months post infection, the fundic region of all *H. suis*-infected BALB/c contained large lymphoid aggregates (Figure 3). For both mice strains, lymphoid aggregates and follicles were mainly composed of T cells (Figure 2I).

Table 3. Aggregates of inflammatory cells and lymphoid follicles in mice and gerbils after experimental infection with *H. suis*

Time pi [a]	Group	Number of animals [b] with inflammatory aggregates (>50 cells) [c]	Mean number of aggregates/animal in this group [c]	LF [e]	EGC [f]
3 weeks	**BALB/c**	1	0.17	0	0
	C57BL/6	1	0.17	0	0
	Gerbil	NA [d]	NA [d]	4	1
9 weeks	**BALB/c**	5	1.33	1	0
	C57BL/6	0	0	0	0
	Gerbil	NA [d]	NA [d]	4	4
8 months	**BALB/c**	6	4.33	2	1
	C57BL/6	4	1.33	1	0
	Gerbil	NA [d]	NA [d]	6	6

[a] pi: post infection
[b] out of 6 animals in each infected group
[c] both parameters were determined on the entire longitudinal H&E stained tissue sections
[d] not applicable
[e] presented are the numbers of animals (out of 6 animals in each infected group) with gastric lymphoid follicles (LF)
[f] presented are the numbers of animals (out of 6 animals in each infected group) with expanding germinal centers (EGC) in gastric lymphoid follicles

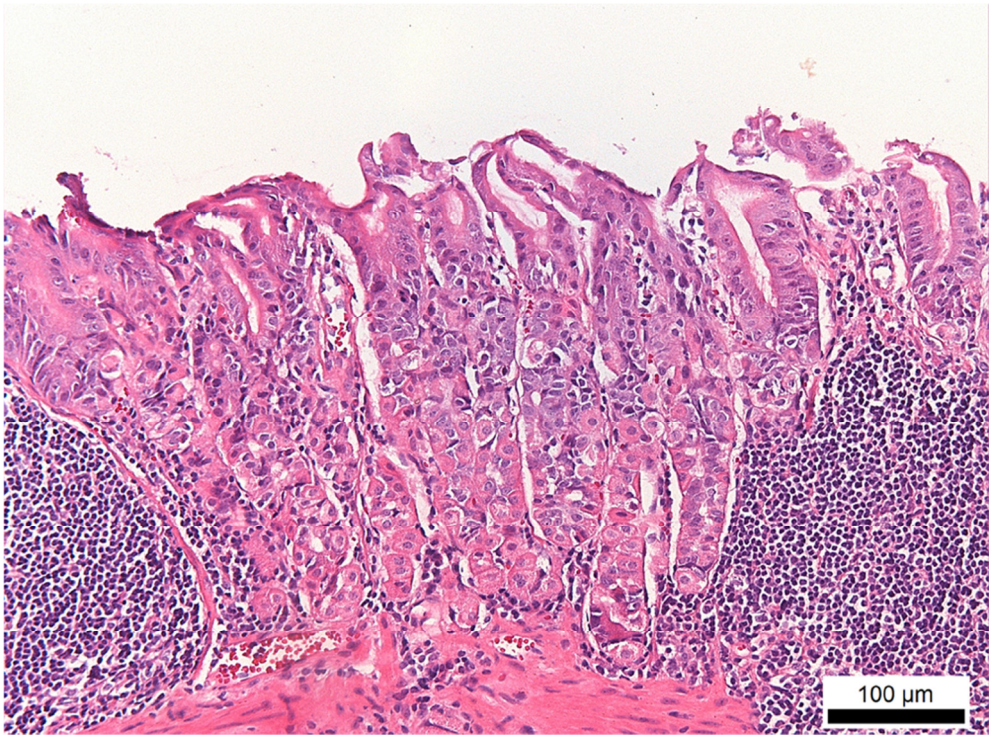

Figure 3: H&E staining of the fundus of a *H. suis*-infected BALB/c mouse. In this animal, infected for 8 months, two large lymphoid aggregates can be seen in the lamina propria mucosae, accompanied by loss of normal mucosal architecture. Original magnification: 200x.

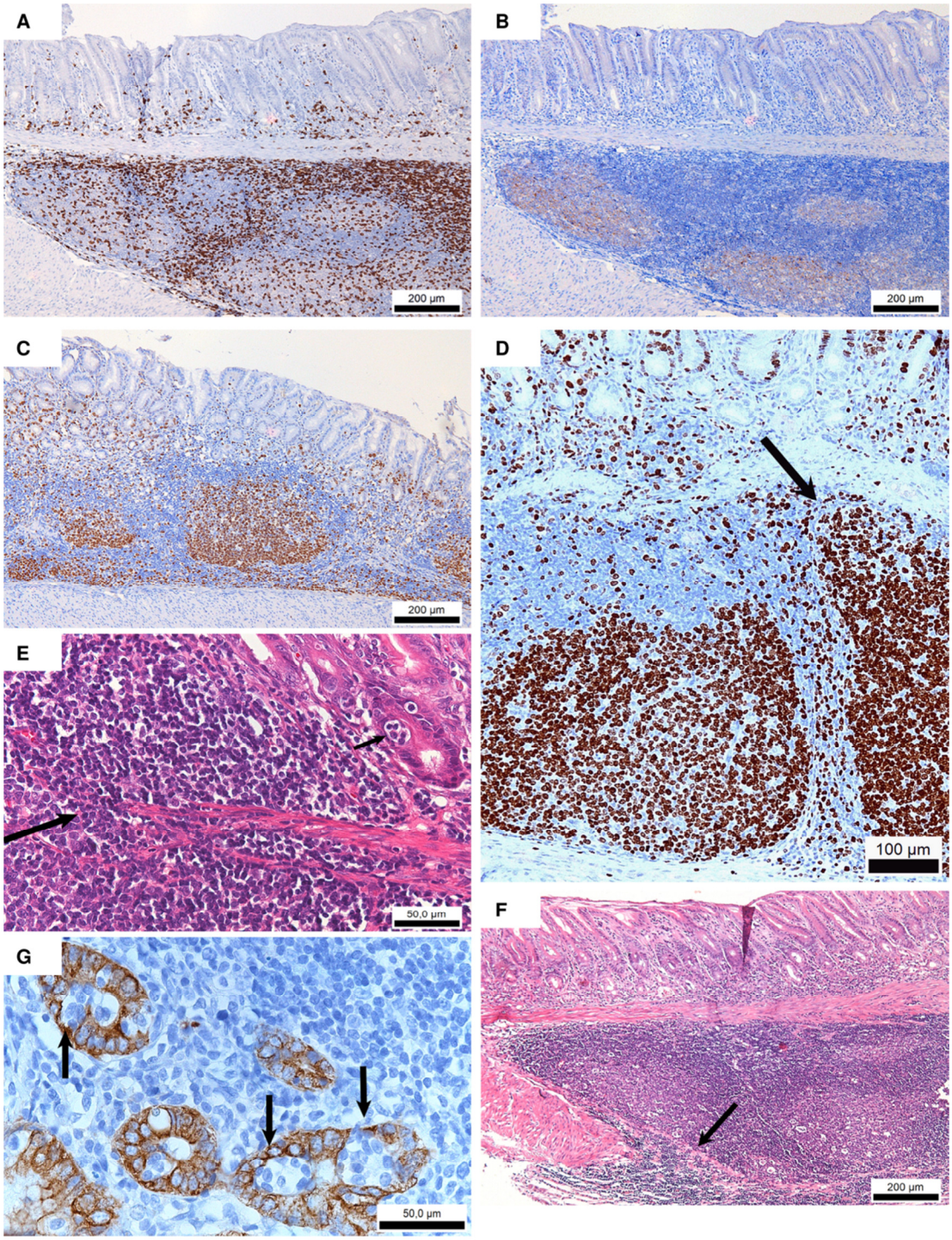

Figure 4: Gastric inflammation in *H. suis*-infected Mongolian gerbils. (A) CD3 staining of the antrum of a gerbil infected with *H. suis* for 8 months, showing T-lymphocytes (brown). Original magnification: 100x. (B) CD20 staining of the antrum of a gerbil infected with *H. suis* for 8 months, showing B lymphocytes (brown) in germinal centers of lymphoid follicles. Original magnification: 100x. (C) Ki67 staining of the antrum of a gerbil infected with *H. suis* for 9 weeks showing distinct proliferation (brown) in the germinal centers of the lymphoid follicles. Original magnification: 100x. (D) Ki67 staining of the antrum of a gerbil infected with *H. suis* for 8 months showing large, hyperproliferative (brown) and irregular (arrow) germinal centers of the lymphoid follicles. Original magnification: 200x. (E) H&E staining of the antrum of a gerbil infected with *H. suis* for 8 months showing lymphocytic infiltration of the lamina muscularis mucosae (large arrow) and a small lymphoepithelial lesion (small arrow). Original magnification: 400x. (F) H&E staining of the antrum of a gerbil infected with *H. suis* for 8 months showing infiltration of the tunica muscularis with mononuclear cells (arrow). Original magnification: 100x. (G) Cytokeratin staining of the antral epithelium (brown) of a gerbil infected with *H. suis* for 8 months showing numerous lymphoepithelial lesions (arrows). Original magnification: 400x.

For *H. suis*-infected Mongolian gerbils, the vast majority of inflammatory cells consisted of T and B lymphocytes. Moreover, diffuse infiltrates and large inflammatory aggregates were very often fused together, which made counting of T and B cell populations virtually impossible. Organization into lymphoid follicles was present in the majority of gerbils at 9 weeks and 8 months post infection (Table 3; Figure 4A and Figure 4B). Image analysis revealed that from 9 weeks of infection onwards, lymphocytic aggregates and follicles in these animals contained at least 60% B cells (CD20 positive and CD3 negative) (Figure 2I and Figure 2J). This fraction was smaller at 3 weeks after infection. In gerbils infected for at least 9 weeks, a distinct B-cell proliferation in the germinal centers of the lymphoid follicles was present (Figure 4C). In gerbils infected for 8 months, these germinal centers were often large, hyperproliferative and irregular (Figure 4D). Moreover, in gerbils infected for 9 weeks and 8 months, severe destruction of the normal antral mucosal architecture with disruption of the lamina muscularis mucosae (Figure 4E) was seen in 4 and in all animals, respectively. In mice, only at 8 months after infection, infiltration and destruction of the lamina muscularis mucosae was detected in 1/3 of the animals. In one gerbil, the tunica muscularis was also invaded by a large number of lymphocytes (Figure 4F). Lymphoepithelial lesions could be detected in the mucosa of 2 gerbils infected with *H. suis* for 8 months (Figure 4E and Figure 4G). At all three timepoints post infection, diffuse infiltration of the antral mucosa with neutrophils was always higher in *H. suis*-infected gerbils compared to control animals (Figure 2H), although this cell type represented only a small fraction of the total population of inflammatory cells. Finally, two primary antibodies (F4/80, Santa Cruz Biotechnology, Inc.; MAC387, Abcam) were used to detect mature macrophages in the gerbils, however without success.

Gastric epithelial cell death

No differences in the average number of apoptotic cells were seen between control and infected animals, for all three animal strains at all timepoints (Figure 5). Transmission electron microscopy revealed that spiral-shaped *H. suis* bacteria were often closely associated with gastric epithelial, mainly parietal cells (Figure 6A) in all three animal strains at all three time points post infection. Frequently, these bacteria were seen surrounded by cellular debris of primary necrotic parietal cells with loss of plasma membrane integrity and formation of necrotic blebs (Figure 6B).

Immunohistochemical staining showed no clear loss of parietal cells in the fundus of infected animals of all three animal strains. However, compared to uninfected animals (Figure

7A), an obvious loss of parietal cells was often detected at the transition zone between fundus and antrum in *H. suis*-infected gerbils at all timepoints post infection (Figure 7B).

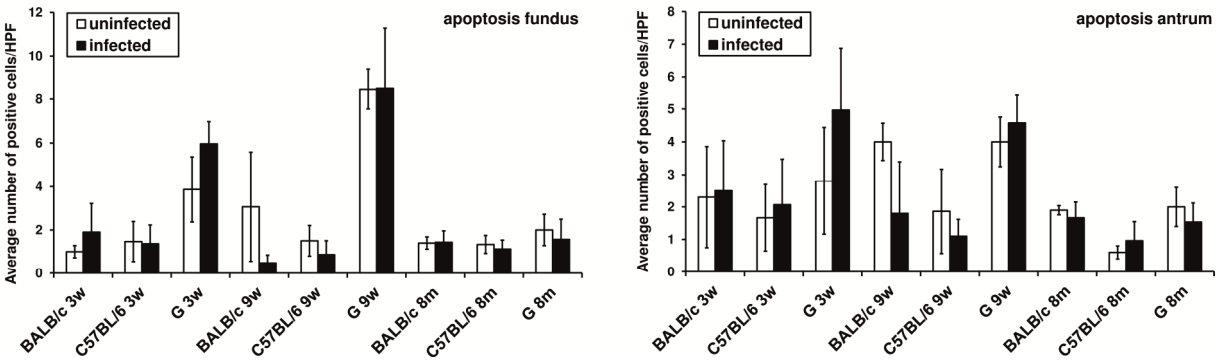

Figure 5: Apoptosis of gastric epithelial cells. For each individual animal, the rate of epithelial cell apoptosis was determined by counting the number of activated caspase-3 positive epithelial cells in five randomly chosen microscopic fields (magnification: x400) at the level of the gastric pits, both in fundus and antrum. Shown are the mean numbers of activated caspase-3-positive epithelial cells in each group. No significant differences were observed between *H. suis*-infected animals compared to uninfected control animals. BALB/c and C57BL/6, mice strains; G, gerbil; 3w, 3 weeks post infection; 9w, 9 weeks post infection; 8m, 8 months post infection.

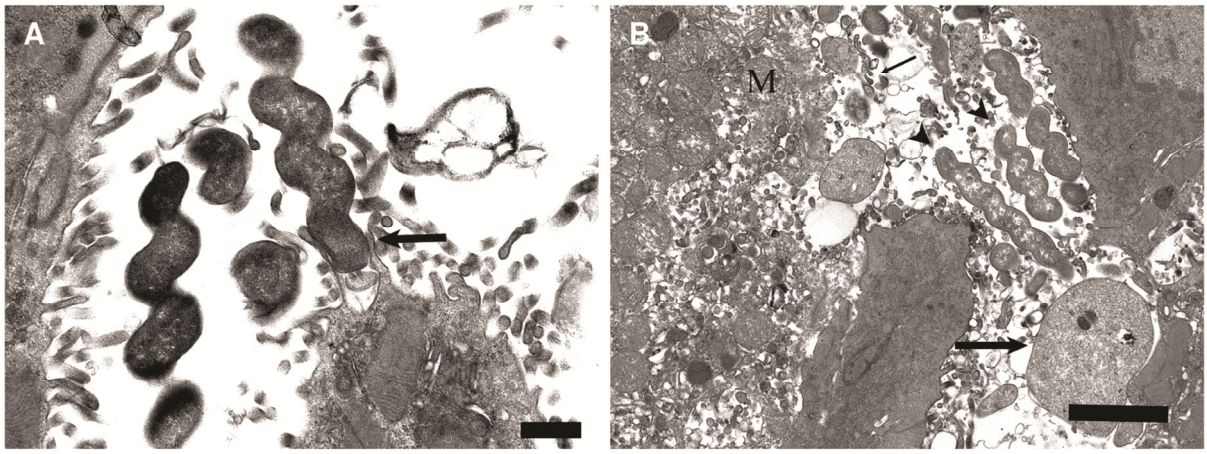

Figure 6. Transmission electron microscopic (TEM) images of BALB/c mice infected with *H. suis*. (A) In a BALB/c mouse infected for 9 weeks, spiral-shaped *H. suis* bacteria are seen in close association with parietal cell microvilli (arrow). Bar = 500 nm. (B) TEM image of a BALB/c mouse infected for 3 weeks with *H. suis* showing *H. suis* bacteria (arrowheads) in between necrotic debris of gastric parietal cells, which can be recognized by the typical abundance of mitochondria (M) and deep folding of the apical membrane. The large arrow indicates the presence of necrotic blebs and the small arrow shows loss of plasma membrane integrity. Bar = 2 µm.

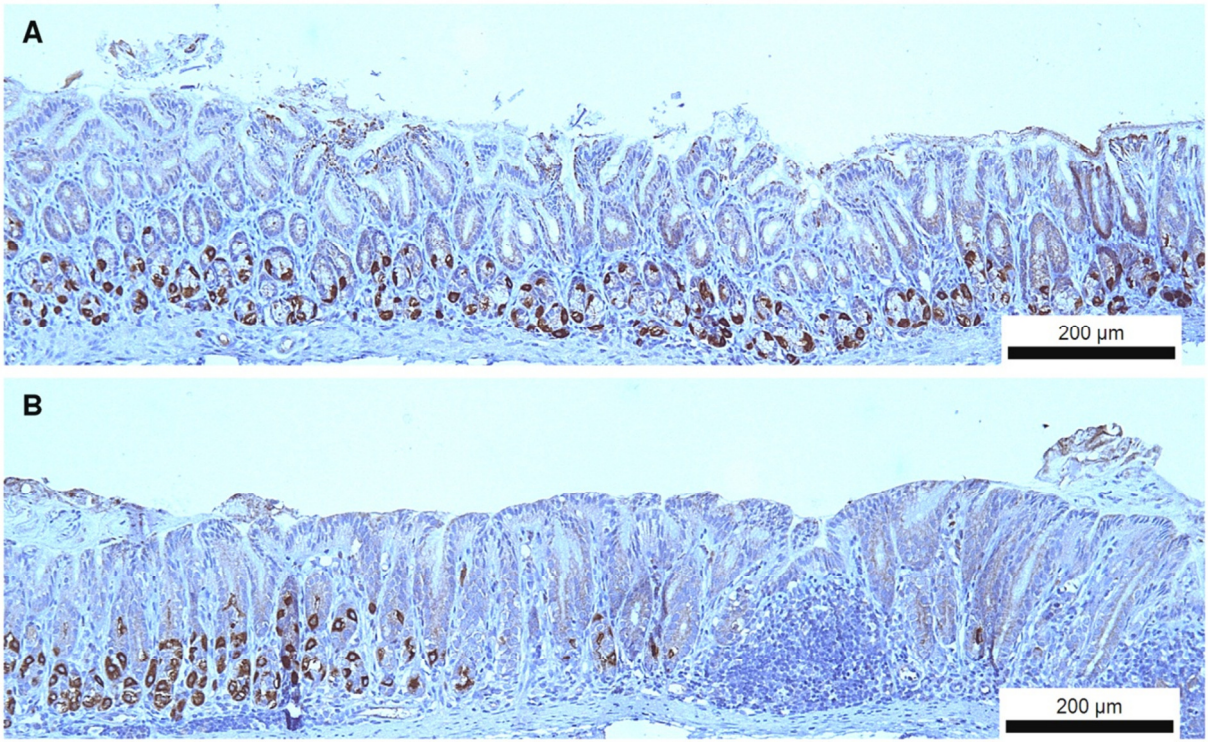

Figure 7. Immunohistochemical staining of the hydrogen potassium ATPase present in gastric parietal cells. (A) Moderate numbers of parietal (brown) cells are still present in the proximal antrum of uninfected Mongolian gerbils. Original magnification: 100x. (B) An abrupt loss of parietal cells is seen in the transition zone between fundus and antrum in Mongolian gerbils infected with *H. suis* for 9 weeks. Original magnification: 100x.

Gastric epithelial cell proliferation

Results of the gastric epithelial cell proliferation scoring are shown in Figure 8. Higher numbers of Ki67-positive, and thus proliferating epithelial cells were seen at 9 weeks and 8 months after infection in both fundus and antrum of *H. suis*-infected BALB/c mice, compared to control animals. Similar results were observed for C57BL/6 mice. In gerbils, a higher proliferation rate was seen in the antrum of infected animals at 8 months after infection. In the fundus of *H. suis*-infected gerbils, no significant increase of epithelial cell proliferation was observed.

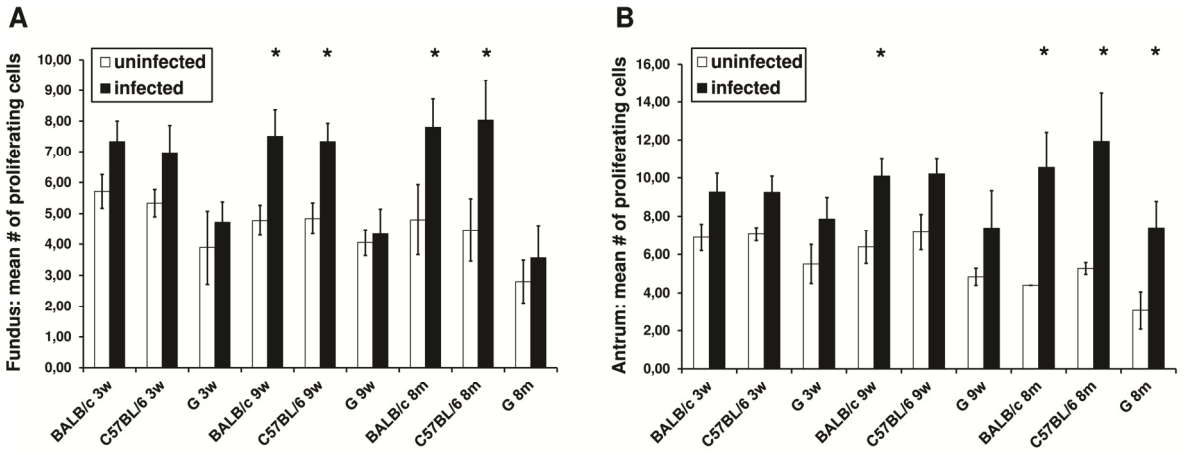

Figure 8: Proliferation of gastric epithelial cells. For each individual animal, the rate of epithelial cell proliferation was determined by counting the number of Ki67 positive epithelial cells in five randomly chosen microscopic fields (magnification: x400) at the level of the gastric pits, both in antrum and fundus. Shown are the mean numbers of Ki67-positive epithelial cells in each group. An * denotes significantly higher values for *H. suis* infected animals compared to uninfected control animals. BALB/c and C57BL/6, mice strains; G, gerbil; 3w, 3 weeks post infection; 9w, 9 weeks post infection; 8m, 8 months post infection.

Discussion

In the present study, at 3 and 9 weeks post infection, increased numbers of mature macrophages were observed in the gastric mucosa of *H. suis* infected mice, mainly in the BALB/c strain. In early *H. pylori* infection, macrophages are known to play an essential role as innate responders to released *H. pylori* factors (Ferrero, 2005; Wilson and Crabtree, 2007). They have been suggested to activate the adaptive immune response by producing factors such as IL-12, stimulating a Th1 response, and IL-10, which is considered a Th2-polarizing cytokine (Meyer et al., 2000; Wilson and Crabtree, 2007).

At 9 weeks and 8 months post infection, a more pronounced lymphocytic infiltration was observed in fundus and antrum of BALB/c mice, compared to C57BL/6 mice. This contrasts with the results of Cinque et al. (2006), who found a higher degree of inflammation in C57BL/6 mice inoculated with gastric mucus from "*H. heilmannii* type 1"-infected pigs, compared to BALB/c mice. Moreover, results similar to those of Cinque et al. (2006) have been reported for mice infected with *H. felis* (Mohammadi et al., 1996; Skagami et al., 1996; De Bock et al., 2005). This suggests that inflammation in these studies was mainly driven by a T-helper (Th) 1 response, since C57BL/6 mice have been described genetically as Th1 responders (Heinzel et al., 1989; Ferrero et al., 2000). In contrast, BALB/c mice are considered predominant Th2 responders. The higher inflammation in BALB/c mice in the present study might therefore indicate that *H. suis* strain 5 elicits a more predominant Th2 response (Heinzel et al., 1989; Ferrero et al., 2000; O'Rourke and Lee, 2003). Indeed, only in

BALB/c mice infected for 8 months, significantly higher levels of B cell accumulation were observed, which suggests a more Th2-polarized response (Mueller et al., 1997; Wilson and Crabtree, 2007). Although this finding was not reflected in the fraction of B cells in lymphoid aggregates and follicles and although no MALT lymphoma-like lesions were seen in these mice in the present study, this might eventually lead to a severe B-cell lymphocytic proliferation and even gastric MALT lymphoma, as shown for BALB/c mice infected for over 18 months with *H. felis* (Enno et al., 1995).

Humans suffering from a "*H. heilmannii"* gastritis have been suggested to develop gastric MALT lymphoma more frequently than those suffering from a *H. pylori* gastritis (Stolte et al., 2002). Interestingly, in the present study, in some gerbils euthanized at 8 months after infection, severe inflammation had evolved to a pathology resembling gastric MALT lymphoma. Similar MALT lymphoma-like lesions have been described in mice infected for at least 18 months with different "*H. heilmannii"*-like strains, isolated *in vivo* in mice (O'Rourke et al., 2004a). Some of these inocula were shown to contain *H. suis* in a separate paper from the same authors (O'Rourke et al., 2004b). Nakamura et al. (2007) also described the development of gastric MALT lymphoma in C57BL/6 mice infected for at least 6 months with an *in vivo* "isolate" that was erroneously designated "*Candidatus* H. heilmannii", but that in fact belonged to the species *H. suis* (Baele et al., 2009). It is not clear why in the present study MALT lymphoma-like lesions were not detected in the mouse models. Differences in *H. suis* strains and duration of infection probably play an important role (O'Rourke et al., 2004a). Other micro-organisms which may have been present in the inoculum used by O'Rourke et al. (2004a) and Nakamura et al. (2007) might also have influenced lesion development (Flahou et al., 2010).

In Mongolian gerbils, inflammation was limited to the antrum and a narrow zone at the forestomach-stomach transition zone. These results are not surprising, since *H. suis* mainly colonizes these stomach regions in Mongolian gerbils, while it tends to colonize both antrum and fundus in mice. In "*H. heilmannii"* infected humans, colonization and inflammation are also mainly localized in the antrum (Stolte et al., 1997). Therefore, the Mongolian gerbil model more closely resembles the human situation. This together with our finding that Mongolian gerbils developed a more severe pathology after *H. suis* infection compared to mice, indicates that the Mongolian gerbil model may be more suitable for studying *H. suis*-host interactions.

In all infected animal strains, a relative absence of neutrophils was observed. Similar findings have been described for mice infected with *H. felis* (Ferrero et al., 2000; De Bock et

al., 2005). However, in the present study, an increase in the number of neutrophils was observed in C57BL/6 mice infected for 8 months. In BALB/c mice, a similar increase was observed, however less pronounced and only at 9 weeks post infection. At 8 months post infection, the level of neutrophil infiltration in this mouse strain had dropped back to basal levels. Possibly, different and fluctuating levels of IL-17 expression, a cytokine which has been shown to be a key regulator of neutrophil infiltration, could play a role, as has been described in long-term *H. pylori* infection studies in mice of different strains (Algood et al., 2007; Shiomi et al., 2008).

In this study we report that *H. suis* induces necrosis of gastric parietal cells, which may have important implications for the development of various gastric pathologies, such as gastric erosion and/or ulcer formation (Dixon, 2001), gastric atrophy and even gastric cancer (Shirin and Moss, 1998). These lesions all have been observed in *"H. heilmannii"*-infected humans (Morgner et al., 1995; Stolte et al., 1997; Debongnie et al, 1998) and are often accompanied by a gastritis (Stolte et al., 1997). The chronic gastritis observed in the present study is possibly caused by direct effects of *H. suis*, but most likely also driven indirectly by necrosis of gastric epithelial cells. Cell necrosis results in the release of cellular contents, including molecules involved in the promotion of inflammation (Fink and Cookson, 2005). Interestingly, mainly parietal cells were affected by necrosis, which has also been described for other non-*H. pylori* helicobacters, such as *H. felis* (De Bock et al., 2006). In the present study, also a clear increase of gastric epithelial cell proliferation was seen in the *H. suis* infected animals. For *H. pylori*, data derived from *in vitro* experiments suggest that this hyperproliferation may be a secondary response to increased cell death in order to maintain cell mass in the mucosa (Shirin and Moss, 1998). In any case, hyperproliferation may result in a shift towards population of gastric epithelium with immature cells, resulting in impaired gastric acid secretion (Dixon, 2001). Both hyperproliferation of the gastric epithelium and reduced gastric acid secretion due to loss of parietal cells may eventually lead to the development of gastric cancer (Shirin and Moss, 1998; Kusters et al., 2006).

In conclusion, our results clearly demonstrate the ability of a pure culture of *H. suis* to cause severe gastric pathology. In humans suffering from gastric disease, the possible involvement of this micro-organism should therefore not be neglected.

Acknowledgments

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Chapter 3

Protective immunization with homologous and heterologous antigens against *Helicobacter suis* challenge in a mouse model

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Abstract

Helicobacter (H.) suis colonizes the stomach of more than 60% of slaughter pigs and is also of zoonotic importance. Recently, this bacterium was isolated *in vitro*, enabling the use of pure cultures for research purposes. In this study, mice were immunized intranasally or subcutaneously with whole bacterial cell lysate of *H. suis* or the closely related species *H. bizzozeronii* and *H. cynogastricus*, and subsequently challenged with *H. suis*. Control groups consisted of non-immunized and non-challenged mice (negative control group), as well as of sham-immunized mice that were inoculated with *H. suis* (positive control group). Urease tests on stomach tissue samples at 7 weeks after challenge infection were negative in all negative control mice, all intranasally immunized mice except one, and in all and 3 out of 5 animals of the *H. cynogastricus* and *H. suis* subcutaneously immunized groups, respectively. *H. suis* DNA was detected by PCR in the stomach of all positive control animals and all subcutaneously immunized/challenged animals. All negative control animals and some intranasally immunized/challenged mice were PCR-negative. In conclusion, immunization using antigens derived from the same or closely related bacterial species suppressed gastric colonization with *H. suis*, but complete protection was only achieved in a minority of animals following intranasal immunization.

Introduction

Helicobacter (H.) pylori infection is the major cause of gastritis and peptic ulcer disease as well as gastric cancer in humans (Marshall and Warren, 1984; Veldhuyzen van Zanten and Sherman, 1994; Dunn et al., 1997). Therapy with proton pump inhibitors and two or three antibiotics, e.g. clarithromycin and amoxicillin, is recommended as a first line treatment (Malfertheiner et al., 2002). Such therapies, however, face increasing problems of antimicrobial resistance (Graham, 1998; Malfertheiner et al., 2007) and recurrence of infection (Kepekci and Kadayifci, 1999). To solve these problems, immunization may be a useful alternative. Different animal models have been developed in order to test such approach (Del Giudice et al., 2001). The immunoproteome of *H. pylori* in the mouse model appears similar to that in human infections, suggesting that the mouse model is a suitable tool for preclinical screening of vaccine candidates (Bumann et al., 2002).

Helicobacter pylori, however, is not the only bacterial pathogen capable of colonizing the human gastric mucosa. Indeed, non-*H. pylori* helicobacters (NHPH) with a typical spiral-shaped morphology have been found in a minority of gastric biopsies (Heilmann and Borchard, 1991; Stolte et al., 1994; Svec et al., 2000; Solnick, 2003). These bacteria were provisionally named "*H. heilmannii"*. However, it has been shown that these NHPH do not represent a single species, but a group of different bacterial species with similar spiral morphology (Trebesius et al., 2001; Haesebrouck et al., 2009). Based on 16S rRNA gene sequences, the "*H. heilmannii*" group was further divided into two types (Solnick et al., 2003). "*H. heilmannii"* type 2 organisms are closely related, if not identical, to the canine and feline *Helicobacter* spp., namely *H. felis* (Lee et al., 1988), *H. bizzozeronii* (Hänninen et al., 1996), *H. salomonis* (Jalava et al., 1997), *'Candidatus* H. heilmannii' (O'Rourke et al., 2004) and two recently described new species, *H. cynogastricus* (Van den Bulck et al., 2006) and *H. baculiformis* (Baele et al., 2008a). The majority of "*H. heilmannii"* infections in humans however, are due to "*H. heilmannii"* type 1 (Trebesius et al., 2001; De Groote et al., 2005; Van den Bulck et al., 2005). It is now accepted that "*H. heilmannii"* type 1 is identical to the recently cultured and described *Helicobacter suis* (Baele et al., 2008b), formerly *"Candidatus* Helicobacter suis*"* (De Groote et al., 1999; O'Rourke et al., 2004), a spiral-shaped bacterium that colonizes the stomach of more than 60 % of slaughter pigs (Mendes et al., 1991; Grasso et al., 1996; Park et al., 2004; Hellemans et al., 2007b). The role of *H. suis* in gastric disease in pigs is still unclear. It has been suggested that this bacterium is associated with gastric

ulceration of the pars oesophagea (Queiroz et al., 1996) and chronic pyloric gastritis in pigs (Mendes et al., 1991; Hellemans et al., 2007a; Kumar et al., unpublished results). Up to this moment, mouse inoculation has been used to grow and maintain this bacterium viable for more than two years starting from infected pig stomach mucosa (Dick et al., 1989; Park et al., 2003). Using the mouse model, studies have been carried out on the control of *H. suis* infection by antibiotic treatment (Hellemans et al., 2005) and by immunization using heterologous antigens from related species (Hellemans et al., 2006).

A succesful *in vitro* culture method has recently been developed for *H. suis* (Baele et al., 2008b).The purpose of the present study was to determine the effect of prophylactic immunization against *H. suis* using antigens from *in vitro* cultured *H. suis*, compared to that obtained using antigens from related species, namely *H. bizzozeronii* and *H. cynogastricus*.

Materials and methods

Animals

Three week-old male *Helicobacter* spp.-free SPF BALB/c mice were purchased from an authorized breeder (HARLAN, Horst, The Netherlands). The animals were housed in groups of five on autoclaved wood shavings in filter top cages. They were fed an autoclaved commercial diet (TEKLAD 2018S, HARLAN) and received autoclaved water *ad libitum* for a period of at least 2 weeks prior to the initiation of the study. At the time of the onset of the study, mice were housed individually according to a random treatment allocation plan. All experiments involving animals were approved by the Animal Care and Ethics Committee of the Faculty of Veterinary Medicine, Ghent University.

Antigens for immunization

H. cynogastricus (LMG 23188^T) and *H. bizzozeronii* (R1051^T) antigens were obtained using two different protocols.

In the first protocol, *H. cynogastricus* and *H. bizzozeronii* were grown in l l or 250 ml vented and baffled shake flasks containing 200 ml and 50 ml, respectively, of porcine brain heart infusion (BHI) broth (Oxoid, Basingstoke, UK) with 10% pig serum and Vitox Supplement (Oxoid). *H. cynogastricus* and *H. bizzozeronii* were seeded at 8.8 x 10^6 and 1 x 10^7 cells/ml, respectively. The flasks were placed in a 37°C incubator and shaken at 120 rpm in a micro-aerobic environment for approximately 20 h to a cell density of ± 1 x 10^8 CFU per

ml. For *H. cynogastricus*, approximately 1050 ml of culture containing 1.16 x 10^{11} cells was centrifuged at 2000 *g* for 30 min and the cell pellet was resuspended in 33.3 ml 0.9% sodium chloride. For *H. bizzozeronii,* approximately 969 ml of culture containing 1.62 x 10^{11} cells was centrifuged at 2000 *g* for 25 min and the cell pellet was resuspended in 50 ml 0.9% sodium chloride. Both suspensions were stored at -80°C until further processing. The frozen cell suspensions were thawed and subjected to 3 passages through a high pressure homogenizer (EmulsiFlex-C5; Avestin Inc., Ottawa, Ontario, Canada) at 17000 PSI. The homogenates were filtered through a 0.45 µm PES filter (pre-filter), followed by a 0.22 µm PES filter.

In the second protocol, *H. bizzozeronii* and *H. cynogastricus* were grown on brain heart infusion (BHI) agar (Oxoid) containing 10 % horse blood, 5 mg amphotericin B/l (Fungizone; Bristol-Myers Squibb, New York, USA), Campylobacter Selective Supplement (Skirrow; Oxoid) and Vitox supplement (Oxoid). Plates were incubated at 37°C in microaerobic conditions. The antigens used for immunization were prepared by harvesting 3-day-old bacterial cultures in sterile phosphate buffered saline (PBS). The bacterial suspension was sonicated 8 times for 30 seconds with a sonicator (Sonicator ultrasonic processor XL 2015; MISONIX, Farmingdale, USA). After centrifugation (5000 g, 5 min, 4°C), the supernatant was filtered through a 0.22-µm pore filter (Schleicher and Schuell, Gent, Belgium) and stored at -70°C.

H. suis strain HS1 (LMG 23995^{T}) was grown on Mueller Hinton agar (Oxoid) containing 10% horse blood, 5 mg amphothericin B/l, Skirrow Supplement (Oxoid) and vitamins (Vitox; Oxoid). In addition, the pH of the agar was adjusted to 5 by adding HCl to a final concentration of approximately 0.05%. Since *H. suis* does not form distinct colonies on dry agar plates, one ml of Mueller Hinton broth (Oxoid) with a pH of 5 was added on top of the agar to obtain biphasic culture conditions. After three days of incubation at 37°C in microaerobic conditions, the Mueller Hinton broth, containing the bacteria, was harvested. Bacteria were washed and concentrated by centrifugation (5000 g, 10 min, 4°C) and suspended in PBS. The bacterial suspension was further homogenized by sonication and processed as for *H. bizzozeronii* and *H. cynogastricus*. For all antigen preparations, the protein concentration was determined using the Lowry assay (Lowry et al., 1951).

Preparation of *H. suis* challenge material

Challenge of the mice was done using *in vivo* isolated *H. suis* from naturally infected pig stomachs. This isolation was performed by mouse inoculation as previously described

(Hellemans et al., 2006). Briefly, *H. suis* infected pig stomachs were collected from the slaughterhouse. Mucosal scrapings were inoculated into mice by stomach tubing. Four serial passages in mice were carried out. *H. suis*-specific PCR (De Groote et al., 2000) confirmed the presence of *H. suis* in the mice stomachs in each passage. Challenge material was prepared from stomach homogenates of mice from the fourth passage. This fourth mouse passage was performed in 10 BALB/c mice. From these animals, the urease-positive stomachs were pooled and homogenized. The homogenate was frozen at -70°C.

Immunization/challenge study design

The mice used for intranasal and subcutaneous immunization were divided into ten groups of five animals each with a set-up as presented in Table 1. Subcutaneous immunization was performed on day 0 followed by two boost immunizations on day 21 and day 42. For this purpose, 100 µg of the sonicated filtrate (in a volume of 54 µl) was mixed in an equal volume with a saponin-based adjuvant (50/50 vol/vol %) and injected subcutaneously at the lower back of the animals. Intranasal immunization was performed at day 21 followed by one boost immunization at day 42. This immunization was done by the application of a total volume of 15 µl containing 100 µg of sonicate or high pressure homogenized filtrate mixed with 5 µg cholera toxin (List, Campbell, California, US) on the external nares of unanaesthetized mice. Group 1 was sham immunized intranasally by application of saline.

On day 70 of the study, the mice were challenged with *H. suis*. Therefore, the frozen stock of *H. suis* infected mice stomach homogenate, prepared as described above, was placed at 37°C for 15 minutes, and each animal (except the animals of group 10) was inoculated intragastrically with 0.3 ml, using a ball-tipped gavage needle. This dose had proven to infect 100% of the inoculated mice in a preliminary experiment.

On day 119-120 of the study, all animals were euthanized by cervical dislocation following isoflurane anaesthesia (IsoFLo; Abbott, Illinois, USA). The non-glandular part of the stomach was discarded, and then the stomach was divided longitudinally into 2 halfs from the esophageal opening to the pylorus. Since *H. suis* does not grow in distinct colonies, quantitative culture of this bacterium is not possible. Therefore, one half of the stomach from each of the animals was used for a semi-quantitative urease assay. From the other half, 4 mm^2 mucosal tissue samples were frozen (-20°C) and used for PCR detection of *H. suis* DNA. One sample was taken both in antrum and fundus.

Serology - ELISA

Just before necropsy, blood was collected by tail bleeding. After centrifugation (10 min, 1000 g, 4°C), serum was frozen at -70°C until further use. Serological responses were assesed by ELISA. In short, 96 well flat bottom plates (Nunc MaxiSorp; Nalge Nunc Int., Rochester, NY, USA), were coated with 200 ng per well of *H. suis, H. bizzozeronii or H. cynogastricus* whole bacterial cell proteins diluted in carbonate coating buffer (24 hours at 4°C). Each plate was blocked with 1% poly-vinyl alcohol in PBS, and the serially diluted serum samples were incubated in duplicates on each antigen coated plate. After incubation at 37°C, all plates were washed and bound antibodies were detected with affinity purified goat anti-mouse IgG peroxidase conjugated (KPL, Gaithersburg, MD, USA) followed by ABTS Peroxidase Substrate System (KPL).

Titers were determined as the reciprocal of the serum dilution that equaled 50% of the mean OD of the wells with positive control serum. For the generation of positive control serum, NIH-Swiss mice (20-30 grams) were injected subcutaneously, 3-times at two-week intervals, with 42 µg protein of whole bacterial cell antigens of *H. suis*, *H. cynogastricus* or *H. bizzozeronii* per dose in TiterMax Gold Adjuvant (Sigma-Aldrich, St. Louis, MO, USA). To collect serum, mice were bled 2 weeks after the last immunization. Sera were diluted 1:400 before adding to the positive control wells.

Urease assay for assessment of colonization of *Helicobacter* bacteria in the mouse stomach

Urease activity in the stomach of mice, typical for gastric *Helicobacter* spp., was assessed using the method of Corthésy-Theulaz et al. (1995) with some modifications. One half of the stomach was immersed in 500 µl of rapid urease test (CUTest; Temmler Pharma, Marburg, Germany) and incubated at 37°C for 3 h. After centrifugation (5 min, 1000 g), the level of urease activity was measured by spectrophotometric analysis at 550 nm. The cut-off value was calculated and corresponded to the mean + 5 x SD of the optical density (OD) values obtained with gastric samples of non-immunized, non-challenged mice.

PCR for detection of *Helicobacter suis* in mouse gastric tissue samples

DNA from mucosal tissue samples was extracted using the DNeasy Tissue kit (Qiagen, Hilden, Germany) according to the instructions of the manufacturer. PCR for the specific detection of *H. suis* was performed as described previously (De Groote et al., 2000).

Statistical analysis

The semi-quantitative urease assay results were compared by a fixed effects model. In a first analysis, antigen was introduced in the fixed effects model to test for the overall effect of antigen. In a second analysis, route of administration of the vaccine was introduced in the model, with adjustment for antigen, to test for the difference between intranasal and subcutaneous administration. Finally, mode of preparation was introduced in the model, with adjustment for antigen, to test for the difference between sonication and high pressure preparation.

The number of PCR-positive animals per group and the anti-*H. suis* IgG antibody titers were compared by the Kruskall-Wallis test. In a first analysis, the difference between the antigens was tested. In a second analysis, route of administration was tested by the Wilcoxon/Mann-Whitney test stratified for antigen. Finally, mode of preparation was tested by the Wilcoxon/Mann-Whitney test stratified for antigen.

Tests were performed at the 5% global significance level, and the significance level for multiple comparisons was adjusted by Bonferroni's technique.

Results

Animals

During the study, 4 animals died from a cause unrelated to the treatment: two animals from group 3, one animal from group 4, and one animal from group 8.

Serology-ELISA

The resulting anti-*H. suis* IgG titers are presented in Table 1. These data show that in all groups, a higher serum IgG titer was observed when compared to both the negative and positive control group ($P<0.05$). This indicates that animals responded to immunization and that homologous as well as heterologous antigens can cause a serological response towards *H. suis* antigens. Similar results were seen for the ELISA tests using *H. bizzozeronii* and *H. cynogastricus* antigens (data not shown). The elevated titers in all three ELISA tests show that cross-reactivity between species has to be taken into account. Only for the subcutaneous immunization route, *H. suis* immunization yielded significantly higher serum anti-*H. suis* IgG titers than immunization with *H. cynogastricus* and *H. bizzozeronii* ($P = 0.01$).

When comparing the two immunization routes in animals that were immunized with the *H. suis* antigens, significantly higher (P = 0.0079) titers in the *H. suis* ELISA were found for the subcutaneously immunized animals compared to the intranasally immunized animals. No significant difference between preparation techniques was found for the anti-*H. suis* IgG antibody titers.

Urease activity of gastric tissue sections

The results of the semi-quantitative urease assays are presented in Table 1. The cut-off value calculated from the non-immunized/non-challenged group was 0.218.

Prophylactic intranasal immunization with *H. bizzozeronii, H. cynogastricus* or *H. suis* antigen preparations resulted in an urease activity that was similar to the non-immunized/non-challenged animals, but significantly lower (P<0.05) as compared to the non-immunized/challenged group. Indeed all intranasally immunized animals (except animal 2 in group 6) had an urease OD value below the cut-off value, so differences between intranasally immunized groups were considered not significant. *H. suis* and *H. cynogastricus* subcutaneously immunized animals showed a significantly lower (P<0.05) urease activity when compared with the non-immunized/challenged group, with 3 out of 5 and all animals, respectively, yielding urease OD values below the cut-off value.

There was a significant effect (P = 0.0131) of the route of immunization on the urease activity. The mean urease activity for intranasal immunization was 0.107, compared to subcutaneous immunization with a value equal to 0.429. There was no significant difference between the mode of preparation of the vaccine (P = 0.567).

PCR analysis of gastric tissue sections

All non-challenged animals were negative in PCR whereas all sham-immunized/challenged animals scored positive in all stomach samples (Table 1).

In the immunized/challenged groups, a clear distinction could be made between the two administration routes (intranasal versus subcutaneous), taking into account the number of PCR-positive animals (P = 0.0486). In all subcutaneously immunized groups all stomach samples – antrum as well as fundus – were positive in PCR. This contrasts clearly with the results from the non-immunized/non-challenged group and is identical to the results from the non-immunized/challenged group. For most of the intranasally immunized groups, however, several immunized animals were negative in PCR (for *H. cynogastricus*, 3/8; for *H. suis*, 2/5; for *H. bizzozeronii*, 1/8).

There was a significant difference in PCR-positivity between the antigens used for immunization (P = 0.0008). In the *H. cynogastricus* and *H. suis* intranasally immunized groups, several animals were PCR-negative and only one animal in each group was positive in both stomach samples. For *H. bizzozeronii* intranasal immunization, most animals were PCR-positive in both stomach samples (group 4) or no animals were PCR-negative (group 5).

There was no significant difference in the number of PCR-positive animals between the two vaccine preparation methods (P = 0.4317). For instance, for the *H. cynogastricus* intranasally immunized group, both homogenization procedures gave comparable results (40% negative animals for high pressure homogenization versus 33.33% for sonication).

Table 1. Intranasal and subcutaneous immunization/challenge experiment: serum anti-*H. suis* IgG antibody titers, semiquantitative urease activity in gastric tissue and PCR results for the specific detection of *H. suis* in murine stomach tissue.

Group	Infection	Vaccine administration route	Antigens used for immunization	Antigen preparation	Antibody titer[a] Median (Min-Max)	Urease activity: mean OD ± SD[b]	PCR-positive mice[b,c]	mice PCR-positive in both antrum and fundus[b,c]
1	*H. suis*	Intranasal	saline	-	<100 (all)	1.630 ± 0.655	5/5	5/5
2	*H. suis*	Intranasal	*H. cynogastricus*	high pressure	227 (197-251)[d]	0.098 ± 0.026[e]	3/5	1/5
3	*H. suis*	Intranasal	*H. cynogastricus*	sonication	172 (145-187)[d]	0.092 ± 0.006[e]	2/3	1/3
4	*H. suis*	Intranasal	*H. bizzozeronii*	high pressure	411 (293-1074)[d]	0.079 ± 0.003[e]	3/4	3/4
5	*H. suis*	Intranasal	*H. bizzozeronii*	sonication	662 (189-1310)[d]	0.094 ± 0.009[e]	5/5	1/5
6	*H. suis*	Intranasal	*H. suis*	sonication	1053 (178-2395)[d,f]	0.136 ± 0.053[e]	3/5	1/5
7	*H. suis*	Subcutaneous	*H. cynogastricus*	sonication	306 (<100-1247)[d,g]	0.122 ± 0.048[e]	5/5	5/5
8	*H. suis*	Subcutaneous	*H. bizzozeronii*	sonication	312 (<100-418)[d,g]	0.826 ± 0.754	4/4	4/4
9	*H. suis*	Subcutaneous	*H. suis*	sonication	5595 (3891-14195)[d,g,f]	0.340 ± 0.224[e]	5/5	5/5
10	-	-	-	-	<100 (all)	0.118 ± 0.020[e]	0/5	0/5

[a] Titers were determined as the reciprocal of the serum dilution that equaled 50% of the mean O.D. of the wells with positive control serum, diluted 1:400.
[b] For urease OD and PCR, the statistical comparisons between vaccine administration route, antigens used for immunization and antigen preparation are discussed in the text.
[c] Number of positive animals/Total no of animals per group.
[d] The IgG antibody titers marked with a [d] are significantly higher compared to the titers of the sham-immunized/infected and non-immunized/non-infected control groups (group 1 and 10, respectively.
[e] Urease OD values differing significantly from the urease OD value of the non-immunized infected control group (group 1).
[f] For immunization with *H. suis* antigens: subcutaneous immunization yields significantly higher serum anti-*H. suis* IgG titers than intranasal immunization.
[g] For subcutaneous immunization: immunization with *H. suis* antigens yields significantly higher serum anti-*H. suis* IgG titers than immunization with *H. cynogastricus* and *H. bizzozeronii* antigens.

Discussion

To our knowledge, this is the first report on immunization against *H. suis* ("*H. heilmannii"* type 1) using a homologous antigen preparation. Since this bacterium was recently cultured and characterized (Baele et al., 2008b), it is now possible to prepare antigens derived from pure *H. suis* cultures.

Many antibacterial vaccines currently used in swine induce only partial protection (Haesebrouck et al., 2004). This protection may be influenced by several factors, including the route of immunization, the antigen composition/preparation, the vaccine formulation, the dose of antigen and the adjuvant used. The present results show that the route of immunization may indeed influence the protective effect of the vaccines used in this study.

In the present study, immunization of mice with sonicated and pressurized whole bacterial cell antigens of *H. cynogastricus* and *H. bizzozeronii* was found to be partially protective against heterologous *H. suis* challenge. In an earlier study using the same challenge model, we showed partial protection against *H. suis* infection using whole bacterial cell antigens from *H. felis* and *H. pylori* (Hellemans et al., 2006). Cross-protection by heterologous vaccines is often attributed to the urease enzyme, given the high degree of amino-acid conservation between urease enzymes from closely related *Helicobacter* species (Ferrero and Labigne, 1993; Ferrero et al., 1994). Although information on the exact species used was not provided, one study showed that "*H. heilmannii"* infection may be prevented by immunization with "*H. heilmannii"* UreB and *H. pylori* UreAB. This confirms that protective immunity against *Helicobacter* infections may be elicited by homologous as well as heterologous *Helicobacter* urease (Dieterich et al., 1999). The protective effect of *H. cynogastricus* and, to a lesser degree, *H. bizzozeronii* immunization found in this study, is probably due to antigens conserved between different *Helicobacter* species with urease and heat shock proteins as possible candidates (Ferrero et al., 1995; Yamaguchi et al., 2000). Based on the sequences of the *ureA, ureB* and *hsp60* gene sequences, *H. bizzozeronii* seems more closely related to *H. suis* than *H. cynogastricus* (Baele et al., 2008b). Therefore, one would expect to obtain better results with a *H. bizzozeronii* immunization. However, *H. cynogastricus* gave better protection than *H. bizzozeronii*. Not yet elucidated immuno-dominant epitopes of specific proteins may play crucial roles.

With respect to immunization route, in *Helicobacter* immunization studies, mucosal application has been the most frequently used method for administration of vaccines. Garhart et al. (2002) have found that intragastric immunization was less effective than intranasal

immunization, with the latter enabling most mice to clear an *H. pylori* infection. Several authors have demonstrated that parenteral immunization with appropriate schedules and formulations also constitutes a valuable approach to reduce *Helicobacter* colonization levels (Guy et al., 1998; Guy et al., 1999; Sanchez et al., 2001). In the present prophylactic immunization study, we demonstrated that a protective response against *H. suis* infection can be induced by administering the vaccine by the intranasal route with homologous (*H. suis*) as well as with heterologous (*H. cynogastricus* and *H. bizzozeronii*) antigens, in some cases leading to complete protection (negative in PCR and rapid urease test). Subcutaneous immunization resulted in a lower level of protection.

In our study, subcutaneous immunization with *H. suis* antigens yielded higher serum anti-*H. suis* IgG antibody titers than homologous intranasal immunization but resulted in a lower level of protection. This is not entirely unexpected, since high levels of antigen-specific circulating antibodies do not protect mice against *H. pylori* challenge (Ermak et al., 1998). Moreover, protection to either *H. felis* or *H. pylori* colonization can occur in the absence of antibodies, as has been shown in B cell-deficient mice, lacking both local and serum antibodies (Blanchard et al., 1999).

This study aimed at showing the use of antigens of *H. bizzozeronii*, *H. cynogastricus* and *H. suis* as protective agents against *H. suis* infection. It was not the intention at this point to study the development of post-immunization gastritis, although this seems an important issue, especially when using sonicates or cell extracts for prophylactic immunization in a mouse model (Michetti et al., 1994). However, the role of this post-immunization gastritis in protection is still unclear, since this phenomenon is absent in Mongolian gerbils protected by oral immunization against *H. pylori* infection (Jeremy et al., 2006). Sutton et al. (2001) have shown that post-immunization gastritis is more pronounced in the *H. felis* immunization and challenge mouse model, as compared to the *H. pylori* immunization and challenge mouse model, but even in the *H. felis* model, it is a transient event which does not produce long-term exacerbation of pathology. The presence of post-immunization gastritis might also be due to persistence of the *H. felis* challenge strain in partially protected mice, since the administration of antibiotics to mice with post-immunization gastritis resulted in a significant decrease in inflammation (Ermak et al., 1997).

These results hold promise for the development of a vaccine using crude antigen preparations against *H. suis* infection in the natural host, i.e. pigs. It may also be worth considering the development of a vaccine for use in humans, especially those people with an increased risk of infection, such as veterinarians, pig farmers and abattoir workers. However,

it remains to be elucidated whether vaccine-induced protection will be sufficient to tip the balance of the persistent *H. suis* infection towards partial protection or even towards a complete elimination.

Acknowledgements

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Chapter 4

Helicobacter suis γ-Glutamyl Transpeptidase Causes Glutathione Degradation-Dependent Gastric Cell Death

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Summary

The recently isolated *Helicobacter (H.) suis* is the most prevalent non-*H. pylori Helicobacter* (NHPH) species colonizing the stomach of humans suffering from gastric disease. Until now, virtually nothing is known about possible *H. suis* virulence factors involved in human gastric pathology. In the present study, we aimed to unravel the mechanism used by *H. suis* to induce gastric epithelial cell damage. *H. suis* lysate induced death of AGS cells (human gastric adenocarcinoma cell line). Inhibition of γ-glutamyl transpeptidase (GGT) activity present in *H. suis* lysate and incubation of AGS cells with purified recombinant *H. suis* GGT showed that this enzyme was largely responsible for the observed cell death. Supplementation with glutathione strongly enhanced the observed effect, demonstrating that *H. suis* GGT-mediated degradation of glutathione and the resulting formation of glutathione degradation products play a direct and active role in the induction of cell death. This was preceded by an increase of extracellular H_2O_2 concentrations, generated in a cell-independent manner and causing lipid peroxidation. In conclusion, *H. suis* GGT-mediated generation of pro-oxidant glutathione degradation products brings on cell damage and actively causes cell death.

Introduction

Helicobacter (H.) pylori is considered to be the primary cause of gastritis, peptic ulcer disease as well as gastric adenocarcinoma and mucosa-associated lymphoid tissue (MALT) lymphoma in humans (Marshall and Warren, 1984; Parsonnet et al., 1991; Parsonnet et al., 1994; Kusters et al., 2006). In 0.2-6% of gastric biopsies, however, large spiral-shaped non-*H. pylori* helicobacters (NHPH) are found (Haesebrouck et al., 2009). Humans infected with the latter have been reported to suffer from gastritis (Stolte et al., 1997), sometimes accompanied by gastric ulcer, gastric MALT lymphoma and gastric cancer (Morgner et al., 1995; Stolte et al.,1997; Debongnie et al., 1998; Morgner et al., 2000). These bacteria have long been referred to as "*H. heilmannii*". In reality, they represent a group of different bacterial species with similar spiral morphology, which are also highly prevalent in different animal species (Haesebrouck et al., 2009). The most prevalent NHPH in humans is *H. suis* (Van den Bulck et al., 2005; De Groote et al., 2005), hosted largely by pigs and only recently isolated *in vitro* (Baele et al., 2008).

Until now, virtually nothing is known about possible *H. suis* virulence factors involved in human gastric pathology (Haesebrouck et al., 2009). Previously, we have shown that a *H. suis* infection induces loss of gastric epithelial, mainly parietal cells in mice and Mongolian gerbil models of human gastric disease (Flahou et al., 2010a). Gastric cell death is considered an important mechanism involved in gastric ulcer formation (Dixon, 2001), gastric atrophy and gastric cancer (Shirin and Moss, 1998). Therefore, in the present study, we investigated the ability of *H. suis* to directly cause gastric epithelial cell death *in vitro*. Additionally, we explored which mechanisms of *H. suis* are involved in this process. In the closely related *H. pylori*, several factors have been shown to induce gastric epithelial cell death, including VacA (Cover et al., 2003), and a γ-glutamyl transpeptidase (GGT) (Shibayama et al., 2003; Kim et al., 2007a; Gong et al., 2010). For the latter, the exact mechanism involved in the induction of gastric epithelial cell apoptosis remains to be elucidated. Besides its role in gastric epithelial cell apoptosis, *H. pylori* GGT has also been shown to play a pivotal role in the upregulation of COX-2 and EGF-related peptide expression and the inhibition of T-cell proliferation (Busiello et al., 2004; Schmees et al., 2007). Membrane-associated GGT activity is often present in eukaryotic cells, where the enzyme plays an important role in the metabolism of glutathione (Orlowski and Meister, 1970), a free thiol maintaining an optimal intracellular redox environment (Circu and Aw, 2010).

In this study, we demonstrate that *H. suis* actively causes apoptosis and necrosis of gastric epithelial cells. We identified *H. suis ggt*, encoding an active GGT, as an important factor involved in death of these cells. The enzyme mediates the disintegration of glutathione into its degradation products. This actively causes an extracellular and cell-independent increase of the oxidative stress burden, leading to lipid peroxidation and finally resulting in gastric epithelial cell death.

Results

Inhibition of GGT activity in *H. suis* whole bacterial cell lysates strongly reduces *H. suis*-induced death of AGS cells

Viability of *H. suis* bacteria is drastically reduced, already after 1 hour of incubation in various cell culture media, including Ham's F12 which is used for cultivation of AGS cells. Therefore, in order to investigate the effect of *H. suis* on the viability of gastric epithelial cells, AGS cells (human gastric adenocarcinoma cell line) were incubated for 20 h with *H. suis* lysate. Flow cytometric and fluorescence microscopic analysis revealed an increase of the percentage of apoptotic cells, positive for active caspase-3 (not shown). After 44 hours of incubation, similar but more pronounced effects were detected, as well as an increase of the percentage of cells showing loss of plasma membrane integrity, detected by propidium iodide (PI) staining. (Figure 1; Figure 2A).

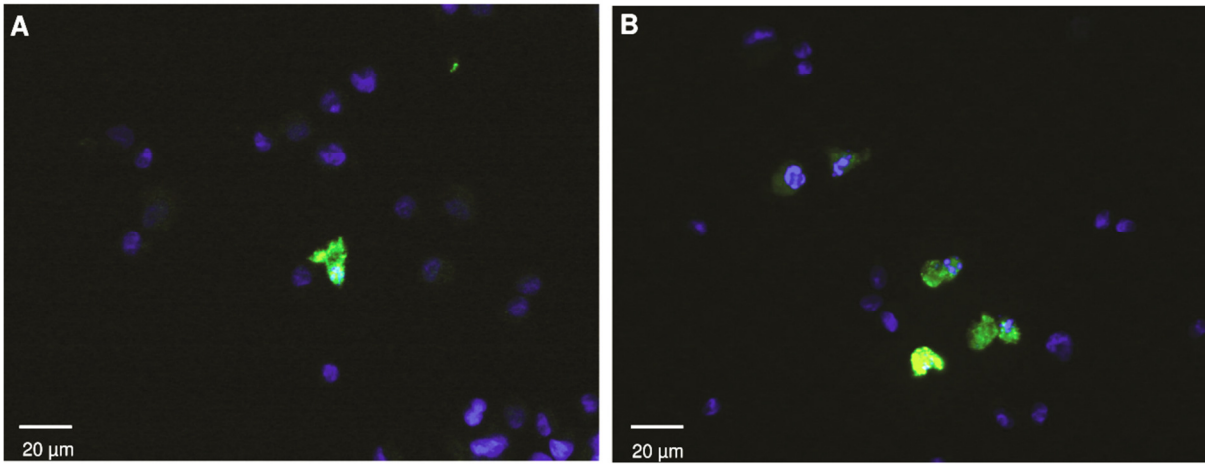

Figure 1. Activated caspase-3 and Hoechst staining of control and *H. suis* lysate treated AGS cells.
Fluorescence microscopic images of activated caspase-3 (green) and nuclear Hoechst staining (blue) of AGS cells. (A) AGS cells treated with HBSS for 44 hours. Only one activated caspase-3 positive cell is observed, reflecting the basal level of apoptosis in normal AGS cell cultures. (B) AGS cells treated with *H. suis* lysate for 44 hours. An increased number (6) of cells positive for activated caspase-3 can be observed. Note that activated caspase-3 positive cells also reveal nuclear condensation and fragmentation, which can typically be observed in apoptotic cells.

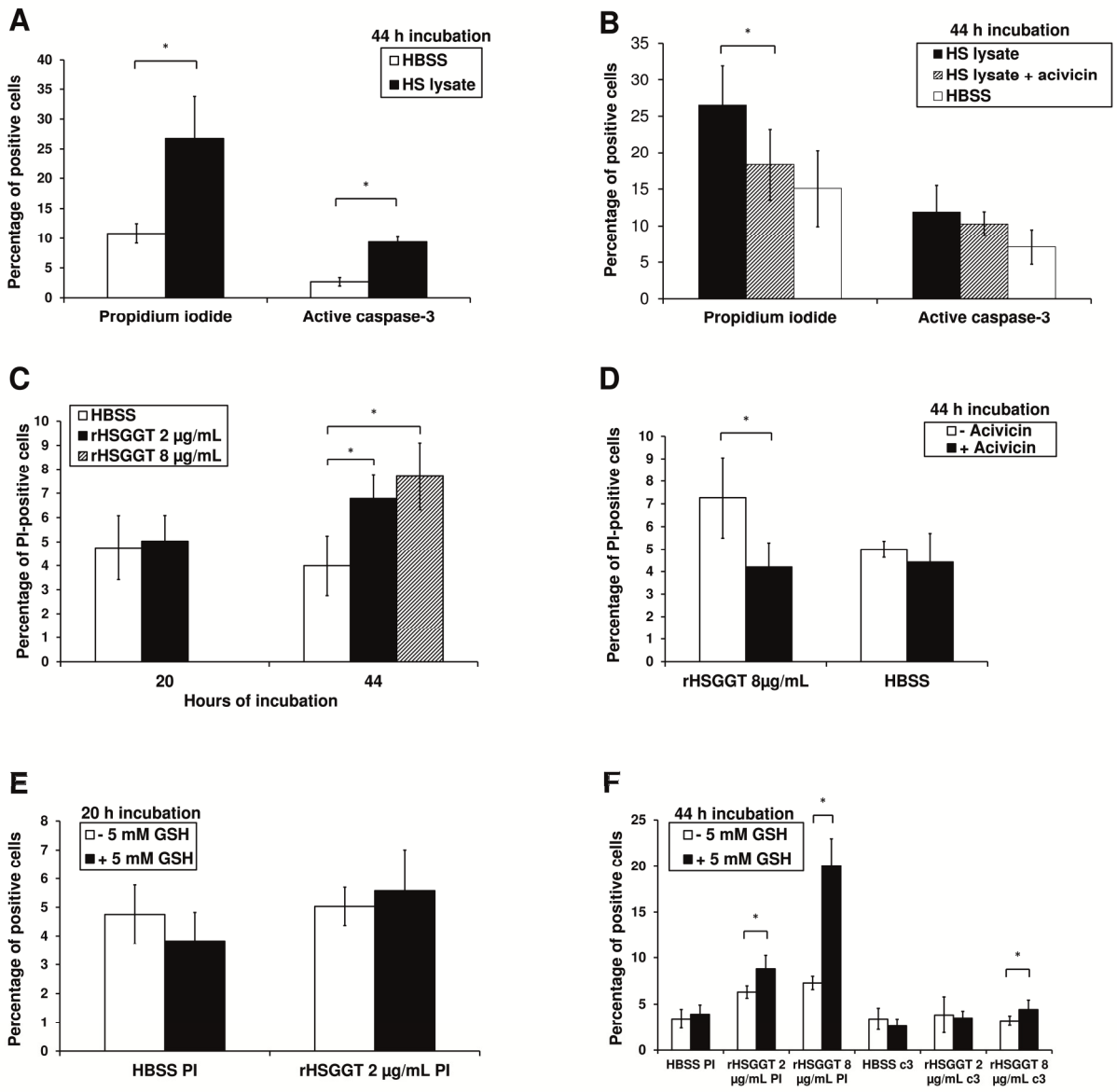

Figure 2. Flow cytometric analysis of cell death of AGS cells.
For each panel, the results of one representative experiment are depicted. HBSS-treated cells served as negative controls. (A) Incubation of AGS cells with *H. suis* whole bacterial cell lysate (HS lysate) induces both an increase in the number of propidium iodide-positive and activated caspase-3-positive cells. (B) Inhibition of GGT activity present in *H. suis* lysate with the known GGT inhibitor acivicin yielded a lower number of PI-positive cells. Also a decrease of the number of activated caspase-3-positive cells could be observed, although not statistically significant. (C) Incubation of AGS cells with recombinant *H. suis* GGT (rHSGGT) induced an increase in the percentage of dead cells, only after 44 hours of incubation. (D) This rHSGGT-induced cell death could be abolished by acivicin-mediated inhibition of GGT activity. (E) Incubation of AGS cells for 20 h with 5 mM GSH and 2 µg/mL rHSGGT did not result in a higher percentage of PI (propidium iodide)-positive cells compared to AGS cells incubated with rHSGGT alone. (F) In contrast, the cell death-inducing capacity of rHSGGT after 44 hours of incubation was enhanced by the addition of 5 mM GSH. Mainly caspase-3 (c3)-independent cell death was induced. * depict relevant statistically significant differences ($P<0.05$).

After 44 hour of incubation, the increase of the percentage of PI-positive cells was higher compared to the increase of cells positive for activated caspase-3, indicating that both apoptosis and necrosis, or another type of caspase-3-independent cell death, were involved. Heat and trypsin treatment of *H. suis* lysate almost completely abolished the cell death-inducing capacity (data not shown), allocating one or more proteins as the inducer(s) of cell death.

Gamma-glutamyl transpeptidase activity was detected in the supernatant fluid of a 24-hour-old biphasic culture of *H. suis* strain 5 (1.8-2.0 U/L). Moreover, GGT activity was detected in whole bacterial cell lysate of this same *H. suis* strain (Figure 3A: 20-30 mU/mg total protein). Lysates of all *H. suis* strains available at present, possess GGT activity in the same order of magnitude (results not shown). This urged us to investigate the possible role of this enzyme in the induction of gastric epithelial cell death. Incubation of *H. suis* lysate with acivicin, a known GGT inhibitor (Schmees et al., 2007), completely abolished GGT activity (Figure 3A) and reduced the *H. suis*-induced caspase-3-independent cell death (Figure 2B). Apoptosis also showed lower values, although statistically not significant. These data suggest that *H. suis* GGT is involved in the induction of gastric epithelial cell death.

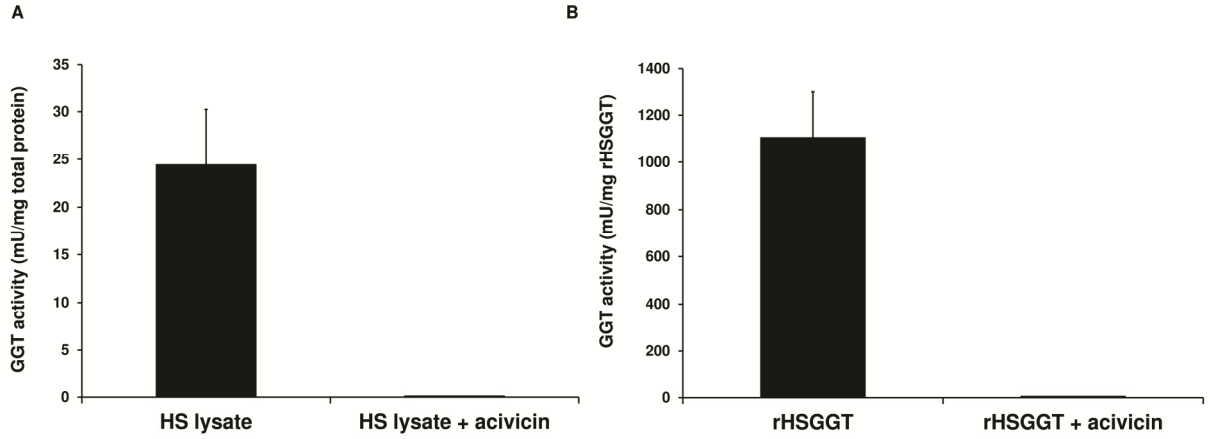

Figure 3. GGT activity detected both in *H. suis* (HS) lysate (A) and recombinant *H. suis* GGT (rHSGGT) (B). This enzymatic activity, expressed as mU/mg total *H. suis* protein or rHSGGT, respectively, was abolished completely after acivicin-treatment (50 µM).

Recombinant *H. suis* GGT causes cell death of AGS cells

To confirm whether *H. suis* GGT is involved in the induction of gastric epithelial cell death, we expressed a recombinant 6xHis-tagged *H. suis* GGT in *E. coli.* After nickel affinity chromatography, the enzyme was purified to homogeneity by gel filtration chromatography. This recombinant *H. suis* GGT (rHSGGT) was highly active in the GGT activity assay, as

shown in Figure 3B. SDS-PAGE analysis showed that this enzyme was purified mainly as a protein with a molecular weight of ~60 kDa, although two discrete protein bands with a molecular weight of ~40 and ~20 kDa, respectively, could also be seen (Figure 4). This suggests that *H. suis* GGT is synthesized as a precursor enzyme with subsequent processing into a large and a small subunit, which has been described for other bacterial GGT's, such as the *H. pylori* and *E. coli* GGT (Suzuki and Kumagai, 2002; Boanca et al., 2006). To test whether rHSGGT retains a long activity in AGS medium, a supernatant aliquot of AGS cells incubated for 20 h with rHSGGT was tested in the GGT activity assay. Results revealed a 2.5-fold increase in activity of the rHSGGT fraction, further confirming that the inactive precursor enzyme is processed into an active 2 subunits-containing enzyme.

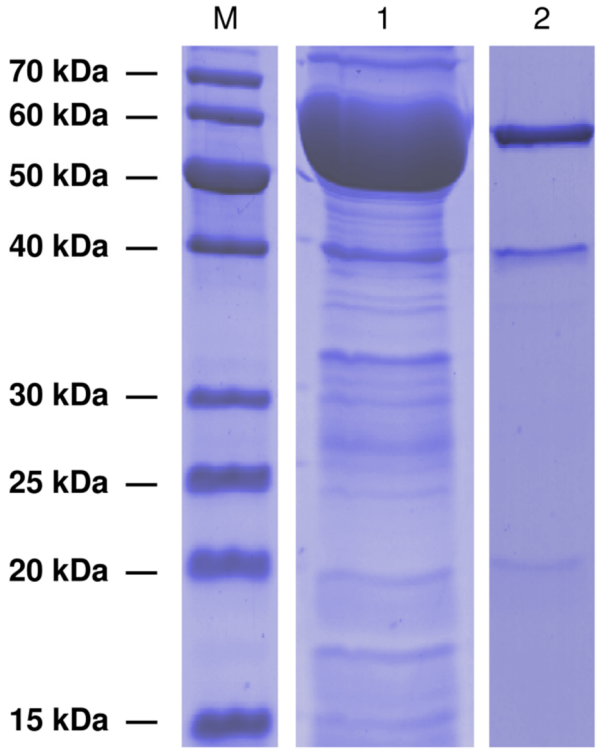

Figure 4. SDS-PAGE analysis of recombinant *H. suis* GGT. Proteins were visualized with Brilliant Blue G - Colloidal staining. Column M: Protein marker with size labelling in kilodalton (kDa) at the left. Column 1 shows the eluate after Ni-affinity chromatography, with a clear band of approximately 60 kDa, representing the unprocessed recombinant *H. suis* GGT (pro-enzyme). Column 2 shows recombinant *H. suis* GGT after gel filtration chromatography, revealing the presence of three protein bands, reflecting the processing of the pro-enzyme (~60 kDa) into a large (~40 kDa) and small (~20 kDa) subunit.

Incubation of AGS cells for 20 h with recombinant *H. suis* GGT showed no statistically significant increase of the number of propidium iodide-positive cells, compared to control cells (Figure 2C). After 44 hours of incubation, higher percentages of PI-positive cells were seen for final rHSGGT concentrations of 2 and 8 µg/mL, compared to control cells

(Figure 2C). An increase of the number of activated caspase 3-positive cells could also be detected, although statistically not significant (results not shown). This was confirmed by light and transmission electron microscopic examination of treated cell cultures. In 44 h HBSS-treated control cultures, the vast majority of cells showed no signs of cell death (Figures 5A and B), but approximately 4% of the cells showed blebbing of the plasma membrane and the presence of apoptotic bodies, of which some contained condensed chromatin (Figures 5C and D). These are all morphological features of apoptosis (Krysko et al., 2008; Kroemer et al., 2009). In cultures treated with 2 or 8 µg/ mL rHSGGT, cells appeared containing large, clear cytoplasmic vacuoles (Figures 5E and F), which is compatible with oncosis or primary necrosis (Krysko et al., 2008; Kroemer et al., 2009). Moreover, these cells did not show morphological signs of apoptosis.

Pretreatment of rHSGGT with 50 µM acivicin rendered GGT activity completely undetectable (Figure 3B). Incubation of AGS cells for 44 h with 8 µg/mL of this inhibited rHSGGT, showed a complete loss of the cell death-inducing capacity of this enzyme (Figure 2D).

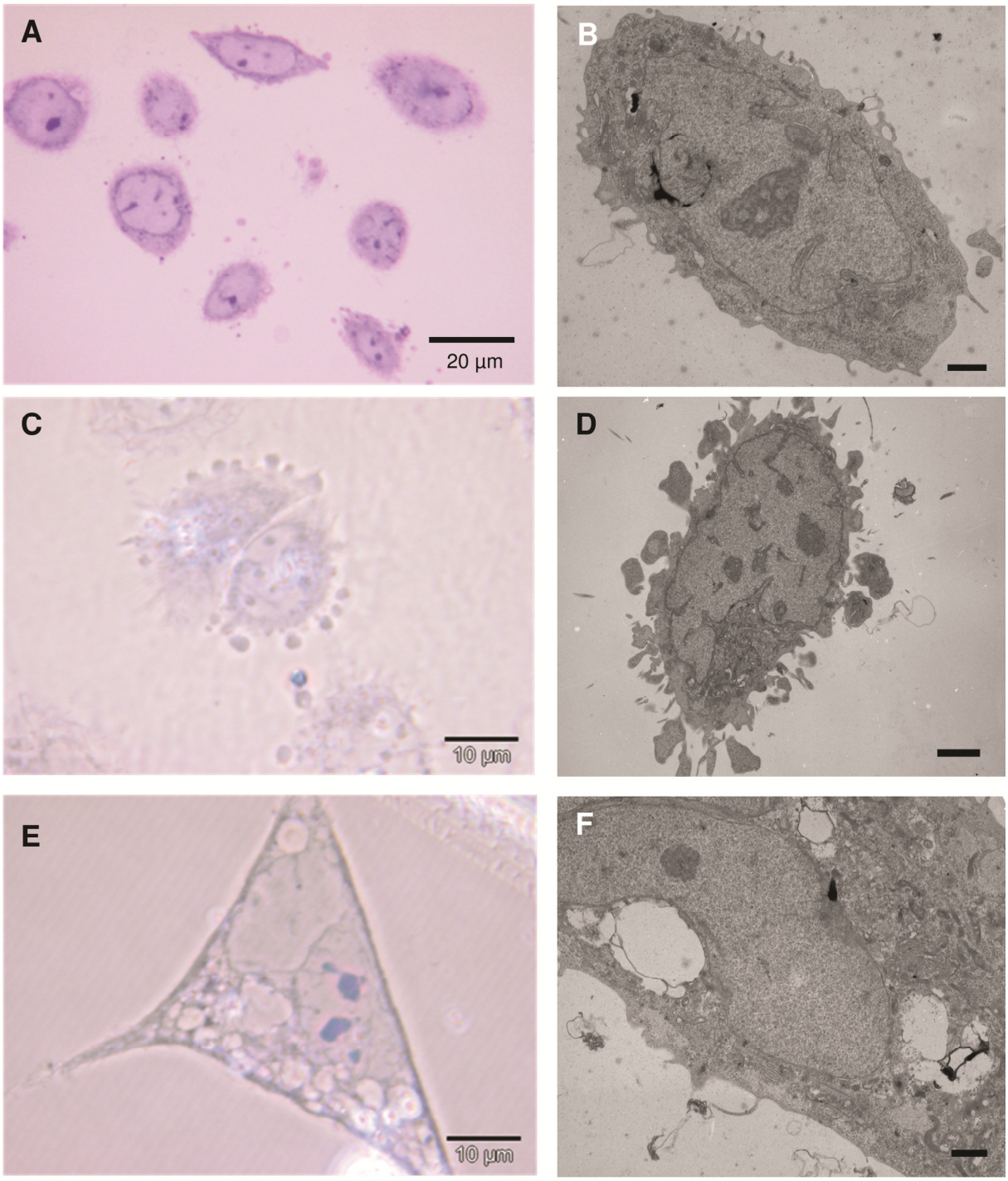

Figure 5. Light and transmission electron microscopic images of treated AGS cells.
(A) and (B) Bright field microscopic (magnification: 630x) and transmission electron microscopic (TEM) image (with bar: 1 µm), respectively, of a control AGS culture treated for 44 h with HBSS, showing normal cells. (C) and (D) Phase contrast image of 3 cells (magnification: 1000x) and TEM image (with bar: 2 µm) of 1 cell, respectively, showing a corona radiata of apoptotic bodies. Approximately 4% of cells in 44 h HBSS-treated control wells showed signs of apoptosis. (E) and (F) Phase contrast image of 1 cell (magnification: 1000x) and TEM image (with bar: 1 µm) of 1 cell, respectively, in an AGS culture treated for 44 hours with 8 µg/mL rHSGGT. In the rHSGGT-treated cultures, numerous cells appeared containing large, clear cytoplasmic vacuoles, compatible with oncosis or primary necrosis. Moreover, these cells did not show morphological signs of apoptosis.

Recombinant *H. suis* GGT causes cell death of AGS cells through extracellular glutathione degradation

Normal basal values of total glutathione in cell culture supernatants of an overnight culture of AGS cells varied between 30 and 140 µM. These concentrations did not alter significantly when AGS cells were incubated with 2 µM rHSGGT for up to 44 hours (not shown).

To detect degradation of extracellular reduced glutathione (GSH) in the AGS model, concentrations of supplemented GSH were monitored in the supernatant of AGS cell cultures in the presence or absence of 2 µg/mL rHSGGT (Figure 6A). Without the addition of rHSGGT, only 9 % of 5 mM supplemented GSH was degraded after 44 hours of incubation, whereas 95% of supplemented GSH was degraded in the presence of rHSGGT. Pre-incubation of rHSGGT with 50 µM acivicin strongly reduced the capacity of the enzyme to degrade GSH. After 20 h of incubation, 5 mM GSH was reduced to approximately 1500 µM GSH, whereas 1 mM GSH in AGS culture supernatant was reduced to only 55 µM (not shown), which equals normal basal GSH values present in AGS cell culture supernatants. For this reason, AGS cells were treated with 2 µg/mL rHSGGT and 5 mM GSH (as opposed to 1 mM) to determine the possible role of a sustained, 44-hour-lasting rHSGGT-mediated GSH degradation in the induction of cell death.

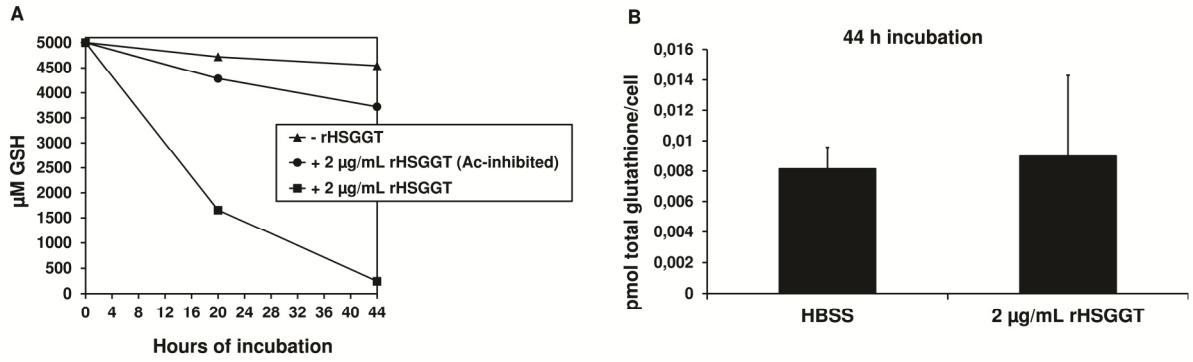

Figure 6. Recombinant *H. suis* GGT catalyzes the degradation of reduced glutathione (GSH).
(A) Monitoring of the degradation of supplemented 5 mM GSH in AGS cell culture supernatant without rHSGGT, with 2 µg/mL rHSGGT and with 2 µg/mL acivicin-inhibited rHSGGT, showing rHSGGT-mediated degradation of GSH in the AGS model used. (B) When AGS cells were treated for 44 hours with rHSGGT, no change of intracellular glutathione concentrations was observed.

Incubation of HBSS-treated AGS cells with 5 mM GSH had no effect on the percentage of PI-positive cells (Figures 2E and F). Moreover, this GSH supplementation induced a mild decrease in the number of cells positive for active caspase-3, revealing an anti-

apoptotic effect of reduced glutathione (Figure 2F). Incubation of AGS cells for 20 h with 5 mM GSH and 2 µg/mL rHSGGT did not result in a higher percentage of PI-positive cells compared to AGS cells incubated with rHSGGT alone (Figure 2E). Longer incubation for 44 hours with 5mM GSH and 2 or 8 µg/mL rHSGGT, however, resulted in a clear increase of the number of PI-positive cells (Figure 2F). Moreover, only a slight increase in the number of apoptotic cells was observed when 5 mM GSH was combined with 8 µg/mL rHSGGT (Figure 2F). These results demonstrate that *H. suis* GGT-mediated degradation of glutathione directly caused mainly caspase-3-independent, and thus non-apoptotic death of AGS cells.

H. suis GGT-mediated glutathione degradation plays an active role in the increase of the extracellular hydrogen peroxide concentration

Because the cleavage of glutathione by plasma membrane GGT of eukaryotic cells has been associated with the extracellular production of hydrogen peroxide (Dominici et al., 1999), we sought to determine whether *H. suis* GGT-mediated glutathione degradation was correlated with an increase of extracellular hydrogen peroxide production. Incubation of AGS cells for 44 hours with 2 µg/mL rHSGGT was accompanied by a mild increase of the extracellular H_2O_2 concentration, as determined with the Amplex Red Hydrogen Peroxide Assay Kit (Invitrogen) (Figure 7A). However, when cells were incubated with 2 µg/mL rHSGGT and 5 mM GSH, even much higher concentrations of extracellular H_2O_2 were observed (Figure 7B). Shorter incubation for 20 hours showed similar results, however less pronounced. As shown in figure 7A, incubation of AGS cells with 5 mM GSH alone, did not yield an increase in extracellular H_2O_2 concentrations, showing that the observed increase of the extracellular H_2O_2 concentration was due to recombinant *H. suis* GGT-mediated degradation of reduced glutathione. The exact same experiments were performed in the same media, however without AGS cells. Comparable results were obtained (Figure 7C), with equal or higher H_2O_2 concentrations in rHSGGT + GSH-containing wells without AGS cells compared to identical wells containing AGS cells. Moreover, acivicin-induced inhibition of the enzymatic activity of rHSGGT completely abolished the observed effect. These data show that the observed increase of the H_2O_2 concentration is an event taking place extracellularly. Finally, an increase of the extracellular H_2O_2 concentration was also observed after incubation of AGS cells for 1 and 20 hours with 200 µg/mL *H. suis* lysate, when compared to HBSS-treated control wells (Figure 7D).

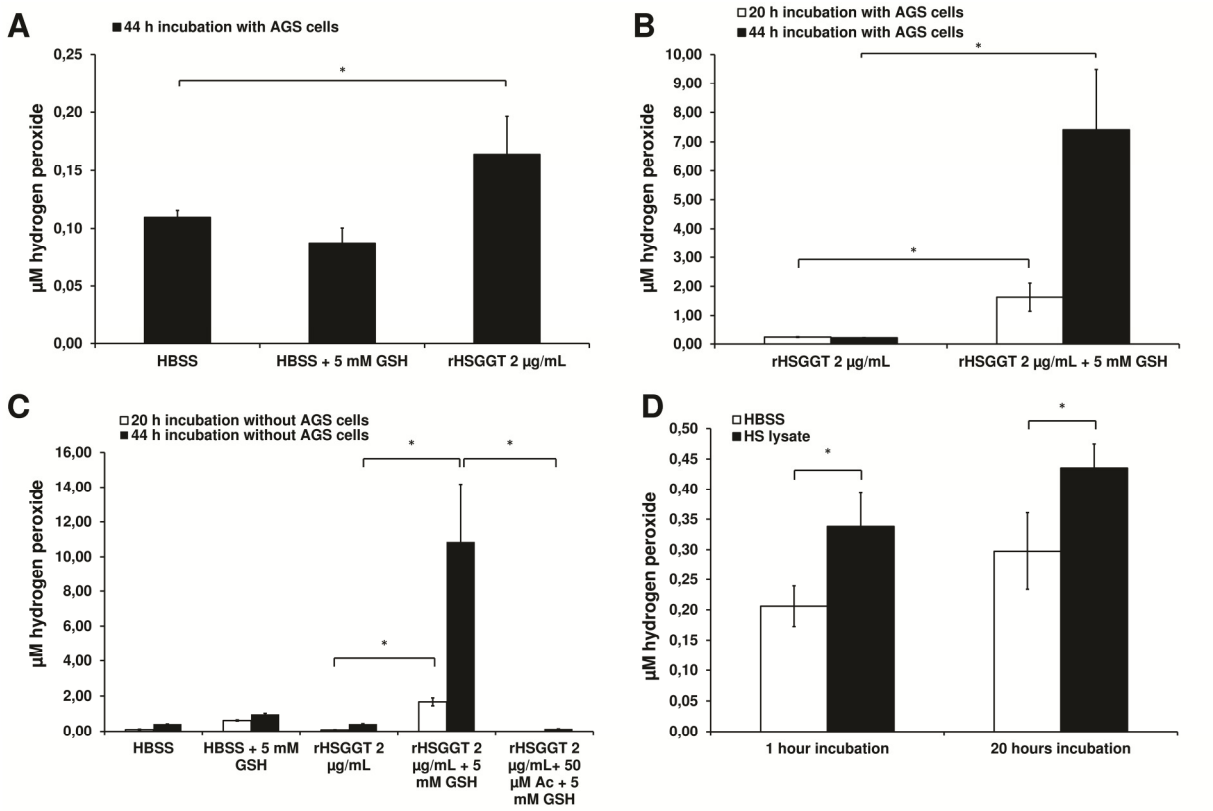

Figure 7. Determination of H_2O_2 concentrations in AGS cell culture supernatants. (A) Incubation of AGS cells for 44 hours with 2 µg/mL rHSGGT resulted in higher H_2O_2 concentrations compared to values in supernatant of control cell cultures, supplemented with HBSS instead of rHSGGT. (B) When a combination of rHSGGT and 5mM GSH was used, this increase was even much higher, both after 20 and 44 hours of incubation. (C) Similar results were obtained in AGS culture medium with the absence of AGS cells. Additionally, pretreatment of rHSGGT with acivicin completely abrogated the observed effect. (D) Incubation of AGS cells for 1 or 20 hours with 200 µg/mL *H. suis* lysate induced an increase of the extracellular H_2O_2 concentrations, compared to HBSS-treated cells. Shown are the mean values of 3 independent experiments or one representative experiment. * depict relevant statistically significant differences ($P<0.05$).

Glutathione degradation-dependent extracellular hydrogen peroxide generation causes lipid peroxidation prior to death of cells

Because *H.* suis GGT-mediated degradation of glutathione resulted in an increase of the extracellular oxidative stress burden, we investigated if these extracellular changes had an impact on the intracellular redox balance, since H_2O_2 is known to be able to permeate the eukaryotic plasma membrane (Bienert et al., 2006; Bienert et al., 2007), with subsequent actions inside the cell, possibly leading to necrotic cell death (Kim et al., 2007b).

When AGS cells were treated for 44 hours with rHSGGT, no change of intracellular glutathione concentrations was observed (Figure 6B). However, after 44 hours of incubation, an increase of membrane lipid peroxidation was detected in AGS cells treated with 2 µg/mL rHSGGT and 5 mM GSH, compared to HBSS-treated cells and cells treated with rHSGGT alone (Figure 8). In cells treated with GSH alone, no increased lipid peroxidation was observed, compared to HBSS-treated control cells, whereas treatment of cells with rHSGGT

alone caused a slight increase of lipid peroxidation, although statistically not significant (Figure 8). These data indicate that this effect is largely caused by rHSGGT-mediated degradation of reduced glutathione. Interestingly, comparable effects were observed after 20 hours of incubation, a point in time at which no increased cell death could yet be observed.

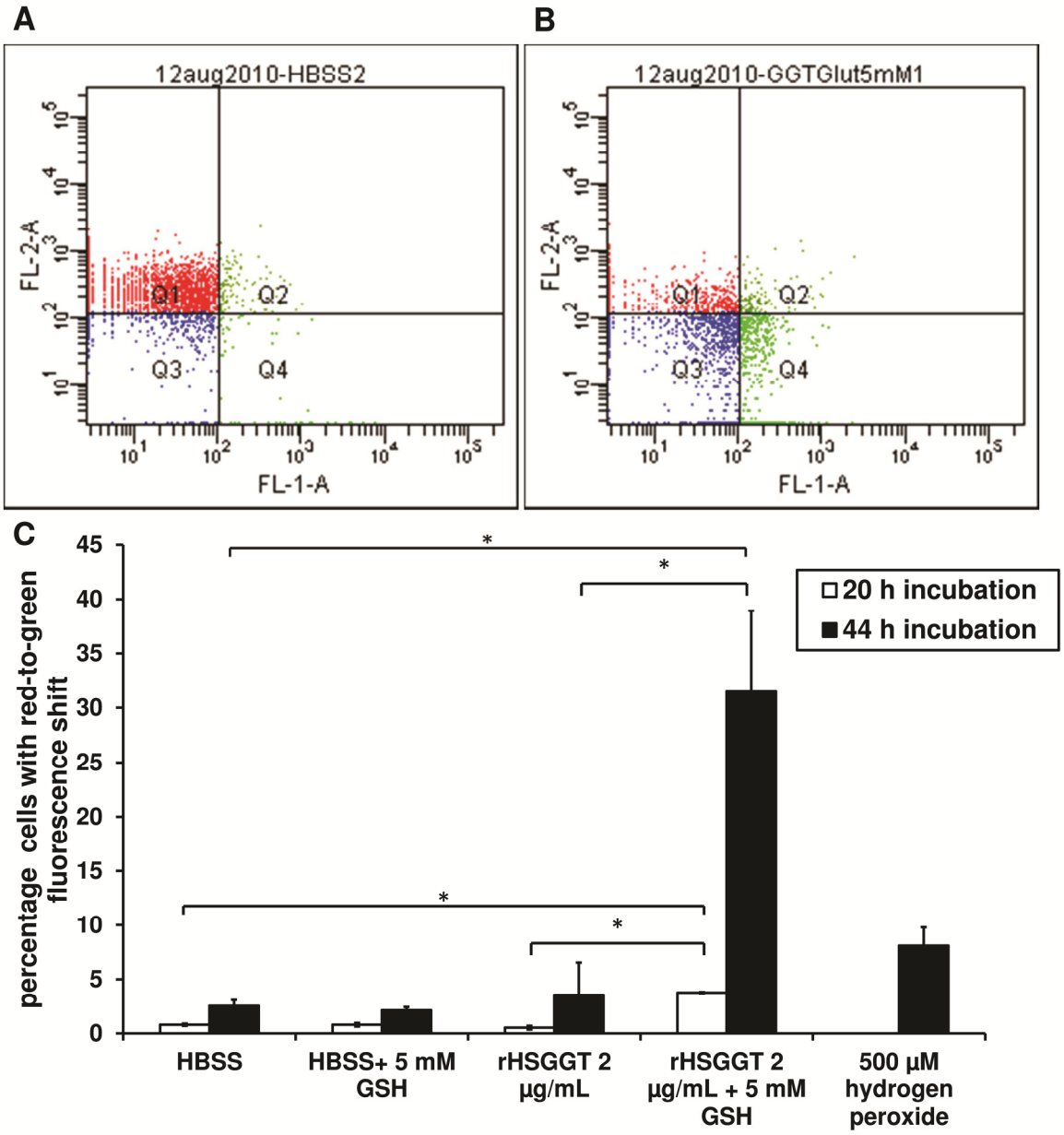

Figure 8. Determination of lipid peroxidation in AGS cells.
Cells were stained with BODIPY 581/591 C_{11}. Upon peroxidation, fluorescence of this dye shifts from red to green. Example of the analysis of the red (FL-2) and green (FL-1) fluorescence intensities of a cell population treated for 44 hours with HBSS (A) and a combination of 2 µg/mL rHSGGT and 5 mM GSH (B). Note the marked decrease of the red fluorescence accompanied by an increased green fluorescence after treatment with rHSGGT and GSH, indicating peroxidation of cellular lipids. (C) Already after 20 hours of incubation with a combination of rHSGGT and GSH, an increase of the percentage of cells showing a fluorescence shift was observed. This lipid peroxidation was even more pronounced after 44 hours of incubation. Shown are the mean results of 3 independent experiments. Incubation for 30 min with 500 µM H_2O_2 was included as a positive control. * depict relevant statistically significant differences.

Mitochondrial respiration is considered one of the main sources of intracellular ROS (Orrenius, 2007; Poyton et al., 2009; Pourova et al., 2010). Therefore, the possibility that this mitochondria-derived ROS could serve as a source of lipid peroxidation was investigated. Already after 24 hours of incubation of AGS cells with a combination of rHSGGT and GSH, higher levels of mitochondrial depolarization were observed, as shown by the decrease in the red/green fluorescence intensity ratio of the cationic dye JC-1 (Figure 9). This loss of mitochondrial membrane potential rather indicates a decrease of mitochondral ROS generation (Starkov and Fiskum, 2003; Brookes et al., 2004).

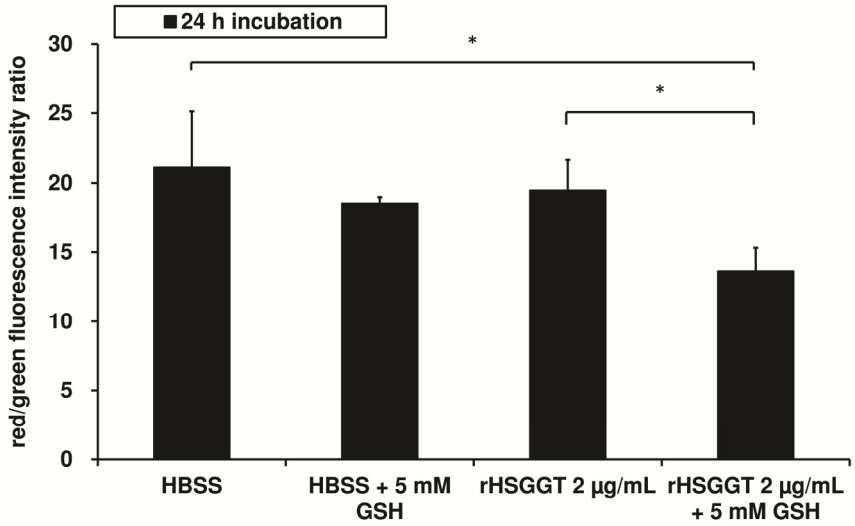

Figure 9. Measurement of mitochondrial membrane potential ($\Delta\Psi_m$) in treated AGS cultures.
Cells were stained with JC-1, a $\Delta\Psi_m$-sensitive probe. A decrease in the red/green fluorescence intensity ratio of this probe reflects mitochondrial depolarization. After 24 hours of incubation of AGS cells with rHSGGT and GSH, a decrease of the mitochondrial membrane potential could be observed. Shown are the mean values of 3 experiments. * depict relevant statistically significant differences.

Discussion

Until now, very little is known about *H. suis* virulence genes (Haesebrouck et al., 2009). In the present study, we showed that *H. suis* is actively involved in the induction of gastric epithelial cell death *in vitro*. These data support previous findings of *H.* suis-induced necrosis of gastric epithelial, mainly parietal cells in mice and Mongolian gerbils (Flahou et al., 2010a). This cell death may have important implications for the development of various gastric pathologies, such as gastric erosion and/or ulcer formation (Dixon, 2001), gastric atrophy and even gastric cancer (Shirin and Moss, 1998). These lesions all have been observed in humans infected with non-*H. pylori* helicobacters (Morgner et al., 1995; Stolte et al., 1997; Debongnie et al., 1998) and are very often accompanied by gastritis (Stolte et al., 1997). Similarly, *H. suis* elicits a strong inflammatory response in experimentally infected

mice and Mongolian gerbils (Flahou et al., 2010a), possibly caused by direct effects of *H. suis*, but most likely also driven indirectly by necrosis of gastric epithelial cells. Cell necrosis results in the release of cellular contents, including molecules involved in the promotion of inflammation (Fink and Cookson, 2005; Vanlangenakker et al., 2008).

Whole-genome screening of *H. suis* strain 5 has revealed the presence of a gene homologous to the *H. pylori ggt*, but also the absence of other virulence factors (Vermoote et al., 2011) involved in *H. pylori*-induced cell death, such as VacA (Cover et al., 2003). Inhibition of GGT activity present in *H. suis* lysate and the use of recombinant *H. suis* GGT revealed an important role for this enzyme in the induction of gastric epithelial cell death in the present study. Similarly, the *H. pylori* GGT has also been identified as a cell death-inducing enzyme (Shibayama et al., 2003).

SDS-PAGE analysis and the relative increase of GGT activity in supernatants of AGS cells incubated for 20 h with recombinant *H. suis* GGT showed that this enzyme is secreted as a pro-form with subsequent processing into a large and a small subunit. This has also been described for other bacterial GGT's, such as the *H. pylori* and *E. coli* GGT (Suzuki and Kumagai, 2002; Boanca et al., 2006). Enzymatic γ-glutamyl transpeptidase activity typically catalyzes the release and transpeptidation of a γ-glutamyl group. This enzyme, also present on the membrane of mammalian cells, plays a role in the degradation and thus metabolism of extracellular glutathione (Orlowski and Meister, 1970). The tripeptide glutathione is an important antioxidant which degrades reactive oxygen species (ROS), including O_2–derived free radicals, as well as O_2–derived nonradical species such as hydrogen peroxide (H_2O_2) (Circu and Aw, 2010). In the present study, we describe the *H. suis* GGT-mediated degradation of reduced glutathione. Supplementation of the extracellular medium with GSH enhanced *H. suis* GGT-induced cell death, suggesting that metabolites of glutathione degradation are directly involved in this process.

In the present study, approximately 2 U/L GGT activity was detected in the supernatant of a 24-hour-old *H. suis* culture, containing a majority of live and motile bacteria. Recombinant *H.suis* GGT was added to AGS cells to reach similar GGT activity levels in AGS cell culture supernatant. It is reasonable to assume that similar levels of GGT activity are present in the region of *H. suis* colonization in the human or porcine stomach. Conflicting reports have been made about the localization of GGT in the highly related *H. pylori*. Some authors suggest a periplasmic localization (Chevalier et al., 1999; Shibayama et al., 2007). In this case, GGT could have free access to the surrounding glutathione (Chevalier et al., 1999; Shibayama et al., 2007), both with or without autolysis of the bacteria, which has been shown

to be an event taking place frequently in *H. pylori* (Marcus and Scott, 2001; Fujita et al., 2005). Other authors suggest, with strong argumentation, that *H. pylori* GGT is in fact a secreted protein (Bumann et al., 2002; Busiello et al., 2004; Schmees et al., 2007). In any case, the conclusion is that *H. pylori* GGT, and by extension most likely also GGT's from other gastric helicobacters, have free access to glutathione, both as a secreted and a periplasmic enzyme.

In the present study, normal basal concentrations of extracellular glutathione were in the micromolar range, which is in accordance with the results of previous studies (Yang et al., 1997). Interestingly, no differences in extracellular concentrations of total glutathione (combined fractions of reduced and oxidized glutathione) were observed between control and *H. suis* GGT-treated cells (results not shown), suggesting that *de novo* synthesis of glutathione and subsequent transport out of the cells take place. Indeed, it is well known that glutathione is synthesized intracellularly and subsequently translocated towards the extracellular space (Griffith and Meister, 1979). By this means, epithelial cells could provide *H. suis* GGT with a continuous supply of its main substrate.

Supplementation of AGS cell cultures with 5 mM GSH, strengthened the observed *H. suis* GGT-mediated increase of extracellular H_2O_2 concentrations and subsequent cell death. In order to produce similar effects *in vivo*, *H. suis* GGT must have access to relatively high concentrations of extracellular glutathione, which is indeed most likely provided by several sources. When cells die by means of necrosis, intracellular contents are released into the surroundings (Vanlangenakker et al. 2008), including large amounts of intracellular glutathione. In the stomach, intracellular glutathione concentrations are high (up to 10 mM), compared to most other tissues (Body et al., 1979; Meister and Anderson, 1983; Mårtensson et al., 1990). Moreover, foods like asparagus, cooked ham and orange juice contain high concentrations of glutathione (Valencia et al., 2001; Kuśmierek and Bald, 2008), resulting in daily uptakes up to more than 100 mg in adult humans (Flagg et al., 1994). Even bile reflux could serve as an important source of extracellular glutathione, since bile contains high levels (up to 6 mM) of GSH (Meister and Anderson, 1983). Several studies suggest that GSH can freely diffuse through the gastric mucus (Mårtensson et al., 1990; Ovrebø et al., 1997), in this way also reaching the fraction of *H. suis* bacteria residing in the mucus layer. All these mechanisms may also be important for *H. pylori* infections, since this bacterium also expresses a functional GGT. Interestingly, Farinati et al. (1996) have described an increase of both the reduced and oxidized glutathione content of gastric mucosa of *H. pylori*-infected humans suffering from chronic nonatrophic gastritis, possibly providing a greater source for

H. pylori GGT-mediated degradation of glutathione. Similar results have been described for total (sum of reduced and oxidized) glutathione contents in the stomach of *H. pylori*-infected Mongolian gerbils (Suzuki et al., 1999). However, these findings are in contrast with the results from another study, describing lower levels of reduced glutathione in *H. pylori*-infected human patients compared to *H. pylori*-negative individuals (Jung et al., 2001).

Deprivation of glutathione is one of the suggested mechanisms of *H. pylori* GGT-mediated cell death (Shibayama et al., 2007). However, in our attempt to reduce the *H. suis* GGT-induced cell death by supplementing cell culture supernatants with reduced glutathione, we observed the opposite effect, a higher number of dead cells. An increase of the extracellular H_2O_2 concentration was observed in AGS cell supernatants of *H. suis* GGT-treated cells. The relevance of this finding was confirmed by the observation that *H. suis* lysate also generated higher extracellular H_2O_2 concentrations, compared to HBSS treatment of cells, despite the presence in *H. suis* of enzymes involved in the neutralization of various ROS, including catalase and superoxide dismutase (Vermoote et al., 2011). The *H.* suis GGT-generated increase of the extracellular H_2O_2 concentration was even much higher when cells were co-incubated with *H. suis* GGT and reduced glutathione. Interestingly, this vast increase was also observed in the absence of AGS cells, showing that the hydrogen peroxide was mainly generated in a cell-independent manner, which contrasts with recently published results describing the *H. pylori* GGT-mediated production of H_2O_2 by AGS cells (Gong et al., 2010).

In the present study, we did not examine the exact mechanism by which H_2O_2 was generated. Several studies have described that plasma membrane-bound GGT of mammalian cells initiates prooxidant reactions through the catabolism of GSH (Dominici et al., 1999; Maellaro et al., 2000). Transpeptidation of the γ-glutamyl group generates the more reactive thiol cysteinyl-glycine, leading to the production of H_2O_2 through the reduction of Fe^{3+} and subsequent production of thiyl radicals and the superoxide anion ($O_2^{\bullet-}$) (Dominici et al., 1999):

$$\mathbf{GSH} \xrightarrow{\text{GGT}} \mathbf{glutamate + GC\text{-}S^{-}\ (cysteinyl\ glycine\ anion) + H^{+}}$$

$$\mathbf{GC\text{-}S^{-} + Fe^{3+}} \rightarrow \mathbf{GC\text{-}S^{\cdot} + Fe^{2+}}$$

$$\mathbf{Fe^{2+} + O_2} \rightarrow \mathbf{Fe^{3+} + O_2^{\cdot -}\ (superoxide\ anion)}$$

$$\mathbf{O_2^{\cdot -} + 2H_2O} \rightarrow \mathbf{O_2 + 2H^{+} + H_2O_2\ (hydrogen\ peroxide)}$$

Moreover, H_2O_2 can generate, through Haber-Weiss and Fenton reactions, the highly reactive hydroxyl radical (Vanlangenakker et al., 2008). So probably, the increased H_2O_2 concentrations also reflect the presence of these other reactive oxygen species or thiyl radicals, responsible for the observed increase of cell death. Anyhow, the observed H_2O_2 concentrations in the present study were relatively low compared to cell death-inducing amounts described in other studies (Hampton and Orrenius, 1997; Zhuang et al., 2008). In these studies, however, these higher H_2O_2 concentrations induced cell death already after 2-6 hours, so longer incubation with lower H_2O_2 concentrations, as in the present study, could explain the induced epithelial cell death. Interestingly, our findings in part contrast with earlier findings reporting the *H. pylori* GGT-induced apoptosis of AGS cells (Shibayama et al., 2003). *In vitro*, the type of H_2O_2-induced cell death has, however, been associated with variations in the concentration of this reactive oxygen species, with the higher concentrations inducing necrosis rather than apoptosis (Hampton and Orrenius, 1997; Krysko et al., 2008). Additionally, both apoptosis and necrosis have been observed in the same cell culture, dependent on a sustained c-Jun N-terminal kinase (JNK) activation due to an enhanced production of ROS (Kamata et al., 2005). In any case, it is well established that increased concentrations of ROS often result in necrotic cell death (Fiers et al., 1999; Duprez et al., 2009; Kroemer et al., 2009).

Twenty hours of incubation of AGS cells with the combination of rHSGGT and GSH did not result in a higher number of dead cells compared to AGS cells treated with rHSGGT alone. At this point in time, however, increased concentrations of extracellular H_2O_2 as well as peroxidation of cellular lipids could be observed. Lipid peroxidation in the plasma membrane often leads to loss of membrane integrity (Vanlangenakker et al., 2008), which is observed in the present study at later points in time compared to the detection of lipid peroxidation. Additionally, in mitochondria, reactive aldehydes derived from lipid peroxidation can affect oxidative phosphorylation and mitochondrial membrane potential (Vanlangenakker et al., 2008), which was also observed in the present study.

In conclusion, for the first time a *H. suis* virulence factor was identified, supporting earlier findings that also non-*H. pylori* helicobacters can cause human gastric disease. We have shown that *H. suis* directly causes gastric epithelial cell death *in vitro*, confirming the results of previous *in vivo* studies (Flahou et al., 2010a). Additionally, we have identified *H. suis* GGT as an important cause of *H. suis*-induced human gastric epithelial cell death *in vitro*. The observed necrosis is enhanced upon *H. suis* GGT-mediated degradation of reduced glutathione, resulting in an increase of extracellular concentrations of H_2O_2, generated by

glutathione degradation products in a cell-independent manner and resulting in cellular lipid peroxidation. This oxidative cell damage finally results in cell death.

Experimental procedures

Bacterial strain for DNA extraction and preparation of whole bacterial cell lysate

H. suis strain HS5, isolated from the gastric mucosa of a sow (Baele et al., 2008), was grown under biphasic culture conditions as described previously (Flahou et al., 2010b). Bacterial genomic DNA was isolated as described by Wilson (1994) and used for expression of recombinant *H. suis* GGT (rHSGGT). For the whole bacterial cell lysate of *H. suis*, bacteria were harvested by centrifugation, washed 2 times with HBSS and resuspended in HBSS. The bacterial suspension was sonicated 8 times for 30 seconds and centrifuged (15000 g, 5 min, 4°C) to remove cellular debris. The supernatant was filtered through a 0.22-µm pore filter (Schleicher and Schuell, Gent, Belgium) and stored at -80°C. The resulting protein concentration was determined with the *RC DC* Protein Assay (Bio-Rad, Hercules, CA, USA).

Preparation of recombinant *H. suis* GGT

Screening of the whole-genome sequence of *H. suis* strain 5 revealed the presence of a 1668 bp gene encoding a γ-glutamyl transpeptidase (GenBank accession number on http://www.ncbi.nlm.nih.gov/Entrez/: GU972556) which was approximately 73% identical to the GGT of *H. pylori* strain 26695 (GenBank accession number on http://www.ncbi.nlm.nih.gov/Entrez/: NP_207909.1) based on the predicted amino acid sequence. The enzyme was expressed in the *E. coli* Expression System with Gateway® Technology (Invitrogen, Carlsbad, CA, USA) as follows. The coding region of the *H. suis* GGT, without the predicted 18 aa signal sequence, was amplified by PCR (forward primer: 5'-CACCATGGCCACTTTGCCTCCTATTAAAGGC-3'; reverse primer: 5'-TTAAAATTCCTTGCGTGGATCTTGAGC-3') using Pwo polymerase with proofreading activity (Roche Applied Science, Mannheim, Germany) according to the guidelines for this enzyme. The resulting PCR product was cloned into the pENTR™/SD/D-TOPO® vector and transferred into the pDEST™17 destination vector. The resulting expression clone was transformed into the chemically competent *E. coli* strain BL21-AI™. A fresh culture was allowed to grow until the OD_{600} reached 0.4, after which 0.2% L-arabinose was added to induce the expression of recombinant *H. suis* GGT (rHSGGT). After incubation at 37°C for 3

hours with shaking, bacteria were harvested by centrifugation (4500g for 20 min). N-terminal 6xHis-tagged rHSGGT was purified on a Ni-sepharose column (His GraviTrap; GE Healthcare Bio-Sciences AB, Uppsala, Sweden) according to the manufacturer's instructions. Bound protein was eluted with 3 mL of elution buffer (20 mM sodium phosphate, 500 mM NaCl, 500 mM imidazole, pH 7.4) and collected in 17 mL HBSS, to prevent precipitation due to high imidazole concentrations. This eluate was concentrated to a final volume of 1.5 mL by ultrafiltration (VIVASPIN 20, 5000 MWCO; Sartorius stedim biotech, Goettingen, Germany), analyzed in the GGT activity assay and by SDS-PAGE with subsequent Brilliant Blue G - Colloidal (Sigma-Aldrich) staining. For further purification, the concentrated eluate was loaded on a Superdex^{TM} 75 gel filtration column (GE Healthcare Bio-Sciences AB), eluted with HBSS and collected as one mL fractions. The purity and GGT activity of five peak fractions were determined by SDS-PAGE and the GGT activity assay (see below), respectively. Since all five fractions were pure on SDS-PAGE and showed GGT activity, they were pooled and stored at -80°C until further use. Protein concentration was determined with the *RC DC* Protein Assay (Bio-Rad).

Cell culture

All *in vitro* cell experiments were performed with AGS cells (human gastric adenocarcinoma cell line; ATCC: CRL-1739). The cells were cultured in Ham's F12 (Invitrogen) supplemented with 10% fetal calf serum (FCS; HyClone, Logan, UT, USA), penicillin and streptomycin (Invitrogen).

Cell treatment

AGS cells were seeded at 2 x 10^4 cells for each well of a 24-well flat-bottom cell-culture plate (Greiner Bio One, Frickenhausen, Germany), allowed to adhere for 6 hours and serum-starved overnight. Prior to each incubation experiment, cells were washed 2 times with HBSS. During the experiments, cells were incubated in Ham's F12 supplemented with penicillin and streptomycin and FCS at a final concentration of 5%. Cell medium was supplemented with *H. suis* whole bacterial cell lysate at a final total protein concentration of 200 µg/mL for 44 hours at 37°C, unless stated otherwise. This corresponds to a calculated theoretical multiplicity of infection (MOI) of approximately 200. To determine whether the cell death-inducing agent was of protein nature, the bacterial lysate was pre-treated with heat (95°C, 1h) or trypsin (1 mg/mL; 2 h, 37°C; Invitrogen), which in turn was inactivated by the addition of 1 mg/mL soybean trypsin inhibitor (Sigma-Aldrich) and 5% FCS prior to

incubation with AGS cells. Alternatively, cell medium was supplemented with recombinant *H. suis* GGT at final protein concentrations of 2 and 8 µg/mL and incubation was performed for 20 and 44 hours at 37°C, unless stated otherwise. For inhibition of GGT activity in both whole bacterial cell lysates and rHSGGT, pre-incubation with 50 µM of acivicin (Enzo life Sciences, Farmingdale, NY, USA), a known GGT inhibitor, was performed for 1 hour at 37°C (Schmees et al., 2007). After this incubation, unbound acivicin was removed from the lysate and rHSGGT by repeated ultrafiltration (Vivaspin 500, 5000 MWCO; Sartorius stedim biotech). In additional experiments, the effect on AGS cells of supplementation with reduced glutathione (GSH) in the presence of recombinant *H. suis* GGT was determined. Therefore, GSH (Sigma-Aldrich, St. Louis, MO, USA) was added to the cell medium at a final concentration of 5 mM. For each experiment, negative control cells were incubated with HBSS which was treated in the same way as *H. suis* lysate or recombinant *H. suis* GGT.

Evaluation of cell death

For the evaluation of cell death, the combined fractions of floating and adherent AGS cells were analyzed. After an initial centrifugation step (1700g, 5 min), cells were resuspended and divided into 2 identical subpopulations. One half was used for the detection of caspase-3 activation as a marker for apoptosis (Fink and Cookson, 2005; De Bock et al., 2006; Galluzzi et al., 2009). Briefly, these cells were fixed with 4% paraformaldehyde for 10 min, followed by permeabilization with 0.1% Triton X-100 in HBSS for 2 min. Cells were incubated with a primary rabbit antibody directed against activated caspase-3 (R&D Systems Europe Ltd) for 1 h at 37°C, followed by an Alexa Fluor 488-conjugated goat anti-rabbit antibody (Invitrogen). Cells treated for 20 h with 0.5 µM staurosporine (Sigma-Aldrich) served as a positive control. The second cell subpopulation was used for the assessment of loss of plasma membrane integrity as a marker for necrosis (Fink and Cookson, 2005; Galluzzi et al., 2009). Briefly, after an initial washing step in HBSS, cell pellets were incubated for 20 min on ice with 1 µg/mL propidium iodide in HBSS. Triton X-100 (0.1%)-treated cells served as a positive control. All analyses were performed on a BD FACSCanto II flow cytometer and processed using FACSDiva software (Becton Dickinson, Erembodegem, Belgium). To confirm active caspase-3 staining of *H. suis* lysate-treated apoptotic cells, some cell suspensions were aditionally analyzed by fluorescence microscopy after counterstaining cell nuclei with Hoechst (100 µM, 15 min, RT). Images were captured using a Cell*M imaging workstation connected to a IX81 fluorescence microscope (Olympus). Except for brightness/contrast adjustments applied to the entire images, images were not digitally

manipulated. Washing steps in HBSS were included at appropriate points in time during all experimental protocols.

To further characterize *H. suis* GGT-induced cell death, rHSGGT-treated cells were examined by light and transmission electron microscopy. Cells were fixed in 4% formaldehyde in a buffer (pH 7.4) containing 0.121 M Na-cacodylate and 1% $CaCl_2$. After post-fixation in 1% osmium tetroxide, cultures were dehydrated in a graded series of ethanol and embedded in LX resin. Semithin sections of 2 µM were stained with toluidine blue and examined by light microscopy. Ultrathin sections were stained with uranyl acetate and lead citrate solutions before examining under a Jeol EX II transmission electron microscope at 80 kV.

GGT activity assay

Detection of GGT activity of *H. suis* whole bacterial cell lysate, *H. suis* culture supernatant or recombinant *H. suis* GGT was based on the method described by Orlowski and Meister (1963). Briefly, reaction buffer consisted of 2.9 mM L-glutamic acid 5-(3-carboxy-4-nitroanilide) (Sigma-Aldrich) as a donor substrate and 100 mM glycyl-glycine (Sigma-Aldrich) as an acceptor in 100 mM Tris buffer (pH 8.25). Bacterial supernatants, whole bacterial cell lysate of *H. suis* or the recombinant *H. suis* GGT were added and the reaction mixture was incubated at 37°C for 5-60 min. The release of p-nitroaniline was determined by measuring the absorbance at 405 nm. Activity was expressed as U/L or mU/mg protein and one unit was defined as the amount of enzyme that catalyzes the formation of 1 µmole of p-nitroaniline per minute under specified conditions.

Glutathione assay

To test the effect of *H. suis* GGT on reduced glutathione (GSH), AGS culture supernatants were supplemented with GSH to a final concentration of 5 or 1 mM GSH. Recombinant *H. suis* GGT was added at a final concentration of 2 µg/mL and incubation was done at 37°C for 44 h. After 0, 20 and 44 hours, an aliquot was removed to determine the concentration of GSH with the Glutathione Assay Kit (Sigma-Aldrich), according to the manufacterer's instructions. This assay is based on the continuous reduction of 5,5'-dithiobis-(2-nitrobenzoic acid) (DTNB) to TNB, which is measured spectrophotometrically at 412 nm. This same Glutathione Assay Kit was used for monitoring basal concentrations of total glutathione in the supernatants of AGS cell cultures. Total glutathione comprises both reduced glutathione (GSH), which is by far the most abundant form (Meister and Anderson, 1983),

and oxidized glutathione disulfide (GSSG). Catabolism of both forms has been shown to be mediated by eukaryotic membrane-bound γ-glutamyl transpeptidase (Meister and Anderson, 1983; Franco et al., 2007).

To determine if *H. suis* GGT activity also influences intracellular levels of total glutathione, the combined fractions of floating and adherent AGS cells were used. After counting cells in a Bürker counting chamber, the cells were pelleted by centrifugation (1700g, 7 min) and lyzed by three freeze/thaw cycles in a 5% 5-sulfosalicylic acid solution. Precipitated proteins were removed by centrifugation (10000g, 10 min) and the supernatant was used for glutathione determination using the Glutathione Assay Kit. The amount of total glutathione was expressed as pmol/cell.

Hydrogen peroxide assay

To detect extracellular H_2O_2 production in supernatants of AGS cell cultures treated or non-treated with *H. suis* whole bacterial cell lysate, rHSGGT or the combination of rHSGGT and 5 mM reduced glutathione, 25 µL of the cell supernatants were collected for processing with the Amplex Red Hydrogen Peroxide Assay Kit (Invitrogen). This assay is based on the horseradish peroxidase-catalyzed oxidation of Amplex Red (10-acetyl-3,7-dihydroxyphenoxazine), a nonfluorescent reagent that becomes highly fluorescent after oxidation by H_2O_2 (Maellaro et al., 2000).

Determination of lipid peroxidation

The possible involvement of previously described treatments in lipid peroxidation was determined by using 4,4-difluoro-5-(4-phenyl-1,3-butadienyl)-4-bora-3*a*,4*a*-diaza-*s*-indacene-3-undecanoic acid (BODIPY 581/591 C_{11}; Invitrogen), a probe which is distributed heterogeneously throughout the cell with predominant staining in the perinuclear region (Drummen et al. 2002). On excitation at 488 nm, this dye yields red emission with a 595 nm maximum. Upon oxidation, its red fluorescence is lost, coinciding with a shift of the emission maximum from 595 nm to 520 nm. This results in an increase of green fluorescence (Drummen et al., 2002; MacDonald et al., 2007).

After incubation of AGS cells for 15 or 39 h with rHSGGT or a combination of rHSGGT and GSH, cell culture plates were briefly centrifuged (1700g, 2 min) to collect both floating and adherent cell populations at the bottom of the wells. The cell supernatant was carefully aspired and kept at 37°C until further use. Cells were detached by brief trypsinization and resuspended in Ham's F12 supplemented with 10% fetal calf serum. After

centrifugation (1700g, 5 min), the cell pellet was resuspended in 200 µL HBSS containing 5 µM of the BODIPY 581/591 C_{11} probe. After incubation for 30 min at 37°C, cells were centrifuged (1700g, 5 min), followed by 2 washing steps in HBSS. Subsequently, the pellet was resuspended in the original cell culture supernatant and incubated for another 5 h at 37°C, with occasional shaking to keep the cells in suspension. Finally, cells were washed twice in HBSS and resuspended in HBSS for analysis of the red and green fluorescence intensity in the FL-2 and FL-1 channel of a BD FACSCanto II flow cytometer. Only cells with unchanged FSC (forward scatter) and SSC (side scatter) values were analyzed. Some control cells were treated with 500 µM H_2O_2 (37°C; 30 min) prior to analysis and served as a positive control.

Mitochondrial membrane potential ($\Delta\Psi_m$) measurement

To investigate the possible role of mitochondria in the generation of ROS in the present study, a $\Delta\Psi_m$-sensitive probe (5,5',6,6'-tetrachloro-1,1',3,3'-tetraethylbenzimidazolylcarbocyanine iodide; JC-1; Invitrogen) was used, since mitochondrial ROS generation is dependent on $\Delta\Psi_m$ (Starkov and Fiskum, 2003; Brookes et al., 2004; Galluzzi et al., 2009). Briefly, AGS cells were treated with HBSS or 2 µg/mL rHSGGT in the presence or absence of 5 mM reduced glutathione. After 24 hours of incubation, the combined fractions of floating and adherent cells were pooled and stained with 10 µg/mL JC-1 in HBSS (37°C, 15 min). Cells were centrifuged (1700g, 5 min), washed twice with HBSS and resuspended in HBSS prior to analysis with a BD FACSCanto II flow cytometer. Upon mitochondrial depolarization, a decrease in the red/green fluorescence intensity ratio of this probe can be observed. Only cells with unchanged FSC (forward scatter) and SSC (side scatter) values, which were considered living cells, were analyzed.

Statistical analysis

All experiments were repeated independently at least in triplicate. All data were analyzed with a Student *t* test or the non-parametric Mann-Whitney rank sum test. *P* values less than 0.05 were considered significant.

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General Discussion

In contrast to *H. pylori*, only very little is known about the exact role in human gastric pathology of non-*H. pylori Helicobacter* (NHPH) species in general and *H. suis* in particular. Nevertheless, the latter is the most prevalent NHPH species in humans suffering from gastric disease (Trebesius et al., 2001; Van den Bulck et al., 2005). No doubt, the very fastidious nature of the bacterium has hampered progress in *H. suis* research. In 2008, our research group was the first to successfully isolate *H. suis in vitro*, opening new doors to investigate the exact role of this bacterium in gastric pathology and to develop possible strategies to control this infection (Baele et al., 2008).

In *Helicobacter pylori* research, rodent models are very often used to investigate bacteria-host interactions. Mainly in Mongolian gerbils, severe *H. pylori*-related gastric pathologies have been reproduced, including gastric ulcer and adenocarcinoma (Watanabe et al., 1998; Ikeno et al., 1999). Additionally, a more rapid development of inflammatory changes is observed in this animal species, when compared to mice (Court et al., 2002). Only very few studies describe an experimental infection of Mongolian gerbils with non-*H. pylori Helicobacter* species, and these are limited to *H. felis* and *H. bizzozeronii* (Court et al., 2002; De Bock et al., 2006a and 2006b). In chapter one and two, we describe the ability of *H. suis* to colonize the Mongolian gerbil stomach. However, preliminary screening of a gerbil population, prior to their use for studying *H. suis* infection, revealed the abundant presence of a yeast, mainly in the antrum. Internally Transcribed rRNA Spacer 2 Region-PCR fragment length and sequence analysis revealed it to be *Kazachstania heterogenica*, previously detected in rodent faeces (Kurtzman et al., 2005). In subsequent experimental infection studies, *K. heterogenica* alone did not elicit significant pathology. However, in *H. suis*-*K. heterogenica* coinfected animals, a markedly enhanced inflammation was observed, compared to *H. suis* monoinfected animals. This indicates a synergism between these two completely different micro-organisms. In humans, some reports have also described a synergism, detrimental to the host, between *H. pylori* and gastric yeasts, as shown by the increased prevalence of gastric ulcers in humans with co-existence of *H. pylori* and *Candida* species in their stomach (Karczewska et al., 2009). Similarly, fungal colonization of gastric ulcers has been shown to impair ulcer healing, accompanied by persistent clinical symptoms (Zwolińska-Wcislo et al., 2001). In contrast, however, some studies describe the beneficial impact of supplemented *Saccharomyces boulardii*, another yeast species, on the classic triple-therapy prescribed for *H. pylori* eradication both in adults and children (Hurduc et al., 2009; Song et al., 2010).

Besides yeast, several bacterial species may also influence the course of a gastric *Helicobacter* infection. Depending on the bacterial species involved and the animal or human host, this may result either in less or more severe gastric lesions.

Bifidobacterium bifidum has been described to reduce *H. pylori* growth and the pathologies caused by this micro-organism (Chenoll et al., 2010). Likewise, *Lactobacillus (L.) plantarum* displays *in vitro* anti-*H. pylori* activity (Rokka et al., 2006) and various *Lactobacillus* spp. have been shown to attenuate, through increased expression of SOCS (suppressor of cytokine signaling), the expression of inflammatory mediators such as TNF-α, IL-8, iNOS and cox-2 in *H. pylori*-treated AGS cells (Lee et al., 2010). Treatment of *H. pylori*-infected mice with *L. fermenti* or *L. acidophilus* decreases the mucosal inflammation and reduces the *H. pylori* colonization density (Cui et al., 2010). Aiba and coworkers (1998) showed that *L. salivarius* exerts an anti-*H. pylori* effect in gnotobiotic mice, however only when *H. pylori* was vastly outnumbered by the *Lactobacillus* strain. The question remains if similar results would have been obtained when using a *H. pylori* strain showing much higher colonization densities, such as the SS1 strain (Lee et al., 1997). Various factors have been suggested to be responsible for the anti-*H. pylori* activity of certain *Lactobacillus* strains, including the production of bacteriocins (Barrett et al., 2007; Deraz et al., 2007; Lin et al., 2009), the mere presence of the *Lactobacillus* cell wall (Rokka et al., 2006) and the production of high quantities of organic acids, of which lactic acid is generally the most abundant one. Indeed, high concentrations of lactic acid have been shown to exert an anti-*H. pylori* activity *in vitro* (Aiba et al., 1998; Lin et al., 2009).

Not all *Lactobacillus* spp. are, however, able to produce equally high concentrations of lactic acid (Aiba et al., 1998; Yoon et al., 2006). Low concentrations of this organic acid may be beneficial for growth of *Helicobacter*. In a recent metabolic profiling study, it was shown that *H. suis* uses lactic acid for metabolic purposes, possibly enhancing colonization and the associated gastric pathology in pigs or humans (Flahou et al., unpublished results). Other *Helicobacter* species such as *H. pylori* can also use lactic acid as a carbon source (Schilling et al., 2002).

Persistent colonization of the stomach mucosa with *Clostridium butyricum* has been achieved after experimental infection of germ-free mice (Takahashi et al., 2000). In the same study, animals were first inoculated *per os* with *H. pylori* and subsequently with *C. butyricum*. This rapidly induced a marked reduction of *H. pylori* bacteria colonizing the gastric mucosa. Additionally, culture supernatant of *C. butyricum* and also butyric acid alone, were shown to inhibit the growth of *H. pylori in vitro*. The question remains if butyric acid-producing

bacteria are also capable of colonizing the gastric mucosa of humans, as compared to the germ-free mice used in the study by Takahashi and coworkers (2000). In pigs, colonization of the pars oesophagea with significant populations of mainly anaerobic bacteria, including *Clostridium* spp., has been decribed (McGillivery et al., 1992). This may lead to the production of butyric acid at this exact location and a subsequent blocking of the development of *H. suis*-induced lesions of the pars oesophagea, as butyrate has been described to protect cells against damage (Bingham et al., 1997; Scharlau et al., 2009). On the other hand, lactobacilli can also colonize the pars oesophagea (McGillivery et al., 1992). It has been suggested that high concentrations of lactic acid (and other organic acids), produced by the metabolism of *Lactobacillus* and other bacteria in the stomach, might play a role in the pathogenesis of gastric ulcer disease in these animals (Argenzio and Eisemann, 1995). Krakowka et al. (1998) have reproduced such lesions in gnotobiotic piglets fed a high-carbohydrate liquid diet and infected experimentally with *Lactobacillus* or *Bacillus* spp..

Infectious agents colonizing the host in tissues other than the stomach may also influence *H. pylori* infection and the host response. Mice colonized in the lower bowel with the enterohepatic *Helicobacter* species *H. bilis* develop less severe gastric lesions compared to non-*H. bilis*-colonized mice after experimental infection with *H. pylori* (Lemke et al., 2009). Similarly, concomitant infection with several parasites, including intestinal helminths and systemic microfilaria, has been described to attenuate *H. pylori*-induced gastritis (Whary and Fox, 2004; Whary et al., 2005; Martin et al., 2010). These effects have mostly been ascribed to anti-inflammatory Th2 or Treg-polarized responses elicited by these other infectious agents.

In conclusion, when performing research on gastric *Helicobacter* infections, one must be aware that other agents colonizing or infecting the host may influence *Helicobacter*-elicited gastric pathology and host responses. On the other hand, interactions between gastric helicobacters and certain other micro-organisms may be utilized in therapeutic strategies by taking advantage of the beneficial effect of certain micro-organisms on gastric *Helicobacter*-elicited pathology.

In chapter 2, we describe the host response in 2 different mouse strains and Mongolian gerbils infected for up to 8 months with *H. suis*. A stronger inflammatory response was elicited in BALB/c mice, compared to C57BL/6 mice. Based on the different preferential T helper response in these 2 mouse strains (O'Rourke and Lee, 2003), this suggests that a more Th2-polarized response is associated with experimental *H. suis* infection. This was confirmed

in a recent study by our research group, describing a mixed Th17/Th2 response in BALB/c mice infected experimentally with *H. suis*, with predominant Th17 polarization (Van Deun et al., submitted). This contrasts with the mixed Th17/Th1 response observed in *H. pylori*-infected mice (Shi et al., 2010). While Shi and coworkers (2010) and other research groups describe an important role for IFN-γ, a signature Th1 cytokine, in *H. pylori*-induced inflammation, we have never observed an upregulation of IFN-γ after experimental *H. suis* infection, neither in BALB/c nor in C57BL/6 mice (Van Deun et al., submitted), the latter nonetheless being a predominant Th1 responder (Ferrero et al., 2000). This contrasts with the results of Cinque et al. (2006), who described a central role for IFN-γ-induced inflammation after experimental inoculation of mice with a pig gastric mucus homogenate, containing *H. suis* bacteria. Other micro-organisms present in this homogenate may, however, have skewed the host reponse.

The development of low-grade B cell MALT lymphoma has been associated with a Th2 response, rather than with a Th1-predominant response (Greiner et al., 1997; Knörr et al., 1999). In Th2 responding BALB/c mice, we indeed observed an increased infiltration of the gastric mucosa with B lymphocytes, although no B cell MALT lymphoma-like lesions were observed. This type of lesions was, however, present in Mongolian gerbils infected for 8 months with *H. suis*. The study of *Helicobacter*-related gastric pathology in hundreds of thousands of humans has revealed that the chance of developing gastric MALT lymphoma is higher in humans infected with NHPH, compared to *H. pylori* (Stolte et al., 1997; Stolte et al., 2002), underlining the suitability of the Mongolian gerbil model for investigating *H. suis*-related gastric pathology in humans.

The suitability of the Mongolian gerbil model for modelling the pathology elicited by *H. suis* in humans, is further evidenced by the striking differences between *H. suis* colonization and inflammation observed in this animal species, compared to both mouse models described in chapter 2. Indeed, in gerbils, *H. suis* colonization and the associated inflammation were observed predominantly in the antrum and in only a very narrow zone at the transition zone between forestomach and stomach, whereas *H. suis* colonizes the entire stomach in mice and elicits inflammation mainly in the fundus in this latter animal species. In humans infected with non-*H. pylori* helicobacters, including *H. suis*, a chronic active gastritis is observed predominantly in the antrum and this correlates well with the colonization pattern of the bacteria, which can not be detected in the fundus (corpus) of the majority of these patients (Heilmann and Borchard, 1991; Debongnie et al., 1995; Stolte et al., 1997). Possibly, these differences could be explained by the absence of the *cag* pathogenicity island in *H. suis*

and other non-*H. pylori* helicobacters (Vermoote et al., 2011 and unpublished results). Indeed, experimental infection studies in gerbils with wild-type and mutant Δ*cag*A and Δ*cag*Y *H. pylori* strains have revealed that all strains elicit a strong inflammation and increased epithelial cell proliferation in the antrum, whereas severe inflammation, huge lymphoid aggregates, parietal cell atrophy, increased proliferation and mucous gland metaplasia in the corpus are mainly observed in WT-infected animals (Rieder et al., 2005; Wiedemann et al., 2009). The latter *cag*PAI-dependent lesions correlate with bacterial colonization levels.

As discussed in the general introduction of this thesis, *H. pylori* induces gastric epithelial cell death through the intervention of various virulence factors. Cell death plays an important role in the development of several gastric pathologies, including gastric ulceration, gastric atrophy and gastric cancer (Shirin and Moss, 1998; Dixon, 2001). Our *in vivo* studies have shown that *H. suis* is also capable of causing gastric epithelial cell death. Different types of cell death can occur in eukaryotes, of which apoptosis seems to be the most important type associated with *H. pylori* infection. In our study, however, no increased apoptosis could be detected. Although a basal apoptotic ratio was detected in both uninfected and infected mice and Mongolian gerbils, an increase of the apoptotic ratio was possibly missed, since active caspase-3 positive cells were often visualized dissociated from the surrounding tissue. It is indeed well-known that tight and adherens junctions are destroyed during apoptosis, leading to cell extrusion, already early in apoptosis (Rosenblatt et al., 2001; Bojarski et al., 2004; Kessler and Müller, 2009). Thus, a substantial fraction of apoptotic gastric epithelial cells must be carried along by the mucus and the peristaltic movement, making their detection *in situ* impossible.

The lack of an increased apoptotic ratio was confirmed by ultrastructural analysis, revealing that *H. suis* bacteria are seldom associated with apoptosis of gastric epithelial cells, whereas they are often surrounded by debris of necrotic parietal cells. Immunohistochemical staining confirmed the loss of parietal cells, mainly at the transition zone between fundus and antrum. Because this cell type is highly specialized and extremely difficult to maintain *in vitro*, we investigated the possible direct effect of *H. suis* on gastric epithelial cell death in AGS cells, derived from a human gastric adenocarcinoma. In chapter 4, we identified *H. suis* γ-glutamyl transpeptidase as an important virulence factor involved in the induction of gastric epithelial cell death. We showed that this enzyme catalyzes the degradation of extracellular glutathione. The resulting degradation products actively cause a cell-independent extracellular generation of H_2O_2, leading to lipid peroxidation ahead of final necrosis. Increased

concentrations of ROS indeed often lead to necrosis (Fiers et al., 1999; Duprez et al., 2009; Kroemer et al., 2009). In our study, increased extracellular ROS may have induced necrosis by inflicting direct and uncontrolled damage to the cellular structures, including the plasma membrane. Alternatively, a strictly controlled and regulated form of necrosis (Vandenabeele et al., 2010) may be responsible for the observed effect. For instance, the generation of extracellular ROS could lead to a sustained c-Jun N-terminal kinase (JNK) activation, leading to necrotic cell death (Kim et al., 2007; Vanden Berghe et al., 2007).

Secretion of hydrochloric acid is the main function of gastric parietal cells. The hydrogen/potassium ATPase, present in the membrane of these cells, is sentenced to pumping acid against a huge gradient, which requires large amounts of ATP (Forte and Zhu, 2010). This important cellular fuel is generated mainly in mitochondria (Brookes et al., 2004), which are indeed abundant in parietal cells. Abundant mitochondrial respiration, however, is also an important source of intracellular ROS (Poyton et al., 2009). The substantial generation of intracellular ROS in parietal cells could thus make them more susceptible to damage inflicted by exogenous ROS, for instance through the activity of *H. suis* GGT. Excessive ROS are generally scavenged by several antioxidants, including reduced glutathione. Higher concentrations of intracellular glutathione are present in gastric mucosa, compared to other tissues (Body et al., 1979; Mårtensson et al., 1990). Together with high concentrations of glutathione in certain foods, this provides a large amount of substrate for the GGT of gastric helicobacters (Figure 1). As a consequence, GGT-mediated degradation of large amounts of extracellular glutathione and subsequent oxidative damaging of cells could thus very well be a mechanism unique to gastric helicobacters. Finally, the massive degradation of glutatione in the gastric lumen by *H. suis* GGT may lead to a depletion of extracellular glutathione in the lumen of the small intestine. This may have an impact on intestinal epithelial cells, which also depend on glutathione for maintaining their health (Mårtensson, 1990) (Figure 1).

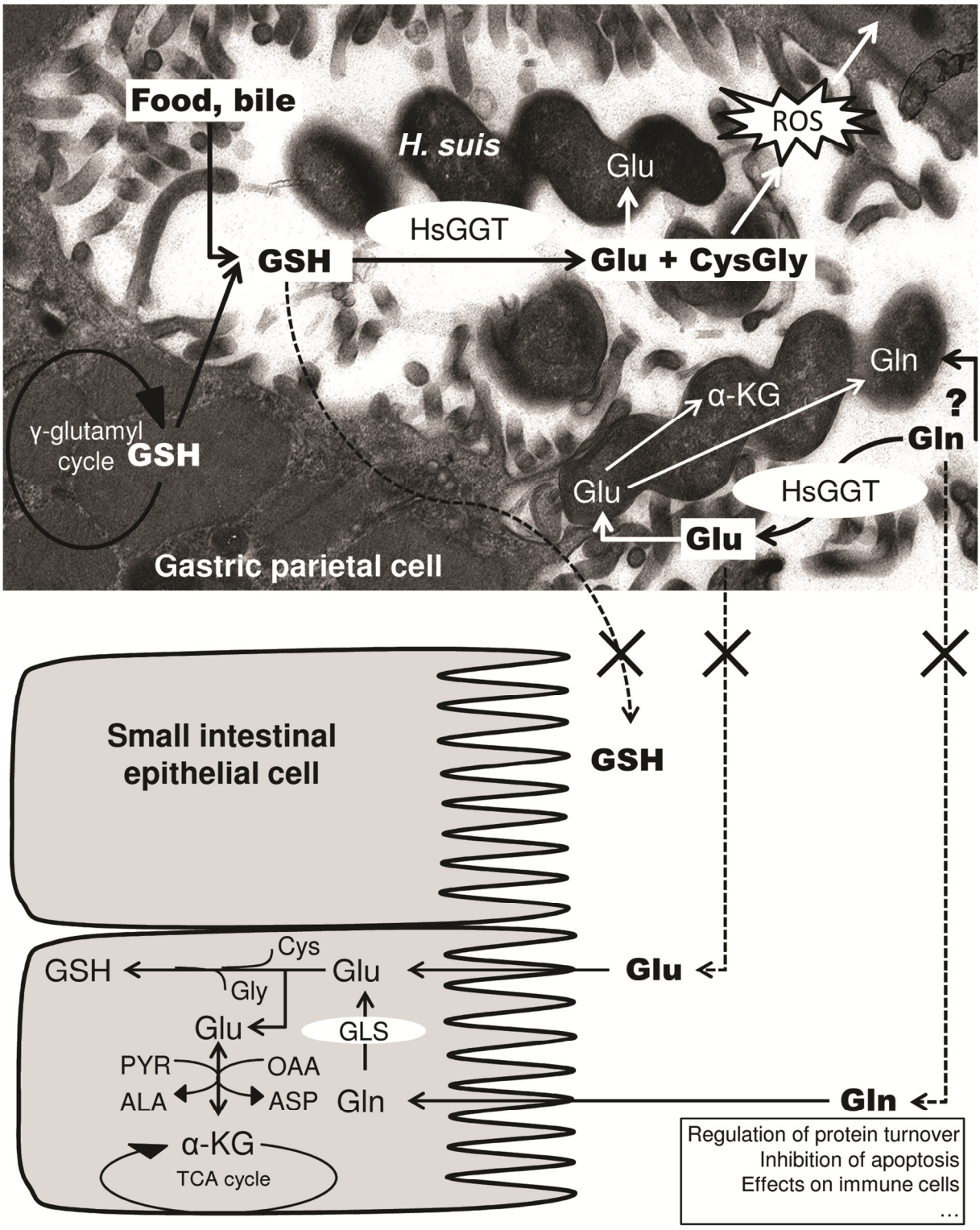

Figure 1. Summary of the proposed roles of *H. suis* GGT in the bacterial metabolism and their possible effects on the gastric and small intestinal epithelium. ALA, alanine; ASP, aspartate; α-KG, α-ketoglutarate; Cys, cysteine; CysGly, cysteinyl glycine; Gln, glutamine; GLS, glutaminase; Glu, glutamate; Gly, glycine; GSH, reduced glutathione; HsGGT, *H. suis* γ-glutamyl transpeptidase; OAA, oxaloacetate; PYR, pyruvate; ROS, reactive oxygen species; TCA, tricarboxylic acid.

GGT-mediated hydrolysis (as opposed to its transpeptidase activity) of glutathione results in the transfer of the γ-glutamyl moiety to water with formation of glutamate (Meister and Anderson, 1983; Shibayama et al., 2007) (Figure 2). Similar to *H. pylori, H. suis* GGT also catalyzes the deamidation of glutamine to glutamate, with formation of ammonia as a by-product (Flahou et al., unpublished results; Shibayama et al., 2007; Leduc et al., 2010) (Figure 2).

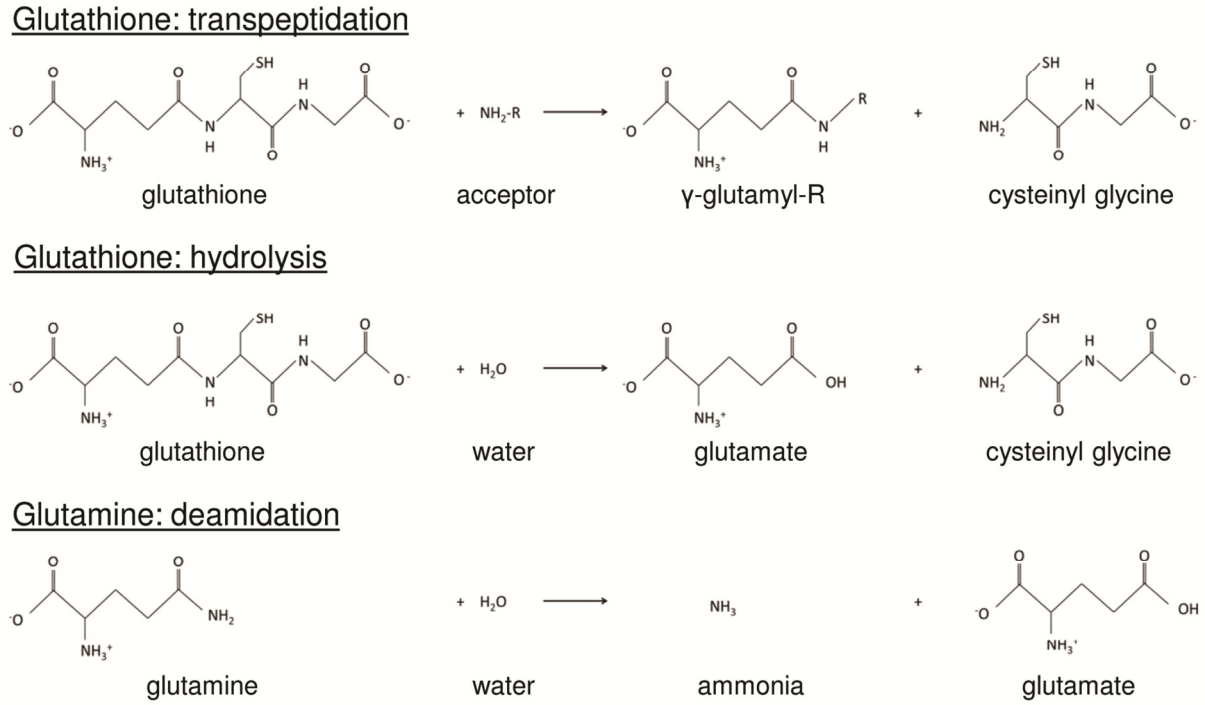

Figure 2. Actions of GGT on glutathione and glutamine.
Shown are GGT-mediated transpeptidation and hydrolysis of glutathione and GGT-mediated deamidation of glutamine.

In addition, unpublished results of a recent metabolic profiling study of *in vitro* grown *H. suis* show a strong depletion of several amino acids in culture medium during *H. suis* culture, including aspartate, asparagine and cysteine. For glutamine (Gln), however, the levels even dropped below the limit of detection and genome analysis of *H. suis* has revealed the presence of several genes involved in the translocation of glutamine across bacterial membranes, revealing an important role for glutamine metabolization by this bacterium (Nohno et al., 1986; Vermoote et al., 2011). For *H. pylori*, glutathione or glutamine-derived glutamate (Glu) is transported to the bacterial cytoplasm by a sole Glu transporter (HP-GltS), also present in the *H. suis* genome (Vermoote et al., 2011). A fraction can directly be used for glutamine synthesis, involving glutamine synthetase, which is also present in the *H. suis* genome (Shibayama et al., 2007; Leduc et al., 2010; Vermoote et al., 2011). More importantly, the

presence of a gene encoding a glutamate dehydrogenase in the *H. suis* genome suggests that the bacterium can channel glutamate to the energy-providing tricarboxylic acid (TCA or Krebs) cycle, by conversion to α-ketoglutarate (Schilling et al., 2002) (Figure 1). Energy may also be provided through several other amino acids and lactate, which are also depleted in the medium during *H. suis* cultivation. Indeed, pyruvate can be generated from several amino acids through the activity of enzymes such as alanine dehydrogenase and from lactate through D-lactate dehydrogenase activity. Both enzymes are present in *H. suis* and the latter suggests possible cross-feeding by lactate-producing bacteria in the stomach. Most likely, *H. suis* can not utilize glucose through glycolysis, as several enzymes, including glucokinase, involved in this process appear to be missing. The *H. suis* genome also lacks most components of the Entner-Doudoroff pathway, which is an alternative pathway for converting glucose to pyruvate, a main supplier of energy through the TCA cycle (Schilling et al., 2002). In *H. pylori*, the ability of the organism to extract energy from hexoses such as glucose also seems impaired (Schilling et al., 2002), although the bacterium has been shown to utilize D-glucose (Mendz et al., 1993). In conclusion, all available data indicate that, as in *H. pylori* (Stark et al., 1997; Schilling et al., 2002) or even more so, glucose metabolism is not an important source of energy for *H. suis,* whereas metabolites as lactate and several amino acids, including glutamine, are fed into the central bacterial metabolism through conversion to pyruvate or α-ketoglutarate.

As mentioned above, *H. suis* GGT has, besides glutathione, another important substrate, L-glutamine, in this way directly competing with epithelial cells for this amino acid (Figure 1). Interestingly, dietary glutamine supplementation attenuates *H. pylori*-induced gastric pathology, both in mice and gerbils (Hagen et al., 2009; Amagase et al., 2010). In the latter animal species, glutamine supplementation inhibits intracellular accumulation of toxic ammonia, possibly due to metabolization of ammonia to urea through glutamine-stimulated arginase activity (Nakamura and Hagen, 2002; Amagase et al., 2010). Also in the intestine, glutamine plays an important cell-protective role. In malnourished rats, glutamine can restore the gut intracellular glutathione stores and villus heights (Belmonte et al., 2007). This is not surprising, as L-glutamate, derived from L-glutamine in mitochondria through glutaminase, is a precursor for glutathione synthesis (Blachier et al., 2009) (Figure 1). In addition, glutamine supplementation in rats has been shown to induce an upregulation of several gut mucosal enzymes involved in glutathione synthesis and antioxidant activity, including glutaminase, membrane-bound γ-glutamyl transpeptidase, γ-glutamylcysteine synthetase and glutathione peroxidase (Kaufmann et al., 2008; Kul et al., 2009). Finally, glutamine starvation of cells *in*

vitro induces a reduced expression of protective Hsp70, through a reduced half-life of Hsp70 mRNA (Roth, 2008).

Besides this protective effect, glutamine also plays an essential role in the metabolism of gastrointestinal epithelial cells. It has been suggested that glutamine dependence is higher for intestinal epithelial cells compared to gastric epithelial cells (Madej et al., 1999). Intracellularly, glutamine can be converted to glutamate, which is, in the presence of oxaloacetate, converted to aspartate and α-ketoglutarate, which can be channeled into the Krebs cycle (Blachier et al., 2009) (Figure 1). Partial oxidation of glutamine to aspartate results in the fast delivery of 9 moles of ATP (Aledo, 2004). In pigs, glutamine was shown to be the only amino acid, out of about 17 dietary amino acids tested, to have a negative net portal balance, indicating a net uptake by portal-drained viscera (Stoll et al., 1997). When pigs grow older, an increase of glutamate dehydrogenase and alanine aminotransferase is observed in the epithelium of the small intestine, indicating that glutamine metabolism becomes more important with increasing age (Madej et al., 1999). Both delivered from the intestinal lumen as well as arterial blood, it is considered a major fuel for rapidly proliferating cells, including enterocytes, lymphocytes and macrophages (Windmueller and Spaeth, 1980; Wu, 2009). Together with other amino acids, including glutamate and aspartate, it is considered a more important fuel for enterocytes, compared to glucose (Windmueller and Spaeth, 1980). Depletion of this enterocyte fuel by *H. suis* GGT may thus result in an impaired intestinal function, for instance leading to a decreased weight gain, as observed in *H. suis*-infected pigs (Kumar et al., 2010). Finally, it has also been shown that glutamine plays an important role in the energy supply and anti-inflammatory functions of various cell types of the immune system, for instance proliferation and IFN-γ secretion of T cells (Newsholme et al., 1999; Roth, 2008).

All these data, summarized in figure 1, reveal an essential role for glutamine in maintaining gastrointestinal health. The fact that gastric helicobacters, including *H. suis,* are ruthless adversaries of epithelial cells when it comes down to incorporating glutamine suggests that glutamine deprivation by GGT activity may also play a significant role in *Helicobacter*-related gastric pathology.

Keeping in mind the adverse effects of *H. suis* GGT, it may be a good approach to target the enzyme by vaccination with the available recombinant *H. suis* GGT. In this way, potential, if any, undesirable side-effects linked to whole-cell protein vaccination could be eliminated and detrimental *H. suis* GGT enzymatic activity on extracellular glutathione and

glutamine can be targeted. For *H. pylori*, the use of GGT as vaccine antigen has indeed proven to be successful (Anderl et al., 2010). Since *H. pylori* GGT is a secreted protein, specific T cells induced by a GGT-based subunit vaccine probably lack the capability of targeting the pathogen. To overcome this, mucosal immunization was done with *H. pylori* GGT, in combination with several outer membrane proteins (Anderl et al., 2010). This immunization strategy induced a strong antibody response to the GGT, blocking its enzymatic activity. Besides inducing gastric epithelial cell death, the *H. pylori* GGT has also been shown to cause inhibition of T cell proliferation (Schmees et al., 2007). As a consequence, mucosal immunization with *H. pylori* GGT results in neutralization of its immunosuppressive effect and subsequent recovery of the full proliferative capacity of T cells upon vaccination with a second antigen (Anderl et al., 2010). Careful examination of the *H. suis* genome may reveal possible additional candidate antigens for use in subunits vaccines, containing recombinant *H. suis* GGT.

Besides the use of subunit vaccines instead of whole bacterial cell lysate for *H. suis* vaccination, as described in chapter 3, other vaccine formulations can be considered. A recent study has revealed that subcutaneous vaccination with *H. suis* lysate and complete Freund's adjuvant against subsequent *H. suis* challenge induces a similar (>150-fold) reduction of colonization compared to intranasal *H. suis* lysate+CT vaccination (Van Deun et al., unpublished results). In contrast, the prophylactic subcutaneous vaccination with *H. suis* lysate and a saponin-based adjuvant described in chapter 3, induced a less protective effect compared to intranasal *H. suis* lysate+CT vaccination, underlining the importance of chosing not only the right antigen and route of administration, but also the right adjuvant.

In conclusion, the results described in this thesis clearly demonstrate that *H. suis* causes severe inflammation in rodent models of human gastric disease. The Mongolian gerbil model is particularly suitable for modelling *H. suis*-induced pathology. However, when performing *in vivo* studies, attention should be paid to the fact that other agents can influence *Helicobacter*-elicited gastric pathology.

Besides lesions related to the influx of various inflammatory cells, *H. suis* infection was also shown to cause necrosis of gastric parietal cells. *In vitro, H. suis* induces both apoptosis and necrosis of human gastric epithelial cells. Other possible cell death-inducing factors remain to be elucidated, for instance through the generation of random mutants by transposon shuttle mutagenesis (Odenbreit et al., 1996 and 1999). As for the induction of gastric epithelial cell necrosis, the *H. suis* γ-glutamyl transpeptidase was shown to play an

essential role, through the degradation of glutathione and subsequent oxidative damaging of cell components. Future experimental infection studies with dietary glutamine or glutathione supplementation and/or experimental infection with a mutant *H. suis* strain lacking GGT will further unravel the complex interplay between glutamine, glutathione, *H. suis* and its host.

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Summary

Already in the early days of *Helicobacter* research, pathologists identified patients where they did not find the typical comma-shaped *Helicobacter (H.) pylori* organisms in their histological sections, but rather large non-*H. pylori* helicobacters with a typical spiral-shaped morphology. In literature, these bacteria are often referred to as "*Helicobacter heilmannii*". Recent investigations, however, have revealed that this group of non-*H. pylori* helicobacters comprises different species which have been detected in the stomach of different animal species. Therefore, at present, the name "*H. heilmannii*" should be reserved to the species as described by Smet and coworkers (published in International Journal of Systematic and Evolutionary Microbiology). The most common gastric non-*H. pylori Helicobacter* species in humans is *Helicobacter suis*, naturally occurring in the stomach of the majority of pigs worldwide. Hitherto, the virulence mechanisms of *H. suis* were largely unknown, in part due to the very fastidious nature of this bacterium. Whole-genome screening of *H. suis* revealed that it lacks some typical virulence factors of *H. pylori*, such as the *cag* pathogenicity island and the vacuolating cytotoxin.

Our research group was the first to successfully isolate *H. suis in vitro* in 2008. Taking advantage of this essential step forward, the general aim of this thesis was to obtain better insights into the pathogenesis of human gastric diseases associated with a *H. suis* infection and to develop possible strategies to control this infection.

In chapter one, we describe the first successful colonization of Mongolian gerbils with a pure culture of *in vitro* grown *H. suis*. This animal model is considered very suitable for investigation of *Helicobacter*-related gastric pathology. However, a preliminary screening of a gerbil population intended for use in *Helicobacter suis* infection studies revealed a natural yeast infection in the stomach of these animals in exactly the same regions as described for gastric helicobacters, including *H. pylori* and *H. felis*. Identification was done by Internally Transcribed rRNA Spacer 2 Region (ITS2) PCR fragment length analysis and revealed it to be *Kazachstania heterogenica*, previously isolated from rodent faeces. To investigate a possible pathologic role of *K. heterogenica*, Mongolian gerbils were infected experimentally with this yeast. In all inoculated animals, high colonization densities were observed, mainly in the antrum, and few pathologic changes were seen in the stomach of infected animals. Next, gerbils free of *K. heterogenica* and gastric helicobacters were infected with *H. suis*, or *K. heterogenica,* or both, to investigate possible interactions between both micro-organisms. Dual infection resulted in a significant increase of inflammation compared to animals infected with *Helicobacter suis* alone, indicating a synergism between both agents. These results

highlight the importance of considering the possible interference of other micro-organisms in experimental gastric *Helicobacter* infection studies.

The second study deals with the interactions between *H. suis* and the gastric mucosa of the host. Therefore, long-term experimental *H. suis* infections were studied in the Mongolian gerbil model, as well as in two mouse models (BALB/c and C57BL/6 mice). Female six-week-old animals, free of *K. heterogenica* and gastric helicobacters, were inoculated with pure *in vitro* grown *H. suis* (strain 5) and sacrificed at 3 weeks, 9 weeks and 8 months after infection. Gastric tissue samples were collected for PCR analysis, histological and ultrastructural examination. In gerbils, bacteria mainly colonized the antrum and a narrow zone in the fundus near the forestomach/stomach transition zone. In both mice strains, bacteria colonized the entire glandular stomach. Transmission electron microscopy revealed an association between *H. suis* colonization and necrosis of parietal cells in all three animal strains. Immunohistochemical staining of the hydrogen/potassium ATPase present on the parietal cell membrane showed that parietal cell loss was most pronounced at the transition zone between fundus and antrum. From 9 weeks after infection onwards, an increased proliferation rate of mucosal epithelial cells was detected in the stomach regions colonized with *H. suis*, as shown by counting of Ki67-positive cells.

At all timepoints, all uninfected control animals showed normal histomorphology and no inflammation. In general, *H. suis*-infected BALB/c mice showed a stronger diffuse inflammation and more abundant inflammatory cell aggregates and lymphoid follicles, compared to C57BL/6 mice. In both mice strains, inflammation was mostly limited to the fundus. At 8 months post infection, a higher number of B cells was detected only in the fundus of BALB/c mice. These data suggest that, in contrast to a *H. pylori* infection, *H. suis* infection is mainly associated with a Th2 response.

Most infected gerbils showed a marked lymphocytic infiltration in the antrum and in the forestomach/stomach transition zone. Diffuse infiltration as well as the presence of large lymphoid aggregates became worse in the course of time. This was accompanied by destruction of the gastric glands and disruption of the lamina muscularis mucosae. From 9 weeks after infection onwards, lymphocytic aggregates contained a majority of CD20-positive B cells, mostly organized in germinal center-presenting lymphoid follicles. In gerbils infected for 8 months, these germinal centers were often large, hyperproliferative and irregular. Additionally, severe destruction of the normal antral architecture at the inflamed sites and development of mucosa-associated lymphoid tissue (MALT) lymphoma-like lesions, such as

lymphoepithelial lesions and infiltration of the tunica muscularis, were observed in some gerbils. The location and the induced pathology, including gastric MALT lymphoma, resemble those observed in humans infected with non-*H. pylori* helicobacters. In conclusion, these findings suggest that the possible involvement of *H. suis* in human gastric disease should not be neglected. Compared to mice, *H. suis*-induced gastric pathology in Mongolian gerbils more closely resembles the human situation, indicating that this model may be more suitable for studying *H. suis*-host interactions.

Prevention and eradication of gastric *Helicobacter* infection are two very important steps to drastically reduce the number of people suffering from gastric disease. Therefore, in the third study, *in vitro* grown *H. suis* was used to vaccinate mice, in a first attempt to evaluate the protective effect of the *in vitro* cultured organisms. BALB/c mice were immunized intranasally or subcutaneously with whole bacterial cell lysate of *H. suis* or the closely related species *H. bizzozeronii* and *H. cynogastricus*, adjuvanted with cholera toxin or a saponin-based adjuvant, respectively. Four weeks after the last immunization, mice were challenged with *H. suis*. Control groups consisted of non-immunized/non-challenged mice (negative control group), as well as of sham-immunized mice that were inoculated with *H. suis* (positive control group). Animals were sacrificed 7 weeks after challenge. In all immunized groups, higher serum anti-*H. suis* IgG titers were observed, compared to both the non-immunized/non-challenged and the sham-immunized/challenged control groups. The highest titers were observed after subcutaneous vaccination with *H. suis* antigens. Urease tests on stomach tissue samples at 7 weeks after challenge infection were negative in all negative control mice and all intranasally immunized mice except one. In all and 3 out of 5 animals of the *H. cynogastricus* and *H. suis* subcutaneously immunized groups, respectively, urease activity values were below the cut-off value. Using a sensitive *H. suis*-specific PCR, *H. suis* DNA was detected in both the fundus and the antrum of all positive control animals and all subcutaneously immunized/challenged animals. All negative control animals and some intranasally immunized/challenged mice were PCR-negative in both stomach regions. In addition, most *H. suis*-positive animals in the intranasally immunized groups were PCR-positive in only one of both stomach regions. In conclusion, immunization using antigens derived from the same or closely related bacterial species suppress gastric colonization with *H. suis*, but complete protection was only achieved in a minority of animals following intranasal immunization.

The results of this vaccination study were promising. The ultimate goal, however, being full protection in all vaccinated animals, the above mentioned vaccinal approach is insufficient. Further progress requires a better understanding of pathogenesis and virulence mechanisms. Regarding the pathogenesis, cell death plays an important role in the development of several *Helicobacter*-associated gastric pathologies, including gastritis, gastric ulceration, gastric atrophy and gastric cancer. In the second study, necrosis of gastric epithelial cells was observed in *H. suis* infected mice and gerbils. Therefore, in a fourth study, we investigated the possible direct effect of *H. suis* on gastric epithelial cell death, using human-derived AGS cells. Fluorescence microscopic and flow cytometric analysis revealed that *H. suis* lysate induces both apoptosis and necrosis. Inhibition of γ-glutamyl transpeptidase (GGT) activity, present in *H. suis* lysate, and incubation of AGS cells with purified recombinant *H. suis* GGT showed that this enzyme was largely responsible for the observed caspase-3-independent cell death. Ultrastructural analysis confirmed that *H. suis* GGT-treated cells show morphological signs of primary necrosis. Supplementation of recombinant *H. suis* GGT-treated cells with glutathione, a strong antioxidant under physiological conditions, strongly enhanced the observed induction of cell death. In addition, we showed that *H. suis* GGT catalyzes the degradation of glutathione. These data demonstrate that *H. suis* GGT-mediated degradation of glutathione and the resulting formation of glutathione degradation products play a direct and active role in the induction of gastric epithelial cell death. Prior to cell death, an increase of the extracellular H_2O_2 concentration, generated in a cell-independent manner by glutathione degradation products, was observed and resulted in lipid peroxidation. The possible involvement of mitochondrial respiration in the generation of oxidative damage was excluded by determining the mitochondrial membrane potential. To our knowledge, this is the first detailed description of a gastric pathogen exploiting an important antioxidant in this way to actively cause damage in the stomach.

In conclusion, the results described in this thesis clearly demonstrate that a pure *in vitro* culture of *H. suis* causes severe gastric pathology in rodent models of human gastric disease. *In vitro, H. suis* γ-glutamyl transpeptidase activity was shown to play an important role in the induction of gastric epithelial cell death. A first attempt to evaluate the protective effect of antigen preparations of *in vitro* cultured *H. suis* showed that vaccination may be a valuable approach to contol *H. suis* infections and related gastric pathology in pigs and humans.

Samenvatting

Al vroeg in de jaren tachtig werd duidelijk dat de overgrote meerderheid van de maagklachten bij de mens veroorzaakt wordt door een bacterie, *Helicobacter pylori*. In een minderheid van de gevallen van mensen met maagklachten echter, kon niet deze bacterie gedetecteerd worden, maar wel grote niet-*H. pylori* helicobacters met een typische spiraalvorm. In de wetenschappelijke literatuur werden deze bacteriën vaak "*Helicobacter heilmannii*" genoemd. Recent onderzoek heeft echter aangetoond dat het in werkelijkheid gaat over een groep van verschillende niet-*H. pylori Helicobacter* soorten die vaak ook aangetroffen worden in de maag bij verschillende diersoorten. Bijgevolg wordt, om verwarring te vermijden, de benaming "*H. heilmannii*" best enkel gebruikt voor de soort die heel recent officieel is erkend en beschreven werd door Smet en collega's (manuscript gepubliceerd in International Journal of Systematic and Evolutionary Microbiology). De vaakst voorkomende niet-*H. pylori Helicobacter* in de maag van mensen met maagklachten is *H. suis*, een bacterie die wereldwijd van nature vaak voorkomt in de maag van varkens. Tot op vandaag waren de virulentiemechanismen van *H. suis* zo goed als ongekend, onder andere door het feit dat deze bacterie bijzonder moeilijk te kweken is onder laboratoriumomstandigheden. Screening van de volledige genoomsequentie van *H. suis* heeft aan het licht gebracht dat de bacterie enkele typische virulentiefactoren mist die wel bij *H. pylori* voorkomen, zoals bijvoorbeeld het *cag* pathogeniciteitseiland en het toxine verantwoordelijk voor celvacuolisatie (VacA).

H. suis werd in 2008 voor het eerst *in vitro* geïsoleerd, aan onze vakgroep. Deze essentiële sprong voorwaarts heeft ons in staat gesteld om onderzoek te verrichten naar de pathogenese van *H. suis*-gerelateerde maagziekten en mogelijke strategieën om deze infecties onder controle te krijgen bij de mens en bij het varken.

In hoofdstuk 1 beschrijven we voor het eerst de succesvolle kolonisatie van de maag van Mongoolse gerbils met een zuivere *in vitro* cultuur van *H. suis*. Deze diersoort wordt beschouwd als een geschikt model om onderzoek te doen naar *Helicobacter*-gerelateerde maagpathologieën. Een steekproef in een gerbilpopulatie die gebruikt zou worden in experimentele *Helicobacter suis* infectieproeven, bracht echter aan het licht dat deze dieren van nature in de maag geïnfecteerd waren met een gist. Het kolonisatiepatroon van dit micro-organisme leunde erg dicht aan bij hetgeen beschreven wordt voor gastrale helicobacters, zoals *H. pylori* en *H. felis.* Internally Transcribed rRNA Spacer 2 Region (ITS) PCR fragment analyse toonde aan dat het hier *Kazachstania heterogenica* betrof, een gist die voorheen reeds uit de faeces van knaagdieren geïsoleerd was. Om na te gaan of *K. heterogenica* een rol speelt

in het ontstaan van maagpathologieën, werden Mongoolse gerbils experimenteel geïnfecteerd met deze gist. In elk van de dieren geïnoculeerd met *K. heterogenica* werd een uitgesproken kolonisatie waargenomen, voornamelijk in het antrum. Bovendien werden geen noemenswaardige pathologische veranderingen gezien in de maag van geïnfecteerde dieren. Vervolgens werden *K. heterogenica*- en gastrale *Helicobacter*-vrije gerbils geïnfecteerd met *H. suis*, *K. heterogenica* of beide, om een mogelijke interactie tussen deze micro-organismen na te gaan. Infectie met *H. suis* en *K. heterogenica* resulteerde in een significante toename van de ontstekingsreactie, in vergelijking met dieren die enkel met *H. suis* geïnfecteerd waren, wat wijst op een synergie tussen beide infectieuze agentia. Deze resultaten tonen het belang aan van rekening te houden met een mogelijke interferentie van andere micro-organismen in experimentele *in vivo* studies met gastrale helicobacters.

In een tweede langetermijnstudie werd de interactie tussen *H. suis* en de maagmucosa van de gastheer onderzocht. Hiervoor werden Mongoolse gerbils en twee muizenstammen (BALB/c en C57BL/6) experimenteel geïnfecteerd met *H. suis*. Vrouwelijke dieren van 6 weken oud en vrij van *K. heterogenica* en van gastrale helicobacters werden geïnoculeerd met een zuivere *in vitro* cultuur van *H. suis* stam 5 en vervolgens geëuthanaseerd na een infectieduur van 3 weken, 9 weken of 8 maanden. Maagstalen werden verzameld voor PCR analyse, histologisch en ultrastructureel onderzoek. In gerbils werd *H. suis* kolonisatie vooral waargenomen in het antrum en een heel smalle zone in de fundus, nabij de overgang tussen de voormaag en de klierzone van de maag. In beide muizenstammen daarentegen werd een duidelijke kolonisatie gezien in het volledige kliergedeelte van de maag (fundus én antrum). Transmissie elektronen microscopisch onderzoek toonde een verband aan tussen *H. suis* kolonisatie en necrose van pariëtale cellen in elk van de drie dierstammen. Immunohistochemische kleuring van de protonpomp (waterstof/kalium ATPase), aanwezig op de buitenste membraan van pariëtale cellen, bracht aan het licht dat het verlies van deze cellen het meest uitgesproken was ter hoogte van de overgangszone tussen fundus en antrum. Vanaf 9 weken na infectie werd een toename gezien van de proliferatie van mucosale epitheliale cellen in de regio's met de meest uitgesproken *H. suis* kolonisatie.

Op elk tijdstip werd in de ongeïnfecteerde controledieren een normaal uitziende maagmucosa waargenomen, zonder noemenswaardige influx van ontstekingscellen. Globaal gezien was de diffuse inflammatie, de aanwezigheid van ontstekingscelaggregaten en lymfoïde follikels meer uitgesproken in BALB/c muizen, in vergelijking met C57BL/6 muizen. In beide muizenlijnen was de inflammatie voornamelijk zichtbaar in lamina propria

mucosae en tunica submucosa van de fundus regio. Acht maanden na experimentele infectie kon een toename van het aantal B lymfocyten waargenomen worden, maar enkel in BALB/c muizen. Deze resultaten doen vermoeden dat *H. suis* voornamelijk een Th2 respons genereert, in tegenstelling tot *H. pylori*.

In de overgrote meerderheid van de experimenteel geïnfecteerde gerbils was een uitgesproken infiltratie met lymfocyten aanwezig in het antrum en ter hoogte van de overgangszone tussen voormaag en maag. Een toename van zowel diffuse infiltratie als aggregaten van lymfoïde cellen was merkbaar naarmate de dieren langer geïnfecteerd waren. Daarnaast kon ook destructie van de maagklieren en onderbreken van de continuïteit van de lamina muscularis mucosae waargenomen worden. Vanaf 9 weken na infectie namen CD20-positieve B cellen de bovenhand in de lymfocytaire aggregaten. Dit ging gepaard met organisatie tot lymfoïde follikels met duidelijke germinale centra die vaak erg groot, hyperproliferatief en onregelmatig van vorm waren. Bovendien was de normale weefselstructuur ter hoogte van de ontstekingsreactie in het antrum vaak niet meer aanwezig en konden ook letsels waaargenomen worden die een sterke gelijkenis vertoonden met lymfoom van het mucosa-geassocieerd lymfoïd weefsel (MALT). Voorbeelden daarvan zijn lymfo-epitheliale letsels en massale lymfocytaire infiltratie van de tunica muscularis. De locatie en de aard van de veroorzaakte pathologieën, met inbegrip van MALT lymfoom, vertonen een sterke gelijkenis met hetgeen gezien wordt bij mensen geïnfecteerd met een niet-*H. pylori Helicobacter* species.

We kunnen dus concluderen dat de rol van *H. suis* in het ontstaan van humane maagpathologieën niet over het hoofd gezien mag worden. Bovendien vertoont de pathologie veroorzaakt door *H. suis* in Mongoolse gerbils meer gelijkenissen met de situatie bij de mens, wat aangeeft dat dit diermodel geschikter is dan het muismodel om de interactie tussen *H. suis* en de gastheer te onderzoeken.

In de strijd tegen chronische infectieuze gastritis, peptische ulcera en MALT lymfoom in de maag van de mens, zijn preventie en eradicatie van gastrale *Helicobacter* infecties twee erg belangrijke stappen. Daarom werd, in een derde studie, een *in vitro* cultuur van *H. suis* gebruikt om muizen te vaccineren, in een poging om voor de eerste maal het beschermende effect van een zuivere *H. suis* cultuur na te gaan. BALB/c muizen werden intranasaal of subcutaan gevaccineerd met een lysaat van *H. suis* of de nauw verwante soorten *H. bizzozeronii* of *H. cynogastricus*. Respectievelijk cholera toxine of een saponine-gebaseerd adjuvans werden aan het vaccin toegevoegd voor intranasale of subcutane vaccinatie. Vier

weken na de laatste immunisatie werden de muizen intragastraal geïnoculeerd met *H. suis*. Niet-geïmmuniseerde/niet-geïnoculeerde muizen werden als negatieve controles ingesloten en muizen geïnoculeerd met *H. suis* na voorafgaande intranasale applicatie van een zoutoplossing in plaats van vaccinatie met antigenen, werden als positieve controledieren ingesloten. Zeven weken na inoculatie met *H. suis* werden de dieren geëuthanaseerd. In alle geïmmuniseerde groepen werden hogere serum anti-*H. suis* IgG antilichaam titers waargenomen, in vergelijking met beide controlegroepen. De hoogste titers werden gedetecteerd in het serum van muizen die subcutaan gevaccineerd waren met *H. suis* antigenen. Bij geen enkel dier uit de negatieve controlegroep en bij slechts één intranasaal geïmmuniseerd dier, werd urease activiteit gedetecteerd in de maag. Ook in de maag van respectievelijk alle en drie van de vijf met *H. cynogastricus* of *H. suis* subcutaan gevaccineerde dieren, werd geen moemenswaardige urease activiteit vastgesteld. Met behulp van een *H. suis*-specifieke PCR kon zowel in de fundus als in het antrum van alle positieve controledieren en alle subcutaan gevaccineerde/*H. suis*-geïnoculeerde dieren DNA van de kiem aangetoond worden. Daarentegen waren alle negatieve controledieren en sommige van de intranasaal gevaccineerde/*H. suis*-geïnoculeerde dieren PCR-negatief in beide maagzones. Bovendien was de meerderheid van de *H. suis*-positieve dieren in intranasaal gevaccineerde groepen PCR-positief in slechts één van beide maagzones. Dit betekent dat immunisatie met antigen bereidingen van dezelfde of nauw verwante *Helicobacter* soorten de kolonisatie van de maag met *H. suis* onderdrukt. Complete bescherming kon slechts waargenomen worden bij een deel van de intranasaal gevaccineerde dieren.

De resultaten van de zonet beschreven vaccinatiestudie zijn veelbelovend. Het ultieme doel is echter een volledige bescherming tegenover *H. suis* kolonisatie op te wekken. Vanuit dit standpunt bekeken, voldoet het vaccinatieprotocol beschreven in hoofdstuk 3 slechts gedeeltelijk. Om verdere vooruitgang op dit vlak te boeken, is een beter begrip van de pathogenese en virulentiemechanismen van *H. suis* infecties onontbeerlijk. Met betrekking tot de pathogenese is het duidelijk dat celdood een belangrijke rol speelt in het ontstaan van allerhande *Helicobacter*-gerelateerde maagpathologieën, zoals gastritis, maagulcers, atrofie van de maagmucosa en maagkanker. In de tweede studie van dit doctoraatsonderzoek hebben we necrose van epitheelcellen in de maag van *H. suis*-geïnfecteerde muizen en gerbils waargenomen. Daarom werd, in een vierde studie, een mogelijk directe rol van *H. suis* in het induceren van epitheliale celsterfte onderzocht, met behulp van de continue AGS cellijn, afkomstig uit de maag van een mens. Fluorescentiemicroscopisch en flowcytometrisch

onderzoek toonde aan dat *H. suis* lysaat zowel apoptose als necrose van AGS cellen veroorzaakt. Inhibitie van γ-glutamyl transpeptidase (GGT) activiteit, aanwezig in dit *H. suis* lysaat, en incubatie van AGS cellen met gepurifieerd recombinant *H. suis* GGT brachten aan het licht dat dit enzyme voornamelijk verantwoordelijk is voor de waargenomen caspase-3-onafhankelijke celdood. Analyse van de ultrastructuur bevestigde vervolgens dat *H. suis* GGT-behandelde cellen morfologische tekenen van primaire necrose vertoonden. Toevoegen van glutathion, een sterk antioxidans onder fysiologische omstandigheden, aan *H. suis* GGT-behandelde cellen bracht een versterking van de geïnduceerde celdood teweeg. Bovendien konden we aantonen dat *H. suis* GGT de afbraak van glutathion katalyseert en dat dit een directe en actieve rol speelt in het veroorzaken van primaire necrose van maagepitheelcellen. Vooraleer celdood optrad, kon een sterke toename van de extracellulaire H_2O_2 concentraties waargenomen worden. Deze stijging werd onafhankelijk van de gastheercel veroorzaakt door afbraakproducten van glutathion en resulteerde in peroxidatie van vetten aanwezig in verschillende structurele componenten van de gastheercel. Een mogelijke rol van mitochondriale respiratie in het ontstaan van oxidatieve celschade kon worden uitgesloten na het bepalen van de mitochondriale membraanpotentiaal. Voor zover bekend, is dit de eerste gedetailleerde beschrijving van een dergelijke uitbuiting van een belangrijk antioxidans door een micro-organisme om weefselschade te veroorzaken in de maag.

Zoals blijkt, tonen de resultaten van deze doctoraatsthesis aan dat een zuivere *in vitro* cultuur van *H. suis* ernstige maagpathologieën kan veroorzaken in knaagdieren, die model kunnen staan voor de ontwikkeling van maagziekten bij de mens. *In vitro* werd aangetoond dat *H. suis* γ-glutamyl transpeptidase activiteit een belangrijke rol speelt in het ontstaan van epitheliale celdood in de maag. Een eerste poging om het beschermend effect van *H. suis* antigen bereidingen na te gaan, toonde aan dat vaccinatie potentieel een goede benadering is om *H. suis* infecties en op die manier ook de gerelateerde maagpathologieën bij mensen en varkens onder controle te krijgen.

Curriculum Vitae

Bram Flahou werd geboren op 5 juni 1981 in Oostende. Na het doorlopen van de studies Grieks-Wetenschappen aan het Sint-Jozefsinstituut in Torhout, startte hij in 1999 de studies Diergeneeskunde aan de Universiteit Gent. In 2005 studeerde hij af als Dierenarts, optie Kleine Huisdieren, met onderscheiding.

Enkele maanden na het behalen van dit diploma, begon hij aan diezelfde universiteit een doctoraatsonderzoek bij de vakgroep Pathologie, Bacteriologie en Pluimveeziekten. In het kader van een Geconcerteerde Onderzoeksactie (GOA) verrichte hij er voornamelijk onderzoek naar de pathogenese van *Helicobacter suis* infecties. Dit onderzoek resulteerde in het huidige proefschrift. Hij beëindigde ook met succes de doctoraatsopleiding.

Bram Flahou is auteur of medeauteur van meerdere publicaties in internationale wetenschappelijke tijdschriften. Hij nam actief deel aan nationale en internationale congressen en was daar meermaals spreker.

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