Role of *Helicobacter suis* and *Fusobacterium gastrosuis* in the pathogenesis of gastric ulcer disease in pigs

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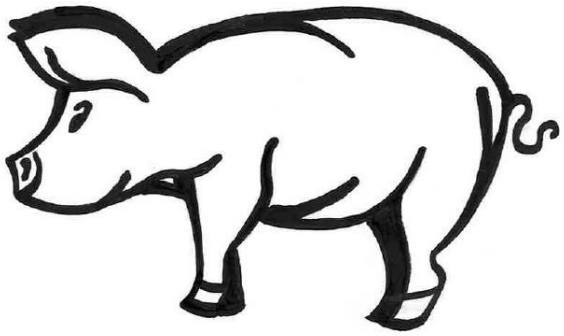
Abbreviation key

16S rRNA	small subunit ribosomal ribonucleic acid
23S rRNA	large subunit ribosomal ribonucleic acid
ACTB	actin beta
AGP	antimicrobial growth promotor
AMOVA	analysis of molecular variance
BA	butyric acid
BFGF	basic fibroblast growth factor
Annexin-V-FITC	annexin-V-fluorescein isothiocyanate
cagA/E/L/M/X	cytotoxin-associated gene A/E/L/M/X
carR	non-coding gene in the genome of *Helicobacter suis*
CCK-B receptor	cholecystokinin type B receptor
CCR4	C-C chemokine receptor type 4
CD3/20/25/40/74/80/86	cluster of differentiation 3/20/25/40/74/80/86
cDNA	complementary DNA
cfu	colony forming units
CLSI	clinical and laboratory standards institute
COX2	cyclooxygenase 2
CpG	cytosine triphosphate deoxynucleotide (C) linked with guanine triphosphate deoxynucleotide (G) by phosphodiester (p)
Ct	threshold cycle
CXCL2/13	chemokine ligand 2/13
cyc5	cyclin 5
D-cells	somatostatin-producing cells
DAB	diaminobenzidine
DEG	database of essential genes
DNA	deoxyribonucleic acid
ECLs	enterochromaffin-like cells
EGF	epidermal growth factor
ELISA	enzyme-linked immunosorbent assay
FAME	whole-cell fatty acid methyl esters
fipA	immunosuppressive protein A
FlaA	flagellin A
FOXP3	forkhead box protein P3

G-cells	gastrin-producing cells
GER	gastroesophageal reflux
GGT	gamma-glutamyl transpeptidase
GIT	gastrointestinal tract
Gln	glutamine
Glu	glutamate
GSH	glutathione
gyrA/B	gyrase A/B
H^+/K^+ ATPase	hydrogen-potassium-adenosinetriphosphatase
H2afz	H2A histone family member Z
H2 receptor	histamine 2 receptor
HBSS+/-	Hank's balanced salt solution with/without calcium
HCl	hydrochloric acid
HE	hematoxylin and eosin
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HGF	hepatocyte growth factor
HMBS	hydroxymethylbilane synthase
HOMOVA	homogeneity of molecular variance
HPRT	hypoxanthine-guanine phosphoribosyltransferase
HRP	horseradish peroxidase
Hsp27/60/72/73	heat shock protein 27/60/72/73 kDa
IgA/G	immunoglobulin A/G
IL-1β/2/4/6/8/10/12/17/23	interleukin 1β/2/4/6/8/10/12/17/23
IFN-γ	interferon gamma
KCNQ1 ion channel	potassium voltage-gated channel subfamily KQT member 1
KYSE-450	human oesophageal squamous cell carcinoma
LCB	locally collinear block
LPS	lipopolysaccharide
M3 receptor	muscarinic acetylcholine receptor
MALT	mucosa associated lymphoid tissue
MHC	major histocompatibility complex
MIC	minimum inhibitory concentration
MKN7	human gastric tubular adenocarcinoma cell line
MLST	multilocus sequencing typing
MOI	multiplicity of infection
mRNA	messenger RNA

Na^+/K^+ ATPase	sodium-potassium adenosine triphosphatase
NAP	neutrophil activating protein
NHPH	non-*Helicobacter pylori* helicobacters
NOX2	nitric oxide synthase 2
OD	optical density
OMP	outer membrane protein
OTU	operational taxonomic unit
PAMPs	pathogen associated molecular patterns
pb1A	platelet binding protein 1A
PCR	polymerase chain reaction
PCV2	porcine circovirus type 2
PI	propidium iodide
PMWS	post-weaning multisystemic wasting syndrome
PPI	proton pump inhibitor
PPIa	peptidyl-prolyl cis-trans isomerase A
ROS	reactive oxygen species
RPL4	ribosomal protein L4
rUreB/rGGT	recombinant UreB/GGT
rRNA	ribosomal ribonucleic acid
RT-PCR	quantitative real time-polymerase chain reaction
SCFA	short chain fatty acids
SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis
SPF	specific pathogen free
SST2 receptor	somatostatin receptor type 2
T5SS	type V secretion system
TBS-T	tris-buffered saline with 0.1% Tween 20
TGFB1	transforming growth factor beta 1
Th1/2/17	T helper cell immune response 1/2/17
TLR	Toll-like receptor
TNF-α	tumor necrosis factor alfa
Tregs	regulatory T cells
ureA/B	urease subunit A/B
vacA/J	vacuolating cytotoxin A/J
VFDB	virulence factor database
vitamin U	S-methylmethionine-sulphonium chloride
ZO1/2	zonula occludens 1/2



General introduction

Partially based on: De Witte, C., Ducatelle, R., Haesebrouck, F., 2018. The role of infectious agents in the development of porcine gastric ulceration. Vet J 236, 56-61.

1. The porcine stomach in health and disease

1.1 Anatomy, histology and physiology of the porcine stomach

The stomach links the oesophagus with the duodenum and its main functions are acidification, protein breakdown and temporary storage of food. In all species, the stomach consists of the cardiac gland zone, the fundic gland zone (corpus or body) and the pyloric gland zone (antrum). The cardiac gland zone is more pronounced in pigs compared to other species such as humans, dogs and cats. In addition, the porcine stomach possesses a non-glandular region, the *Pars oesopaghea*, a small rectangular area around the opening of the oesophagus covered by a stratified squamous epithelium (Figure 1) (Haesebrouck *et al.*, 2009).

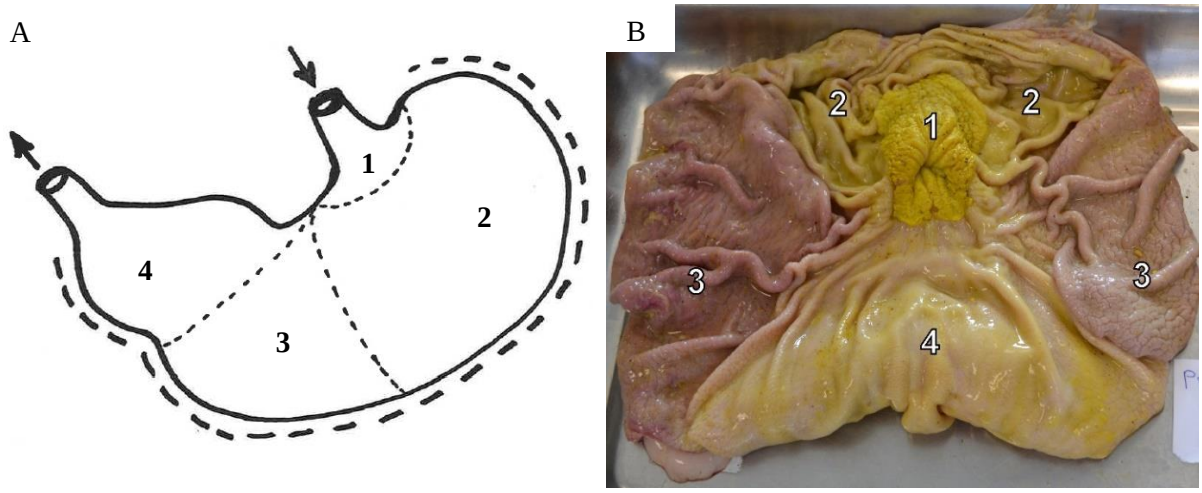


Figure 1: Anatomy of the porcine stomach. (A) Schematic overview of a closed stomach. (B) When the stomach is opened along the greater curvature (indicated by the dotted line on figure A), the different stomach regions can be seen: 1 = *Pars oesophagea*, 2 = cardiac gland zone, 3 = fundic gland zone, 4 = pyloric gland zone.

Histologically, the stomach wall can be divided, from the inside to the outside, into 4 layers: tunica mucosa, tunica submucosa, tunica muscularis and tunica serosa. The tunica mucosa contains the gastric pits and glands and consists, from the inside to the outside, of 3 layers: lining epithelium, lamina propria and lamina muscularis mucosae (Figure 2) (Eurell *et al.*, 2006). The epithelium of the glandular part of the stomach is of the single, columnar type and secretes products essential for the digestion of food. Gastric pits and glands are formed by invagination of the epithelium into the lamina propria. The surface epithelium and gastric pits mainly consist of mucous cells, responsible for production of the mucus layer which protects the underlying mucosa against pathogens and hydrochloric acid (HCl). The gastric glands

consist of specialized cell types, which differ depending on the stomach region. The cardiac gland zone mainly consists of glands producing mucus and bicarbonate. The fundic gland zone consists, from the neck to the base of the gland, of several specialized cell types: mucous neck cells, parietal cells, chief cells, enterochromaffin-like cells (ECLs) and enteroendocrine cells (Figure 2). Finally, the pyloric gland zone consists of mucus producing cells, enteroendocrine cells, such as gastrin- and somatostatin-cells (G- and D-cells, respectively) and a limited number of parietal cells (Soybel *et al.*, 2005). A brief overview of the main function and regulation of these specialized cells is presented in the paragraph below.

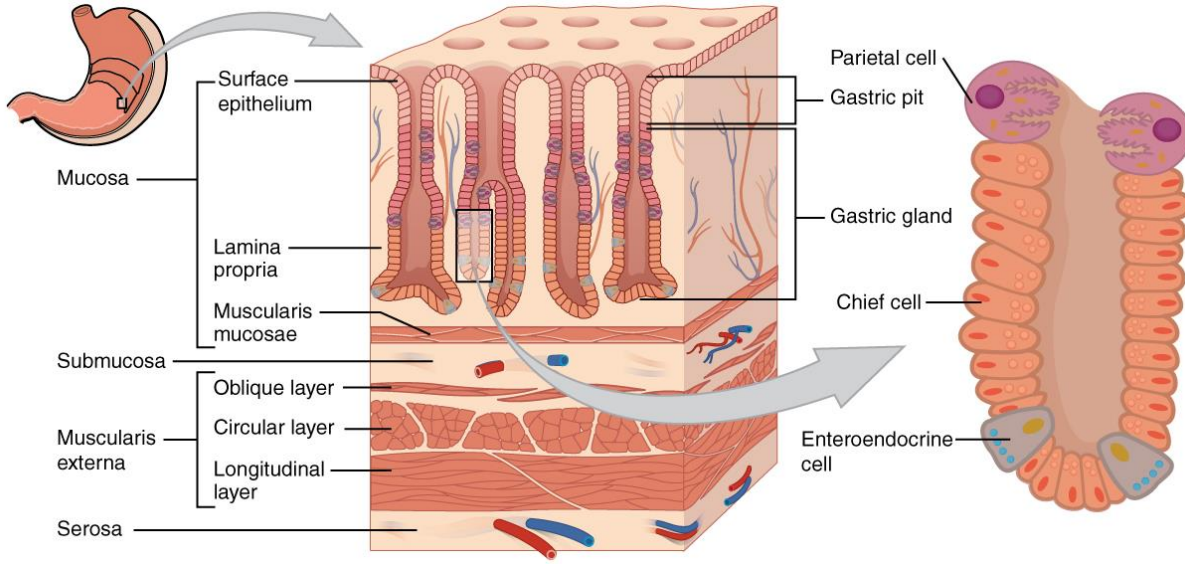


Figure 2: Schematic representation of the histology of the gastric mucosa. In the epithelium, gastric pits lead to gastric glands. The gastric glands (shown enlarged on the right) contain different cell types that secrete a variety of substances, including HCl, which activates the protein-digesting enzyme pepsin (Adapted from https://cnx.org/contents/FPtK1zmh@8.108:O9dvCxUQ@3/The-Stomach)

The surface mucous and bicarbonate producing cells both function as a buffer against HCl. Mucous neck cells secrete a thin layer of acidic mucus which is different from the overlying alkaline mucus produced by the surface epithelium. Pepsinogen is produced by chief cells and HCl converts pepsinogen into its active form pepsin. Parietal cells are responsible for the production of HCl, which requires activity of the H^+/K^+ ATPase proton pump and KCNQ1 ion channel. Gastrin is produced by G-cells and stimulates gastric acid secretion through binding with CCK-B receptors present on parietal cells and ECLs. Acetylcholine, released from intramural neurons, also stimulates gastric acid secretion through binding on M3-receptors on both parietal cells and ECLs. After binding with gastrin/acetylcholine,

ECLs produce histamine which stimulates the production of gastric acid through binding with H2-receptors on parietal cells. Conversely, somatostatin, produced by D-cells downregulates the function of parietal cells and ECLs, inhibiting gastric acid secretion (Calam, 1999).

The porcine stomach can be divided in an upper compartment (i.e. *Pars oesophagea* and cardiac gland zone) and distal compartment (i.e. fundic and pyloric gland zone). In the upper compartment, food is temporarily stored and has a solid appearance, while it becomes more liquid due to acidification and protein breakdown in the distal compartment. In a normal porcine stomach, no mixing of luminal content takes place between both compartments. The pH of the upper compartment is 5-7 due to the presence of bicarbonate in saliva and cardiac gland secretions. In the distal compartment, however, the pH is very low (2-3) due to HCl production by parietal cells. Any factor contributing to a breakdown of this pH gradient between both compartments may lead to an increased contact of the *Pars oesophagea* with distally produced HCl, pepsin and/or bile salts. Since this non-glandular part is not protected by mucus, it is highly susceptible to irritation. Chronic insult of the *Pars oesophagea* results in hyperkeratosis, as a form of protection of the surface epithelium against the irritation (Haesebrouck *et al.*, 2009). When hyperkeratosis persists, nutrients can no longer diffuse from the lamina propria to the epithelial cells, resulting in cellular apoptosis. In addition, hydrogen protons from HCl enter and acidify the epithelial cell cytoplasm. Due to acidification, the function of cell membrane-bound Na^+/K^+ ATPase is disrupted, resulting in an accumulation of sodium ions and secondary water in the cell cytoplasm. Acute cellular swelling and hydropic degeneration occurs, which finally results in necrosis. As a result of this epithelial cell death, erosions are formed. In erosive lesions, the underlying basement membrane remains intact and re-epithelialization occurs rapidly. However, when the irritation continues, the basement membrane is disrupted and ulcers are formed (Krakowka and Ellis, 2006).

1.2 Gastric ulceration

Ulceration of the upper, non-glandular part of the stomach has been described with increasing frequency since the late 1950s as a result of pig production intensification (Queiroz *et al.*, 1996). Now, it is a common disease entity of pigs worldwide, with prevalences of up to 93%. Although lesions can occur at any age, pigs at slaughter age (i.e. 6-8 months old) and sows around the time of parturition are most frequently affected (Zimmerman *et al.*, 2012). Both the prevalence and severity of lesions can vary greatly between different pig herds. The reason for this is not clear, although feeding and management strategies may sometimes be involved.

The disease outcome is mainly subclinical, with only the most severe ulcers resulting in development of clinical signs of anaemia (i.e. paleness, tremor, slow gate, anorexia, melena, dyspnoea) or sudden death due to significant blood loss and/or perforation of the ulcers through the stomach wall resulting in fatal peritonitis. The average mortality rate due to gastric ulceration is around 1-2%, although higher rates have been reported sporadically, mainly in sows (Melnichouk, 2002). Excessive scar formation during healing may cause stenosis of the oesophageal opening into the stomach, resulting in vomiting shortly after feed intake and a decreased body condition (Friendship, 2004). Even less severe lesions have been associated with decreased weight gain of up to 75 g/day, decreased feed intake and most likely also pain. Therefore, economic losses as well as animal welfare issues are of major importance (Haesebrouck *et al.*, 2009).

1.3 Evaluation of lesions

At necropsy, the presence of lesions needs to be confirmed by examination of the stomach, with special attention to the upper, non-glandular region. For this purpose, the stomach should be opened along the greater curvature, inverted and rinsed. So far, different scoring systems have been proposed to grade lesions, taking into account presence and extent of rough and thickened mucosa, erosions, ulcers and/or scar tissue. Often, the mucosa of the non-glandular region has a yellow appearance as a result of bile staining (Figure 3). Autopsy of pigs that died acutely of ulceration may reveal presence of a variable amount of clotted or fresh blood in the stomach, large erosive lesion(s) and/or pale mucosae in combination with a good body condition score (Thomson and Friendship, 2012).

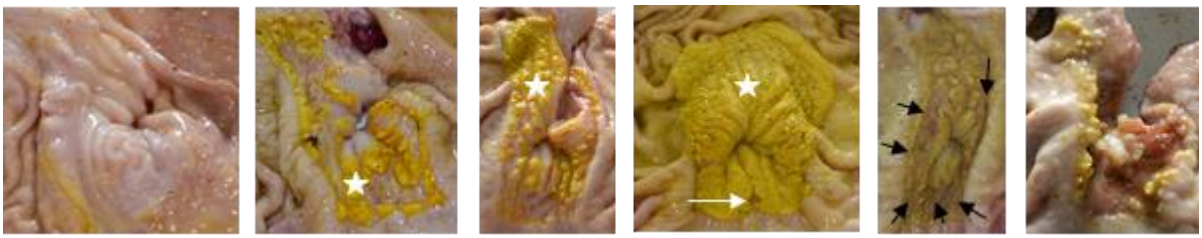


Figure 3: Mucosa of the *Pars oesophagea* showing different morphologies. From left to right: normal mucosa; mild hyperkeratosis (indicated by the white star); severe hyperkeratosis; severe hyperkeratosis and presence of erosion (indicated by the white arrow); severe hyperkeratosis and several erosions (indicated by black arrows); hyperkeratosis and severe ulceration.

Histologically, the thickened mucosa is characterized by the presence of parakeratosis, a thickened keratin layer, papillae elongation and/or swollen cells. The pattern of erosion and ulceration shows presence of epithelial separation, necrotic debris and formation of granulation tissue. In the lumen of an erosion/ulcer, exudate consisting of neutrophils, eosinophils and mononuclear cells is often found. Usually, ulcers penetrate down to the submucosa, but may sporadically advance to the tunica muscularis and/or serosa (Queiroz *et al.*, 1996; Thomson and Friendship, 2012).

2. Etiology of porcine gastric ulceration

The mechanism behind porcine gastric ulceration is not yet exactly known. The disease is clearly of multifactorial origin. In general, factors that increase the fluidity of the gastric content cause a breakdown of the pH gradient and are considered to be risk factors. Well known examples of such risk factors are small particle size of feed, pelleting of feed and interruption of feed intake (Zimmerman *et al.*, 2012). Apart from feeding and management strategies, other factors such as infectious agents, genetic background, hormonal changes and gastric microbiota composition have also been suggested to play a role in ulceration of the non-glandular part of the porcine stomach (Haesebrouck *et al.*, 2009).

2.1 Feed and management

Overall, small particle size of the feed increases stomach content fluidity and speeds up gastric emptying time, resulting in an increased contact of the *Pars oesophagea* with HCl, pepsin and/or bile salts. Several factors affect feed particle size: type of grain, milling procedure and processing. Grains such as wheat and maize are more likely to shatter during grinding and result in a finer particle size compared to oats or barley. The use of a hammer mill results in more grinding and finer particles compared to a roller

mill. Compared to mash or crumbled feed, pelleting further reduces feed particle size and has been associated with severe lesions (Thomson and Friendship, 2012; De Jong *et al.*, 2016). It has been stated that when the mean particle size of the feed is smaller than 700 μm, there is a significant risk for development of stomach lesions (Friendship, 2004). The amount of small particles is also important, as an increased risk for development of stomach ulceration has been shown when 30% of the feed particles are smaller than 400 μm. Increasing the proportion of coarse particles, however, does not exhibit an ulceroprotective effect (Grosse Liesner *et al.*, 2009).

Dry or liquid feeding does not seem to influence the development of porcine gastric ulceration (Mößeler *et al.*, 2012), although some reported a higher incidence of gastric ulceration during dry feeding compared to liquid feeding (Scott *et al.*, 2007). Fermentation of the feed has been sporadically associated with gastric ulceration, although the reason for this is not clear (Missotten *et al.*, 2015).

The method of feeding is considered to be as important as feed structure. A major risk factor is interruption of the feed intake, as it results in a more fluid stomach content. Significant increases in prevalence and severity of stomach lesions have been described in pigs fasted for 24h before slaughter (Swaby and Gregory, 2012). Most often, mechanical problems or human errors are responsible for an interruption of the feed intake. Other contributing factors may be acute diseases, heat stress and periods of transition in feeding patterns, for example transfer of gilts from the finishing to the breeding herd or sows around time of parturition (Thomson and Friendship, 2012). Some studies reported an increased risk for developing gastric ulcers when feeding ad libitum compared to restricted feeding (Blackshaw *et al.*, 1980; Robertson *et al.*, 2002). In these studies, however, diets with a small particle size were fed, making it difficult to separate the effect of ad libitum feeding and particle size.

Stress may also induce fasting. Different types of stress have been associated with porcine gastric ulceration: forced immobilization, slatted floors, un-enriched environment, social stress from mixing, tail biting, rearing in confinement, high stocking densities, environmental changes and transfer to unfamiliar environment (Hessing *et al.*, 1992; Lawrence *et al.*, 1998; Berg *et al.*, 2005; Swaby and Gregory, 2012). Increased plasma levels of the stress hormone cortisol have been described in pigs with severe stomach lesions. This indicates that, apart from fasting, production of cortisol may also affect

stomach lesion development (Hessing *et al.*, 1992). Nevertheless, parenteral injections of cortisol could not induce stomach ulceration (Jensen *et al.*, 1996) and induced intermittent stress was not associated with an increased prevalence of gastric ulceration (Jensen *et al.*, 1996). These discrepancies might be caused by differences in data interpretation and/or difficulty to asses stress in pigs. Increased plasma cortisol levels may also be a direct consequence of lesion development, as gastric ulceration is likely to be painful and stressful for the pig.

2.2 *Helicobacter suis:* an infectious agent possibly involved in porcine gastric ulceration

An increased gastric acid secretion, as a result of *Helicobacter pylori* infection, has been associated with peptic ulcers in human patients (Calam, 1999). Pigs are frequently infected with a pathogen closely related to *H. pylori*, namely *H. suis*. This pathogen colonizes the fundic and pyloric gland zone of the porcine stomach, but not the *Pars oesophagea*. Although ulceration of the non-glandular region of the porcine stomach is clearly different from peptic ulcers in humans (which are found in stomach tissue containing glands and in the duodenum), it was hypothesized that an infection with *H. suis* may result in secretion of excessive amounts of gastric acid in the distal part of the stomach, leading to increased contact of the non-glandular part of the stomach with HCl (Haesebrouck *et al.*, 2009).

2.2.1 Characterization and cultivation

In 1990, the first description of large, spiral-shaped bacteria colonizing the surface mucosa and pits of the pyloric gland zone of pig stomachs was made (Queiroz *et al.*, 1990). Initially, '*Gastrospirillum suis*' was proposed as a name, but further characterization showed that this organism belonged to the genus *Helicobacter* (De Groote *et al.*, 1999) and a new name, '*Candidatus* H. suis' was proposed. In 2008, the first *in vitro H. suis* isolate was obtained from the stomach of pigs at slaughter age (Baele *et al.*, 2008).

H. suis is a Gram-negative, micro-aerophilic and non-sporulating bacterium. It has a typical spiral-shaped morphology with 3 to 8 dense turns and an average of 6 turns. The bacterium is around 2.3-6.7 μm long and 0.9-1.2 μm wide. Due to the presence of 4-10 flagella at both ends of the cell, *H. suis* is highly motile and able to move through the stomach mucus layer (Baele *et al.*, 2008) (Figure 4).

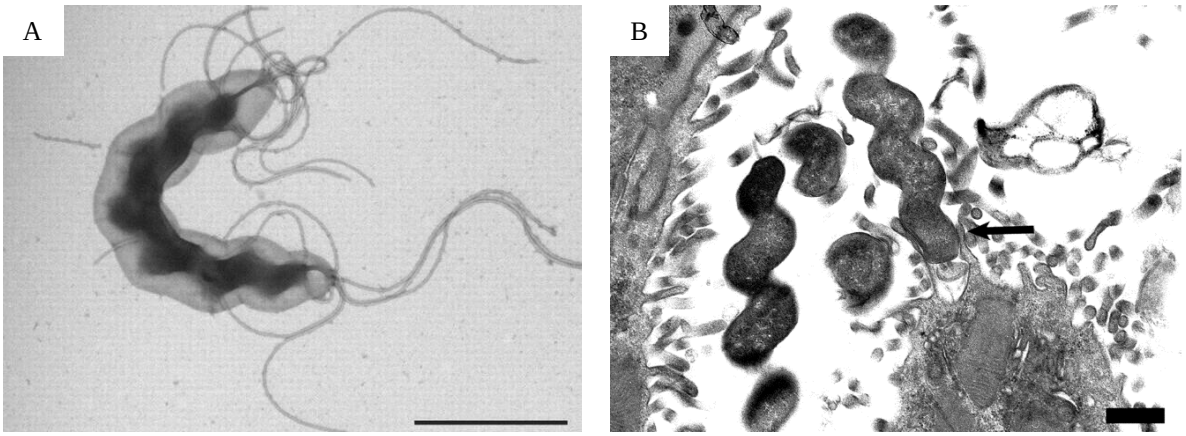


Figure 4: Transmission electron microscopy images of *H. suis* bacteria. (A) Bipolar flagella of *in vitro* cultured *H. suis.* Scale bar represents 2 µm (Baele *et al.*, 2008). (B) Typical spiral-shaped *H. suis* bacteria colonizing the gastric mucosa of BALB/c mice. Scale bar represents 500 nm (Flahou *et al.*, 2010).

Due to its fastidious nature, *H. suis* requires specific growth conditions for isolation and cultivation (Baele *et al.*, 2008). For isolation, the stomach should be rinsed and submersed in 1% HCl for 1h to prevent overgrowth by other bacteria. The mucus is then scraped off, after which it is slightly liquefied with Brucella broth (pH 5) supplemented with 20% fetal calf serum, Campylobacter selective supplement and Vitox supplement and inoculated on biphasic medium. The biphasic medium consists of Brucella agar supplemented with 20% fetal calf serum, 0.1% activated charcoal, 0.01% amphotericin B/l, 0.001% crystal violet, Campylobacter selective supplement and Vitox supplement. The pH of the agar should be adjusted to 5 by adding HCl to a final concentration of 0.05%. Finally, Brucella broth (pH 5) is added on top. When isolating *H. suis* from experimentally infected mice, supplementation of linezolid (5 µg/ml) to the Brucella agar plates has been shown to be successful in inhibiting proliferation of contaminants, such as lactobacilli which are highly present in the murine stomach (Blaecher *et al.*, 2017). The same biphasic medium is used for *H. suis* cultivation, but without supplementation of activated charcoal, crystal violet or linezolid. All strains need to be cultured under biphasic and humidified microaerobic conditions (37°C; 85% N_2, 10% CO_2, 5% O_2) at 37°C. Growth and viability of growing *H. suis* cultures should be checked every 2 days by light microscopic examination. When sufficient bacteria are present ($\geq 2 \times 10^7$ bacteria/ml), the *H. suis* containing broth can be divided onto 2-4 fresh agar plates. Finally, pure cultures of *H. suis* can be established by growing bacteria as

individual colonies on 1% Brucella agar plates, after which they are purified and enriched as described above. This may, however, affect the bacterial phenotype and genotype (Liang *et al.*, 2015).

2.2.2 Phylogeny

To date, the genus *Helicobacter* consists of 46 identified species. Helicobacters can be divided into 2 major groups: the gastric species colonizing the stomach and the enterohepatic species colonizing the liver and/or intestines of their host. *H. pylori* is the best-studied and most prevalent *Helicobacter* species colonizing the human stomach. The other, non-*H. pylori* helicobacters (NHPH) colonize the gastro-intestinal tract of several animal species, going from terrestrial and aquatic mammals to reptiles, amphibians, birds, rodents and fish (Haesebrouck *et al.*, 2009). *Helicobacter* spp. colonization may lead to development of gastric and/or enterohepatic pathologies and some NHPH have zoonotic potential (Table 1).

H. suis belongs to the gastric NHPH and can be distinguished from other *Helicobacter* spp. by sequencing of genes encoding 16S and 23S ribosomal RNA (16S rRNA and 23S rRNA, respectively), urease subunit A and B (ureA/B), gyrase B (gyrB) and heat shock protein 60 kDa (Hsp60) (Baele *et al.*, 2008). Its phylogenetic relationship with other *Helicobacter* spp. is shown in Figure 5.

In a recent study, it has been shown that *H. suis* in pigs possibly originates from non-human primates (Flahou *et al.*, 2017). A possible host jump from macaques to pigs happened between 100,000 and 15,000 years ago, after which domestication may have had a significant impact on the spread of *H. suis* in the pig population.

Table 1: *Helicobacter* spp. and their pathogenic significance for humans and animals (Adapted from (Flahou *et al.*, 2016))

Taxon	Natural hosts	Zoonotic potential	Disease associations
Gastric *Helicobacter* spp.			
'*Candidatus* H. bovis'	Cattle	Yes	Unknown
'*Candidatus* H. homininae'	Chimpanzee, gorilla	Unknown	Unknown
H. acinonychis	Cheetah, tiger, lion	Unknown	Severe chronic gastritis
H. ailurogastricus	Cat	Unknown	Asymptomatic, gastritis, peptic ulcers, MALT-lymphoma
H. baculiformis	Cat	Unknown	Unknown
H. bizzozeronii	Cat, dog	Yes	Asymptomatic, gastritis, peptic ulcers, MALT-lymphoma
H. cetorum	Whale, dolphin	Unknown	Asymptomatic, gastritis, peptic ulcers
H. cynogastricus	Dog	Unknown	Unknown
H. felis	Dog, cat, cheetah, New Guinea wild dog, rabbit	Yes	Asymptomatic, gastritis, peptic ulcers, MALT-lymphoma
H. heilmannii	Dog, cat, cheetah, bobcat, tiger, lynx, leopard, puma	Yes	Asymptomatic, gastritis, peptic ulcers, MALT-lymphoma
H. mustelae	Ferret	Unknown	Asymptomatic, gastritis, peptic ulcers, gastric cancer, MALT-lymphoma
H. pylori	Human	/	Asymptomatic, gastritis, peptic ulcers, gastric cancer, MALT-lymphoma
H. salomonis	Cat, dog, rabbit	Yes	Asymptomatic, gastritis, peptic ulcers, MALT-lymphoma
H. suis	Pig, mandrill monkey, rhesus macaque, crab-eating macaque	Yes	Asymptomatic, gastritis, peptic ulcers, MALT-lymphoma
Enterohepatic *Helicobacter* spp.			
'*Candidatus* H. colifelis'	Cat	Unknown	Unknown
H. anseris	Goose	Unknown	Unknown
H. apri	Wild boar	Unknown	Unknown
H. aurati	Hamster	Unknown	Asymptomatic, hepatic and intestinal disease
H. bilis	Mouse, rat, gerbil, dog, cat, sheep	Yes	Asymptomatic, hepatic and intestinal disease
H. brantae	Goose	Unknown	Unknown
H. callitrichis	Marmoset	Unknown	Unknown
H. canadensis	Bird, pig	Yes	Unknown
H. canicola	Dog	Unknown	Unknown
H. canis	Dog, cat	Yes	Asymptomatic, diarrhea, hepatitis, gastroenteritis

Taxon	Natural hosts	Zoonotic potential	Disease associations
Enterohepatic *Helicobacter* spp.			
H. cholecystus	Hamster	Unknown	Asymptomatic, hepatic and intestinal disease
H. cinaedi	Hamster, rat, cat, dog, rhesus monkey, baboon	Yes	Asymptomatic, hepatic and intestinal disease
H. equorum	Horse	Unknown	Unknown
H. fennelliae	Dog	Yes	Asymptomatic, enteritis, proctitis, proctocolitis
H. ganmani	Mouse	Yes	Asymptomatic, hepatic and intestinal disease
H. hepaticus	Mouse, gerbil	Yes	Asymptomatic, hepatic and intestinal disease
H. himalayensis	Marmot	Unknown	Unknown
H. jaachi	Marmot	Unknown	Unknown
H. japonicus	Mouse	Unknown	Typhlocolitis, lower bowel carcinoma
H. macacae	Rhesus monkey, baboon	Unknown	Asymptomatic, chronic colitis, intestinal adenocarcinoma
H. marmotae	Woodchuck, cat	Unknown	Asymptomatic, hepatitis, typhlocolitis
H. magdeburgensis	Mice	Unknown	Asymptomatic, hepatic and intestinal disease
H. mastomyrinus	Rodents	Unknown	Asymptomatic, hepatic and intestinal disease
H. mesocricetorum	Hamster	Unknown	Asymptomatic, hepatic and intestinal disease
H. muricola	Wild mouse	Unknown	Asymptomatic, hepatic and intestinal disease
H. muridarum	Mouse, rat	Unknown	Asymptomatic, hepatic and intestinal disease
H. pamatensis	Bird, pig, cat	Yes	Unknown
H. pullorum	Poultry	Yes	Asymptomatic, hepatitis, diarrhea, enteritis
H. rodentium	Mouse, rat	Unknown	Asymptomatic, hepatic and intestinal disease
H. saguini	Cotton-top tamarin	Unknown	Asymptomatic, ulcerative colitis, typhlocolitis and dysplasia
H. suncus	House musk shrew	Unknown	Unknown
H. trogontum	Rat, pig, sheep	Unknown	Asymptomatic, hepatic and intestinal disease
H. typhlonius	Mouse, rat	Unknown	Asymptomatic, hepatic and intestinal disease
H. valdiviensis	Wild birds	Unknown	Unknown

Candidatus = *Helicobacter* species that has not yet been isolated and cultivated *in vitro*

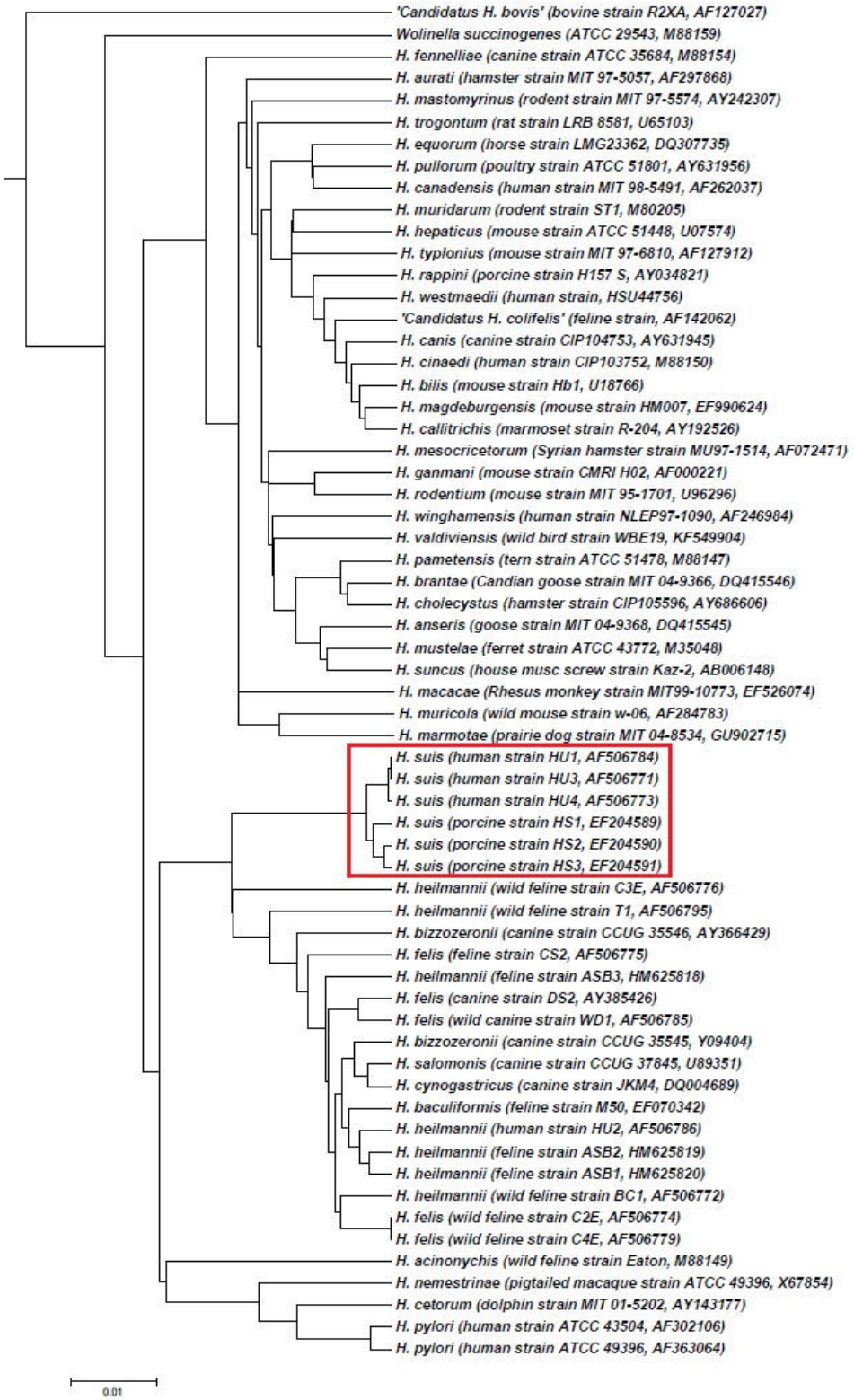


Figure 5: Phylogenetic tree based on the near-complete *16S rRNA* gene sequences from all gastric and enterohepatic *Helicobacter* species described so far. *H. suis* strains are highlighted in a frame (Flahou *et al.*, 2016).

2.2.3 Prevalence in pigs

H. suis colonizes the gastric mucosa of domestic pigs worldwide. Prevalence rates may vary from 8 to 95% depending on geographic area, diagnostic tools, management and hygiene standards, but in general, a prevalence of 60% or more has been described in pigs at slaughter age (i.e. 6-8 months old). The incidence seems to increase with the pig's age. In a Belgian study, the prevalence in suckling pigs was very low (i.e. 2%) and gradually increased to 25%, 80% and 90% in nursery pigs, pigs at slaughter age and adult pigs, respectively (Hellemans *et al.*, 2007a). The rapid increase after weaning suggests the presence of protective antibodies in the sow's milk. Most likely, few pigs are infected by *H. suis* before weaning and regrouping of weaned pigs may favour the spread of *H. suis* from infected to non-infected pigs, which further continues during the fattening period. Transmission most likely occurs via the oral-oral route through saliva or via the gastric-oral route through vomiting or regurgitation (Haesebrouck *et al.*, 2009), as *H. suis* DNA has been shown in the saliva and stomach content of pigs (Casagrande Proietti *et al.*, 2010). Up till now, *H. suis* DNA has not been detected in feces, indicating that fecal-oral spread between pigs is negligible.

2.2.4 Zoonotic significance

The most frequently detected pathogen in humans with gastric complaints is the curve-shaped *H. pylori*. Apart from *H. pylori*, spiral-shaped NHPH have been described to colonize the human gastric mucosa as well. These NHPH have been demonstrated in 0.2-6% of human patients with gastric complaints undergoing a gastric biopsy, although this is most likely an underestimation of their true prevalence. *H. suis* comprises 13.9 to 78.5% of human NHPH infections, making it the most prevalent gastric NHPH in humans. NHPH infections have been associated with development of chronic gastritis, peptic ulcers and mucosa associated lymphoid tissue (MALT)-lymphoma. The risk for developing MALT-lymphoma is higher during NHPH infection compared to *H. pylori* infection (Haesebrouck *et al.*, 2009). Interestingly, a high prevalence of *H. suis* DNA (i.e. 27%) was detected in gastric biopsies from human patients with idiopathic parkinsonism (Blaecher *et al.*, 2013). *H. suis* was shown to affect the blood-cerebrospinal fluid barrier resulting in inflammation and neurodegeneration in a mouse model (Gorlé *et al.*, 2017).

Clinical signs associated with NHPH infections in humans are acute or chronic epigastric pain and nausea. Other symptoms may be hematemesis, recurrent dyspepsia, irregular defecation frequency and variable stool consistency, vomiting, heartburn, dysphagia and decreased appetite. Nevertheless, these symptoms are atypical and cannot be distinguished from other noxa inducing gastric pathologies. Gastroscopy may reveal a variety of lesions, ranging from a normal to slightly hyperemic mucosa to mucosal edema and to multiple erosions and ulcerations in the pyloric gland zone and/or duodenum (Haesebrouck *et al.,* 2009). Histologically, the inflammation induced by *H. suis* is characterized by lymphocytic exudation into gastric foveolae, sometimes mixed with plasma cells. Occasionally, lymphocytic aggregates and/or epithelial metaplasia have been described (Joosten *et al.*, 2013). Gastritis induced by NHPH is less severe compared to *H. pylori* associated inflammation (Stolte *et al.,* 1997).

It is not exactly known how *H. suis* is transmitted to humans, but most likely, it occurs through direct or indirect contact with infected pigs. Living in close proximity to pigs as well as intense contact have indeed been identified as important risk factors, as a higher *H. suis* incidence has been described in pig farmers and people working in slaughterhouses (Stolte *et al.*, 1994; Haesebrouck *et al.,* 2009). A pig veterinarian suffering from reflux oesophagitis and dyspepsia presented *H. suis* colonization and inflammation (Joosten *et al.,* 2013). MLST analysis of *H. suis* from this human host revealed a very close relationship with porcine *H. suis* strains, suggesting that the infection originated through close contact of the veterinarian with pigs (Liang *et al.,* 2013). *H. suis* presence has been demonstrated in commercial pork and the bacterium is able to survive for at least 48h in minced pork (De Cooman *et al.*, 2014), indicating that handling or consumption of raw or undercooked pork might be a source of human infection as well. An additional transmission route might be contaminated water, as *Helicobacter* spp. are able to survive in water (Azevedo *et al.*, 2008). However, this requires further investigation. Finally, the role of wild mice as vectors might be considered as well, since laboratory mice are easily colonized by most gastric NHPH (Haesebrouck *et al.*, 2009; Whary and Fox, 2004).

2.2.5 Diagnosis of *H. suis* infections

2.2.5.1 *Pigs*

H. suis infections in pigs can be detected by several methods such as isolation, histology, immunohistochemistry, rapid urease testing and molecular microbiological methods. The procedure for isolation requires a whole pig stomach, as described under 2.2.1. Since isolation is time consuming, labor intensive and not always successful (Baele *et al.*, 2008), other methods are more often used for routine diagnosis. For histology, immunohistochemistry and molecular microbiological methods, gastric biopsies should be taken. This can be done by gastroscopy, although sampling of the stomach after slaughter is preferred for economic reasons. The rapid urease testing requires at least a part of the fundic and pyloric gland zone of the porcine stomach.

So far, detection of *H. suis* DNA is considered to be the most sensitive and specific method (De Groote *et al.*, 2000). Amplification and sequencing of the *16S rRNA* gene by the use of polymerase chain reaction (PCR) is sufficient to distinguish *H. suis* from other helicobacters. Other genes, such as *ureA, ureB* and *Hsp60* may also be used. By using a quantitative real time (RT)-PCR based on the *ureA* gene, *H. suis* presence as well as quantification can be determined (Blaecher *et al.*, 2013). When combining ethidium bromide monoazide treatment and RT-PCR, viable *H. suis* bacteria can be distinguished from dead bacteria, allowing a more reliable quantification of *H. suis* bacteria present in the stomach (De Cooman *et al.*, 2013). Below, the sensitivity and specificity of other diagnostic methods are compared with (RT-)PCR.

The typical spiral-shaped morphology of *H. suis* can be easily detected by gastric biopsy staining with haematoxylin eosin (HE), Giemsa or silver. The sensitivity of this method (42%) is relatively low compared to (RT-)PCR, especially in stomachs with a low colonization rate of *H. suis*. Microscopic detection of immunohistochemically labelled *H. suis* bacteria shows a higher sensitivity (i.e. 72%). The rapid urease test can also be used for detection of *H. suis*, although specificity and sensitivity seem to depend on the incubation time. A short incubation time shows a specificity and sensitivity of 93% and 55%, respectively, while a prolonged incubation time shows a specificity of 72% and a sensitivity of 74% (De Groote *et al.*, 2000).

Currently, no method has been developed which detects *H. suis* antibodies in the serum or *H. suis* antigens in the faeces. Furthermore, *H. suis* DNA is not detectible in faeces, indicating that this non-invasive method cannot be used for routine diagnosis of *H. suis* infections in pigs. Investigation of saliva for presence of *H. suis* DNA might be considered as an alternative method, although it is not known how these numbers are related to *H. suis* colonization rate in the stomach (Casagrande Proietti *et al.*, 2010).

2.2.5.2 *Humans*

Isolation cannot be used for routine diagnosis of NHPH infections in humans due to their fastidious nature. Attempts to isolate *H. suis* from human gastric biopsies have been unsuccessful. So far, only the somewhat less fastidious *H. felis* and *H. bizzozeronii* have been isolated (Kivistö *et al.*, 2010; Wüppenhorst *et al.*, 2013). Direct isolation from biopsies is hampered by the low colonization density of NHPH in the human stomach (Blaecher *et al.*, 2013), while isolation after mouse passage is often hampered by overgrowth of contaminants, such as *Lactobacillus* spp. Recently, it has been shown that addition of linezolid to the culture medium can inhibit *Lactobacillus* spp. overgrowth (Blaecher *et al.*, 2017).

Commercial non-invasive tests to diagnose NHPH infections are currently not available. The urea breath test, frequently used to diagnose *H. pylori* infection, is often falsely negative due to the focal colonization of NHPH predominantly in the pyloric gland zone, compared to the more diffuse colonization of *H. pylori* over the entire stomach mucosa (Stolte *et al.*, 1997; Matsumoto *et al.*, 2014).

Analysis of gastric biopsies by molecular microbiological methods and/or histology is necessary to confirm presence of NHPH infection. Due to the focal colonization of NHPH, several gastric biopsies should be analysed. For histological analysis, a HE staining is most often used. The spiral-shaped NHPH can be distinguished from the curve-shaped *H. pylori*. Nevertheless, this method does not allow identification to species level (Haesebrouck *et al.*, 2009). Detection of the *16S rRNA* gene is frequently used for differentiation between gastric *Helicobacter* species (Jani L O'Rourke *et al.*, 2004). Although this method allows identification of *H. suis*, it cannot distinguish between the canine and feline NHPH (i.e. *H. felis, H. bizzozeronii, H. salomonis, H. heilmannii* sensu stricto, *H. cynogastricus, H.*

baculiformis, *H. ailurogastricus*) (Baele *et al.*, 2004). For differentiation between the majority of these species, sequencing of the *urease A* and *B* genes, the *Hsp60* gene and the *gyrB* gene is useful, as well as is whole-cell protein profiling if pure *in vitro* cultures are available (O'Rourke *et al.*, 2004; Haesebrouck *et al.*, 2009). Whole-genome sequencing is, however, necessary to differentiate between *H. heilmannii* sensu stricto and the closely related *H. ailurogastricus* (Joosten *et al.*, 2015). After detection of potential NHPH associated DNA, additional sequencing of the positive PCR amplicons is necessary, as cross-reactivity with host DNA and DNA from other gastric NHPH may occur. Recently, a carR probe-based RT-PCR method has been validated that allows detection and quantification of *H. suis* in gastric tissue samples without the need for sequencing. This method exhibits a high degree of diagnostic specificity and analytical sensitivity and no cross-reactivity was detected with human, porcine, non-human primate and murine DNA nor with DNA from other bacteria including *Helicobacter* spp. and *Campylobacter* spp. (Blaecher *et al.*, 2017).

2.2.6 Association with gastric pathologies

In young pigs and pigs at slaughter age, *H. suis* mainly colonizes the pyloric gland zone, while in adult pigs, *H. suis* can also be found in the fundic gland zone (Hellemans *et al.*, 2007a). *H. suis* colonization induces inflammation of the gastric mucosa and has been associated with a decreased daily weight gain and ulceration of the *Pars oesophagea*.

2.2.6.1 *Gastritis*

Both in natural and experimental infected pigs, *H. suis* infection has been associated with chronic gastritis. Although inflammation can be found in all glandular zones, the pyloric gland zone is most often affected (Mendes *et al.*, 1991; Queiroz *et al.*, 1996; Park *et al.*, 2000; Hellemans *et al.*, 2007b; De Bruyne *et al.*, 2012). Histological analysis shows presence of diffuse lymphocytic/plasmacytic infiltration, lymphocytic aggregates, lymphoid follicles and occasionally neutrophilic infiltrates (Hellemans *et al.*, 2007b; De Bruyne *et al.*, 2012). De Bruyne *et al.* demonstrated that pure *in vitro* cultures of *H. suis* do not only cause gastritis but also reduce daily weight gain in experimentally infected pigs, which may lead to substantial economic losses (De Bruyne *et al.*, 2012).

Rodent models are often used to study *H. suis* infection associated pathologies. Specific pathogen free (SPF) inbred C57BL/6 and BALB/c mice have been shown to be useful models for the study of *Helicobacter*-related gastric diseases (O'Rourke and Lee, 2003). As in pigs, experimental *H. suis* infection in mice leads to gastritis. Apart from inflammation, *H. suis* infections in mice have also been associated with necrosis of parietal cells and increased mucosal epithelial cell proliferation (O'Rourke *et al.*, 2004; Nakamura *et al.*, 2007; Flahou *et al.*, 2010). Furthermore, gastric lymphoid nodules, hyperplasia and metaplasia of the gastric mucosa have been described after long term (i.e. >18 months) infection of mice with *H. suis* (O'Rourke *et al.*, 2004; Park *et al.*, 2008). Differences in gastric inflammation and colonization after *H. suis* infection have been found between C57BL/6 and BALB/c mouse strains. A more pronounced gastric inflammation has been described in the *H. suis*-infected BALB/c mice compared to C57BL/6 mice, whereas a higher colonization density in the pyloric and fundic gland zone has been noted in *H. suis*-infected C57BL/6 mice compared to BALB/c mice (Flahou *et al.*, 2010).

2.2.6.2 *Gastric ulceration*

Several studies have found a positive correlation between the presence of *H. suis* in the glandular part of the stomach and the prevalence and severity of lesions in the *Pars oesophagea* (Barbosa *et al.*, 1995; Yeomans and Kolt, 1996; Grasso *et al.*, 1996; Queiroz *et al.*, 1996; Cantet *et al.*, 1999; Phillips *et al.*, 2000; Choi *et al.*, 2001; Roosendaal *et al.*, 2002; Silva *et al.*, 2002; Szeredi *et al.*, 2005; Appino *et al.*, 2006; Yamasaki *et al.*, 2009), indicating that this bacterium might play a role in the development of porcine gastric ulceration. In other studies, however, this association was not found (Melnichouk *et al.*, 1999; Accioly *et al.*, 2000; Park *et al.*, 2000; Mall *et al.*, 2004; Casagrande Proietti *et al.*, 2010). These discrepancies might be due to different sampling techniques, different detection techniques for presence of *H. suis*, differences in virulence between *H. suis* strains and/or presence or absence of other contributing factors such as specific feeding and management strategies.

As correlation does not necessarily imply a causal relationship, attempts have been made to induce gastric lesions in experimentally infected pigs. Hellemans *et al.* (2007b) showed a trend towards more severe lesions in 6-weeks old pigs orally inoculated with mouse stomach homogenate containing *H. suis*

(Hellemans *et al.*, 2007b). In contrast, Krakowka and Ellis (2006) did not find gastric lesions in 3-days old pigs orally inoculated with mouse stomach homogenate containing "*H. heilmannii*-like organisms" (Krakowka and Ellis, 2006). The results of these studies are, however, difficult to interpret since no pure *Helicobacter* cultures were used to inoculate the pigs and in the study of Krakowka and Ellis (2006), helicobacters were not identified to the species level. Other bacteria present in stomach homogenate may have influenced the results and *in vivo* passages of *Helicobacter* spp. isolates in mice may affect the virulence and pathogenicity of the strains (Hellemans *et al.*, 2007b).

Since 2008, pure *in vitro* isolates of *H. suis* are available (Baele *et al.*, 2008), enabling more reliable *H. suis*-infection trials. When 6-weeks old piglets were inoculated with a pure culture of *H. suis*, hyperkeratosis and ulcers were present in the *Pars oesophagea* of *H. suis*-infected pigs, while sham-inoculated pigs showed no or only mild lesions (Haesebrouck *et al.*, 2009). In a more extensive study, several *H. suis*-infected 6-weeks old pigs developed severe lesions. Nevertheless, the sham-inoculated pigs also showed presence of lesions, although less severe compared to the infected group (De Bruyne *et al.*, 2012).

These experimental infection studies indicate that *H. suis* plays a role in gastric ulceration in pigs, but it is clearly not the only factor involved.

An increase of gastric mucus with free acidic groups was detected in pigs with gastric ulceration, indicating a reduced resistance to HCl which may have increased the likelihood of porcine gastric ulceration (Barbosa *et al.*, 1995). However, no consistent association could be observed between mucin degradation, *H. suis* infection and lesions in the *Pars oesophagea* (Mall *et al.*, 2004). Furthermore, since the *Pars oesophagea* is not protected by mucus, it is unlikely that gastric mucin alterations play a major role in the development of ulceration of this non-glandular stomach part.

When comparing the genome of *H. suis* with the well-known pathogen *H. pylori*, several homologs of *H. pylori* virulence associated genes are found. These include *DNA polymerase III subunit beta, urease* and *gamma-glutamyl transpeptidase* (*GGT*) which encode proteins associated with gastric epithelial cell death as well as *plasminogen-binding protein A* and *B* which have been associated with a delay in healing

of gastric lesions (Vermoote *et al.*, 2011b). *In vitro* studies confirmed that *H. suis* GGT can induce apoptosis and necrosis in human gastric epithelial cells (Flahou *et al.*, 2011). Since *H. suis* does not colonize the *Pars oesophagea* (Haesebrouck *et al.*, 2009), it seems unlikely that noxious substances produced by the bacterium will reach the non-glandular part in sufficient concentrations to cause direct epithelial cell damage (Yeomans and Kolt, 1996). They may, however, affect the viability and function of cells involved in gastric acid secretion, such as parietal cells, and influence gastric acid secretion in this way.

Pigs experimentally infected with *H. suis* develop inflammation of the fundic and pyloric gland zone as well as a 10% decrease in daily weight gain (De Bruyne *et al.*, 2012). The decrease in weight gain could be due to loss of appetite as a result of pain and inflammation, which may lead to a more fluid gastric content, breakdown of the pH gradient and irritation of the *Pars oesophagea* due to increased contact with HCl and pepsin (Zimmerman *et al.*, 2012). Furthermore, as described for *H. pylori*, increased expression of pro-inflammatory cytokines has been associated with alterations in gastric acid secretion (Calam, 1999).

Interestingly, *H. suis* is often found near and inside the canaliculi of parietal cells in naturally and experimentally infected pigs (De Bruyne *et al.*, 2012; Zhang *et al.*, 2016), experimentally infected Mongolian gerbils and mice (Flahou *et al.*, 2010) and in human patients (Joo *et al.*, 2007). In naturally *H. suis*-infected rhesus monkeys, a significant increase in gastric acid secretion in combination with colonization near parietal cells has been demonstrated (Dubois *et al.*, 1991), indicating a direct or indirect effect of *H. suis* on the acid-producing parietal cell function. In addition, *H. suis* has been associated with degenerative changes, necrosis and loss of parietal cells in humans and in rodent models of gastric disease (Joo *et al.*, 2007; Flahou *et al.*, 2010). *In vitro* incubation of porcine parietal cells with viable *H. suis* bacteria or *H. suis* lysate results in a decreased cell viability as well (Zhang *et al.*, 2016). However, when analysing the stomach of pigs naturally infected with *H. suis*, no parietal cell loss could be demonstrated (Zhang *et al.*, 2016). Host factors, which are absent in *in vitro* studies, might influence the effect of a *H. suis* infection on porcine parietal cells. The effect on parietal cells *in vivo* might also

differ between different host species, as the host reaction may vary between host species (Bosschem *et al.*, 2016, 2017).

Zhang *et al.* (2016) showed an upregulated expression of H^+/K^+ ATPase in naturally *H. suis*-infected pigs. The parietal cell H^+/K^+ ATPase is involved in gastric acid production. This study indicates that *H. suis* may disturb homeostasis of parietal cells, which may affect gastric acid secretion. Furthermore, Sapierzynski *et al.* (2007) showed an increased number of gastrin producing G-cells and a decreased number of somatostatin producing D-cells in *H. suis*-infected pigs. Since gastrin stimulates and somatostatin suppresses gastric acid production through their association with CCK-B and SST2 receptors on parietal cells, respectively, gastric acid secretion might be upregulated. However, other studies could not find an upregulation of gastrin in the stomach tissue nor in the serum of *H. suis*-infected pigs (Bunn *et al.*, 1981; Silva *et al.*, 2002).

In conclusion, these results indicate that *H. suis* infection may affect gastric acid secretion through different mechanisms, such as an alteration in the number and/or function of parietal cells and G- or D-cells (Figure 6). This may play a role in the pathogenesis of gastric ulcer disease in pigs, but the exact mechanism remains to be determined (Haesebrouck *et al.*, 2009). Furthermore, the conflicting results may suggest that other contributing factors are involved in the development and/or maintenance of porcine gastric ulceration.

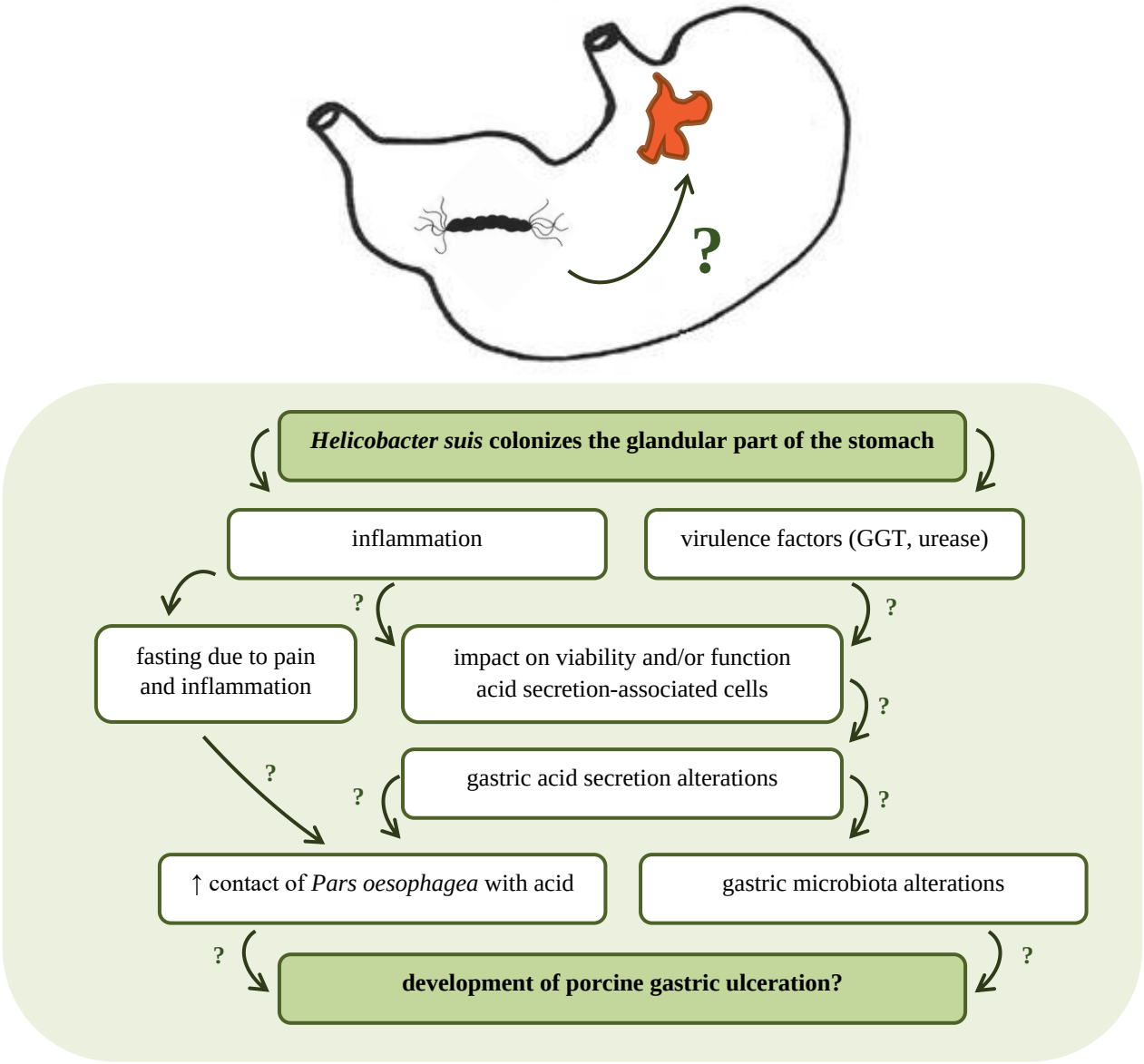


Figure 6: An overview of potential effects of a *H. suis* infection on gastric acid secretion and lesion development in pigs.

2.2.7 Host immune response to *H. suis* infection

High prevalence rates of *H. suis* have been described in pigs at slaughter age and adult pigs, which might indicate that the host immune response is not able to eliminate *H. suis* from the stomach resulting in life-long infections (Flahou *et al.*, 2012). Compared to *H. pylori*, little information is available on the host immune response against *H. suis* infections and most information was obtained from rodent models (Flahou *et al.*, 2010; Flahou *et al.*, 2012; Bosschem *et al.*, 2017). The use of different animal species as experimental hosts, however, may influence the results. For example, C57BL/6 and BALB/c mice are predominant T helper 1 and 2 (Th1 and Th2, respectively) effector cell responders, respectively (Flahou *et al.*, 2010). Furthermore, whole mucus or stomach homogenate has often been used for experimental infection, which may affect the results as well.

2.2.7.1 *Innate immune response*

In *H. suis*-infected BALB/c and C57BL/6 mice, an infiltration with mononuclear and polymorphonuclear cells in the lamina propria and/or the tunica submucosa has been described (Flahou *et al.*, 2010; Bosschem *et al.*, 2016). More specifically, infiltration of macrophages can be seen at 3 weeks post-infection. At 9 weeks post-infection the number of neutrophils and T cells increases and at 8 months post-infection large lymphoid aggregates are formed (Flahou *et al.*, 2010).

Recently, Bosschem *et al.* (2017) demonstrated that *H. suis* induces a semimaturation of porcine monocyte-derived dendritic cells, characterized by increased expression of CD25, CD80/86 and CD40, but impaired expression of MHC class II molecules on the surface of these cells. It was suggested that this impaired dendritic cell response may elicit the expansion of regulatory T cells (Tregs), which may help to establish a chronic infection as Tregs are immune-suppressive and tolerogenic. In mice, however, *H. suis* stimulation of murine dendritic cells increased the expression of dendritic cell associated CD40, CD80, CD86 and MHC class II, which resulted in an increased secretion of IL-6 and IL-23, inducing the differentiation of Th17 effector cells from naïve T cells (Bosschem *et al.*, 2017).

Currently, no information is available on how innate immune cells recognize *H. suis*, for example by pathogen-associated molecular patterns (PAMPs) recognized by Toll-like receptors (TLR). *In vitro* studies have shown that *H. pylori* stimulates pro-inflammatory gene expression via binding of its NAP

and Hsp60 on TLR-2, LPS on TLR-4, flagellin on TLR-5, RNA on TLR-7/8 and DNA on TLR-9 (Smith, 2014). However, contradicting results have been published. For example, *H. pylori* LPS has been found to activate inflammatory signalling in human epithelial cells via TLR-2, rather than the classical LPS sensor, TLR-4. In addition, others have shown that the major *H. pylori* flagellin, FlaA, is not recognized by TLR-5, thereby failing to induce the NF-κB inflammation cascade (Smith, 2014). Further research is required to verify if *H. suis* antigens, similar to *H. pylori,* contribute to the activation of the host immune response.

2.2.7.2 *Acquired immune response*

Several studies have shown that *H. suis* infection induces a T cell response in rodent models. C57BL/6J mice inoculated with homogenized pig or mouse stomach mucosa developed a Th1 predominant immune response, characterized by increased levels of IFN-γ (Cinque *et al.*, 2006; Mimura *et al.*, 2011). Another study showed enhanced levels of IFN-γ and IL-10 in C57BL/6J mice inoculated with homogenates from mouse stomach, suggesting presence of a Th1/Treg immune response (Park *et al.*, 2008). In these studies, however, presence of other micro-organisms in stomach homogenates, used as inoculum, may have influenced the results.

For the first time, Flahou *et al.* (2012) demonstrated that infection in Th2-prone BALB/c mice as well as Th1-prone C57BL/6 mice with pure *in vitro* isolated strains of *H. suis* resulted in increased expression of IL-4 and IL-10, but not of IFN-γ, suggesting that a Th2/Treg immune response was induced (Flahou *et al.*, 2012). Experimental infections with pure *H. suis* cultures in Mongolian gerbils showed increased expression of IFN-γ, suggesting a Th1 response (Bosschem *et al.*, 2015; Zhang *et al.*, 2015). In conclusion, the type of immune response differs between different animal host species.

Presence of Th17 cells and/or increased levels of IL-17 has been demonstrated in the stomachs of *H. suis*-infected BALB/c and C57BL/6J mice and Mongolian gerbils, indicating that *H. suis* infection induces a Th17 immune response as well (Flahou *et al.*, 2012; Vermoote *et al.*, 2013; Bosschem *et al.*, 2015, 2016; Zhang *et al.*, 2015). The Th17 response was significantly higher in BALB/c mice compared to C57BL/6 mice (Flahou *et al.*, 2012), possibly due to the overall higher inflammatory response in BALB/c mice compared to C57BL/6 mice. Moreover, a negative correlation between *H. suis*

colonization and the expression of IL-17 has been reported (Flahou *et al.*, 2012; Vermoote *et al.*, 2013; Bosschem *et al.*, 2015). This might be caused by the high inflammatory response in BALB/c mice, which might induce a less favourable environment for *H. suis*, thereby resulting in a lower colonization density (Flahou *et al.*, 2012). In addition, increased levels of IL-17 were positively correlated with protection against *H. suis* infection challenge in mice (Vermoote *et al.*, 2012).

H. suis infections in mice and gerbils have also been associated with induction of a Treg immune response, which is characterized by increased expression levels of IL-10 (Vermoote *et al.*, 2012, 2013, Bosschem *et al.*, 2015, 2016; Zhang *et al.*, 2015). Indeed, elevated expressions of IL-10 and of the *FOXP3* gene were observed in *H. suis*-infected BALB/c mice (Zhang *et al.*, 2015). In addition, a positive correlation between IL-10 expression levels and *H. suis* colonization density has been described in BALB/c mice (Flahou *et al.*, 2012; Vermoote *et al.*, 2012, 2013).

In line with the evoked immune response in *H. suis*-infected mice, increased expression levels of IL-17 and IL-10 have been observed in the pyloric and fundic gland zone, respectively, of natural *H. suis*-infected pigs. On the other hand, upregulated expressions of Th2 marker IL-4 and Th1 marker IFN-γ were not observed. These findings might indicate the presence of a Th17/Treg host immune response in *H. suis*-infected pigs (Bosschem *et al.*, 2017).

Several studies indicate that B lymphocytes (B cells) might play a role in the pathogenesis of *H. suis* infection as well (Vermoote *et al.*, 2012; Zhang *et al.*, 2015). BALB/c mice and Mongolian gerbils infected with pure cultures of *H. suis* showed a clear (sub)mucosal infiltration with B cells at 6 months post infection (Zhang *et al.*, 2015). These infiltrates were often organized into lymphoid follicles characterized by large, irregular and hyperproliferative B cell-containing germinal centres (Zhang *et al.*, 2015). Similarly, inoculation of C57BL/6 mice with *H. suis* containing inoculum derived from pig stomach evoked infiltration of B cells and Th cells, mainly located around the gastric lymphoid follicles, and their numbers were positively correlated with the duration of *H. suis* infection (Yamamoto *et al.*, 2011). Finally, another study showed that *H. suis* induced lymphoid follicles were characterized by the presence of both B cells and Th cells. Development of such lymphoid follicles could not be detected in *H. suis*-infected IFN-γ deficient mice, indicating that IFN-γ might play a role in the formation of *H. suis*

induced gastric lymphoid follicles (Mimura *et al.*, 2011). CXCL13, a chemokine attracting B cells, might also play a role, since a pronounced upregulation of CXCL13 has been found in *H. suis*-infected Mongolian gerbils and 6-8 months old pigs compared to non-infected ones (Zhang *et al.*, 2015; Bosschem *et al.*, 2017).

2.2.8 Control of *H. suis* infection

2.2.8.1 *Antimicrobial therapy*

Over the last 20 years, the most recommended first line treatment for *H. pylori* infected human patients was the standard triple therapy, consisting of a combination of a proton pump inhibitor (PPI) and 2 antibiotics (clarithromycin + amoxicillin or metronidazole) twice a day for 1-2 weeks. Nevertheless, therapy failure is increasingly reported due to clarithromycin resistance. It has been recommended that when the clarithromycin resistance rate in a geographical area exceeds 15%, metronidazole or levofloxacin should replace clarithromycin in the triple therapy (i.e. PPI + amoxicillin + metronidazole or levofloxacin). Non-bismuth quadruple therapies such as sequential therapy (5 days PPI + amoxicillin followed by 5 days metronidazole + clarithromycin or tetracycline or levofloxacin) or concomitant therapy (PPI + amoxicillin + metronidazole + clarithromycin for 14 days) may also be used. In areas of high dual clarithromycin and metronidazole resistance, bismuth quadruple therapy is the recommended first-line treatment (i.e. PPI + bismuth salt + tetracycline + metronidazole or levofloxacin or furazolidone). If bismuth is not available, levofloxacin, rifabutin and high dose dual (PPI + amoxicillin) treatments can be considered. If tetracycline is not available in high dual resistance areas, bismuth-containing quadruple therapy combining furazolidone plus metronidazole or amoxicillin plus metronidazole can be considered, as well as bismuth plus triple therapy (PPI + amoxicillin + clarithromycin or levofloxacin) (Malfertheiner *et al.*, 2017).

Antimicrobial treatment of gastric NHPH infections in humans is based on clinical experience and mostly, treatment schemes successful in eradicating *H. pylori* are also used to treat NHPH infections (Morgner *et al.*, 2000; Kaklikkaya *et al.*, 2002). A *H. suis*-infected veterinarian was successfully treated with a triple therapy, consisting of amoxicillin, clarithromycin and pantoprazole for 10 days (Joosten *et al.*, 2013). Similar therapeutic protocols, however, are not indicated in *H. suis*-infected pigs since this is

expensive, labor intensive and antibiotic use may favour spread of antimicrobial resistance in pathogens as well as in bacteria belonging to the microbiota.

Due to their fastidious nature and limited number of *in vitro* isolates, few data is available on the antimicrobial susceptibility and acquired resistance of gastric NHPH species. Moreover, since *H. suis* only grows in a biphasic medium with an acidic pH, standard antimicrobial susceptibility assays cannot be used for minimum inhibitory concentration (MIC) determinations. In 2011, Vermoote *et al.* developed a combined Brucella agar and broth dilution method to analyse the antimicrobial susceptibility pattern of *H. suis* (Vermoote *et al.*, 2011a). In brief, a twofold dilution of the antimicrobial agent is added to the Brucella agar and broth, with final concentrations ranging from 0.03 to 128 µg/ml. In total, 5 x 10^7 bacteria/ml are added to the broth and incubated during 48h under microaerobic conditions. After incubation, *H. suis* is quantified using a RT-PCR where the *ureA* gene is amplified. The MIC is determined as the lowest concentration of antimicrobial for which ΔCt is at least 1 Ct higher than ΔcCt (ΔCt = Ct after incubation - Ct before incubation of the antimicrobial exposed strains; ΔcCt = Ct after incubation - Ct before incubation of the controls; Ct = threshold cycle value). This is the lowest concentration of antimicrobial with at least 50% less bacterial growth compared to controls without antimicrobials. Using this method, Vermoote *et al.* determined the susceptibility of 9 *H. suis* strains isolated from pigs for the following antimicrobials: ampicillin, ceftiofur, clarithromycin, enrofloxacin, gentamicin, lincomycin, metronidazole, tetracycline and tylosin. A monomodal distribution of MICs was observed for all antimicrobials, with the exception of enrofloxacin which showed a bimodal distribution, indicating acquired resistance in one *H. suis* strain. This strain showed an AGT → AGG (Serine → Arginine) substitution at codon 99 of *gyrA* gene, which may be responsible for the resistance. Moreover, high MICs were observed for ampicillin and metronidazole, although with monomodal distribution. The MICs of ampicillin for *H. suis* were higher than those observed for *H. felis, H. bizzozeronnii, H. salomonis* and *H. pylori* strains, which might indicate a lower intrinsic susceptibility of *H. suis* for this antimicrobial agent compared to other gastric helicobacters (Van den Bulck *et al.*, 2005). It has been hypothesized that *H. bizzozeronii* and *H. felis* isolates with MICs of metronidazole of 8–16 µg/ml are resistant to this antimicrobial agent (Van den Bulck *et al.*, 2005).

Similarly, the MICs of metronidazole of the *H. suis* strains mostly fell in this higher MIC range, indicating a lower susceptibility to this antibiotic as well. Nevertheless, as no specific clinical breakpoints for antimicrobials against *H. suis* are available, prediction of the clinical efficacy based on *in vitro* testing solely remains difficult. Furthermore, only a limited number of *H. suis* strains were investigated in this study and additional tests are needed to determine if these results reflect the susceptibility of the *H. suis* population (Vermoote *et al.*, 2011a).

The efficacy of antimicrobials to eliminate *H. suis* infection has been tested in mouse models. A triple-agent therapy with lansoprazole (15 mg/kg), clarithromycin (50 mg/kg) and amoxicillin (50 mg/kg) two times daily for 1 week cleared *H. suis* infection from all C57BL/6J mice inoculated with homogenized mouse stomach mucosa. Moreover, gastric MALT lymphoma-like lesions nearly disappeared in the treated mice 4 months after therapy (Matsui *et al.*, 2008). In another study, BALB/c mice inoculated with 2 different *H. suis* isolates were given a combination of amoxicillin (750 mg/kg) and omeprazole (40 mg/kg) three times daily for 2 weeks. A marked decrease in the excretion of *H. suis* DNA was observed 6 days after the onset of treatment and the level remained low until the end of the experiment, indicating a suppression of *H. suis*. Moreover, after euthanasia at 8 days after cessation of the treatment, all mice challenged with one of the isolates tested negative for *H. suis* presence in the stomach, while only 2 from the 10 mice challenged with the other isolate tested negative. These discrepancies may have been caused by differences in sensitivity for amoxicillin between both *H. suis* isolates (Hellemans *et al.*, 2005). So far, no antimicrobial trials have been performed in experimentally or naturally *H. suis*-infected pigs.

2.2.8.2 *Vaccination*

In mouse models, prophylactic immunization followed by challenge with *H. pylori*, *H. felis* or *H. suis* has been shown to induce a more severe gastritis compared to non-immunized, challenged mice (Sutton *et al.*, 2001; Bosschem *et al.*, 2015). This phenomenon hampers vaccine development and so far, no vaccine formulation is available which completely protects the host against *Helicobacter* spp. infections.

Compared to *H. pylori* and *H. felis*, only a limited number of studies investigated immunization strategies against other gastric NHPH. In mouse models, urease has been shown to induce protection in

immunization studies against *H. pylori* (Kleanthous *et al.*, 1998) and *H. felis* infection challenge (Michetti *et al.*, 1994). Furthermore, as urease is highly conserved among gastric helicobacters, immunization with heterologous urease may induce antigenic cross-protection between *Helicobacter* species (Michetti *et al.*, 1994). Indeed, a decreased fecal excretion of *H. suis* DNA has been demonstrated in BALB/c mice vaccinated intranasally and subcutaneously with whole bacterial cell lysate of *H. pylori* or *H. felis* and subsequently inoculated with pig stomach mucosa containing *H. suis*. As whole bacterial cell lysates also contain other proteins apart from urease, for example conserved heat shock proteins, presence of such proteins may have contributed to the observed cross-protection as well. However, complete clearance was not achieved, as *H. suis* DNA was still detected in the stomach at 16 weeks post challenge (Hellemans *et al.*, 2006). In another study, BALB/c mice were vaccinated intranasally and subcutaneously with whole bacterial cell lysates of *H. suis*, *H. cynogastricus* and *H. bizzozeronnii* after which they were challenged with *H. suis*. Despite high serum IgG titers in all vaccinated mice, complete protection was only achieved in a minority of mice following intranasal immunization (Flahou *et al.*, 2009).

Since *in vitro* cultivation of *H. suis* is laborious, it may not yield sufficient antigen. Furthermore, whole-cell lysates may contain both protective antigens and antigens suppressing protection, which might result in insufficient protection against *H. suis* challenge. Therefore, Vermoote *et al.* compared the protective efficacy of subunit vaccine candidates with that of whole cell lysate in BALB/c mice. The protective efficacy of intranasal vaccination of BALB/c mice with *H. suis* UreB, recombinantly expressed in *Escherichia coli* (rUreB), was compared with that of *H. suis* lysate. Intranasal immunization with rUreB resulted in a significant decrease of the bacterial load in the gastric mucosa, but could not prevent *H. suis* colonization. Immunization with whole-cell lysate resulted in a significant reduction of the bacterial load, with 50% of the mice negative for presence of *H. suis* DNA, indicating higher levels of protection compared to immunization with rUreB. Significantly higher expression levels of IFN-γ and IL-17 were detected in rUreB and lysate immunized compared to sham immunized mice, while IL-10 expression levels were significantly lower in the lysate-immunized group compared to rUreB and sham immunized mice. Moreover, increased levels of IFN-γ and IL-17 as well as decreased levels of IL-10 were positively

correlated with protection against *H. suis* infection challenge in a mouse model (Vermoote *et al.*, 2012). It was hypothesized that, in order to obtain a degree of protection similar to the one induced by whole-cell lysate, additional *H. suis* antigens should be included in subunit vaccines. Indeed, simultaneous and consecutive intranasal immunization with the recombinant *H. suis* GGT (rGGT) and rUreB induced better protection against a subsequent *H. suis* challenge compared to immunization with single proteins in a mouse model. A complete clearance was observed in around 50% of the mice which was similar to the protection induced by a whole-cell lysate. Also in this study, the degree of protection was correlated with increased levels of TNF-α, IFN-γ and IL-17 as well as decreased levels of IL-10. A weak, but significant ($p < 0.05$, $\rho = -0.235$) correlation was observed between decreased bacterial load and increased specific serum IgG. This indicates that a Th1/Th17 response in combination with antibody responses are involved in the protective immunity against *H. suis* infections. Post-vaccination immunization gastric inflammation was less severe in mice vaccinated with a combination of rUreB and rGGT compared to mice vaccinated with whole-cell lysate. Nevertheless, increased mortality of 10-40% was observed in the sham, lysate and antigen-immunized groups. This might be due to the intranasal use of cholera toxin as adjuvant, which might have caused swelling of the nasal cavity mucosae, resulting in oxygen deficiency (Vermoote *et al.*, 2013).

Bosschem *et al.* investigated the impact of different adjuvants on protection against *H. suis* infection challenge in a mouse model (Bosschem *et al.*, 2015). Several vaccine adjuvants (i.e. CpG-DNA, Curdlan, Freund's Complete and Incomplete, Cholera toxin and CCR4 antagonist) were administered either subcutaneously, sublingually or intranasally along with *H. suis* whole-cell lysate, after which the mice were challenged with *H. suis*. Subcutaneous and sublingual immunization with Freund's complete + lysate and intranasal immunization with cholera toxin + lysate resulted in a significant decrease of colonizing *H. suis* bacteria in the stomach, although adverse effects such as post-immunization gastritis/pseudo-pyloric metaplasia and increased mortality were observed. In the groups immunized subcutaneously with CCR4 antagonist + lysate, a similar protection level was observed, but gastric pseudo-pyloric metaplasia was less severe or even absent. In general, an inverse correlation was

observed between IFN-γ, IL-4 and IL-17 expression levels and *H. suis* colonization density, whereas lower IL-10 expression levels were observed in partially protected animals.

The post-immunization gastritis observed in prophylactic *Helicobacter* spp. vaccination studies is most likely a immunopathological response of the host to residual *Helicobacter* bacteria. Especially whole bacterial cell lysates/sonicates and the use of cholera toxin as adjuvant have been linked with a pronounced gastritis. It has been hypothesized that the development of post-immunization gastritis is critical for the elimination of the bacteria, as a clear correlation between the severity of post-immunization gastritis and reduction of bacterial load has been demonstrated (Garhart *et al*., 2002; Vermoote *et al*., 2012, 2013; Bosschem *et al*., 2015). However, the protective role of post-immunization gastritis is unclear, since this phenomenon is absent in Mongolian gerbils protected by oral immunization against *H. pylori* infection (Jeremy *et al*., 2006). The disparity in gastritis between *H. pylori* and *H. felis* infected mice and immunized/challenged mice has also been described to disappear, showing that this is most likely a transient event (Garhart *et al*., 2002; Sutton *et al*., 2007). Finally, post-immunization gastritis was found to be negligible in mice vaccinated with single proteins and mice immunized with combinations of rUreB and rGGT, although in these groups a significant reduction or even clearance of *H. suis* was achieved (Vermoote *et al*., 2013). Long term experimental studies will have to be performed in order to determine whether this post-immunization gastritis is transient and if it also occurs in the natural hosts of *H. suis.*

Although these studies show promising results, it is still unclear how to promote the induction of protective immunity with the final goal to develop a successful vaccine. Further research should focus on a better understanding of the innate and adaptive immune responses towards a *H. suis* infection in pigs, with emphasis on the role of dendritic cells and the development of safe and efficient vaccine adjuvants.

2.3 Infectious agents, other than *H. suis*, possibly involved in porcine gastric ulceration

2.3.1 Other *Helicobacter* species

Krakowka *et al.* (2005) described a *Helicobacter* sp. present in the stomach of young pigs, with a curve-shaped morphology different from the tightly coiled *H. suis.* This agent seemed to be morphologically similar to, but antigenically different from *H. pylori*. Three-day-old piglets experimentally infected with these *H. pylori*-like bacteria and fed a carbohydrate rich diet, developed lesions of the *Pars oesophagea*. Only mild lesions were present when the *H. pylori*-like infected pigs received a normal diet, indicating a synergistic effect between bacteria and diet (Krakowka and Ellis, 2006). The authors hypothesized that the *H. pylori*-like bacteria produced short chain fatty acids (SCFA) from the carbohydrates and/or that growth of *H. pylori*-like bacteria was promoted by the diet. Unfortunately, as far as we know, no taxonomic studies or genome sequences have been published for this *Helicobacter* species. In any case, fermentative capacities of *Helicobacter* species are, generally speaking, low and the number of *H. pylori*-like bacteria did not seem to differ between the groups. The exact identity of this agent and its possible involvement in gastric ulcer disease remains unclear.

2.3.2 *Lactobacillus* and *Bacillus* species

Argenzio and Eisemann (1996) described that undissociated SCFA may induce gastric epithelial cell death by inhibiting cellular osmoregulation resulting in cell swelling and necrosis. It was hypothesized that microbial production of SCFA, such as acetic acid, may contribute to the pathogenesis of porcine gastric ulceration (Argenzio and Eisemann, 1996; Argenzio, 1999). Erosions and ulcers of the non-glandular part of the stomach were present in gnotobiotic pigs given a carbohydrate rich diet in combination with a mono-infection with a pure culture of a *Lactobacillus* sp. or *Bacillus* sp. It was hypothesized that the inoculated bacteria were able to ferment the carbohydrates with production of SCFA (Krakowka *et al.*, 1998). Nevertheless, the role of SCFA remains highly controversial. Although coarsely ground diets stimulate the production of SCFA in the non-glandular part of the stomach of pigs, they have been shown to exert a protective effect against gastric ulcers. Conversely, finely ground and pelleted diets may favour ulceration while production of SCFA is much less or not stimulated (Argenzio, 1999). Moreover, to the best of our knowledge, adding SCFA to the feed or drinking water of pigs, has

not been associated with an increased risk for development of gastric ulcers. Unfortunately, in the study of Krakowka *et al.* (1998) the inoculated *Lactobacillus* sp. and *Bacillus* sp. were not identified to the species-level. It remains to be determined whether some specific species belonging to these genera might stimulate ulcer development, while others are harmless. Most *Lactobacillus* spp. are indeed considered to be beneficial for the host health and high numbers colonize the mucosa of the *Pars oesophagea* of pigs (Su *et al.*, 2008; Mann *et al.*, 2014). It is also possible that only some SCFA favour ulcer development, while others may even exert a protective effect. Butyrate, for instance, has been shown to protect intestinal cells against damage (Van Immerseel *et al.*, 2010), while lactic acid, produced by lactobacilli, may prevent infection or colonization by pathogens (Su *et al.*, 2008).

2.3.3 Yeast and fungi

Tannock and Smith (1970) described a pronounced presence of *Candida albicans* and *C. slooffii* in parakeratotic and necrotic epithelial tissues of the *Pars oesophagea* of pigs. The authors hypothesized that these indigenous yeasts could only colonize the non-glandular part of the stomach after removal of the resident autochthonous *Lactobacillus* spp., for example after antibiotic treatment. It is not clear whether these colonizing *Candida* spp. were a contributing factor to gastric ulceration or that presence of gastric lesions merely facilitated colonization by these yeasts. Further studies are necessary to investigate potential mechanisms of yeast induced gastric ulceration.

Kazachstania slooffiae colonizes in great numbers the porcine gastro-intestinal tract and seems to be beneficial for pig health by maintaining a well-balanced microbiota and by increasing the amount of SCFA (Urubschurov *et al.*, 2011, 2017). Similarly, the pyloric gland zone of Mongolian gerbils is naturally colonized by the closely related *K. heterogenica*. When these animals were, however, co-experimentally infected with *K. heterogenica* and *H. suis*, a significant increase in the severity of gastritis was demonstrated compared to gerbils infected only with *H. suis* (Flahou *et al.*, 2010a). It is not known whether pigs co-infected with *H. suis* and *K. slooffiae* may develop more severe gastric pathologies.

2.3.4 Acute infectious diseases

Acute respiratory infections have been associated with increased likelihood of gastric lesions (Dionissopoulos *et al.*, 2001; Zimmerman *et al.*, 2012). These infections are often characterized by inappetence resulting in increased fluidity of the gastric content. Also, increased levels of histamine, which promote inflammation and stimulate gastric acid secretion by direct binding to parietal cells, might be involved. In general, it can be assumed that each acute infectious disease may contribute to the development of porcine gastric ulceration. For example, pigs affected by porcine reproductive respiratory syndrome, post-weaning multisystemic wasting syndrome (PMWS), swine influenza, porcine contagious pleuropneumonia or porcine dermatitis nephropathy syndrome often present gastric lesions (Casagrande Proietti *et al.*, 2010; Busch and Nielsen, 2016; Detmer, 2016). Although presence of these lesions may be accidental (Casagrande Proietti *et al.*, 2010), large amounts of porcine circovirus type 2 (PCV2) antigen have been found in epithelial cells and debris in the glandular stomach zone of pigs affected by PMWS (Corrêa *et al.*, 2008). This may indicate a role of PCV2 in the development of porcine gastric ulceration, although further studies are necessary to confirm this hypothesis.

2.3.5 Parasites

Although larval stages of *Sarcophaga bullata* and *Musca domestica*, *Ollanus tricuspis* and *Gnathostoma hispidum* have been sporadically linked with gastric ulceration in pigs, these findings are most likely accidental (Qureshi *et al.*, 1978). *Hyostrongylus rubidus* may also cause irritation due to its attachment to the fundic mucosa, resulting in erosion and ulceration (Dodd *et al.*, 1960; Banga-Mboko *et al.*, 2003). Although parasitic lesions may be common in free range, outdoor reared pigs, the prevalence is expected to be low in conventional, indoor pigs farms (Zimmerman *et al.*, 2012).

Some studies demonstrated an association between *Ascaris suum* infection and gastric lesions in pigs. When 8-week-old pigs were inoculated twice with 2 weeks interval with infective *A. suum* eggs, hyperkeratosis, erosion and ulceration of the non-glandular zone of the stomach was induced (Rivera and Gaafar, 1976). Similarly, *Pars oesophageal* lesions developed when 3-months old pigs were moved to a new location, probably due to a second invasion with *A. suum* larvae (Qureshi *et al.*, 1978). Since pigs only developed gastric lesions after a second exposure and since *A. suum* induces a strong antigenic

response, the host-immune response might play a role in the development of these ulcerations. Furthermore, migration of *A. suum* larvae through liver and lungs causes cell damage, inducing histamine release. Consequently, an increased gastric acid secretion might further irritate the *Pars oesophagea* (Häni and Indermühle, 1979). Nevertheless, autopsy records from commercial pigs did not reveal a clear association between presence of *A. suum* infection and gastric ulceration. These discrepancies may be explained by the presence of contributing factors and/or the lower number of *A. suum* in field conditions (Häni and Indermühle, 1979).

2.4 Other factors contributing to porcine gastric ulceration

2.4.1 Gender, genetics and keratinization pattern

Martino *et al.* demonstrated that when pigs were kept up to 170 kg, barrows had a higher risk for developing gastric ulcers compared to gilts (Di Martino *et al.*, 2013). It was hypothesized that production of estrogen may be ulceroprotective, as already shown in female mice (Shimozawa *et al.*, 2006). Nevertheless, others could not find an effect of gender in pigs slaughtered at around 100 kg (Elbers *et al.*, 1995a; Robertson *et al.*, 2002). These discrepancies might be caused by a different hormonal production in younger animals (Di Martino *et al.*, 2013).

Selection for lean pigs, fast growth and thin back fat has been associated with an increased risk for developing gastric ulceration (Berruecos and Robison, 1972; Grondalen and Vangen, 1974). Furthermore, a strong litter-effect has been shown, indicating that genetic predisposition might play a role in the development of porcine gastric ulceration (Hessing *et al.*, 1992).

When analysing the keratin pattern in normal and ulcerated *Pars oesophagea* of pigs, keratin 6 was more expressed in pigs with ulceration. Nevertheless, keratin 16, which forms a natural pair with keratin 6, was absent in both normal and ulcerated tissue. In addition, keratin 10, a marker for hyperkeratosis, could not be detected in pigs with and without ulceration. It is unclear if keratin patterns may play a role in the susceptibility of the *Pars oesophagea* for development of lesions (Roels *et al.*, 1997).

2.4.2 Microbiota

The microbiota is a complex ecosystem of commensal, pathogenic and symbiotic micro-organisms which reside in or on their host. These micro-organisms may include bacteria, archaea, protists, fungi as well as viruses. The most diverse and dense microbiota can be found in the gastrointestinal tract (GIT), which harbors over 10^{14} bacterial cells (Saltzman *et al.*, 2018). Compared to the lower GIT, the stomach and small intestine contain relatively low numbers of micro-organisms ($10^3 – 10^5$ bacteria/g or ml content) due to the low pH and rapid flow, respectively (Mackie *et al.*, 1999). The GIT microbiota plays an important role in nutrient utilization, absorption and metabolism as well as in host immunity modulation and protection against pathogens, thereby affecting host health and production efficiency (Metzler-Zebeli *et al.*, 2013). Even though culture based systems have been important in determining the major groups of bacteria present in the GIT, it has been estimated that at least 50% of the GIT microbiota cannot be cultured (Kim and Isaacson, 2015). By using high throughput DNA sequencing of *16S rRNA* genes (i.e. metagenomics), the composition and diversity of the microbiota can be determined more accurately.

For a long time, the gastric environment was considered to be sterile. Since the discovery of *H. pylori* in 1982, several studies have described the presence of other bacteria residing in the stomach. Studies using metagenomics to analyze the gastric microbiota of pigs are, however, still infrequent. In the past, the gastric content of pigs has been investigated more intensively than the gastric mucosa. Nevertheless, their bacterial composition is quite different, as resident bacteria are closely associated with the mucosa, while transient bacteria are more part of the content (Nava and Stappenbeck, 2011). In addition, the majority of the studies focused on the gastric microbiota of weaned pigs rather than pigs at slaughter age or adult pigs. All these factors make it difficult to define a normal, healthy gastric microbiota in pigs (Motta *et al.*, 2017). Below, a brief overview of bacteria described to be present in the pig stomach is given.

Several bacteria have been detected in the stomach content of pigs: *Lactobacillus* spp.; *Streptococcus* spp.; *Enterococcus* spp.; *Clostridium* cluster I, IV, IX, XIV; *Bifidobacterium* spp.; *Bacteroides* spp.; *Prevotella* spp.; *Porphyromonas* spp.; *Helicobacter* spp.; *Campylobacter* spp.; *Fusobacterium* spp. and

Enterobacteriaceae (Castillo *et al.*, 2007; Metzler-Zebeli *et al.*, 2013). In a recent study, Motta *et al.* compared the gastric microbiota composition of the stomach content and the mucosa of the cardiac, fundic and pyloric gland zone of weaned pigs (Motta *et al.*, 2017). The dominant phylum was Proteobacteria in both content and mucosal samples, while the second phylum was Firmicutes for the content samples and Actinobacteria for mucosal samples. Significant differences in community structure were found between the mucosal and the content samples, but not between the different mucosal regions. The gastric content was characterized at genus level by *Pasteurella, Streptococcus, Lactobacillus* and *Lactococcus*, while the gastric mucosa was characterized by *Herbiconiux, Moritella* and *Brevundimonas*. In contrast with the glandular stomach regions, Firmicutes represents the dominant phylum in mucosal samples from the non-glandular *Pars oesophagea*. This discrepancy can be explained by the high abundance of *Lactobacillus* spp. adhering to the mucosa of the *Pars oesophagea*. The composition of lactobacilli communities can greatly differ between pigs, while various *Lactobacillus* spp. may colonize the *Pars oesophagea* of the same pig. Apart from *Lactobacillus*, other genera such as *Prevotella, Escherichia, Bacteroides, Pseudomonas, Acinetobacter, Clostridium* can also be detected in the *Pars oesosphagea*, indicating a high diversity and species richness in this stomach region (Mann *et al.*, 2014).

A stable GIT microbiota inhibits proliferation of opportunistic pathogens, thereby preventing development of gastro-intestinal disorders (Castillo *et al.*, 2007). Factors such as stress and feed composition, however, may induce an imbalance of the GIT microbiota. For example, weaning induces a decrease in the proportion of *Lactobacillus* populations in the upper GIT (i.e. stomach, jejunum and ileum), resulting in a higher abundance of pathogenic bacteria in these GIT regions, such as *S. suis* (Su *et al.*, 2008). High dietary calcium-phosphorus levels, fermented feed and coarsely ground diets decrease the abundance of *Enterobacteriaceae*, while increasing the numbers of *Lactobacillaceae* at all GIT sites (Van Winsen *et al.*, 2001; Sander *et al.*, 2012; Mann *et al.*, 2014). Lactobacilli are hypothesized to prevent infection or colonization of pathogens of the GIT by competition for nutrients and epithelial binding sites and by production of antimicrobial factors, such as lactic acid and bacteriocins (Su *et al.*, 2008). Moreover, *Lactobacillus* spp. may contribute to a low pH in the GIT. It has been stated that a

decreased abundance of this population may result in an increased pH which on its turn may allow proliferation of pathogenic bacteria (Su *et al.*, 2008). Similarly, it can be hypothesized that an impaired gastric acid secretion creates an environment which is less hostile for bacteria, enabling the establishment of a specific microbiota. Changes in the gastric microbiota of pigs have been suggested to play a role in the development of ulceration of the *Pars oesophagea*, although further investigation is necessary.

3. Measures to control porcine gastric ulceration

As the outcome of porcine gastric ulceration is mainly subclinical, early diagnosis followed by adequate treatment is difficult. In general, pale and weak pigs should be separated from pen mates to prevent bullying and eating should be encouraged, for example by providing diets with a high fat content. Severely anemic pigs as well as pigs with severe ulceration or stenosis of the oesophageal opening into the stomach are most often euthanized, as treatment is expensive, laborious and often unsuccessful. In case of valuable breeding animals, however, pharmaceuticals such as antacida and omeprazole and/or blood transfusions in combination with parenteral injections of iron and vitamin B might be employed (Thomson and Friendship, 2012).

Prevention is considered to be the most appropriate approach to reduce porcine gastric ulceration. Risk factors associated with severe gastric ulceration, however, are often beneficial from the economic point of view. For example, finely ground diets are associated with severe ulceration, but also with improved feed conversion ratios. Steps to reduce gastric ulceration should be carefully balanced between economic considerations and animal welfare problems (Thomson and Friendship, 2012).

In general, feeding and management factors should be optimized to control porcine gastric ulceration. Sudden changes in feeding or environment as well as overcrowding result in stress and/or anorexia which contributes to the development of stomach lesions. Good management practices should allow to minimize occurrence of these risk factors. Feed intake should be closely monitored as anorexia may result in severe ulceration (Thomson and Friendship, 2012).

Increased stomach content firmness results in maintenance of the pH gradient and has been associated with a normal stomach mucosa. Feeding coarsely ground feed contributes to a firm stomach content and is considered to be ulceroprotective (Mößeler *et al.*, 2012). Introducing such diet even decreases the severity of lesions present in pigs that were previously fed a diet with fine particle size (Ayles *et al.*, 1996; Millet *et al.*, 2012). Nevertheless, feeding a coarsely ground diet is accompanied by decreased digestibility of the feed and reduced weight gain compared to fine and/or pelleted diets (Millet *et al.*, 2012). The adverse effect on weight gain, however, seems to be limited when rotating pellets and coarsely ground meal diets, while maintaining an ulceroprotective effect (De Jong *et al.*, 2016).

High fiber diets are also hypothesized to decrease stomach content firmness and retention time. The addition of fibers to finely ground diet has been shown to reduce the risk of lesion development (Kopinski and McKenzie, 2007; Paulk *et al.*, 2015), although others did not find such association (Grosse Liesner *et al.*, 2009). In these studies, however, fibers were added after the milling process making it difficult to separate the effect of particle size distribution and fiber content. When fibers were added before milling, a coarsely ground diet rich in fibers showed a higher ulceroprotective effect compared to coarsely ground diet low in fibers or finely ground diet rich in fibers (Millet *et al.*, 2012). Nevertheless, increased fiber content was accompanied by reduced digestibility of the feed and inferior carcass yield (Millet *et al.*, 2012).

Providing straw has been associated with a decreased risk for development of gastric ulcers due to increased stomach content firmness, prolonged stomach retention time and/or reduced stress levels. While some studies showed a protective effect of 70 g straw/day (Di Martino *et al.*, 2013), others only saw beneficial effects when at least 500 g/day of straw was provided (Herskin *et al.*, 2016). In another study, 300 g/day seems to be beneficial, while increasing amounts were less ulceroprotective (Jensen *et al.*, 2017). This indicates that straw may be a potential strategy to reduce, but not eliminate, *Pars oesophagea* ulceration in fattening pigs in a commercial setting.

Yamaguchi *et al*. showed that diets containing 0.1-0.5% sodium polyacrylate are retained longer in the stomach, resulting in a firm stomach content and decreased risk for development of ulceration. Furthermore, addition of sodium polyacrylate improved weight gain and feed conversion (Yamaguchi

et al., 1981). Conversely, Alaviuhkola *et al.* could not demonstrate an ulceroprotective effect of 0.2% sodium polyacrylate (Alaviuhkola *et al.*, 1993). Differences in diet and/or pig breed may have contributed to these discrepancies.

Micronutrients, such as zinc and vitamins A, B, C, D and E, as well as antioxidants, such as selenium and melatonin, are hypothesized to confer robustness to the non-glandular epithelium, thereby reducing the risk of developing lesions. Although supplementation seems to be beneficial when pigs are deficient for these nutrients, for example due to decreased feed intake during weaning, clear evidence of their protective effect is missing (Nafstad and Tollersrud, 1967; Bubenik *et al.*, 1998). On the other hand, high concentrations of vitamin A, copper and zinc have been associated with increased severity of stomach lesions (Southern *et al.*, 1993; Lawrence *et al.*, 1997; Coates *et al.*, 1998). Addition of 400 ppm S-methylmethionine-sulphonium chloride (vitamin U) has been reported to reduce the prevalence and severity of stomach lesions by 50% compared to pigs without supplementation (Elbers *et al.*, 1995b). In another study, 200 ppm vitamin U did not prevent increase in lesion severity in pigs with a low initial lesion score, while it tended to reduce lesion severity in pigs with a high initial lesion score, although not significantly (Kopinski *et al.*, 2007). Further research is necessary to investigate the beneficial effects of vitamin U supplementation.

Human patients suffering from gastric ulceration are successfully treated with gastric acid secretion-inhibitors (Shim and Kim, 2017). Similarly, Friendship *et al.* showed that supplementation of 40 mg omeprazole in the feed resulted in a higher gastric pH which prevented some, but not all, of the tissue damage in the *Pars oesophagea* related to feed withdrawal (Friendship *et al.*, 2000). Conversely, supplementation of 30 mg lansoprazole, which inhibits H^+/K^+ ATPase similar to omeprazole, could not prevent tissue damage during fasting (Melnichouk *et al.*, 1999). The addition of H_2-antagonists, which also inhibit gastric acid secretion, could not reduce the ulcerogenic effects induced by finely ground diets (Hedde *et al.*, 1985). In another study, the addition of alkaline buffers such as $NaHCO_3$ and $KHCO_3$ resulted in a decreased incidence and severity of stomach lesions in pigs (Wondra *et al.*, 1995). These studies indicate that stomach lesions may be prevented by reducing the gastric HCl secretion and/or by neutralizing the effect of HCl, although further research is necessary.

As porcine gastric ulceration is of multifactorial origin, it is unlikely that one single pharmaceutical, feeding or management technique will completely prevent development of lesions. Most likely, a combination of optimal feeding strategies and good management practices is necessary. An equilibrium of the gastric microbiota should be maintained, although so far, the composition of a normal, healthy microbiota is not yet defined nor how it can be influenced. In conclusion, no ideal solution exists and further investigation is needed to solve this complex disease entity.

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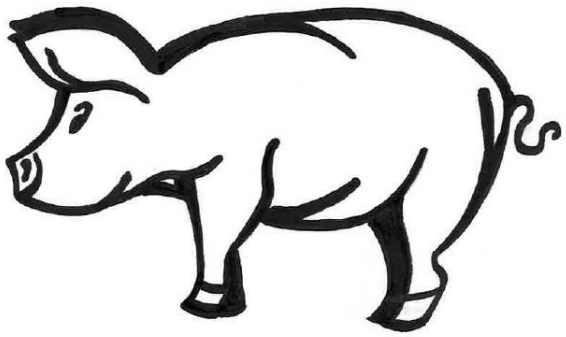


Scientific aims

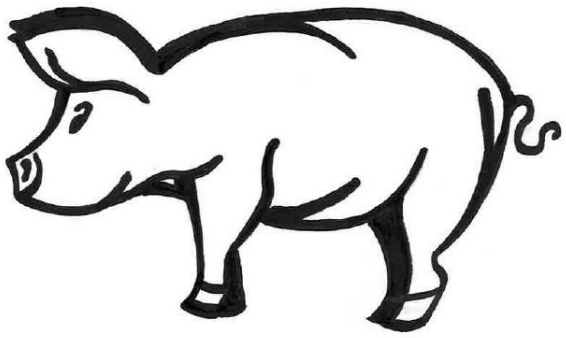
Gastric ulcera are of major importance in swine production, however, its pathogenesis is largely unknown. Furthermore, there are no preventive measures which completely protect pigs from gastric lesions. The **general aim** of this thesis was to investigate the role of pathogens in the development of porcine gastric ulceration, which may ultimately facilitate the development of effective control measures.

Several studies have attributed a role to *Helicobacter suis* in the development of porcine gastric lesions. It is not completely clear how *H. suis* influences lesion development, but alterations in gastric acid secretion may be involved. Therefore, our **first specific aim** was to obtain further insights in the mechanisms involved in persistence of *H. suis* in the porcine stomach and in its effects on gastric acid secretion and lesion development.

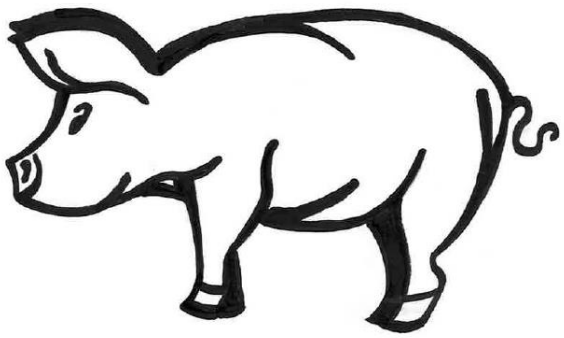
An impaired gastric acid secretion, induced by *H. suis*, may favour the establishment of a specific gastric microbiota. Indeed, an unidentified *Fusobacterium* sp. was abundantly present in the gastric microbial community of *H. suis*-infected pigs. Since most *Fusobacterium* spp. are associated with (pyo)necrotic infections, it was hypothesized that this novel species could play a role in the development of gastric ulceration. Our **second specific aim** was to isolate and characterize this new *Fusobacterium* sp., designated *F. gastrosuis*, in order to enable further research into its pathogenicity.

Our **third specific aim** was to obtain further insights in the influence of a naturally acquired *H. suis* infection on the microbiota of the *Pars oesophagea* of the porcine stomach and in the pathogenic potential of *F. gastrosuis*. The ability of *F. gastrosuis* to induce cell death *in vitro* was determined and its genome was analysed for the presence of genes encoding putative virulence factors.

Since an inhibitory action of bambermycin has been described towards *H. pylori* and as this bacterium is closely related to *H. suis*, it was hypothesized that bambermycin might inhibit *H. suis* colonization and, as a result, infection-induced gastric pathologies as well. Therefore, our **fourth specific aim** was to determine the effect of in feed medication with this antibiotic on the course of a *H. suis* infection, the host response and the gastric microbiota.



Experimental studies



Chapter 1: *Helicobacter suis* induces changes in gastric inflammation and acid secretion markers in pigs of different ages

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Abstract

Gastric mRNA expression of markers for acid secretion and inflammation and presence of gastric ulceration was studied in naturally *Helicobacter suis*-infected and non-infected 2-3 months old, 6-8 months old and adult pigs. In *H. suis*-infected 2-3 months old pigs, IL-8 and IL-1β transcript levels were upregulated in the pyloric gland zone, indicating an innate immune response. A similar response was demonstrated in the fundic gland zone of adult pigs, potentially due to a shift of *H. suis* colonization from the pyloric to the fundic gland zone. A Treg response in combination with decreased expressions of IL-8, IL-17A and IFN-γ was indicated to be present in the *H. suis*-infected 6-8 months old pigs, which may have contributed to persistence of *H. suis*. In *H. suis*-infected adult pigs, a Treg response accompanied by a Th17 response was indicated, which may have played a role in the decreased number of *H. suis* bacteria in the stomach of this age group. The decreased G-cell mass and upregulated expression of somatostatin indicated decreased acid secretion in *H. suis*-infected 6-8 months old pigs. In *H. suis*-infected adult pigs, upregulation of most markers for gastric acid secretion and increased G-cell mass was detected. Presence of severe hyperkeratosis and erosions in the non-glandular part of the stomach were mainly seen in the *H. suis*-positive groups. These results show that *H. suis* infection affects the expression of markers for acid secretion and inflammation and indicate that these effects differ depending on the infection phase.

Keywords: Helicobacter suis – pig – stomach – ulceration – gastric acid secretion – immune response

Introduction

Gastric ulceration is a common disease entity of pigs worldwide, with prevalences of up to 93% (Haesebrouck *et al*., 2009). Although the disease outcome is often subclinical, animal welfare issues as well as economic losses due to decreased daily weight gain, decreased feed intake and sudden death, are of major importance (Haesebrouck *et al*., 2009). The etiology seems to be multifactorial. Indeed, several factors, including diet particle size (Ayles *et al*., 1996), management (Herskin *et al*., 2016), gastric microbiota composition, infection with *Helicobacter suis* (Hellemans *et al*., 2007b; De Bruyne *et al*., 2012), and hormonal changes (Bubenik *et al*., 1998) have been hypothesized to be involved. The pathogenesis of porcine gastric ulceration, however, remains largely unknown (Haesebrouck *et al*., 2009). What we do know is that, in contrast to other animal species, in pigs gastric ulcers develop almost exclusively in the *Pars oesophagea*, a small area around the opening of the oesophagus which does not contain glands. Since this stomach region is not protected by mucus, it is highly susceptible to irritation with for instance hydrochloric acid, produced in the fundic gland zone of the porcine stomach (Haesebrouck *et al*., 2009). Chronic insult of the *Pars oesophagea* results in hyperkeratosis, erosion and finally ulceration.

Pigs are commonly infected with the zoonotic pathogen *H. suis* (Haesebrouck *et al*., 2009). This pathogen mainly colonizes the fundic and pyloric gland zone of the porcine stomach, inducing inflammation and a decreased daily weight gain (De Bruyne *et al*., 2012). It has been hypothesized that alterations in hydrochloric acid production in the glandular region of the stomach, associated with chronic *H. suis* infections, may play a role in the pathogenesis of swine gastric ulceration (Hellemans *et al*., 2007b; Haesebrouck *et al*., 2009; De Bruyne *et al*., 2012). Hellemans *et al*. demonstrated a tropism of *H. suis* for the gastric acid producing parietal cells. Histological analysis of the stomach of *H. suis*-infected pigs at slaughter age, has revealed that these bacteria are often found in close vicinity of parietal cells and even inside the canaliculi of these cells (Hellemans *et al*., 2007b). In addition, *H. suis* can cause degenerative changes and necrosis of parietal cells in porcine, human and rodent models of gastric disease (Joo *et al*., 2007; Flahou *et al*., 2010). Recent reports indicated that *H. suis* may disturb homeostasis of porcine parietal cells and affect their expression of genes encoding H^+/K^+ ATPase

(Zhang *et al*., 2016). The latter is an enzyme typically associated with parietal cells and is involved in gastric acid production by these cells. Not only parietal cells, but also gastrin producing G-cells and somatostatin producing D-cells can be altered during *H. suis* infection (Sapierzynski *et al*., 2007). Gastrin stimulates and somatostatin suppresses gastric acid production through their association with CCK-B and SST2 receptors on parietal cells, respectively, suggesting that *H. suis* infection may indeed affect gastric acid secretion through different mechanisms.

The main objectives of this study were to obtain further insights in the mechanisms involved in persistence of *H. suis* in the porcine stomach and in its effects on gastric acid secretion and lesion development. This was studied in naturally *H. suis*-infected pigs during the acute and chronic phases of infection. Therefore, the mRNA expression of different cytokines, chemokines and markers for gastric acid secretion was studied, the parietal cell, D-cell and G-cell mass was analyzed and the severity of *Pars oesophageal* lesions was determined in *H. suis*-infected and non-infected 2-3 months old pigs, 6-8 months old pigs and adult sows.

Material and methods

Sampling of porcine stomachs

Sixty-eight stomachs of 6-8 months old pigs and 60 stomachs of adult sows (1-3 years old) were collected over a period of 10 months from 2 slaughterhouses in Flanders, Belgium. The pigs originated from different herds. The stomachs of the 6-8 months old pigs had also been used in a previous study (Bosschem *et al*., 2017). In addition, stomachs of 34, 2-3 months old pigs were collected from 2 different pig herds (17 samples from each herd). The stomachs were transported immediately to the laboratory and stored at 4°C until further examination within 2h. The stomachs were opened along the greater curvature and rinsed with sterile tap water. Based on the method of Hessing (Hessing *et al*., 1992), mucosal lesions of the *Pars oesophagea* were scored as follows: score 0 for normal mucosa, score 1 for mild hyperkeratosis covering less than 50% of the surface, score 2 for severe hyperkeratosis covering more than 50% of the surface, score 3 for hyperkeratosis with few erosions, score 4 for hyperkeratosis with several erosions and score 5 for hyperkeratosis with many erosions or ulceration. Using autoclaved

tweezers and scalpels, biopsies of 40-50 mg consisting of mucosa and submucosa were taken from the *Pars oesophagea* as well as from the cardiac, fundic and pyloric gland zone for quantification of *H. suis* DNA by real time quantitative (RT)-PCR. In addition, biopsies consisting of mucosa and submucosa were taken from the fundic and pyloric gland zones to determine mRNA expression levels of genes encoding host factors (markers) involved in gastric acid secretion and inflammation. In order to correlate altered markers with gastritis and the number of parietal cells, D-cells and G-cells, biopsies consisting of mucosa, submucosa and tunica muscularis were taken from fundic and pyloric gland zones, fixed in 10% phosphate-buffered formalin and used for histopathology and immunohistochemistry.

H. suis *quantification*

DNA was extracted from the biopsies of each stomach region, using the Isolate II Genomic DNA Kit (Bioline, Taunton, USA), according to the instructions of the manufacturer. The presence of *H. suis* DNA was determined using a species-specific, RT-PCR based on the *ureA* gene (Blaecher *et al*., 2013). The copy number of the obtained amplicons was calculated and converted to the number of *H. suis* bacteria per mg gastric tissue, by including 10-fold dilutions of an external standard consisting of a 1,236 bp segment of the *ureAB* gene cluster from *H. suis* strain HS5 (O'Rourke *et al*., 2004).

Histopathology and immunohistochemistry

The biopsies were embedded in paraffin, sectioned at 5 μm, rehydrated, deparaffinized, stained with haematoxylin and eosin, dehydrated and finally mounted with a coverslip for light microscopic evaluation. The severity of gastritis was scored according to the Updated Sydney System with some modifications (Dixon *et al*., 1996; De Bruyne *et al*., 2012). Both diffuse infiltration with inflammatory cells and the presence of lymphoid aggregates and lymphoid follicles in the mucosa and submucosa were taken into consideration. The infiltration of mononuclear and polymorphonuclear cells was scored as follows: score 0 for absence of infiltration, score 1 for mild infiltration, score 2 for moderate infiltration and score 3 for marked infiltration. In addition, the formation of lymphoid follicle formation was scored as follows: score 0 for absence of lymphoid aggregates, score 1 for presence of a small number of lymphoid aggregates ($n < 5$), score 2 for a large number of lymphoid aggregates ($n \geq 5$)

and/or the presence of 1 organized lymphoid follicle and score 3 for the presence of at least 2 organized lymphoid follicles. Based on the scoring of the diffuse infiltration with inflammatory cells and the presence of lymphoid aggregates and lymphoid follicles, an overall gastritis score was obtained. Therefore, the average score was calculated for each *H. suis*-negative and -positive age group and this for the pyloric and fundic gland zone. When an overall score of $0 \leq n \leq 1$; $1 < n \leq 2$ or $2 < n \leq 3$ was obtained, the gastritis was considered as mild, moderate and severe, respectively.

To determine the number of parietal cells, D-cells and G-cells, 3 consecutive sections of 5 µm were cut from the paraffin embedded tissues. After rehydration and deparaffinization, 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) to block endogenous peroxidase activity and with 30% goat serum (30 min) to block non-specific reactions. Parietal cells were identified by immunohistochemical staining for the H^+/K^+ ATPase using a mouse monoclonal antibody (1/200; Abcam Ltd, Cambridge, United Kingdom) and a biotinylated goat anti-mouse IgG antibody (1/200; Agilent Technologies, Santa Clara, California, USA) (Zhang *et al*., 2016). D-cells and G-cells were identified by immunohistochemical staining using a rabbit polyclonal anti-somatostatin and anti-gastrin antibody, respectively (1/600; Agilent Technologies, Santa Clara, California, USA) and a biotinylated goat anti-rabbit IgG antibody (1/600; Agilent Technologies, Santa Clara, California, USA). After rinsing, the sections were incubated with a streptavidin-biotin-HRP complex (Agilent Technologies, Santa Clara, California, USA) (Sapierzynski *et al*., 2007). The color was developed with diaminobenzidine tetrahydrochloride (DAB) and H_2O_2. Finally, positive D-cells and G-cells were counted in five randomly chosen high power fields (magnification: × 400), both in the fundic and pyloric gland zone. The average number of positive cells per high power field was then calculated for each pig in both stomach regions. As a positive control for the parietal cell staining, the fundic gland zone of a non-*H. suis* infected pig was used, as this zone is known to contain large numbers of these cells (Sapierzynski *et al*., 2007). The pyloric gland zone of this pig was used as a positive control for D-cells and G-cells staining. This zone indeed contains large numbers of these cell types (Sapierzynski *et al*., 2007). Negative controls to confirm the specificity of the secondary antibodies were obtained by incubating the sections without the primary antibodies. In

addition, the cardiac gland zone was also used as a negative control, as this stomach region is known to contain only mucus and bicarbonate producing cells (Sapierzynski *et al*., 2007).

Expression analysis of markers for inflammation and gastric acid secretion

RNA was extracted from the gastric biopsies using the RNeasy Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. The obtained RNA concentrations were measured using a NanoDrop spectrophotometer (Isogen Life Science, Utrecht, The Netherlands), after which the concentration of all samples was adjusted to 1 µg/µl, followed by cDNA synthesis using the iScript™ cDNA Synthesis Kit (Bio-Rad, Hercules, California, USA). Expression of genes encoding host factors involved in gastric acid secretion (H^+/K^+ ATPase, Sonic Hedgehog, KCNQ1, gastrin, the cholinergic muscarinic M3 receptor, somatostatin, the histamine H2 receptor and the gastrin CCK-B receptor), mucosal integrity (claudin 18) and inflammation (IL-4, IL-8, IL-10, IL-17A, IL-1β, IFN-γ and CXCL13) was analyzed. HPRT, Cyc5 and ACTB have been shown to have a stable mRNA expression and were therefore included as reference genes (Zhang *et al*., 2016). All primer sequences are shown in the Additional file 1. The mRNA expression levels of the reference and target genes were quantified using RT-PCR, as described earlier (Flahou *et al*., 2012). No-template control reaction mixtures were included and all samples were run in duplicate. The threshold cycle (Ct)-values were first normalized to the geometric mean of the Ct-values of the reference genes. Fold changes were calculated using ΔΔCt method with the means of Ct-values from the *H. suis* negative pigs. Finally, for each target gene, the results were expressed as fold changes of *the* mRNA expression of *H. suis* positive pigs relative to mRNA expression levels of *H. suis* negative pigs and this for each age group separately.

Statistical analysis

Statistical analysis was performed using SPSS statistics 24 (IBM, New York, USA). Differences in severity of *Pars oesophageal* lesions, severity of gastritis, number of parietal cells, D-cells and G-cells and fold changes of the markers for gastric acid secretion and inflammation between the *H. suis*-negative and *H. suis*-positive groups were investigated using the non-parametric Kruskal-Wallis H test with Bonferroni correction. A p-value ≤ 0.05 was considered to be significant. Correlations between mucosal

lesions, severity of gastritis, number of parietal cells, D-cells and G-cells, fold changes and the number of *H. suis* bacteria were examined using the Pearson correlation coefficient. Differences were considered statistically significant at $p \leq 0.05$.

Results

H. suis *prevalence and association with mucosal lesions, gastritis and number of parietal cells, D-cells and G-cells*

Two-3 months old pigs

The prevalence of *H. suis* was 47% (16/34) and the average number of *H. suis* bacteria per mg tissue was higher in the pyloric gland zone than in the other stomach regions ($p < 0.001$) (Additional file 2). Almost all pigs had *H. suis* DNA in the fundic and pyloric gland zone (= 15/16). On gross examination, all pigs showed an intact mucosa or mild hyperkeratosis of the *Pars oesophagea* (Table 1), with moderate gastritis in the fundic and pyloric gland zone (Additional file 3). The scores for *Pars oesophageal* lesions and gastritis were not significantly different between the *H. suis*-negative and *H. suis*-positive pigs. Similarly, the number of G-cells and D-cells in the pyloric gland zone did not differ between the *H. suis*-negative and *H. suis*-positive group (Figures 1A and B). A small number of G-cells and D-cells was detected in the fundic gland zone of the pigs, varying from 0-3 per high power field and independent from the *H. suis* status (data not shown). In the fundic gland zone of both *H. suis*-infected and non-infected pigs, the number of parietal cells was high in each high power field (> 800/field), making the counting impossible. A small number of parietal cells was detected in the pyloric gland zone of the pigs, varying from 0-2 per high power field and independent from the *H. suis* status (data not shown). For the *H. suis*-positive pigs, statistical analysis did not reveal significant correlations between mucosal lesions, gastritis and the number of *H. suis* bacteria. Analysis of gene expression, histopathology and immunohistochemistry was done on samples from all pigs in this age category (see below).

Six-8 months old pigs

H. suis was detected in 55/68 (81%) of the investigated stomachs. The average number of *H. suis* bacteria per mg tissue was similar for the fundic and pyloric gland zone (Additional file 2). *H. suis* DNA was detected in the fundic and pyloric gland zone of all *H. suis*-infected pigs. Severe hyperkeratosis and erosions were only seen in the *H. suis*-positive group (Table 1). The number of D-cells did not differ between the *H. suis*-negative and *H. suis*-positive group, whereas the number of G-cells was decreased in the *H. suis*-positive group ($p = 0.054$) (Figures 1A and B). A small number of G-cells and D-cells was detected in the fundic gland zone of the pigs, varying from 0-3 per high power field and independent from the *H. suis* status (data not shown). In the fundic gland zone of both *H. suis*-infected and non-infected pigs, the number of parietal cells was high in each high power field (> 800/field), making the counting impossible. A small number of parietal cells was detected in the pyloric gland zone of the pigs, varying from 0-2 per high power field and independent from the *H. suis* status (data not shown). No significant correlations were detected between the number of *H. suis* bacteria and severity of gastritis. All *H. suis*-negative pigs ($n = 13$), 5 pigs with > 1000 *H. suis* bacteria/mg tissue in the fundic gland zone, 5 pigs with > 1000 *H. suis* bacteria/mg tissue in the pyloric gland zone and 5 pigs with < 100 *H. suis* bacteria/mg tissue in both the fundic and pyloric gland zone were selected for analysis of gene expression, histopathology and immunohistochemistry.

Adult sows

H. suis was detected in the stomach of 55/60 sows (92%). In contrast with the other age groups, the average number of *H. suis* bacteria per mg tissue was higher in the fundic gland zone than in the other stomach regions ($p < 0.01$) (Additional file 2). All *H. suis*-infected sows had *H. suis* DNA in the fundic and pyloric gland zone. Ulceration was mainly found in the *H. suis*-positive sows, although this was not significantly different from the *H. suis*-negative group (Table 1). No significant differences were detected in the severity of gastritis between the *H. suis*-negative and *H. suis*-positive pigs (Additional file 3). A significant positive correlation was found, however, between the number of *H. suis* bacteria per mg gastric tissue and lymphoid infiltration in the fundic gland zone ($p < 0.001$). The number of D-

cells did not differ between the *H. suis*-negative and *H. suis*-positive group, while the number of G-cells was increased in the *H. suis*-positive group ($p = 0.002$) (Figures 1A and B, Additional file 4). A small number of G-cells and D-cells was detected in the fundic gland zone of the pigs, varying from 0-3 per high power field and independent from the *H. suis* status (data not shown). In the fundic gland zone of both *H. suis*-infected and non-infected pigs, the number of parietal cells was high in each high power field (> 800/field), making the counting impossible. A small number of parietal cells was detected in the pyloric gland zone of the pigs, varying from 0-2 per high power field and independent from the *H. suis* status (data not shown). All *H. suis*-negative sows ($n = 5$) were selected for gene expression, histopathological and immunohistochemical analysis. From the *H. suis*-positive group, these analyses were performed on 20 stomachs, selected as follows: 5 sows with > 1000 *H. suis* bacteria/mg tissue in both the fundic and pyloric gland zone, 5 sows with > 1000 *H. suis* bacteria/mg tissue in the fundic gland zone, 5 sows with > 1000 *H. suis* bacteria/mg tissue in the pyloric gland zone and 5 sows with < 100 *H. suis* bacteria/mg tissue in both the fundic and pyloric gland zone.

Table 1: General overview of the score distribution of lesions (%) in the *Pars oesophagea* of pigs of different ages.

Age group	***Pars oesophagea* – lesion score**					
	0 (%)	**1 (%)**	**2 (%)**	**3 (%)**	**4 (%)**	**5 (%)**
2-3 months old (*n* = 34)	38	44	18	0	0	0
H. suis-positive (*n* = 16)	37	44	19	0	0	0
H. suis-negative (*n* = 18)	39	44	17	0	0	0
6-8 months old (*n* = 68)	2	27	50	13	3	5
H. suis-positive (*n* = 55)	0	13	61	17	4	6
H. suis-negative (*n* = 13)	8	92	0	0	0	0
Adult sows (*n* = 60)	0	5	20	15	10	50
H. suis-positive (*n* = 55)	0	5	20	13	9	53
H. suis-negative (*n* = 5)	0	0	20	40	20	20

Score 0 = normal mucosa, 1 = mild hyperkeratosis covering less than 50% of the surface, 2 = severe hyperkeratosis covering more than 50% of the surface, 3 = hyperkeratosis with few erosions, 4 = hyperkeratosis with several erosions, 5 = hyperkeratosis with many erosions or ulceration, *n* = total number of investigated pigs' stomachs per age group. The data are shown as the percentage of pigs showing a certain lesion score.

Comparison of the different age groups

The scores given for *Pars oesophageal* lesions were significantly different between each age group ($p < 0.001$), with more severe lesions in adult sows, followed by 6-8 months old pigs. In contrast, the scores for lymphoid infiltration and lymphoid follicle formation did not differ significantly between the age groups, nor did the number of parietal cells, G-cells and D-cells (Figures 1A and B, Additional file 3). Although the prevalence of *H. suis* progressively increased with age, the number of *H. suis* bacteria per mg tissue decreased with age, especially in the pyloric gland zone ($p < 0.05$; Figure 2). Significantly higher scores for lymphoid infiltration and lymphoid follicle formation were found in the pyloric gland zone compared to the fundic gland zone ($p < 0.005$), independent from the *H. suis* status, and this in all age groups.

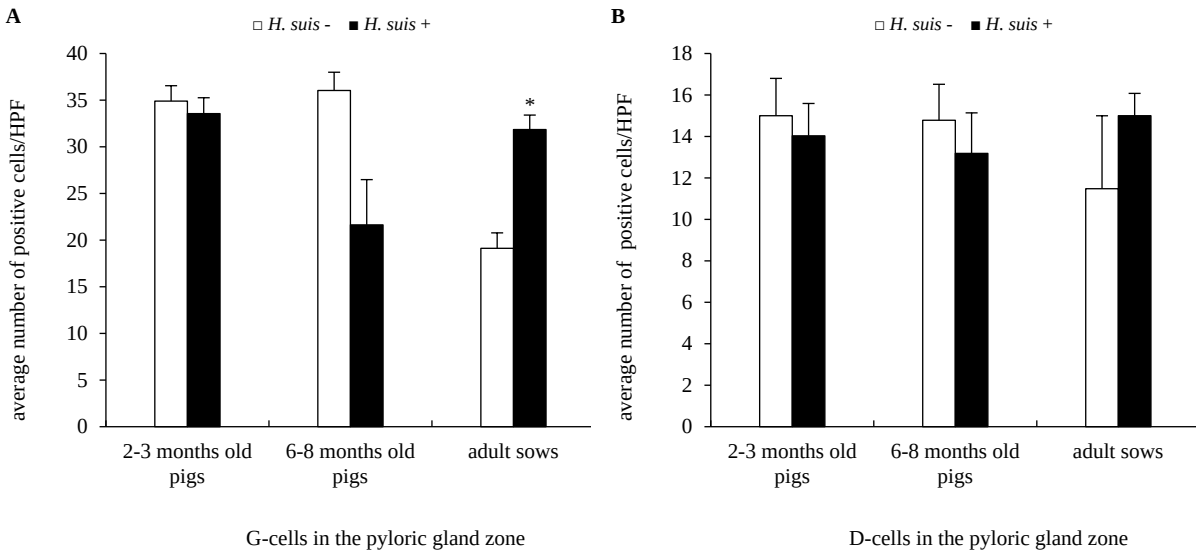

Figure 1: The number of G-cells (A) and D-cells (B) present in the pyloric gland zone of *H. suis*-negative (−) and *H. suis*-positive (+) pigs of different ages. Data are shown as the average number of G-positive and D-positive cells of each age group with standard deviation. Statistical differences were calculated using the non-parametric Kruskal–Wallis H test. * Significant differences between the *H. suis*-negative and *H. suis*-positive pigs ($p < 0.01$); HPF, high power field.

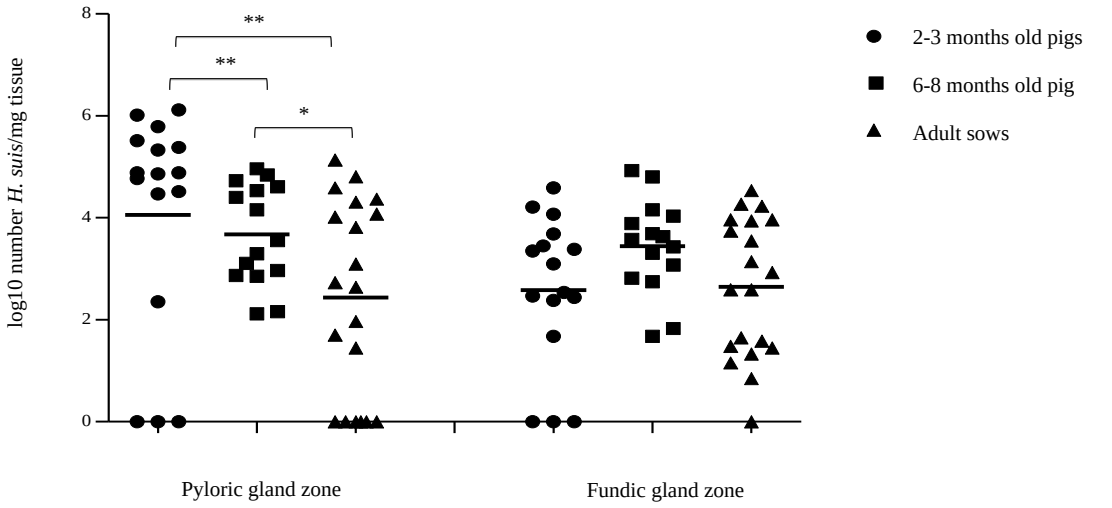

Figure 2: Comparison of the number of *H. suis* bacteria in the fundic and pyloric gland zone of pigs of different ages. Data are shown as log10 values of the number H. suis bacteria per mg tissue. Pigs in which no *H. suis* infection was detected, were set as 0. Individual pigs are shown as figures around the mean (lines). Statistical differences were calculated using the non-parametric Kruskal–Wallis H test. *$p < 0.05$; **$p < 0.001$ significant differences between the *H. suis*-positive age groups.

Gene expression analysis of markers for inflammation

Two-3 months old pigs

Compared to the non-infected group, the mRNA expression of CXCL13 was significantly upregulated in the fundic and pyloric gland zones of the *H. suis*-infected group ($p = 0.027$ and < 0.001, respectively), as well as the IL-8 and IL-1β transcript levels in the pyloric gland zone ($p = 0.001$ and 0.034, respectively). In contrast, IL-17A was significantly downregulated in the pyloric gland zone ($p = 0.039$) (Figures 3A and B, Additional file 5). Since significant correlations were found between both, the altered fold changes of IL-8, IL-17A, IL-1β and CXCL13 were more pronounced in pigs with a high number of *H. suis* bacteria per mg gastric tissue (Additional file 6).

Six-8 months old pigs

Upregulated expression of inflammatory cytokines was as described in the study of Bosschem *et al*. (Bosschem *et al*., 2017). In brief, compared to the non-infected group, in the *H. suis*-positive group, significant upregulations of IL-10 and CXCL13 were detected in the fundic gland zone ($p = 0.047$ and 0.011, respectively). The expressions of IL-4 in the fundic gland zone and that of IL-4, IL-17A and CXCL13 in the pyloric gland zone were also upregulated, although not significantly. A significant downregulation of IL-8 and IL-17A was detected in the fundic gland zone ($p = 0.040$ and 0.029, respectively), while IFN-γ was significantly downregulated in both fundic and pyloric gland zone ($p < 0.001$ and $= 0.005$, respectively) (Figures 3A and B, Additional file 5). Since significant correlations were found between both, the altered fold changes of IL-4, IL-8, IL-10, IL-17A, IFN-γ and CXCL13 were more pronounced in pigs with > 1000 *H. suis* bacteria/mg gastric tissue (Additional file 6).

Adult sows

Compared to the non-infected sows, in the *H. suis*-infected adult sows, the mRNA expression of IL-8 and IL-1β was significantly upregulated in the fundic gland zone ($p = 0.018$ for IL-8 and 0.037 for IL-1β), while IL-10 and IL-17A were upregulated in the pyloric gland zone of *H. suis*-infected sows

(p = 0.019 and 0.042, respectively). Although not significantly, increased IL-10 and IL-17A mRNA expression was also detected in the fundic gland zone and in the pyloric gland zone for IFN-γ and CXCL13 (Figures 3A and B, Additional file 5). Since significant correlations were found between both, the altered fold changes of IL-8, IL-10, IL-17A, IL-1β, IFN-γ and CXCL13 were more distinct in pigs with > 1000 *H. suis* bacteria/mg gastric tissue (Additional file 6).

Gene expression analysis of markers for gastric acid secretion

Two-3 months old pigs

Compared to the non-infected group, in the *H. suis*-infected 2-3 months old pigs, the majority of the markers for gastric acid secretion were not altered, except for a significant downregulated expression of the M3-receptor in the pyloric gland zone (p = 0.027). The expression of KCNQ1 was upregulated in the fundic gland zone, while somatostatin was downregulated in the pyloric gland zone, although not significantly (Figures 4A and B, Additional file 7). Since significant correlations were found between both, the altered fold changes of KCNQ1, M3-receptor and somatostatin were more pronounced in pigs with > 1000 *H. suis* bacteria/mg per mg gastric tissue (Additional file 8). In addition, since significant correlations were found between both markers, the altered fold change of somatostatin was more pronounced in pigs with a high expression of CXCL13 and IL-1β (Additional file 9).

Six-8 months old pigs

Compared to the non-infected group, in the *H. suis*-infected group claudin 18, gastrin, M3 receptor and CCK-B receptor were significantly downregulated in the fundic gland zone (p = 0.022, 0.040, 0.002 and 0.004, respectively), whereas Sonic Hedgehog and somatostatin mRNA levels were significantly upregulated in the pyloric gland zone (p = 0.048 and 0.007, respectively). The H^+/K^+ ATPase expression in the pyloric gland zone was upregulated as well, although not significantly. In the pyloric gland zone of 9 *H. suis*-infected pigs, the expression of gastrin was upregulated (Figures 4A and B, Additional file 7), while for 6 *H. suis*-infected pigs the expression was not altered. Since significant correlations were found between both, the altered fold changes of H^+/K^+ ATPase, Sonic Hedgehog,

claudin 18, gastrin, M3 receptor, somatostatin and CCK-B receptor were more pronounced in pigs with > 1000 *H. suis* bacteria/mg gastric tissue (Additional file 8). In addition, since significant correlations were found between both markers, the altered fold changes of claudin 18, M3 receptor, somatostatin and CCK-B receptor were more pronounced in pigs with lower expressions of IL-8, IL-17A and IFN-γ (Additional file 9).

Adult sows

Compared to the non-infected sows, most markers for gastric acid secretion were upregulated in the *H. suis*-infected sows. The expression of genes encoding H^+/K^+ ATPase, claudin 18, H2 receptor and CCK-B receptor were significantly upregulated in the fundic and pyloric gland zone of the *H. suis*-infected sows (p = 0.049, 0.002, 0.019, 0.049, 0.002, 0.012, 0.015 and 0.012, respectively). In addition, KCNQ1 and gastrin transcript levels were significantly upregulated in the fundic gland zone (p = 0.012 and < 0.001, respectively), whereas a significant downregulated mRNA expression of the M3 receptor in the pyloric gland zone was noticed (p = 0.049). An upregulated expression of Sonic Hedgehog was also detected in the fundic and pyloric gland zone, although not significant (Figures 4A and B, Additional file 7). Since significant correlations were found between both, the altered fold changes of genes encoding H^+/K^+ ATPase, Sonic Hedgehog, claudin 18, KCNQ1 and CCK-B receptor were more pronounced in pigs with > 1000 *H. suis* bacteria/mg gastric tissue (Additional file 8). In addition, since significant correlations were found between both markers, the altered fold changes of genes encoding H^+/K^+ ATPase, Sonic Hedgehog, claudin 18, gastrin and CCK-B receptor were more pronounced in pigs with high expressions of IL-10, IL-17A and IFN-γ (Additional file 9). Furthermore, since significant positive correlations were found between both, an increased G-cell number may have contributed to the increased expression of gastrin in both fundic and pyloric gland zone (r = 0.562, p = 0.003; r = 0.465, p = 0.022, respectively).

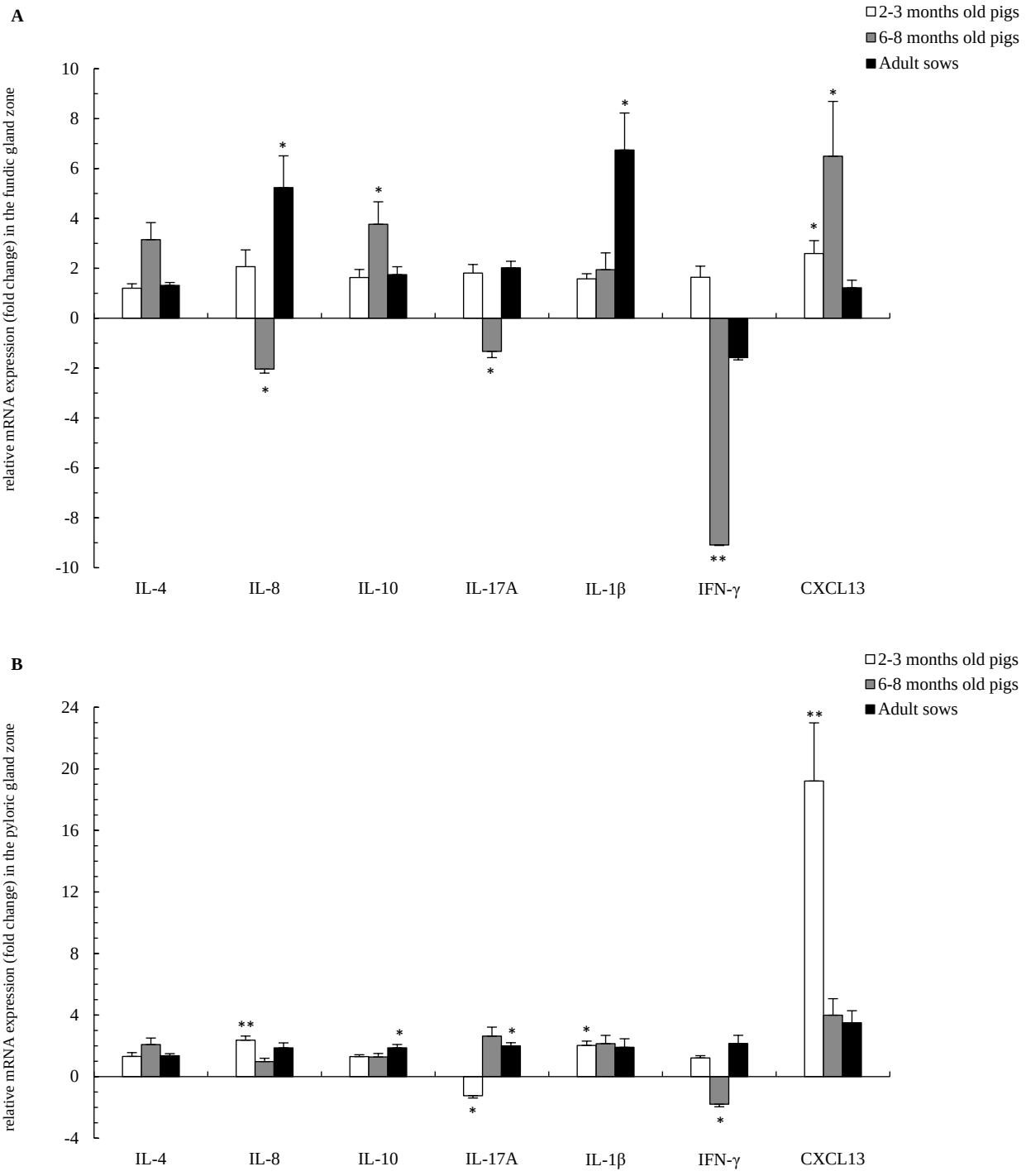

Figure 3: General overview of gene expression analysis of markers for inflammation in the fundic (A) and pyloric (B) gland zone of *H. suis*-infected pigs of different ages. The data are presented as fold changes in gene expression normalized to 3 reference genes and relative to a *H. suis*-negative control group which is considered as 1. The fold changes are shown as means with the standard error of the mean. Statistical differences were calculated using the non-parametric Kruskal–Wallis H test. *$p < 0.05$; **$p < 0.001$ significant differences between the *H.suis*-positive pigs and *H. suis*-negative pigs.

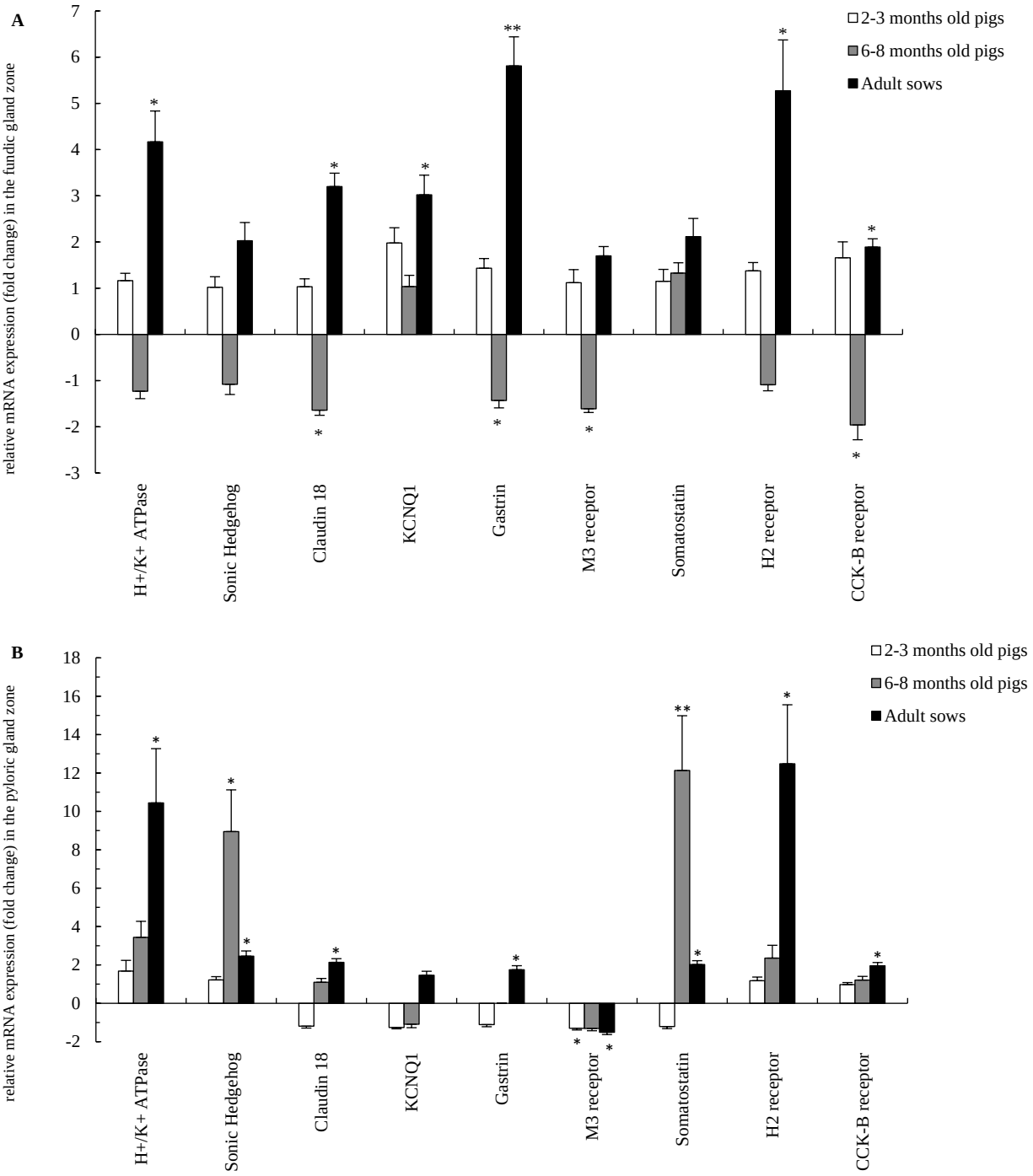

Figure 4: General overview of gene expression analysis of markers for gastric acid secretion in the fundic (A) and pyloric (B) gland zone of *H. suis*-infected pigs of different ages. The data are presented as fold changes in gene expression normalized to 3 reference genes and relative to a *H. suis*-negative control group which is considered as 1. The fold changes are shown as means with the standard error of the mean. The average fold change of gastrin in the pyloric gland zone of 6–8 months old pigs is not shown, since these values were too high (231.97 ± 64.63). Statistical differences were calculated using the non-parametric Kruskal–Wallis H test. *$p < 0.05$; **$p < 0.001$ significant differences between the *H. suis*-positive pigs and *H. suis*-negative pigs.

Discussion

In the present study, the prevalence of *H. suis* was 47% in 2-3 months old pigs and increased to 81% in pigs at slaughter age, which is in line with the results of previous studies (Roosendaal *et al*., 2000; Choi *et al*., 2001; Park *et al*., 2004).

The prevalence of *H. suis* was very high in adult animals, indicating that the host immune response is not able to clear the infection. In a recent study, Bosschem *et al*. (Bosschem *et al*., 2017) showed that *H. suis* induces a semimaturation of porcine monocyte-derived dendritic cells, characterized by increased expression of CD25, CD80/86 and CD40, but impaired expression of MHC class II molecules on the surface of these cells. It was suggested that this impaired dendritic cell response may elicit the expansion of Treg cells, which may help to establish a chronic infection as Treg cells are immune-suppressive and tolerogenic (Kao *et al*., 2010). Indeed, a tolerogenic immune response was indicated to be present in this study, since the Treg cell-associated cytokine IL-10 was upregulated in both *H. suis*-infected 6-8 months old pigs and adult sows. In addition, the downregulated expressions of IL-8, IL-17A and especially IFN-γ indicated presence of an immune-suppressive environment in the *H. suis*-infected 6-8 months old pigs, which may have contributed to the establishment of a chronic infection. In the *H. suis*-infected adult sows, however, the mRNA expression of the Th17 cell-associated IL-17A was upregulated. We also found a shift in colonization of *H. suis* from the pyloric gland zone during the more acute phase of the infection (2-3 months old pigs) to the fundic gland zone in the more chronic phase of the infection (adults sows) in combination with upregulated expressions of IL-8 and IL-1β. Taken together, these findings suggest that shortly after colonization, the immune response is suboptimal, contributing to the persistence of *H. suis* infection. Later a more pronounced immune response is seen, which may result in lower numbers of *H. suis* bacteria in that stomach region. Indeed, although the prevalence of *H. suis* was highest in adult sows, the average number of *H. suis* bacteria per mg tissue decreased with age, as was also observed in other studies (Flahou *et al*., 2010). The presence of a specific Treg/Th17 response should be confirmed in future studies, where the expansion of Treg and Th17 cells is directly assessed by the use of staining or flow cytometry.

Interestingly, the expression of CXCL13 was upregulated in *H. suis*-infected pigs of each age group. Since this chemokine attracts B-lymphocytes, its upregulation may be important for the development of a specific local immune response towards *H. suis,* but this requires further research (Bosschem *et al*., 2017). The upregulation of CXCL13 has also been demonstrated in *H. suis*-infected mice (Zhang *et al*., 2015) and has been linked with the development of mucosa associated lymphoid tissue (MALT)-lymphomas in *Helicobacter* sp. infected human patients (Zhang *et al*., 2015). MALT-lymphoma lesions were not detected in the present study and, as far as we know, have not been described in pigs.

In 2-3 months old pigs, the average number of *H. suis* bacteria was the highest in the pyloric gland zone, indicating that *H. suis* colonization starts in this stomach region, as already suggested by others (Hellemans *et al*., 2007a). In adult sows, the average number of *H. suis* bacteria was the highest in the fundic gland zone, indicating a shift in colonization to that region in animals infected during longer periods of time, which is similar to the findings of Hellemans *et al*. (Hellemans *et al*., 2007a). It appears that when *H. suis* colonizes the stomach epithelium, it triggers an innate immune response in that region, characterized by upregulated expression of the pro-inflammatory cytokines IL-8 and IL-1β in the pyloric gland zone of 2-3 months old pigs and in the fundic gland zone of adult sows.

Severe hyperkeratosis and erosions were only seen in the *H. suis*-infected 6-8 months old pigs and not in non-infected pigs of this age group. In adult sows, ulceration was also mainly found in the *H. suis*-positive animals, although this was not significantly different from the *H. suis*-negative group, which may be due to the low number of non-infected sows available. In this field study, interpretation of results is further complicated by variation between herds of other factors that may play a role in development of gastric pathologies such as diet, feeding strategy and management (Haesebrouck *et al*., 2009). Nevertheless, our findings provide further evidence that *H. suis* may be one of the factors playing a role in the pathogenesis of gastric ulceration in pigs. A similar conclusion was drawn from results of an experimental infection study in pigs (De Bruyne *et al*., 2012). Interestingly, severe lesions in the *Pars oesophagea* were more frequently found in adult sows compared to the other age groups, indicating that ulceration is a long-term process which may affect the majority of the adult pigs.

It is not yet clear how exactly *H. suis* might influence ulcer development in the *Pars oesophagea*, but alterations in gastric acid secretion might be involved. No clear effects on the markers for gastric acid secretion or number of parietal cells, D-cells and G-cells, and no lesions in the *Pars oesophagea* were detected in the *H. suis*-infected 2-3 months old pigs (acute phase of infection). In a later phase of infection (6-8 months *H. suis*-infected pigs), the markers for gastric acid secretion were downregulated, the gene encoding somatostatin was upregulated and the number of G-cells was decreased, indicating inhibition of gastric acid secretion. In this age group, lesions in the *Pars oesophagea* were present in several animals. The prevalence of severe lesions was extremely high in *H. suis*-infected adult sows (chronic phase of infection). Markers for gastric acid secretion were upregulated and the number of G-cells was increased in this age group, indicating increased gastric acid secretion. We hypothesize that decreased gastric acid secretion in the glandular part of the stomach may affect the composition of the *Pars oesophageal* microbiota which may affect development of lesions in this non-glandular part of the stomach. Indeed, higher numbers of a recently described *Fusobacterium* species, designated *F. gastrosuis,* were detected in the *Pars oesophagea* of *H. suis*-infected 6-8 months old pigs than in non-infected pigs of the same age group (**Experimental studies: chapter 2**). Increased production of gastric acid during the chronic phase of infection might further aggravate severity of lesions in this stomach region, which is not protected by mucus. Further studies in which for instance the gastric microbiota and pH are determined in *H. suis*-infected and non-infected pigs, are necessary to confirm or reject this hypothesis.

Several mechanisms might be involved in altered gastric acid secretion in *H. suis*-infected animals. A clear parietal cell loss, as described in *H. suis*-infected Mongolian gerbils and mice (Joosten *et al*., 2013; Zhang *et al*., 2016), was not seen in the *H. suis*-infected pigs, although small differences in the number of these cells between the infected and non-infected animals cannot be excluded since counting was impossible in the fundic gland zone. The expression of genes encoding H^+/K^+ ATPase was, however, altered. As this enzyme is typically associated with gastric acid production by parietal cells, this shows that the function of these cells was affected. This is also indicated by altered expression of H2-, M3- and CCK-B receptors, although these receptors are also found on enterochromaffin cells, which were

not studied here. The exact mechanism behind the effect of a *H. suis* infection on gastric acid secretion by parietal cells is not clear and requires further studies. Since *H. suis* is often found in close proximity to these host cells, a direct effect of this bacterium on the parietal cells might be involved. Indirect effects probably also play a role since the number of G-cells and/or the expression of gastrin was decreased or enhanced in pigs with downregulated and upregulated expression of H^+/K^+ ATPase, respectively. In *H. pylori* infections, increased gastric acid secretion has been associated with increased expression of genes encoding IL-8 and IL-1β (Haruma *et al*., 1995; Calam, 1996, 1999; Lehmann *et al*., 1996; Beales *et al*., 1997). Expression of genes encoding these cytokines was upregulated in the fundic gland zone of adult sows with upregulated expression of genes encoding H^+/K^+ ATPase. Literature dealing with the effect of IL-1β is, however, controversial as Beales and Calam (Beales and Calam, 1998) demonstrated that IL-1β inhibits acid secretion in cultured parietal cells.

In *H. suis*-infected 6-8 months old pigs and adult sows, expression of genes encoding Sonic Hedgehog was upregulated in the fundic and pyloric gland zone. Since Sonic Hedgehog is involved in the regeneration of damaged epithelium (Feng *et al*., 2012), this may indicate a compensation for epithelial loss induced by *H. suis* in these gastric regions. Disruption of the gastric epithelium, followed by regeneration was further suggested by the downregulated expression of genes encoding claudin 18, an important tight junction protein of the stomach (Caron *et al*., 2015), in *H. suis*-infected 6-8 months old pigs and its upregulation in adult sows. The increased IL-10 and IL-17A transcript levels in *H. suis*-infected adult sows may have promoted the regeneration of the gastric epithelium as well, as both cytokines are associated with intestinal barrier restoration (Lee, 2015).

To summarize, we revealed an increased prevalence of *H. suis* and a shift of colonization towards the fundic gland zone in adult sows, while the number of *H. suis* bacteria per mg tissue decreased with age. Gastric erosion and ulceration were more frequently detected in *H. suis*-infected pigs. During the more acute phase of the infection, an innate immune response was indicated to be present, followed by a Treg and Th17 response in pigs colonized during longer periods of time. While no clear alterations in the markers for gastric acid secretion were detected in 2-3 months old pigs, a decrease and increase were found in 6-8 months old pigs and adult sows, respectively. These results indicate that *H. suis* affects the

expression of markers for gastric acid secretion and inflammation and indicate that these effects differ, depending on the infection phase. An overview of the main results and conclusions of the present study is presented in Figure 5.

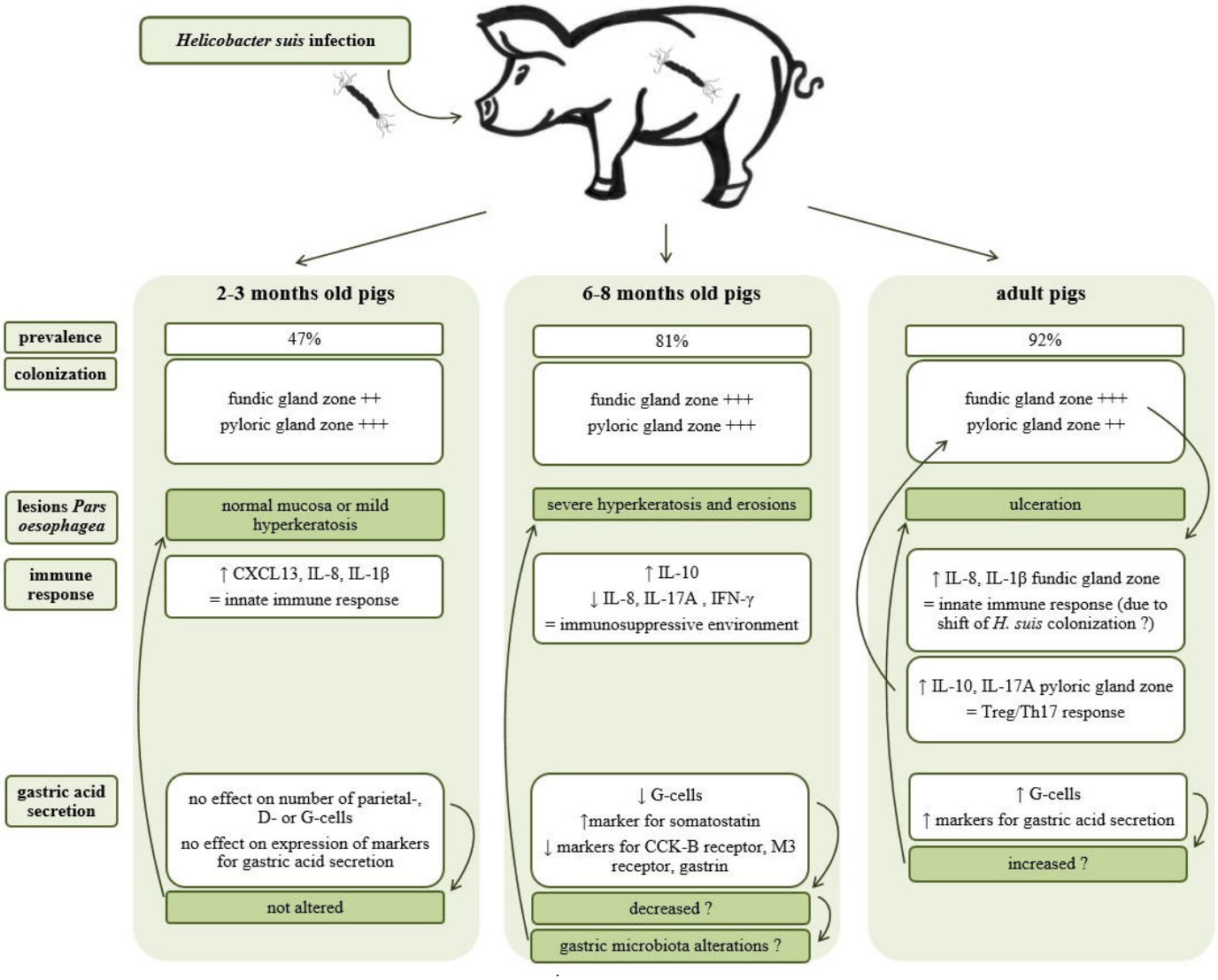

Figure 5: An overview of the main effects of a *H. suis* infection on gastric acid secretion and lesion development in pigs of different ages. *H. suis* affects the expression of markers for gastric acid secretion and inflammation and these effects may differ, depending on the infection phase.

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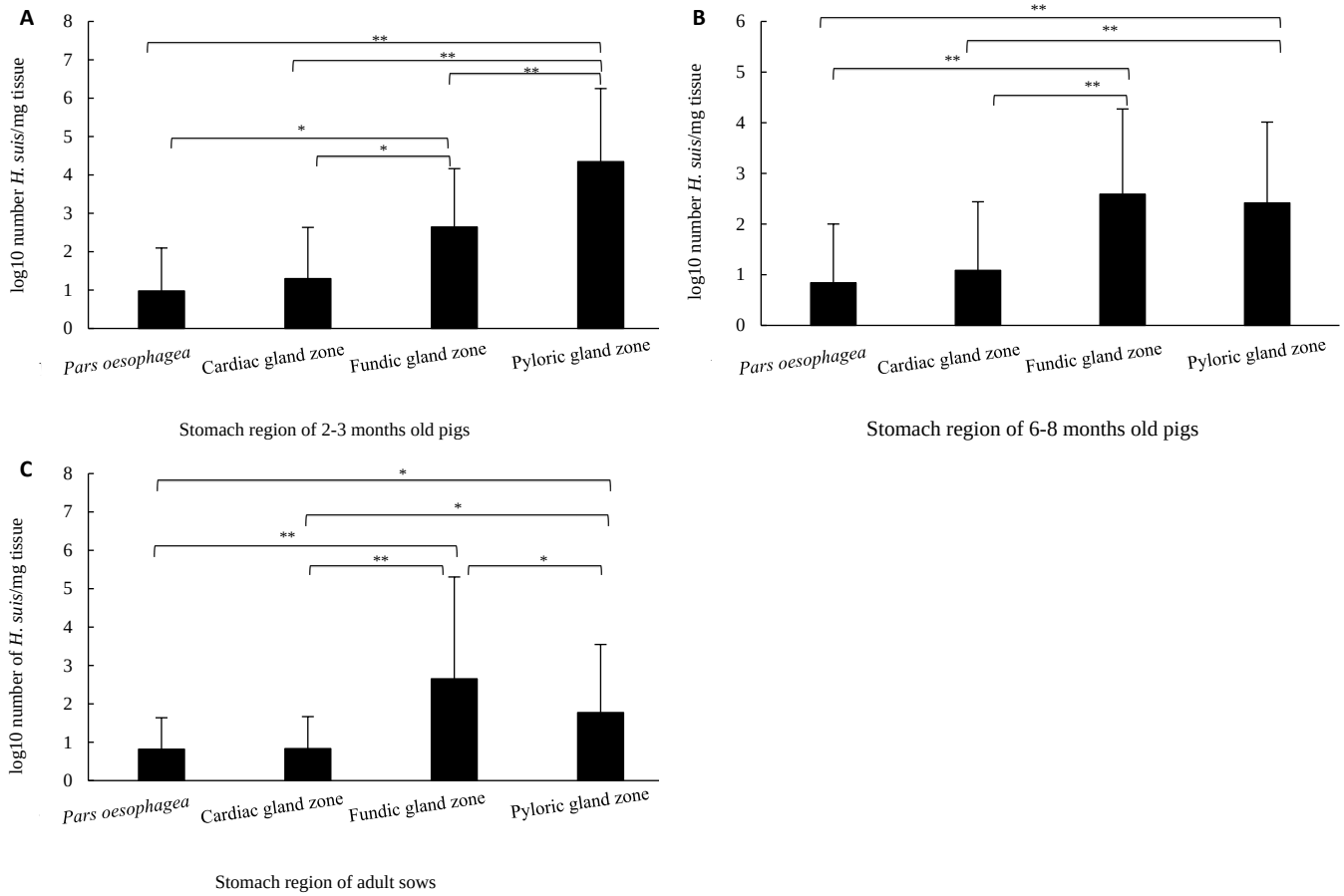
Additional file 1: List of primers used in quantitative RT-PCR for gene expression analysis of markers for gastric acid secretion and inflammation.

Primer	Sequence	Reference
HPRT-forward	5'-GTG ATA GAT CCA TTC CTA TGA CTG TAG A-3'	(Duvigneau *et al*., 2005)
HPRT-reverse	5'-TGA GAG ATC ATC TCC ACC AAT TAC TT-3'	(Duvigneau *et al*., 2005)
Cyc5-forward	5'-CCT GAA CAT ACG GGT CCT G-3'	(Lee *et al*., 2004)
Cyc5-reverse	5'-AAC TGG GAA CCG TTT GTG TTG-3'	(Lee *et al*., 2004)
ACTB-forward	5'-CTC TTC CAG CCC TCC TTC CT-3'	(Daudelin *et al*., 2011)
ACTB-reverse	5'-GCG TAG AGG TCC TTC TTC CTG ATG T-3'	(Daudelin *et al*., 2011)
H^+/K^+ ATPase -forward	5'-GCA TAT GAG AAG GCC GAG AG-3'	(Bosi *et al*., 2006)
H^+/K^+ ATPase-reverse	5'-TGG CCG TGA AGT AGT CAG TG-3'	(Bosi *et al*., 2006)
Sonic Hedgehog-forward	5'-TGA CCC CTT TAG CCT ACA AGC A-3'	(Zhang *et al*., 2016)
Sonic Hedgehog-reverse	5'-TGG GGG TGA GTT CCT TAA ATC G-3'	(Zhang *et al*., 2016)
Claudin 18-forward	5'-CAT GGG TGG GAT GGT GCA GA-3'	(Zhang *et al*., 2016)
Claudin 18-reverse	5'-CGG CAA GCG ATG CAC ATC AT-3'	(Zhang *et al*., 2016)
KCNQ1-forward	5'-CTC CGT GGT CTT CAT CCA C-3'	(Soma *et al*., 2011)
KCNQ1-reverse	5'-GTA GCT GCC GAA CTC CAC TT-3'	(Soma *et al*., 2011)
Gastrin-forward	5'-TCC TCA GCA CTG CGG CGG-3'	(Dall'Aglio *et al*., 2013)
Gastrin-reverse	5'-ATG GAG GAG GAA GAA GAA GC-3'	(Dall'Aglio *et al*., 2013)
M3 receptor-forward	5'-AAC AAT GAT GCT GCT GCC-3'	(Mansfield *et al*., 2005)
M3 receptor-reverse	5'-GTG ATC TGA CTT CTG GTC TTC-3'	(Mansfield *et al*., 2005)
Somatostatin-forward	5'-GTC CTG GCT **C**TG GGC GGT GTC A-3'	Adjusted from (Takaishi and Wang, 2007)
Somatostatin-reverse	5'-TGC AGC TCC AGC CTC AT**T** TC**A** T-3'	Adjusted from (Takaishi and Wang, 2007)
H2 receptor-forward	5'-CCA CCA TCA GGG AGC ACA A-3'	(Osawa *et al*., 2005)
H2 receptor-reverse	5'-AGG G**G**A ACC AGC AGA TGA TGA A-3'	Adjusted from (Osawa *et al*., 2005)
CCK-B receptor-forward	5'-CGC CAT CTG CCG ACC ACT GC-3'	(Zhang *et al*., 2016)
CCK-B receptor-reverse	5'-TTG GCT GTC GCT GTC ACT GT-3'	(Zhang *et al*., 2016)
IL-1β-forward	5'-GGC CGC CAA GAT ATA ACT GA-3'	(Collado-Romero *et al*., 2010)
IL-1β-reverse	5'-GGA CCT CTG GGT ATG GCT TTC-3'	(Collado-Romero *et al*., 2010)
IL-4-forward	5'-GAC ACA AGT GCG ACA TCA CC-3'	(Bosschem *et al*., 2016)
IL-4-reverse	5'-AGC TCC ATG CAC GAG TTC TT-3'	(Bosschem *et al*., 2016)
IL-8-forward	5'-TTC GAT GCC AGT GCA TAA ATA-3'	(Collado-Romero *et al*., 2010)
IL-8-reverse	5'-CTG TAC AAC CTT CTG CAC CCA-3'	(Collado-Romero *et al*., 2010)
IL-10-forward	5'-GAT ATC AAG GAG CAC GTG AAC TC-3'	(Daudelin *et al*., 2011)
IL-10-reverse	5'-GAG CTT GCT AAA GGC ACT CTT C-3'	(Daudelin *et al*., 2011)
IL-17A-forward	5'-CTC TCG TGA AGG CGG GAA TC-3'	(Jiang *et al*., 2016)
IL-17A-reverse	5'-GTA ATC TGA GGG CCG TCT GG-3'	(Jiang *et al*., 2016)
IFN-γ-forward	5'-AGG TTC CTA AAT GGT AGC TCT GGG-3'	(Daudelin *et al*., 2011)
IFN-γ-reverse	5'-AGT TCA CTG ATG GCT TTG CGC T-3'	(Daudelin *et al*., 2011)
CXCL13-forward	5'-GAT CTT TCC CAT CCA AGC AA-3'	(Bosschem *et al*., 2016)
CXCL13-reverse	5'-AAC GCA AAT GGT CAG TAG GG-3'	(Bosschem *et al*., 2016)

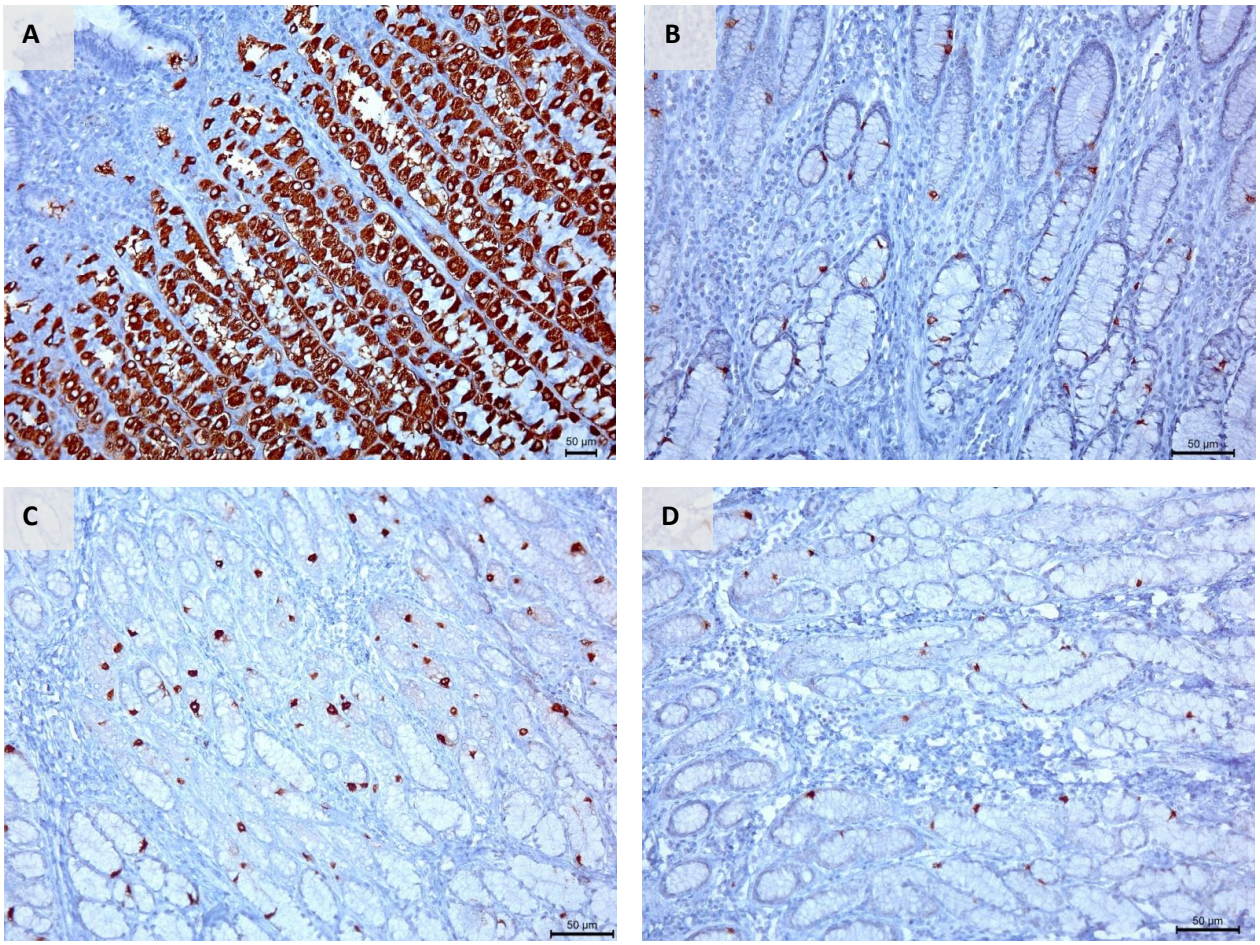


Additional file 2: The number of *H. suis* bacteria in the different stomach regions of 2-3 months old pigs (A), 6-8 months old pigs (B) and adult sows (C). Data are shown as log10 values of the average number of *H. suis* bacteria per mg tissue with standard deviation. Statistical differences were calculated using the non-parametric Kruskal-Wallis H test. *, $p < 0.01$; **, $p < 0.001$ significant differences between the stomach regions.

Additional file 3: General overview of the average scores of infiltration with inflammatory cells and lymphoid follicle formation in the fundic and pyloric gland zone of pigs of different ages

Age group	Inflammatory cell infiltration		Lymphoid follicles	
	Fundic gland zone	**Pyloric gland zone**	**Fundic gland zone**	**Pyloric gland zone**
2-3 months old (n=34)	1.6 ± 0.3	2.0 ± 0.7	1.0 ± 0.5	1.5 ± 0.4
H. suis-positive (n=16)	1.6 ± 0.3	2.0 ± 0.6	1.3 ± 0.4	1.7 ± 0.3
H. suis-negative (n=18)	1.5 ± 0.5	2.1 ± 0.4	1.0 ± 0.5	1.3 ± 0.4
6-8 months old (n=68)	1.8 ± 0.6	2.1 ± 0.7	1.0 ± 0.6	1.7 ± 1.0
H. suis-positive (n=55)	1.7 ± 0.6	2.1 ± 0.6	1.0 ± 0.6	1.8 ± 1.0
H. suis-negative (n=13)	2.1 ± 0.6	1.9 ± 0.8	1.2 ± 0.4	1.3 ± 0.9
Adult sows (n=60)	1.7 ± 0.6	2.1 ± 0.7	1.2 ± 0.7	1.6 ± 0.6
H. suis-positive (n=55)	1.7 ± 0.7	2.1 ± 0.7	1.3 ± 0.7	1.6 ± 0.6
H. suis-negative (n=5)	1.8 ± 0.4	2.4 ± 0.5	1.0 ± 0.7	1.2 ± 0.4

Gastritis was scored based on infiltration with inflammatory cells / lymphoid follicle formation, with score 0 = absence of infiltration / absence of lymphoid aggregates, 1 = mild infiltration / small number of lymphoid aggregates (n < 5), 2 = moderate infiltration / large number of lymphoid aggregates (n > 5) or presence of 1 organized lymphoid follicle, 3 = marked infiltration / at least 2 organized lymphoid follicles, n = total number of investigated pigs' stomachs per age group. The data are shown as the average of the administered scores with standard deviation.



Additional file 4: Microscopic visualization of the parietal cells (A), D-cells (B) and G-cells (C–D) in the porcine stomach using immunohistochemistry. (A) H^+/K^+ ATPase staining of the fundic gland zone of a *H. suis*-positive adult sow, showing parietal cells (brown). No clear parietal cell loss was detected. Original magnification ×100, scale bar: 50 µm. (B) Somatostatin staining of the pyloric gland zone of a *H. suis*-positive adult sow, showing D-cells (brown). Original magnification ×200, scale bar: 50 µm. (C) Gastrin staining of the pyloric gland zone of a *H. suis*-positive adult sow, showing G-cells (brown). Original magnification ×200, scale bar: 50 µm. (D) Gastrin staining of the pyloric gland zone of a *H. suis*-negative sow, showing G-cells (brown). Original magnification ×200, scale bar: 50 µm. The number of G-cells in the *H. suis*-negative sow (D) is lower than observed in the *H. suis*-positive sow (C).

Additional file 5: Overview of the relative fold changes of altered markers for inflammation in *H. suis*-infected pigs of different ages.

Age group	Gene	Relative fold change	*p*-value
2-3 months old pigs			
Fundic gland zone	CXCL13	2.59 ± 0.52	0.027
Pyloric gland zone	IL-8	2.37 ± 0.27	0.001
	IL-17A	0.81 ± 0.15	0.039
	IL-1β	2.03 ± 0.28	0.034
	CXCL13	19.21 ± 3.78	< 0.001
6-8 months old pigs			
Fundic gland zone	IL-4	3.15 ± 0.68	0.183
	IL-8	0.49 ± 0.16	0.040
	IL-10	3.77 ± 0.90	0.047
	IL-17A	0.75 ± 0.25	0.029
	IFN-γ	0.11 ± 0.02	< 0.001
	CXCL13	6.49 ± 2.20	0.011
Pyloric gland zone	IL-4	2.08 ± 0.42	0.095
	IL-17A	2.63 ± 0.58	0.128
	CXCL13	3.99 ± 0.17	0.196
	IFN-γ	0.56 ± 0.17	0.005
Adult sows			
Fundic gland zone	IL-8	5.23 ± 1.27	0.018
	IL-10	1.75 ± 0.32	0.243
	IL-17	2.02 ± 0.26	0.148
	IL-1β	6.74 ± 1.48	0.037
Pyloric gland zone	IL-10	1.87 ± 0.22	0.019
	IL-17A	2.00 ± 0.21	0.042
	IFN-γ	3.50 ± 0.78	0.371
	CXCL13	2.16 ± 0.53	0.169

The data are presented as fold changes in gene expression normalized to 3 reference genes and relative to the *H. suis*-negative control group which is considered as 1. The fold changes are shown as means with the standard error of the mean. Statistical differences were calculated using the non-parametric Kruskal-Wallis H test. A *p*-value lower than 0.05 is considered to be significant.

Additional file 6: Overview of important correlations between markers for inflammation and the number of *H. suis* bacteria in pigs of different ages.

	Fundic gene expression and the number of *H. suis* bacteria in the fundic gland zone		**Antral gene expression and the number of *H. suis* bacteria in the pyloric gland zone**	
Age group	**Gene**		**Gene**	
2-3 months old pigs	CXCL13	r = 0.307	IL-8	r = 0.514
		p = 0.023		p < 0.001
			IL-17A	r = -0.335
				p = 0.011
			IL-1β	r = 0.371
				p = 0.005
			CXCL13	r = 0.584
				p < 0.001
6-8 months old pigs	IL-4	r = 0.224	IL-4	r = 0.259
		p = 0.120		p = 0.08
	IL-8	r = -0.279	IL-17A	r = 0.197
		p = 0.059		p = 0.176
	IL-10	r = 0.288	IFN-γ	r = -0.339
		p = 0.046		p = 0.017
	IL-17A	r = -0.315	CXCL13	r = 0.293
		p = 0.027		p = 0.050
	IFN-γ	r = -0.465		
		p = 0.002		
	CXCL13	r = 0.419		
		p = 0.004		
Adult sows	IL-8	r = 0.371	IL-8	r = 0.320
		p = 0.013		p = 0.039
	IL-10	r = 0.192	IL-10	r = 0.255
		p = 0.187		p = 0.091
	IL-17A	r = 0.226	IL-17A	r = 0.277
		p = 0.120		p = 0.066
	IL-1β	r = 0.185	IFN-γ	r = 0.380
		p = 0.213		p = 0.012
			CXCL13	r = 0.255
				p = 0.091

r = Pearson correlation coefficient, calculated using SPSS Statistics 24. A r-value close to 1 indicates a strong, positive correlation, whereas a r-value of −1 indicates a strong, negative correlation. A p-value lower than 0.05 is considered to be significant.

Additional file 7: Overview of relative fold changes of altered markers for gastric acid secretion in *H. suis*-infected pigs of different ages.

Age group	Gene	Relative fold change	*p*-value
2-3 months old pigs			
Fundic gland zone	KCNQ1	1.98 ± 0.33	0.088
Pyloric gland zone	M3-receptor	0.77 ± 0.09	0.027
	Somatostatin	0.83 ± 0.11	0.091
6-8 months old pigs			
Fundic gland zone	Claudin 18	0.61 ± 0.11	0.022
	Gastrin	0.70 ± 0.16	0.040
	M3 receptor	0.62 ± 0.08	0.002
	CCK-B receptor	0.51 ± 0.32	0.004
Pyloric gland zone	H^+/K^+ ATPase	3.44 ± 0.83	0.106
	Sonic Hedgehog	8.95 ± 2.17	0.048
	Somatostatin	12.13 ± 2.85	0.007
Adult sows			
Fundic gland zone	H^+/K^+ ATPase	4.17 ± 0.67	0.049
	Sonic Hedgehog	2.03 ± 0.39	0.477
	Claudin 18	3.20 ± 0.29	0.002
	KCNQ1	3.02 ± 0.43	0.012
	Gastrin	5.81 ± 0.63	< 0.001
	Somatostatin	2.12 ± 0.39	0.447
	H2 receptor	5.27 ± 1.10	0.019
	CCK-B receptor	1.89 ± 0.18	0.049
Pyloric gland zone	H^+/K^+ ATPase	10.44 ± 2.83	0.002
	Sonic Hedgehog	2.46 ± 0.27	0.060
	Claudin 18	2.13 ± 0.19	0.012
	Gastrin	1.75 ± 0.21	0.068
	M3 receptor	0.66 ± 0.12	0.049
	Somatostatin	2.02 ± 0.20	0.071
	H2 receptor	12.48 ± 3.07	0.015
	CCK-B receptor	1.96 ± 0.17	0.012

The data are presented as fold changes in gene expression normalized to 3 reference genes and relative to the *H. suis*-negative control group which is considered as 1. The fold changes are shown as means with the standard error of the mean. Statistical differences were calculated using the non-parametric Kruskal-Wallis H test. A *p*-value lower than 0.05 is considered to be significant.

Additional file 8: Overview of important correlations between markers for gastric acid secretion and the number of *H. suis* bacteria in pigs of different ages.

	Gene expression and the number of *H. suis* bacteria in the fundic gland zone		**Gene expression and the number of *H. suis* bacteria in the pyloric gland zone**	
Age group	**Gene – stomach region**		**Gene – stomach region**	
2-3 months old pigs	KNCQ1 - fundic gland zone	r = 0.401	Somatostatin - pyloric gland zone	r = -0.287
		p = 0.002		p = 0.029
	Somatostatin - pyloric gland zone	r = -0.313	M3 receptor - pyloric gland zone	r = -0.173
		p = 0.018		p = 0.188
6-8 months old pigs	Claudin 18 – fundic gland zone	r = -0.327	Claudin 18 – fundic gland zone	r = -0.285
		p = 0.022		p = 0.045
	Gastrin – fundic gland zone	r = -0.279	Gastrin – fundic gland zone	r = -0.272
		p = 0.066		p = 0.074
	M3 receptor – fundic gland zone	r = -0.445	M3 receptor – fundic gland zone	r = -0.356
		p = 0.002		p = 0.012
	CCK-B - fundic gland zone	r = -0.469	CCK-B - fundic gland zone	r = -0.392
		p = 0.001		p = 0.006
	H^+/K^+ ATPase – pyloric gland zone	r = 0.293	H^+/K^+ ATPase – pyloric gland zone	r = 0.245
		p = 0.045		p = 0.098
	Gastrin – pyloric gland zone	r = 0.250	Gastrin – pyloric gland zone	r = 0.338
		p = 0.089		p = 0.022
	Sonic Hedgehog – pyloric gland zone	r = 0.366	Sonic Hedgehog – pyloric gland zone	r = 0.402
		p = 0.015		p = 0.007
	Somatostatin – pyloric gland zone	r = 0.323	Somatostatin – pyloric gland zone	r = 0.418
		p = 0.026		p = 0.004
Adult sows	Claudin 18 – fundic gland zone	r = 0.260	H^+/K^+ ATPase – fundic gland zone	r = 0.410
		p = 0.074		p = 0.007
	CCK-B receptor – pyloric gland zone	r = 0.263	KCNQ1 – fundic gland zone	r = 0.365
		p = 0.070		p = 0.015
			Sonic Hedgehog – fundic gland zone	r = 0.368
				p = 0.016
			CCK-B receptor – fundic gland zone	r = 0.259
				p = 0.086
			CCK-B receptor - pyloric gland zone	r = 0.269
				p = 0.074

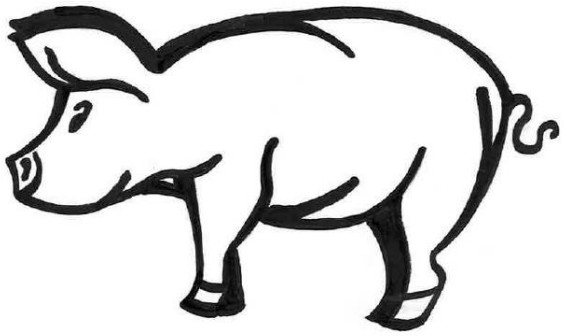
r = Pearson correlation coefficient, calculated using SPSS Statistics 24. A r-value close to 1 indicates a strong, positive correlation, whereas a r-value of -1 indicates a strong, negative correlation. P-values lower than 0.05 are considered to be significant.

Additional file 9: Correlations of altered markers for gastric acid secretion with the number of *H. suis* bacteria and with the altered markers for inflammation in H. suis-infected pigs of different age groups.

Age group	**Markers for gastric acid secretion**	**Correlation with the number of *H. suis* bacteria**		**Correlation with markers for inflammation**	
		Fundic gland zone	**Pyloric gland zone**	**Fundic gland zone**	**Pyloric gland zone**
2-3 months old					
Fundic gland zone	KCNQ1	$r = 0.401$; $p = 0.002$	/	/	/
Pyloric gland zone	M3 receptor	/	$r = -0.173$; $p = 0.188$	/	/
	Somatostatin	$r = -0.313$; $p = 0.018$	$r = -0.287$; $p = 0.029$	CXCL13 $r = -0.529$; $p = 0.042$	IL-1β $r = -0.443$; $p = 0.086$ CXCL13 $r = -0.617$; $p = 0.011$
6-8 months old					
Fundic gland zone	Claudin 18	$r = -0.327$; $p = 0.022$	$r = -0.285$; $p = 0.045$	IL-8 $r = 0.500$; $p = 0.058$ IFN-γ $r = 0.579$; $p = 0.024$	/
	Gastrin	$r = -0.279$; $p = 0.066$	$r = -0.272$; $p = 0.074$	/	/
	M3 receptor	$r = -0.445$; $p = 0.002$	$r = -0.356$; $p = 0.012$	/	/
	CCK-B receptor	$r = -0.469$; $p = 0.001$	$r = -0.392$; $p = 0.006$	IL-8 $r = 0.526$; $p = 0.044$ IL-17A $r = 0.754$; $p = 0.001$ IFN-γ $r = 0.523$; $p = 0.046$	/
Pyloric gland zone	H^+/K^+ ATPase	$r = 0.293$; $p = 0.045$	$r = 0.245$; $p = 0.098$	/	/
	Sonic Hedgehog	$r = 0.366$; $p = 0.015$	$r = 0.402$; $p = 0.007$	/	/
	Gastrin	$r = 0.250$; $p = 0.089$	$r = 0.338$; $p = 0.022$	/	/
	Somatostatin	$r = 0.323$; $p = 0.026$	$r = 0.418$; $p = 0.004$	IL-17A $r = -0.429$; $p = 0.098$	/

Age group	Markers for gastric acid secretion	Correlation with the number of *H. suis* bacteria		Correlation with markers for inflammation	
		Fundic gland zone	Pyloric gland zone	Fundic gland zone	Pyloric gland zone
Adult sows					
Fundic gland zone	H^+/K^+ ATPase	/	$r = 0.410$; $p = 0.007$	/	/
	Sonic Hedgehog	/	$r = 0.368$; $p = 0.016$	IL-17A $r = 0.575$; $p = 0.008$	IFN-γ $r = 0.512$; $p = 0.021$
	Claudin 18	$r = 0.260$; $p = 0.074$	/		IL-17A $r = 0.470$; $p = 0.037$ IFN-γ $r = 0.468$; $p = 0.037$
	KCNQ1	/	$r = 0.365$; $p = 0.015$	/	/
	Gastrin	/	/	/	/
	Somatostatin	/	/	/	/
	H2 receptor	/	/	/	/
	CCK-B receptor	/	$r = 0.259$; $p = 0.086$	/	/
Pyloric gland zone	H^+/K^+ ATPase	/	/	IL-10 $r = 0.554$; $p = 0.014$	/
	Sonic Hedgehog	/	/	/	IL-10 $r = 0.678$; $p = 0.001$
	Claudin 18	/	/	/	IL-10 $r = 0.502$; $p = 0.024$
	Gastrin	/	/	/	IL-10 $r = 0.426$; $p = 0.061$
	M3 receptor	/	/	/	/
	Somatostatin	/	/	/	/
	H2 receptor	/	/	/	/
	CCK-B receptor	$r = 0.263$; $p = 0.070$	$r = 0.269$; $p = 0.074$	/	IL-10 $r = 0.454$; $p = 0.045$

r = Pearson correlation coefficient, calculated using SPSS Statistics 24. A *r*-value close to 1 indicates a strong, positive correlation, whereas a *r*-value of −1 indicates a strong, negative correlation. *P*-values lower than 0.05 are considered to be significant. / = no clear correlation, yes = correlation with H. suis colonization rate (see additional files 6 and 8 for the *r*- and *p*-values)



Chapter 2: Detection, isolation and characterization of *Fusobacterium gastrosuis* sp. nov. colonizing the stomach of pigs

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Abstract

Nine strains of a novel *Fusobacterium* sp. were isolated from the stomach of 6–8 months old and adult pigs. The isolates were obligately anaerobic, although they endured 2h exposure to air. Phylogenetic analysis based on *16S rRNA* and *gyrase B* genes demonstrated that the isolates showed high sequence similarity with *Fusobacterium mortiferum, Fusobacterium ulcerans, Fusobacterium varium, Fusobacterium russii* and *Fusobacterium necrogenes,* but formed a distinct lineage in the genus *Fusobacterium*. Comparative analysis of the genome of the type strain of this novel *Fusobacterium* sp. confirmed that it is different from other recognized *Fusobacterium* spp. DNA-DNA hybridization, fingerprinting and genomic %GC determination further supported the conclusion that the isolates belong to a new, distinct species. The isolates were also distinguishable from these and other *Fusobacterium* spp. by phenotypical characterization. The strains produced indole and exhibited proline arylamidase and glutamic acid decarboxylase activity. They did not hydrolyse esculin, did not exhibit pyroglutamic acid arylamidase, valine arylamidase, α-galactosidase, β- galactosidase, β-galactosidase-6-phosphate or α-glucosidase activity nor produced acid from cellobiose, glucose, lactose, mannitol, mannose, maltose, raffinose, saccharose, salicin or trehalose. The major fatty acids were C16:0 and C18:1w9c. The name *Fusobacterium gastrosuis* sp. nov. is proposed for the novel isolates with the type strain CDW1(T) (=DSM 101753(T) = LMG 29236(T)). We also demonstrated that *Clostridium rectum* and *Fusobacterium mortiferum* represent the same species, with nomenclatural priority for the latter.

Key words: *Fusobacterium* – *Fusobacterium gastrosuis* - pig – stomach – *Clostridium rectum*

Introduction

Fusobacteria have been described as anaerobic, non-motile, non-sporulating, fastidious Gram-negative rods that produce butyric acid as major end product of their metabolism (Staley and Whitman, 2010; Markey, 2013). The genus currently consists of 15 recognized species (Citron, 2002; Conrads *et al*., 2004; Gharbia *et al*., 2012; Euzéby, 2017). Although Fusobacteria are normal constituents of the oropharyngeal, gastrointestinal and genital microbiota, they are the second most frequently isolated anaerobic microbial group from clinical samples of both human and animal origin, especially from cases of pyonecrotic infections (Rowland *et al*., 1987). Considering their fastidious nature, this reported detection frequency still may be an underestimation of the true frequency. In human patients, *Fusobacterium* spp. have been described to play a role in gingivitis and dental plaque formation (Bartlett and Finegold, 1972; Chow and Guze, 1974; George *et al*., 1981; Moore-Gillon *et al*., 1984; McGuire *et al*., 2014), whereas in pigs they are associated with lameness and facial skin necrosis (Hampson *et al*., 2010; Zhou *et al*., 2010; Cameron, 2012). In cattle and sheep they are involved in necrotic laryngitis and footrot (Langworth, 1977; Hampson *et al*., 2010; Praet *et al*., 2015). In horses they may be associated with necrotic oral and lower respiratory tract diseases as well as intra-abdominal abscesses (Hampson *et al*., 2010).

Ulceration of the non-glandular *Pars oesophagea* of the stomach is very common in pigs and can lead to discomfort, pain, decreased daily weight gain and even sudden death. *Helicobacter suis*, *Lactobacillus* sp. and *Bacillus* sp. have all been suggested to play a role in the development of gastric ulceration. Nevertheless, the exact etiology of this disease still is a matter of debate and is clearly multifactorial (Krakowka *et al*., 1998; Hellemans *et al*., 2007; Haesebrouck *et al*., 2009). Results of a recent metagenomic analysis of the gastric microbiota of 20 pigs of 6–8 months old showed that an unidentified *Fusobacterium* sp. was abundantly present, representing up to 20% of the gastric microbial community. Compared to *H. suis*-negative animals, higher numbers of this *Fusobacterium* sp. were detected in *H. suis*-infected animals (unpublished results).

The main aim of the present study was to isolate and characterize this putative new *Fusobacterium* sp. This is required to enable further research into its possible pathogenic significance and role in the pathogenesis of ulceration of the non-glandular part of the stomach in pigs. Therefore, *Fusobacterium* isolates obtained from porcine stomachs were characterized phenotypically and genotypically. The new *Fusobacterium* sp. showed the highest sequence similarity with *F. mortiferum, F. russii, F. ulcerans, F. varium, F. necrogenes* and, surprisingly, *Clostridium rectum*. Although clostridia are Gram-positive bacteria capable of producing endospores, in previous studies it was described that *C. rectum* is closely related to *Fusobacterium* spp. (Collins *et al*., 1994; Bolivar *et al*., 2012; Lee *et al*., 2016). The second aim of the present study was, therefore, to try and solve this inconsistency in the classification of *C. rectum*.

Material and methods

Isolation from porcine stomachs

Thirty five stomachs of 6–8 months old pigs and 25 stomachs of adult sows were collected over a period of 8 months from different slaughterhouses in Flanders, Belgium. The stomachs were transported immediately to the laboratory and stored at 4°C until further examination within 2h. The stomachs were opened along the greater curvature one at the time and rinsed with sterile tap water. Swabs were taken from each stomach region (*Pars oesophagea*; cardiac, fundic and pyloric gland zone), streaked on Columbia agar plates (Oxoid, Basingstoke, United Kingdom) supplemented with 5% defibrinated sheep blood (E&O laboratories, Bonnybridge, Scotland), 100 mg/l Neomycin, 5 mg/l Vancomycin and 1 mg/l Erythromycin (Sigma–Aldrich, Saint Louis, Missouri), and incubated anaerobically for 3 days at 37°C. Based on previous descriptions of the colony morphology of *Fusobacterium* spp. (Sutter and Finegold, 1971; Rowland *et al*., 1987; Brazier *et al*., 1991), colonies of interest were purified on Columbia agar plates supplemented with 5% defibrinated sheep blood and incubated anaerobically for 3 days at 37°C. A Gram-staining was performed on purified cultures. When this staining revealed Gram- negative rods, several colonies were suspended in 200 µl of an in-house bacterial preservation medium (Hellemans *et al*., 2007) for storage at −70°C. In order to identify the bacterium at species level, DNA was extracted

using PrepMan Ultra Sample Preparation Reagent (Life Technologies, Carlsbad, California) according to the manufacturer's instructions. The near complete *16S rRNA* gene was amplified with αβ-NOT and wMB primers (Baele *et al*., 2001) and sequenced by GATC Biotech, Supremerun sequencing (Constance, Germany). The obtained sequences were analyzed with Vector NTI (Life Technologies, Carlsbad, California). Finally, a comparison was made between the 16S rRNA sequences of the isolates and the previously detected *Fusobacterium* spp. (pig gastric microbiota metagenomic analysis, unpublished results). Colonies were considered as the putative new *Fusobacterium* sp. of interest when at least 99% identity was obtained.

Determination of species with high sequence similarities

A consensus sequence of the *16S rRNA* gene of the 9 isolates was obtained using the BioEdit Sequence Alignment Editor and ClustalIW Multiple Alignment tools (Ibis Biosciences, California, United States) in order to identify potential nucleotide differences between the isolates. The 16S rRNA sequences of the 9 isolates were blasted using EZ taxon database of EZBioCloud (ChunLab, Korea) and the species showing the highest sequence similarities were selected for further characterization.

The following type strains of *F. mortiferum, F. russii, F. ulcerans, F. varium, F. necrogenes* and *C. rectum* were obtained from the Culture Collection of University of Göteborg (CCUG) and Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSMZ) bacterial culture collection: CCUG 14475T, CCUG 45924, CCUG 50053T, CCUG 4858T, CCUG 4949T and DSM 1295T, respectively. In order to perform the characterization tests, the organisms were grown on Columbia agar plates supplemented with 5% defibrinated sheep blood for 3 days at 37°C.

Genotypic characterization

Both *16S rRNA* and *gyrase B (gyrB)* genes were selected for phylogenetic analysis. The *16S rRNA* gene of the 9 isolates and the species showing high sequence similarities, were amplified and sequenced as described above. The *gyrB* gene was amplified using UP-1 and UP-2r primers as described previously (Yamamoto and Harayama, 1995), except that 35 cycles were used with an annealing temperature of

57°C. A consensus sequence of the 9 isolates was also created for the *gyrB* gene, as described above for the *16S rRNA* gene, to determine the sequence similarity. The sequences of both genes were compared with those in the NCBI database using the BLAST search tool. The available *16S rRNA* and *gyrB* gene sequences of the type strains of all recognized *Fusobacterium* spp., were selected for phylogenetic analysis. A multiple alignment was performed using MUSCLE (EMBL-EBI, Cambridge, United Kingdom) with Gblocks as alignment curation. A phylogenetic tree was created using PhyML (ATGC, Montpellier, France) with the maximum likelihood method and a bootstrap value of 1000 to estimate the robustness of the topology of the tree. Finally, the 16S rRNA and gyrB trees were visualized using TreeDyn (GEMI Bioinformatics, Montpellier, France). Maximum parsimony and neighbour-joining algorithm based trees were compared with the maximum likelihood based tree in order to determine the closest phylogenetic neighbours and conserved roots in a reliable way. Finally, gyrB derived amino-acid trees were constructed and compared to the nucleotide based trees.

Isolate CDW1 was chosen as type strain. The genomic DNA G + C content of this strain and *F. mortiferum*, *F. necrogenes* and *C. rectum* were determined (Cleenwerck *et al*., 2010) and DNA–DNA hybridizations were performed (Ezaki *et al*., 1989). Repetitive sequence-based PCR fingerprinting with the (GTG)5 primer (Švec *et al*., 2010) was also performed to confirm the non-clonal nature of the 9 isolates.

In order to sequence the genome of isolate CDW1, it was cultivated on Columbia agar plates supplemented with 5% defibrinated sheep blood and incubated anaerobically for 3 days at 37°C. Subsequently, genomic DNA was extracted using the Gentra Puregene Yeast/Bact. Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. The genome was sequenced using SMRT Technology PacBio RS II (GATC Biotech, Constance, Germany), with an average genome coverage of 100 and a run mode of 240 min movie. Gene finding and automatic annotation were performed using the Rapid Annotation Subsystems Technology (RAST) server (Aziz *et al*., 2008; Overbeek *et al*., 2014). The available annotated draft and complete genomes of different *Fusobacterium* spp. were obtained from the NCBI database and selected for further phylogenetic comparison. After analyzing these annotated genome assemblies, pangenomes were created using the rapid large-scale prokaryote pan

genome analysis (Roary) tool (Page *et al*., 2015). Briefly, the annotated proteins from all isolates were used for a BLASTP all-versus-all sequence similarity search. From the BLASTP output, groups of orthologous proteins were predicted using the Orthagogue and MCL software (Ekseth *et al*., 2014). Orthologous groups with exactly one representative protein from each of the input strains were considered to be part of the *Fusobacterium* core genome. This obtained core genome alignment was then used for phylogenetic tree construction using PhyML (ATGC, Montpellier, France) with maximum likelihood method and a bootstrap value of 1000. Finally, the tree was visualized using the interactive tree of life (iTOL) tool (http://itol.embl.de/). Using the Genome-to-Genome Distance calculator (GGDC; http://ggdc.dsmz.de), whole-genome distances were determined in order to assess the degree of DNA-DNA hybridization between isolate CDW1 and other *Fusobacterium* spp. In addition, the average nucleotide identity (ANI) values were obtained using the online "average nucleotide identity calculator" tool (enveomics.ce.gatech.edu/ani/index). In order to assess genomic changes, for example due to recombination, the multiple genome alignment tool (Mauve) was used (The Darling lab, http://darlinglab.org/mauve/mauve.html). This tool identifies conserved regions internally free from genome rearrangements which are referred to as Locally Collinear Blocks (LCBs).

Phenotypic characterization

API 20A, Rapid ID 32A and API ZYM systems (Biomérieux, Marcy l'Etoile, France) were used to test substrate utilization and enzymes properties of the isolates and the species showing the highest sequence similarities (i.e. *F. mortiferum, F. russii, F. ulcerans, F. varium, C. rectum* and *F. necrogenes*). The instructions of the manufacturer were followed, with the exception that API ZYM was incubated anaerobically. Whole-cell fatty acid methyl esters (FAME) composition of the isolates and their phylogenetic neighbours were determined using an Agilent Technologies 6890N gas chromatograph (Agilent Technologies, Santa Clara, United States). Extraction and analysis of the FAME were performed according to the recommendations of the Microbial Identification System, Sherlock version 3.10 (MIDI, Delaware, United States). Peaks of the FAME profiles were identified using the TSBA50 identification library version 5.0 (MIDI, Delaware, United States). For the characterization tests, the

organisms were grown under identical conditions, i.e. on Columbia agar plates supplemented with 5% defibrinated sheep blood for 3 days at 37°C under anaerobic conditions.

GenBank/EMBL/DDBJ accession numbers

The GenBank/EMBL/DDBJ accession numbers of the *16S rRNA* gene sequences of *Fusobacterium gastrosuis* DSM 101753T (=LMG 29236T = CDW1T), *F. mortiferum* CCUG 14475T, *F. necrogenes* CCUG 4949T, *C. rectum* DSM 1295T, *F. varium* CCUG 4858T, *F. ulcerans* CCUG 50053T and *F. russii* CCUG 45924 are LN906797.1, LT574675.1, LT574677.1, LT574676.1, LT594100.1, LT594101.1 and LT594099.2 respectively. The gyrB sequences were deposited under the accession numbers LN906798.1, LT574675.1, LT574677.1, LT574676.1, LT594103.1, LT594104.1 and LT594102.1, respectively.

The GenBank/EMBL/DDBJ accession number of the complete and closed genome of *F. gastrosuis* sp. nov. DSM 101753T (=LMG 29236T = CDW1T) is LT607734.1.

Results and discussion

Isolation of 9 strains of the putative new Fusobacterium *sp.*

Nine isolates of the putative new *Fusobacterium* sp. were obtained: 6 from pigs at slaughter age (CDW1–6) and 3 from adult sows (CDW7–9). CDW1–CDW4 and CDW7–CDW9 originated from the non-glandular *Pars oesophagea* and CDW 5–6 from the cardiac and pyloric gland zone, respectively (Rowland *et al*., 1987). All isolates formed circular, white, slightly elevated colonies of approximately 0.4 cm in diameter surrounded by a very narrow zone of complete hemolysis, often only observed under the colony (Additional file 1). The isolates were obligately anaerobic, although they endured 2h of exposure to air. Gram-staining revealed 1.5–2 µm long and 0.3–0.5 µm wide Gram-negative rods with rounded ends, non-capsulated, with presence of swelling and globular forms and occasionally long filaments. The species showing high sequence similarity, namely *F. mortiferum, F. russii, F. ulcerans, F. varium, C. rectum* and *F. necrogenes*, also appeared as Gram-negative rods. In addition, *C. rectum* showed presence of a low number of endospores.

Genotypic characterization reveals a clear distinction between the novel Fusobacterium *sp. and other* Fusobacterium *spp.*

The near complete 16S rRNA consensus sequence was 1364 bp long and showed presence of only 2 wobbles, demonstrating that the isolates showed 99% similarity with each other. Based on the 16S rRNA sequences of the 9 isolates, *F. mortiferum, F. ulcerans, F. varium, F. russii, F. necrogenes* and *C. rectum* were identified as the species with the highest sequence similarities, showing average values of 96%, 96%, 96%, 96%, 95% and 95%, respectively. These low similarities already suggested that the isolated *Fusobacterium* sp. represented a new species.

Apart from the *16S rRNA* gene, the *gyrB* gene was selected for genotypic characterization as housekeeping genes often possess high discriminatory power for phylogenetic analysis (Cleenwerck *et al*., 2010). The sequence of this gene was available for the majority of *Fusobacterium* spp. in the NCBI database, in strong contrast with other housekeeping genes that were only available for a few species. Additionally, the *16S rRNA* gene already showed similarity values below 97% with other *Fusobacterium* spp. For those reasons 1 additional housekeeping gene was sequenced in order to confirm the results obtained by 16S rRNA sequencing. The gyrB consensus sequence was 1066 bp long with presence of 21 wobblers. The 9 isolates showed 98–99% similarity with each other and 84%, 75%, 75%, 74%, 74% and 72% similarity with *F. russii, F. mortiferum, C. rectum, F. necrogenes, F. varium* and *F. ulcerans*, respectively.

Phylogenetic trees based on *16S rRNA* and *gyrB* genes demonstrated that the isolates formed a distinct lineage in the genus *Fusobacterium* and this clustering was further supported by bootstrap values of 99–100% (Figs. 1 and 2). The different methods for construction of the trees resulted in similar clustering and positioning of roots, however, the neighbour-joining method yielded higher bootstrap values than the maximum likelihood. As indicated by the lower similarities, gyrB seemed to be more sensitive than 16S rRNA to differentiate the novel *Fusobacterium* sp. from other *Fusobacterium* spp. In contrast with the conserved *16S rRNA* gene, protein encoding genes, such as *gyrB*, evolve faster and are therefore more useful to discriminate among closely related species (Praet *et al*., 2015). The gyrB derived amino-

acid trees (Additional file 2) were comparable with the nucleotide based tree, although seemingly less reliable for further (sub)species identification. For example, *F. periodonticum*, *F. nucleatum* subspecies, *F. canifelinum* and *F. naviforme* clustered together as one group.

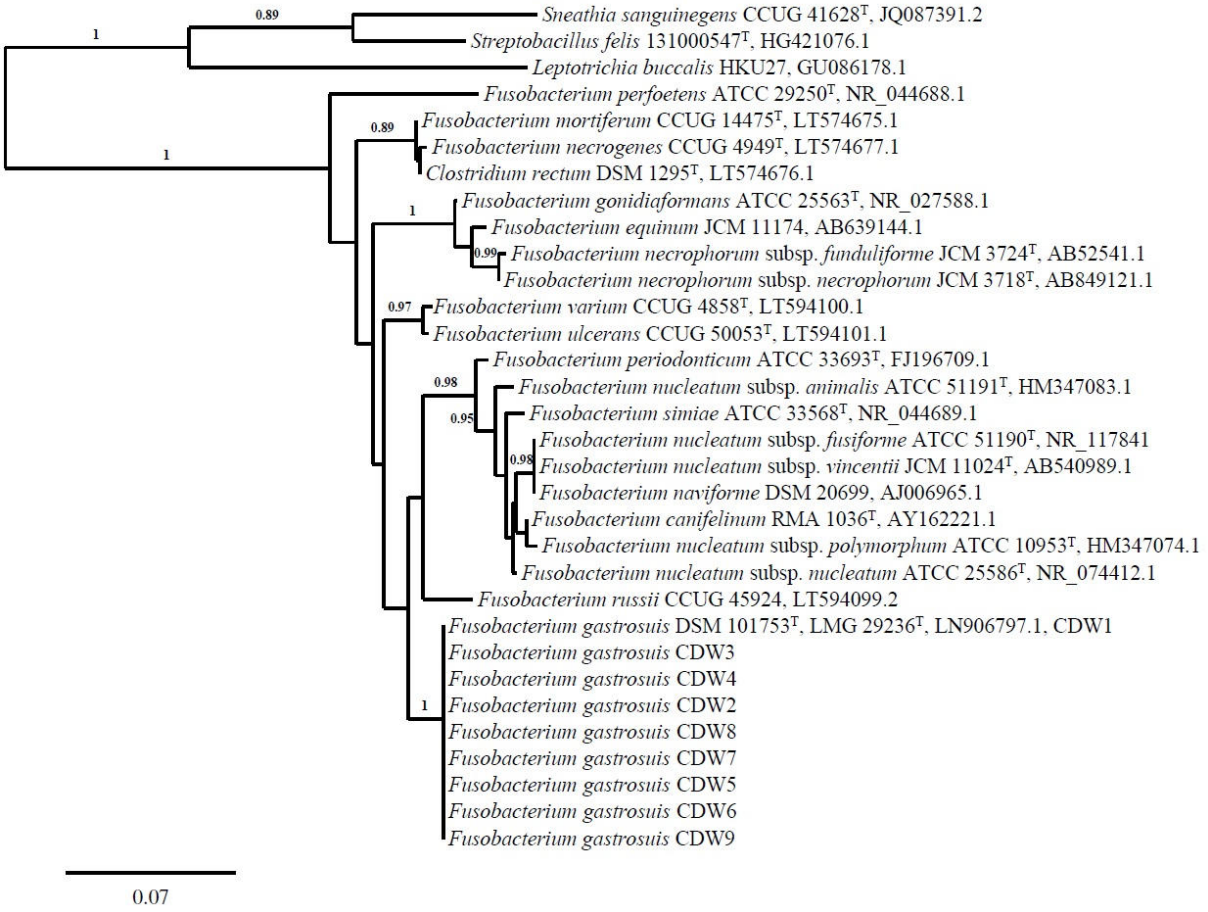

Figure 1: Phylogenetic tree based on 16S rRNA sequences and maximum likelihood method shows the genetic relationships between *Fusobacterium* spp. and the 9 isolates of *Fusobacterium gastrosuis* sp. nov. (CDW1–9). The scale-bar represents 7% differences in nucleotide sequences; bootstrap values (≥0.7) of 1000 replicates are displayed next to the corresponding branch and GenBank accession numbers are included. *Leptotrichia buccalis* HKU27, *Sneathia sanguinegens* CCUG 41628T and *Streptobacillus felis* 131000547T were used as outgroups. All present nodes were coincident in the tree generated with the neighbor algorithm.

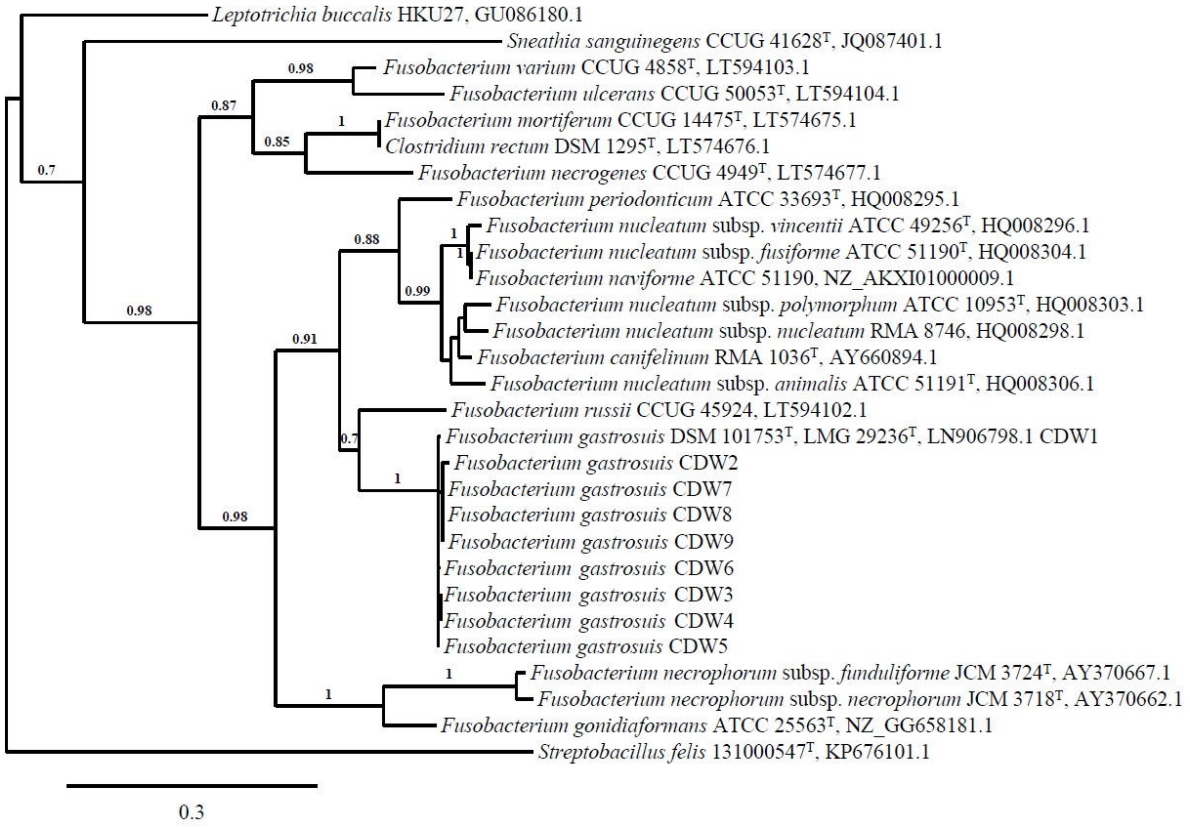

Figure 2: Phylogenetic tree based on gyrB sequences and maximum likelihood method shows the genetic relationships between *Fusobacterium* spp. and the 9 isolates of *Fusobacterium gastrosuis* sp. nov. (CDW1-9). The scale-bar represents 30% differences in nucleotide sequences; bootstrap values (≥0.7) of 1000 replicates are displayed next to the corresponding branch and GenBank accession numbers are included. *Leptotrichia buccalis* HKU27, *Sneathia sanguinegens* CCUG 41628T and *Streptobacillus felis* 131000547T were used as outgroups. All present nodes were coincident in the tree generated with the neighbor algorithm.

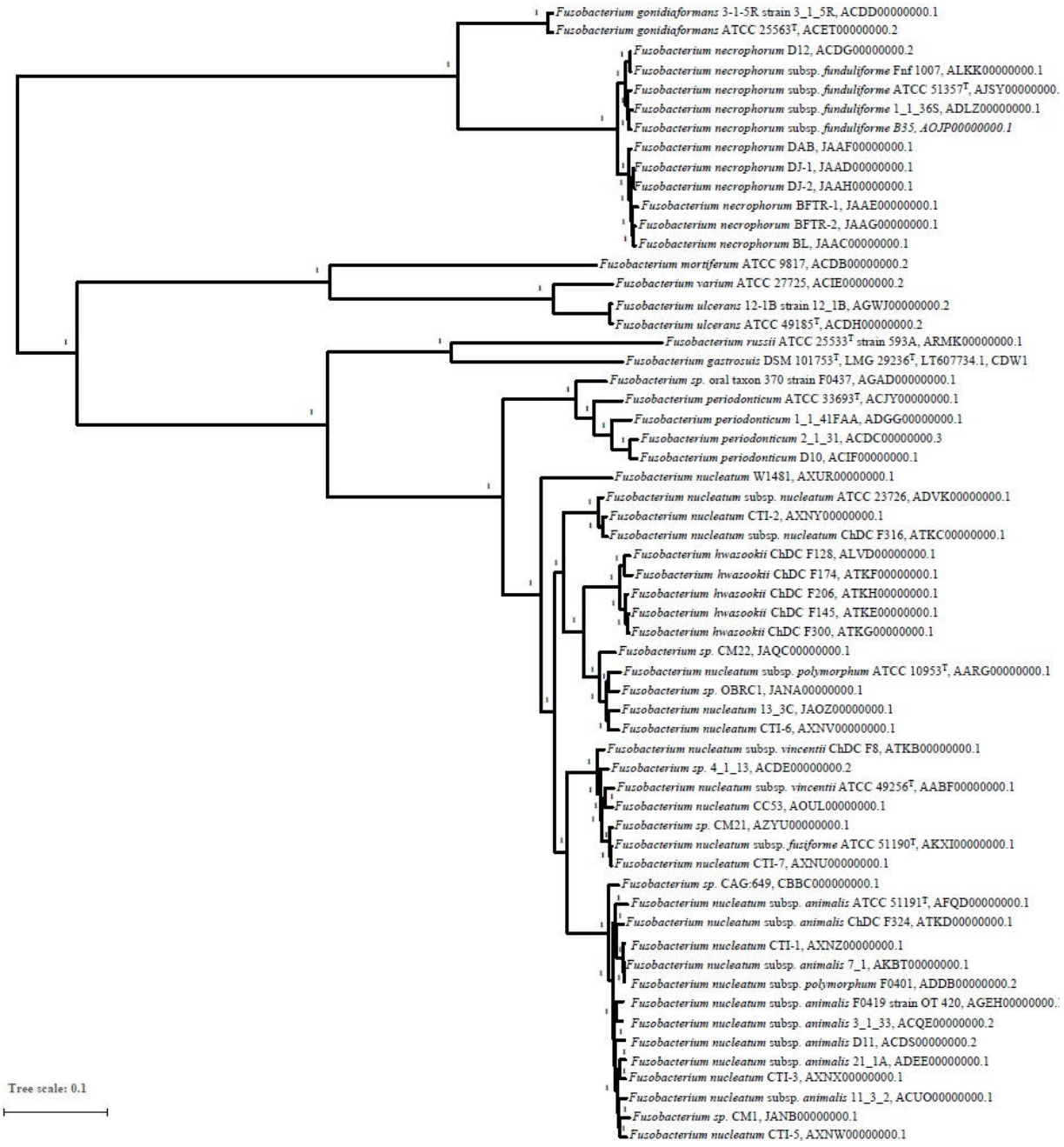

Figure 3: Phylogenetic tree based maximum likelihood method and on 486 aligned and concatenated core genes of *Fusobacterium* spp. shows the genetic relationships between *Fusobacterium* spp. and the type strain of *Fusobacterium gastrosuis* sp. nov. (CDW1). The scale-bar represents 10% differences in nucleotide sequences; bootstrap values (≥0.7) of 1000 replicates are displayed next to the corresponding branch and GenBank accession numbers are included.

Isolate CDW1 was chosen as type strain since this was the first isolated strain and all isolates showed similar genotypic and phenotypic properties. *F. mortiferum* was selected for genomic G + C content determination, since this species showed the highest sequence similarity with the 16S rRNA sequences of the isolates. Since other studies had shown that *C. rectum* clustered together with *F. necrogenes* (Collins *et al*., 1994; Bolivar *et al*., 2012; Lee *et al*., 2016), these 2 species were also selected for further genotypic characterization. The genomic G + C contents were 28.5, 29.9, 29.7 and 29.7 mol% for the novel *Fusobacterium* sp., *F. necrogenes* CCUG 4949T, *F. mortiferum* CCUG 14475T and *C. rectum* DSM 1295T, respectively. The 16S rRNA and gyrB sequences and GC% were identical for *F. mortiferum* CCUG 14475T and *C. rectum* DSM 1295T, strongly suggesting that these presented the same species. The repetitive sequence-based PCR fingerprinting with the (GTG)5 primer showed a banding pattern for 7 of the 9 isolates (CDW 1, CDW 3–5, CDW 7–9), *F. mortiferum, F. necrogenes* and *C. rectum*. It was not possible to obtain a banding pattern for strains CDW 2 and 6 using the (GTG)5 primer or other primers. Analysis confirmed that the 7 isolates were different from each other, since no identical pattern was obtained (Additional file 3). This was not surprisingly as all isolates were obtained from different pigs that were sampled on different days from 2 distinct slaughterhouses. Although no banding pattern was obtained for strain CDW 2 and 6, they were most likely also non-clonal strains since they were obtained from different stomachs and since the gyrB sequence similarity between the 9 isolates was not 100%. The level of DNA–DNA hybridization between *F. mortiferum* CCUG 14475T and *C. rectum* DSM 1295T was 95.5% (reciprocal hybridization values of 95 and 96%) and DNA fingerprinting showed an identical pattern, demonstrating that these are the same species.

To further identify these isolates as a distinct species, whole genome sequencing was performed. The complete and closed genome of the type strain of the new *Fusobacterium* sp. was 1.82 Mb large, with following characteristics: 28.2% GC, 1771 coding sequences, 255 subsystems, 359 hypothetical proteins and 64 RNAs. The phylogenetic tree based on 486 core genes confirmed that the novel *Fusobacterium* sp. formed a distinct lineage in the *Fusobacterium* genus, supported by bootstrap values of 100% (Figure 3). DNA–DNA hybridization parameters, assessed by calculating whole-genome distances, varied between 12 and 40%, which is lower than the threshold of 70% for belonging to the same species (Moore

et al., 1987). In addition, using logistic regression this resulted in a probability of 0.0–2.86% that the putative new *Fusobacterium* sp. belonged to a recognized *Fusobacterium* species or subspecies. The ANI values between the new *Fusobacterium* sp. and other *Fusobacterium* spp. varied between 75 and 80%, which is below the generally accepted threshold of 95% for belonging to the same species (Konstantinidis and Tiedje, 2005). Finally, when aligning the genome of the new *Fusobacterium* sp. with other *Fusobacterium* spp., LCBs values varied between 120 and 250. When comparing isolate CDW1 with its closest phylogenetic neighbour, *F. russii*, it was clear that the genomes were sufficiently different, showing a LCB value of 143 (Additional file 4). As genome sequences are not yet available for some *Fusobacterium* spp., the results of this genomic analysis should be considered as incomplete. However, they provide further evidence that the isolated *Fusobacterium* sp. belongs to a new species.

Phenotypic characterization confirms the distinction of the novel Fusobacterium *sp.*

Substrate utilization and enzyme properties were identical for all 9 isolates. These isolates and *F. mortiferum, F. russii, F. varium, F. ulcerans, C.* rectum *and F. necrogenes* did not exhibit urease, catalase, nitrate reduction, gelatin hydrolysis, α-fucosidase, β-glucosidase, β-glucuronidase, α-mannosidase, lipase (C14) or trypsin activity.

Activity of acid phosphatase, alkaline phosphatase and esterase (C4, although weakly) was detected. They did not produce acid from arabinose, melezitose, rhamnose or sorbitol. Differences in properties between the isolates and *F. mortiferum, F. russii, F. ulcerans, F. varium, C. rectum* and *F. necrogenes* are presented in Table 1. The cellular fatty acid profiles are presented in Table 2.

Table 1: Phenotypic features for distinguishing *Fusobacterium gastrosuis* sp. nov. (9 isolates) from other *Fusobacterium* spp. with high sequence similarity. All data were obtained in this study. The organisms were grown under identical conditions, i.e. on Columbia agar plates supplemented with 5% defibrinated sheep blood for 3 days at 37°C.

Test	***F.mortiferum***	***C.rectum***	***F.necrogenes***	***F.russii***	***F.ulcerans***	***F.varium***	***F.gastrosuis* sp. nov.**
Indole production	-	-	-	-	-	+	+
Esculin hydrolysis	+	+	+	+	+	+	-
Cystine arylamidase	-	W	-	-	-	W	$-^{W}$
Histidine arylamidase	-	-	-	+	+	+	V
Proline arylamidase	-	-	-	-	-	-	+
Pyroglumtaic acid arylamidase	+	+	-	+	+	+	-
Alanine arylamidase	-	-	-	-	+	+	-
Arginine arylamidase	-	-	-	W	+	+	-
Glutamyl glutamic acid arylamidase	-	-	-	+	-	+	-
Glycine arylamidase	-	-	-	-	-	W	-
Leucine arylamidase	-	-	-	W	+	+	-
Leucyl glycine arylamidase	-	-	-	-	-	W	-
Phenylalanine arylamidase	-	-	-	W	-	+	-
Serine arylamidase	-	-	-	-	+	+	-
Tyrosine arylamidase	-	-	-	-	W	+	-
Valine arylamidase	-	-	-	-	-	-	$-^{W}$
α-galactosidase	W	W	W	-	-	-	-
β-galactosidase	+	+	W	-	-	-	-
β-galactosidase 6 Phosphate	+	+	+	-	-	-	-
α-glucosidase	-	-	W	-	-	-	-
N-acetyl-β-glucosaminidase	-	-	-	-	-	-	$-^{W}$
Cellobiose acidification	+	-	-	-	-	-	-
Glucose acidification	+	+	+	+	+	+	-
Glycerol acidification	-	-	-	W	-	-	-
Lactose acidification	+	+	W	-	-	-	-
Mannitol acidification	-	-	W	-	-	-	-
Mannose acidification	+	+	W	-	+	+	-
Maltose acidification	+	+	-	+	+	-	-
Raffinose acidification	+	+	-	-	-	-	-
Saccharose acidification	+	+	-	-	-	-	-
Salicin acidification	+	+	W	-	-	-	-
Trehalose acidification	W	W	W	-	-	-	-
Xylose acidification	-	-	-	-	W	W	-
Glutamic acid decarboxylase	-	-	-	+	+	+	+
Naphtol-AS-BI-phosphohydrolase	+	+	W	-	-	-	W
Esterase lipase (C8)	-	-	-	-	-	-	W
α-chymotrypsin	-	-	-	-	-	-	V

+ = positive, - = negative, W = weakly positive, $-^{W}$ = most strains are negative and some weakly positive, V = some strains are positive and others negative.

Table 2: Cellular fatty acid profiles, expressed as percentage of the total cellular fatty acids, of *Fusobacterium gastrosuis* sp. nov. and the species with high sequence similarity. All data were obtained in this study. The organisms were grown under identical conditions, i.e. on Columbia agar plates supplemented with 5% defibrinated sheep blood for 3 days at 37°C.

Cellular fatty acid	***F.mortiferum***	***C.rectum***	***F.necrogenes***	***F.russii***	***F.ulcerans***	***F.varium***	***F.gastrosuis* sp. nov.**
Saturated							
C12 : 0	ND	T	1.6	7.6	T	T	1.5
C14 : 0	13.7	12.5	17.2	11.5	17.1	13.5	10.1
C16 : 0	27.9	22.0	13.5	15.2	ND	ND	22.0
C17 : 0	ND	T	ND	ND	ND	3.5	T
C18 : 0	5.0	6.6	6.4	1.8	1.4	ND	5.3
Unsaturated							
C13 : 1 AT 12-13	ND	0.6	1.5	ND	1.5	2.9	ND
C16 : 1ω9c	1.8	1.7	ND	3.2	2.8	2.1	6.2
C16 : 1ω5c	T	T	ND	ND	ND	ND	1.2
C17 : 1ω8c	ND	T	ND	ND	T	ND	ND
C18 : 1ω9c	13.5	15.1	11.5	9.3	7.2	1.5	12.0
C18 : 1ω7c	4.1	2.8	1.1	ND	1.0	ND	2.1
C18 : 1ω6c	ND	1.0	ND	ND	ND	ND	1.0
Hydroxyl							
C12 : 0 3-OH	1.0	T	ND	1.1	T	1.4	ND
C15 : 0 3-OH	ND	ND	ND	ND	ND	ND	T
C16 : 0 3-OH	ND	T	1.4	T	T	ND	1.2
Summed features							
Summed feature 1	T	1.5	5.5	ND	4.7	5.1	ND
Summed feature 2	14.6	15.9	22.4	40.5	27.1	52.0	18.9
Summed feature 3	12.2	10.2	8.4	7.0	13.0	9.7	14.5
Summed feature 4	2.5	3.1	5.9	ND	7.1	3.3	ND
Summed feature 5	2.0	1.5	1.9	1.5	T	ND	1.7

ND = not detected. T = values below 1% have no taxonomic evidence and were considered as traces. Summed features = groups of fatty acids that cannot be separated by the MIDI System: summed feature 1 = C13 : 0 3-OH, iso-C15 : 1 H, iso-C15 : 1 I; summed feature 2 = C14 : 0 3-OH, iso-C16 : 1 I; summed feature 3 = C16 : 1ω7c, iso-C15 : 0 2-OH; summed feature 4 = iso-C17 : 1 I, anteiso-C17 : 1 B; summed feature 5 = C18 : 2ω6,9c, anteiso-C18 : 0.

Reclassification of C. rectum

Phylogenetic analysis and phenotypical characterization revealed virtually no differences between *C. rectum* DSM 1295T and *F. mortiferum* CCUG 14475T. Gram-staining revealed the presence of endospores for *C. rectum* DSM 1295T, but not for *F. mortiferum* CCUG 14475T. However, analysis of the *F. mortiferum* (ACDB00000000.2) genome by RAST showed presence of coding sequences for spore maturation protein A and B (spmA, spmB) and spore photoproduct lyase (SPL) (Popham *et al*., 1995). The SPL coding sequence was also detected in the novel *Fusobacterium* sp. Similarly, another study demonstrated the presence of the putative septation protein in *F. nucleatum* (Onyenwoke *et al*., 2004). Due to the ability of endospore formation of *C. rectum* and due to the presence of genes associated with spore formation in other *Fusobacterium* spp., the *Fusobacterium* taxon should be emended to take into account the possibility of spore formation, as already stated by Collins *et al*., *C. rectum* DSM 1295T might thus be considered as a variant of *F. mortiferum* that not only contains, but also expresses genes associated with spore formation (Collins *et al*., 1994).

Although not all available *C. rectum* type strain subcultures were tested in the present study, similar results can be expected since the 16S rRNA sequence of *C. rectum* DSM 1295T showed 99.9% similarity with ATCC 25751T (=NCIMB 10651T, accession number X77850.1) and JCM 1412T (accession number LC053839.1) and subcultures of the *C. rectum* type strain that are present in other bacteria collections originated from ATCC 25751T. In addition, the characteristics originally described for *C. rectum* VPI 2488T (Heller, 1922; W E Moore and Holdeman, 1972; Whitman and Parte, 2011) were almost identical to the phenotypic traits of DSM 1295T obtained in the present study, with the following additions. The API ZYM showed negative reactions for leucine arylamidase, valine arylamidase, cystine arylamidase, trypsin, α-chymotrypsin, N-acetyl-β-glucosaminidase, α-mannosidase, α-fucosidase, β-glucosidase, β-glucuronidase and esterase lipase (C8), but positive reactions for alkaline phosphatase, esterase (C4, although weakly) and naphtol-AS-BI-phosphohydrolase. Tests for enzyme activities by use of Rapid ID 32 A showed positive reactions for β-galactosidase 6 phosphatase, pyroglutamic acid arylamidase, but not for arginine dihydrolase, α-arabinosidase, glutamic acid decarboxylase, α-fucosidase, arginine arylamidase, proline arylamidase, leucyl glycine arylamidase, phenylalanine

arylamidase, leucine arylamidase, tyrosine arylamidase, alanine arylamidase, glycine arylamidase, histidine arylamidase, glutamyl glutamic acid arylamidase, serine arylamidase and urease. The API 20 A showed a negative reaction for catalase activity. Deviations from the original description were that *C. rectum* DSM 1295T produced acid, although weakly, from maltose, mannose, raffinose and trehalose, but this was most likely due to differences in sensitivity of the applied test procedures. The genotypic and phenotypic similarities with the original *C. rectum* strain strongly indicate that the present results were not caused by a contamination in the lineage maintained at the DSMZ collection and that a subculture of the original *C. rectum* VPI 2488T, which is no longer available, was used.

These findings were similar to those of Lee and colleagues who demonstrated a close phylogenetic relationship between *C. rectum* ATCC 25751T and *F. mortiferum* DSM 19809T (Lee *et al*., 2016). Additionally, the 16S rRNA sequence based tree (Release LTPs123) obtained from the SILVA database also showed clustering of *C. rectum* NCIMB 10651T within the *Fusobacterium* genus (Yarza, 2015).

As already suggested in several other studies (Collins *et al*., 1994; Wiegel *et al*., 2006; Bolivar *et al*., 2012; Gharbia *et al*., 2012; Lee *et al*., 2016), the classification of *C. rectum* must be revised. Data from our and previous studies show that *F. mortiferum* and *C. rectum* must be considered as heterotypic synonyms. Phylogenetically, these bacteria belong to the genus *Fusobacterium* and therefore the name *F. mortiferum* has nomenclatural priority. An emended species description is presented below.

Description of Fusobacterium gastrosuis *sp. nov.*

F. gastrosuis sp. nov. (gas. tro. su'is., Gr. n. gaster gastros, stomach; L. n. sus suis, a pig; L. gen. n. gastrosuis, from the stomach of a pig). After anaerobic incubation on Columbia agar plates supplemented with 5% defibrinated sheep blood for 3 days at 37°C, the colonies are circular, white with a translucent border, slightly elevated with a smooth edge and 0.4 cm in diameter with presence of a narrow zone of complete hemolysis. Gram-staining of these colonies shows 1.5–2 µm long and 0.3–0.5 µm wide Gram- negative rods with rounded ends, non-capsulated with presence of swelling and globular forms and occasionally long filaments. The strains produce indole and exhibit proline arylamidase and glutamic acid decarboxylase activity. They do not hydrolyse esculin, do not exhibit pyroglutamic acid

arylamidase, valine arylamidase, α-galactosidase, β-galactosidase, β-galactosidase-6-phosphate or α-glucosidase activity nor produce acid from cellobiose, glucose, glycerol, lactose, mannitol, mannose, maltose, raffinose, saccharose, salicin or trehalose. The major fatty acids are C16:0 and C18:1w9c. The type strain, CDW1(T) (=DSM 101753(T) = LMG 29236(T)), was isolated from the *Pars oesophagea* of a 6-months old pig, in Ghent (Belgium) in 2014. The G + C content of the DNA of the type strain is 28.2 mol%.

Transfer of C. rectum (Heller, 1922; W E Moore and Holdeman, 1972) to F. mortiferum (Harris, 1905; W.E. Moore and Holdeman, 1972)

Phylogenetic analysis and phenotypic characterization indicate that *C. rectum* and *F. mortiferum* should be considered as a single species. The names *C. rectum* (Heller, 1922; W E Moore and Holdeman, 1972) and *F. mortiferum* (Harris, 1905; W.E. Moore and Holdeman, 1972) can be considered as heterotopic synonyms. According to Rule 15 and 17 of the Bacteriological Code, we conclude that *C. rectum* should be given the name *F. mortiferum*, with strain ATCC 25557(T) (=350A(T), CCUG 14475(T), DSM 19809(T), VPI 4123A(T)) as type strain.

Emended description of F. mortiferum

F. mortiferum (mor.ti'fer.um., L. neut. adj. mortiferum, death-bringing, death-bearing)

The description is as given for *F. mortiferum* (type strain = CCUG 14475(T), 350A(T), ATCC 25557(T), DSM 19809(T), VPI 4123A(T)) (Whitman and Parte, 2011) with the following additions. The API ZYM shows negative reactions for leucine arylamidase, valine arylamidase, cystine arylamidase, trypsin, α-chymotrypsin, N-acetyl- β-glucosaminidase, α-mannosidase, α-fucosidase, β-glucosidase, β-glucuronidase and esterase lipase (C8), but positive reactions for alkaline phosphatase, esterase (C4, although weakly) and naphtol-AS-BI-phosphohydrolase. Tests for enzyme activities by use of Rapid ID 32 A show positive reactions for β-galactosidase 6 phosphatase, pyroglutamic acid arylamidase, but not for arginine dihydrolase, α-arabinosidase, glutamic acid decarboxylase, α-fucosidase, arginine arylamidase, proline arylamidase, leucyl glycine arylamidase, phenylalanine arylamidase, leucine

arylamidase, tyrosine arylamidase, alanine arylamidase, glycine arylamidase, histidine arylamidase, glutamyl glutamic acid arylamidase, serine arylamidase and urease. The API 20 A shows a negative reaction for catalase. The major fatty acids (>10% of the total fatty acids) are C14:0, C16:0 and C18:1w9c. Some strains (=ATCC 25751, NCIB 10651, DSM 1295, JCM 1412) possess the possibility to produce endospores that are oval, subterminal and swell the cell.

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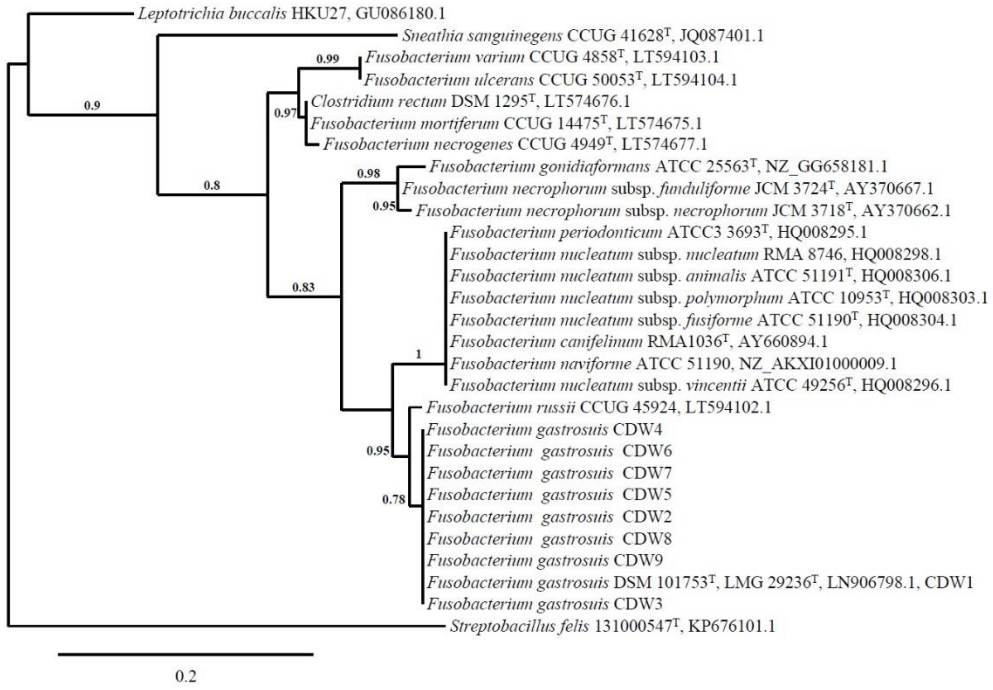
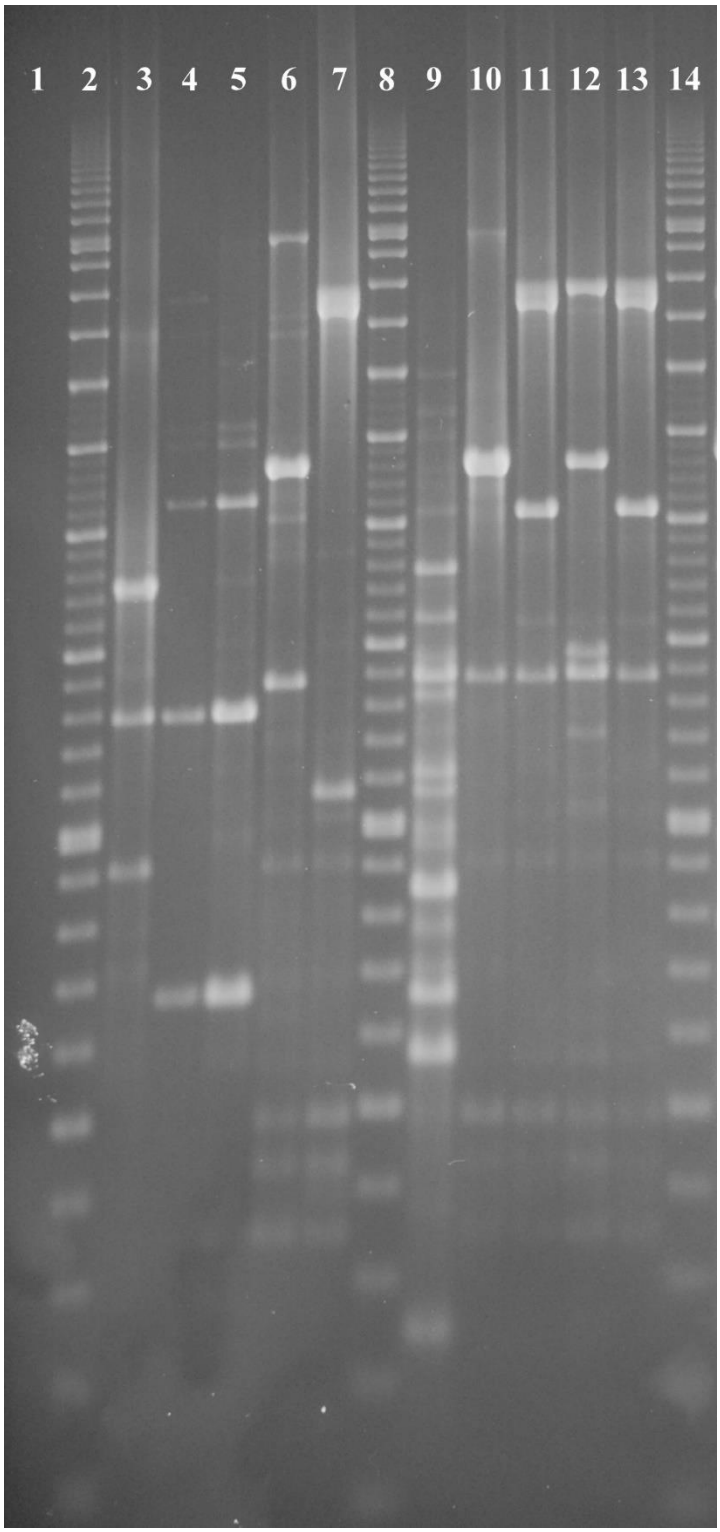
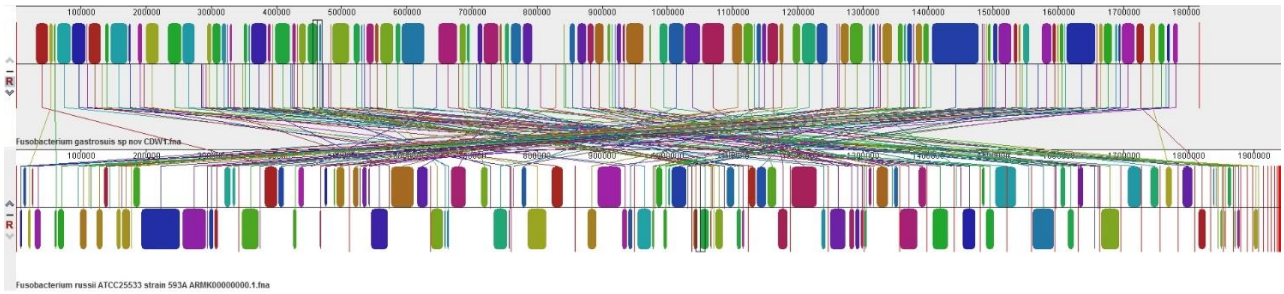
Additional file 1: Colony morphology (left image) of *Fusobacterium gastrosuis* sp. nov., purified on Columbia agar plates supplemented with 5% defibrinated sheep blood, showing a very narrow zone of complete hemolysis, often only observed under the colony (right image).



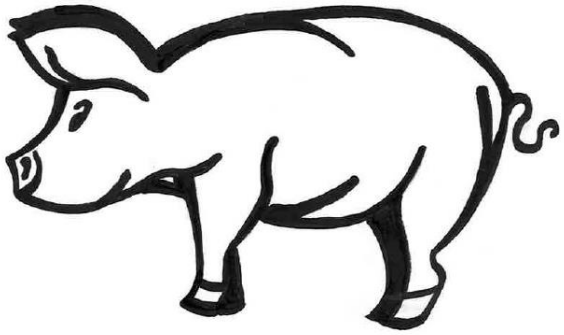
Additional file 2: Phylogenetic tree based on gyrB derived amino-acid sequences and maximum likelihood method shows the genetic relationships between *Fusobacterium* spp. and the 9 isolates of *Fusobacterium gastrosuis* sp. nov. (CDW1-9). The scale-bar represents 20% differences in nucleotide sequences; bootstrap values (≥0.7) of 1000 replicates are displayed next to the corresponding branch and GenBank accession numbers are included. *Leptotrichia buccalis* HKU27, *Sneathia sanguinegens* CCUG 41628T and *Streptobacillus felis* 131000547T were used as outgroups. All present nodes were coincident in the tree generated with the neighbor algorithm.



Additional file 3: Repetitive sequence-based PCR fingerprinting patterns with the $(GTG)_5$ primer of *F. necrogenes*, *F. mortiferum*, *C. rectum* and *F. gastrosuis* sp. nov. 1 = negative control, 2 = 1 kb ladder, 3 = *F. necrogenes* CCUG 4858^T, 4 = *F. mortiferum* CCUG 14475^T, 5 = *C. rectum* DSM 1295^T, 6 = *F. gastrosuis* CDW1, 7 = *F. gastrosuis* CDW3, 8 = 1 kb ladder, 9 = *F. gastrosuis* CDW4, 10 = *F. gastrosuis* CDW5, 11 = *F. gastrosuis* CDW7, 12 = *F. gastrosuis* CDW8, 13 = *F. gastrosuis* CDW9, 14 = 1 kb ladder.



Additional file 4: Pairwise genome alignment of *Fusobacterium gastrosuis* sp. nov. CDW1 with its closest phylogenetic neighbour *F. russii* shows that the genomes were sufficiently different, with presence of 143 Locally Collinear Blocks. The multiple genome alignment tool (Mauve) software tool was used to align the genomes.



Chapter 3: Characterization of the non-glandular gastric region microbiota in *Helicobacter suis*-infected versus non-infected pigs identifies a potential role for *Fusobacterium gastrosuis* in gastric ulceration

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Abstract

Helicobacter suis has been associated with development of gastric ulcers in the non-glandular part of the porcine stomach, possibly by affecting gastric acid secretion and altering the gastric microbiota. *Fusobacterium gastrosuis* is highly abundant in the gastric microbiota of *H. suis*-infected pigs and it was hypothesized that this micro-organism could play a role in the development of gastric ulcerations. The aim of this study was to obtain further insights in the influence of a naturally acquired *H. suis* infection on the microbiota of the non-glandular part of the porcine stomach and in the pathogenic potential of *F. gastrosuis*. Although infection with *H. suis* had no effect on microbial diversity, richness and population evenness of the non-glandular porcine stomach microbiota, it influenced the relative abundance of several taxa at phylum, family, genus and species level. *H. suis*-infected pigs showed a significantly higher colonization rate of *F. gastrosuis* in the non-glandular gastric region compared to non-infected pigs. *In vitro,* viable *F. gastrosuis* strains as well as their lysate induced death of both gastric and oesophageal epithelial cell lines. These gastric cell death inducing bacterial components were heat-labile. Genomic analysis revealed that genes are present in the *F. gastrosuis* genome with sequence similarity to genes described in other *Fusobacterium* spp. that encode factors involved in adhesion, invasion and induction of cell death as well as in immune evasion. We hypothesize that, in a gastric environment altered by *H. suis*, colonization and invasion of the non-glandular porcine stomach region and production of epithelial cell death inducing metabolites by *F. gastrosuis*, play a role in gastric ulceration. This hypothesis remains to be confirmed and the underlying molecular mechanism to be elucidated in pigs experimentally co-infected with *H. suis* and *F. gastrosuis*.

Key words: microbiota – *Pars oesophagea* – porcine gastric ulceration – *Helicobacter suis* – *Fusobacterium gastrosuis*

Introduction

Gastric ulceration is a common disease entity of pigs worldwide, with prevalences of up to 93%. It may result in decreased daily weight gain, decreased feed intake and sudden death, leading to significant economic losses and animal welfare issues. The aetiology is multifactorial. Diet particle size, management and infection with pathogens are factors that have been hypothesized to be involved (**General introduction: Etiology of porcine gastric ulceration**). The exact pathophysiological mechanism behind porcine gastric ulceration, however, is not completely clear. In marked contrast with human patients and several other animal species, gastric ulcers do not develop in the glandular part of the porcine stomach, but are almost exclusively found in the *Pars oesophagea*, a small area around the opening of the oesophagus which does not contain glands. Since this stomach region is not protected by mucus, it is highly susceptible to irritation by hydrochloric acid produced in the distal gland zone of the porcine stomach. Chronic irritation of the *Pars oesophagea* results in hyperkeratosis, erosion and finally ulceration (reviewed by Haesebrouck *et al*., 2009).

Helicobacter suis is a zoonotic bacterium that colonizes the gastric mucosa of pigs worldwide. Results of recent studies indicate that *H. suis* infection plays a role in porcine gastric ulcer disease, probably by affecting gastric acid secretion through alteration of the number and/or function of parietal, D- and G-cells (Zhang *et al*., 2016; **Experimental studies: chapter 1**). Impaired gastric acid secretion, induced by *H. suis*, may favor the establishment of specific gastric microbiota. Our metagenomics study of pooled samples from the different stomach regions (i.e. *Pars oesophagea*, cardiac, fundic and pyloric gland zones) from *H. suis*-positive and -negative 6-8 months old pigs revealed that a novel *Fusobacterium* sp., designated *F. gastrosuis*, was highly abundant in the gastric microbial community of *H. suis*-infected animals (**Experimental studies: chapter 2**).

Fusobacteria are normal constituents of the oropharyngeal, gastrointestinal and genital microbiota of a wide range of animal species, going from mammals to birds and fish. Nevertheless, they are also frequently isolated from clinical samples of both human and animal origin, especially from cases of pyonecrotic infections (McGuire *et al*., 2014). Infections with *Fusobacterium* spp. have been linked to a wide range of pathologies. Furthermore, genome analysis of *Fusobacterium* spp. showed the presence

of a large and diverse set of virulence associated genes, such as leukotoxin and immunosuppressive protein A (fipA), which have been associated with cell death and immuno-evasion, respectively (Ang *et al*., 2014). Additionally, some *Fusobacterium* spp. were able to adhere to and actively invade host cells without the aid of other factors, due to the presence of genes encoding adhesins and membrane-related proteins, while others only aggravated necrosis when tissue damage was initiated by other microorganisms or environmental factors (McGuire *et al*., 2014).

We hypothesize that, in a gastric environment altered by *H. suis*, colonization and invasion of the *Pars oesophagea* and production of epithelial cell death inducing metabolites by *F. gastrosuis* may play a role in gastric ulceration.

The overall aim of the current study was to obtain further insights in the influence of a naturally acquired *H. suis* infection on the microbiota of the *Pars oesophagea* of the porcine stomach and in the pathogenic potential of *F. gastrosuis* upon co-infection. Therefore, the ability of *F. gastrosuis* to induce cell death in oesophageal and gastric epithelial cell lines was first determined and its genome was also analysed for the presence of genes encoding putative virulence factors which may be involved in adhesion, invasion and host cell death as well as in immuno-evasion. Secondly, the colonization rate of *F. gastrosuis* and its abundance in the microbiota of the *Pars oesophagea* was compared in *H. suis*-infected and non-infected pigs.

Material and Methods

Study 1: Microbiota composition of the Pars oesophagea

Sampling of porcine stomachs

Ten *H. suis*-positive and 10 *H. suis*-negative stomachs of 6-8 months old pigs used in another study from our group were further analysed (Bosschem *et al*., 2017). Using autoclaved tweezers and scalpels, a biopsy of 40-50 mg consisting of mucosa and submucosa was taken from the *Pars oesophagea* for gastric microbiota analysis and subsequent *F. gastrosuis* quantification by quantitative real time (RT)-PCR was realized. In addition, a second biopsy consisting of mucosa and submucosa was taken from

the *Pars oesophagea* to determine mRNA expression levels of genes encoding host factors (markers) involved in inflammation, hyperkeratosis and ulceration.

Gastric microbiota analysis

DNA was extracted from the gastric biopsy of the *Pars oesophagea* using the DNeasy Blood & Tissue Kit (Qiagen, Hilden, Germany) according to the instructions of the manufacturer. *16S rRNA* gene amplicon pyrosequencing was performed using the Roche GS-Junior Genome Sequencer as described previously (Rodriguez *et al*., 2015). The obtained 16S rDNA sequence reads were processed using MOTHUR (software package v1.35), Pyronoise algorithm and UCHIME algorithm for alignment and clustering, denoising and chimera detection, respectively (Schloss *et al*., 2009; Haas *et al*., 2011). The obtained read sets were compared to a reference dataset of aligned sequences of the corresponding region derived from the SILVA database (v1.15) of full-length rRNA sequences implemented in MOTHUR (Pruesse *et al*., 2007). The final reads were clustered into operational taxonomic units (OTUs) using the nearest neighbor algorithm using MOTHUR with a 0.03 distance unit cut-off. A taxonomic identity was attributed to each OTU by comparison with the SILVA database using a 80% homogeneity cut-off. When a taxonomic identification lower than 80% was obtained, the taxonomic level was labelled with the first defined level from higher level followed by the label 'unclassified'. Finally, the unique sequences for each OTU were compared with the SILVA data set using the BLASTN algorithm. For each OTU, a consensus taxonomic identification was given when less than 1% of mismatch with the aligned sequence was obtained. In the final metadata table, the following labelling was used: the population is identical to a taxonomically defined species and is labelled 'genus_species'; the population is identical to a reference sequence belonging to a still undefined species and is labelled 'genus_NCBI accession number'; the sequence is not identical to any known sequence and is labelled with the corresponding OTU number.

In order to determine the effect of *H. suis* on the gastric microbiota composition, the *H. suis*-negative and *H. suis*-positive groups were compared.

Subsampled datasets were obtained and evaluated in MOTHUR to estimate the richness, microbial diversity and population evenness by using the Chao1 estimator, Simpson's reciprocal index and Simpson's evenness index, respectively (Chao and Bunge, 2002; Chao and Shen, 2003). Population structure and community membership were assessed with MOTHUR using distance matrix based on Bray-Curtis dissimilarity index. Differences in functional profiles of gastric bacterial communities were analysed by mapping taxa into several phenotypes (i.e. metabolism, Gram staining, sporulation,…) using METAGENassist (Arndt *et al*., 2012). Only the phenotypes detected in more than 50% of the samples were included for further analysis.

Statistical analysis

Statistical differences in microbial diversity, richness and population evenness between the groups were investigated using non-parametric Kruskal-Wallis tests with Tukey post-hoc tests using PRISM 7 (Graphpad Software). Using MOTHUR, community composition differences were investigated using Analysis of molecular variance (AMOVA) and homogeneity of molecular variance (HOMOVA). In order to highlight statistical differences in relative bacterial abundance between the groups, non-parametric Kruskal-Wallis tests with Tukey post-hoc tests and Benjamini-Hochberg False Discovery Rate were performed using the STAMP software. Differences were considered statistically significant at a corrected *p*-value of less than 0.05.

Study 2: F. gastrosuis *colonization*

Sampling of porcine stomachs

Sixty-eight stomachs of 6-8 months old pigs and 60 stomachs of adult sows (1-3 years old) were collected over a period of 10 months from 2 slaughterhouses in Flanders, Belgium. The pigs originated from different herds. Stomachs of 46, 2-3 months old pigs were collected from 2 different pig herds (23 samples from each herd). The stomachs of all pigs had been previously used in **Experimental studies: chapter 1**. Based on the method of Hessing (Hessing *et al*., 1992), mucosal lesions of the *Pars oesophagea* were scored as follows: score 0 for normal mucosa, score 1 for mild hyperkeratosis encompassing less than 50% of the surface, score 2 for severe hyperkeratosis encompassing more than

50% of the surface, score 3 for hyperkeratosis with few erosions, score 4 for hyperkeratosis with several erosions and score 5 for hyperkeratosis with many erosions or ulceration. Using autoclaved tweezers and scalpels, biopsies of 40-50 mg consisting of mucosa and submucosa were taken from the *Pars oesophagea* as well as from the cardiac, fundic and pyloric gland zone for quantification of *F. gastrosuis* DNA by RT-PCR. In addition, a swab from the oral cavity was taken from each 2-3 months old pig using a MasterAmp™ Buccal Brush (Epicentre, Madison, USA), as well as biopsies of 40-50 mg consisting of mucosa and submucosa from jejunum and colon.

Quantification of F. gastrosuis *by RT-PCR*

DNA was extracted from the gastric biopsies from the *Pars oesophagea,* cardiac, fundic and pyloric gland zone from the different age groups and from the jejunum and colon biopsies from the 2-3 months old pigs using the DNeasy Blood & Tissue Kit (Qiagen, Hilden, Germany) according to the instructions of the manufacturer. In addition, DNA was extracted from the oral cavity swab using PrepMan Ultra Sample Preparation Reagent (Life Technologies, Carlsbad, California) according to the manufacturer's instructions. The DNA extracted from the *Pars oesophagea* biopsies sampled for gastric microbiota analysis in study 1 was also included for *F. gastrosuis* quantification.

The presence of *F. gastrosuis* DNA was determined using a species-specific, RT-PCR based on the *gyrase B* (*gyrB*) gene (**Experimental studies: chapter 2**). The copy number of the obtained amplicons was calculated and converted to the number of *F. gastrosuis* bacteria per mg gastric tissue, by including 10-fold dilutions of an external standard. For generating the standard, part of the *gyrB* gene (1,212 bp) from *F. gastrosuis* strain CDW1 was amplified using UP-1 and UP-2r primers as previously described (**Experimental studies: chapter 2**). The standard consisted of 10-fold dilutions, starting at 10^8 PCR amplicons, for each 10 µl of reaction mixture. Two microliter of extracted DNA template was added to 10 µl reaction mixture, consisting of 0.25 µl of both primers (0.5 pmol/µl) located within the 1,212 bp fragment (to yield a 158 bp PCR product), 3.5 µl HPLC water and 6 µl SensiMix™ SYBR No-ROX (Bioline Reagents Ltd, London, UK). Sense primer was GB2_F: 5′-GCA GCT CAA AGA GCA AGA GAA GCA-3′. Anti-sense primer was GB2_R: 5′-CTT CCC TGC TTT GCA GAA CCT CC-3′. The cycling conditions were initial denaturation at 95°C for 10 min, followed by 47 cycles of 95°C for 20

s, 60 °C for 30 s and 72 °C for 30 s after which the total fluorescence of the samples was measured. Both standards and samples were run in duplicate on a CFX384™ RT-PCR System with a C1000 Thermal Cycler (Bio-Rad, Hercules, California, USA).

Expression of markers for inflammation and ulceration

RNA was extracted from the gastric biopsies of the *Pars oesophagea* using the RNeasy Mini Kit (Qiagen, Hilden, Germany), according to the manufacturer's instructions. The obtained RNA concentrations were measured using a NanoDrop spectrophotometer (Isogen Life Science, Utrecht, The Netherlands), after which the concentration of each sample was adjusted to 1 μg/μl, followed by cDNA synthesis using the iScript™ cDNA Synthesis Kit (Bio-Rad). Expression analysis was then performed for genes encoding host factors involved in inflammation ((interleukin (Il)-1β IL-6, IL-8, IL-10, CXCL13, interferon (IFN)-γ and tumor necrosis factor (TNF)-α) as well as in hyperkeratosis and ulceration (claudin 1, 2, 3, 4 and 18; keratin 6A; heat shock protein (Hsp) 27, 72 and 73; epidermal growth factor (EGF); basic fibroblast growth factor (BFGF); hepatocyte growth factor (HGF); transforming growth factor beta 1 (TGFB1); cyclooxygenase 2 (COX2); nitric oxide synthase 2 (NOX2); CXCL2; occludin; zonula occludens (ZO) 1 and 2). The housekeeping genes HMBS, Cyc, RPL4 and HPRT1 were shown to have a stable mRNA expression and therefore included as reference genes (Nygard *et al*., 2007; Flahou *et al*., 2012). All primer sequences are shown in Additional file 1. The mRNA expression levels of the reference and target genes were quantified using a RT-PCR, as previously described (Flahou *et al*., 2012). No-template-control reaction mixtures were included and all samples were run in duplicate. The Ct-values were first normalized to the geometric mean of the Ct-values of the reference genes. Fold changes were calculated using ΔΔCt method with mean of Ct-values from the *H. suis*-negative group. Finally, for each target gene, the results were expressed as fold changes of the mRNA expression of the *H. suis*-positive group relative to mRNA expression level of the *H. suis*-negative group.

Statistical analysis

Statistical analysis was performed using SPSS statistics 24 (IBM, New York, USA). Differences in severity of *Pars oesophageal* lesions, number of colonizing *F. gastrosuis* bacteria and fold changes of the markers for inflammation, hyperkeratosis and ulceration were investigated using the non-parametric Kruskal-Wallis H test with Bonferroni correction. A p-value ≤ 0.05 was considered to be significant. Correlations between mucosal lesions, fold changes and the number of *F. gastrosuis* bacteria were examined using the Pearson correlation coefficient. Differences were considered statistically significant at $p \leq 0.05$.

Study 3: Effect of F. gastrosuis *on gastric epithelial cell lines*

Bacterial strains

F. gastrosuis strains CDW1, CDW3, CDW6 and CDW8 were used for the *in vitro* cell experiments as previously described (**Experimental studies: chapter 2**). All strains were isolated from the *Pars oesophagea* of pigs suffering from gastric ulceration.

Cell lines and culture conditions

All *in vitro* cell experiments were performed with MKN7 (human gastric tubular adenocarcinoma cell line, Riken Cell Bank, Japan) and KYSE-450 (human oesophageal squamous cell carcinoma, ACC 387; Deutsche Sammlung von Mikroorganismen und Zellkulturen) cells. The MKN7 cells were cultured in 89% RPMI medium 1640 (supplemented with 1 mM L-glutamine, Invitrogen, Waltham, MA, USA), 10% fetal calf serum (FCS; HyClone, Logan, Utah, USA) and 1% penicillin-streptomycin (10,000 U/ml, Invitrogen). The KYSE-450 cells were cultured in 44.5% RPMI medium 1640 (supplemented with 1mM L-glutamine, Invitrogen), 44.5% Ham's F12 medium (supplemented with 1 mM L-glutamine, Invitrogen), 10% FCS (HyClone) and 1% penicillin-streptomycin (10,000 U/ml, Invitrogen). Penicillin-streptomycin was not added to the media for *in vitro* cell experiments with viable *F. gastrosuis* bacteria. The cells were seeded in plastic tissue culture flasks (VWR, Radnor, Pennsylvania, USA), maintained in a humidified incubator at 37°C under 5% CO_2 and passaged at least twice a week using 1% trypsin solution consisting of 88% trypsin diluent (8 g NaCl, 0.2 g KCl, 0.12 g KH_2PO_4, 0.91 g Na_2HPO_4 and

4 ml 0.5% phenol red solution/1000 ml aqua dest), 10% trypsin stock solution (Invitrogen) and 2% EDTA (2 g/100 ml trypsin diluent).

Cell-death inducing effects of F. gastrosuis *lysates*

Both MKN7 and KYSE-450 cell lines were incubated with *F. gastrosuis* lysates from strains CDW1, CDW3, CDW6 and CDW8 to determine cytotoxicity. To prepare the *F. gastrosuis* lysate, bacteria were incubated anaerobically for 2 days on Columbia blood agar plates (Oxoid, Basingstoke, United Kingdom). After incubation, they were harvested by centrifugation (368 g, 10 min, 4°C), washed twice with Hank's Balanced Salt Solution without calcium and magnesium (HBSS-) (Invitrogen) and resuspended in HBSS- until an optical density (OD) of 2.9 at 660 nm was obtained (i.e. approximately 1 x 10^8 bacteria/ml). The bacterial suspension was sonicated 20 times for 30 s and centrifuged (15,000 g; 5 min, 4°C) to remove cellular debris. The supernatant was then filtered through a 0.22 µm pore filter (Schleicher and Schuell, Munich, Germany) and stored at −80°C. The resulting protein concentration was determined with the Pierce BCA protein assay kit (ThermoFisher Scientific, Waltham, Massachusetts, USA), according to the manufacturer's instructions. The MKN7 and KYSE-450 cell lines were seeded at a concentration of 8 x 10^4 cells/ml and 1 x 10^5 cells/ml, respectively, in 6-well flat-bottom cell-culture plates (Greiner Bio One, Frickenhausen, Germany) and incubated at 37°C and 5% CO_2 until a monolayer was obtained after 48h. Each well was washed twice with Hank's Balanced Salt Solution with calcium and magnesium (HBSS+) to remove dead cells caused by the trypsin treatment and/or seeding. As previously described, each *F. gastrosuis* lysate was then added in different concentrations (50, 200 and 500 µg/ml, corresponding to a calculated theoretical multiplicity of infection (MOI) of approximately 5, 20 and 50 respectively) and incubated for 0, 24, 36 and 48h (Flahou *et al*., 2011). As a positive control, *F. necrophorum* subps. *necrophorum* lysate (50, 200 and 500 µg/ml) was used. Untreated cells of both cell lines were included as negative controls, as well as positive controls for necrosis and apoptosis using 0.1% Triton X-100 and 1 µM staurosporine, respectively. Additional controls were used to quantify and compensate for spectral bleed-through. All conditions were performed in triplicate. After incubation, the cells were trypsinized and washed 3 times with HBSS- to remove cellular debris. Next, the cells were stained incubated for 10 min in the dark at room temperature

with a labelling solution (10 mM 4-(2-hydroxyethyl)-1-piperazine ethanesulfonic acid (HEPES), 140 mM NaCl and 5 mM $CaCl_2$) containing Annexin-V-fluorescein isothiocyanate (1/50 diluted Annexin-V-FITC; 11828681001, Sigma-Aldrich) and propidium iodide (1µg/ml PI ; P4864, Sigma-Aldrich). Acquisition and analysis was performed on a CytoFLEX flow cytometer using the CytExpert 2.0 software (Beckman Coulter, Indianapolis, Indiana, USA). Following terms were used to describe the different subpopulations: viable cells (Annexin-V-FITC negative, PI negative), early apoptotic cells (Annexin-V-FITC positive, PI negative), late apoptotic/necrotic cells (Annexin-V-FITC positive, PI positive) and late necrotic cells (Annexin-V-FITC negative, PI positive) (Broekaert *et al*., 2016). A laser line of 488 nm was used with emission filters 525/40 and 585/42 for detection of annexin and propidium iodide, respectively. Before each measurement, calibration was performed to exclude instrument-related fluorescence intensity changes over time using CytoFLEX daily QC fluorospheres (Beckman Coulter). A minimum number of 10,000 events in the single, viable cell gate was analysed for each sample. The gating strategy for both cell lines is presented in Additional files 2-3.

Simultaneously, the presence of apoptosis and/or necrosis was verified using light microscopy. The cells were harvested and cytospins were prepared by centrifugation at 55 g for 5 min. Slides were air-dried, fixed in methanol and stained with Hemacolor (Merck, Darmstadt, Germany).

Cell-death inducing effect of viable F. gastrosuis *bacteria*

Prior to infection, the viability of *F. gastrosuis* grown and harvested as described above was determined. Approximately 5 x 10^8 bacteria/ml cell medium were added to 6-well flat-bottom cell-culture plates and incubated for 0, 6, 12, 24 and 36h at 37°C and 5% CO_2. After incubation, the colony forming units (cfu) were determined by serial dilution and plating on Columbia agar plates (Oxoid, Basingstoke, United Kingdom) supplemented with 5% defibrinated sheep blood (E&O laboratories, Bonnybridge, Scotland). The plates were incubated anaerobically for 3 days at 37 °C.

Both MKN7 and KYSE-450 cell lines were incubated with viable *F. gastrosuis* bacteria from strains CDW1, CDW3, CDW6 and CDW8 to determine the effect on cell death. The bacteria were incubated and harvested until an OD of 2.9 was obtained, as described above. After obtaining a monoculture of

MKN7 and KYSE-450 cell lines (± 5 x 10^5 cells/ml), these were inoculated with the different *F. gastrosuis* strains at a MOI of 5, 20 and 50 (i.e. corresponding to approximately 50, 200 and 500 µg/ml lysate, respectively) and incubated for 0, 2, 6 and 12h. The percentages of viable, apoptotic and necrotic cells were determined using the CytoFLEX as described above.

Properties of cell death inducing F. gastrosuis *component(s)*

To determine whether the cell death inducing component(s) were of protein nature, *F. gastrosuis* bacteria were pre-treated with pronase, trypsine or proteinase K (all at 1 mg/ml, 2h, 37°C) (Sigma-Aldrich) prior to incubation with MKN-7 and KYSE-450 cells. Bacteria were also pre-treated with paraformaldehyde (1%, 1h, room temperature) to determine if cell death inducing agents were associated with the bacterial surface. Finally, *F. gastrosuis* bacteria and lysates were heat treated (100°C, 10 min) to investigate their heat-lability. The percentages of viable, apoptotic and necrotic cells were flowcytometrically determined as described above. Negative as well as positive controls (i.e. untreated *F. gastrosuis* bacteria and lysates, respectively) were included. The viability of *F. gastrosuis* bacteria after protease, paraformaldehyde and heat treatment was verified by incubation on Columbia blood agar plates (37°C, 48-72h, anaerobic) after which bacterial growth was compared with that of untreated *F. gastrosuis* bacteria.

Activation of the pyroptotic pathway

Similarly as shown for *F. nucleatum* (Bui *et al*., 2016), activation of the pyroptotic pathway was investigated by determining caspase-1 and IL-1β expression using Western blot analysis.

Both MKN-7 and KYSE-450 cell lines were incubated either for 0, 24, 36 and 48h with 50, 200 and 500 µg/ml *F. gastrosuis* lysates from strains CDW1, CDW3, CDW6, or with 5, 20 and 50 MOI *F. gastrosuis* bacteria from strains CDW1, CDW3, CDW6 for 0, 6, 12, 24 and 36h as described. After incubation, total cell extracts were obtained using RIPA buffer supplemented with a protease inhibitor cocktail, as previously described (Lamote *et al*., 2007). Protein concentration was determined with the Pierce BCA protein assay kit, according to the manufacturer's instructions (ThermoFisher). After the samples were boiled for 5 min at 95°C, a total of 100 µg was suspended in 200 µl loading buffer (i.e. 24 ml of 0.5 M

Tris pH 6.8, 20 ml of 10% SDS, 10 ml glycerol, 5 ml of 0.5% bromophenol blue and 48 ml aqua dest) and loaded on a 10% self-prepared denaturing polyacrylamide gel. Pageruler™ plus prestained protein ladder with bands from 10 to 250 kDa (ThermoFisher) was used for molecular mass determination. Separation under reducing conditions was followed by blotting on a 0.45 µm nitrocellulose membrane (Bio-Rad). After blocking in Tris-buffered saline with 0.1% Tween 20 (TBS-T) and 5% milk powder for 1 h, overnight incubation with the primary anti-IL1β (0.1 µg/ml; AF-201-NA, R&D systems, Minnesota, USA) and anti-caspase 1 (0.1 µg/ml; AB207802, Abcam, Cambridge, United Kingdom) in TBS-T with 5% milkpowder was carried out. Membranes were washed with TBS-T and horseradish peroxidase-conjugated donkey-anti-goat-antibody, respectively goat-anti-rabbit-antibody (each at 1:5000 in TBS-T with 5% milk powder) were each applied for 1h. The blots were washed and protein bands were immunodetected by enhanced chemiluminescence, using Supersignal West Dura Extended Duration Substrate (ThermoFisher). The specificity of both primary antibodies was evaluated by dot immunoblot analysis using recombinant human IL-1β/IL-1F2 protein (201-LB, R&D Systems) and recombinant human caspase-1 protein (AB39901, Abcam). These recombinant proteins also served as positive controls for the western blot analysis. Untreated MKN-7 and KYSE-450 cells served as negative control.

Statistical analysis

Statistical analysis was performed using SPSS statistics 24 (IBM, New York, USA). Differences in the percentage of viable, early apoptotic and late apoptotic/necrotic cells and cellular debris were investigated using the non-parametric Kruskal-Wallis H test with Bonferroni correction. A *p*-value ≤ 0.05 was considered to be significant.

Potential virulence associated genes in F. gastrosuis

Bacterial strain

The proteome of the *F. gastrosuis* type strain CDW1 has been previously determined (LT607734.1) (**Experimental studies: chapter 2**). In the current study, its proteome was further investigated for presence of genes encoding putative virulence factors which may be involved in adhesion, invasion and host cell death as well as immunoevasion

Essential genes

The database of essential genes (DEG) version 15.2 consists of 53,885 essential protein-coding genes and 786 essential non-coding sequences from 48 prokaryotes and 26 eukaryotes. BLASTp search was performed for the proteome of *F. gastrosuis* against bacterial proteins of DEG with cut-off parameters of 1E-05 e-value and bit score of 100.

Potential virulence associated genes

Screening for presence of virulence associated genes was performed using the VirulentPred tool. The proteome of *F. gastrosuis* was also blasted against Pfam domains associated with virulence using the Pfam search tool. In addition, the proteome of *F. gastrosuis* was blasted against protein sequences from the virulence factor database (VFDB) core dataset (setA) with default parameters. This database contains 1,796 virulence factors and 2,599 curated virulence factor-related genes obtained from 74 bacterial pathogens. The query sequences having hit with cut-off bit score >100 were considered as potential virulence factors. Finally, data on virulence factors from other *Fusobacterium* spp. reported in the literature was collected (Kapatral *et al*., 2002; Karpathy *et al*., 2007; Ang *et al*., 2014; Kumar *et al*., 2016), as well as data on genes characteristic for active invasion (McGuire *et al*., 2014). Homology search of these proteins was performed to find close matches in *F. gastrosuis* using BLASTP. The query sequences having an E-value < 1e-6 and an identity > 30% over at least 100 amino acids were hypothesized to have a similar structure and function.

Results

Study 1: Microbiota composition of the Pars oesophagea

Sufficient sequencing reads were obtained from the *Pars oesophagea* of all pigs (n=20). Pyrosequencing yielded between 7,479 and 7,500 reads per sample. A total of 284,815 final reads were attributed to 3,837 species level OTUs. Chimeric sequences represented 5-10% of the total sequencing reads and were excluded from the analysis.

In general, total bacterial community analysis showed that the most dominant phylum in the porcine *Pars oesophagea* was Proteobacteria (56%), followed by Firmicutes (30%), Bacteroidetes (10%), Fusobacteria (3%) and Actinobacteria (1%). The relative abundance of other phyla was below 0.1%. On family level, following populations (i.e. >1%) were dominant: *Pasteurellaceae* (33%), *Clostridiaceae*_1 (11%), *Lactobacillaceae* (10%), *Enterobacteriaceae* (9%), *Prevotellaceae* (7%), *Comamonadaceae* (4%), *Veillonellaceae* (3%), *Moraxellaceae* (3%), *Campylobacteraceae* (2%), *Fusobacteriaceae* (2%), *Neisseriaceae* (2%), *Porphyromonadaceae* (2%) and *Streptococcaceae* (2%). The major genera (i.e. >1%) were *Actinobacillus* (29%), *Clostridium*_sensu_stricto_1 (10%), *Lactobacillus* (10%), *Escherichia* (7%), *Pasteurellaceae*_unclassified (4%), *Pelomonas* (4%), *Moraxella* (3%), *Prevotella*_7 (3%), *Alloprevotella* (2%), *Campylobacter* (2%), *Fusobacterium* (2%), *Neisseriaceae*_unclassified (2%), *Salmonella* (2%), *Streptococcus* (2%) and *Veillonella* (2%). The average gastric bacterial community composition at the phylum, family and genus level present in the *H. suis*-positive and -negative groups is represented in Figure 1, while the bacterial community composition of the *Pars oesophagea* of each individual pig is shown in Additional file 4.

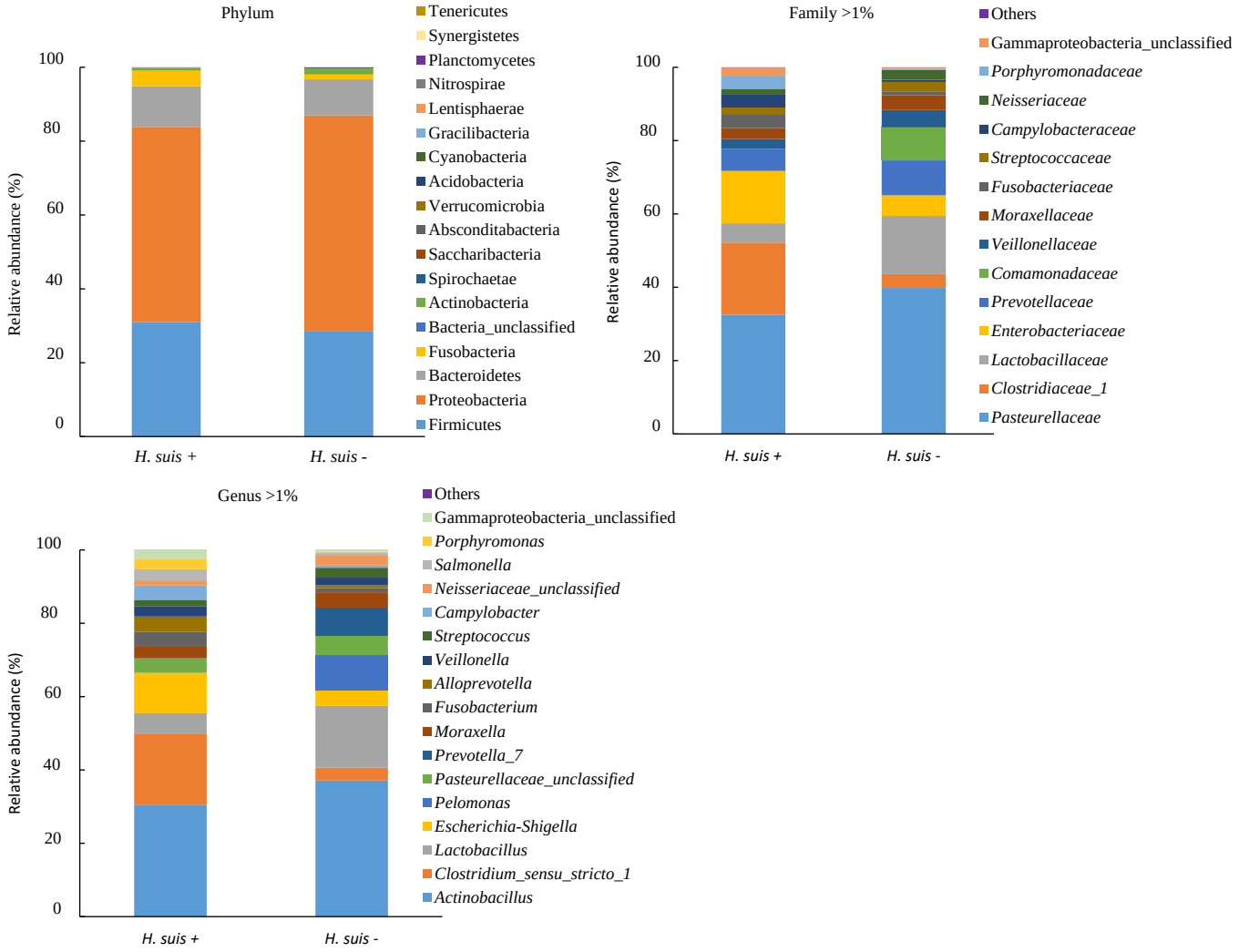

Figure 1: Average bacterial community compositions in the *Pars oesophagea* of *H. suis*-positive and -negative pigs. The cumulated histograms show the relative abundance of the identified taxa at phylum, family and genus level in the *Pars oesophagea* of *H. suis*-positive (n=10) and -negative (n=10) pigs. At family and genus level, taxa with a relative abundance <1% are merged in the category 'others'. The unclassified populations correspond to defined groups of the genus level for which a taxonomical classification assignation to the genus could not be attributed. These populations are therefore labelled with the first defined superior hierarchical taxonomic level followed by '_unclassified' to prevent confusion

Infection with *H. suis* had no effect on microbial diversity, richness and population evenness (Additional file 5). Furthermore, Unifrac-weighted analysis as well as AMOVA and HOMOVA did not reveal significant differences regarding community structure and composition of the groups. Population structure and community membership, as determined by Bray-Curtis dissimilarity index, were also not different between the groups.

In general, phenotypic analysis of the microbiota of the porcine *Pars oesophagea* revealed the presence of 19 metabolic phenotypes of which ammonia oxidizer, dehalogenation, nitrite reducer, sulfate reducer, sulfide oxidizer, chitin degradation, nitrogen fixation and xylan degrader were the most abundant, each accounting for 85%, 70%, 53%, 51%, 48%, 47%, 12% and 9% of the bacterial community, respectively. In the *Pars oesophageal* microbiota of all pigs, non-sporulating and Gram-negative bacteria were more abundant than sporulating and Gram-positive bacteria, respectively (38% vs 15% and 67% vs 26%, respectively). The *H. suis*-negative pigs showed a relative lower abundance of sporulating, nitrogen fixating and propionate metabolizing bacteria compared to the *H. suis*-positive pigs, although this difference was not significant (9% vs 21%, $p = 0.075$; 8% vs 17%, $p = 0.063$; 4% vs 12%, $p = 0.064$; respectively). Furthermore, *H. suis*-negative pigs showed a relative higher abundance of autotrophic bacteria compared to *H. suis*-positive pigs, which was borderline significant (68% vs 44%, $p = 0.052$).

Infection with *H. suis* influenced the relative abundance of several taxa at phylum, family, genus and species level, either significantly or borderline significantly. A detailed overview is presented in Table 1. In general, *H. suis*-positive pigs showed a relative higher abundance of the following taxa with at phylum level more Fusobacteria, at family level more *Fusobacteriaceae, Porphyromonadaceae, Bacteroidaceae, Clostridiaceae*_1, and at genus level more *Fusobacterium, Porphyromonas, Porphyromonadaceae*_unclassified, *Bacteroides, Alloprevotella, Clostridium_sensu_stricto_1, Leuconostoc, Pasteurella* and *Gammaproteobacteria*_unclassified. A relative lower abundance was detected for the following taxa with at family level less *Actinobacteria*_unclassified, *Bifidobacteriaceae, Corynebacteriaceae, Coriobacteriaceae, Streptococcaceae* and Gammaproteobacteriaceae_unclassified, at genus level less *Actinobacteria*_unclassified, *Bifidobacterium,* Corynebacteriaceae_unclassified, Lachnospiraceae_UCG-007,

*Lachnospiraceae*_XPB1014_group, *Staphylococcus, Aerococcus, Selenomonas, Megasphaera, Mitsuokella* and *Veillonellaceae*_unclassified.

The relative abundance of *F. gastrosuis* was higher in the *Pars oesophagea* of *H. suis*-positive pigs compared to *H. suis*-negative pigs (3% vs 0.9%, $p = 0.068$). This was confirmed by RT-PCR, as *H. suis*-infected pigs showed a significantly higher colonization rate of *F. gastrosuis* in the *Pars oesophagea* compared to non-infected pigs ($p < 0.05$) (Additional file 6D).

Table 1: Overview of the main differences in relative abundance of taxa at phylum, family, genus and species level in the *Pars oesophagea* of the *H. suis*-positive and -negative pigs.

Level	Taxa	*H. suis* +	*H. suis* -	*p*-value	Corrected *p*-value
Phylum	Fusobacteria	4.20 ± 1.02	1.26 ± 0.51	0.018	0.190
Family	*Fusobacteriaceae*	3.50 ± 0.83	0.94 ± 0.36	0.015	0.060
	Porphyromonadaceae	3.25 ± 0.51	0.19 ± 0.08	0.002	0.059
	Bacteroidaceae	0.25 ± 0.09	0.03 ± 0.02	0.028	0.206
	*Clostridiaceae*_1	18.02 ± 7.23	3.51 ± 1.44	0.045	0.262
	Gammaproteobacteriaceae_*unclassified*	2.02 ± 0.73	0.35 ± 0.19	0.021	0.177
	Actinobacteria_unclassified	0.00 ± 0.00	0.03 ± 0.01	0.005	0.051
	Bifidobacteriaceae	0.00 ± 0.00	0.13 ± 0.06	0.005	0.054
	Corynebacteriaceae	0.02 ± 0.01	0.27 ± 0.18	0.060	0.285
	Coriobacteriaceae	0.01 ± 0.01	0.10 ± 0.05	0.049	0.283
	Streptococcaceae	1.62 ± 0.84	2.38 ± 0.98	0.137	0.475
Genus	*Fusobacterium*	3.49 ± 0.83	0.94 ± 0.36	0.009	0.075
	Porphyromonas	2.51 ± 1.24	0.17 ± 0.07	0.007	0.073
	*Porphyromonadaceae*_unclassified	0.67 ± 0.34	0.02 ± 0.01	0.042	0.246
	Bacteroides	0.25 ± 0.09	0.03 ± 0.02	0.021	0.154
	Alloprevotella	3.66 ± 1.58	0.70 ± 0.35	0.034	0.219
	*Clostridium*_sensu_stricto_1	16.93 ± 6.80	3.03 ± 1.19	0.035	0.221
	Leuconostoc	0.02 ± 0.01	0.00 ± 0.00	<0.001	0.019
	Pasteurella	0.03 ± 0.01	0.00 ± 0.00	0.003	0.042
	*Gammaproteobacteria*_unclassified	2.02 ± 0.73	0.35 ± 0.19	0.029	0.199
	*Actinobacteria*_unclassified	0.00 ± 0.00	0.03 ± 0.01	0.003	0.040
	Bifidobacterium	0.00 ± 0.00	0.13 ± 0.06	0.008	0.072
	Corynebacteriaceae_unclassified	0.00 ± 0.00	0.01 ± 0.00	0.038	0.236
	*Lachnospiraceae*_UCG-007	0.00 ± 0.00	0.02 ± 0.01	<0.001	0.015
	*Lachnospiraceae*_XPB1014_group	0.00 ± 0.00	0.03 ± 0.02	0.004	0.045
	Staphylococcus	0.00 ± 0.00	0.02 ± 0.01	<0.001	0.054
	Aerococcus	0.00 ± 0.00	0.11 ± 0.06	0.009	0.078
	Selenomonas	0.00 ± 0.00	0.30 ± 0.15	<0.001	0.036
	Megasphaera	0.02 ± 0.01	1.14 ± 0.70	0.014	0.113
	Mitsuokella	0.01 ± 0.01	0.20 ± 0.08	0.011	0.091
	*Veillonellaceae*_unclassified	0.01 ± 0.01	0.42 ± 0.32	0.048	0.272
Species	*Fusobacterium gastrosuis*	3.11 ± 0.78	0.91 ± 0.35	0.015	0.068
	*Porphyromonas*_16S_OTU40	0.96 ± 0.37	0.04 ± 0.02	<0.001	0.037
	*Porphyromonas*_16S_OTU24	1.36 ± 0.86	0.09 ± 0.04	0.041	0.595
	*Porphyromonas*_16S_OTU293	0.05 ± 0.02	0.01 ± 0.00	0.068	0.817
	*Porphyromonadaceae*_unclassified_16S_OTU1254	0.02 ± 0.01	0.00 ± 0.00	<0.001	0.349
	*Porphyromonadaceae*_unclassified_16S_OTU61	0.50 ± 0.27	0.01 ± 0.01	0.041	0.600
	*Porphyromonadaceae*_unclassified_16S_OTU212	0.10 ± 0.07	0.00 ± 0.00	0.082	0.930
	*Bacteroides*_16S_OTU103	0.19 ± 0.09	0.02 ± 0.01	0.041	0.598
	Bacteroidales_unclassified_16S_OTU977	0.01 ± 0.00	0.00 ± 0.00	0.016	0.335

Level	Taxa	*H. suis* +	*H. suis* -	*p*-value	Corrected *p*-value
Species	*Alloprevotella*_16S_OTU50	0.74 ± 0.37	0.02 ± 0.01	0.026	0.475
	*Alloprevotella*_16S_OTU1438	0.02 ± 0.01	0.00 ± 0.00	0.069	0.822
	*Clostridium*_sensu_stricto_1_GQ249583	7.32 ± 2.86	1.26 ± 0.50	0.045	0.651
	*Leuconostoc*_16S_OTU708	0.02 ± 0.01	0.00 ± 0.00	<0.001	0.047
	Pasteurella multocida	0.03 ± 0.01	0.00 ± 0.00	0.004	0.114
	*Gammaproteobacteria*_unidentified_16S_OTU46	0.30 ± 0.17	1.93 ± 0.69	0.045	0.424
	Bifidobacterium AB034094	0.00 ± 0.00	0.07 ± 0.03	0.035	0.537
	Bifidobacterium thermophilum	0.00 ± 0.00	0.05 ± 0.03	0.005	0.141
	*Streptococcus*_16S_OTU295	0.01 ± 0.01	0.08 ± 0.04	0.083	0.936
	*Lachnospiraceae*_UCG-007_16S_OTU727	0.00 ± 0.00	0.02 ± 0.01	<0.001	1.278
	*Lachnospiraceae*_XPB1014_group_AB506358	0.00 ± 0.00	0.03 ± 0.02	0.003	0.093
	Staphylococcus EU341210	0.00 ± 0.00	0.01 ± 0.00	0.016	0.304
	Aerococcus suis	0.00 ± 0.00	0.09 ± 0.05	0.003	0.092
	Selenomonas bovis	0.00 ± 0.00	0.30 ± 0.15	<0.001	0.046
	Megasphaera KF842421	0.00 ± 0.00	0.15 ± 0.08	<0.001	0.034
	*Megasphaera*_16S_OTU1576	0.00 ± 0.00	0.02 ± 0.01	<0.001	0.051
	*Megasphaera*_16S_OTU1120	0.00 ± 0.00	0.04 ± 0.01	0.011	0.260
	Megasphaera elsdenii	0.01 ± 0.01	0.82 ± 0.53	0.027	0.491
	*Mitsuokella*_16S_OTU1562	0.00 ± 0.00	0.01 ± 0.00	0.031	0.521
	Mitsuokella AB506409	0.00 ± 0.00	0.03 ± 0.01	0.041	0.602
	Mitsuokella AM500804	0.00 ± 0.00	0.03 ± 0.02	<0.001	0.060
	Mitsuokella DQ797043	0.00 ± 0.00	0.08 ± 0.04	0.033	0.510
	*Veilonellaceae*_unclassified_16S_OTU715	0.00 ± 0.00	0.03 ± 0.01	<0.001	0.035
	*Veilonellaceae*_unclassfied_16S_OTU1085	0.00 ± 0.00	0.02 ± 0.01	0.049	0.717

The data are presented as the mean relative abundance of the taxa with the standard error of the mean. Statistical differences were calculated using the non-parametric Kruskal-Wallis tests with Tukey post-hoc tests and Benjamini-Hochberg False Discovery Rate were performed using STAMP. A corrected *p*-value lower than 0.05 is considered to be significant.

Study 2: F. gastrosuis *colonization in the porcine gastro-intestinal tract and impact of* H. suis *infection*

In the 2-3 months old pigs, the highest abundance of *F. gastrosuis* was found in the oral cavity, followed by the *Pars oesophagea*, independent from the *H. suis* status ($p < 0.05$) (Additional file 7). All pigs' stomachs tested positive for presence of *F. gastrosuis* and the average number of bacteria per mg tissue was higher in the *Pars oesophagea* than in the other stomach regions ($p < 0.01$) (Additional file 6). The number of *F. gastrosuis* bacteria was lower in the 2-3- compared to the 6-8 months old pigs and adult sows for each stomach region ($p < 0.01$), while no significant differences were observed between the latter groups (Additional file 8).

The number of *F. gastrosuis* bacteria in the oral cavity was highly and positively correlated with the number in the *Pars oesophagea* ($p < 0.001$). The number of *F. gastrosuis* in the *Pars oesophagea* was highly and positively correlated with each of the numbers in the cardiac, fundic and pyloric gland zones, as well as in the jejunum ($p < 0.001$).

The number of *F. gastrosuis* bacteria did not differ between the *H. suis*-negative and -positive 2-3 months old pigs, while a trend was observed towards higher number colonizing the *Pars oesophagea* of *H. suis*-positive 6-8 months old pigs compared to the non-infected age-matched group, although this difference was not significant. The *H. suis*-infected adult sows showed significantly lower number of *F. gastrosuis* colonizing the *Pars oesophagea* compared to the non-infected age-matched group ($p < 0.05$).

Gene expression analysis of markers for inflammation and ulceration

Despite several attempts, including the use of different primer pairs, no mRNA expression was detected of the genes encoding claudin 4, NOX2, IL-6 and TNFα in the porcine *Pars oesophagea*.

Compared to the *H. suis*-negative pigs, the mRNA expression of claudin 18, Hsp 72 and IL-8 was upregulated in the *Pars oesophagea* of *H. suis*-positive pigs, although either borderline or not significantly ($p = 0.050$, 0.211 and 0.139, respectively). In contrast, claudin 2, claudin 3 and CXCL2 gene expressions were either significantly or not significantly downregulated ($p = 0.043$, 0.093 and 0.065, respectively) (Additional files 9-10).

The fold changes of claudin 18, Hsp 72, keratin 6A and ZO1 were more pronounced in pigs with a high number of *F. gastrosuis* bacteria per mg gastric tissue of the *Pars oesophagea*, since positive correlations were found between both. Conversely, the fold changes of claudin 3, BFGF, CXCL2 and IL-1β were less pronounced in pigs with a high number of *F. gastrosuis* bacteria per mg gastric tissue due to negative correlations between both (Additional file 11).

Study 3: Cell-death inducing effects of F. gastrosuis *on gastric epithelial neoplastic cell lines*

F. gastrosuis was able to survive in an aerobic environment up to 12h. After this incubation time, the viability of *F. gastrosuis* was significantly decreased and after 36h incubation, viable F. gastrosuis bacteria could no longer be detected (data not shown).

Cell-death inducing effects on F. gastrosuis *on MKN-7 cell line*

Compared to the negative control, the viability of MKN-7 cells hardly decreased with an average of 3% after 24h incubation with the different concentrations of *F. gastrosuis* lysate, but significantly decreased with an average of 32% and 65% after 36h and 48h incubation, respectively ($p < 0.05$) (Figure 2A). After 2h, 6h and 12h incubation with the different concentrations of viable *F. gastrosuis* bacteria, cell viability significantly decreased with an average of 23%, 24% and 34%, respectively, compared to the negative control ($p < 0.05$) (Figure 2B).

Overall, the cell viability significantly decreased with increasing concentrations of *F. gastrosuis* lysate and MOI as well as with prolonged incubation time compared to the negative control ($p < 0.05$) (Additional file 12-14). Although the observed decreases in cell viability were systematically caused by both an increase in apoptosis and late apoptosis/necrosis, the percentage of early apoptotic cells increased after short incubation time and/or low concentrations of *F. gastrosuis* lysate and MOI, while the percentage of late apoptotic/necrotic cells increased after longer incubation time and/or higher concentrations of *F. gastrosuis* lysate and MOI (Additional file 12-14). Morphological features of apoptosis (i.e. plasma membrane blebbing, pyknosis) and necrosis (i.e. cell swelling, cytoplasmic vacuoles) reflecting these differences were observed on Hemacolor staining (Additional file 14).

Compared to *F. gastrosuis* lysate, more variation was observed between the 4 *F. gastrosuis* strains after incubation with viable *F. gastrosuis* bacteria. CDW1 lysate had the strongest effect on cell viability, CDW3 the weakest ($p < 0.05$). Conversely, viable CDW1 bacteria had the weakest effect on cell viability, CDW6 the strongest ($p < 0.05$).

In general, cell viability was significantly higher after incubation with the 4 *F. gastrosuis* strains compared to the positive control *F. necrophorum* subsp. *necrophorum* ($p < 0.05$). However, a significantly lower viability was observed for following conditions: at 50 µg lysate after 24h, 36 h and 48h for CDW1 and CDW3, and after 48h also for CDW6 and CDW8; at 200 µg lysate CDW1, CDW6 and CDW8 after 48h; at 50 MOI viable bacteria after 6h and 12h for CDW1 and after 12h also for CDW6 and CDW8 ($p < 0.05$).

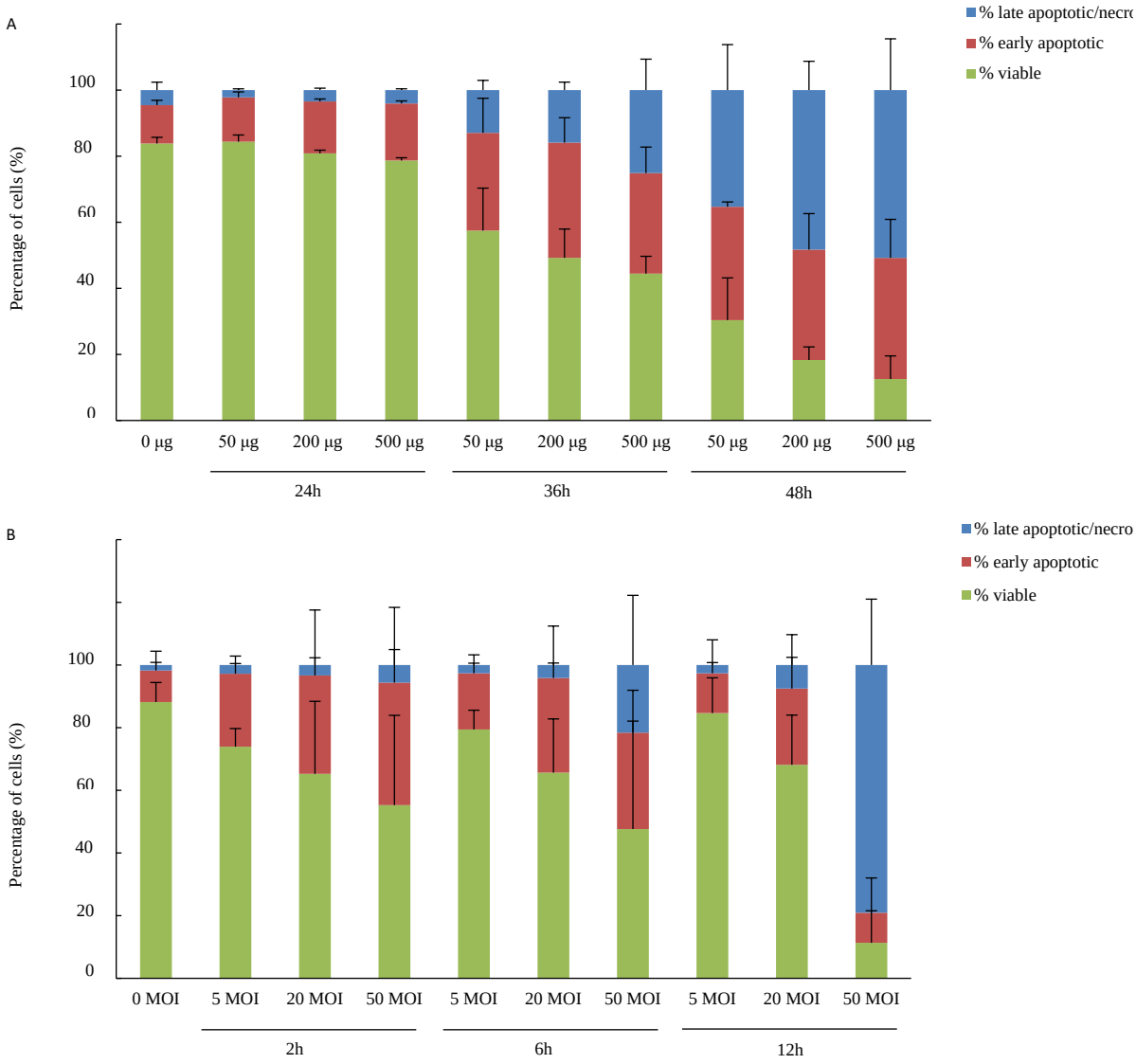

Figure 2: Percentage of viable, early apoptotic, late apoptotic/necrotic MKN-7 cells after incubation with different concentrations of *F. gastrosuis* lysate (A) and viable *F. gastrosuis* bacteria (B). Data are shown as the average (4 strains, 3 replications per strain) percentages of viable, early apoptotic and late apoptotic/necrotic MKN-7 cells with standard deviation. (A) The cells were incubated for 24, 36 and 48 with 50, 200 and 500 µg *F. gastrosuis* lysate (4 strains). (B) The cells were incubated for 2, 6 and 12h with 5, 20 and 50 MOI viable *F. gastrosuis* bacteria (4 strains).

Cell-death inducing effects on F. gastrosuis *on KYSE-450 cell line*

The viability of KYSE-450 cells was significantly decreased with an average of 4%, 10%, 14% and 37% after 24h, 36h, 48h and 72h incubation, respectively, with the different concentrations of *F. gastrosuis* lysate compared to the negative control ($p < 0.05$) (Figure 3A). Compared to the negative control, cell viability hardly decreased with an average of 3% after 2h incubation with the different concentrations of viable *F. gastrosuis* bacteria, but significantly decreased with an average of 8% and 15% after 6h and 12h incubation, respectively ($p < 0.05$) (Figure 3B).

Overall, the cell viability significantly decreased with increasing concentrations of *F. gastrosuis* lysate and MOI as well as with prolonged incubation time compared to the negative control ($p < 0.05$) (Additional file 15-17). Although the observed decreases in cell viability were systematically caused by both an increase in early apoptosis and late apoptosis/necrosis, the percentage of early apoptotic cells increased after short incubation time and/or low concentrations of *F. gastrosuis* lysate and MOI, while the percentage of late apoptotic/necrotic cells increased after longer incubation time and/or higher concentrations of *F. gastrosuis* lysate and MOI. Morphological features of apoptosis (i.e. plasma membrane blebbing) and necrosis (i.e. cell swelling, cytoplasmic vacuoles) reflecting these differences were observed on Hemacolor staining (Additional file 17).

In contrast to the MKN-7 cell line, a small subpopulation of Annexin-V-FITC negative and PI positive KYSE-450 cells was detected after incubation with *F. gastrosuis* viable bacteria and lysates. This subpopulation was defined as late necrotic and increased with increasing concentrations of *F. gastrosuis* lysate and MOI as well as with prolonged incubation time compared to the negative control ($p < 0.05$) (Additional file 15-17).

A similar effect on cell viability was seen after incubation with lysate obtained from the different *F. gastrosuis* strains. Conversely, more variance was seen after incubation with viable *F. gastrosuis* bacteria, where CDW1 had the weakest effect on cell viability and CDW6 the strongest ($p < 0.05$).

In general and in contrast to the MKN-7 cell line, the cell viability was lower after incubation with the 4 *F. gastrosuis* lysates compared to the positive control *F. necrophorum* subsp. *necrophorum*. A

significantly lower viability was observed for the following conditions: at 50 µg lysate after 24h and 72h for CDW1, CDW3, CDW6 and CDW8, after 36h and 48h also for CDW6 and after 48h also for CDW1 and CDW3; at 200 µg lysate after 24h, 48h and 72h for CDW1 and CDW3 and after 72h also CDW6 and CDW8; at 500 µg lysate after 24h, 48h and 72h for CDW1 and after 48h and 72h also for CDW3 and CDW6 ($p < 0.05$).

In general and similar to the MKN-7 cell line, the cell viability was significantly higher after incubation with the 4 viable *F. gastrosuis* strains compared to the positive control *F. necrophorum* subsp. *necrophorum* bacteria ($p < 0.05$). However, a significantly lower viability was observed for following conditions: at 5 MOI viable bacteria after 6h for CDW3 and CDW6 and after 12h also for CDW6; at 20 MOI viable bacteria after 6h and 12h for CDW6; at 50 MOI after 6h for CDW6 and after 12h also for CDW3 ($p < 0.05$).

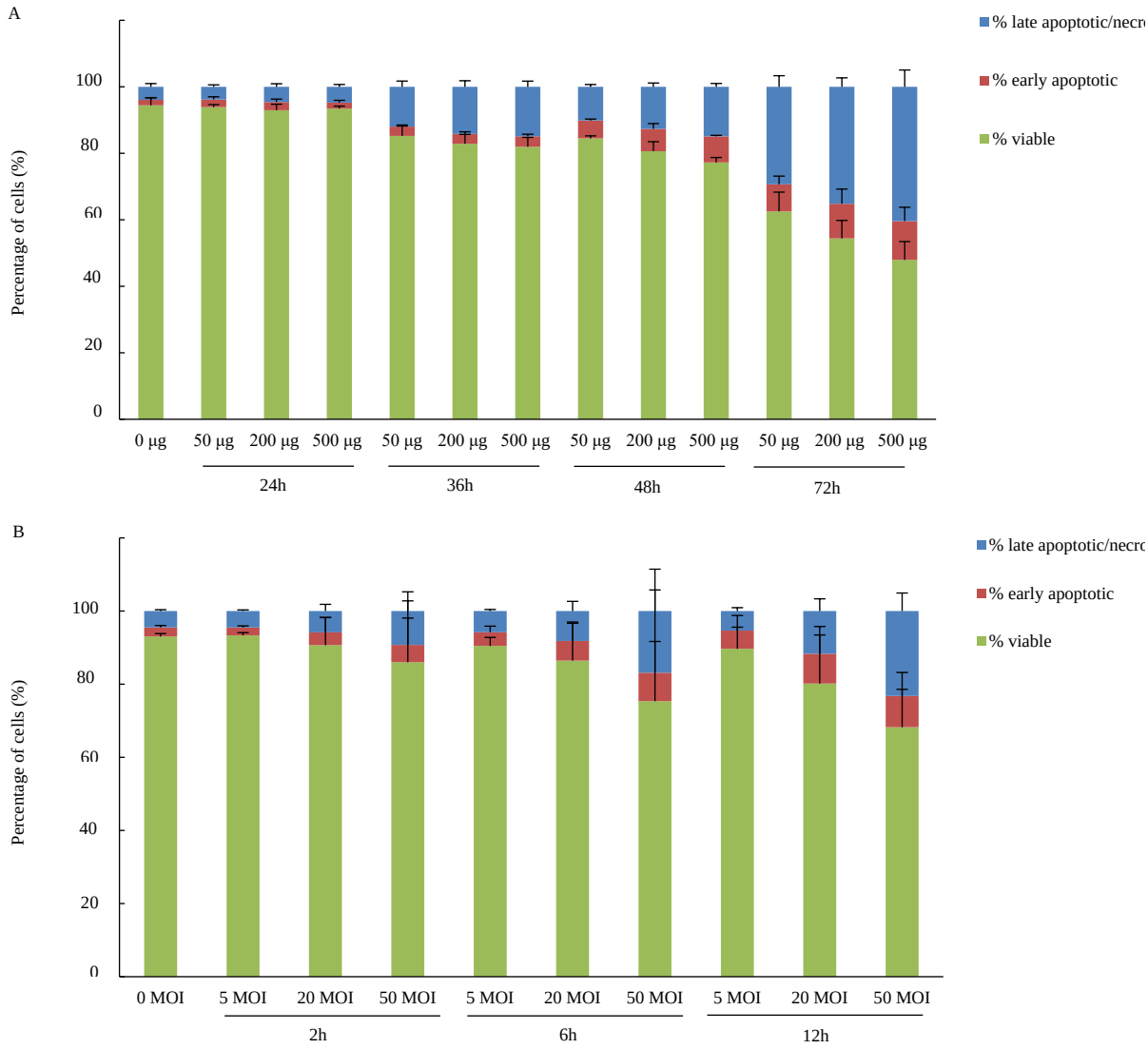

Figure 3: Percentage of viable, early apoptotic, late apoptotic/necrotic KYSE-450 cells after incubation with different concentrations of *F. gastrosuis* lysate (A) and viable *F. gastrosuis* bacteria (B). Data are shown as the average (4 strains, 3 replications per strain) percentages of viable, early apoptotic and late apoptotic/necrotic KYSE-450 cells with standard deviation. (A) The cells were incubated for 24, 36, 48 and 72h with 50, 200 and 500 µg *F. gastrosuis* lysate (4 strains). (B) The cells were incubated for 2, 6 and 12h with 5, 20 and 50 MOI viable *F. gastrosuis* bacteria (4 strains). The percentage late apoptotic/necrotic cells (i.e. Annexin-V-FITC positive and PI positive) contains a small subpopulation of Annexin-V-FITC negative and PI positive cells defined as late necrotic cells. See additional files 15-16 for more detailed information.

Properties of cell death inducing F. gastrosuis *component(s)*

Heat treatment completely abolished the cell-death inducing capacity of the 4 *F. gastrosuis* viable bacteria and lysates. Proteinase K treatment completely abolished the cell-death inducing capacity of viable *F. gastrosuis* bacteria, while no effects were seen for the trypsine and pronase treatments. Variable effects were seen for the paraformaldehyde treatment, namely a complete loss, partial loss (10-30%) or no loss of cell-death inducing capacities, and this independent from the strains (Table 2). *F. gastrosuis* bacteria remained viable after the different protease treatments and bacterial growth was similar to the untreated *F. gastrosuis* bacteria, while paraformaldehyde and heat treatment resulted in complete loss of viability.

Table 2: Overview of the effect of heat, protease and formaldehyde treatment on cell-death inducing capacity of the 4 *F. gastrosuis* strains.

Treatment	**Loss of cell-death inducing capacity**	
	***F. gastrosuis* viable bacteria**	***F. gastrosuis* lysate**
Heat	+	+
Proteinase K	+	/
Trypsine	-	/
Pronase	-	/
Paraformaldehyde	+/-	/

+ = complete loss of cell-death inducing capacity, - = no loss of cell-death inducing capacity, +/- = variable effects on cell-death inducing capacity, / = not determined

Activation of the pyroptotic pathway

All samples tested negative for presence of pro- and active IL-1β and caspase-1 proteins on western blot analysis, while bands were obtained for the positive controls (data not shown).

Proteome analysis

A search for DEG homologous sequences in the proteome of *F. gastrosuis* yielded 1,048 hits. Obtained hits have been reported to be involved in structural organization, nutrient uptake, pathogenesis, antibiotic resistance and other processes essential for the survival of *F. gastrosuis*.

Using the VirulentPred tool, 922 genes, of which 370 encoded hypothetical proteins, were predicted to be associated with virulence. Of the entire *F. gastrosuis* proteome, 51 proteins showed a close match with Pfam domains associated with virulence, antibiotic resistance and immune-evasion. About 162

proteins of the proteome showed significant hits against VFDB. The obtained hits have been reported to be involved in adhesion, invasion, immune-evasion, capsule formation, resistance to antibiotics and uptake of magnesium, zinc and iron. Some examples of these potential virulence factors are: (i) Hemolysin - lyses erythrocytes and creates anaerobic environment at the site of infection, stimulates the production of IL-1β and TNFα; (ii) ABC transporters – facilitates iron uptake, related with antibiotic resistance; (iii) Lipooligosaccharides synthesis – involved in immune system evasion, attachment to epithelial tissue, mediator of the proinflammatory response; (iiii) Cytolysin - forms pores in cell membrane, induces apoptosis, promotes cellular invasion, triggers iNOS and cytokine release (Additional file 19). In total, 232 proteins showed a close match with virulence factors of other *Fusobacterium* spp. reported in the literature. Some examples of these matches are: (i) Immunosuppressive protein (fipA) – inhibits T-cell activation; (ii) Haemagglutinin - attachment to epithelial cells; (iii) Outer membrane proteins - mediate adherence to other pathogenic bacteria and host cells, suppress host immune system, induce cell death in lymphocytes; (iiii) Serine protease - degrades the extracellular matrix proteins fibrinogen and fibronectin as well as collagen I and collagen IV, contribute to damage of periodontal tissues, helps the evasion of the immune system. Finally, *F. gastrosuis* showed presence of genes associated with active invasion of host cells.

Discussion

The gastro-intestinal microbiota plays an important role in host metabolism, nutrition, immunity and protection against pathogens (Castillo *et al*., 2007; Metzler-Zebeli *et al*., 2013). Disturbance of this delicate system is strongly correlated with the onset and progression of a wide range of pathologies (Cho and Blaser, 2012). Alterations in the gastric microbial community may play a role in porcine gastric lesion development (**General introduction: Etiology of porcine gastric ulceration**). However, due to technical limitations and the fact that the stomach was long considered inhospitable, limited information is available on the gastric microbiota composition of pigs (Motta *et al*., 2017). For the first time, the microbiota of the upper, non-glandular part of the stomach of 6-8 months old pigs was determined, as well as the impact on it of a natural *H. suis* infection.

Proteobacteria, Firmicutes, Bacteroidetes, Fusobacteria and Actinobacteria were the most abundant phyla present in the *Pars oesophagea* of 6-8 months old pigs, irrespective of the *H. suis* status. These phyla have also been shown to dominate the gastric microbiota of weaned pigs (Su *et al*., 2008; Mann *et al*., 2014). Nevertheless, their relative abundance may differ, as Proteobacteria was the most dominant phylum in the *Pars oesophagea* of 6-8 months old pigs in the current study, while Firmicutes has been shown to dominate this stomach region in weaned pigs (Mann *et al*., 2014). This apparent discrepancy between both studies might be explained by the age difference of the pigs. In suckling pigs, *Lactobacillus* spp., which form a major part of the Firmicutes phylum, colonize the upper, non-glandular part of the stomach in large numbers. After weaning, the diet changes from sow milk to hard food, resulting in a gradual decrease of *Lactobacillus* spp. (Su *et al*., 2008). Indeed, diet can greatly affect the microbial community, as bacterial enterotypes are known to cluster based on the dietary abundance of animal protein relative to carbohydrate (Cho and Blaser, 2012). Proteobacteria have also been shown to dominate stomach content as well as the cardiac, fundic and pyloric mucosa of weaned pigs, suggesting that other factors may affect the relative abundance of phyla, such as management and sampling sites or techniques (Motta *et al*., 2017). Furthermore, the microbiota composition can also vary substantially within different herds and within pigs from the same herd, especially at the lower taxonomic levels. These factors complicate both the straightforward comparison of study results and the unequivocal definition of the gastric microbial community of healthy pigs (Cho and Blaser, 2012; Kernaghan *et al*., 2012).

Fusobacteriaceae are part of the core microbiota of the soft palate tonsils in pigs (Lowe *et al*., 2012; Pena Cortes *et al*., 2018) and some members, such as *F. necrophorum*, have been associated with stomatitis and tooth root abscesses (Zimmerman *et al*., 2012). In the current study on the group of the 2-3 months old pigs, the highest colonization rate of *F. gastrosuis* was found in the oral cavity, indicating that the oropharynx, and potentially its tonsils, may function as a natural reservoir for this bacterium. *F. gastrosuis* may enter the stomach through ingestion of food, water or saliva, after which it may opportunistically colonize the upper, non-glandular *Pars oesophagea*. Future studies are necessary to verify if *F. gastrosuis* does belong to the oral microbiota and/or if it may induce oropharyngeal

pathologies. Interestingly, recent metagenomic studies have also shown presence of *F. gastrosuis* in the nasal and oral microbiota of dogs (Bernard Taminiau, 2018, personal communication) and in the faeces of humans (De Paepe, 2018), indicating that *F. gastrosuis* is able to colonize a wide range of mammalian hosts, as has been described for *F. nucleatum* and *F. necrophorum* (McGuire *et al*., 2014). Nevertheless, although unlikely, it might still be possible that *F. gastrosuis* was merely transient in these three hosts.

Recent studies indicate that *H. suis* infection plays an important role in porcine gastric ulceration, probably by affecting gastric acid secretion and influencing the gastric microbiota (**Experimental studies: chapter 2**; Sapierzynski *et al*., 2017). In these studies, also high numbers of *F. gastrosuis* were detected in the gastric microbial community of *H. suis*-infected 6-8 months old pigs with a downregulation of markers for gastric acid secretion (**Experimental studies: chapter 1-2**). In the present study, *16S rRNA* gene amplicon pyrosequencing revealed a higher relative abundance of *F. gastrosuis* in the *Pars oesophageal* microbiota of *H. suis*-infected 6-8 months old pigs compared to non-infected pigs which was confirmed by RT-PCR. Analysis of many more pig stomachs of the same age group by RT-PCR showed a high *F. gastrosuis* colonization rate in the *Pars oesophagea* of *H. suis*-infected pigs as well, although the difference with non-infected pigs became less pronounced. This apparent discrepancy may have been caused by variation between herds or other factors such as diet, feeding strategy and management. Nonetheless, more stomachs should be investigated to confirm the increased *F. gastrosuis* colonization rate in *H. suis*-infected pigs. In marked contrast with our group of 6-8 months old pigs, the numbers of *F. gastrosuis* bacteria did not differ between *H. suis*-infected and non-infected 2-3 months old pigs. This may have been caused by the absence of gastric acid secretion alterations during the first phase of a *H. suis* infection i.e. in the 2-3 months old pigs as previously reported (**Experimental studies: chapter 1**). The lower numbers of *F. gastrosuis* colonizing the *Pars oesosphagea* of *H. suis*-infected adult sows compared to non-infected sows might be a consequence of the upregulation of gastric acid secretion during the chronic phase of infection i.e. in the *H. suis*-infected adult sows as again previously reported (**Experimental studies: chapter 1**). Combining the results of the current and our previous studies, we hypothesize that during the earlier phases of a *H. suis* infection (i.e. in the 6-8 months old pigs), gastric acid secretion is downregulated, resulting in higher numbers of

F. gastrosuis in the non-glandular part of the stomach which may facilitate the initiation of lesion development. During the more chronic phase of the infection (i.e. in the adult sows), gastric acid secretion is stimulated which may further aggravate lesion severity in the non-glandular part of the stomach which is not protected against acid due to the lack of mucus. Combined *F. gastrosuis* and *H. suis* infections in an experimental model complementing the observations obtained in naturally infected animals are, however, necessary to confirm or reject this working hypothesis.

Since no commercial porcine gastric cell line exists and as the epithelium of the oesophagus from humans and the *Pars oesophagea* of pigs is very similar (Ziegler *et al*., 2016), human-derived cell lines were used in the present study to further investigate *in vitro* the pathogenic traits of *F. gastrosuis*. Overall, cell death was clearly induced by both the viable *F. gastrosuis* bacteria and their lysate in the two selected cell lines. As lesions of the *Pars oesophagea* are characterized by the presence of swollen epithelial cells and necrotic debris (Queiroz *et al*., 1996; Thomson and Friendship, 2012), these *in vitro* cell death data further highlight a potential role of *F. gastrosuis* in the development of porcine gastric ulceration. Finally, it would be interesting to evaluate the effect of *F. gastrosuis* on mucosal explants of the porcine *Pars oesophagea*, as these target species explants comprise several additional host cell and host cell - environment interactions lacking in the relatively simple gastric cell line set-up that may be important in the elucidation of porcine gastric ulceration development.

In contrast to the cell death mechanism reported for *F. nucleatum* in a human gingival cell line (Bui *et al*., 2016), the current study did not observe indications of pyroptotic pathway (i.e. pro-inflammatory programmed cell death) activation in epithelial cells incubated with *F. gastrosuis*, characterized by the absence of the pro-inflammatory caspase-1 and IL-1β specific protein bands. It is possible that other caspases were activated through either the intrinsic or extrinsic apoptotic pathway. These complex cell-death inducing pathways needs to be further elucidated, for example by analyzing the expression of different caspase family members through flow cytometry (Fink and Cookson, 2005).

The mechanisms by which *F. gastrosuis* induces host cell death are unknown. Our data suggest that heat labile proteins most likely play a role, as heat- and proteinase K-treated *F. gastrosuis* completely lost their cell death inducing potential. In contrast, pronase and trypsin treatments did not affect this cell

death inducing capacity of *F. gastrosuis*, similar as described for other *Fusobacterium* spp. (Kolenbrander *et al*., 1995). This may be explained by differences in enzymatic specificity, as trypsin specifically cleaves the peptide bonds at the carboxyl terminus of lysine and arginine residues, while proteinase K non-specifically cleaves at aromatic, aliphatic as well as hydrophobic amino acid residues (Mótyán *et al*., 2013). The activity of pronase is also broad, yet the cell-death inducing capacity of *F. gastrosuis* was unaffected after the latter treatment. We hypothesize that other, currently unknown, factors may have further contributed here. For example, genes associated with capsule and biofilm formation as found in the proteome of *F. gastrosuis* may have contributed to this resistance against proteases such as pronase. As for the effect of formaldehyde treatment, widely used as fixation agent which slowly cross-links proteins in the bacterial cell wall, variable results were obtained. This indicates that cell-death inducing metabolites are, at least partly, associated with the bacterial surface and that their expression may be affected by environmental signals such as cell-contact. Nevertheless, this interpretation should be made with caution.

The current study identified that outer membrane proteins (OMPs) homologous to autotransporter secretion systems (type V) and/or ABC transporters are present in the proteome of *F. gastrosuis.* In analogy with a previous study, these OMPs might induce cell-death (Kaplan *et al*., 2010). Indeed, bacterial secretion systems are associated with host cell death through active transport of effector metabolites (Henderson and Nataro, 2001) like hemolysin, leukotoxin and protease, all pore-forming proteins that either directly destroy host cells at high concentrations or activate apoptosis through induction of host plasma membrane changes at lower concentrations. The presence of serine protease and hemolysin homologs in the proteome of *F. gastrosuis,* as well as an increased percentage necrosis when epithelial cells were incubated with high *F. gastrosuis* concentrations support their potential role in the induction of cell death. Other putative virulence factors present in the proteome of *F. gastrosuis* may also have contributed to host cell death. Homologs of methionine gamma lyase, cystathionine gamma- and beta lyase were found to be associated with host tissue destruction through production of volatile sulfur products (Nagaraja and Chengappa, 1998; Chukwu *et al*., 2014), while gamma-glutamyl transferase (Flahou *et al*., 2011) and genes associated with butyrate production (Karpathy *et al*., 2007)

have both been associated with host cell death through the production of reactive oxygen species (ROS). Last but not least, some *Fusobacterium* spp. possess genes encoding a diverse set of adhesins and membrane-related proteins allowing to actively enter host cells even in the absence of tissue damage (McGuire *et al*., 2014). Analysis of the *F. gastrosuis* genome revealed the presence of such genes, supporting host colonization, host immune system evasion and deeper tissue penetration. Additional studies are necessary to determine if these putative virulence factors are expressed, secreted as well as functional. It would be interesting to create mutants lacking genes encoding these putative virulence-associated factors in order to investigate their roles in the pathogenic significance of *F. gastrosuis*.

In conclusion, high numbers of *F. gastrosuis* were demonstrated in the oropharynx and *Pars oesophagea* of pigs. It was also demonstrated that *F. gastrosuis* induces epithelial cell death and that genes are present in the genome of this bacterium with sequence similarity to genes encoding factors involved in adhesion, invasion and induction of cell death as well as immune evasion. We hypothesize that, in a gastric environment altered by *H. suis*, colonization and invasion of the *Pars oesophagea* and production of epithelial cell death inducing metabolites by *F. gastrosuis* cause ulceration. Experimental studies in pigs infected with *H. suis* and *F. gastrosuis* are necessary to confirm this hypothesis.

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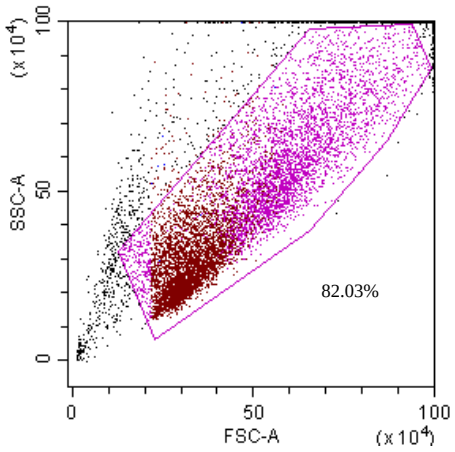
Additional file 1: List of primers used in quantitative RT-PCR for gene expression analysis of porcine housekeeping genes and markers for inflammation, hyperkeratosis and ulceration.

Primer	Sequence	Reference
HPRT-forward	5'-GGA CTT GAA TCA TGT TTG TG-3'	(Nygard *et al*., 2007)
HPRT-reverse	5'-CAG ATG TTT CCA AAC TCA AC-3'	(Nygard *et al*., 2007)
Cyc-forward	5'-CCT GAA CAT ACG GGT CCT G-3'	(Zhang *et al*., 2016)
Cyc-reverse	5'-AAC TGG GAA CCG TTT GTG TTG-3'	(Zhang *et al*., 2016)
HMBS-forward	5'-AGG ATG GGC AAC TCT ACC TG-3'	(Nygard *et al*., 2007)
HMBS-reverse	5'-GAT GGT GGC CTG CAT AGT CT-3'	(Nygard *et al*., 2007)
RPL4-forward	5'-CAA GAG TAA CTA CAA CCT TC-3'	(Nygard *et al*., 2007)
RPL4-reverse	5'-GAA CTC TAC GAT GAA TCT TC-3'	(Nygard *et al*., 2007)
Claudin 1-forward	5'-GAT GCG GAT GGC TGT CAT TG-3'	This study
Claudin 1-reverse	5'-CCA GAA GGC AGA GAG AAG CA-3'	This study
Claudin 2-forward	5'-GGC TCT CTA CTT GGG CAT CA-3'	This study
Claudin 2-reverse	5'-ACT CTT GGC TTT GGG TGG TT-3'	This study
Claudin 3-forward	5'-GCC AAA GCC AAG ATC CTC TAC-3'	This study
Claudin 3-reverse	5'-GGA CTG GTC TCG GAT GCA A-3'	This study
Claudin 4-forward	5'-GAC TCA CCG GAA GCT GTG TTC-3'	This study
Claudin 4-reverse	5'-GAA GAG AGG CTT TTC ACC GC-3'	This study
Claudin 18-forward	5'-CCC TGA TGA TCG TGG GCA TA-3'	This study
Claudin 18-reverse	5'-ACT CCA GCG ATT GTG CAA AG-3'	This study
Keratin 6-forward	5'-CTC AGG AGT AAC CCA GTA CCA-3'	This study
Keratin 6-reverse	5'-TTG GTG TCC AGG ACC TTG TT-3'	This study
Heat shock protein 27-forward	5'-TCT CGG AGA TCC AGC AGA CT-3'	This study
Heat shock protein 27-reverse	5'-GGA AAT GAA GCC GTG CTC AT-3'	This study
Heat shock protein 72-forward	5'-AGC GGT ACA AGT CGG AAG AT-3'	This study
Heat shock protein 72-reverse	5'-TGA TCA CCT CCT GAC ACT TGT-3'	This study
Heat shock protein 73-forward	5'-GTG CTC ATT CAG GTT TAT GAA GGT-3'	This study
Heat shock protein 73-reverse	5'-CCT GTG CTC TTA TCC ACA GC-3'	This study
Epidermal growth factor-forward	5'-ACA GCC CTG AAA TGG ATA GAG A-3'	This study
Epidermal growth factor -reverse	5'-CTC CCT CTG TCT GTC CAA TAG A-3'	This study
Basic fibroblast growth factor-forward	5'-GAG TGT GTG CAA ACC GTT ATC T-3'	This study
Basic fibroblast growth factor-reverse	5'-TGC CAC ATA CCA ACT GGA GTA-3'	This study
Hepatocyte growth factor-forward	5'-CAA TCC AGA GGT ACG CTA CGA-3'	This study
Hepatocyte growth factor-reverse	5'-TCC CAA CGC TGA CAA ATC TTG-3'	This study
Transforming growth factor beta-forward	5'-AGC TCC ACG GAG AAG AAC TG-3'	This study
Transforming growth factor beta -reverse	5'-AGT GTC TAG GCT CCA GAT GTA G-3'	This study
Cyclooxygenase 2-forward	5'-CTT CCA AGA CGC CAC TTC AC-3'	This study
Cyclooxygenase 2-reverse	5'-CTT GGG CAT CCA TTG TGC TA-3'	This study
Nitric oxide synthase 2-forward	5'-CGA GGC AAA CAC CAC ATT CA-3'	This study
Nitric oxide synthase 2-reverse	5'-TGC TGC TGA GAG CTT TGT TG-3'	This study
CXCL2-forward	5'-TGG TCA GGA AGT TTG TCT CAA C-3'	This study
CXCL2-reverse	5'-TCC GCT AAA GCT ACA GCA GTA-3'	This study
Occludin-forward	5'-ATC ACT ACT GCG TGG TGG AT-3'	This study
Occludin-reverse	5'-GGG CTG CTC GTC ATA AAT ACG-3'	This study
Zonula occludin 1-forward	5'-GCT CCT GGA TTT GGA TTT GGA-3'	This study
Zonula occludin 1-reverse	5'-ACC ATT GCA ACT CGG TCA TT-3'	This study
Zonula occludin 2-forward	5'-AGC TCC AGG AAG CAC AGA AT-3'	This study
Zonula occludin 2-reverse	5'-TCC TCT GGG ATC CTG ATA AAG TC-3'	This study
IL6-forward	5'-TGT CGA GGC TGT GCA GAT TA-3'	This study
IL6-reverse	5'-GTG GTG GCT TTG TCT GGA TT-3'	This study
IL8-forward	5'-CAG AGC CAG GAA GAG ACT AGA A-3'	This study
IL8-reverse	5'-GGC ATC GAA GTT CTG CAC TTA-3'	This study
IL10-forward	5'-GGG TTG CCA AGC CTT GTC-3'	This study
IL10-reverse	5'-CTC CAC GGC CTT GCT CTT-3'	This study
CXCL13-forward	5'-TGA GGT TCA CAC TGG GAT CTC-3'	This study
CXCL13-reverse	5'-CCC AGG AGG CCA GAT TTG AA-3'	This study
IL1β-forward	5'-GGA AGT GAT GGC TAA CAA TGG T-3'	This study
IL1β-reverse	5'-GGC TTC TCC TTT GCC ACA AT-3'	This study
IFNγ-forward	5'-TTT CAG CTT TGC GTG ACT TTG-3'	This study
IFNγ-reverse	5'-CAC TCT CCT CTT TCC AAT TCT TCA-3'	This study
TNFα-forward	5'-CCC AAG GAC TCA GAT CAT CGT-3'	This study
TNFα-reverse	5'-TGT CCC TCG GCT TTG ACA T-3'	This study

A



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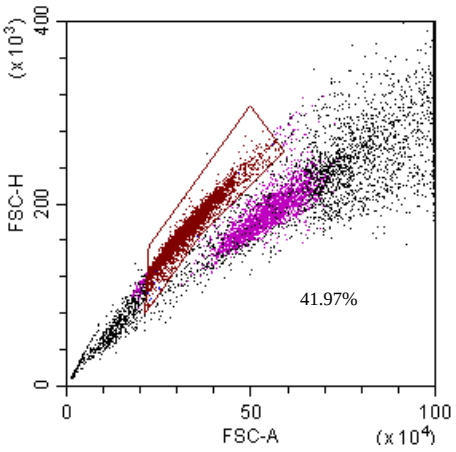
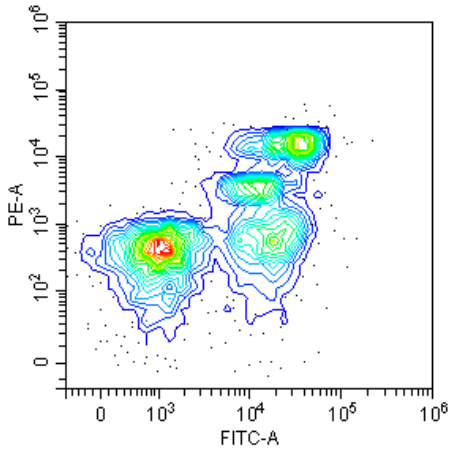


C

Gain settings	
FSC	30
SCC	35
FITC	5
PE	7

Compensation matrix		
Channel	**-FITC%**	**-PE%**
FITC		0.00
PE	41.07	

D



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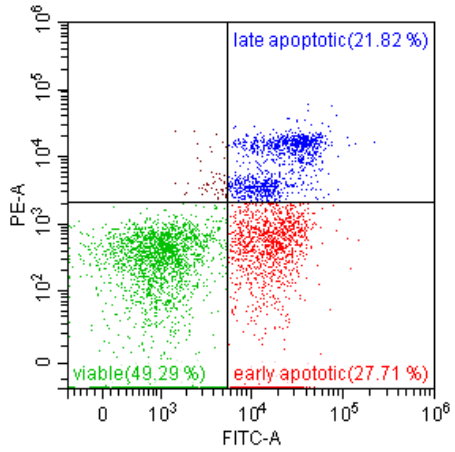
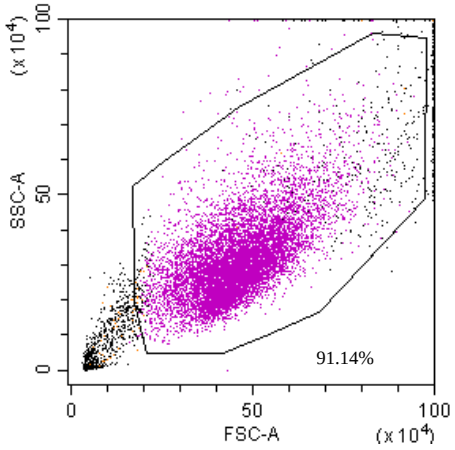


Additional file 2: Gating strategy of the MKN-7 cell line. (A) FSC-A/SSC-A represents the distribution of cells in the light scatter based on their size and intracellular complexity, respectively. The cells of interest are gated excluding debris. (B) FSC-A/FSC-H allows discrimination between single cells and doublets, single cells are gated. (C) Gain settings and compensation matrix. (D-E) FITC-A/PE-A identifies the selective subpopulations: viable (Annexin-V-FITC negative, PI negative), early apoptotic (Annexin-V-FITC positive, PI negative) and late apoptotic/necrotic (Annexin-V-FITC positive, PI positive) cells.

A



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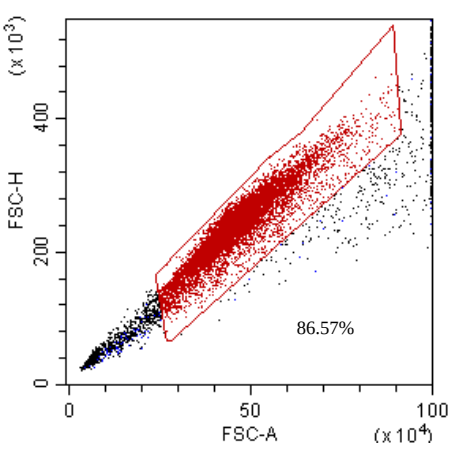

C

Gain settings	
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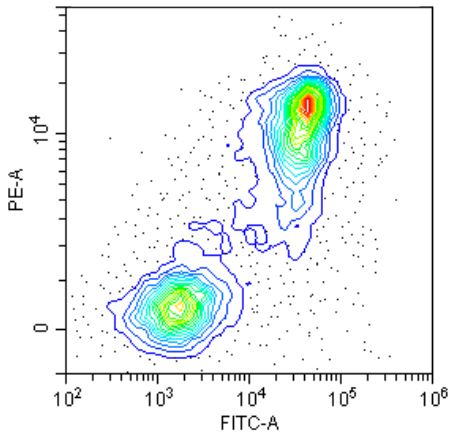
Compensation matrix		
Channel	**-FITC%**	**-PE%**
FITC		0.00
PE	41.07	

D



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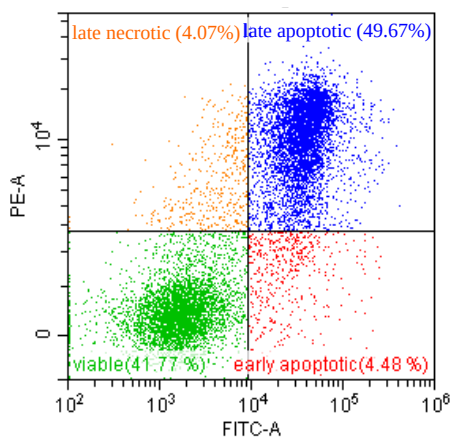
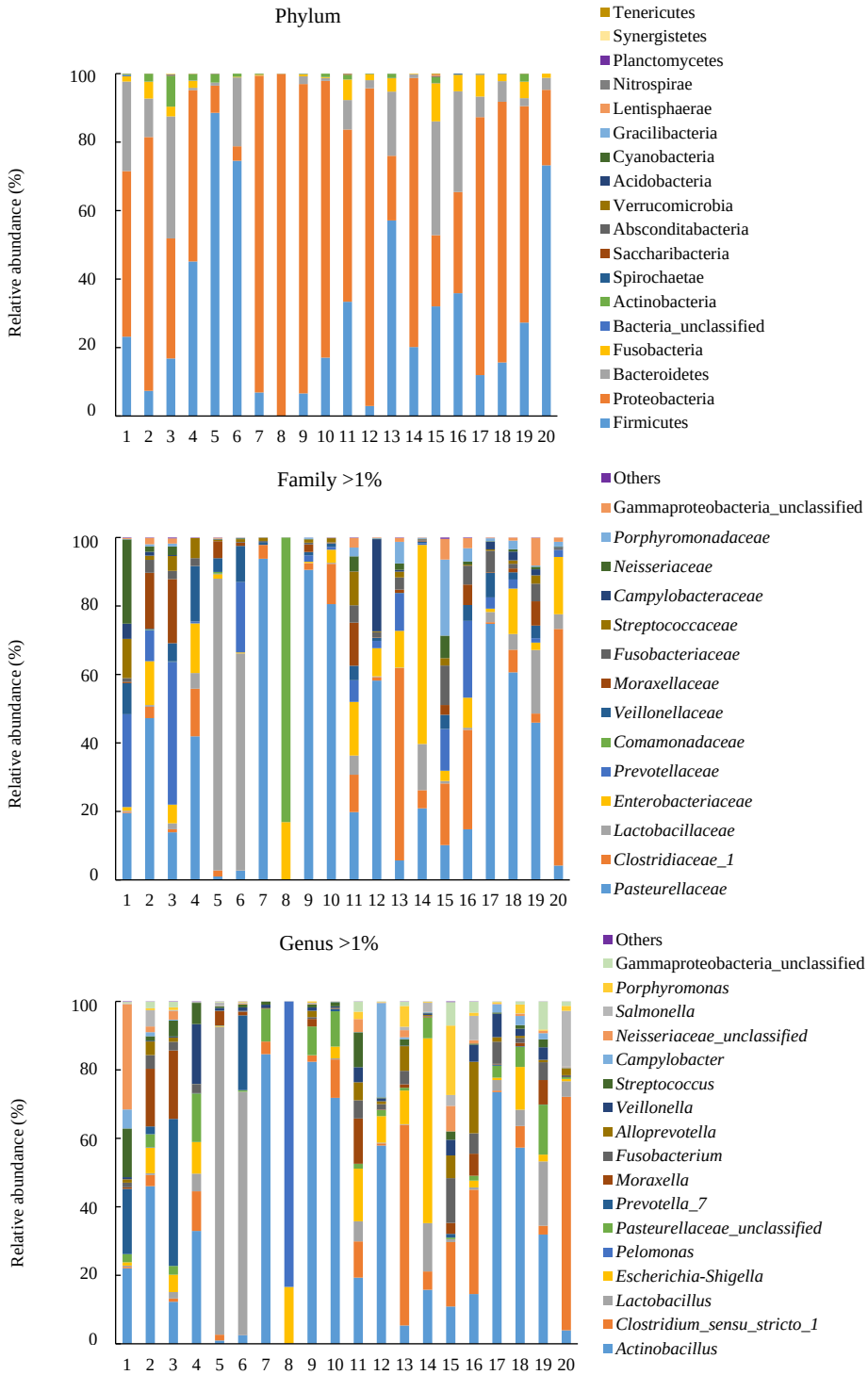
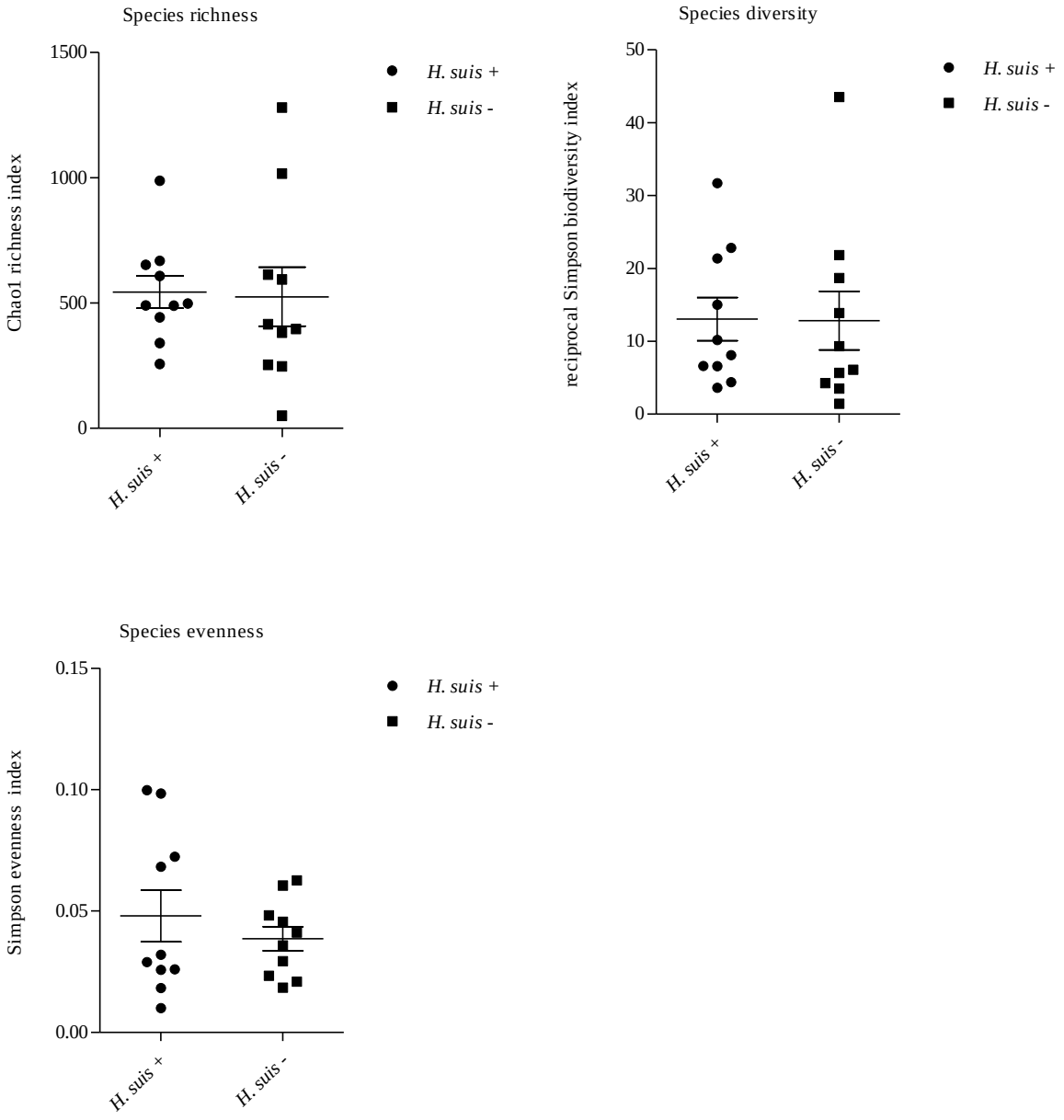


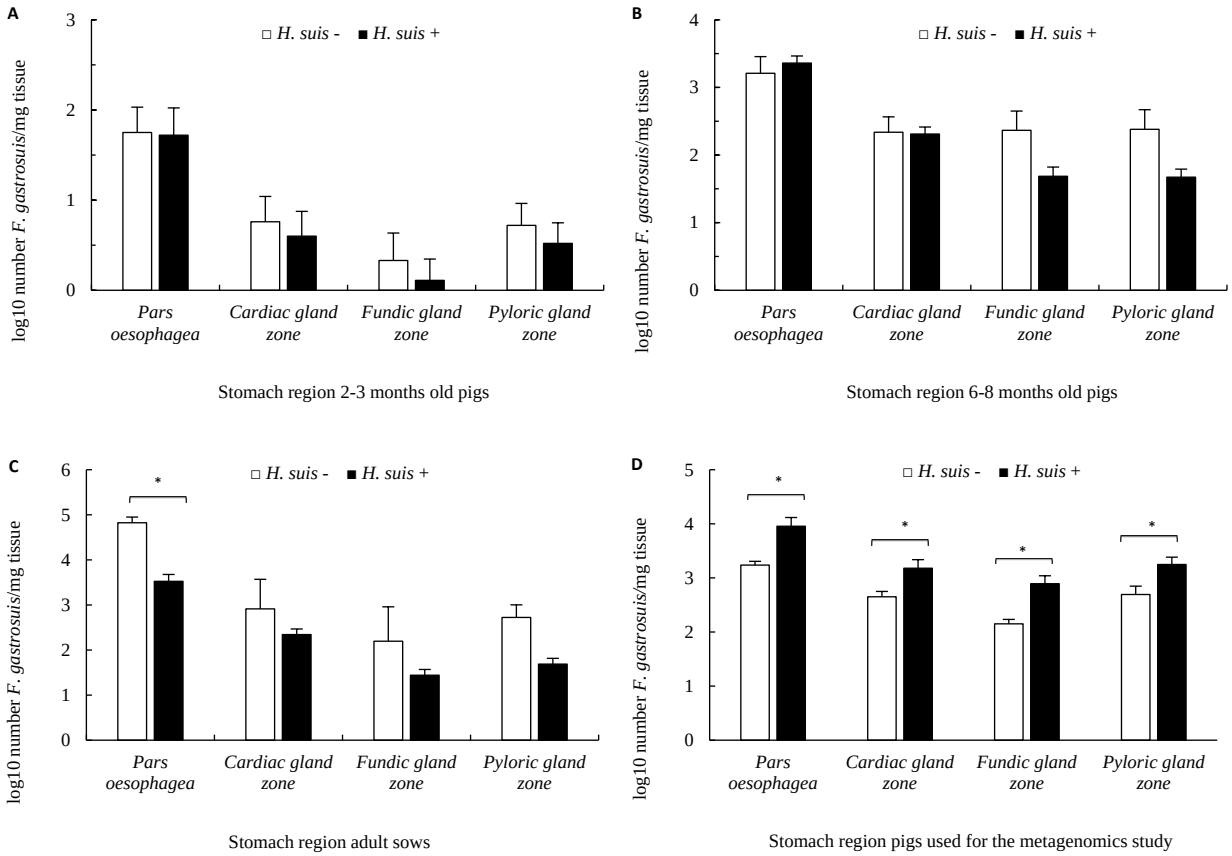
Additional file 3: Gating strategy of the KYSE-450 cell line. (A) FSC-A/SSC-A represents the distribution of cells in the light scatter based on their size and intracellular complexity, respectively. The cells of interest are gated excluding debris. (B) FSC-A/FSC-H allows discrimination between single cells and doublets, single cells are gated. (C) Gain settings and compensation matrix. (D-E) FITC-A/PE-A identifies the selective subpopulations: viable (Annexin-V-FITC negative, PI negative), early apoptotic (Annexin-V-FITC positive, PI negative), late apoptotic/necrotic (Annexin-V-FITC positive, PI positive) and late necrotic (Annexin-V-FITC negative, PI positive) cells.



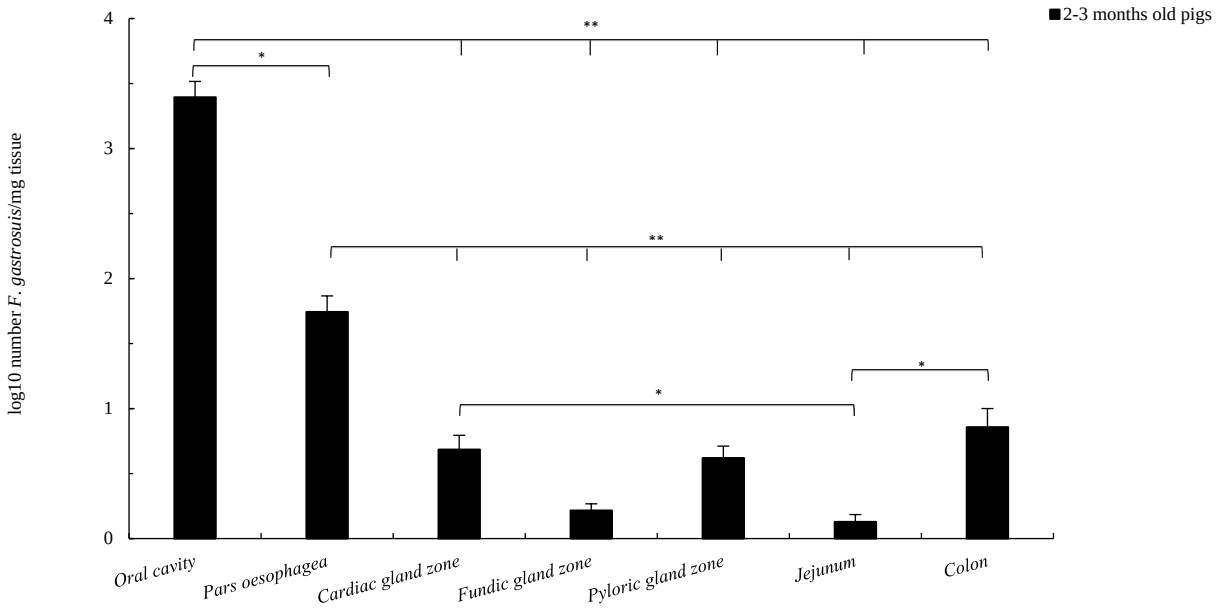
Additional file 4: Bacterial community compositions present in the *Pars oesophagea* of each individual pig. The cumulated histograms show the relative abundance of the identified taxa at phylum, family and genus level. At family and genus level, taxa with a relative abundance <1% are merged in the category "others". 1-10 = *H. suis*-negative pigs, 11-20 = *H. suis*-positive pigs. The unclassified populations correspond to defined groups of the genus level for which a taxonomical classification assignation to the genus cannot be attributed. These populations are therefore labelled with the first defined superior hierarchical taxonomic level followed by "_unclassified" to prevent confusion.



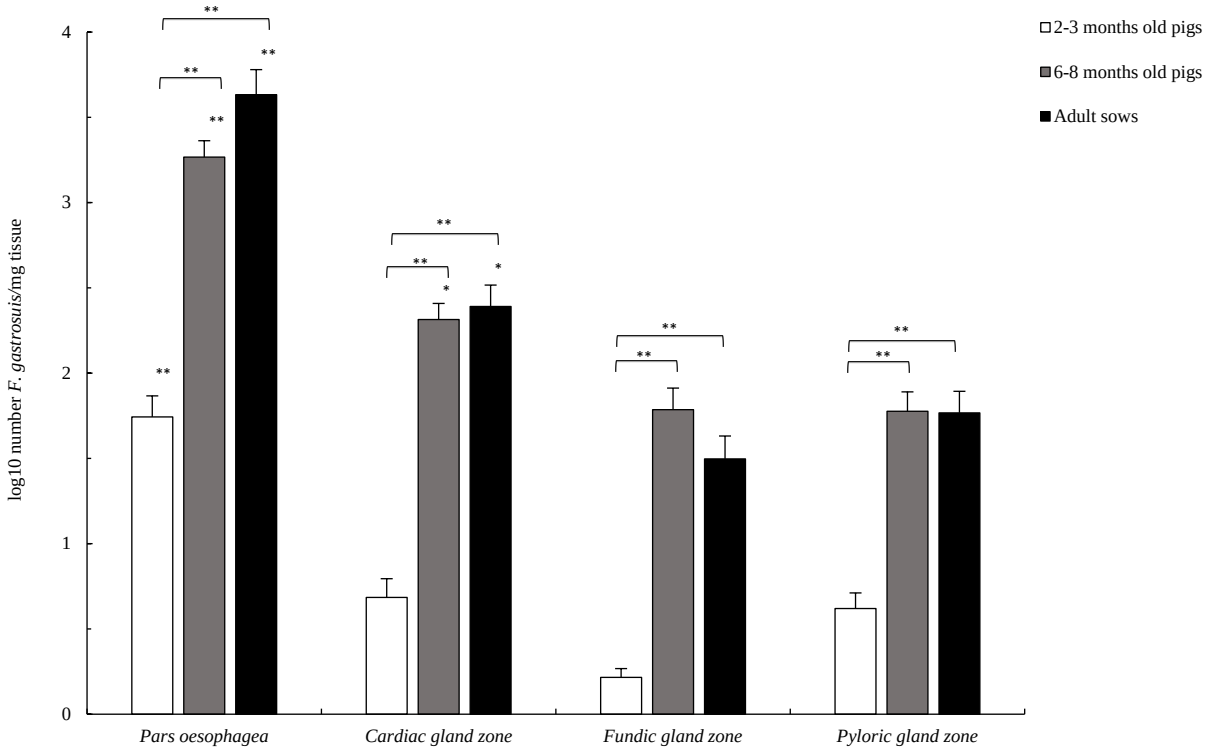
Additional file 5: Overview of the bacterial richness, diversity and evenness of the *Pars oesophagea* of *H. suis*-positive and -negative pigs. The data are represented as scatter plots: each dot represents a pig, the middle line represents the median and the whiskers represent the standard error or the mean.



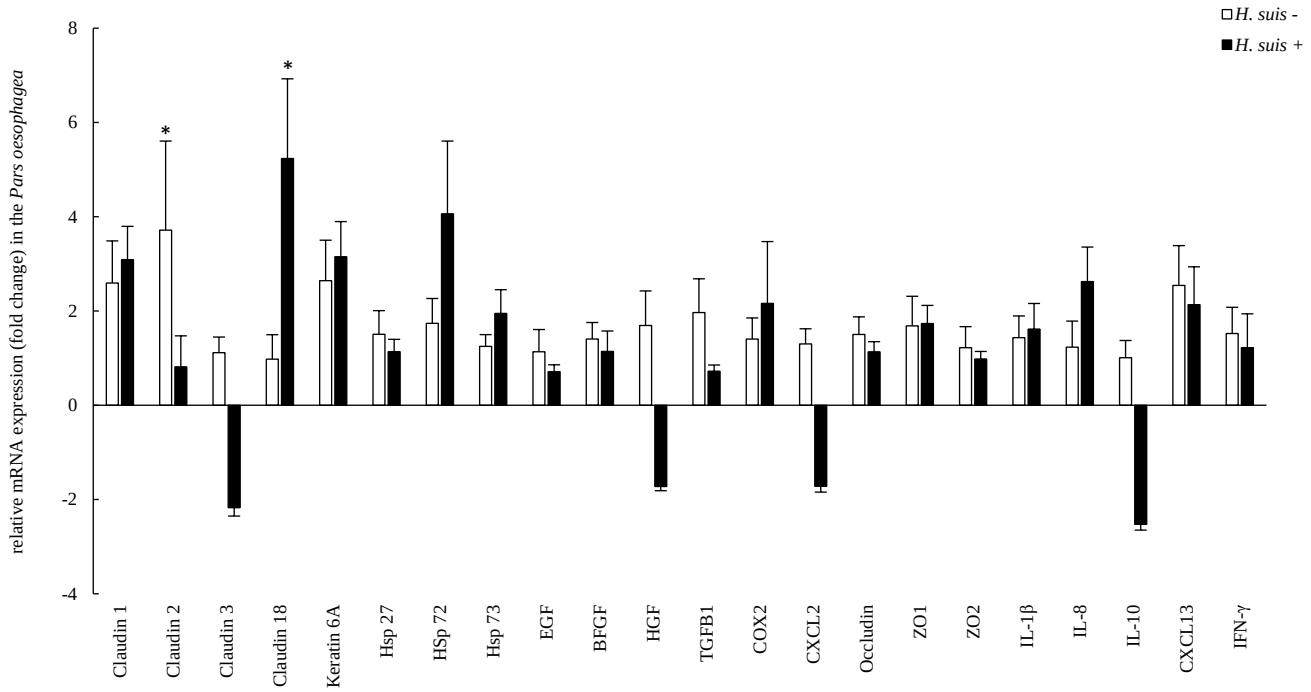
Additional file 6: The number of *F. gastrosuis* bacteria in the different stomach regions of *H. suis*-positive and -negative 2-3 months old pigs (A), 6-8 months old pigs (B), adult sows (C) and the pigs used for the metagenomics study (D). Data are shown as log10 values of the average number of *F. gastrosuis* bacteria per mg tissue with standard deviation. Statistical differences were calculated using the non-parametric Kruskal-Wallis H test. *, $p < 0.05$.



Additional file 7: The number of *F. gastrosuis* bacteria in the oral cavity and gastro-intestinal tract of 2-3 months old pigs. Data are shown as log10 values of the average number of *F. gastrosuis* bacteria per mg tissue with standard deviation. Statistical differences were calculated using the non-parametric Kruskal-Wallis H test. *, $p < 0.05$; **, $p < 0.001$ significant differences between the regions.



Additional file 8: The number of *F. gastrosuis* bacteria in the different stomach regions of 2-3 months old pigs, 6-8 months old pigs and adult sows. Data are shown as log10 values of the average number of *F. gastrosuis* bacteria per mg tissue with standard deviation. Statistical differences were calculated using the non-parametric Kruskal-Wallis H test. *, $p < 0.05$; **, $p < 0.001$ significant differences between the stomach regions. Significant differences between the age groups are indicated with brackets.

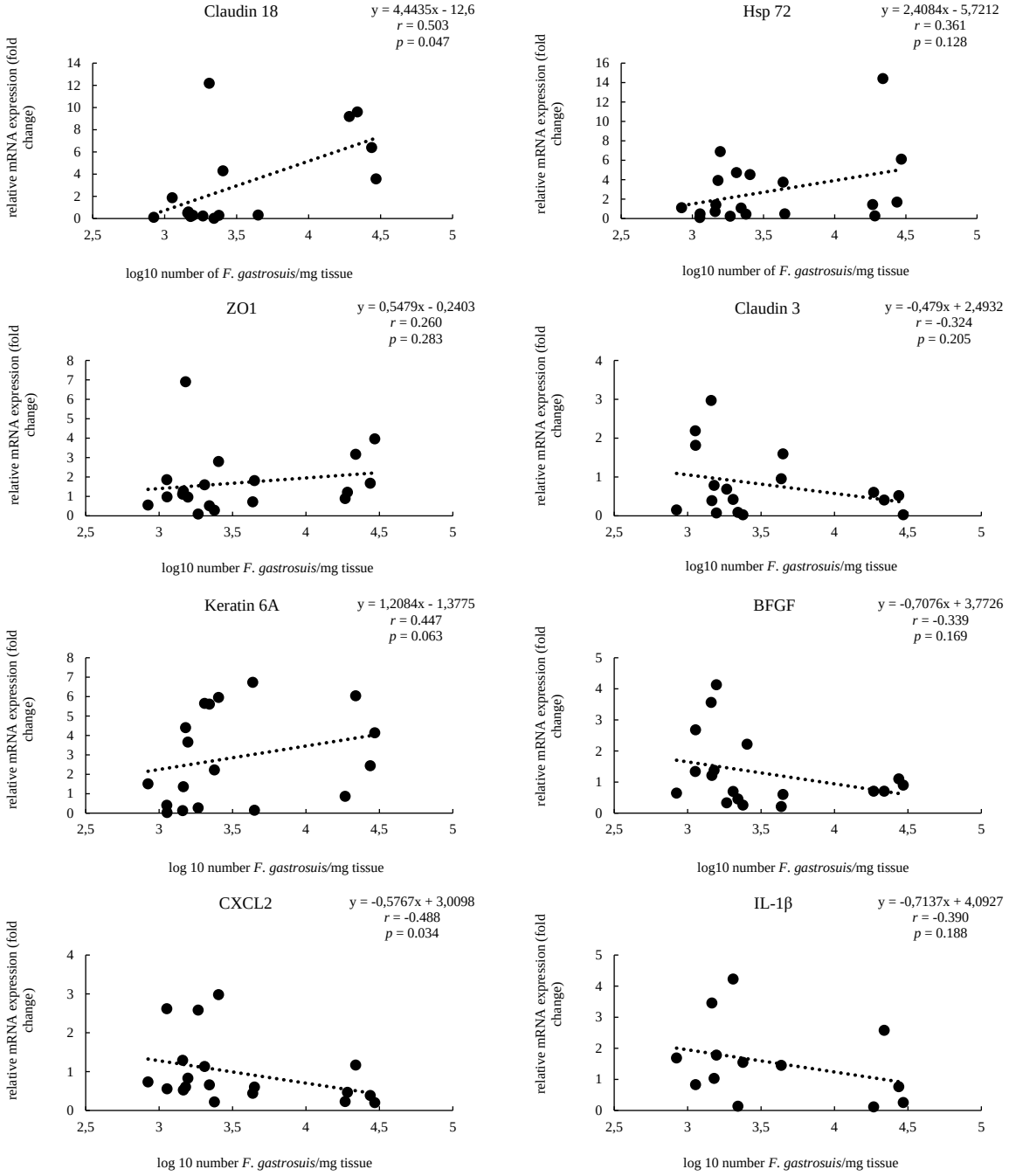


Additional file 9: General overview of gene expression analysis of markers for inflammation and ulceration in the *Pars oesophagea* of *H. suis*-negative and -positive pigs. The data are presented as fold changes in gene expression normalized to 3 reference genes and relative to a *H. suis*-negative control pigs. The fold changes are shown as means with the standard error of the mean. Statistical differences were calculated using the non-parametric Kruskal-Wallis H test. *, $p < 0.05$; significant differences between the *H. suis*-positive pigs and -negative pigs.

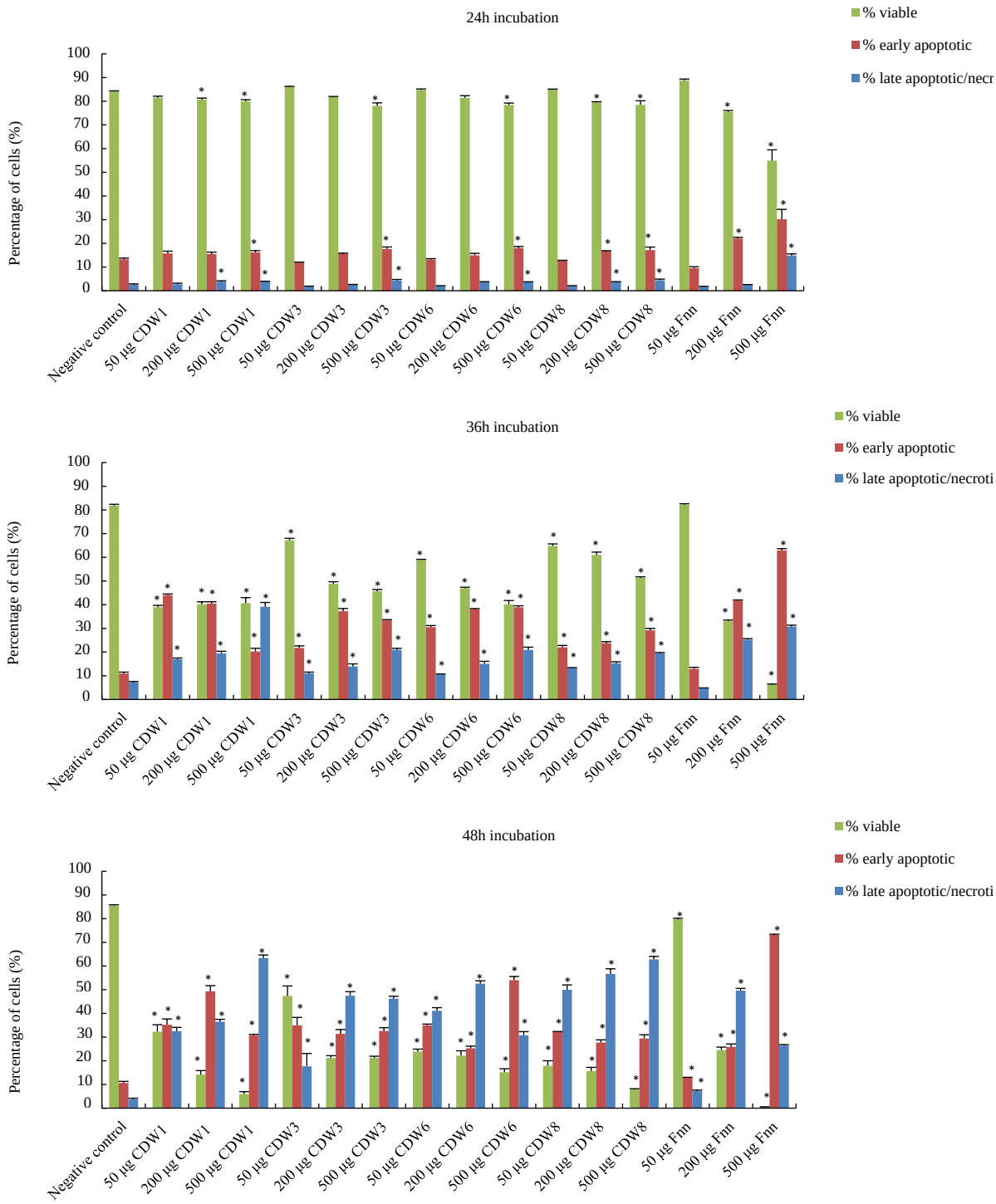
Additional file 10: Overview of relative fold changes of altered markers for inflammation and ulceration in the *Pars oesophagea* of *H. suis*-positive pigs.

Gene	**Relative fold change**	***p*-value**
Claudin 18	5.23 ± 1.69	0.050
Hsp 72	4.06 ± 1.55	0.211
IL-8	2.62 ± 0.73	0.139
Claudin 2	0.81 ± 0.66	0.043
Claudin 3	0.46 ± 0.18	0.093
CXCL2	0.58 ± 0.13	0.065

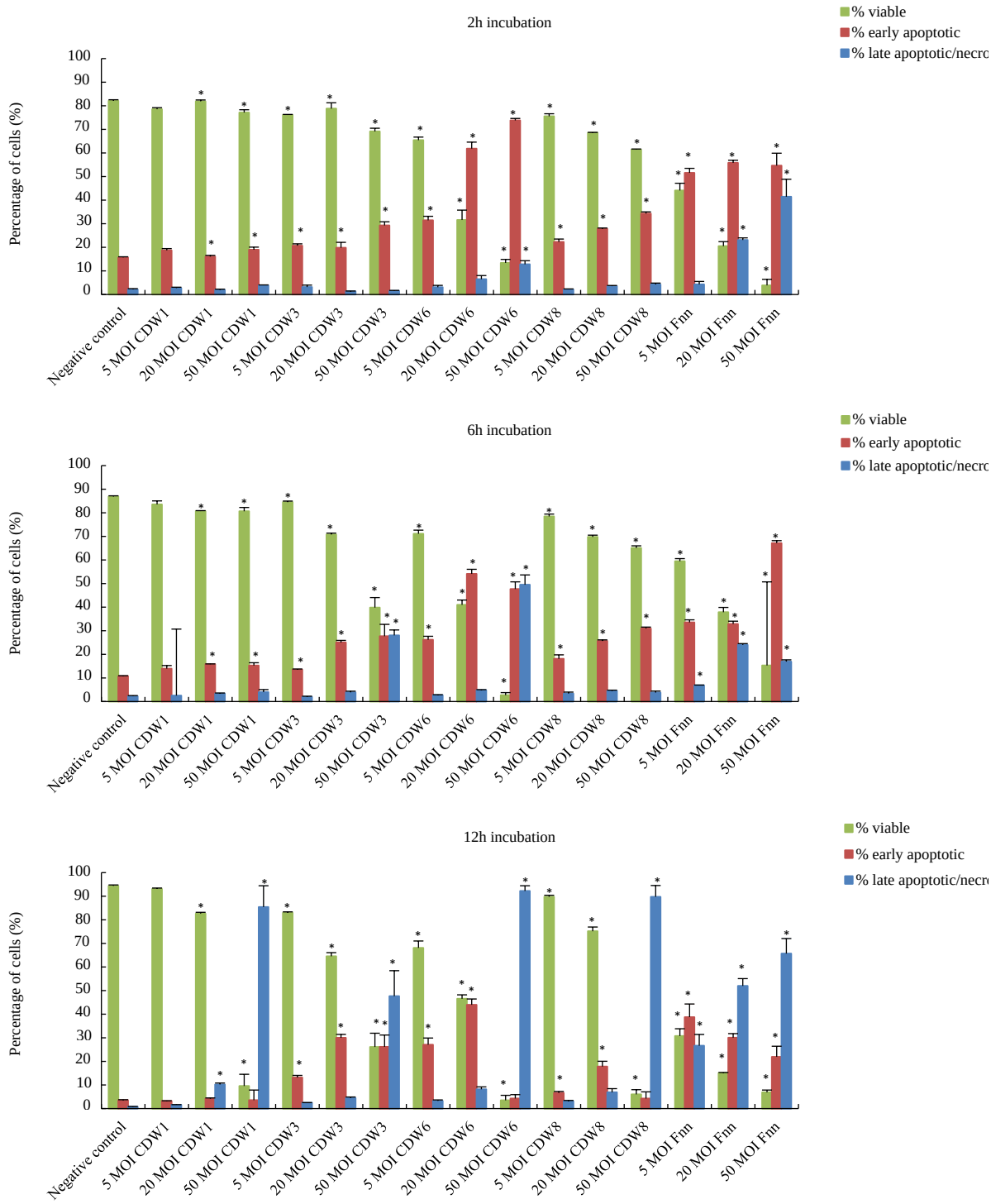
The data are presented as fold changes in gene expression normalized to 3 reference genes and relative to the *H. suis*-negative pigs. The fold changes are shown as means with the standard error of the mean. Statistical differences were calculated using the non-parametric Kruskal-Wallis H test. A *p*-value lower than 0.05 is considered to be significant.



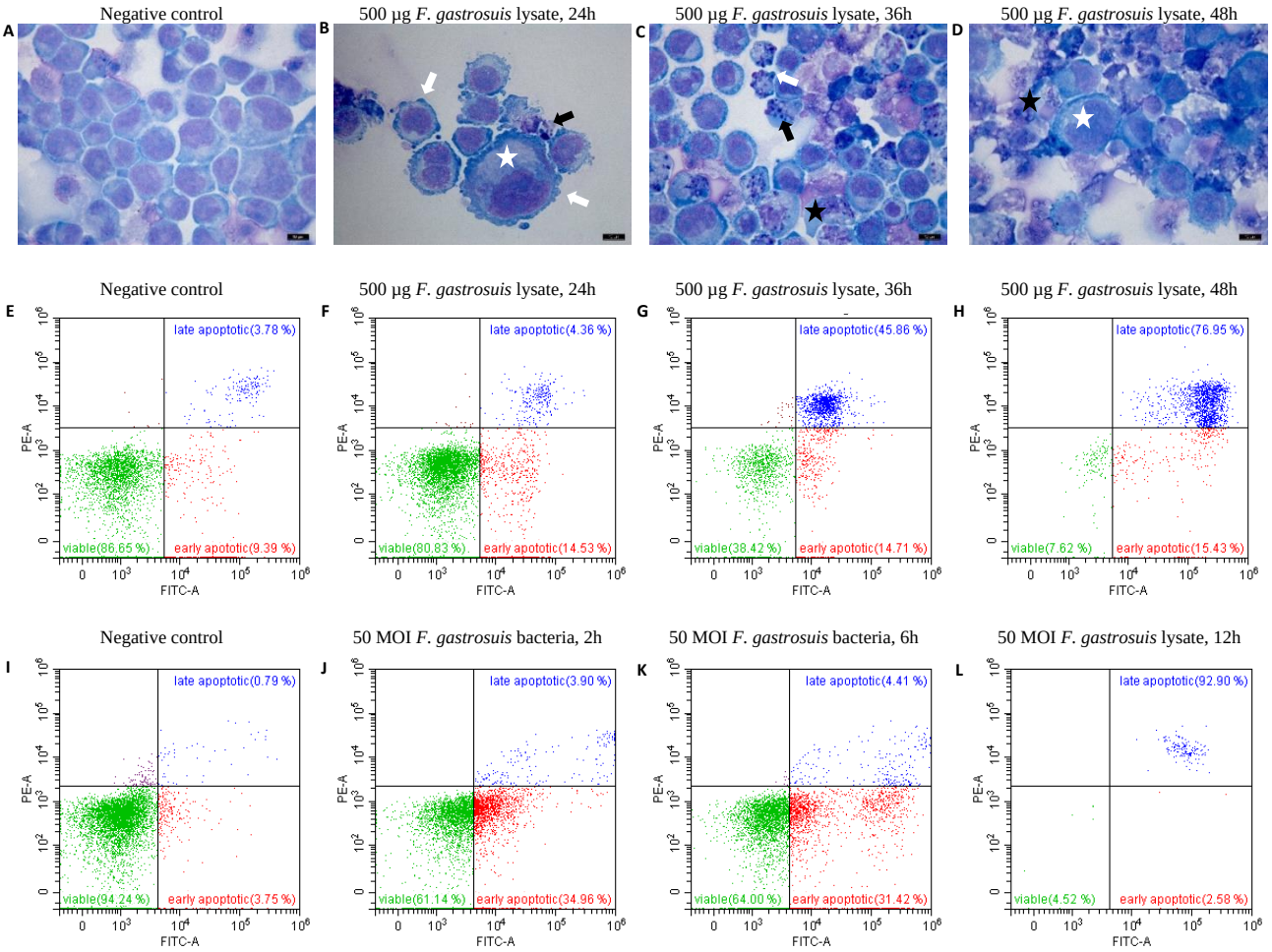
Additional file 11: Correlation between the number of colonizing *F. gastrosuis* bacteria per mg gastric tissue and the expression of markers for inflammation and ulceration. The data are presented as a scatter plot: each dot represents the individual data of a pig. The trendline shows the relationship between the relative mRNA expression of a marker for inflammation/ulceration and the log10 values of the number of *F. gastrosuis* bacteria per mg gastric tissue. y = equation of the trendline. *r* = Pearson correlation coefficient, calculated using SPSS Statistics 24. A *r*-value close to 1 indicates a strong, positive correlation, whereas a *r*-value of −1 indicates a strong, negative correlation. *P*-values lower than 0.05 are considered to be significant.



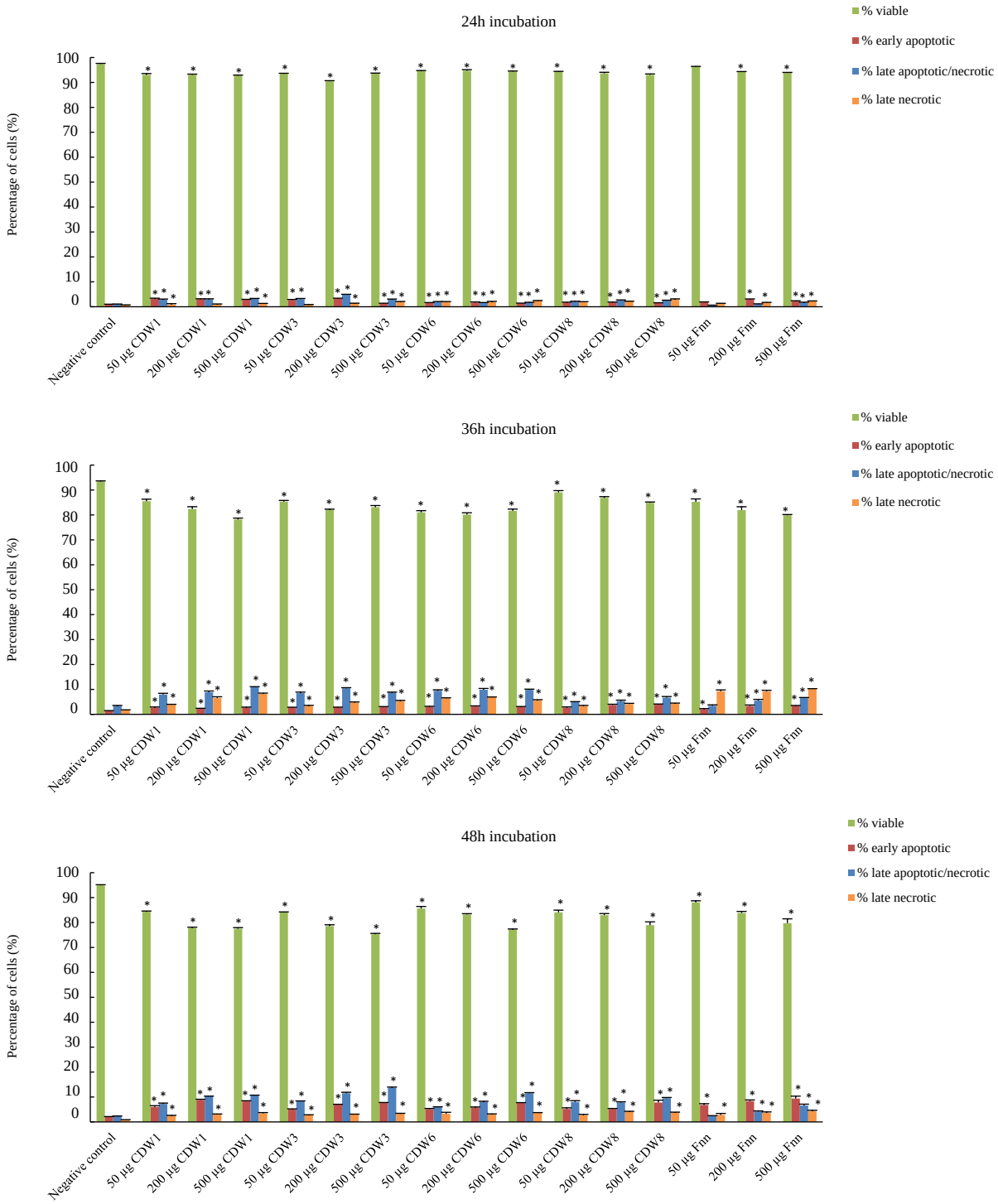
Additional file 12: Percentage of viable, early apoptotic and late apoptotic/necrotic MKN-7 cells after incubation with *F. gastrosuis* lysate. Data are shown as the average (n=3) percentages of viable (green), early apoptotic (red) and late apoptotic/necrotic (blue) MKN-7 cells with standard deviation. The cells were incubated for 24, 36 and 48h with 50 µg, 200 µg and 500 µg *F. gastrosuis* lysate (4 strains, CDW1, 3, 6 and 8) and *F. necrophorum* subsp. *necrophorum* (Fnn) as positive control. * Significant differences between the negative control and cells incubated with each bacterial lysate ($p < 0.05$).

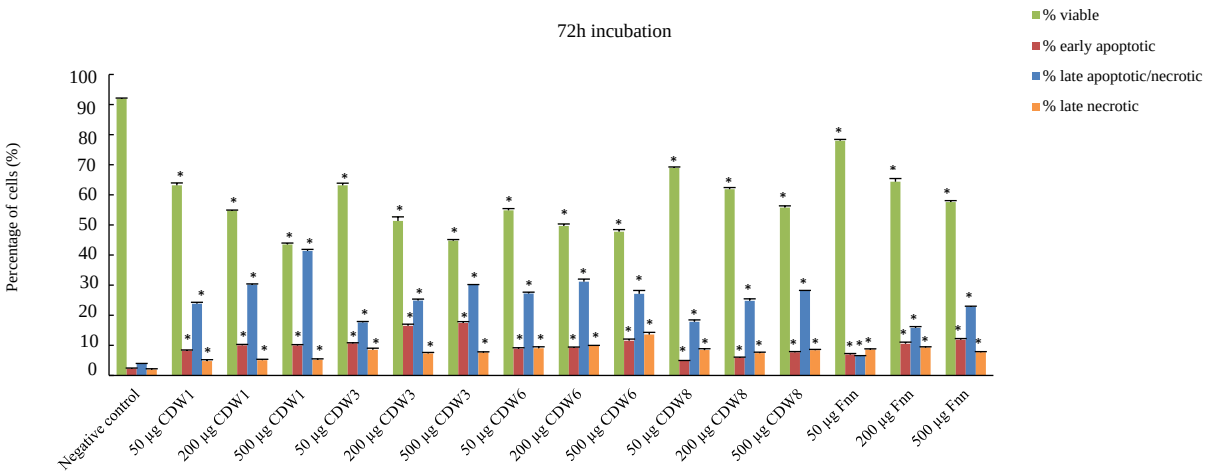


Additional file 13: Percentage of viable, early apoptotic and late apoptotic/necrotic MKN-7 cells after incubation with viable *F. gastrosuis* bacteria. Data are shown as the average (n=3) percentages of viable (green), early apoptotic (red) and late apoptotic/necrotic (blue) MKN-7 cells with standard deviation. The cells were incubated for 2, 6 and 12h with 5 MOI, 20 MOI and 50 MOI viable *F. gastrosuis* bacteria (4 strains, CDW1, 6, 6 and 8) and *F. necrophorum* subsp. *necrophorum* (Fnn) as positive control. * Significant differences between the negative control and cells incubated with each bacterial strain ($p < 0.05$).

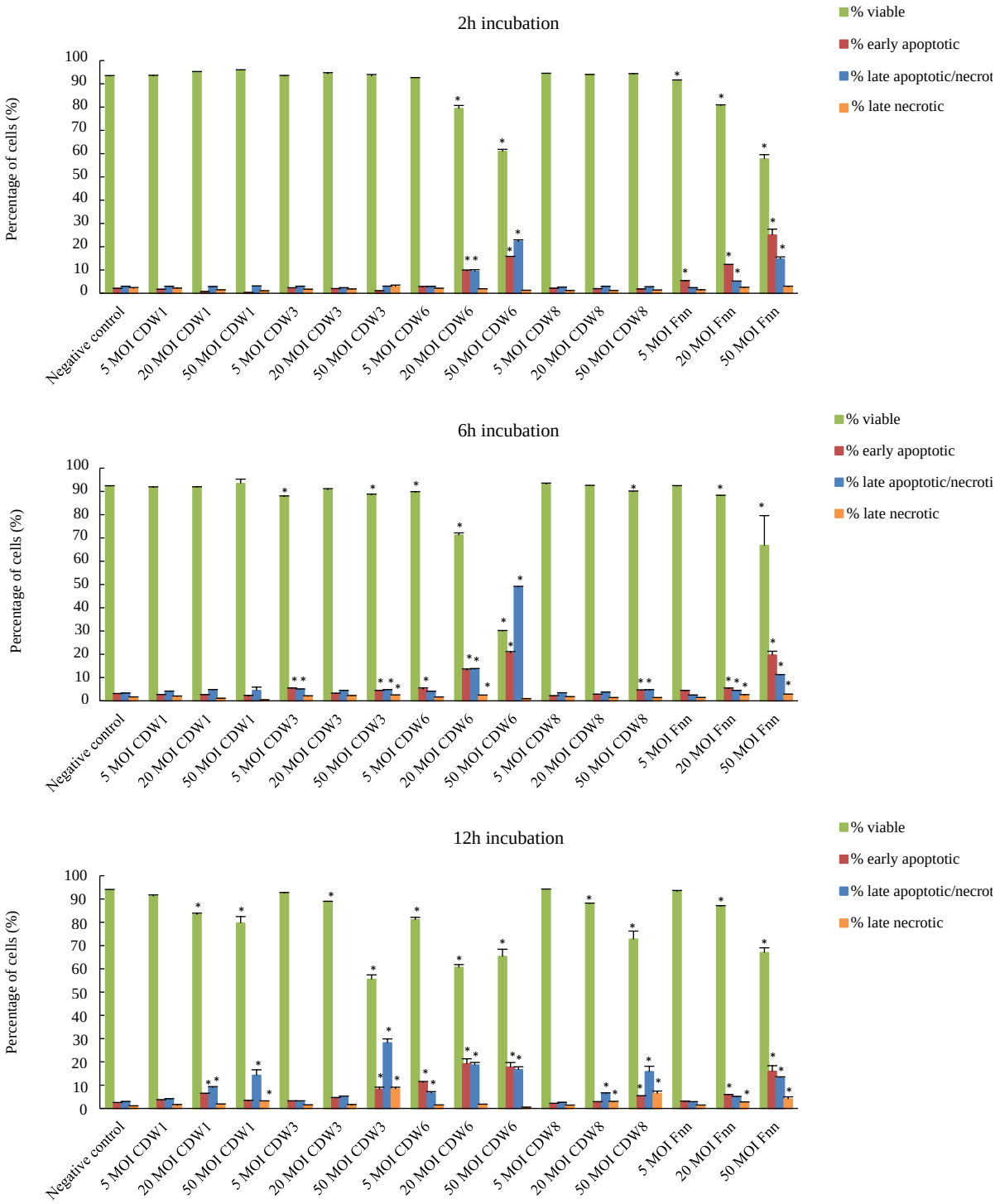


Additional file 14: (A-D) Hemacolor staining of MKN-7 cells incubated (A) without *F. gastrosuis* for 48h and (B-D) with 500 µg *F. gastrosuis* strain CDW1 incubated for (B) 24h, (C) 36h and (D) 48h. Following morphologic features can be seen: plasma membrane blebbing (white arrow), cell swelling (white star), pyknosis (black arrow), cytoplasmic vacuoles (black star). Original magnification x400, scale bar represents 10 µm. (E-L) Representative population plots displaying viable (green, Annexin-V-FITX negative, PI negative), early apoptotic (red, Annexin-V-FITX positive, PI negative), late apoptotic/necrotic (blue, Annexin-V-FITX positive, PI positive) cells of MKN-7 cells incubated (E) without *F. gastrosuis* lysate for 48h; (F-H) with 500 µg *F. gastrosuis* strain CDW1 for (F) 24h; (G) 36h and (H) 48h; (I) without viable *F. gastrosuis* bacteria for 12h; (J-L) with 50 MOI *F. gastrosuis* bacteria strain CDW8 for (J) 2h; (K) 6h and (L) 12h. Y-axis: propidium iodide (PE) signal intensity; X-axis: Annexin-V-fluorescein isothiocyanate (FITC) signal intensity. The percentage of population plots is presented in the corresponding gate.

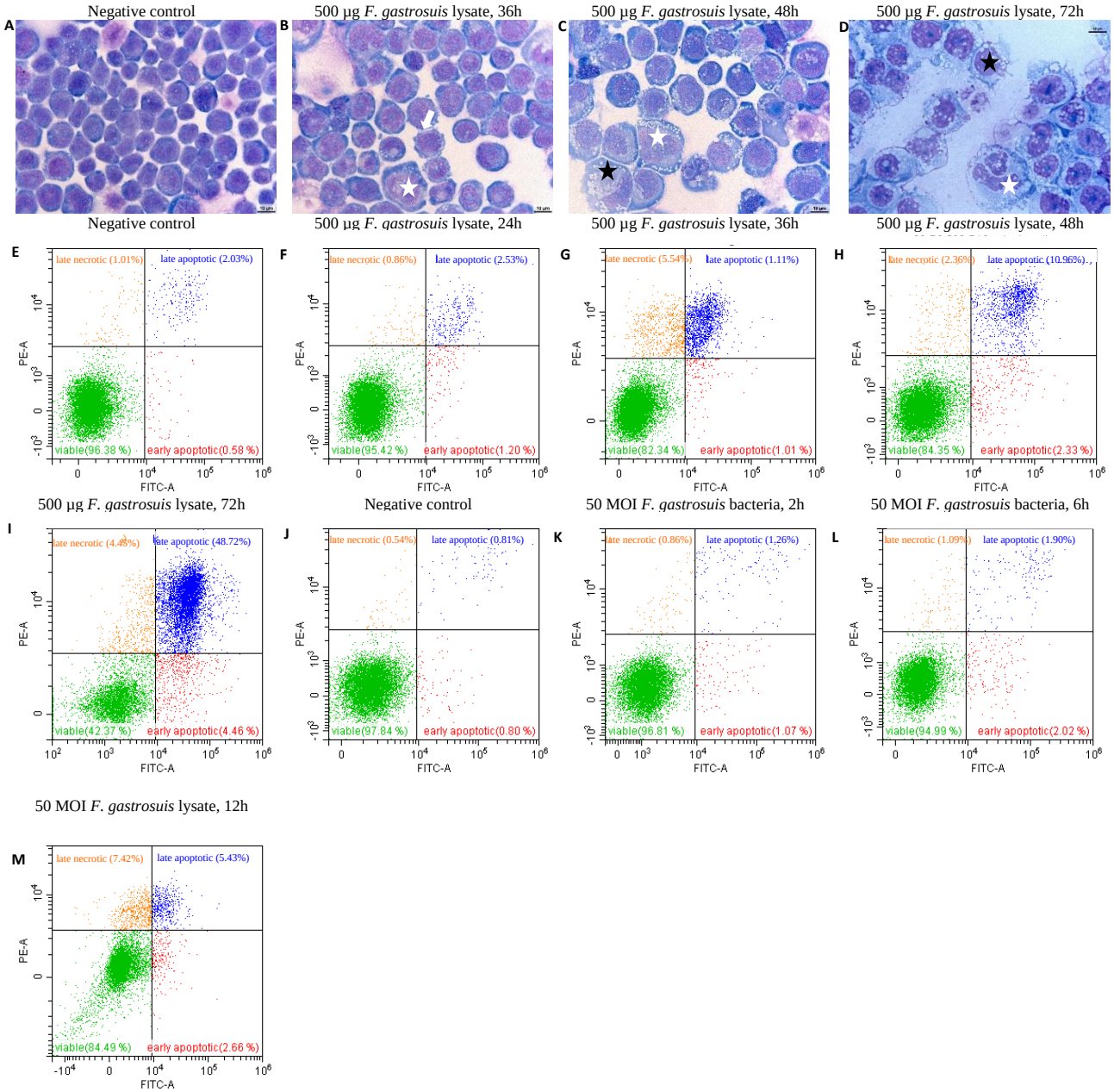

24h incubation
% viable
% early apoptotic
% late apoptotic/necrotic
% late necrotic
Percentage of cells (%)
0
10
20
30
40
50
60
70
80
90
100
Negative control
50 µg CDW1
200 µg CDW1
500 µg CDW1
50 µg CDW3
200 µg CDW3
500 µg CDW3
50 µg CDW6
200 µg CDW6
500 µg CDW6
50 µg CDW8
200 µg CDW8
500 µg CDW8
50 µg Fnn
200 µg Fnn
500 µg Fnn
36h incubation
% viable
% early apoptotic
% late apoptotic/necrotic
% late necrotic
Percentage of cells (%)
Negative control
50 µg CDW1
200 µg CDW1
500 µg CDW1
50 µg CDW3
200 µg CDW3
500 µg CDW3
50 µg CDW6
200 µg CDW6
500 µg CDW6
50 µg CDW8
200 µg CDW8
500 µg CDW8
50 µg Fnn
200 µg Fnn
500 µg Fnn
48h incubation
% viable
% early apoptotic
% late apoptotic/necrotic
% late necrotic
Percentage of cells (%)
Negative control
50 µg CDW1
200 µg CDW1
500 µg CDW1
50 µg CDW3
200 µg CDW3
500 µg CDW3
50 µg CDW6
200 µg CDW6
500 µg CDW6
50 µg CDW8
200 µg CDW8
500 µg CDW8
50 µg Fnn
200 µg Fnn
500 µg Fnn



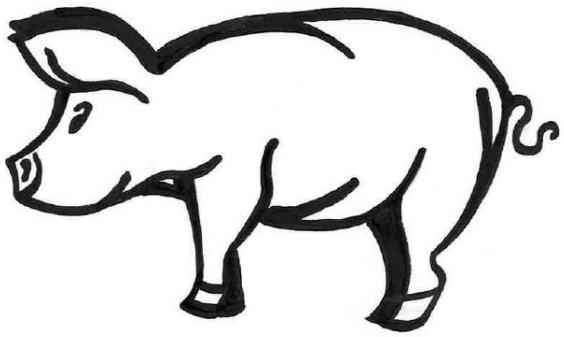
Additional file 15: Percentage of viable, early apoptotic, late apoptotic/necrotic and late necrotic KYSE-450 cells after incubation with *F. gastrosuis* lysate.Data are shown as the average (n=3) percentages of viable (green), early apoptotic (red), late apoptotic/necrotic (blue) and late necrotic (orange) KYSE-450 cells with standard deviation. The cells were incubated for 24, 36, 48 and 72h with 50 µg, 200 µg and 500 µg *F. gastrosuis* lysate (4 strains, CDW1, 3,6 and 8) and *F. necrophorum* subsp. *necrophorum* (Fnn) as positive control. * Significant differences between the negative control and cells incubated with each bacterial lysate ($p < 0.05$).



Additional file 16: Percentage of viable, early apoptotic, late apoptotic/necrotic and late necrotic KYSE-450 cells after incubation with viable *F. gastrosuis* bacteria. Data are shown as the average (n=3) percentages of viable (green), early apoptotic (red), late apoptotic/necrotic (blue) and late necrotic (orange) KYSE-450 cells with standard deviation. The cells were incubated for 2, 6 and 12h with 5 MOI, 20 MOI and 50 MOI viable *F. gastrosuis* bacteria (4 strains, CDW1, 3, 6 and 8) and *F. necrophorum* subsp. *necrophorum* (Fnn) as positive control. * Significant differences between the negative control and cells incubated with each bacterial strain ($p < 0.05$).



Additional file 17: (A-D) Hemacolor staining of KYSE-450 cells incubated (A) without *F. gastrosuis* for 48h and (B-D) with 500 µg *F. gastrosuis* strain CDW1 incubated for (B) 36h, (C) 48h and (D) 72h. Following morphologic features can be seen: plasma membrane blebbing (white arrow), cell swelling (white star), cytoplasmic vacuoles (black star). Original magnification x400, scale bar represents 10 µm. (E-M) Representative population plots displaying viable (green, Annexin-V-FITC negative, PI negative), early apoptotic (red, Annexin-V-FITC positive, PI negative), late apoptotic/necrotic (blue, Annexin-V-FITC positive, PI positive) cells and late necrotic (orange, Annexin-V-FITC negative, PI positive) of KYSE-450 cells incubated (E) without *F. gastrosuis* lysate for 72h; (F-I) with 500 µg *F. gastrosuis* strain CDW1 for (F) 24h; (G) 36h; (H) 48h and (I) 72h; (J) without viable *F. gastrosuis* bacteria for 12h; (K-M) with 50 MOI *F. gastrosuis* bacteria strain CDW8 for (K) 2h; (L) 6h and (M) 12h. Y-axis: propidium iodide (PE) signal intensity; X-axis: Annexin-V-fluorescein isothiocyanate (FITC) signal intensity. The percentage of population plots is presented in the corresponding gate.



Chapter 4: In-feed bambermycin medication induces anti-inflammatory effects and prevents parietal cell loss without influencing *Helicobacter suis* colonization in the stomach of mice

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Abstract

The minimum inhibitory concentration of bambermycin on 3 porcine *Helicobacter suis* strains was shown to be 8 µg/ml. The effect of in-feed medication with this antibiotic on the course of a gastric infection with one of these strains, the host response and the gastric microbiota was determined in mice, as all of these parameters may be involved in gastric pathology. In *H. suis*-infected mice which were not treated with bambermycin, an increased number of infiltrating B cells, T cells and macrophages in combination with increased expressions of IL-8, IL-10, and TNF-α was demonstrated, as well as a decreased parietal cell mass. Compared to this non-treated, infected group, in *H. suis*-infected mice medicated with bambermycin, gastric *H. suis* colonization was not altered, but a decreased number of infiltrating T cells, B cells and macrophages as well as downregulated expressions of IL-1β, IL-8, IL-10 and IFN-γ were demonstrated and the parietal cell mass was not affected. In bambermycin treated mice that were not infected with *H. suis*, the number of infiltrating T cells and expression of IL-1β were lower than in non-infected mice that did not receive bambermycin. Gastric microbiota analysis indicated that the relative abundance of bacteria that might exert unfavorable effects on the host was decreased during bambermycin supplementation. In conclusion, bambermycin did not affect *H. suis* colonization, but decreased gastric inflammation and inhibited the effects of a *H. suis* infection on parietal cell loss. Not only direct interaction of *H. suis* with parietal cells, but also inflammation may play a role in death of these gastric acid producing cells.

Keywords: *Helicobacter suis* – stomach – parietal cells - microbiota – inflammation – bambermycin

Introduction

Helicobacter suis is a Gram-negative, spiral-shaped bacterium found in the stomach of up to 90% of pigs at slaughter age, causing gastritis and a decreased daily weight gain (Hellemans *et al*., 2007; De Bruyne *et al*., 2012). In addition, infection with this bacterium has been associated with ulceration of the non-glandular part of the porcine stomach, most likely through induction of gastric acid secretion alterations and by influencing the gastric microbiota (**Experimental studies: chapter 1**). Apart from pigs, *H. suis* is the most prevalent gastric non-*H. pylori Helicobacter* species in humans. *H. suis* infection in humans has been associated with gastritis, peptic ulcers and mucosa-associated lymphoid tissue (MALT) lymphoma (Haesebrouck *et al*., 2009). Direct or indirect contact with *H. suis*-infected pigs or consumption of contaminated water or raw or undercooked pork may be a source of human infection (De Cooman *et al*., 2013). Since rodent models are easily colonized with *H. suis*, the role of wild mice as vectors might also be considered.

In general, *Helicobacter* spp. infected human patients are treated with a combination of a proton pump inhibitor and two or three antibiotics selected from clarithromycin, amoxicillin, metronidazole, tetracycline and/or levofloxacin (Malfertheiner *et al*., 2012). A similar therapeutic protocol is not indicated in *H. suis*-infected pigs since this is expensive, labor intensive and antibiotic use may favor spread of antimicrobial resistance in pathogens as well as in bacteria belonging to the microbiota (Vermoote *et al*., 2012). In addition, no vaccine formulation is available which completely protects pigs against *H. suis* infection (Haesebrouck *et al*., 2004), making development of an alternative therapy desirable (Haesebrouck *et al*., 2009). Recently, an inhibitory action of bambermycin, a glycolipid antibiotic, has been described towards *H. pylori* strains (Tseng *et al*., 2014). As *H. pylori* is closely related to *H. suis*, it can be hypothesized that bambermycin might inhibit *H. suis* as well.

Bambermycin (synonyms: flavophospholipol, moenomycin, flavomycin) disrupts bacterial cell wall synthesis (Butaye *et al*., 2003) and is mainly active against Gram-positive bacteria and to some extent against Gram-negative bacteria such as *Pasteurella* spp., *Brucella* spp. and *H. pylori* (Butaye *et al*., 2003). Due to its high molecular weight, bambermycin is not absorbed in the gastro-intestinal tract after

oral administration. Until 2006, it was used as a growth promoter in animal feed in the European Union (Butaye *et al*., 2003). The growth promoting effects of bambermycin supplementation have been linked with a better equilibrium of the gastro-intestinal microbiota which might be due to a reduced colonization of pathogens, such as *Salmonella enterica*, *Clostridium perfringens* and *Fusobacterium* spp. (Stutz and Lawton, 1984; Bolder *et al*., 1999; Butaye *et al*., 2003; Edwards *et al*., 2005), while bacteria considered to have beneficial effects, such as *Lactobacillus* spp., are not affected. The effect on *S. enterica* and *C. perfringens* in the intestinal tract, however, contrasts with the relative insensitivity of these species to bambermycin *in vitro* (Butaye *et al*., 2003). Although bambermycin was frequently used as a feed additive for over 50 years, acquired resistance, transfer of resistance or cross-resistance with other antimicrobials has not yet been reported (Butaye *et al*., 2003). It has been described that bambermycin selectively inhibits the growth of *Escherichia coli* and *S. enterica* harboring resistance plasmids and decreases the conjugation transfer frequency of resistance plasmids in *E. coli*, *S. enterica* and *Staphylococcus aureus* (George *et al*., 1982; Riedl *et al*., 2000; Van den Bogaard *et al*., 2002).

The main objective of this study was to determine the effect of bambermycin supplementation on the course of a *H. suis* infection, the host response and the gastric microbiota, as all these parameters may be involved in gastric pathology. BALB/c mice were used as an experimental model, as in previous studies we showed that these rodents can easily be colonized with *H. suis*, resulting in gastritis, epithelial cell hyperproliferation and necrosis of parietal cells (Flahou *et al*., 2010), which are also representing the main characteristics of a human and porcine infection with this agent.

A second objective of our study was to determine if in-feed medication with bambermycin might be useful to control *H. suis* infections in pigs.

Materials and methods

Minimum Inhibitory Concentration (MIC) of bambermycin on H. suis *strains*

H. suis *strains*

H. suis strains HS1, HS8 and P13/26 were isolated from the gastric mucosa of pigs from different herds according to the method described by Baele *et al.* (Baele *et al*., 2008). The strains were shown to be genetically different by multilocus sequence typing (Liang *et al*., 2013).

All strains were cultured under biphasic and microaerobic conditions at 37°C. The biphasic medium consisted of Brucella agar (BD, Franklin Lakes, New Jersey, USA) supplemented with 20% fetal calf serum (GE Healthcare Life Sciences, Logan, USA), 5 mg amphotericin B/l (Sigma-Aldrich, Saint Louis, Missouri, USA), Campylobacter selective supplement (Oxoid) and Vitox supplement (Oxoid). The pH of the agar was adjusted to 5 by adding HCl to a final concentration of approximately 0.05%. Finally, Brucella broth (BD, pH 5) was added on top. Isolates were passaged twice to ensure reliable growth. After incubation, the bacteria were harvested and the concentration was determined using an improved Neubauer counting chamber (Sigma-Aldrich).

Determination of MIC-values of bambermycin

The MIC of bambermycin for *H. suis* strains HS1, HS8 and P13/26 was determined according to the method described by Vermoote *et al*. (Vermoote *et al*., 2011). In brief, a combined Brucella agar and broth dilution method in 24-well plates was used. Twofold dilutions of bambermycin (flavomycin, Huvepharma, Antwerp, Belgium) were added to the agar and broth, with final concentrations ranging from 0.03 to 128 µg/mL. In total, 5×10^7 bacteria/mL were added to the broth and incubated during 48h under microaerobic conditions. After incubation, *H. suis* was quantified using a quantitative real-time (RT)-PCR where the *ureA* gene was amplified. The MIC was determined as the lowest concentration of bambermycin for which ΔC_t was at least 1 C_t higher than ΔcC_t (ΔC_t = C_t after incubation - C_t before incubation of the bambermycin exposed strains; ΔcC_t = C_t after incubation - C_t before incubation of the

controls; C_t = threshold cycle value) (Vermoote *et al*., 2011). This is the lowest concentration of bambermycin with at least 50% less bacterial growth compared to controls without bambermycin.

S. aureus ATCC 29213 was included as a reference strain. For this strain, 4 different MIC assays were performed: the broth microdilution procedure according to the CLSI standards, the method described by Butaye *et al*. (Butaye *et al*., 2000; Watts *et al*., 2008), the *H. suis* susceptibility assay conditions at pH 5 and the same conditions but at pH7.

Effect of bambermycin supplemented diet on a H. suis *infection in mice*

Ethic statement

The *in vivo* experimental protocol was approved by the Ethical Committee of the Faculty of Veterinary Medicine, Ghent University, Belgium (EC 2015/131; November 17th, 2015).

Animals and experimental design

Forty-eight specific-pathogen-free (SPF) female, five-week-old BALB/cOlaHsd mice were purchased from Harlan NL (Horst, the Netherlands). All animals tested negative for presence of *Helicobacter* spp. by the use of a *Helicobacter* genus specific PCR. The animals were randomly divided into 6 groups, each consisting of 8 mice. The groups were assigned as followed: non-*H. suis* infected mice fed a control diet without bambermycin (*H. suis*-negative control group; group 1), non-*H. suis* infected mice fed a 32 ppm bambermycin supplemented diet (group 2), non-*H. suis* infected mice fed a 64 ppm bambermycin supplemented diet (group 3), *H. suis*-infected mice fed a control diet without bambermycin (*H. suis*-positive control group, group 4), *H. suis*-infected mice fed a 32 ppm bambermycin supplemented diet (group 5) and *H. suis*-infected mice fed a 64 ppm bambermycin supplemented diet (group 6). For each group, the mice were housed on autoclaved soft wood shavings in 2 separate filter-top cages to minimize cage-effect (i.e. 4 mice per cage). Drinking water was provided ad libitum. The animals were monitored several times a day during the whole experiment. Enrichment was provided in the form of paper tissues,

mouse houses and other homemade available enrichment products. All animals were exposed to a 12:12 light:dark cycle in the same stable under controlled environmental conditions.

One week after arrival, groups 4-6 were inoculated twice with *H. suis* strain HS1 with a 48h interval. Under brief isoflurane anesthesia and using a ball-tipped gavage needle, 300 µl Brucella broth (pH 5) containing 7×10^7 bacteria of HS1 was administered intragastrically. Groups 1-3 received an equal volume of Brucella broth (pH 5). The mice were held in an upright position until they regained consciousness, to minimize the risk of reflux. Starting from 1 week after inoculation, the mice were fed ad libitum a control diet (groups 1 and 4), a diet supplemented with 32 ppm bambermycin (groups 2 and 5) or a diet supplemented with 64 ppm bambermycin (groups 3 and 6). The bambermycin supplemented diets were identical to the control diet, with the exception of being supplemented with 32 or 64 ppm bambermycin and with a variable amount of corn starch, used to correct minor differences in the energy content (Research Diets Inc., New Brunswick, USA). Finally, 9 weeks after the second inoculation, the mice were euthanized by cervical dislocation under deep isoflurane anesthesia. The stomachs were removed and opened along the major curvature. Four longitudinal strips were taken from the forestomach to the duodenum. One strip was fixed in 10% phosphate-buffered formalin and used for histopathology and immunohistochemistry. The other strips were used for DNA- and RNA-extraction.

Histopathology and immunohistochemistry

The formalin fixed longitudinal strip from the stomach was embedded in paraffin, sectioned at 5 µm, rehydrated and deparaffinized.

For each stomach, one of the sections was stained with haematoxylin and eosin, dehydrated and finally mounted with a coverslip for light microscopic evaluation. The severity of gastritis was scored according to the Updated Sydney System with some modifications (Dixon *et al*., 1996; De Bruyne *et al*., 2012). Both diffuse infiltration with inflammatory cells and the presence of lymphoid aggregates and lymphoid follicles in the mucosa and submucosa were taken into consideration. The diffuse infiltration of mononuclear and polymorphonuclear cells was scored as follows: score 0 for absence of infiltration,

score 1 for mild infiltration, score 2 for moderate infiltration and score 3 for marked infiltration. In addition, the formation of lymphoid follicles was scored as follows: score 0 for absence of lymphoid aggregates, score 1 for presence of a small number of lymphoid aggregates ($n < 5$), score 2 for a large number of lymphoid aggregates ($n > 5$) and/or the presence of 1 organized lymphoid follicle and score 3 for the presence of at least 2 organized lymphoid follicles. Based on the scoring of the diffuse infiltration with inflammatory cells and the presence of lymphoid aggregates and lymphoid follicles, an overall gastritis score was obtained. Therefore, the average score was calculated for each group. When an overall score of $0 < n \leq 1$; $1 < n \leq 2$ or $2 < n \leq 3$ was obtained, the gastritis was considered as mild, moderate and severe, respectively.

The other sections were used to determine *H. suis* colonization density, to study the presence of lymphoepithelial lesions, to analyze the number of infiltrating T cells, B cells and macrophages and, finally, to determine the number of parietal cells, necrotic cells and replicating cells. After rehydration and deparaffinization, heat-induced antigen retrieval was performed in citrate buffer (pH 6) using a microwave oven. Slides were incubated with 3% H_2O_2 in methanol (5 min) to block endogenous peroxidase activity and 30% goat serum (30 min) to block non-specific reactions. Negative controls to confirm the specificity of the secondary antibodies were obtained by incubating the sections without the primary antibodies.

H. suis colonization was visualized using a polyclonal genus-specific rabbit anti-*H. pylori* antibody (1/320; DakoCytomation, Glostrup, Denmark). For detection of infiltrating T- and B cells, a polyclonal rabbit anti-CD3 antibody (1/100; DakoCytomation, Glostrup, Denmark) and a polyclonal rabbit anti-CD20 antibody (1/25; Thermo Scientific, Fremont, USA) were used, 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 (Agilent Technologies, Santa Clara, California, USA) and the color was developed with diaminobenzidine tetrahydrochloride (DAB) and H_2O_2.

To determine the infiltrating macrophages, a primary antibody directed against the F4/80 surface marker (1/50; Santa Cruz Biotechnology, Santa Cruz, USA) was used. Detection was done using a rat ABC staining system (Santa Cruz Biotechnology). Apoptotic cells were identified by immunohistochemical staining 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 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 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 CD3 and CD20 staining. A monoclonal mouse anti-cytokeratin antibody (1/50; DakoCytomation) was used to highlight lymphoepithelial lesions.

H. suis colonization density was scored according to the updated Sydney System (Dixon *et al*., 1996). T cells, B cells, macrophages, apoptotic cells, replicating cells and parietal cells were counted in five randomly chosen High Power Fields (magnification: ×400). The average number of positive cells per High Power Field was then calculated for each mouse.

Quantification of H. suis *by RT-PCR*

DNA was extracted from the second gastric tissue strip using the Isolate II Genomic DNA Kit (Bioline, Taunton, USA), according to the instructions of the manufacturer. The presence of *H. suis* DNA was determined using a species-specific, RT-PCR based on the *ureA* gene (Blaecher *et al*., 2013). The copy number of the obtained amplicons was calculated and converted to the number of *H. suis* bacteria per mg gastric tissue, by including 10-fold dilutions of an external standard consisting of a 1,236 bp segment of the *ureAB* gene cluster from *H. suis* strain HS5, as described previously (O'Rourke *et al*., 2004).

Expression of markers for inflammation and gastric acid secretion

RNA was extracted from the third gastric tissue strip using the RNeasy Mini Kit (Qiagen, Hilden, Germany), according to the manufacturer's instructions. The obtained RNA concentrations were measured using a NanoDrop spectrophotometer (Isogen Life Science, Utrecht, The Netherlands), after which the concentration of each sample was adjusted to 1 μg/μl, followed by cDNA synthesis using the iScript™ cDNA Synthesis Kit (Bio-Rad, California, USA). Expression analysis was then performed for genes encoding host factors involved in gastric acid secretion (H^+/K^+ ATPase, Sonic Hedgehog, KCNQ1, gastrin, the cholinergic muscarinic M3 receptor, somatostatin, the histamine H2 receptor and the gastrin CCK-B receptor) and in inflammation (IL-4, IL-6, IL-8, IL-10, IL-17, Il-1β, IFN-γ and TNFα). The housekeeping genes *PPIa*, *H2afz* and *HPRT* were shown to have a stable mRNA expression and therefore included as reference genes (Flahou *et al*., 2012). All primer sequences are shown in Additional file 1. The mRNA expression levels of the reference and target genes were quantified using a RT-PCR, as described earlier (Flahou *et al*., 2012). No-template-control reaction mixtures were included and all samples were run in duplicate. The Ct-values were first normalized to the geometric mean of the Ct-values of the reference genes. Fold changes were calculated using ΔΔCt method with mean of Ct-values from the control groups (groups 1 and 4). Finally, for each target gene, the results were expressed as fold changes of the mRNA expression of groups 2-4 and 5-6 relative to mRNA expression levels of the *H. suis*-negative control group and the *H. suis*-positive control group, respectively.

Gastric microbiota composition

DNA was extracted from the fourth gastric tissue strip using the DNeasy Blood & Tissue Kit (Qiagen) according to the instructions of the manufacturer. 16S rRNA amplicon pyrosequencing was performed using the Roche GS-Junior Genome Sequencer as described previously by Rodriguez *et al.* (Rodriguez *et al*., 2015). The obtained 16S rRNA sequence reads were processed using MOTHUR (software package v1.35), Pyronoise algorithm and UCHIME algorithm for alignment and clustering, denoising and chimera detection, respectively (Schloss *et al*., 2009; Haas *et al*., 2011). The obtained read sets were

compared to a reference dataset of aligned sequences of the corresponding region derived from the SILVA database (v1.15) of full-length rRNA sequences implemented in MOTHUR (Pruesse *et al.*, 2007). The final reads were clustered into operational taxonomic units (OTUs) using the nearest neighbor algorithm using MOTHUR with a 0.03 distance unit cutoff. A taxonomic identity was attributed to each OTU by comparison with the SILVA database using a 80% homogeneity cutoff. When a taxonomic identification lower than 80% was obtained, the taxonomic level was labelled with the first defined level from higher level followed by the label "unclassified". Finally, the unique sequences for each OTU were compared with the SILVA data set using the BLASTN algorithm. For each OTU, a consensus taxonomic identification was given when less than 1% of mismatch with the aligned sequence was obtained. In the final metadata table, the following labelling was used: the population is identical to a taxonomically defined species and is labelled "genus_species"; the population is identical to a reference sequence belonging to a still undefined species and is labelled "genus_NCBI accession number"; the sequence is not identical to any known sequence and is labelled with the corresponding OTU number.

In order to determine the effect of *H. suis* and/or bambermycin supplementation on the gastric microbiota composition, different groups were compared with each other:

(i) *H. suis*-negative mice without bambermycin supplementation (i.e. group 1) (n = 5) vs. *H. suis*-negative mice with bambermycin supplementation (i.e. groups 2-3) (n = 7), *H. suis*-positive mice without bambermycin supplementation (i.e. group 4) (n = 4) and *H. suis*-positive mice with bambermycin supplementation (n = 9) (i.e. groups 5-6)

(ii) *H. suis*-negative mice (i.e. groups 1-3) vs. *H. suis*-positive mice (i.e. groups 4-6)

(iii) Mice without bambermycin supplementation (i.e. groups 1 and 4) vs. mice with bambermycin supplementation (i.e. groups 2-3 and 5-6).

Subsampled datasets were obtained and evaluated in MOTHUR to estimate the richness, microbial diversity and population evenness by using the Chao1 estimator, Simpson's reciprocal index and Simpson's evenness index, respectively (Chao and Bunge, 2002; Chao and Shen, 2003). Population

structure and community membership were assessed with MOTHUR using distance matrix based on Bray-Curtis dissimilarity index. Differences in functional profiles of gastric bacterial communities were analyzed by mapping taxa into several phenotypes (i.e. metabolism, Gram staining, sporulation,…) using METAGENassist (Arndt *et al*., 2012). Only the phenotypes detected in more than 50% of the samples were included for further analysis.

Statistical analysis

Statistical analysis was performed using SPSS statistics 24 (IBM, New York, USA). Differences in histopathology and fold changes of the markers for gastric acid secretion and inflammation were investigated using the non-parametric Kruskal-Wallis test with Bonferroni correction for multiple comparisons. Correlations between histopathology, fold changes and the number of *H. suis* bacteria were examined using the Pearson correlation coefficient. Differences were considered statistically significant at a corrected *p*-value of less than 0.05.

Statistical differences in microbial diversity, richness and population evenness between the groups were investigated using non-parametric Kruskal-Wallis tests with Tukey post-hoc tests using PRISM 7 (Graphpad Software). Using MOTHUR, community composition differences were investigated using Analysis of molecular variance (AMOVA) and homogeneity of molecular variance (HOMOVA). In order to highlight statistical differences in relative bacterial abundance between the groups, non-parametric Kruskal-Wallis tests with Tukey post-hoc tests and Benjamini-Hochberg False Discovery Rate were performed using the STAMP software. Differences were considered statistically significant at a corrected *p*-value of less than 0.05.

Results

MIC of bambermycin on H. suis *strains*

For all investigated *H. suis* strains, the MIC of bambermycin was 8 μg/ml. For the reference strain *S. aureus* ATCC 29213 the MIC-value was 0.12 μg/ml when tested according to the method described by Butaye *et al*. (Butaye *et al*., 2000; Watts *et al*., 2008) and 0.5 μg/ml when tested according to the CLSI standards, while MIC-values of 1 and 2 μg/ml were obtained using the *H. suis* susceptibility assay conditions at pH 7 and 5, respectively.

Effect of bambermycin supplementation on H. suis *colonization in mice*

Non-*H. suis* infected groups 1, 2 and 3 tested negative for presence of *H. suis* as determined by RT-PCR and immunohistochemical staining, while all *H. suis*-infected groups 4, 5 and 6 tested positive for *H. suis*. In both bambermycin-supplemented diet groups, the number of colonizing *H. suis* bacteria per mg gastric tissue as well as the colonization density was not altered compared to the *H. suis*-positive control group without bambermycin supplementation (Additional file 2). In all *H. suis*-infected groups, *H. suis* bacteria were often found in the lumen of the gastric glands and in close proximity of parietal cells (Additional file 3).

Effect of bambermycin supplementation on H. suis*-infection associated pathologies in mice*

Inflammation

An overview of the defined immune cell populations of each group is shown in Figure 1.

The *H. suis*-positive control group 4 showed significantly higher numbers of infiltrating B cells and macrophages compared to the *H. suis*-negative control group 1 ($p = 0.016$ and 0.003, respectively) (Figure 2). Furthermore, a trend towards a higher number of infiltrating T cells was seen in all *H. suis*-infected groups 4-6 compared to the non-infected groups 1-3, although this was not significant (Figures 1 and 2).

Compared to the *H. suis*-positive control group 4, a lower number of infiltrating B cells and macrophages was demonstrated in the 32 ppm bambermycin supplemented, *H. suis*-infected group 5 ($p = 0.068$ and 0.001, respectively) as well as in the 64 ppm bambermycin supplemented, *H. suis*-infected group 6 ($p = 0.003$ and 0.005, respectively) (Figure 2). In comparison to both groups that did not receive bambermycin (groups 1 and 4), all bambermycin-supplemented diet groups showed significantly lower scores for infiltration with inflammatory cells as well as infiltrating T cells ($p < 0.005$ and < 0.001, respectively) (Figures 1 and 2). No significant differences were detected in the formation of lymphoid follicles between the groups.

The infiltration with inflammatory cells was positively correlated with the number of infiltrating T cells and macrophages, while the number of infiltrating T cells was positively correlated with the number of infiltrating B cells and macrophages (Additional file 4). No correlations were found between the number of inflammatory cells and the number of colonizing *H. suis* bacteria per mg gastric tissue.

Positive correlations were found between the number of infiltrating macrophages and the expression of IL-8Kc, IL-8Li, IL-10, IL-17, IFN-γ, TNF-α and IL-1β; between the number of infiltrating T cells and the expression of IL-8Li, IL-10 and IL-1β and between the number of infiltrating B cells and the expression of IL-4, IL-10 and IL-12 (Additional file 4).

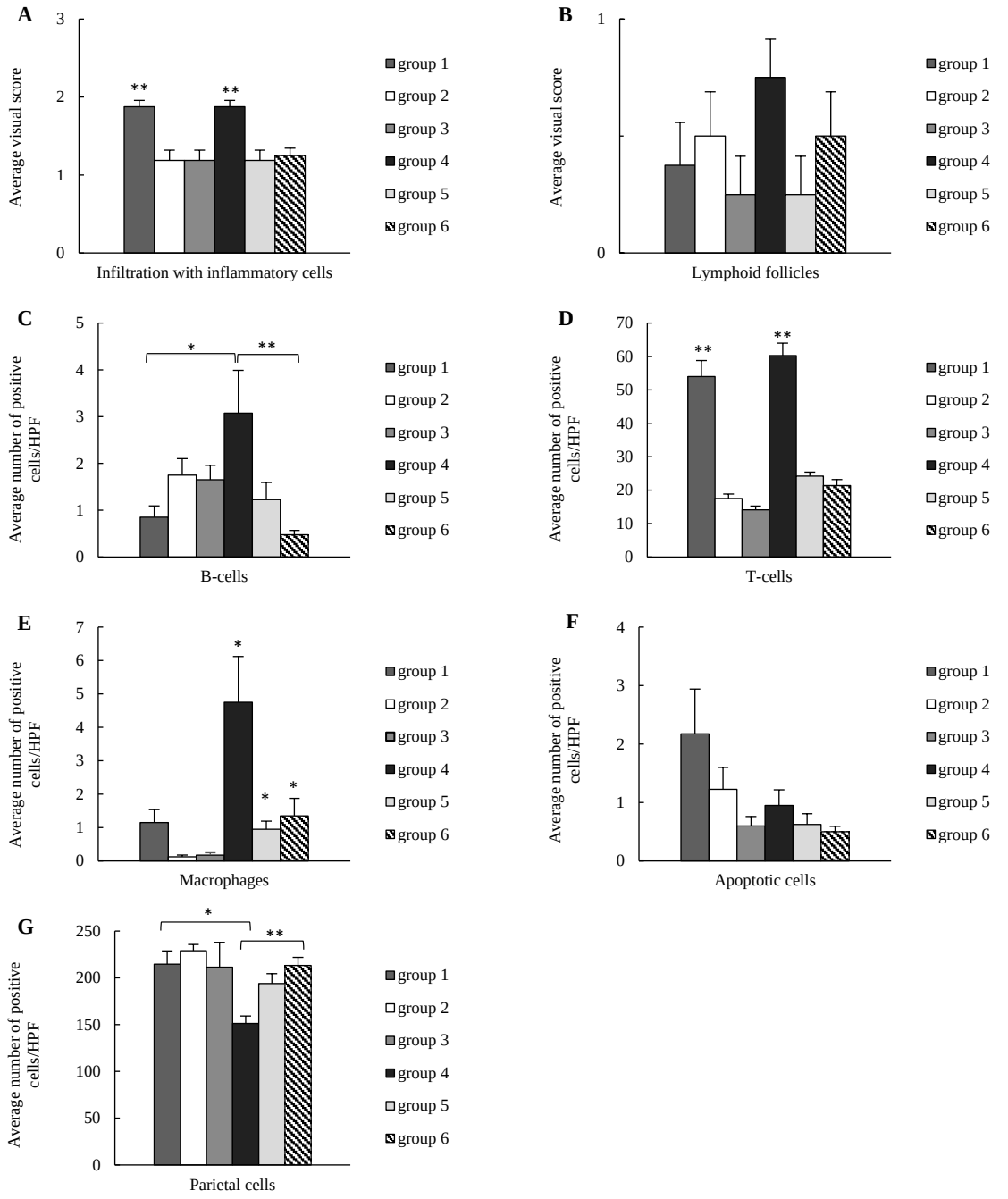

Figure 1: Scores for infiltration with inflammatory cells (A) and lymphoid follicle formation (B); number of infiltrating B cells (C), T cells (D), macrophages (E); number of apoptotic cells (F) and parietal cells (G) in bambermycin supplemented and non-supplemented groups. (A-B). Data are shown as the average scores for infiltration with inflammatory cells / lymphoid follicle formation, with standard deviation, of group 1-6. (C-G) Data are shown as the average number of positive cells per High Power Field (HPF), with standard deviation, belonging to a defined cell population, including B cells, T cells, macrophages, necrotic cells and parietal cells, of group 1-6. Statistical differences were calculated using the non-parametric Kruskal-Wallis H test SPSS statistics 24. * Significant differences between the groups ($p < 0.05$), ** significant differences between the groups ($p < 0.005$). Group 1 = *H. suis*-negative control group without bambermycin supplementation; group 2 = 32 ppm bambermycin supplemented, non-*H. suis* infected group; group 3 = 64 ppm bambermycin supplemented, non-*H. suis* infected group; group 4 = *H. suis*-positive control group without bambermycin supplementation; group 5 = 32 ppm bambermycin supplemented, *H. suis*-infected group; group 6 = 64 ppm bambermycin supplemented, *H. suis*-infected group.

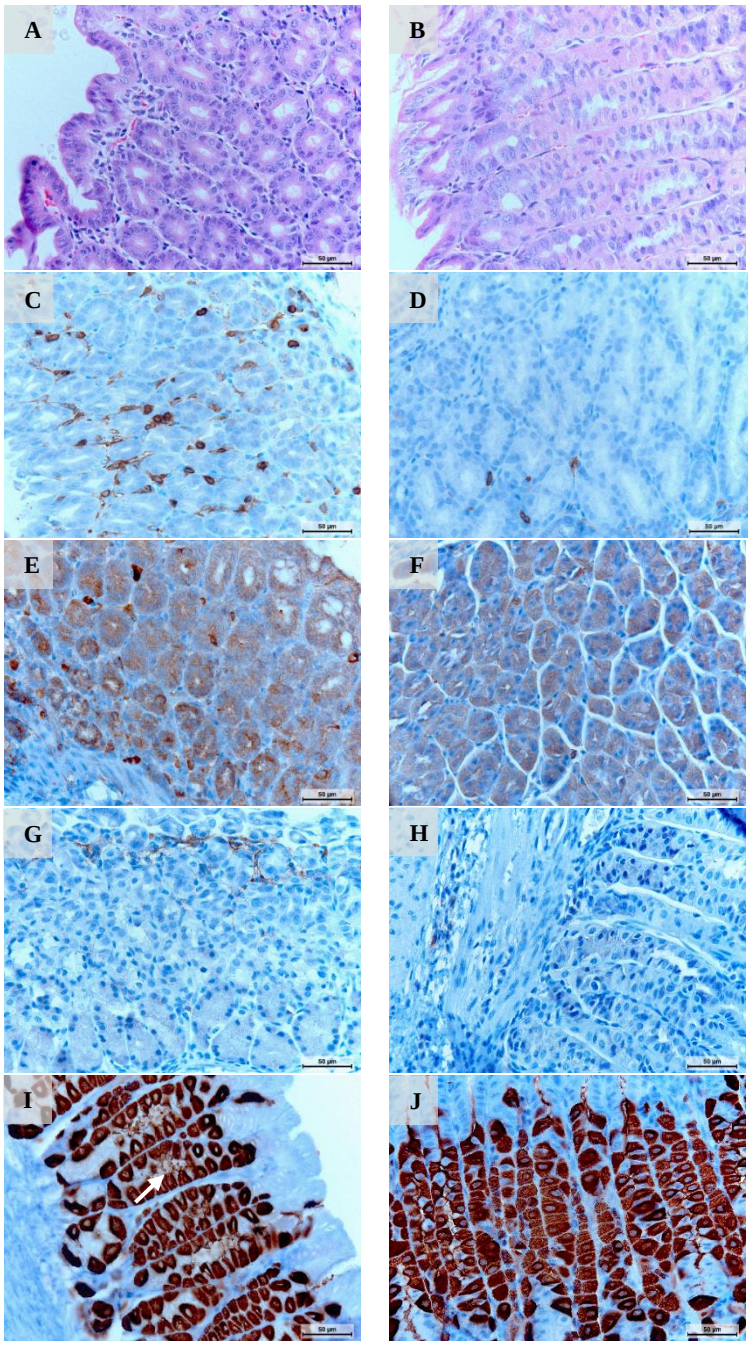

Figure 2: Microscopic visualization of immune cells, apoptotic cells, proliferative cells and parietal cells in the mouse stomach. Original magnification: x400, scale bar: 50 µm. (A-B): HE-staining showing infiltration with mononuclear and polymorphonuclear cells in *H. suis*-infected mice without (A) and with bambermycin (B) supplementation. The infiltration is less severe during bambermycin supplementation. (C-D): CD3-staining showing infiltration with T cells (brown) in *H. suis*-infected mice without (C) and with bambermycin (D) supplementation. The infiltration is less severe during bambermycin supplementation. (E-F): CD20-staining showing infiltration with B cells (dark brown) in mice infected with (E) and without *H. suis* (F). The infiltration is more severe during *H. suis* infection. (G-H): F4/80 staining showing infiltration with macrophages (brown) in mice infected with (G) and without (H) *H. suis*. The infiltration is more severe during *H. suis* infection. (I-J): H^+/K^+ ATPase -staining showing parietal cells (brown) in mice infected with (I) and without (J) *H. suis*. The number of parietal cells is lower during *H. suis* infection. *H. suis* bacteria can be seen in the lumen of the gastric glands (arrow).

Gastric epithelial cell proliferation and death

An overview of the defined cell populations of each group is shown in Figure 1.

The *H. suis*-positive control group 4 showed significantly lower numbers of parietal cells compared to the *H. suis*-negative control group 1 ($p = 0.032$) (Figures 1 and 2). The 64 ppm bambermycin supplemented, *H. suis*-infected group 6, however, showed significantly higher numbers of parietal cells compared to the *H. suis*-positive control group 4 ($p = 0.038$). This was also the case for the 32 ppm bambermycin supplemented, *H. suis*-infected group 5, although not significant. The number of apoptotic cells and epithelial cell proliferation did not differ between the groups.

Negative correlations were detected between the number of parietal cells and the number of infiltrating T cells, B cells, macrophages and inflammatory cells in general (Additional file 4).

Effect of bambermycin supplementation on the expression of markers for inflammation

The fold changes of altered markers for inflammation are represented in Figure 3 and Additional file 5.

The *H. suis*-positive control group 4 showed upregulated expressions of IL-4, IL-6, IL-8M, IL-8Li, IL-10, and TNF-α compared to the *H. suis*-negative control group 1 ($p = 0.091$, 0.115, 0.298, 0.002, 0.012 and 0.065, respectively). In addition, IL-17 and IL-23 were significantly downregulated ($p < 0.001$) (Additional file 5, Figure 3). Both bambermycin supplemented, *H. suis*-infected groups 5 and 6 showed upregulated expressions of IL-4, IL-6 and IL-8Li in comparison with the *H. suis*-negative control group 1 (fold change = 3.24 ± 0.89, 2.95 ± 0.64 and 3.25 ± 0.54; $p = 0.118$, 0.066 and 0.002, respectively for group 5; fold change=1.71 ± 0.48, 2.38 ± 0.51 and 3.44 ± 1.03; $p = 0.970$, 0.066 and 0.015, respectively for group 6), while IL-17 and IL-23 transcript levels were downregulated (fold change = 0.05 ± 0.02 and 0.15 ± 0.05; $p < 0.001$ and < 0.001, respectively for group 5; fold change = 0.03 ± 0.01 and 0.22 ± 0.07; $p < 0.001$ and $= 0.001$, respectively for group 6). The expression of TNF-α, however, was not altered during bambermycin supplementation in the *H. suis*-infected groups 5 and 6 in comparison with the *H. suis*-negative control group 1. Since positive correlations were found between both, the

altered fold changes of IL-4, IL-8Li, IL-17 and IL-23 were more pronounced in mice with a higher number of colonizing *H. suis* bacteria per mg gastric tissue (Additional file 4).

Compared to the *H. suis*-positive control group 4, the mRNA expressions of IL-8M and IFN-γ were downregulated in the 32 ppm bambermycin supplemented, *H. suis*-infected group 5 ($p = 0.100$ and 0.015, respectively), as well as the IL-8M, IL-10 and IFN-γ transcript levels in the 64 ppm bambermycin supplemented, *H. suis*-infected group 6 ($p = 0.001$, 0.077, 0.253, respectively).

In both bambermycin supplemented, *H. suis*-negative groups 2 and 3, the expression of IL-1β was significantly downregulated compared to the *H. suis*-negative control group 1 ($p = 0.028$ and 0.015, respectively). A similar observation was found for the 64 ppm bambermycin supplemented, *H. suis*-positive group compared to the *H. suis*-negative control group (fold change = 0.72 ± 0.13; $p = 0.065$).

Effect of bambermycin supplementation on the expression of markers for gastric acid secretion

The fold changes of altered markers for gastric acid secretion are presented in Figure 4 and Additional file 6.

In summary, the *H. suis*-positive control group 4 showed upregulated expressions of KCNQ1 and the CCK-B receptor in comparison with the *H. suis*-negative control group 1 ($p = 0.119$ and 0.032, respectively). In addition, the bambermycin supplemented groups 2-3 and 5 showed upregulated expressions of H^+/K^+ ATPase, Sonic Hedgehog, KCNQ1, M3 receptor and/or CCK-B receptor in comparison with the *H. suis*-negative and/or positive control groups 1 and 4 (Figure 4, Additional file 6).

Positive correlations were found between the altered expressions of KCNQ1, CCK-B receptor and somatostatin and the expressions of IL-8 and IL-10 (Additional file 4).

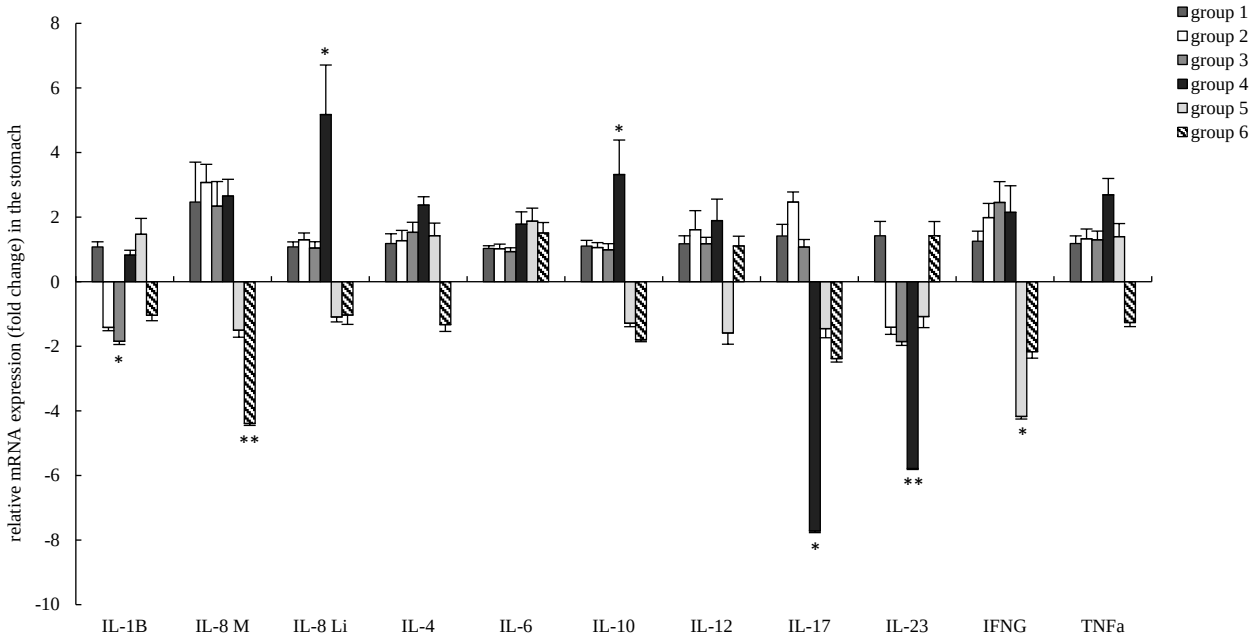

Figure 3: General overview of relative fold changes of altered markers for inflammation in the bambermycin-supplemented and non-supplemented groups. The data are presented as fold changes in gene expression normalized to 3 reference genes and relative to control groups 1 and 4 (i.e. group 2-4 relative to group 1 and group 5-6 relative to group 4) which are considered as 1. The fold changes are shown as means with the standard error of the mean. Statistical differences were calculated using the non-parametric Kruskal-Wallis H test SPSS statistics 24. * Significant differences with the control group ($p < 0.05$), ** significant differences with the control group ($p < 0.005$). Group 1 = *H. suis*-negative control group without bambermycin supplementation; group 2 = 32 ppm bambermycin supplemented, non-*H. suis* infected group; group 3 = 64 ppm bambermycin supplemented, non-*H. suis* infected group; group 4 = *H. suis*-positive control group without bambermycin supplementation; group 5 = 32 ppm bambermycin supplemented, *H. suis*-infected group; group 6 = 64 ppm bambermycin supplemented, *H. suis*-infected group.

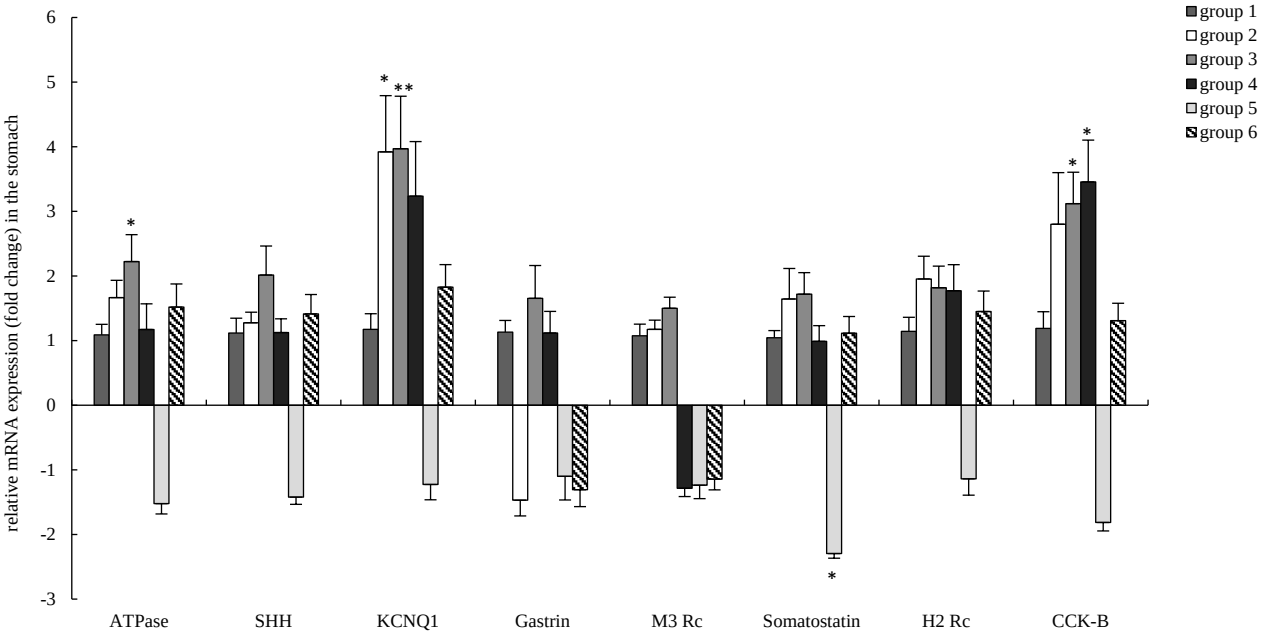

Figure 4: General overview of relative fold changes of altered markers for gastric acid secretion in the bambermycin-supplemented and non-supplemented groups. The data are presented as fold changes in gene expression normalized to 3 reference genes and relative to control groups 1 and 4 (i.e. group 2-4 relative to group 1 and group 5-6 relative to group 4) which are considered as 1. The fold changes are shown as means with the standard error of the mean. Statistical differences were calculated using the non-parametric Kruskal-Wallis H test SPSS statistics 24. * Significant differences with the control group ($p < 0.05$), ** significant differences with the control group ($p < 0.005$). Group 1 = *H. suis*-negative control group without bambermycin supplementation; group 2 = 32 ppm bambermycin supplemented, non-*H. suis* infected group; group 3 = 64 ppm bambermycin supplemented, non-*H. suis* infected group; group 4 = *H. suis*-positive control group without bambermycin supplementation; group 5 = 32 ppm bambermycin supplemented, *H. suis*-infected group; group 6 = 64 ppm bambermycin supplemented, *H. suis*-infected group.

Effect of bambermycin supplementation and/or H. suis *infection on the gastric microbiota composition in mice*

Despite several attempts, including altering the number of pyrosequencing cycles, no sequencing reads could be obtained from 3 mice of the *H. suis*-negative control group 1; 5 of group 2; 4 of group 3; 4 of group 4; 4 of group 5 and 3 of group 6. The other 25 samples yielded sufficient reads after pyrosequencing and were selected for further analysis. Pyrosequencing yielded between 400 and 4,000 reads per sample (Additional file 7). A total of 70,118 final reads were attributed to 1,290 species level OTUs for the 25 samples. Chimeric sequences represented 20% of the total sequencing reads and were thus excluded from the analysis.

In general, total bacterial community analysis showed that the most dominant phylum in the murine stomach was Firmicutes contributing up to 92%, followed by Bacteroidetes (4%) and Proteobacteria (4%). The relative abundance of other phyla was below 0.1%. On the family level, following populations (i.e. >0.5%) were dominant in the murine stomach: *Lactobacillaceae* (63%), *Clostridiaceae* (9%), *Erysipelotrichaceae* (6%), *Streptococcaceae* (4%) and *Porphyromonadaceae* (4%). The major genera (i.e. >0.5%) were *Lactobacillus* (63%), *Clostridium* sensu stricto (9%), unclassified *Erysipelotrichaceae* (5%), *Lactococcus* (4%), *Parabacteroides* (4%) and *Turicibacter* (2%). In 2 bambermycin supplemented, *H. suis*-positive mice (i.e. one of the 32 ppm supplemented group 5 and one of the 64 ppm supplemented group 6), however, Proteobacteria represented the major phylum (97%). *Helicobacteriaceae* (11%) and *Helicobacter* (11%) also represented dominant populations in the murine stomach, but only in the *H. suis*-positive groups 4-6. The average gastric bacterial community composition at the phylum, family and genus level present in the different groups is represented in Figures 5A-C, while the bacterial community composition of each individual mice is shown in Additional files 8A-C.

Bambermycin supplementation and/or infection with *H. suis* had no effect on microbial diversity, richness and population evenness (Additional files 9A-C). Furthermore, Unifrac weighted analysis as well as AMOVA and HOMOVA did not reveal significant differences regarding community structure and composition of the groups. Population structure and community membership, as determined by Bray-Curtis dissimilarity index, was also not different between the groups.

Phenotypic analysis of the murine gastric microbiota in general revealed the presence of 12 metabolic phenotypes of which ammonia oxidizer, dehalogenation, chitin degradation, xylan degrader, sulfide oxidizer and dinitrogen-fixing were the most abundant, each accounting for 78%, 78%, 14%, 7%, 7% and 6% of the gastric bacterial community, respectively. In the gastric microbiota of all mice, non-sporulating and Gram-positive bacteria were more abundant than sporulating and Gram-negative bacteria, respectively (70% vs 10% and 72% vs 0.5%, respectively). Presence of *H. suis* infection did not influence the phenotypic features of the gastric bacterial community. The bambermycin supplemented groups, however, showed significant lower abundance of sporulating and chitin degrading

bacteria compared to the groups without bambermycin supplementation (3.0% vs 19.7%, $p = 0.027$ and 7.9% vs 23.7%, $p = 0.027$, respectively).

The taxa *Helicobacteriaceae*, *Helicobacter* and *H. suis* at family, genus and species level, respectively, were only present in the *H. suis*-infected groups (11%, $p < 0.05$). Infection with *H. suis* did not influence the relative abundance of other taxa at phylum, family or genus level. At species level, *Christensenella* sp. EF603775 was only present in the *H. suis*–negative mice (0.041%), although this difference was not significant ($p = 0.223$). In general, mice that received bambermycin (i.e. groups 2, 3, 5 and 6) showed a relative lower abundance of the following taxa:

- At phylum family, genus and species level: Firmicutes_unclassified
- At phylum, family and genus level: Bacteria_unclassified
- At family level: *Clostridiaceae*_1
- At genus level: *Clostridiaceae*_1_unclassified, *Coprococcus*, *Turicibacter*, *Clostridium*_sensu_stricto_1
- At species level: *Coprococcus*_EF099198, *Coprococcus*_16S_OTU119, *Clostridiales*_Family_XIII_AB702776, *Clostridiales*_Family_XIII_16S_OTU162, *Clostridiales*_Family_XIII_EF604613, *Clostridiaceae*_1_16S_OTU75, *Clostridiaceae*_1_16S_OTU107, *Firmicutes*_16S_OTU195, *Firmicutes*_16S_OTU37, *Firmicutes*_16S_OTU43, *Firmicutes*_16S_OTU594, *Turicibacter*_EF406660, *Turicibacter*_DQ015666 and *Turicibacter*_EF406615 (Additional file 10).

The relative abundance of these taxa did not differ between the groups receiving different doses of in-feed bambermycin medication.

Interestingly, the relative abundance of these taxa was positively correlated with infiltration with inflammatory cells, T cells, B cells and macrophages as well as the expression of IFN-γ, gastrin and somatostatin, while negatively correlated with the number of parietal cells and the expression of H2 receptor, CCK-B receptor, H^+/K^+ ATPase and KCNQ1 (Additional file 11).

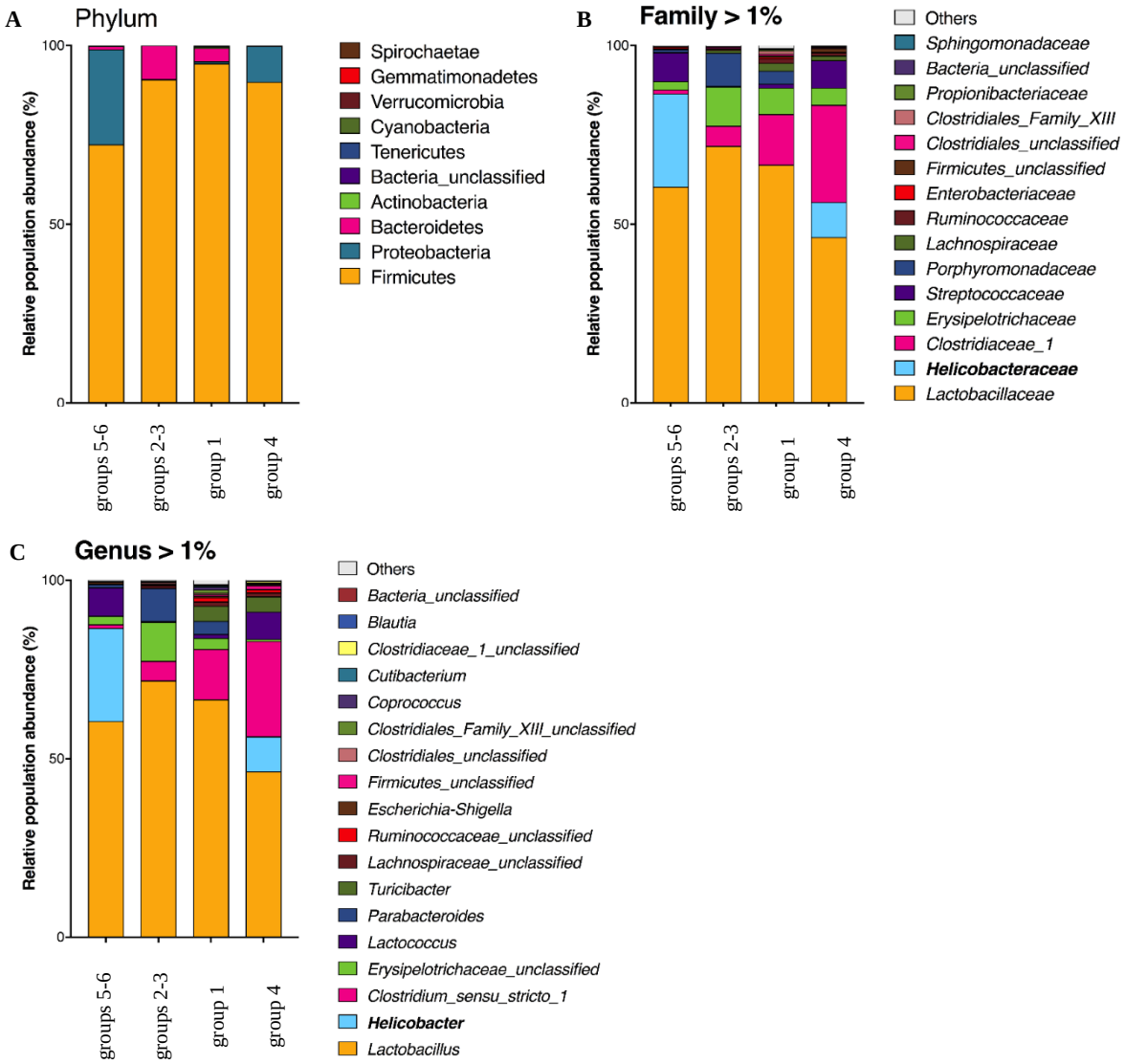

Figure 5 Average gastric bacterial community compositions present in the bambermycin-supplemented and non-supplemented groups. The cumulated histograms show the relative abundance of the identified taxa at phylum (A), family (B) or genus (C) level. At family and genus level, taxa with a relative abundance <1% are merged in the category "others". Group 1 = *H. suis*-negative control group without bambermycin supplementation; group 2 = 32 ppm bambermycin supplemented, non-*H. suis* infected group; group 3 = 64 ppm bambermycin supplemented, non-*H. suis* infected group; group 4 = *H. suis*-positive control group without bambermycin supplementation; group 5 = 32 ppm bambermycin supplemented, *H. suis*-infected group; group 6 = 64 ppm bambermycin supplemented, *H. suis*-infected group. The unclassified populations correspond to defined groups of the genus level for which a taxonomical classification assignation to the genus cannot be attributed. These populations are therefore labelled with the first defined superior hierarchical taxonomic level followed by "_unclassified" to prevent confusion.

Discussion

In the present study, an infection with *H. suis* elicited increased infiltration with inflammatory cells such as B cells, macrophages and T cells. These findings are in line with previous studies where *H. suis* infection was associated with development of severe gastritis in experimentally and naturally infected pigs and experimentally infected mice and Mongolian gerbils (Flahou *et al*., 2010; Bosschem *et al*., 2015; Zhang *et al*., 2015). Since macrophages produce several factors, such as IL-10, which provoke a Th2 response (Duque and Descoteaux, 2014) and since B cells promote the expansion of Th2 cells (Gajewski *et al*., 1991), infiltration with these inflammatory cells may have contributed to a Th2 response in *H. suis*-infected mice observed in previous studies (Flahou *et al*., 2010; Bosschem *et al*., 2015; Zhang *et al*., 2015).

The expression of IL-17, however, was downregulated in the *H. suis*-infected mice, in contrast with other studies where a clear IL-17 upregulation and a mixed Th2/Th17 response was present in *H. suis*-infected mice (Flahou *et al*., 2010, 2012; Bosschem *et al*., 2015, 2016, 2017). This might be caused by the absence of an IL-23 upregulation, a cytokine stimulating Th17 cell expansion (Ouyang *et al*., 2008). As Flahou *et al*. showed an inverse correlation between the *H. suis* colonization rate and the expression of IL-17 (Flahou *et al*., 2012), the observed high number of colonizing *H. suis* bacteria per mg gastric tissue might have contributed to the downregulation of IL-17. Still, it is not clear why a Th17 response was absent in this study.

BALB/c mice experimentally infected with *H. pylori* and C57BL/6 mice experimentally infected with *H. felis*, showed a decreased number of *Lactobacillus* spp. and an increased number of *Clostridium* spp., *Bacteroides* spp., *Prevotella* spp., *Eubacterium* spp., *Ruminococcus* spp., *Streptococcus* spp. and *E. coli* in their stomach (Aebischer *et al*., 2006; Schmitz *et al*., 2011). In another study, however, *H. pylori* infected C57BL/6 mice did not show gastric microbiota alterations (Tan *et al*., 2007). In the present study, *H. suis* infection was not associated with a shift in the gastric microbiota. Often, the used mouse substrain is not mentioned and although the same strain can be used in different studies, the substrain may be different. These differences in genetic background may contribute to discrepancies between

studies (Fontaine and Davis, 2016). Differences in virulence between *Helicobacter* spp., stage of infection, and/or influence of the host immune response may also contribute to the discrepancies (Suzuki *et al*., 2016). In future studies, it would be interesting to investigate a more long term effect of a *H. suis* infection on the gastric microbiota composition.

Since *H. suis* only grows in a biphasic medium with an acidic pH, standard antimicrobial susceptibility assays cannot be used for MIC determinations. The use of the combined agar and broth dilution method (Vermoote *et al*., 2011) may have influenced the results, as Butaye *et al*. demonstrated that medium composition has a clear impact on bambermycin activity (Butaye *et al*., 2000). For example, addition of sheep blood, hemoglobin, albumin, casein and starch as well as variations in pH and inoculum size affect the activity of bambermycin *in vitro* (Butaye *et al*., 2000, 2003). Although for MIC determinations, a strict standardization of the test medium is preferable (Butaye *et al*., 2000), no optimal medium exists to test bambermycin susceptibility of fastidious micro-organisms, such as *H. suis*. In comparison with the method described by Butaye *et al*. (Butaye *et al*., 2000), the MIC endpoints of the reference strain *S. aureus* ATCC 29213 were 2, 3 and 4 times higher when using the broth microdilution procedure according to CLSI standards and the *H. suis* susceptibility assay conditions at pH 5 and 7, respectively. The presence of dextrose, casein and/or other components in Brucella broth may have contributed to the decreased activity of bambermycin.

To the best of our knowledge, no susceptibility data of bambermycin on *H. suis* has been published. In the present study, all *H. suis* strains showed a MIC-value of 8 µg/ml. Despite this relatively low MIC endpoint, bambermycin supplementation did not affect the *H. suis* colonization rate *in vivo*. As no specific clinical breakpoints for bambermycin against *H. suis* are available, prediction of the clinical efficacy based on *in vitro* testing solely is difficult. This is further complicated by the fact that, even *in vitro*, different components in the medium such as proteins, starch and lipid substances, may highly influence the antibacterial activity of bambermycin (Butaye *et al*., 2000). The gastric environment is far more complex than this *in vitro* environment, making it almost impossible to predict the activity of bambermycin in the stomach from results of MIC determinations.

Despite the absence of an effect on the *H. suis* colonization rate, parietal cell loss during *H. suis* infection was countered when bambermycin was supplemented. A decreased parietal cell mass has also been shown in *H. suis*-infected BALB/c mice and Mongolian gerbils (Flahou *et al*., 2010; Zhang *et al*., 2015). It has been postulated that the loss of parietal cells might be due to a direct interaction of *H. suis* with these cells, since the bacterium can cause degenerative changes and necrosis of parietal cells in pigs, humans and rodent models (Joo *et al*., 2007; Flahou *et al*., 2010) and *H. suis* is able to directly interfere with cultured parietal cells, causing a significant impairment in cell viability (Zhang *et al*., 2016). Production of gamma-glutamyl transpeptidase (GGT) by *H. suis* has been linked with these degenerative changes and/or impairment in cell viability (Zhang *et al*., 2015). It remains to be determined if bambermycin affects this direct interaction of the bacterium with parietal cells, without influencing its colonization capacity. The inverse correlation seen in the present and other studies (Zhang *et al*., 2016), between severity of gastric inflammation and the number of parietal cells indicates that not only direct interaction of this bacterium with these host cells, but also inflammation may play a role in parietal cell death in *H. suis*-infected hosts.

Several studies attribute a role to *H. suis* in the development of hyperkeratosis and ulceration of the non-glandular stratified squamous epithelium of the *Pars oesophagea* of the porcine stomach, although *H. suis* does not colonize this region (Haesebrouck *et al*., 2009). It is not completely clear how exactly *H. suis* influences ulcer development, but alterations in gastric acid secretion may be involved, indicated by the results described in **Experimental studies: chapter 1**. Indeed, in *H. suis*-infected 6-8 months old pigs with severe hyperkeratosis and erosions of the non-glandular part of the stomach, expression of markers for gastric acid secretion was downregulated. It was hypothesized that decreased gastric acid secretion may affect the composition of the *Pars oesophageal* microbiota. Indeed, compared to non-infected, 6-8 months old pigs with no obvious lesions, higher numbers of *Fusobacterium gastrosuis* (**Experimental studies: chapter 2**), were detected in the *Pars oesophagea* of *H. suis*-infected pigs with hyperkeratosis and erosions of the *Pars oesophagea* and downregulated markers for gastric acid secretion. In the present study, bambermycin supplementation resulted in an upregulation of several markers for gastric acid secretion and seemed to counter the parietal cell mass loss during *H. suis*

infection. It remains to be determined if bambermycin affects gastric ulcer development in *H. suis*-infected pigs.

Although an improved feed conversion and growth has been demonstrated in pigs, cattle and poultry during bambermycin supplementation, the effect on gastro-intestinal inflammation had not been investigated before (Hagsten *et al*., 1980; De Schrijver *et al*., 1991; Parks *et al*., 2001; Sharifi *et al*., 2012). Here, the increased infiltration with macrophages, T cells and B cells and upregulated expressions of IL-8M, IL-10, TNF-α and IFN-γ in *H. suis*-infected mice was countered when bambermycin was supplemented in the diet, even though this antibiotic apparently did not influence *H. suis* colonization. Similarly, in the stomach of the non-*H. suis* infected mice treated with bambermycin, a decreased T cell and macrophage infiltration was observed as well as a downregulated expression of the pro-inflammatory cytokine IL-1β, further indicating that bambermycin may alter the function of inflammatory cells resulting in a more tempered host immune response. The mechanism behind this anti-inflammatory effect is not clear. It has been hypothesized that macrolides, cyclines and streptogramins may accumulate in phagocytic cells, reducing the production of pro-inflammatory cytokines (Niewold, 2007). Macrolides may also inhibit T cell maturation and proliferation (Menconi *et al*., 2014). It is not known if similar mechanisms are involved for bambermycin.

In the present study, bambermycin supplementation did not cause major shifts in *Lactobacillus* spp., which have been considered to exert a beneficial effect in the intestinal tract (Butaye *et al*., 2003). On the other hand, the presence of bacterial species belonging to *Firmicutes*, *Turicibacter*, *Coprococcus*, *Clostridiaceae*, *Clostridiales* family XIII and *Clostridium senso stricto* 1 taxa was positively correlated with infiltration of inflammatory cells as well as expression of markers for inflammation. This might indicate that some bacteria belonging to these taxa might exert unfavorable effects on the host. Since the relative abundance of these taxa was decreased during bambermycin supplementation, an altered gastric microbiota composition may also have contributed to the anti-inflammatory effect of bambermycin. Nevertheless, as most of these species are yet unknown, further research is necessary to confirm or deny this hypothesis. Finally, ammonia oxidizers accounted for 78% of the gastric bacterial community. These bacteria produce nitric oxide, which may exert damaging effects and contribute to

gastritis. It has been shown that bambermycin exhibits an anti-oxidative effect by scavenging free nitric oxide radicals *in vitro* (Kabploy, Krittika; Bunyapraphatsara and Morales, Noppawan Phumala Paraksa, 2015), which may also play a role in the observed anti-inflammatory effect of in-feed bambermycin medication.

In conclusion, bambermycin supplementation did not affect *H. suis* colonization, but did decrease gastric inflammation and inhibited the effects of a *H. suis* infection on parietal cell loss. Not only direct interaction of *H. suis* with parietal cells, but also inflammation may play a role in death of these gastric acid producing cells.

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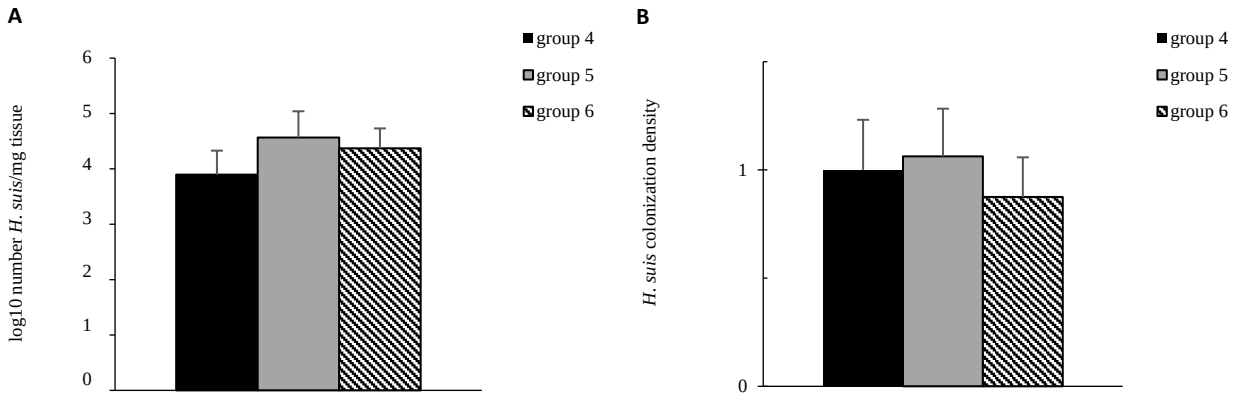
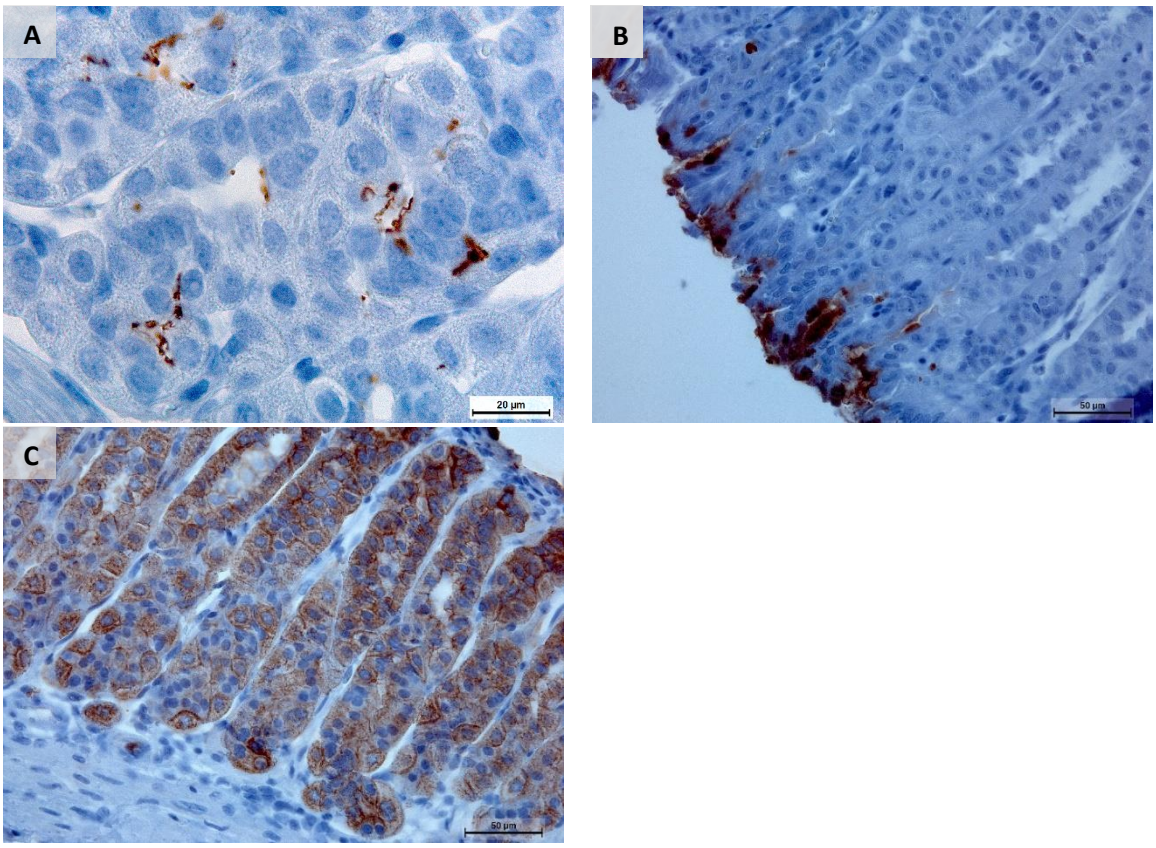
Additional file 1: List of primers used in quantitative RT-PCR for gene expression analysis of markers for gastric acid secretion and inflammation.

Primer	Sequence	Reference
Sonic Hedgehog-forward	5'-AGC AGG TTT CGA CTG GGT CT-3'	(Fukuhara *et al*., 2014)
Sonic Hedgehog-reverse	5'-GCC ACG GAG TTC TCT GCT T-3'	(Fukuhara *et al*., 2014)
Somatostatin-forward	5'-GTC CTG GCT TTG GGC GGT GTC A-3'	(Takaishi and Wang, 2007)
Somatostatin-reverse	5'-TGC AGC TCC AGC CTC ATC TCG T-3'	(Takaishi and Wang, 2007)
Histamine 2 receptor-forward	5'-GCC ACC ATC AGA GAA CAC AAA G-3'	Adjusted from (Osawa *et al*., 2005)
Histamine 2 receptor-reverse	5'-AAG GAA ACC AGC AGA CGA TGA A-3'	Adjusted from (Osawa *et al*., 2005)
Muscarinic 3 receptor-forward	5'-ACC AAG CTA CCC TCC TCA GA-3'	Adjusted from (Kitazawa *et al*., 2009)
Muscarinic 3 receptor-reverse	5'-GAC AGT TGT CAC GGT CAT CC-3'	(Kitazawa *et al*., 2009)
Gastrin-forward	5'-AGC GCC AGT TCA ACA AGC T-3'	(Du *et al*., 2013)
Gastrin-reverse	5'-CCA AAG TCC ATC CAT CCG TAG-3'	(Du *et al*., 2013)
Cholecystokinin B receptor-forward	5'-CTG GCT GTC GCT TGC ATG CC-3'	Adjusted from (Zhang *et al*., 2016)
Cholecystokinin B receptor-reverse	5'-GCA GTG GTC GGC AGA TGG CG-3'	Adjusted from (Zhang *et al*., 2016)
H^+/K^+ ATPase-forward	5'-AGA TGT CCT CAT CCG CAA GAC AC-3'	(Fukuhara *et al*., 2014)
H^+/K^+ ATPase-reverse	5'-CAG CCA ATG CAG ACC TGG AA-3'	(Fukuhara *et al*., 2014)
KCNQ1-forward	5'-AAC AGA ATT GTC AAG TTC CTC-3'	(Jain *et al*., 2006)
KCNQ1-reverse	5'-AGA CTG AAG GTG CCA TTG-3'	(Jain *et al*., 2006)
H2afz-forward	5'-GGT ATC ACC CCT CGT CAC TT-3'	(Flahou *et al*., 2010)
H2afz-reseverse	5'-TCA GCG ATT TGT GGA TGT GT-3'	(Flahou *et al*., 2010)
HPRT-forward	5'-CAG GCC AGA CTT TGT TGG AT-3'	(Flahou *et al*., 2010)
HPRT-reverse	5'-TTG CGC TCA TCT TAG GCT TT-3'	(Flahou *et al*., 2010)
PPIa-forward	5'-AGC ATA CAG GTC CTG GCA TC-3'	(Flahou *et al*., 2010)
PPIa-reverse	5'-TTC ACC TTC CCA AAG ACC AC-3'	(Flahou *et al*., 2010)
IL1β-forward	5'-CAC CTC ACA AGC AGA GCA CAA G-3'	(Catrysse *et al*., 2016)
IL1β-reverse	5'-GCA TTA GAA ACA GTC CAG CCC ATA C-3'	(Catrysse *et al*., 2016)
IL8KC-forward	5'-GCT GGG ATT CAC CTC AAG AA-3'	(Liu *et al*., 2016)
IL8KC-reverse	5'-TCT CCG TTA CTT GGG GAC AC-3'	(Liu *et al*., 2016)
IL8MIP-forward	5'-TGC CTG AAG ACC CTG CCA AGG-3'	(Liu *et al*., 2016)
IL8MIP-reverse	5'-GTT AGC CTT GCC TTT GTT CAG-3'	(Liu *et al*., 2016)
IL8Lix-forward	5'-CTC AGT CAT AGC CGC AAC CGA GC-3'	(Liu *et al*., 2016)
IL8Lix-reverse	5'-CCG TTC TTT CCA CTG CGA GTG C-3'	(Liu *et al*., 2016)
IL4-forward	5'-GGT CTC AAC CCC CAG CTA GT-3'	(Castiglioni *et al*., 2015)
IL4-reverse	5'-GCC GAT GAT CTC TCT CAA GTG AT-3'	(Castiglioni *et al*., 2015)
IL6-forward	5'-TAG TCC TTC CTA CCC CAA TTT CC-3'	(Castiglioni *et al*., 2015)
IL6-reverse	5'-TTG GTC CTT AGC CAC TCC TTC-3'	(Castiglioni *et al*., 2015)
IL10-forward	5'-CTG GAC AAC ATA CTG CTA ACC G-3'	(Huang *et al*., 2015)
IL10-reverse	5'-GGG CAT CAC TTC TAC CAG GTA A-3'	(Huang *et al*., 2015)
IL12-forward	5'-ACCTGTGACACGCCTGAAGAAG-3'	(Ichikawa *et al*., 2012)
IL12-reverse	5'-TGT GGA GCA GCA GAT GTG AGT G-3'	(Ichikawa *et al*., 2012)
IL17-forward	5'-CAG GAC GCG CAA ACA TGA-3'	(Lee *et al*., 2011)
IL17-reverse	5'-GCA ACA GCA TCA GAG ACA CAG AT-3'	(Lee *et al*., 2011)
IL23-forward	5'-CAC CTC CCT ACT AGG ACT CAG C-3'	(Tortola *et al*., 2012)
IL23-reverse	5'-TGG GCA TCT GTT GGG TCT-3'	(Tortola *et al*., 2012)
IFNγ-forward	5'-CTG ACC TAG AGA AGA CAC AT-3'	(Flahou *et al*., 2012)
IFNγ-reverse	5'-GGT CAG TGA AGT AAA GGT AC-3'	(Flahou *et al*., 2012)
TNFα-forward	5'-ACC CTG GTA TGA GCC CAT ATA C-3'	(Liu *et al*., 2016)
TNFα-reverse	5'-ACA CCC ATT CCC TTC ACA GAG-3'	(Liu *et al*., 2016)



Additional file 2: Number and colonization density of *H. suis* in groups 4-6. (A) Number of *H. suis* bacteria per mg gastric tissue of group 4-6. Data are shown as log10 values of the average of number of *H. suis* bacteria per mg tissue with standard deviation. (B) Colonization density of *H. suis* in the stomach of group 4-6. Data are shown as the average of the colonization score for each group with standard deviation. Group 4 = *H. suis*-positive control without bambermycin supplementation; group 5 = 32 ppm bambermycin supplemented, *H. suis*-infected group; group 6 = 64 ppm bambermycin supplemented, *H. suis*-infected group.



Additional file 3: Immunohistochemical *Helicobacter* (A), caspase-3 (B) and KI-67 (C) staining of a mouse stomach, showing *H. suis* colonization, apoptotic cells and replicating cells, respectively. (A) *H. suis* bacteria (brown) present in the glands of the antrum of a stomach of a *H. suis*-positive control mouse not treated with bambermycin. Original magnification: ×400, scale bar 20 µm. (B) Apoptotic cells (brown) present in a stomach of a *H. suis*-negative mouse treated with bambermycin. Original magnification: ×400, scale bar 50 µm. (C) Replicating cells (brown) present in a stomach of a *H. suis*-negative mouse treated with bambermycin. Original magnification: ×400, scale bar 50 µm.

Additional file 4: Overview of important correlations

Number of cells	**Number of infiltrating cells**
Infiltration with inflammatory cells	T-cells *r* = 0.691 *p*-value < 0.001 Macrophages *r* = 0.259 *p*-value = 0.076
T-cells	B-cells *r* = 0.326 *p*-value = 0.024 Macrophages *r* = 0.543 *p*-value < 0.001
Parietal cells	Infiltration with inflammatory cells *r* = -0.222 *p*-value = 0.129 T-cells *r* = -0.375 *p*-value = 0.009 B-cells *r* = -0.278 *p*-value = 0.055 Macrophages *r* = -0.264, *p*-value = 0.070
Number of infiltrating cells	**Expression of markers for inflammation**
T-cells	IL-8Li *r* = 0.237 *p*-value = 0.117 IL-10 *r* = 0.323 *p*-value = 0.048 IL-1β *r* = 0.228 *p*-value = 0.120
B-cells	IL-4 *r* = 0.260 *p*-value = 0.085 IL-10 *r* = 0.554 *p*-value < 0.001 IL-12 *r* = 0.518 *p*-value < 0.001
Macrophages	IL-8Kc *r* = 0.389 *p*-value = 0.007 IL-8Li *r* = 0.659 *p*-value < 0.001 IL-10 *r* = 0.401 *p*-value = 0.013

Number of infiltrating cells	**Expression of markers for inflammation**
Macrophages	IL-17 *r* = 0.284 *p*-value = 0.053 IFN-γ *r* = 0.413 *p*-value = 0.005 TNF-α *r* = 0.248 *p*-value = 0.093 IL-1β *r* = 0.293 *p*-value = 0.044
Gene expression of markers for inflammation	**Number of *H. suis* bacteria**
IL-4	*r* = 0.337 *p*-value = 0.024
IL-6	*r* = 0.517 *p*-value < 0.001
IL-8Li	*r* = 0.274 *p*-value = 0.069
IL-17	*r* = -0.264 *p*-value = 0.073
IL-23	*r* = -0.201 *p*-value = 0.180
Gene expression of markers for gastric acid secretion	**Gene expression of markers for inflammation**
KCNQ1	IL-8Kc *r* = 0.290 *p*-value = 0.050 IFN-γ *r* = 0.300 *p*-value = 0.054
CCK-B receptor	IL-8Kc *r* = 0.381 *p*-value = 0.010 IL-8M *r* = 0.257 *p*-value = 0.092 IL-8Li *r* = 0.427 *p*-value = 0.004 IL-10 *r* = 0.252 *p*-value = 0.138 TNF-α *r* = 0.214 *p*-value = 0.162
Somatostatin	IL-8M *r* = 0.413 *p*-value = 0.004 IFN-γ *r* = 0.383 *p*-value = 0.010

r = Pearson correlation coefficient, calculated using SPSS Statistics 24. A *r*-value close to 1 indicates a strong, positive correlation, whereas a *r*-value of -1 indicates a strong, negative correlation. *P*-values lower than 0.05 are considered to be significant.

Additional file 5: Overview of the relative fold changes of altered markers for inflammation in the bambermycin-supplemented and non-supplemented groups.

Group	Gene	Relative fold change	*p*-value
2			
	IL-1β	0.71 ± 0.10	0.028
3			
	IL-1β	0.54 ± 0.10	0.015
4			
	IL-4	2.37 ± 0.26	0.091
	IL-6	1.78 ± 0.38	0.115
	IL-8M	2.65 ± 0.52	0.298
	IL-8Li	5.18 ± 1.53	0.002
	IL-10	3.32 ± 1.07	0.012
	IL-17	0.13 ± 0.05	<0.001
	IL-23	0.17 ± 0.03	<0.001
	TNF-α	2.69 ± 0.51	0.065
5			
	IL-8M	0.67 ± 0.22	0.100
	IFN-γ	0.24 ± 0.08	0.015
6			
	IL-8M	0.23 ± 0.06	0.001
	IL-10	0.56 ± 0.06	0.077
	IFN-γ	0.46 ± 0.20	0.253

The data are presented as fold changes in gene expression normalized to 3 reference genes and relative to control groups 1 and 4 (i.e. group 2-4 relative to group 1 and group 5-6 relative to group 4) which are considered as 1. The fold changes are shown as means with the standard error of the mean. Statistical differences were calculated using the non-parametric Kruskal-Wallis H test SPSS statistics 24. A *p*-value lower than 0.05 is considered to be significant. Group 2 = 32 ppm bambermycin supplemented, non-*H. suis* infected group; group 3 = 64 ppm bambermycin supplemented, non-*H. suis* infected group; group 4 = *H. suis*-positive control group without bambermycin supplementation; group 5 = 32 ppm bambermycin supplemented, *H. suis*-infected group; group 6 = 64 ppm bambermycin supplemented, *H. suis*-infected group.

Additional file 6: Overview of the relative fold changes of altered markers for gastric acid secretion in the bambermycin-supplemented and non-supplemented groups.

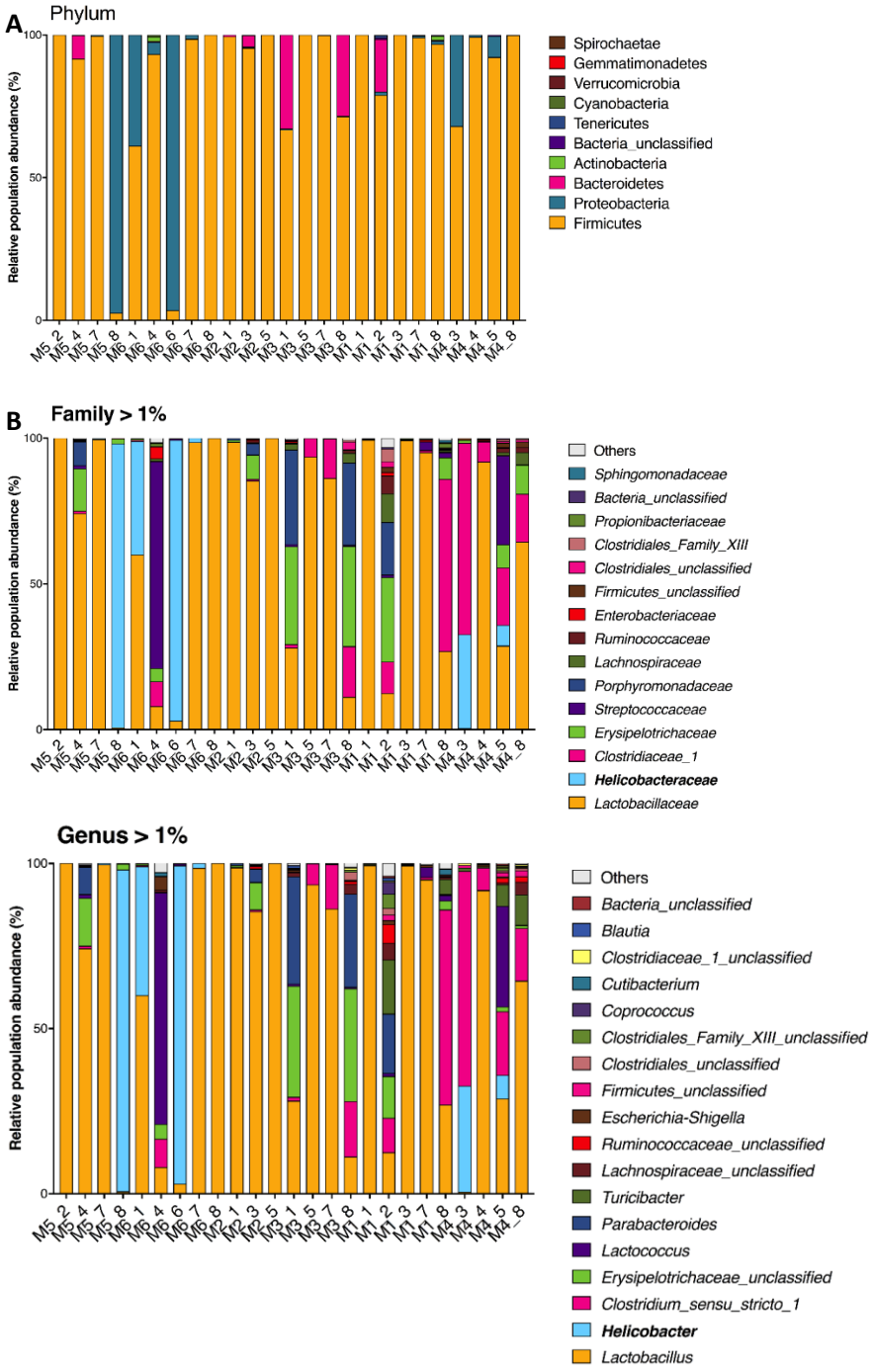
Group	Gene	Relative fold change	*p*-value
2	KCNQ1	3.92 ± 0.87	0.023
	CCK-B receptor	2.80 ± 0.80	0.214
	Gastrin	0.68 ± 0.24	0.156
3	H^+/K^+ ATPase	2.22 ± 0.42	0.125
	Sonic Hedgehog	2.01 ± 0.45	0.163
	KCNQ1	3.97 ± 0.81	0.014
	CCK-B receptor	3.12 ± 0.49	0.035
	M3 receptor	1.50 ± 0.17	0.181
4	KCNQ1	3.24 ± 0.84	0.119
	CCK-B receptor	3.46 ± 0.65	0.032
5	CCK-B receptor	0.67 ± 0.22	0.117
	Somatostatin	0.63 ± 0.35	0.011

The data are presented as fold changes in gene expression normalized to 3 reference genes and relative to control groups 1 and 4 (i.e. group 2-4 relative to group 1 and group 5-6 relative to group 4) which are considered as 1. The fold changes are shown as means with the standard error of the mean. Statistical differences were calculated using the non-parametric Kruskal-Wallis H test SPSS statistics 24. A *p*-value lower than 0.05 is considered to be significant. Group 2 = 32 ppm bambermycin supplemented, non-*H. suis* infected group; group 3 = 64 ppm bambermycin supplemented, non-*H. suis* infected group; group 4 = *H. suis*-positive control group without bambermycin supplementation; group 5 = 32 ppm bambermycin supplemented, *H. suis*-infected group; group 6 = 64 ppm bambermycin supplemented, *H. suis*-infected group.

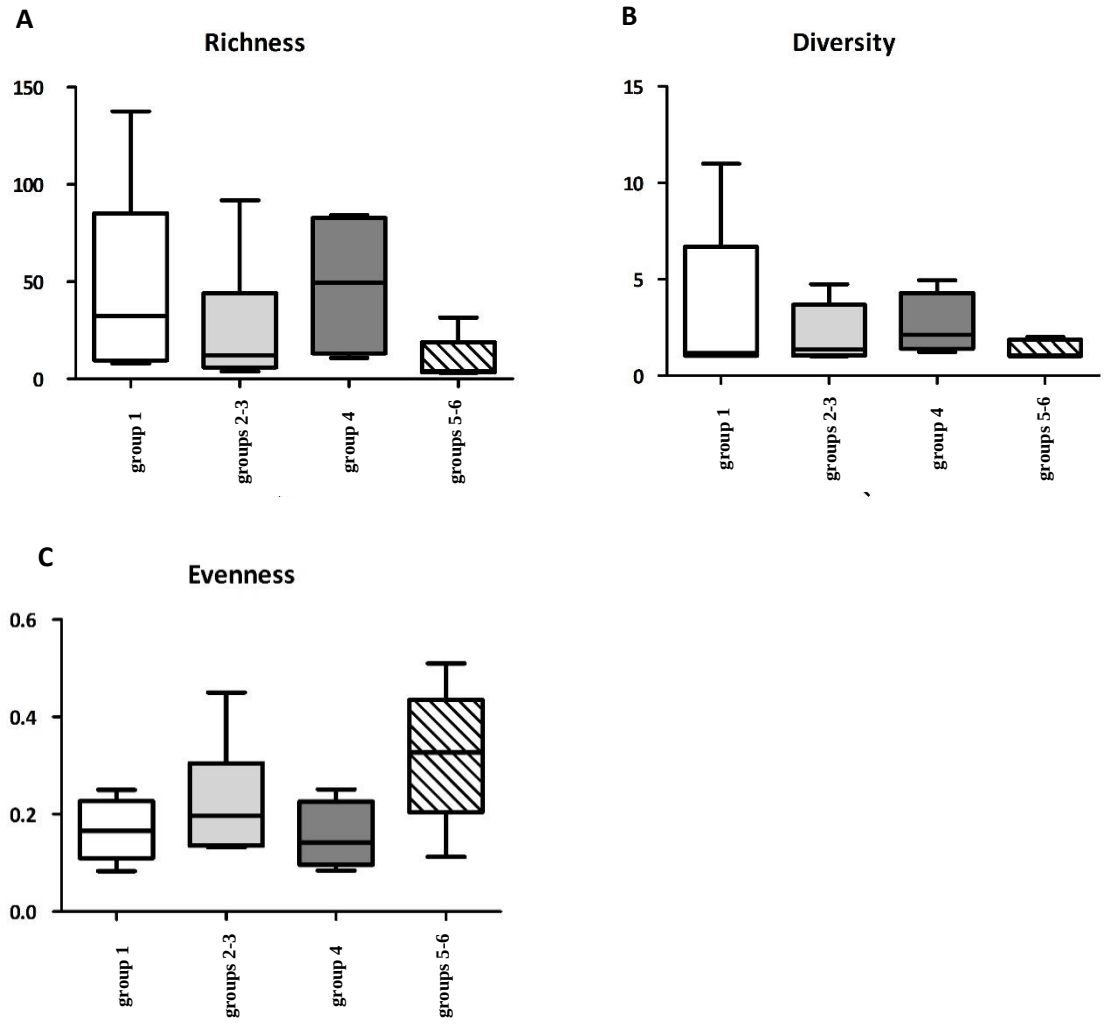
Additional file 7: Overview of the number of pyrosequencing reads for each mice.

Mice	Group	Number of reads
M1_1	1	3,776
M1_2	1	2,835
M1_3	1	3,771
M1_7	1	3,527
M1_8	1	3,806
M2_1	2	3,603
M2_3	2	3,020
M2_5	2	3,916
M3_1	3	2,586
M3_5	3	3,269
M3_7	3	2,861
M3_8	3	2,450
M4_3	4	913
M4_4	4	3,004
M4_5	4	1,433
M4_8	4	2,430
M5_2	5	3,918
M5_4	5	2,839
M5_7	5	3,690
M5_8	5	355
M6_1	6	839
M6_4	6	3,734
M6_6	6	411
M6_7	6	3,219
M6_8	6	3,895

Group 1 = *H. suis*-negative control group without bambermycin supplementation; group 2 = 32 ppm bambermycin supplemented, non-*H. suis* infected group; group 3 = 64 ppm bambermycin supplemented, non-*H. suis* infected group; group 4 = *H. suis*-positive control group without bambermycin supplementation; group 5 = 32 ppm bambermycin supplemented, *H. suis*-infected group; group 6 = 64 ppm bambermycin supplemented, *H. suis*-infected group.



Additional file 8: Bacterial community compositions present in the stomach of each individual mouse. The cumulated histograms show the relative abundance of the identified taxa at phylum (A), family (B) or genus (C) level. At family and genus level, taxa with a relative abundance <1% are merged in the category "others". M1_ = group 1 = *H. suis*-negative control group without bambermycin supplementation; M2_ = group 2 = 32 ppm bambermycin supplemented, non-*H. suis* infected group; M3_ = group 3 = 64 ppm bambermycin supplemented, non-*H. suis* infected group; M4_ = group 4 = *H. suis*-positive control group without bambermycin supplementation; M5_ = group 5 = 32 ppm bambermycin supplemented, *H. suis*-infected group; M6_ = group 6 = 64 ppm bambermycin supplemented, *H. suis*-infected group. The unclassified populations correspond to defined groups of the genus level for which a taxonomical classification assignation to the genus cannot be attributed. These populations are therefore labelled with the first defined superior hierarchical taxonomic level followed by "_unclassified" to prevent confusion.



Additional file 9 Overview of the gastric bacterial richness, diversity and evenness of the bambermycin-supplemented and non-supplemented groups. Gastric bacterial richness (A), diversity (B) and evenness (C). The data are represented as box plots: the bottom and top of the box represent the first and the third quartile, the line in the box represents the median and the whiskers represent the minimum and maximum values. Group 1 = *H. suis*-negative control group without bambermycin supplementation; group 2 = 32 ppm bambermycin supplemented, non-*H. suis* infected group; group 3 = 64 ppm bambermycin supplemented, non-*H. suis* infected group; group 4 = *H. suis*-positive control group without bambermycin supplementation; group 5 = 32 ppm bambermycin supplemented, *H. suis*-infected group; group 6 = 64 ppm bambermycin supplemented, *H. suis*-infected group.

Additional file 10: An overview of the main differences in relative abundance of taxa at phylum, family, genus and species level in the bambermycin-supplemented and non-supplemented groups.

Level	Taxa	Bambermycin +	Bambermycin -	*p*-value
Phylum	Bacteria_unclassified	0.00 ± 0.00	0.12 ± 0.06	0.025
Family	Firmicutes_unclassified	0.05 ± 0.02	0.64 ± 0.26	0.048
	*Clostridiaceae*_1	3.07 ± 1.37	24.08 ± 10.30	0.300
	Bacteria_unclassified	0.00 ± 0.00	0.12 ± 0.06	0.048
Genus	*Clostridiaceae*_1_unclassified	0.04 ± 0.03	0.23 ± 0.11	0.445
	*Firmicutes*_unclassified	0.05 ± 0.02	0.64 ± 0.26	0.024
	Turicibacter	0.00 ± 0.00	3.12 ± 1.39	0.018
	*Clostridium*_sensu_stricto_1	3.02 ± 1.34	23.85 ± 10.25	0.243
	Bacteria_unclassified	0.00 ± 0.00	0.12 ± 0.06	0.018
	Coprococcus	0.00 ± 0.00	0.11 ± 0.05	0.014
Species	*Coprococcus*_EF099198	0.00 ± 0.00	0.49 ± 0.44	0.021
	*Coprococcus*_16S_OTU119	0.00 ± 0.00	0.07 ± 0.03	0.031
	*Clostridiales*_Family_XIII_AB702776	0.00 ± 0.00	0.17 ± 0.15	0.023
	*Clostridiales*_Family_XIII_16S_OTU162	0.00 ± 0.00	0.07 ± 0.05	0.035
	*Clostridiales*_Family_XIII_EF604613	0.00 ± 0.00	0.48 ± 0.41	0.131
	*Clostridiaceae*_1_16S_OTU75	0.00 ± 0.00	0.08 ± 0.05	0.033
	*Clostridiaceae*_1_16S_OTU107	0.01 ± 0.01	0.05 ± 0.03	0.469
	*Firmicutes*_16S_OTU195	0.00 ± 0.00	0.01 ± 0.01	0.035
	*Firmicutes*_16S_OTU37	0.00 ± 0.00	0.14 ± 0.07	0.039
	*Firmicutes*_16S_OTU43	0.00 ± 0.00	0.18 ± 0.09	0.090
	*Firmicutes*_16S_OTU594	0.00 ± 0.00	0.02 ± 0.01	0.126
	*Turicibacter*_EF406660	0.00 ± 0.00	3.96 ± 2.12	0.058
	*Turicibacter*_DQ015666	0.00 ± 0.00	0.02 ± 0.01	0.138
	*Turicibacter*_EF406615	0.00 ± 0.00	0.08 ± 0.06	0.104
		***H. suis* +**	***H. suis* -**	***p*-value**
Species	*Christensenella*_EF603775	0.00 ± 0.00	0.041 ± 0.019	0.223

The data are presented as the mean relative abundance of the taxa with the standard error of the mean. Statistical differences were calculated using the non-parametric Kruskal-Wallis tests with Tukey post-hoc tests and Benjamini-Hochberg False Discovery Rate were performed using STAMP. A *p*-value lower than 0.05 is considered to be significant.

Additional file 11: Overview of important correlations between the gastric bacterial community and the number of inflammatory cells, parietal cells and expression of markers for inflammation and gastric acid secretion

	Taxa	**Expression of markers for inflammation, gastric acid secretion and number of infiltrating cells**
Family	Firmicutes_unclassified	Gastrin *r* = 0,672 *p*-value < 0,001 Infiltration with inflammatory cells *r* = 0,444 *p*-value = 0,026 Parietal cells *r* = -0,414 *p*-value = 0,040 T-cells *r* = 0,529 *p*-value = 0,007 B-cells *r* = 0,561 *p*-value = 0,003
	*Clostridiaceae*_1	Gastrin *r* = 0,538 *p*-value = 0,007 IFNg *r* = 0,556 *p*-value = 0,009 Parietal cells *r* = -0,516 *p*-value = 0,008 B-cells *r* = 0,447 *p*-value = 0,025
	Bacteria_unclassified	Gastrin *r* = 0,398 *p*-value = 0,054 Infiltration with inflammatory cells *r* = 0,356 *p*-value = 0,080 T-cells *r* = 0,472 *p*-value = 0,017 B-cells *r* = 0,362 *p*-value = 0,075
Genus	*Firmicutes*_unclassified	Gastrin *r* = 0,672 *p*-value = 0 IFNg *r* = 0,432 *p*-value = 0,050 Infiltration with inflammatory cells *r* = 0,444 *p*-value = 0,026 Parietal cells *r* = -0,414 *p*-value = 0,040 T-cells *r* = 0,529 *p*-value = 0,007 B-cells *r* = 0,561 *p*-value = 0,003
	*Clostridiaceae*_1_unclassified	Gastrin *r* = 0,526 *p*-value = 0,008 Somatostatin *r* = 0,351 *p*-value = 0,085 IFNg *r* = 0,565 *p*-value = 0,008 Parietal cells *r* = -0,510 *p*-value = 0,009

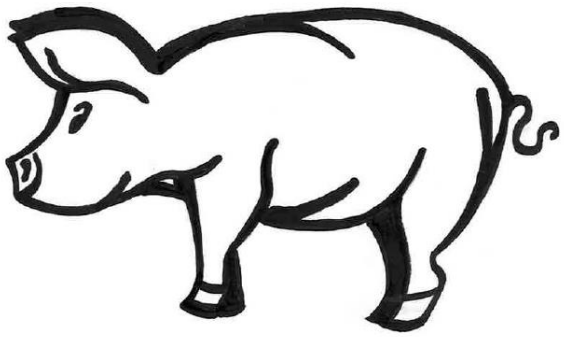
	Taxa	Expression of markers for inflammation, gastric acid secretion and number of infiltrating cells
Genus	*Clostridiaceae*_1_unclassified	B-cells *r* = 0,513 *p*-value = 0,009
	*Clostridium*_sensu_stricto_1	B-cells *r* = 0,513 *p*-value = 0,009 IFNg *r* = 0,556 *p*-value = 0,009 Parietal cells *r* = -0,516 *p*-value = 0,008 B-cells *r* = 0,447 *p*-value = 0,025
	Bacteria_unclassified	Gastrin *r* = 0,398 *p*-value = 0,054 Infiltration with inflammatory cells *r* = 0,356 *p*-value = 0,080 T-cells *r* = 0,472 *p*-value = 0,017 B-cells *r* = 0,362 *p*-value = 0,075
	Turicibacter	Gastrin *r* = 0,548 *p*-value = 0,006 IFNg *r* = 0,403 *p*-value = 0,070 Infiltration with inflammatory cells *r* = 0,599 *p*-value = 0,002 T-cells *r* = 0,741 *p*-value < 0,001 B-cells *r* = 0,389 *p*-value = 0,054 Marcophages *r* = 0,505 *p*-value = 0,01
	Coprococcus	KCNQ1 *r* = -0,502 *p*-value = 0.015 Gastrin *r* = 0,391 *p*-value = 0,059 H2Rc *r* = -0,450 *p*-value = 0,024 Infiltration with inflammatory cells *r* = 0,621 *p*-value = 0,001 T-cells *r* = 0,600 *p*-value = 0,002 B-cells *r* = 0,347 *p*-value = 0,09
Species	*Coprococcus*_EF099198	KCNQ1 *r* = -0,441 *p*-value = 0,035 H2Rc *r* = -0,372 *p*-value = 0,067 T-cells *r* = 0,485 *p*-value = 0,014

	Taxa	Expression of markers for inflammation, gastric acid secretion and number of infiltrating cells
Species	*Coprococcus*_EF099198	B-cells *r* = 0,374 *p*-value = 0,066
	*Coprococcus*_16S_OTU119	T-cells *r* = 0,485 *p*-value = 0,014 B-cells *r* = 0,374 *p*-value = 0,066 H2Rc *r* = -0,458 *p*-value = 0,021 Infiltration with inflammatory cells *r* = 0,621 *p*-value = 0,001 T-cells *r* = 0,620 *p*-value = 0,001
	*Clostridiales*_Family_XIII_AB702776	Infiltration with inflammatory cells *r* = 0,486 *p*-value = 0,014 T-cells *r* = 0,469 *p*-value = 0,018 B-cells *r* = 0,381 *p*-value = 0,060 Macrophages *r* = 0,412 *p*-value = 0,041
	*Clostridiales*_Family_XIII_16S_OTU162	H^+/K^+ ATPase *r* = -0,417 *p*-value = 0,038 KCNQ1 *r* = -0,458 *p*-value = 0,028 H2Rc *r* = -0,448 *p*-value = 0,025 Infiltration with inflammatory cells *r* = 0,486 *p*-value = 0,014 T-cells *r* = 0,416 *p*-value = 0,039 B-cells *r* = 0,381 *p*-value = 0,060
	*Clostridiales*_Family_XIII_EF604613	H^+/K^+ ATPase *r* = -0,367 *p*-value = 0,071 Infiltration with inflammatory cells *r* = 0,412 *p*-value = 0,041 T-cells *r* = 0,395 *p*-value = 0,051 B-cells *r* = 0,386 *p*-value = 0,057
	*Clostridiaceae*_1_16S_OTU75	Gastrin *r* = 0,355 *p*-value = 0,088 Infiltration with inflammatory cells *r* = 0,35 *p*-value = 0,087 T-cells *r* = 0,506 *p*-value = 0,010 B-cells *r* = 0,495 *p*-value = 0,012

	Taxa	Expression of markers for inflammation, gastric acid secretion and number of infiltrating cells
Species	*Clostridiaceae*_1_16S_OTU75	Macrophages *r* = 0,495 *p*-value = 0,012
	*Clostridiaceae*_1_16S_OTU107	IFNg *r* = 0,468 *p*-value = 0,032 Parietal cells *r* = -0,581 *p*-value = 0,002 B-cells *r* = 0,466 *p*-value = 0,019 Macrophages *r* = 0,507 *p*-value = 0,010
	*Firmicutes*_16S_OTU195	Gastrin *r* = 0,437 *p*-value = 0,033 IFNg *r* = 0,373 *p*-value = 0,096 Infiltration with inflammatory cells *r* = 0,555 *p*-value = 0,001 T-cells *r* = 0,455 *p*-value = 0,022 Macrophages *r* = 0,355 *p*-value = 0,081
	*Firmicutes*_16S_OTU37	Gastrin *r* = 0,512 *p*-value = 0,011 Infiltration with inflammatory cells *r* = 0,685 *p*-value < 0,001 T-cells *r* = 0,665 *p*-value < 0,001 B-cells *r* = 0,347 *p*-value = 0,090 Macrophages *r* = 0,439 *p*-value = 0,028
	*Firmicutes*_16S_OTU43	Gastrin *r* = 0,428 *p*-value = 0,037 H2Rc *r* = -0,349 *p*-value = 0,088 IFNg *r* = 0,470 *p*-value = 0,032 Parietal cells *r* = -0,371 *p*-value = 0,068 B-cells *r* = 0,557 *p*-value = 0,004
	*Firmicutes*_16S_OTU594	H^+/K^+ ATPase *r* = -0,348 *p*-value = 0,088 H2Rc *r* = -0,362 *p*-value = 0,075
	*Firmicutes*_16S_OTU594	Infiltration with inflammatory cells *r* = 0,412 *p*-value = 0,041 T-cells *r* = 0,389 *p*-value = 0,055

	Taxa	Expression of markers for inflammation, gastric acid secretion and number of infiltrating cells
Species	*Firmicutes*_16S_OTU594	B-cells *r* = 0,369 *p*-value = 0,069 Macrophages *r* = 0,339 *p*-value = 0,098
	*Turicibacter*_EF406660	Gastrin *r* = 0,548 *p*-value = 0,006 IFNg *r* = 0,403 *p*-value = 0,070 Infiltration with inflammatory cells *r* = 0,599 *p*-value = 0,002 T-cells *r* = 0,741 *p*-value < 0,001 B-cells *r* = 0,389 *p*-value = 0,054 Macrophages *r* = 0,505 *p*-value = 0,010
	*Turicibacter*_DQ015666	H^+/K^+ ATPase *r* = -0,347 *p*-value = 0,090 H2Rc *r* = -0,365 *p*-value = 0,073 Infiltration with inflammatory cells *r* = 0,412 *p*-value = 0,041 T-cells *r* = 0,384 *p*-value = 0,058 B-cells *r* = 0,364 *p*-value = 0,074
	*Turicibacter*_EF406615	KCNQ1 *r* = -0,395 *p*-value = 0,062 Gastrin *r* = 0,387 *p*-value = 0,062 H2Rc *r* = -0,469 *p*-value = 0,018 Infiltration with inflammatory cells *r* = 0,486 *p*-value = 0,014 T-cells *r* = 0,530 *p*-value = 0,006
	*Christensenella*_EF603775	IL-6 *r* = -0,490 *p*-value = 0,015 IL-17 *r* = 0,362 *p*-value = 0,082 IL-23 *r* = 0,452 *p*-value = 0,023 TNF-α *r* = -0,423 *p*-value = 0,040

r = Pearson correlation coefficient, calculated using SPSS Statistics 24. A *r*-value close to 1 indicates a strong, positive correlation, whereas a *r*-value of -1 indicates a strong, negative correlation. *P*-values lower than 0.05 are considered to be significant.



General discussion

"Omnium rerum principia parva sunt" (Cicero)

"The beginnings of all things are small"

The upper, non-glandular part of the porcine stomach, *Pars oesophagea,* is often affected by lesions, such as hyperkeratosis, erosion and ulceration. Abattoir surveillance programs have shown that the prevalence of these lesions on national pig population level can vary from 5 up to 100% (Hessing *et al*., 1992; Guise *et al*., 1997; Robertson *et al*., 2002; Swaby and Gregory, 2012; Omotosho *et al*., 2016; Gottardo *et al*., 2017). Not much is known, however, on the presence and severity of gastric lesions in Belgium, as only few abattoir surveys have been published and the most recent one dates back from 1995 (Hessing *et al*., 1992; Roels *et al*., 1995). Incorporation of routine screening methods in Belgian slaughterhouses will not only result in a better monitoring of porcine gastric lesions, it can also function as welfare criterion, since stress has been associated with increased severity of gastric lesions (Nielsen and Ingvartsen, 2000). The additional time to perform this screening would depend on the standard operating procedures in the abattoir. Often, pigs' stomachs are opened and washed in the gut room before export, which allows quick inspection of the *Pars oesophagea* with minimum handling and interference. However, if stomachs are discarded without emptying the content, 1 to 2 minutes would be necessary to inspect each stomach (Nielsen and Ingvartsen, 2000). The same scoring system should be used in each slaughterhouse and needs to include presence of lesions as well as formation of scar tissue, as pre-existing ulcers may have healed prior to slaughter. Caution is necessary during abattoir screening, as transport and holding overnight at the abattoir may increase severity of gastric lesions due to stress and/or fasting (Lawrence *et al*., 1998; Amory *et al*., 2006).

In chapter 1, lesions were found in 62% of the 2-3 months old pigs, in 98% of the 6-8 months old pigs and in 100% of the adult sows. Compared to previous Belgian surveys, 35% more 6-8 months old pigs were affected with gastric lesions, due to an increased percentage of severe hyperkeratosis, while for adult sows this was 32%, due to an increased frequency of severe ulceration (Hessing *et al*., 1992; Roels *et al*., 1995). This confirms the overall trend towards a higher frequency of gastric lesions since the late 1950s which may be a result of pig production intensification (Queiroz *et al*., 1996; Swaby and Gregory, 2012). In the Netherlands, however, no distinct trend has been observed since 1990. There, the number of pig stomachs with an intact mucosa of the *Pars oesophagea* remains low, as well as the number of pigs with severe gastric ulceration (Geudeke and Houben, 2018). The presence and severity of lesions

can thus greatly vary between herds and geographical regions (Thomson and Friendship, 2012). Differences in management and feeding strategies may contribute to these discrepancies, although we did not obtain such information. For future studies, it is thus recommended to obtain background information when sampling pigs' stomachs. It cannot be excluded that differences in virulence exist between *Helicobacter suis* strains or other bacteria possibly involved in gastric lesion development such as the novel pathogen *Fusobacterium gastrosuis*, described in this thesis.

Although we only investigated a limited number of pig stomachs (< 200), they most likely originated from different pig farms, as stomachs were collected over a period of 2 years from 2 slaughterhouses located in different geographical regions. Our results indicate that gastric lesions are highly prevalent in today's Belgian pig industry. Furthermore, as gastric lesions may result in a decreased daily weight gain, decreased feed intake, sudden death and most likely also pain (De Bruyne *et al*., 2012), these high prevalence rates may imply major economic losses as well as animal welfare issues (Haesebrouck *et al*., 2009a).

Despite the high prevalence and economic impact of porcine gastric lesions, the exact mechanism behind this disease entity is largely unknown and clearly multifactorial. Furthermore, there are no preventive measures which completely protect pigs from gastric lesions (Thomson and Friendship, 2012). In this thesis, we aimed to gain new insights in the role of *H. suis* and *F. gastrosuis* in the development of porcine gastric lesions, which may ultimately facilitate the development of effective control measures.

New insights in the persistence of *H. suis* in the stomach

Pigs are commonly infected with the zoonotic pathogen *H. suis*, with prevalence rates of up to 95% (**chapter 1**) (Melnichouk *et al*., 1999; Roosendaal *et al*., 2002). Infection has been associated with gastritis, decreased daily weight gain and ulceration of the non-glandular part of the stomach in pigs, as well as with gastric disease in humans (Haesebrouck *et al*., 2009a). Its adaptation to the acidic environment as well as evasion of the host immune response contributes to a persistent infection of the gastric mucosa (Flahou *et al*., 2010; De Bruyne *et al*., 2012; Bosschem *et al*., 2017). Several immune-evasion mechanisms have been described for *H. pylori*, including presence of a regulatory T cell (Treg)

immune response, but these are mainly attributed to the presence of virulence factors VacA and CagA, which are absent in *H. suis* (Vermoote *et al*., 2011). It is important to determine the mechanisms by which *H. suis* evades the host immune system as well, since this may lead to development of potential strategies to prevent progression of chronic infection and development of gastric pathologies.

In chapter 1 and 4, a clear upregulation of the cytokine IL-10 was shown in the gastric mucosa of *H. suis*-infected 6-8 months old pigs, adult sows and BALB/c mice, which is in line with the findings of previous studies (Flahou *et al*., 2012; Bosschem *et al*., 2017). Since IL-10 is one of the most important cytokines produced by Treg cells and as these cells are immune-suppressive and tolerogenic (Bosschem *et al*., 2017), this response may contribute to the development of a chronic and persistent infection of the gastric mucosa. Indeed, when IL-10 deficient mice are experimentally infected with *H. pylori* or *H. felis*, they develop a more severe gastritis and immune response, resulting in clearance of infection (Matsumoto *et al*., 2005). Inhibition of the Treg immune response can be explored as a potential strategy to eliminate *H. suis* from the stomach, provided that severe inflammation can be avoided at the same time. A CCR4-antagonist, which inhibits Treg migration, could be used as adjuvant in *H. suis* vaccines, as it induces less pseudo-pyloric metaplasia compared to BALB/c mice immunized with cholera toxin or Freund's complete adjuvant, while maintaining good protection levels against bacterial colonization (Bosschem *et al*., 2015). However, further investigation is required since blocking Treg activity might evoke auto-immune manifestations (Bayry *et al*., 2014).

The molecular mechanisms by which *H. suis* elicits a Treg response are, however, unclear. Bosschem *et al*. showed that *H. suis* induces a semi maturation of porcine monocyte-derived dendritic cells *in vitro*, characterized by impaired expression of antigen presenting MHC class II molecules on the cell surface. It was suggested that this impaired dendritic cell response may elicit the expansion of Treg cells, as shown for *H. pylori*, resulting in a persistent and chronic gastric infection (Bosschem *et al*., 2017). Since *H. pylori* urease impairs folding and correct transport of MHC II molecules by binding to CD74 and as this enzyme is highly conserved among gastric helicobacters (Beswick *et al*., 2006), *H. suis* urease may play a similar role in inducing a Treg immune response.

In contrast with the porcine model, however, complete maturation of murine bone marrow-derived dendritic cells was induced by *H. suis*, followed by Th17 cell proliferation and production of IL-17 (Bosschem *et al*., 2017). Despite the absence of an impaired dendritic cell response, IL-10 is also upregulated in *H. suis*-infected mice (**chapter 4**) (Flahou *et al*., 2012; Bosschem *et al*., 2015, 2016), indicating that other mechanisms contribute to Treg cell expansion. For example in *H. pylori* infected humans, a high concentration of TGF-β has been shown to inhibit the production of IL-23, thereby hampering Th17 cell expansion and favouring Treg cell proliferation (Freire de Melo *et al*., 2012). This might explain why IL-23 and IL-17 were downregulated and why IL-10 was upregulated in *H. suis*-infected BALB/c mice (**chapter 4**), although we did not determine the expression of TGF-β. In addition, CagA positive *H. pylori* strains can subvert gastric epithelial cells as mediators to inhibit T cell proliferation and to promote expansion of Treg cells by increased expression of the T cell co-inhibitory molecule B7-H7 on their cell surface (Lina *et al*., 2014). It would be interesting to investigate if similar mechanisms are present during *H. suis* infection.

Apart from IL-10, the chemokine CXCL13 was significantly upregulated in *H. suis*-infected 2-3 months old pigs, 6-8 months old pigs and adult sows (**chapter 1**). Although this was not determined **in chapter 4**, others have shown increased expression of CXCL13 in the gastric mucosa of *H. suis*-infected mice and gerbils as well (Bosschem *et al*., 2016). As Treg cells are recruited by CXCL13 (Cook *et al*., 2014), increased transcript levels of this chemokine may have contributed to the development of a tolerogenic response. Furthermore, since this chemokine attracts B-lymphocytes, its upregulation might play a role in the development of a specific, local immune response towards *H. suis* in both pigs and rodents (Bosschem *et al*., 2017). Nevertheless, both theories require further research. At the moment, it is unclear by which mechanisms CXCL13 secretion is induced. Most likely, it is a part of the innate immune response, induced by *H. suis*, as dendritic cells as well as monocytes are the main source of CXCL13 production (Legler *et al*., 1998).

The presence of Treg cell expansion in *H. suis*-infected pigs and mice, however, still needs to be directly confirmed in future studies. For example, after Treg cell isolation from the gastric mucosa, local draining lymph nodes and/or spleen, the presence of Treg cell specific antigens FOXP3, CD3, CD4, CD25 and

CD127 can be assessed using flow cytometry. In order to determine Treg cell function, intracellular cytokines can be assessed using flow cytometry as well (IL-10, TGF-β). Determination of the Treg cell function is necessary as effector T cells may also upregulate CD25 and FOXP3, resulting in an overestimation of the Treg cell population when relying on antigen expression solely (Cappione, 2016).

Apart from a potential association between urease and semi-maturation of dendritic cells, other virulence factors may contribute to the establishment of a persistent and chronic infection. Analysis of the genome of *H. suis* shows presence of genes involved in sialic acid biosynthesis (*neuA*, *neuB* and *wecB*), which may contribute to evasion of the host complement system, as well as genes encoding enzymes involved in oxidative-stress resistance (*napA*, *sodB*, *katA*, *mutS*, *mdaB* and peroxiredoxin coding sequence), which may function as a defence mechanism against the host immune response (Vermoote *et al*., 2011). In addition, *H. suis* gamma-glutamyl transferase (GGT) has been shown to inhibit proliferation and cytokine production of lymphocytes *in vitro* through deprivation of extracellular glutamine (Gln), a conditional essential amino acid important for normal cell function and maintenance, and hydrolysis of glutathione (GSH), an important antioxidant (Zhang *et al*., 2013). It would be interesting to experimentally infect mice or pigs with *H. suis* mutants lacking these potential virulence factors to investigate their role in evasion of the host immune response. As such, this may lead to recognition of potential intervention targets to prevent the progression of chronic infection.

New insights in the role of *H. suis* in stomach lesion development

Severe hyperkeratosis and erosions were exclusively found in the *H. suis*-infected 6-8 months old pigs and not in the non-infected pigs of this age group. In adult sows, ulceration was also mainly found in the *H. suis*-positive animals, indicating that *H. suis* might be involved in the development of gastric lesions (**chapter 1**). As positive correlations have been shown between the presence of *H. suis* in the acid secreting, glandular part of the stomach and the presence and severity of lesions in the upper, non-glandular *Pars oesophagea* (Hellemans *et al*., 2007; Haesebrouck *et al*., 2009b; De Bruyne *et al*., 2012), alterations in hydrochloric acid production, induced by *H. suis*, might play a role in the pathogenesis of porcine gastric lesions. For the first time, evidence for this hypothesis was obtained in **chapter 1**, as *H. suis* infection was associated with alterations in the expression of markers for gastric acid secretion and

G-cell mass, which may affect gastric acid secretion. Nevertheless, as increased mRNA expression levels not always result in increased protein expression and secretion (Greenbaum *et al*., 2003), our results need to be confirmed at the protein level by ELISA, western blot or multiplex bead-based immunoassay. In addition, the gastric pH should be measured to verify presence of gastric acid secretion alterations.

Direct as well as indirect mechanisms can be involved in the altered gastric acid secretion in *H. suis*-infected animals. It is necessary to verify the presence of these mechanisms and to identify virulence factors inducing these alterations, as this may lead to development of effective control measures of porcine gastric lesions.

A clear parietal cell loss has been shown in *H. suis*-infected Mongolian gerbils and mice which may result in a decreased gastric acid secretion capacity (**chapter 4**) (Flahou *et al*., 2010; Zhang *et al*., 2015). Parietal cell viability can be directly affected by *H. suis*, as viable *H. suis* bacteria and bacterial lysate have been shown to induce parietal cell death *in vitro* (Zhang *et al*., 2016). Although GGT induces apoptosis in gastric epithelial cells *in vitro* (Flahou *et al*., 2011), a mutant lacking the gene encoding GGT did not affect the viability of porcine parietal cells *in vitro*, indicating that other bacterial metabolites may be involved in parietal cell death (Zhang *et al*., 2016). The host immune response might affect parietal cell viability as well, as negative correlations were found between the number of parietal cells and inflammatory cell infiltration in *H. suis*-infected BALB/c mice (**chapter 4**). In contrast, no parietal cell loss was observed in the *H. suis*-infected pigs of the different age groups (**chapter 1**), similar to the findings of others (Zhang *et al*., 2016). Host factors, which are absent in *in vitro* studies, might influence the effect of *H. suis* infection on porcine parietal cells. The effect on parietal cells *in vivo* might also differ between different host species, since the host reaction may vary between host species. Infection of non-natural hosts (i.e. rodents) is indeed associated with more severe gastric pathologies (Flahou *et al*., 2017). As pigs are the natural host of *H. suis*, the results obtained in pigs might be more relevant than those obtained in rodents. Nevertheless, we could not exclude small differences in the number of these cells between the infected and non-infected pigs, since counting was impossible in the fundic gland zone due to the high area percentage of parietal cells (> 800/high power field). Automatic

cell counting might be a solution to overcome this problem, for example by use of ImageJ software.

Despite the seeming lack of impact on parietal cell mass, we showed altered expression levels of H^+/K^+ ATPase, KCNQ1, H2-, M3- and CCK-B receptors during *H. suis* infection in both pigs and mice (**chapter 1 and 4**). As these markers are associated with gastric acid production by parietal cells, this may indicate that the function of these cells was affected. Since the bacterium is often found in the vicinity of or inside the canaliculi of parietal cells of pigs, non-human primates and experimentally infected rodents (**chapter 1 and 4**) (Dubois *et al*., 1991; Joo *et al*., 2007; Flahou *et al*., 2010), a direct effect of *H. suis* on the parietal cell function could be involved. Recently, Zhang *et al*. showed that *H. suis* is able to adhere to parietal cells *in vitro* (Zhang *et al*., 2016). Parietal cell binding with *H. suis* or its bacterial products may induce intracellular signalling cascades, thereby affecting gene expression. One of these bacterial products might be GGT, as mice infected with *H. suis* Δ GGT showed no parietal cell loss and no downregulation of H^+/K^+ ATPase expression, in contrast with mice infected with *H. suis* wild type. Nevertheless, the downregulated expression of H^+/K^+ ATPase might have been a direct consequence of parietal cell death and not of gene expression alterations. Furthermore, no differences were seen between Mongolian gerbils infected with *H. suis* Δ GGT and *H. suis* wild type (Zhang *et al*., 2015) and, as already mentioned, *H. suis* Δ GGT did not affect the viability of porcine parietal cells *in vitro* (Zhang *et al*., 2016). The exact role of GGT in gastric acid secretion alterations seems to depend on the host and remains to be further determined.

For *H. pylori*, members of the cag pathogenicity island (CagA, CagE, CagL and CagM) have been described to downregulate the expression of H^+/K^+ ATPase through activation of the NF-κB pathway (Saha *et al*., 2010; Hammond *et al*., 2015). In addition, VacA disrupts apical membrane-cytoskeletal interactions in parietal cells, thereby affecting HCl secretion (Wang *et al*., 2008). Although the genome of *H. suis* possesses two members of the *H. pylori* cag pathogenicity island (CagE and CagX), the majority of the genes are missing, indicating that the cag protein transporter secretion system is not functional. Furthermore, *H. suis* possesses a paralog of the *H. pylori* VacA, but its functionality is not clear as it lacks a VacA signal sequence (Vermoote *et al*., 2011). *In vitro* and *in vivo* studies with knockout mutants of *H. suis* VacA paralog, CagE and/or CagX could clarify the functionality of these

H. pylori homologs and their potential role in gene expression alterations.

Indirect effects probably also play a role since the number of G-cells and/or the expression of gastrin and somatostatin were decreased or enhanced in pigs with downregulated and upregulated expression of H^+/K^+ ATPase, respectively. It has been hypothesized that *H. pylori* urease induces local alkalinisation of the gastric mucosa by increasing ammonia levels, which stimulates gastrin release and inhibits somatostatin release (Calam *et al*., 1999). Indeed, inverse correlations have been found between luminal ammonia levels and somatostatin concentration in *H. pylori*-infected humans (Kaneko *et al*., 1992). In addition, chronic high ammonia has been shown to exert a trophic effect on G-cell mass in *H. pylori* infected human patients (Waldum *et al*., 2016). Nevertheless, the role of urease remains controversial, as neither increasing *H. pylori* ammonia production by intragastric infusion of urea (Chittajallu *et al*., 1991), nor inhibiting ammonia production by acetohydroxamic acid (el Nujumi *et al*., 1991) could induce serum gastrin alterations. Besides urease, *H. pylori* produces N-alpha-methyl histamine, which has been shown to promote gastrin release from G-cells via histamine H2 receptor binding (Bliss *et al*., 1999). Nevertheless, as N-alpha-methyl histamine can also bind to histamine H3 receptors on enterochromaffin cells, thereby inhibiting histamine release (Prinz *et al*., 1993), its impact on gastric acid secretion is unclear. Finally, *H. pylori* LPS can stimulate gastric acid secretion through upregulation of histamine, although this was observed in only 1 out of 5 strains (Padol *et al*., 2001). Since *H. suis* also possesses urease, LPS and probably N-alpha-methyl histamine, these factors might play a role in the altered gastric acid secretion during *H. suis* infection, but further research is necessary.

The host immune response might also indirectly affect parietal cell function. In *H. pylori* infections, increased gastric acid secretion has been associated with increased expression of genes encoding IL-8 and IL-1β (Calam, 1996, 1999; Beales *et al*., 1997). Expression of genes encoding these cytokines was upregulated in the fundic gland zone of adult sows with upregulated expression of genes encoding H^+/K^+ ATPase (**chapter 1**). Furthermore, positive correlations were found between the expression of inflammatory cytokines (IL-8, IL-10, IL-17, IFN-γ) and markers for gastric acid secretion (H^+/K^+ ATPase, KCNQ1, CCK-B receptor) in both *H. suis*-infected pigs and BALB/c mice (**chapter 1 and 4**). Nevertheless, the role of the host immune response remains controversial, as Beales and Calam

demonstrated that IL-1β inhibits acid secretion in cultured parietal cells (Beales and Calam, 1998). To further determine whether changes in gastric acid secretion during *H. suis* infection may be due to a direct effect of *H. suis* on parietal cells and/or due to the activation of the host immune response, porcine parietal cells can be co-cultured with *H. suis* or with *H. suis* activated cells of the immune system.

In contrast to our findings, others did not find alterations in serum/tissue gastrin or G-cell mass in *H. suis*-infected 5-8 months old pigs (Bunn *et al*., 1981; Silva *et al*., 2002). Furthermore, while we have shown a decreased G-cell mass in *H. suis*-infected 6-8 months old pigs, Sapierzynski *et al*. found an increased G-cell mass and decreased D-cell mass in *H. suis*-infected pigs of the same age group, although not in all of them (Sapierzynski *et al*., 2007). These discrepancies might be explained by the use of different detection techniques for presence of *H. suis* (carbol fuchsin staining vs. RT-PCR), differences in virulence between *H. suis* strains, variation between herds and/or presence or absence of other factors that may play a role in the development of gastric pathologies such as diet, feeding strategy and management. We also investigated more markers for gastric acid secretion, apart from gastrin and somatostatin, as well as a large number of pigs' stomachs of different ages, to increase the chance to detect gastric acid secretion alterations.

Apart from HCl, increased contact of the *Pars oesophagea* with distally produced pepsin or bile salts may also result in irritation and formation of lesions (Rokkjaer *et al*., 1979). Elevated serum levels of the pepsin precursor pepsinogen have been shown in pigs with *Pars oesophageal* lesions, suggesting increased luminal pepsin concentration. In human patients, increased levels of pepsin and acid are associated with severe peptic ulceration and *H. pylori* has been shown to increase pepsinogen secretion from human peptic cells *in vitro* (Lorente *et al*., 2002). Not VacA or CagA, but LPS and/or other proteins of the bacterial cell wall are hypothesized to stimulate pepsinogen secretion (Young *et al*., 1992). It remains to be determined if *H. suis* infection also augments pepsin levels, resulting in irritation of the *Pars oesophagea.*

Synergism between *H. suis*, gastric microbiota and porcine gastric lesions

The gastro-intestinal microbiota plays an important role in host metabolism, nutrition, immunity and protection against pathogens (Castillo *et al*., 2007; Metzler-Zebeli *et al*., 2013). Disturbance of this delicate system may result in an overgrowth of pathogens and development of gastro-intestinal pathologies (Cho and Blaser, 2012). It can be hypothesized that an impaired gastric acid secretion during *H. suis* infection favours the establishment of a specific gastric microbiota which, on its turn, might affect porcine gastric lesion development. Indeed, we have shown for the first time that the relative abundance of gastric bacterial species belonging to *Fusobacteriaceae*, *Porphyromonadaceae*, *Bacteroidaceae*, *Clostridiaceae*_1 and *Gammaproteobacteriaceae* was higher in *H. suis*-infected pigs, while lower for species belonging to *Actinobacteria*, *Bifidobacteriaceae*, *Corynebacteriaceae*, *Coriobacteriaceae* and *Streptococcaceae* (**chapter 3**). Interestingly, a novel *Fusobacterium* sp., designated *F. gastrosuis* (**chapter 2**), was highly abundant in the gastric microbial community of the *Pars oesophagea* of *H. suis*-infected animals. It comprised up to 20% of this community in these animals. We hypothesized that this novel bacterium could play a role in the development of gastric lesions, since other *Fusobacterium* spp. are associated with development of (pyo)necrotic infections in other tissues of other host species (McGuire *et al*., 2014). Most of the other gastric bacterial species, however, are yet unknown and since only a limited number of animals were investigated, further research is necessary to investigate their potential role in development of gastric pathologies.

Other organisms (f.e. yeasts, parasites) might also affect lesion development in synergism with *H. suis*, although their presence was not determined in our study. For example, co-infection with *H. pylori* and *Candida* spp. has been associated with an increased prevalence of gastric ulceration in humans (Karczewska *et al*., 2009). Fungal colonization of gastric ulcers also impairs ulcer healing, accompanied by persistent clinical symptoms (Zwolinska-Wcisło *et al*., 2001). *Candida* spp. are normal inhabitants of the oropharynx and large intestines, but transient in the stomach and small intestines. As such, *H. pylori* infection may facilitate gastric colonization of these yeasts. Other mechanisms besides a decreased gastric acid secretion are more likely to be involved, as *Candida* spp. are able to survive and grow at a pH of 2 (Sherrington *et al*., 2017). For example, binding of *Helicobacter* spp. to gastric

epithelial cells triggers inflammatory cell infiltration and production of pro-inflammatory cytokines. As this innate immune response is not specific, the viability of other gastric bacteria may be affected and/or nutrients may be released during collateral damage to host cells (Maldonado-Contreras *et al*., 2011). Furthermore, *Helicobacter* spp. urease has been hypothesized to alter gastric microbiota composition, as it provides substrates, such as ammonia and bicarbonate, for the growth of surrounding gastric bacteria (Williams *et al*., 1996). Indeed, ammonia-oxidizing bacteria were abundant metabolic phenotypes of the gastric microbiota in 6-8 months old pigs and BALB/c mice (**chapter 3-4**). Similarly, presence of *C. albicans* and *C. slooffii* in parakeratotic and necrotic epithelial tissues of the *Pars oesophagea* of pigs has been described, although it remains to be determined whether these colonizing *Candida* spp. were a contributing factor to gastric ulceration in synergism with *H. suis* or that presence of gastric lesions merely facilitated colonization by these yeasts (Tannock and Smith, 1970). Synergism has already been shown between *H. suis* and *Kazachstania heterogenica*, a yeast which naturally colonizes the pyloric gland zone of Mongolian gerbils. When these animals were co-infected with *K. heterogenica* and *H. suis*, a significant increase in the severity of gastritis was demonstrated compared to gerbils infected only with *H. suis* (Flahou *et al.*, 2010a). It is not known whether pigs co-infected with *H. suis* and *K. slooffiae* may develop more severe gastric pathologies.

A decreased gastric acid secretion may not only affect the microbiota of the stomach, but other gastro-intestinal sites as well. The use of acid suppressive agents has been associated with development of enteric infections with *Salmonella* spp., *Campylobacter* spp., *Shigella* spp. and *Clostridium difficile*, especially in immunocompromised human patients (Dial, 2009; Kwok *et al*., 2012). In the oesophageal microbiota, *Comamonadaceae* were decreased following proton pump inhibitor treatment, while *Clostridiaceae, Lachnospiraceae, Microccocaceae, Actinomycetaceae* and unclassified families from Clostridia, Lactobacillales and Gemellales were increased (Amir *et al*., 2014). An increased abundance of *Lactobacillus* spp. and *Veillonella* spp. has also been observed in the lower intestine of human patients with chronic atrophic gastritis. Due to the decreased gastric acid secretion, these oropharyngeal bacteria were most likely able to survive gastric passage after which they colonized the lower intestines (Kanno *et al*., 2009). Impaired gastric acid secretion also hampers protein digestion in the stomach, resulting in

an increase of unabsorbed nutrients in the lower intestines. Bacterial fermentation of these malabsorbed proteins results in the production of harmful metabolites (H_2S, NH_3) associated with the development of colonic cancer and ulcerative colitis (Evenepoel *et al*., 1998). Similarly in pigs, post weaning diarrhoea has been associated with a high gastric pH, allowing intestinal proliferation of enterotoxigenic *E. coli* (Rhouma *et al*., 2017). An intact gastric acid barrier is thus important for maintenance of gastro-intestinal health. It remains to be determined if the decreased gastric acid secretion in *H. suis*-infected 6-8 months old pigs also affects the microbial composition of other gastro-intestinal sites and whether this is associated with development of pathologies.

Gastro-oesophageal reflux (GER) disease in human patients and lesions of the *Pars oesophagea* in pigs share some similarities. Both are associated with increased gastric acid secretion in the glandular part of the stomach induced by *Helicobacter* spp. infection, resulting in inflammation and lesion development. Furthermore, the *Pars oesophagea* of the porcine stomach is a continuation of the distal oesophagus into the stomach and the epithelium of the oesophagus of humans and pigs is very similar (Ziegler *et al*., 2016). In healthy humans, the microbiota of the distal oesophagus resembles that of the oropharynx, with *Streptococcus* as dominant genus as well as other genera, such as *Fusobacterium*, *Neisseria*, *Haemophilus* and *Prevotella* (Walker and Talley, 2014). Acid reflux and metaplasia during GER, however, causes a significant decrease in Gram-positive bacteria and an increase in *Enterobacteriaceae* and other Gram-negative anaerobic/microaerophilic bacteria, including *Fusobacterium* (Yang *et al*., 2009). The Gram-negative predominant microbiota then further contributes to inflammation and lesion development of the oesophagus, as increased concentrations of LPS, present in the cell wall of Gram-negative bacteria, promote relaxation of the upper oesophageal sphincter and delayed gastric emptying (Walker and Talley, 2014). Synergism between increased gastric acid and alterations in the *Pars oesophageal* microbiota might have contributed to the development of severe lesions in *H. suis-infected* adult sows. It would be interesting to determine the microbiota of the *Pars oesophagea* of adult pigs chronically infected with *H. suis*.

It is possible that other microorganisms colonizing or infecting the gastric mucosa may enhance the outcome of gastric pathologies induced by *H. suis*. When performing *in vivo* experimental infection studies with *H. suis,* one should be aware of potential interactions with other micro-organisms and, preferably, gastric microbiota analysis should be included. In addition, changes in gastric acid secretion may have an impact on the microbiota of other gastro-intestinal sites, which may lead to the development of a wide range of pathologies.

Pathogenic significance of *F. gastrosuis*

Fusobacterium spp. can aggravate necrosis when tissue damage is initiated by other microorganisms or environmental factors. For example, fighting or environmental induced lesions of the porcine skin, mouth and/or hoofs can be secondary infected with *F. necrophorum,* resulting in skin necrosis, necrotic stomatitis and formation of hoof abscesses, respectively (Zhou *et al*., 2010; Cameron *et al*., 2012). **In chapter 3**, we have shown that viable *F. gastrosuis* bacteria as well as lysate can induce apoptosis and/or necrosis of epithelial cell lines. It was also demonstrated that colonization of the *Pars oesophagea* was increased in *H. suis*-infected animals with alterations in gastric acid secretion and with gastric ulceration, giving further evidence for the involvement of *F. gastrosuis* in the development of *Pars oesophageal* lesions. We hypothesize that, in a gastric environment altered by *H. suis*, colonization and invasion of the *Pars oesophagea* and production of epithelial cell death inducing metabolites by *F. gastrosuis* cause ulceration. Possibly, heat labile proteins variably expressed on the bacterial surface play a role, as heat and proteinase K treated *F. gastrosuis* bacteria completely lost their ability to induce host cell death, while variable results were obtained for paraformaldehyde treated bacteria. Further studies are necessary to characterize virulence factors of *F. gastrosuis* inducing apoptosis and/or necrosis. For example, after separation of *F. gastrosuis* proteins present in the lysate by ion exchange chromatography and by size exclusion chromatography, the different fractions can be added to cell lines, after which the proteins responsible for the observed apoptosis and/or necrosis can be identified using SDS-PAGE and mass spectrometry. Mutants lacking putative virulence genes can then be constructed, after which their adhering, invading and cell-death inducing capacity can be studied in gastric cell lines as well as in *Pars oesophagea* explants and compared to the parent strain. Finally, a combined infection model with *F.*

gastrosuis and *H. suis* should be developed.

Some *Fusobacterium* spp. possess genes encoding a diverse set of adhesins and membrane-related proteins allowing them to enter host cells actively, even in the absence of tissue damage initiated by other agents (McGuire *et al*., 2014). Analysis of the genome of *F. gastrosuis* showed presence of such genes (**chapter 3**), indicating that this bacterium is also able to actively invade gastric epithelial cells. It would be interesting to confirm if *F. gastrosuis* indeed invades host cells and by which mechanisms, for example by the use of invasion assays and/or light microscopy. *In vitro* studies have already shown that *F. nucleatum* closely adheres to human gingival epithelial cells using lectin-like interactions, after which the bacterium actively invades host cells using a zipping mechanism, resulting in engulfment of the bacteria in intracellular vacuoles. This mechanism requires participation of host actin, microtubules, signal transduction and energy metabolism (Han *et al*., 2000). After internalization, *F. nucleatum*, *F. necrophorum* and *F. mortiferum* have been shown to multiply intracellularly for up to 8h (Kahraman Gursoy *et al*., 2008). As the use of an actin formation inhibitor markedly reduced the number of living intracellular bacteria, it was hypothesized that these *Fusobacterium* spp. are able to re-arrange actin filaments to form a barrier-like structure around its invasion vacuole, thereby preventing fusion with lysosomes as described for *E. coli* and *Salmonella* spp. (Ribet and Cossart, 2015). After replication, *Fusobacterium* spp. were released in the medium and formed co-aggregations with each other, prolonging their survival in an aerobic environment. Some strains were even able to re-enter the host cells (Kahraman Gursoy *et al*., 2008). Presence of a VacJ homolog, an important virulence factor of *Shigella flexneri* and *E. coli*, in the proteome of several *Fusobacterium* spp., including *F. gastrosuis*, might indicate that these bacteria are also able to spread intercellularly, without using the extracellular environment (Karpathy *et al*., 2007). These mechanisms most probably contribute to the pathogenicity of *Fusobacterium* spp., as they allow host immune system evasion and spread to deeper tissues (Han *et al*., 2000).

Several mechanisms might play a role in the epithelial cell death induced by *F. gastrosuis*. DNA and/or mitochondrial damage may be directly induced by *F. gastrosuis* and/or its metabolites, resulting in activation of the intrinsic apoptotic pathway (Fink and Cookson, 2005). Proteome analysis of *F.*

gastrosuis showed presence of GGT. As this enzyme hydrolyses GSH, reactive oxygen species (ROS) may be produced. Transpeptidation of the gamma-glutamyl group generates the more reactive thiol cysteinyl-glycine, leading to production of H_2O_2 through reduction of Fe^{3+} and subsequent production of thiyl radicals and superoxide anion (Flahou *et al*., 2011). These ROS can then induce apoptosis and/or oncosis through molecular changes to lipids, proteins and DNA (Djavaheri-Mergny *et al*., 2002). Furthermore, *F. gastrosuis* also shows presence of butyric acid (BA) producing genes. BA, a major extracellular metabolite of periodontopathic bacteria, has been shown to induce apoptosis in different cell types mediated by ROS production in the mitochondria and by ceramide production (Ochiai and Kurita-Ochiai, 2009). Nevertheless, BA has also been shown to play a protective role in the development of irritable and non-specific inflammatory bowel disease (Borycka-Kiciak *et al*., 2017). Moquet *et al*. have demonstrated that the beneficial effect of BA on gut health of broilers depends on the gastro-intestinal region considered. When high concentrations of BA are present in the stomach and small intestine, regions where BA production is normally limited, inflammation and dysbiosis is induced in colon and caecum. Conversely, high production of BA in colon and caecum, regions where BA production is substantial, reduces inflammation and promotes host health (Moquet *et al*., 2018). The role of this short chain fatty acid in porcine gastric lesion development remains to be determined.

Currently, different secretion systems (including type I, II, III, IV, V and VI) have been found in bacteria that mediate host cell death through formation of membrane pores after which bacterial effector proteins are actively transferred into the host cells (Henderson and Nataro, 2001). Although genome analysis of *F. nucleatum* as well as *F. gastrosuis* revealed absence of the ubiquitous type III and IV secretion systems, presence of a conserved family of outer membrane proteins (OMPs) was shown, showing homology with type Va secretion systems (T5SS) or autotransporters (**chapter 3**) (Kaplan *et al*., 2010). OMPs of *F. nucleatum* are suggested to induce cell death through a contact-based mechanism rather than through secretion of effector proteins, as cell free *F. nucleatum* membranes were able to induce cell death in epithelial cells to similar levels as intact bacteria. The exact mechanisms behind this contact-based cell death are, however, unknown. It has been hypothesized that OMPs may induce apoptosis by direct delivery of death signals through contact with host cell surface death receptors, or by aggregation

of host cells, mediated by *F. nucleatum*, allowing host cell death receptors to be cross-linked with their ligands on adjacent cells (Kaplan *et al*., 2005). Nevertheless, a potential role of pore forming proteins and/or injection of bacterial effector proteins cannot be excluded, as genes encoding proteases, leukotoxin and hemolysin have been found in *Fusobacterium* spp. and have been associated with host cell death *in vitro* (Bachrach *et al*., 2004; Miao *et al*., 2010). Most likely, several mechanisms are associated with host cell death induced by *Fusobacterium* spp. Since we also demonstrated presence of T5SS, protease and hemolysin homologs in the proteome of *F. gastrosuis*, these virulence factors might be involved in the observed epithelial cell death. Further research is necessary to determine whether or not these putative proteins are expressed, secreted and/or functional, as well as their roles in bacterial pathogenicity.

Immune cells as well as epithelial cells are able to recognize conserved elements of a wide range of pathogens, also known as pathogen-associated molecular patterns (PAMPs), through presence of pattern recognition receptors (PRRs) on their cell surface. Toll-like receptors (TLRs) are a well-known family of PRRs. Binding of PAMPs on TLRs activates intracellular signalling cascades leading to NF-κB activation and to initiation of the innate immune response (Salaun *et al*., 2007). Indeed, *in vitro* studies have shown that *F. nucleatum* LPS and lipoprotein binding to TLR-2 and -4, respectively, as well as internalized *F. gastrosuis* DNA binding to endosomal TRL-9 results in the production of TNF-α, IL-6 and IL-8 (Gonzalez *et al*., 2010; Park *et al*., 2014). Apart from immune response activation, TLRs binding may activate the apoptotic program in host cells through activation of caspase 8 and subsequently caspase 3/6/9 (Salaun *et al*., 2007). It might be possible that PAMPs of *F. gastrosuis* binding to TLRs also plays a role in inducing epithelial cell death.

In conclusion, *F. gastrosuis* might be able to adhere and actively invade gastric epithelial cells, resulting in host cell death. Furthermore, presence of other genes encoding proteins involved in intercellular spread, extracellular matrix degeneration, iron uptake, antibiotic resistance, capsule and biofilm formation may further contribute to the pathogenic significance of *F. gastrosuis*. A schematic overview of potential interactions between *F. gastrosuis* and host cells is represented in **Figure 1**.

Several studies have described a synergism between *Fusobacterium* spp. and other micro-organisms in the onset of pathologies. During the initial stages of periodontitis, *Streptococcus* spp. colonize the tooth enamel and root surface, which sets the stage for *F. nucleatum*. Due to its ability to connect with other bacteria, *F. nucleatum* serves as a 'bridge' between these early colonizers and late periodontopathic colonizers, such as *Porphyromonas gingivalis* and *Aggregatibacter actinomycetemcomitans*, leading to dental plaque formation and lesion development (Kapatral *et al*., 2002; Li *et al*., 2015). Co-infection with *F. nucleatum* also enhances the adhering and invasive capacities of *P. gingivalis* and *A. actinomycetemcomitans* to human gingival cells *in vitro* (Saito *et al*., 2008; Li *et al*., 2015). These synergistic effects are mediated by the presence of adhesins on *F. nucleatum* cell surface. In addition, *in vitro* studies have shown that *H. pylori* co-aggregates with oral *Fusobacterium* spp. (Andersen *et al*., 1998) and a higher number of human patients with periodontitis had a coexistent presence of *H. pylori* in the stomach and in dental plaque compared with non-periodontitis patients (Al Asqah *et al*., 2009). It is therefore hypothesized that dental plaque may function as a reservoir for *H. pylori*, and potentially a source of transmission or reinfection for the stomach (Al Asqah *et al*., 2009). As *H. suis* and *F. gastrosuis* are both present in the oropharynx (unpublished results, **chapter 3**), similar interactions may occur between both species. Presence of genes encoding proteins associated with adhesion and biofilm formation in the proteome of *F. gastrosuis* seems to strengthen this hypothesis, although further investigation remains necessary.

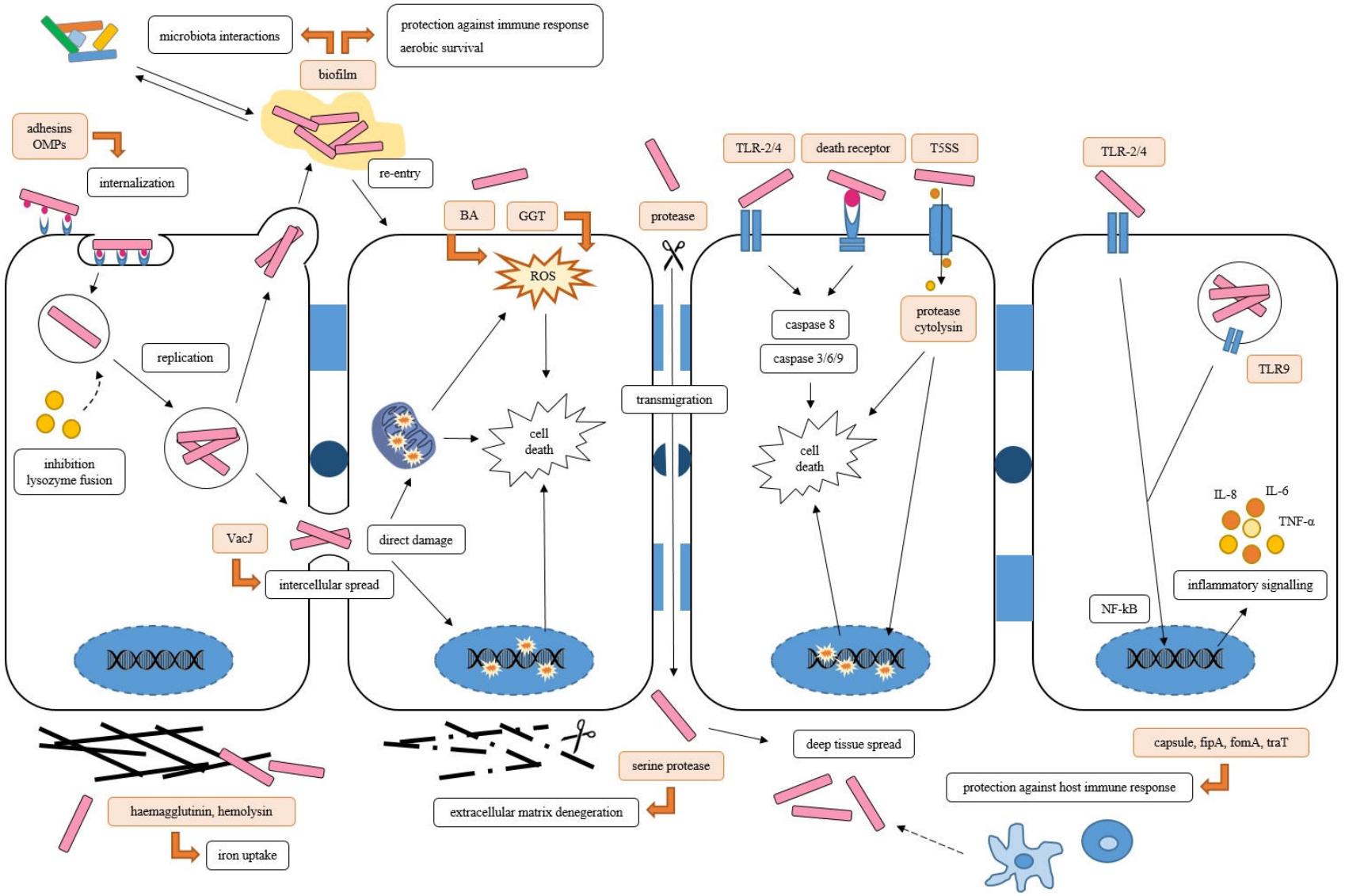


Figure 1: A schematic overview of potential interactions between *F. gastrosuis* and gastric epithelial cells. BA = butyric acid, GGT = gamma-glutamyl transferase, OMPs = outer membrane proteins, ROS = reactive oxygen species, T5SS = type V secretion system, TLR = toll-like receptor, VacJ = vacuolating cytotoxin J

Control measures against porcine gastric lesions

Although porcine gastric lesions are associated with significant economic losses, as well as animal welfare issues, no ideal solution exists which completely protects pigs from lesion development (Thomson and Friendship, 2012). The results from **chapter 1-3** lead to the hypothesis that *H. suis* is involved in gastric pathology through its effects on gastric acid secretion and on the gastric microbiota composition. The novel pathogen, *F. gastrosuis,* might also play a role by inducing epithelial cell death. Inhibition of colonization by these bacteria and/or their induced pathologies can be considered as potential control measures against porcine gastric lesions.

Recently, an inhibitory action of bambermycin, a glycolipid antibiotic, has been described towards *H. pylori* strains (Tseng *et al*., 2014). Despite the relatively low MIC values of bambermycin on porcine *H. suis* strains, gastric *H. suis* colonization was not affected by bambermycin supplementation *in vivo* (**chapter 4**). Still, a decreased number of infiltrating T cells, B cells and macrophages as well as downregulated expressions of IL-1β, IL-8, IL-10 and IFN-γ were demonstrated and the parietal cell mass was not affected in contrast with *H. suis-infected* mice without bambermycin supplementation. It might be possible that in-feed medication of bambermycin counters the decreased gastric acid secretion observed in *H. suis*-infected 6-8 months old pigs. As such, no overgrowth of *F. gastrosuis* would occur and the *Pars oesophagea* would remain intact. In addition, *F. gastrosuis* colonization might be directly affected by bambermycin, as it has been shown that bambermycin supplementation reduces gastro-intestinal colonization of *Fusobacterium* spp. in sheep (Edwards *et al*., 2005). Experimental infection studies in pigs with and without bambermycin supplementation would be necessary to confirm or reject this hypothesis.

Bambermycin has been widely used as antimicrobial growth promotor (AGP) in animal feed. Despite its use for over 50 years, acquired resistance, transfer of resistance or cross-resistance with other antimicrobials has not yet been reported (Butaye *et al*., 2003). Beneficial effects of AGPs are often linked with a better equilibrium of the gastro-intestinal microbiota. Our and other studies indeed show that bambermycin supplementation results in a decreased relative abundance of pathogens, such as *Clostridium* spp., while bacteria considered to have beneficial effects, such as *Lactobacillus* spp., are

not affected (Butaye *et al*., 2003; Edwards *et al*., 2005). In addition, AGPs are hypothesized to decrease gastro-intestinal inflammation, thereby reducing metabolic cost which results in improved feed conversion and growth rate of the host, although no actual proof has been obtained so far (Niewold *et al*., 2007). For the first time, we have shown that bambermycin also decreases inflammatory cell infiltration and pro-inflammatory cytokine expression in the stomach. It has been hypothesized that macrolides, cyclines and streptogramins may accumulate in phagocytic cells, reducing the production of pro-inflammatory cytokines (Niewold *et al*., 2007). Macrolides may also inhibit T cell maturation and proliferation (Menconi *et al*., 2014). It is not known if similar mechanisms are involved for bambermycin. Finally, alterations in the gastric microbiota composition (i.e. a decreased abundance of potential pathogens) may have contributed to the anti-inflammatory effect of bambermycin as well. The increasing problem of antimicrobial resistance, however, has led to an overall ban of AGPs in animal feed in the European Union (Butaye *et al*., 2003). Despite its safety and beneficial effects, it is unlikely that bambermycin will be approved as prophylactic in-feed medication against porcine gastric lesions.

The combination of ulcer-healing drugs, for example proton pump inhibitors, and antibiotics to eradicate *H. pylori* has been shown to speed up ulcer healing in human patients with *H. pylori* positive duodenal ulcer compared to ulcer-healing drugs alone. Furthermore, this combination therapy was effective in preventing recurrence of duodenal ulcers (Ford *et al*., 2016). In pigs, however, medication with an ulcer-healing drug (lansoprazole) and an antibiotic (azithromycin) could not prevent development of gastric ulceration during fasting (Melnichouk *et al*., 1999). This discrepancy might be explained by high MIC values of azithromycin against *H. suis* (unpublished results), indicating that this antibiotic is not effective enough *in vivo*. In addition, the use of ulcer-healing drugs may have led to an increased gastric pH and overgrowth of opportunistic pathogens, contributing to lesion development. The potential use of antibiotics in prevention and/or healing of porcine gastric ulcers is thus not clear. Nevertheless, a widespread control strategy based on antibiotics is not indicated in *H. suis-infected* pigs since this is expensive, labor intensive and antibiotic use may favour spread of antimicrobial resistance in pathogens as well as in bacteria belonging to the microbiota. Indeed, recent studies in our laboratory have shown mutations in the *16S rRNA* and *gyrA* genes of porcine *H. suis* isolates with acquired resistance to

tetracycline and fluoroquinolones, respectively, but not in the non-human primate isolates. Remarkably, MICs of ampicillin were higher for porcine *H. suis* isolates than for isolates obtained from non-human primates and this was associated with mutations in the *pbp1A* gene. It is possible that the frequent use of these antimicrobial classes in the pig industry may have resulted in the development of acquired resistance, similar as described for clarithromycin resistant *H. pylori* strains, although further investigation is necessary (unpublished results).

A curative eradication method based on antibiotics may, however, be used to obtain pigs free from *H. suis* and *F. gastrosuis* and, consequently, gastric lesions. Especially closed system pig farms with a high lesion morbidity and/or mortality might profit from this one-time treatment. De Bruyne *et al*. showed the feasibility of medicated early weaning to obtain *H. suis*-negative pigs. In these animals, fewer lesions and less severe gastritis were observed compared to experimentally *H. suis*-infected pigs (De Bruyne *et al*., 2012). In-feed use of bambermycin as supportive treatment might also be considered, as it decreased gastric inflammation and inhibited the effects of a *H. suis* infection on parietal cell loss. Furthermore, bambermycin supplementation is regarded as safe, since no acquired resistance, transfer of resistance or cross-resistance has yet been reported despite its frequent use for over 50 years (Butaye *et al*., 2003).

Feeding coarsely ground feed is considered to be ulceroprotective and even decreases the severity of lesions present in pigs that were previously fed a diet with fine particle size (Ayles *et al*., 1996; Millet *et al*., 2012; Mößeler *et al*., 2012). These beneficial effects are mainly attributed to increased stomach content firmness and maintenance of the gastric pH gradient. A better equilibrium of the gastro-intestinal microbiota might also play a role, as the number of lactic acid bacteria and bacterial diversity was increased in the stomach and jejunum of pigs fed a coarsely ground diet compared to a standard finely ground pelleted diet, while the number of *Enterobacteriaceae* was decreased in caecum and colon (Canibe *et al*., 2005). Furthermore, a decreased risk for *Salmonella enterica* infection has been associated with coarsely ground diets compared to fine diets (Thomson and Friendship, 2012). Millet *et al*. showed a decreased amount of urease positive stomachs and a lower percentage of gastric lesions in pigs fed a coarsely ground diet compared to finely ground diet. Since urease testing gives an indication of the metabolic activity of *H. suis*, these results indicate that the feed structure may affect *H. suis*

infection induced gastric pathologies, such as ulceration (Millet *et al*., 2012). It would be interesting to further investigate the effect of coarsely ground diets on *H. suis* colonization rate and its protective effect on gastric acid secretion alterations and lesion development induced by *H. suis* and/or *F. gastrosuis*.

Nevertheless, feeding a coarsely ground diet is accompanied by decreased digestibility of the feed and reduced weight gain compared to fine and/or pelleted diets (Millet *et al*., 2012). These adverse effects seem to be limited when rotating finely and coarsely ground meal diets, while maintaining an ulceroprotective effect (De Jong *et al*., 2016). Adding organic acids and/or enzymes may also allow the use of coarse feed particle size as a means of reducing gastric ulcers, while maintaining acceptable feed conversion (Thomson and Friendship, 2012). In some countries like Denmark, the presence of *S. enterica* in pigs is economically penalized, which can make it advantageous to the producer to avoid *Salmonella* in the animals by feeding more coarsely ground feed, even though it is associated with lower growth performance (Alban *et al*., 2012).

GGT is an important virulence factor of gastric *Helicobacter* species. This enzyme hydrolyses its 2 substrates, i.e. GSH and Gln, into glutamate (Glu), after which Glu supplies energy for the bacterium. The first substrate, i.e. the tripeptide GSH, is a highly abundant free thiol in eukaryotic cells and plays an important role in cell cycle regulation and apoptosis. It is also an important anti-oxidant which degrades ROS (Ricci *et al*., 2014). High intracellular GSH concentrations are present in the gastric mucosa, as a form of protection against the high amount of ROS produced by mitochondria. The latter is as a direct result of the energy-demanding production of HCl. Together with presence of GSH in food and bile reflux, this provides a large amount of substrate for the GGT of *H. suis*. The other GGT substrate, Gln, is one of the most abundant amino acids in alimentary protein and it is abundant in the bloodstream and the intestinal lumen as well. Gln plays a crucial role in digestion, absorption and retention of nutrients, in maintenance of the epithelial barrier and in energy supply and anti-inflammatory functions of immune cells (Flahou *et al*., 2011; Zhang *et al*., 2013). Furthermore, Gln is a major fuel for enterocytes, especially for those of the small intestine (Ricci *et al*., 2014). It can be stated that the depletion of GSH and Gln affects host health (Flahou *et al*., 2011; Zhang *et al*., 2013). Indeed, *H. suis* GGT has been shown to induce gastric epithelial cell death and to inhibit T cell

proliferation and function *in vitro*, which may contribute to development of gastric lesions and immunosuppression, respectively (Flahou *et al*., 2011; Zhang *et al*., 2013). Degradation of GSH and Gln in the gastric lumen by *H. suis* GGT may also result in a depletion of these amino acids in the intestinal tract, affecting cell function, cell viability and integrity of the intestinal barrier. This may explain why intestinal leakage, associated with systemic inflammation and changes in the blood-cerebrospinal fluid integrity barrier, has been observed in *H. suis*-infected mice (Gorlé *et al*., 2017). Finally, **in chapter 3**, a GGT homolog was found in the proteome of *F. gastrosuis*, indicating that this bacterium might also hydrolyse GSH and Gln for energy. It is possible that the synergism between *H. suis* and *F. gastrosuis* GGT results in a massive depletion of GSH and Gln, thereby greatly affecting host health.

Oral supplementation of Gln has been shown to temper *H. suis* induced gastritis and epithelial proliferation in Mongolian gerbils, while GSH supplementation even resulted in a return to inflammation and proliferation baseline levels (De Bruyne *et al*., 2016). De Bruyne *et al*. also showed a pronounced reduction of gastritis and downregulated expression of IL-8 in experimentally *H. suis*-infected pigs fed a diet supplemented with 4% Gln compared to *H. suis*-infected pigs receiving the standard diet. IL-8 was also downregulated in pigs fed a 1% Gln diet, although gastritis was not tempered (De Bruyne *et al*., 2013). It would be interesting to verify if Gln and/or GSH supplementation also reduces severity of gastritis in naturally *H. suis*-infected pigs. As gastric inflammation may also affect gastric acid secretion and since GGT has been shown to alter parietal cell function and viability *in vitro* (Zhang *et al*., 2013), Gln and/or GSH supplementation might also be ulceroprotective. Furthermore, as we also found GGT in the proteome of *F. gastrosuis*, Gln and/or GSH supplementation might counter *F. gastrosuis* induced gastric pathologies as well. The addition of Gln and/or GSH to the feed may thus have several benefits for pig health and it can be easily manufactured and provided to the animals.

Vaccination is a potentially valuable approach to prevent *H. suis* infections and to manage gastric inflammation and ulceration in pigs. However, prophylactic immunization followed by challenge with *H. pylori*, *H. felis* or *H. suis* has been shown to induce a more severe gastritis compared to non-immunized, challenged mice (Sutton *et al*., 2001; Bosschem *et al*., 2015). This phenomenon hampers

vaccine development and so far, no vaccine formulation is available which completely protects the host against *Helicobacter* spp. infections. In recent years, attention has turned to self-replicating mRNA vaccination. Compared to the more conventional vaccination methods, antigens are produced de novo by host cells and are suggested to reduce the risk for post-immunization gastritis (Chahal *et al*., 2016). Furthermore, mRNA is expressed very efficiently in dividing as well as non-dividing cells, it does not contain antibiotic resistance genes and its expression is limited in time. It would be interesting to investigate the potential of self-replicating mRNA vaccines on the course of a *H. suis* infection. A potential strategy could be inhibition of the Treg cell expansion, as its immunosuppressive properties contribute to a persistent infection of the gastric mucosa.

Several studies have investigated the efficacy of probiotics on *H. pylori* eradication and improvement of *H. pylori* related gastric pathologies. Probiotics are defined as "live microorganisms which, when administered in adequate amounts, confer a health benefit on the host" (Mack *et al*., 2005). Frequently used probiotic strains are *Lactobacillus, Bifidobacterium* and *Saccharomyces* and their mode of action relies on competition for nutrients and adhesion to cell receptors, antimicrobial activity by secreting short chain fatty acids and antibacterial substances, as well as modulation of host immune system and microbiota composition (Goderska *et al*., 2018). In prospective human studies, probiotic monotherapy decreased *H. pylori* colonization density with 2% up to 64%, although the effect seemed to be strain dependent. Probiotic monotherapy has also been shown to eradicate *H. pylori* in up to 32.5% of the human patients. Nevertheless, as no data was obtained after treatment, reinfection could not be excluded (Boltin *et al*., 2016). The use of probiotics as adjuvants to antibiotics seems to improve *H. pylori* eradication efficiency and to reduce side effects, such as diarrhoea. The benefits are, however, strain-specific and more significant with relatively ineffective antibiotic regimens (Boltin *et al*., 2016). Although the use of probiotics during *Helicobacter* spp. infections is promising, the optimal probiotic species, dose and treatment duration are yet to be determined. Finally, experimental studies in rats showed protective as well as therapeutic effects of the use of probiotic *Lactobacillus* strains on gastric lesion development. It was hypothesized that probiotic supplementation results in an increased

production of prostaglandins, growth factors and anti-inflammatory cytokines and a decrease of apoptosis. Nevertheless, clinical trials in both humans and pigs are necessary to confirm these results.

Conclusions

Lesions of the upper, non-glandular part of the stomach are highly prevalent in the current Belgian pig population and may lead to significant economic losses as well as animal welfare issues. In order to develop effective control measures, it is important to identify the risk factors involved in gastric lesion development.

Several studies have attributed a role to *H. suis* in the development of ulceration of the upper, nonglandular *Pars oesophagea*, although *H. suis* does not colonize this stomach region. It is not completely clear how *H. suis* influences lesion development, but our studies indicate that alterations in gastric acid secretion may be involved. During the acute phase of a *H. suis* infection, the gastric acid secretion is unaffected and so no irritation occurs of the *Pars oesophagea*. Later on, a decreased gastric acid secretion in the glandular part of the stomach affects the composition of the *Pars oesophageal* microbiota, which may affect development of lesions in this non-glandular part of the stomach. Indeed, higher numbers of *F. gastrosuis* were detected in the *Pars oesophagea* of *H. suis*-infected 6-8 months old pigs than in non-infected pigs of the same age group. *In vitro* experiments showed that viable *F. gastrosuis* bacteria as well as bacterial lysate induced massive epithelial cell death and genome analysis showed presence of several, potential virulence genes. Finally, increased production of gastric acid during the chronic phase of infection might further aggravate severity of lesions in this stomach region, which is not protected by mucus. An overview of the main results and conclusions of this thesis is presented in Figure 2.

Our results lead to the hypothesis that *H. suis* is involved in gastric pathology through its effects on gastric acid secretion and on the gastric microbiota composition. We hypothesize that, in a gastric environment altered by *H. suis*, colonization and invasion of the *Pars oesophagea* and production of epithelial cell death inducing metabolites by the novel pathogen *F. gastrosuis* cause lesions of this stomach region. Experimental studies in pigs infected with *H. suis* and *F. gastrosuis* would be necessary to confirm these hypotheses. Inhibition of colonization by these bacteria and/or their induced pathologies can be considered as potential control measures against porcine gastric ulceration.

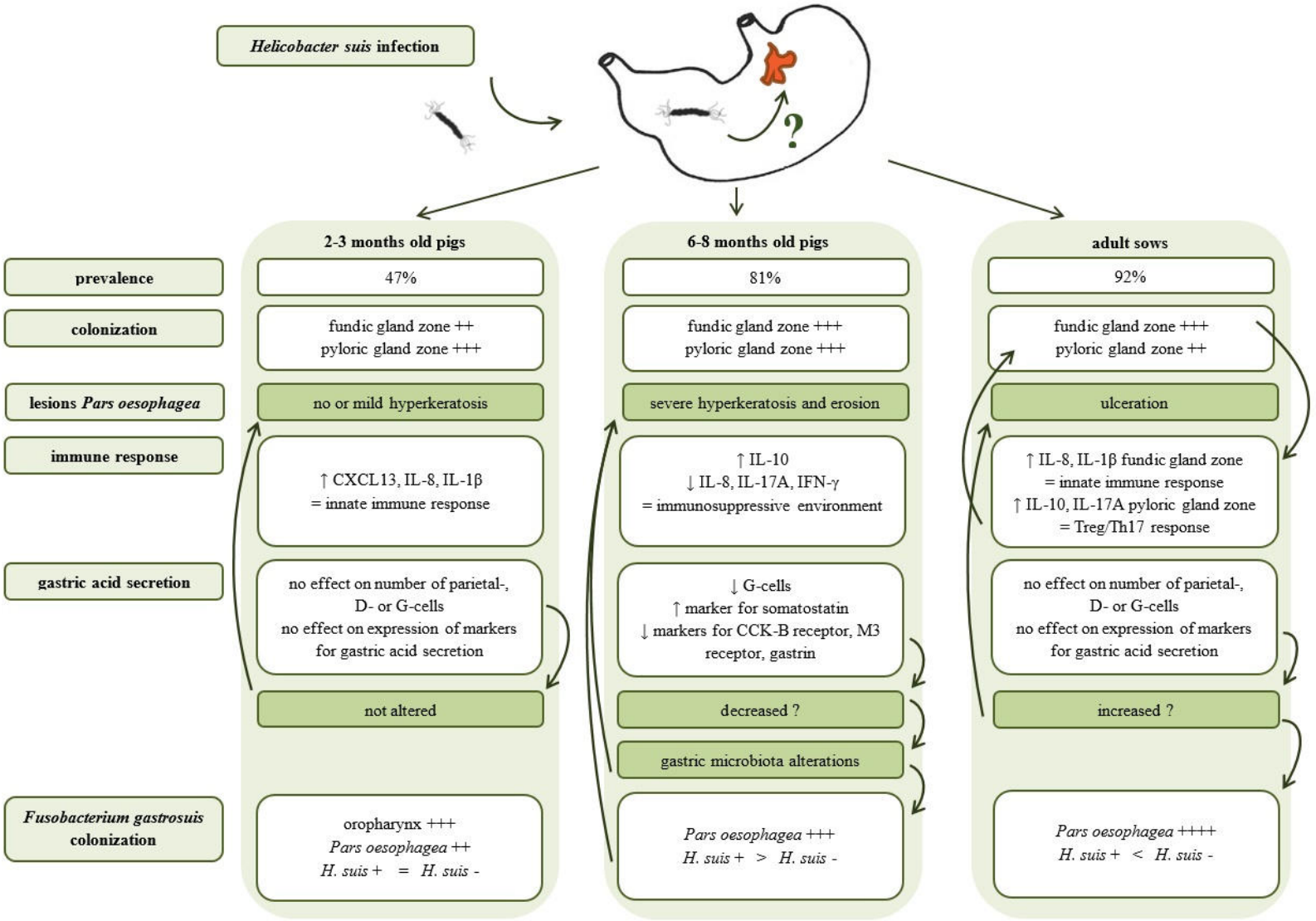


Figure 2: A diagram summarizing the main conclusions of this thesis. Our results lead to the hypothesis that *H. suis* is involved in gastric pathology through its effects on gastric acid secretion and on the gastric microbiota composition. The novel pathogen, *F. gastrosuis*, might also play a role by inducing epithelial cell death.

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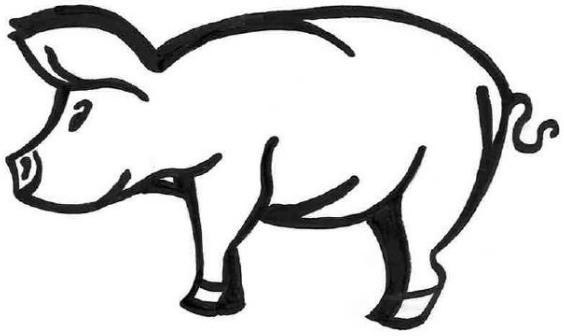


Summary

Gastric ulceration is a common problem in intensive pig production, with prevalences of up to 93%. It may result in decreased daily weight gain, decreased feed intake and sudden death, leading to significant economic losses and animal welfare issues. In contrast with human patients and several other animal species, gastric ulcers do not develop in the glandular part of the porcine stomach, but are almost exclusively found in the *Pars oesophagea*, a small area around the opening of the oesophagus which does not contain glands. Since this stomach region is not protected by mucus, it is highly susceptible to irritation with for instance hydrochloric acid, produced in the fundic and pyloric gland zone of the porcine stomach. Chronic insult of the *Pars oesophagea* results in hyperkeratosis, erosion and, eventually, ulceration. The aetiology is complex and multifactorial. Diet particle size, management and infection with pathogenic bacteria, such as *Helicobacter suis*, have been hypothesized to be involved. The exact mechanism behind porcine gastric ulceration is, however, not completely clear. Furthermore, there are no preventive measures which completely protect pigs from lesion development.

The general aim of this thesis was to gain new insights in the role of *H. suis* and a novel bacterial species described in this thesis, *Fusobacterium gastrosuis*, in the development of porcine gastric ulceration, which may ultimately facilitate the development of effective control measures.

Several studies have attributed a role to *H. suis* in the development of lesions of the *Pars oesophagea*, although *H. suis* does not colonize this region. It has been hypothesized that an infection with *H. suis* may result in secretion of excessive amounts of gastric acid in the distal part of the stomach, leading to increased contact, and consequently irritation, of the upper, non-glandular *Pars oesophagea* with hydrochloric acid. Therefore, the aim of **chapter 1** was to obtain further insights in the mechanisms involved in persistence of *H. suis* in the porcine stomach and in its effects on gastric acid secretion and lesion development. This was studied in naturally *H. suis*-infected pigs during the acute and chronic phases of infection (i.e. *H. suis*-infected and non-infected 2-3 months old pigs, 6-8 months old pigs and adult sows). In *H. suis*-infected 2–3 months old pigs, IL-8 and IL-1β transcript levels were upregulated in the pyloric gland zone, indicating an innate immune response. A similar response was demonstrated in the fundic gland zone of adult pigs, potentially due to a shift of *H. suis* colonization from the pyloric to the fundic gland zone. A Treg response in combination with decreased expressions of IL-8, IL-17A

and IFN-γ was indicated to be present in the *H. suis*-infected 6–8 months old pigs, which may have contributed to persistence of *H. suis*. In *H. suis*-infected adult pigs, a Treg response accompanied by a Th17 response was indicated, which may have played a role in the decreased number of *H. suis* bacteria in the stomach of this age group. No clear effects on markers for gastric acid secretion or numbers of acid secretion-associated cells and no lesions were detected in the *Pars oesophagea* in 2–3 months old pigs infected with *H. suis* (i.e. acute phase of infection). In a later phase of infection (i.e. 6–8 months old pigs infected with *H. suis*), markers for gastric acid secretion were downregulated, genes encoding somatostatin were upregulated and the number of G cells was decreased, indicating inhibition of gastric acid secretion. In this age group, severe hyperkeratosis and erosion of the *Pars oesophagea* were exclusively present in the *H. suis*-infected group. In adult sows, the prevalence of severe lesions was very high (i.e. chronic phase of infection) and the most severe lesions were mainly found in the *H. suis*-infected group. In the *H. suis*-infected adult pigs, markers for gastric acid secretion were upregulated and the number of G cells was increased, indicating increased gastric acid secretion.

It was hypothesised that, in a later phase of *H. suis* infection (i.e. 6–8 months old pigs), a decreased gastric acid secretion in the glandular part of the stomach may affect the composition of the microbiota in the *Pars oesophagea*, which may affect lesion development in this non-glandular part of the stomach. Indeed, in a recent metagenomics study it was demonstrated that an unidentified *Fusobacterium* sp. was abundantly present, representing up to 20% of the gastric microbial community of 6-8 months old pigs. Compared to *H. suis*-negative animals, higher numbers of this *Fusobacterium* sp. were detected in *H. suis*-infected animals.

Since most *Fusobacterium* spp. can aggravate necrosis when tissue damage is initiated by other micro-organisms or environmental factors, it was hypothesized that the novel *Fusobacterium* sp. could play a role in the development of porcine gastric ulceration. To enable further research into its pathogenicity, we decided to isolate and characterize this *Fusobacterium* sp. **in chapter 2**. In total, 9 strains were isolated from the stomach of 6-8 months old pigs and adult sows, from which 7 originated from the *Pars oesophagea* of pigs with lesions. Phenotypic, phylogenetic and genomic analysis showed that this novel species, designated *F. gastrosuis*, can be clearly differentiated from its nearest phylogenetic neighbours

F. mortiferum, F. russii, F. ulcerans, F. varium, F. necrogenes and, surprisingly, *Clostridium rectum.* In the same study, we also demonstrated that *C. rectum* and *F. mortiferum* represent the same species, with nomenclatural priority for the latter.

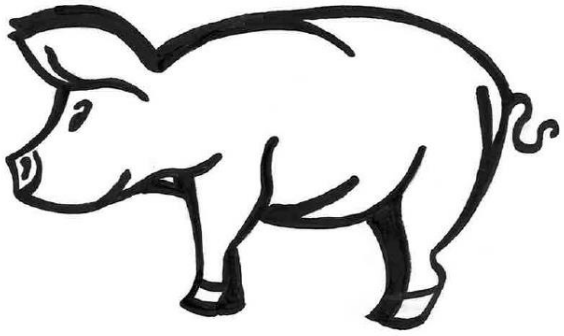
The pathogenic significance of *F. gastrosuis* was further investigated **in chapter 3.** It was demonstrated that *F. gastrosuis* induces epithelial cell death and that genes are present in the genome of this bacterium with sequence similarity to genes encoding factors involved in adhesion, invasion and induction of cell death as well as immune evasion. In addition, colonization of the *Pars oesophagea* was increased in *H. suis*-infected animals with alterations in gastric acid secretion and with gastric lesions, giving further evidence for the involvement of *F. gastrosuis* in the development of *Pars oesophageal* lesions. We hypothesize that, in a gastric environment altered by *H. suis*, colonization and invasion of the *Pars oesophagea* and production of epithelial cell death inducing metabolites by *F. gastrosuis* cause lesions of this stomach region. Experimental studies in pigs infected with *H. suis* and *F. gastrosuis* would be necessary to confirm this hypothesis.

The results from **chapter 1-3** lead to the hypothesis that *H. suis* is involved in gastric pathology through its effects on gastric acid secretion and on the gastric microbiota composition. The novel pathogen, *F. gastrosuis*, might also play a role by inducing epithelial cell death. Inhibition of colonization by these bacteria and/or their induced pathologies can be considered as potential control measures against porcine gastric ulceration.

Since an inhibitory action of bambermycin has been recently described towards *H. pylori* and as this bacterium is closely related to *H. suis*, the effect of in-feed medication with this antibiotic on the course of a *H. suis* infection, the host response and the gastric microbiota was investigated **in chapter 4** using a mouse model. Despite the relatively low minimum inhibitory concentration (8 µg/ml) of bambermycin on porcine *H. suis* strains, gastric *H. suis* colonization was not affected by bambermycin supplementation in-feed. Still, a decreased number of infiltrating T cells, B cells and macrophages as well as downregulated expressions of IL-1β, IL-8M, IL-10 and IFN-γ were demonstrated and the parietal cell mass was not affected in contrast with *H. suis*-infected mice without bambermycin supplementation. Also in bambermycin treated mice that were not infected with *H. suis*, the number of infiltrating T cells

and expression of IL-1β were lower than in non-infected mice that did not receive bambermycin. Gastric microbiota analysis indicated that the relative abundance of bacteria that might exert unfavorable effects on the host was decreased during bambermycin supplementation, further highlighting beneficial effects of bambermycin. It might be possible that in-feed medication of bambermycin counters the decreased gastric acid secretion observed in *H. suis*-infected 6-8 months old pigs. As such, no overgrowth of *F. gastrosuis* would occur and the *Pars oesophagea* would remain intact. Experimental infection studies in pigs with and without bambermycin supplementation would be necessary to confirm or reject this hypothesis.

In conclusion, during the early phase of a *H. suis* infection, the gastric acid secretion seems to be unaffected and so no irritation occurs of the *Pars oesophagea*. Later on, a decreased gastric acid secretion in the glandular part of the stomach may allow expansion of *F. gastrosuis* in the upper, non-glandular region, which may affect development of lesions through induction of epithelial cell death. Finally, increased production of gastric acid during the chronic phase of infection might further aggravate severity of lesions in this stomach region, which is not protected by mucus. In-feed use of bambermycin as supportive treatment against porcine gastric ulceration might be considered, as it decreased gastric inflammation and inhibited the effects of a *H. suis* infection on parietal cell loss. A widespread, prophylactic use of antibiotics in the pig industry, however, will probably not be accepted, since it might favour spread of antimicrobial resistance in pathogens as well as in bacteria belonging to the microbiota.



Samenvatting

Maagulcera komen frequent en wereldwijd voor bij varkens, met prevalenties tot 93%. Deze aandoening kan aanleiding geven tot een daling van de dagelijkse gewichtsaanzet, een daling in voederopname en plotse sterfte, met economische verliezen en dierwelzijnsproblemen tot gevolg. In tegenstelling tot de mens en andere diersoorten, ontwikkelen de ulcera zich zelden in het kliergedeelte van de varkensmaag, maar komen ze bijna uitsluitend voor in de *Pars oesophagea*, het gedeelte van de varkensmaag dat zich rondom de opening van de oesophagus bevindt en geen klieren bevat. Aangezien de *Pars oesophagea* niet beschermd wordt door mucus, is deze regio zeer gevoelig voor irritatie, door bijvoorbeeld maagzuur geproduceerd in het distale kliergedeelte (i.e. fundus en antrum). Chronische irritatie van de *Pars oesophagea* resulteert in het ontstaan van hyperkeratose, erosie en uiteindelijk ulceratie. De etiologie van maagletsels is complex en multifactorieel. Voeder met een fijne partikelgrootte, management en infectie met pathogene bacteriën, zoals *Helicobacter suis*, spelen mogelijks een rol. Het exacte ontstaansmechanisme van maagulcera is echter niet gekend. Daarnaast bestaan er geen preventieve maatregelen die varkens volledig kunnen beschermen tegen het ontstaan van maagletsels.

Het algemene doel van dit doctoraatsonderzoek was om nieuwe inzichten te verkrijgen in de rol van *H. suis* en *Fusobacterium gastrosuis* in het ontstaan van maagulcera bij varkens. *F. gastrosuis* is een nieuwe bacteriesoort die tijdens dit doctoraatsonderzoek beschreven werd. Deze bevindingen kunnen bijdragen tot de ontwikkeling van effectieve controle strategieën.

Verscheidene studies hebben reeds een rol toegekend aan *H. suis* in het ontstaan van letsels in de *Pars oesophagea*, hoewel *H. suis* deze maagregio niet koloniseert. Een hypothese die naar voor gebracht werd, was dat een infectie met *H. suis* aanleiding geeft tot een verhoogde zuursecretie in het distale gedeelte van de maag, wat kan resulteren in een verhoogd contact van het proximale, niet-klier gedeelte met maagzuur. Het doel van **hoofdstuk 1** was om meer inzicht te verkrijgen in de persistentie van *H. suis* in de maag alsook in de impact van *H. suis* op de maagzuursecretie en het ontstaan van maagletsels. Hiervoor werden varkens die al dan niet geïnfecteerd waren met *H. suis* onderzocht tijdens de acute en de chronische fase van deze infectie (i.e. *H. suis* geïnfecteerde en niet-geïnfecteerde 2-3 maand oude varkens, 6-8 maand oude varkens en volwassen zeugen). Verhoogde IL-8 en IL-1β expressie levels werden aangetoond in het antrum van met *H. suis* geïnfecteerde 2-3 maand oude varkens. Een

gelijkaardige respons werd vastgesteld in de fundus van volwassen varkens, mogelijks veroorzaakt door een shift in *H. suis* kolonisatie van antrum naar fundus. Een Treg respons in combinatie met gedaalde expressie levels van IL-8, IL-17A en IFN-γ werd aangetoond in met *H. suis* geïnfecteerde 6-8 maand oude varkens, wat zou kunnen bijdragen tot persistentie van *H. suis.* Bij met *H. suis* geïnfecteerde volwassen varkens werd, naast een Treg respons, ook een Th17 respons gedetecteerd, wat kan geleid hebben tot een daling van het aantal *H. suis* bacteriën in de maag van deze leeftijdsgroep. Er werd geen duidelijk effect vastgesteld op de merkers voor zuursecretie of het aantal met zuursecretie-geassocieerde cellen en er werden geen letsels van de *Pars oesophagea* aangetoond in 2-3 maand oude varkens geïnfecteerd met *H. suis* (i.e. acute infectie fase). In een later stadium van de infectie (i.e. 6-8 maand oude varkens geïnfecteerd met *H. suis*) duidden genexpressiestudies erop dat de zuursecretie gedaald en somatostatine secretie gestegen was. Ook het aantal G-cellen was verminderd, wat eveneens indicatief is voor een gedaalde maagzuursecretie. In diezelfde leeftijdsgroep werd ook ernstige hyperkeratose en erosie van de *Pars oesophagea* vastgesteld en dit enkel in de met *H. suis* geïnfecteerde dieren. De prevalentie van ernstige maagletsels was zeer hoog bij volwassen varkens (i.e. chronische fase van infectie), waarbij de meest ernstige letsels hoofdzakelijk gevonden werden bij met *H. suis* geïnfecteerde dieren. Bij de met *H. suis* geïnfecteerde volwassen varkens werd eveneens een verhoogde expressie van merkers voor zuursecretie aangetoond, alsook een stijging in de G-cel massa, indicatief voor een verhoogde maagzuursecretie.

Een mogelijke hypothese is dat de daling in zuursecretie in het distale kliergedeelte van de maag de microbiota samenstelling van de *Pars oesophagea* beïnvloedt, wat vervolgens een rol zou kunnen spelen bij het ontstaan van letsels bij met *H. suis* geïnfecteerde 6-8 maand oude varkens (later stadium van infectie). In een recente metagenomische studie werd een overvloedige aanwezigheid aangetoond van een ongekend *Fusobacterium* sp., dat tot 20% van de gastrale microbiota vertegenwoordigde bij 6-8 maand oude varkens. Varkens die geïnfecteerd waren met *H. suis* vertoonden ook een hoger aantal van deze *Fusobacterium* sp. in vergelijking met niet-geïnfecteerde dieren.

Aangezien necrose versterkt kan worden door de meeste *Fusobacterium* spp. wanneer weefselschade reeds geïnitieerd is door andere micro-organismen of omgevingsfactoren, werd de hypothese naar voor gebracht dat de nieuwe *Fusobacterium* sp. een rol zou kunnen spelen bij het ontstaan van maagulcera bij varkens. Om verder onderzoek naar de pathogeniciteit van deze bacterie toe te laten, werd beslist om de kiem te isoleren en te karakteriseren **in hoofdstuk 2**. In totaal werden 9 stammen geïsoleerd uit de maag van 6-8 maand oude varkens en volwassen zeugen, waarvan 7 stammen afkomstig waren uit de *Pars oesophagea* van varkens met letsels. Fenotypische, fylogenetische en genoom analyse toonden aan dat deze nieuwe species, aangeduid als *F. gastrosuis*, duidelijk onderscheiden kan worden van zijn meest verwante fylogenetische buren *F. mortiferum, F. russii, F. ulcerans, F. varium, F. necrogenes* en, opmerkelijk, *Clostridium rectum*. In diezelfde studie toonden we evenwel aan dat *C. rectum* en *F. mortiferum* dezelfde species vertegenwoordigen, met prioriteit van de nomenclatuur voor de laatste.

Het pathogeen belang van *F. gastrosuis* werd verder onderzocht **in hoofdstuk 3**. Zowel levende *F. gastrosuis* bacteriën als lysaat konden celdood induceren in epitheliale cellijnen. Een hogere graad van *F. gastrosuis* kolonisatie werd aangetoond in de *Pars oesophagea* van met *H. suis* geïnfecteerde varkens met een wijziging in de maagzuursecretie en met letsels, wat een bijkomend argument is dat *F. gastrosuis* een rol kan spelen bij het ontstaan van maagletsels. Finaal werden in het genoom van deze bacterie genen aangetoond waarvan de sequentie gelijkenissen vertoonde met deze van genen coderend voor factoren betrokken in adhesie, invasie en celdood alsook immuno-evasie bij andere bacteriën. Het is mogelijk dat, in een gastrale omgeving gewijzigd door *H. suis* infectie, kolonisatie en invasie van de *Pars oesophagea* en productie van celdood inducerende metabolieten door *F. gastrosuis* maagletsels veroorzaakt. Deze hypothese kan bevestigd worden door experimentele infectie van varkens met *H. suis* en *F. gastrosuis*.

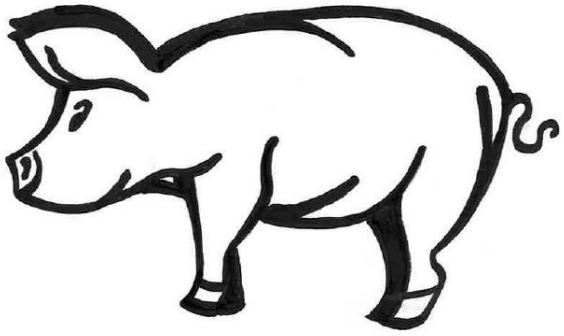
De resultaten van hoofdstuk 1-3 leiden tot de hypothese dat *H. suis* een rol speelt in het ontstaan van maagletsels door wijzigingen in de maagzuursecretie, wat een invloed kan hebben op de samenstelling van de gastrale microbiota. De nieuwe, pathogene bacterie, *F. gastrosuis*, kan door inductie van epitheliale celdood bijdragen tot het ontstaan van deze letsels. Inhibitie van kolonisatie door deze

bacteriën en/of de geïnduceerde pathologieën kunnen beschouwd worden als mogelijke controle maatregelen tegen het ontstaan maagulcera bij varkens.

Recent werd een inhiberend effect van bambermycine aangetoond tegenover *H. pylori*. Aangezien deze bacterie nauw verwant is met *H. suis,* werd **in hoofdstuk 4** het effect nagegaan van voedermedicatie met dit antibioticum op het verloop van een *H. suis* infectie, op de reactie van de gastheer en op de gastrale microbiota samenstelling en dit bij BALB/c muizen. Ondanks de relatief lage minimum inhibitorische concentratie (8 µg/ml) van bambermycine tegenover *H. suis* isolaten uit varkens, was de kolonisatie van *H. suis* ongewijzigd na bambermycin supplementatie. Wel werd een daling van het aantal infiltrerende T cellen, B cellen en macrofagen alsook een verminderde expressie van IL-1β, IL-8M, IL-10 en IFN-γ aangetoond. Bovendien was het aantal pariëtale cellen ongewijzigd in vergelijking met muizen die experimenteel geïnfecteerd werden met *H. suis* en niet behandeld werden met bambermycine. Ook in de niet-geïnfecteerde muizen die met bambermycine behandeld werden, werd minder T cel infiltratie en een gedaalde expressie van IL-1β aangetoond in vergelijking met de niet-geïnfecteerde muizen die niet behandeld werden met bambermycine. Analyse van de gastrale microbiota toonde ook een daling aan van het relatieve aandeel van bacteriën met een mogelijke negatieve impact op de gastheer, wat de voordelige effecten van bambermycine verder bevestigt. Het zou dus kunnen dat bambermycine de daling in zuursecretie tegengaat, die voorkomt bij 6-8 maand oude varkens die geïnfecteerd zijn met *H. suis*. Op deze manier ontstaat er geen overgroei van *F. gastrosuis* en blijft de mucosa van de *Pars oesophagea* intact. Experimentele infectie studies bij varkens die al dan niet behandeld worden met bambermycine zijn echter noodzakelijk om deze hypothese verder te onderzoeken.

Kort samengevat, duiden de resultaten van dit doctoraatsonderzoek erop dat tijdens de eerste stadia van een *H. suis* infectie de maagzuursecretie ongewijzigd is, waardoor er geen irritatie ontstaat van de *Pars oesophagea.* In een later stadium daalt de maagzuursecretie in het distale kliergedeelte, resulterend in een wijziging van de microbiota en een stijging van het aantal *F. gastrosuis* bacteriën in het proximale, niet-klier gedeelte van de maag. *F. gastrosuis* speelt mogelijks een rol in de initiatie van maagletsels door inductie van epitheliale celdood. De maagzuursecretie stijgt evenwel tijdens de meer chronische

fase van een *H. suis* infectie, wat kan resulteren in een verdere irritatie en ergere letsels in de *Pars oesophagea* die niet beschermd wordt door mucus. Toevoeging van bambermycine aan het voeder zou kunnen gebruikt worden als ondersteunende therapie om wijzigingen in maagzuursecretie en inflammatie, veroorzaakt door een *H. suis* infectie, tegen te gaan. Een wijdverspreid, profylactisch gebruik van antibiotica zal echter niet aanvaard worden, aangezien dit de verspreiding van antimicrobiële resistentie in pathogene alsook bacteriën behorend tot de microbiota in de hand kan werken.



Curriculum Vitae

Chloë De Witte was born on December 11, 1990 in Izegem, Belgium. After finishing her studies Latin-Mathematics at Prizma Campus College in Izegem in 2008, she started studying Veterinary Medicine at Ghent University, Belgium. In 2014, she obtained her Master's degree in Veterinary Medicine, option Research, with summa cum laude. She also received the price for the best master thesis, option Research and was awarded the price of the Faculty of Veterinary Medicine for the best graduation results.

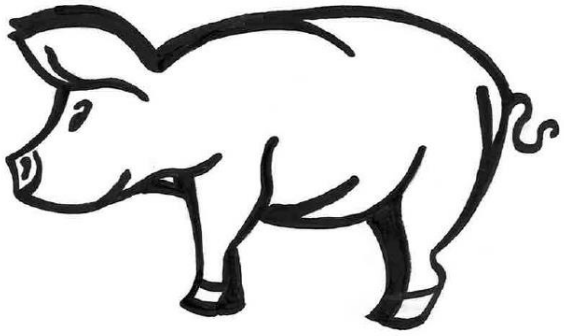
In October 2014, Chloë De Witte started her PhD research at the Department of Pathology, Bacteriology and Avian Diseases, Faculty of Veterinary Medicine, financed by the Special Research Fund of Ghent University (BOF, 01D20414). This research project focussed on the role of infectious agents in the development of porcine gastric ulceration, a highly prevalent and economic important disease entity of pigs worldwide. She also obtained the certificate of the Doctoral Training Programme of Life Sciences and Medicine.

Chloë De Witte is (co-)author of several papers in international peer-reviewed journals. She also gave multiple presentations at (inter)national conferences, where she obtained awards for the best poster/oral presentation. She actively participated in the practical sessions for the course 'Bacteriology and Mycology', 3rd Bachelor of Veterinary Medicine and she presented lectures on bacterial infections of the porcine gastro-intestinal tract for the course 'Bacterial and fungal infectious diseases and zoonoses', 1st Master of Veterinary Medicine.

Chloë De Witte werd geboren op 11 december 1990 te Izegem, België. Na het beëindigen van haar studies secundair onderwijs, richting Latijn-Wiskunde, aan het Prizma Campus College in Izegem, startte ze in 2008 met de studies Diergeneeskunde aan de Universiteit Gent, België. In 2014 studeerde ze af als Dierenarts, optie Onderzoek, met grootste onderscheiding. Daarbij won ze de prijs voor de beste master thesis, optie Onderzoek, en kreeg ze de prijs van de Faculteit Diergeneeskunde voor de beste studie resultaten.

In oktober 2014 startte ze een doctoraatsonderzoek aan de Vakgroep Pathologie, Bacteriologie en Pluimveeziekten, Faculteit Diergeneeskunde, gefinancierd door een beurs van het Bijzonder Onderzoeksfonds van de Universiteit Gent (01D20414). Hierbij werd onderzoek verricht naar de rol van bacteriën in het ontstaan van maagzweren bij varkens, een frequent voorkomende en economisch belangrijke ziekte van varkens wereldwijd. De doctoraatsopleiding 'Doctoral School of Life Sciences and Medicine' werd ook met succes voltooid.

Chloë De Witte is (mede)auteur van meerdere publicaties in internationale wetenschappelijke tijdschriften. Ze presenteerde ook meermaals op (inter)nationale congressen, waarbij prijzen voor de beste poster en orale presentatie werden gewonnen. Daarnaast werkte ze ook actief mee tijdens de practica 'Bacteriologie en Mycologie', Bachelor of Science in de Diergeneeskunde en gaf ze hoorcolleges omtrent bacteriële infecties van het spijsverteringstelsel van het varken in 'Bacteriële en mycotische ziekten en zoönosen', Master of Science in de Diergeneeskunde.



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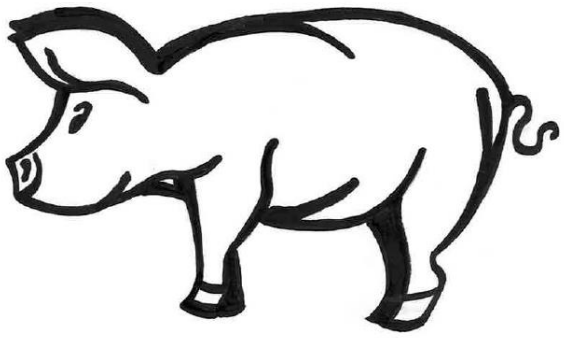

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