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Effects of antimicrobial feed additives as pre-harvest intervention measure to reduce *Campylobacter coli* in pigs

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List of abbreviations for introduction and discussion

AFLP	Amplified fragment length polymorphism
AhpC	Alkyl hydroperoxide reductase C
C.	*Campylobacter*
cdt	Cytolethal distending toxin
*cad*F	*Campylobacter* adhesion to fibronectin F
*cia*B	*Campylobacter* invasion antigen B
cfu/KbE	Colony forming units/Koloniebildende Einheiten
E. coli	*Escherichia coli*
E. faecium	*Enterococcus faecium*
fAFLP	Fluorescent AFLP
GALT	Gut-associated lymphoid tissues
GIT	Gastrointestinal tract
H_2O_2	Hydrogen peroxide
HSPs	Heat shock proteins
KatA	Catalase A
L.	*Lactobacillus*
LAB	Lactic acid bacteria
MIC	Minimal inhibition concentration
MLN	Mesenteric lymph node
MLST	Multilocus sequence typing
NP	Nanoparticles
$O_2\cdot^-$	Superoxide anions
$OH\cdot$	Hydroxyl radicals
PerR	Peroxidase response regulator
PP	Peyer's Patch
PWD	Post-weaning diarrhoea
ROS	Reactive oxygen species
Ig	Immunoglobulin
SodB	Superoxide dismutase B
ST	Sequence type
ZnO	Zinc oxide

Chapter 1: General introduction

Campylobacter (*C.*) bacteria are a major cause of foodborne diarrhoeal diseases in humans and the most common cause of gastroenteritis worldwide, with *C. jejuni* and *C. coli* as the most important species. The principal reservoir of thermophilic *Campylobacter* spp. is the gastrointestinal tract (GIT) of warm-blooded mammals and birds. *Campylobacter* spp. differ significantly in their prevalence in animal species. While chickens are known to be the main source for *C. jejuni*, pigs serve as principal reservoir for *C. coli* (Keller et al., 2007). Thus, food of animal origin, particularly poultry and pork, has been implicated in human campylobacteriosis (Gürtler et al., 2005; Moore et al., 2005).

The prevalence of *C. coli* in pigs ranges between 50–100% with excretion levels of up to 10^7 colony forming units (cfu) per g faeces (Alter et al., 2005b; Jensen et al., 2006). The high prevalence and colonisation rate of *Campylobacter* spp. in pigs before slaughter is of concern and constitutes a potential contamination source for pork with subsequent implications for human health (Wong et al., 2007). In fact, risk factor studies indicate that up to 10% of pork meat samples at retail are contaminated with *Campylobacter* spp. (Pezzotti et al., 2003; Whyte et al., 2004). Moreover, *C. coli* accounts for approximately 10% of the human *Campylobacter* infections and the consumption of raw minced meat was identified as specific risk factor for *C. coli* infections (Gillespie et al., 2002). Although, the impact of human *C. coli* infections derived from the consumption of contaminated pork is substantial, still little is known about the *C. coli* ecology within pigs.

The high incidence of human *C. coli* infections through the consumption of contaminated pork, emphasises the need for proper intervention strategies to control and prevent the *Campylobacter* spp. colonisation in pigs. As *Campylobacter* spp. do not grow outside the host, the reduction at the end of the food chain is best achieved if the colonisation of live animal can be prevented or reduced (Wagenaar et al., 2008). Preventing the *Campylobacter* spp. colonisation in pig herds is difficult as they are natural inhabitants of the porcine intestinal microbiota and typically colonise their host at young age. Therefore, a reduction in the numbers of *Campylobacter* spp. just before slaughter is more feasible. Reduced faecal *C. coli* counts might help to minimise the carcass contamination risk at the slaughter plant and subsequently might reduce the incidence of human *C. coli* infections (Malakauskas et al., 2006).

An effective pre-harvest intervention measure to reduce the *Campylobacter* spp. levels in livestock might be the administration of antimicrobial feed additives. Dietary supplementation of probiotics and pharmacological concentrations of trace elements seems to be promising as they have been demonstrated to alter the intestinal microbiota composition in pigs. The

probiotic strain *Enterococcus faecium* (*E. faecium*) NCIMB 10415 as well as pharmacological concentrations of the trace element zinc, usually applied as zinc oxide (ZnO), have been demonstrated to promote the growth and decrease the incidence of diarrhoea, mainly by decreasing the pathogenic load (Melin and Wallgren, 2002; Pollmann et al., 2005; Taras et al., 2006). Although the antibacterial properties of both feed additives are well established and despite their widespread use as growth promoter in livestock, the mechanisms of action are not as yet well understood. Major mechanisms related to the antagonistic effect of probiotics on various microorganisms include the competition for nutrients, the competition for attachment sites, and the production of antimicrobial compounds (Bermudez-Brito et al., 2012). The main antimicrobial factor of ZnO contributes to the production of reactive oxygen species (ROS). ROS are highly active molecules that are able to damage DNA and proteins and eventually leads to the death of bacterial cells (Kumar et al., 2011; Rhaghupati et al., 2011; Xie et al., 2011). The antimicrobial activity of both feed additives against *C. jejuni* has already been observed *in vitro* (Ghareeb et al., 2012; Liedtke and Vahjen, 2012). However, the inhibitory activity of *E. faecium* NCIMB 10415 and ZnO against *C. coli* has not been investigated so far.

The first goal of the present thesis was to conduct an animal trial to gain a deeper insight into the *C. coli* excretion dynamics, the colonisation within different gut section and the translocation ability to extra-gastrointestinal sites of pigs (**Chapter 4**). The localisation of *C. coli* within the pigs is important in order to prevent the *C. coli* contamination of pork during the slaughter process. This model further served as starting point to test, whether the antimicrobial feed additives, such as high doses of ZnO and *E. faecium* NCIMB 10415 as pre-harvest intervention measure can reduce the faecal *C. coli* load in pigs (**Chapter 5 & 6**). To make a better use of these feed additives in pig production, it is necessary to understand the mechanism of action against pathogenic bacteria. In this sense, studies on the antimicrobial action of *E. faecium* NCIMB 10415 and ZnO against *C. coli* were performed (**Chapter 5 & 6**).

Chapter 2: Literature review

2.1 *Campylobacter* spp.

2.1.1 History and taxonomy

Today known as one of the principal causes of foodborne infections in developed and developing countries, *Campylobacter* spp. were not recognised as human pathogen until the 1970s. Sebald and Véron (1963) proposed the genus *Campylobacter* in 1963. However, Butzler et al. (1973) were first able to selectively isolate *Campylobacter* cells from humans with diarrhoea. Little later Skirrow (1977) announced *Campylobacter* enteritis a 'new disease'.

As illustrated in **Figure 2-1** *Campylobacter*, along with *Arcobacter*, *Dehalospirillum*, and *Sulfurospirillum* form the family *Campylobacteraceae* (Vandamme and De Ley, 1991). Together with the families of *Helicobacteraceae* and *Hydrogenimonaceae* they build the order *Campylobacterales*, which is included in the class of *Epsilonproteobacteria* and the overall *Proteobacteria* phylum (Garrity et al., 2005). The genus *Campylobacter* consists of a large and diverse group of bacteria currently comprising 25 species (http://www.bacterio.net/campylobacter.html; status: October 2014).

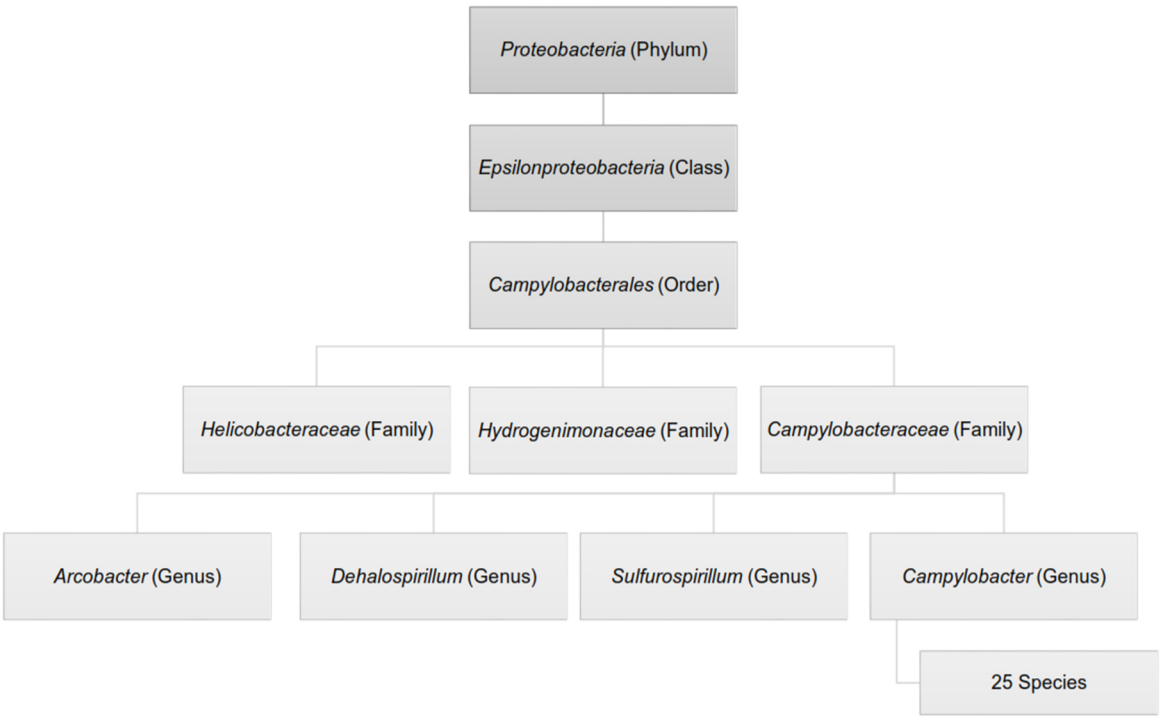

Figure 2-1: *Campylobacter* spp. taxonomy

The species most commonly associated with human infection are *C. jejuni* followed by *C. coli*, and *C. lari*, but other *Campylobacter* species, including the non-thermophilic *C. fetus* and *C. upsaliensis,* are also known to occasionally cause human infection (EFSA, 2014).

2.1.2 Morphology and physiology

Campylobacter spp. are gram-negative bacteria and motility is reached by either unipolar or bipolar flagella. Members of the genus are morphologically diverse, including spiral, helix or comma shapes with a characteristic corkscrew-like motility. Exposure to different stresses results in a remarkable morphological shift from spiral-shaped cells to coccoid forms that may be an evolutionary strategy enabling survival between the hosts. However, this is usually associated with a loss of culturability (Tangwatcharin et al., 2006). *Campylobacter* spp. require complex nutritional environments and have fastidious growth requirements (Park, 2002). Growth is achieved under microaerobic conditions of 5–10% oxygen and 1–10% carbon dioxide (Bolton and Coates, 1983). The optimum pH for growth is 6.5–7.5 and *Campylobacter* spp. do not grow in culture below pH 4.9 and are being killed readily at pH values less than this (Park, 2002). *Campylobacter* spp. have a restricted temperature growth range and whilst they grow optimally between 37 °C and 42 °C, the organisms do not grow at temperatures below 30 °C or above 45 °C, but survival under other environmental conditions is possible (Skirrow and Benjamin, 1980; Park, 2002). *Campylobacter* species with an optimal growth temperature of 42 °C are termed thermophilic *Campylobacter* with *C. jejuni*, *C. coli*, *C. lari*, and *C. upsaliensis* as their representatives and mainly associated with human infections. *Campylobacter* spp. cannot ferment sugars as they lack the enzyme 6-phosphofructokinase (Parkhill et al., 2000). For energy production it relies on organic acids and sulphite, which are metabolic products of other bacteria found in the intestine of mammals and birds. *Campylobacter* spp. are also capable for metabolising amino acids as energy and carbon source. Therefore, the main reservoir of thermophilic *Campylobacter* spp. is the intestine of warm-blooded animals (Horrocks et al., 2009).

2.1.3 Reservoir

Members of the genus *Campylobacter* occupy highly diverse habitats. *Campylobacter* spp. are ubiquitous in the environment, wild birds, rodents, other wild life and domesticated animals. Even marine mammals can carry these organisms in their intestine (Man, 2011). Moreover, surface water and soil contaminated with excreta from infected wild animals or the runoff from livestock are also a major source for *Campylobacter* spp. (Jones, 2001; Schonberg-Norio et

al., 2004). However, the principal reservoir of thermophilic *Campylobacter* spp. is the intestinal tract of mammals. Thus, food of animal origin like poultry, pigs, cattle, and sheep serve as main source for human *Campylobacter* infections. Chickens are known to be the main source for *C. jejuni*, while pigs serve as principal reservoir for *C. coli*. Shellfish, such as cockles, mussels, scallops and oysters are also linked to be a reservoir for *Campylobacter* spp., mainly *C. lari* (Whyte et al., 2004; Wilson and Moore, 1996). Moreover, wild birds like pigeon and sea gulls are assumed to be the main reservoir for *C. lari* (Broman et al., 2002; Waldenstrom et al., 2010). *Campylobacter* spp. have also frequently been isolated from asymptomatic companion animals (Acke et al., 2009; Chaban et al., 2010). Thermophilic *Campylobacter* spp. can also be recovered from insects in environments heavily contaminated with the faeces of colonised livestock (Nichols, 2005; Guerin et al., 2007). Humans do not represent a natural host as colonisation of thermophilic *Campylobacter* spp. leads to intestinal infections.

2.1.4 General stress response

Most foodborne bacterial pathogens are considered to be relatively robust organisms. However, *Campylobacter* possesses fastidious growth requirements and is highly susceptible to environmental stress. Thermophilic *Campylobacter* spp. do not grow at temperatures below 30 °C or above 42 °C, are very sensitive to desiccation, low pH and osmotic stress. Genome sequence analysis revealed that *Campylobacter* spp. lack many of the adaptive response mechanisms that can be correlated with resistance to stress in other foodborne pathogens. For example it lacks RpoS, the general stress/stationary phase sigma factor and other heat shock and oxygen regulators that are present in many gram-negative bacteria (Park, 2002). However, mechanisms for stress resistance and survival in the environment have not been well understood in *Campylobacter* spp. It is assumed, that they must have evolved alternative strategies to promote survival that involves genetic variation.

2.1.4.1 Oxidative stress response

Unlike other foodborne pathogens, *Campylobacter* spp. are apparently fragile organisms that are unable to grow in the presence of air, multiply outside the animal host and are highly susceptible to a number of environmental stresses (Park, 2002). During its lifecycle *Campylobacter* spp. are exposed to highly variable oxygen concentrations. As a microaerophilic bacterium, *Campylobacter* spp. require low levels of molecular oxygen for proper growth due to its dependence on an oxygen-dependent ribonucleotide reductase (Sellars et al., 2002). Most research is done on *C. jejuni* as it is the major cause of

campylobacteriosis and little is known about the stress response in *C. coli*. *C. jejuni* possess a broad and complex battery of enzymes for defence against oxidative stress. However, *Campylobacter* spp. lack many stress mechanisms that other gram-negative foodborne pathogens like *Salmonella* spp. and *Escherichia coli* (*E. coli*) exhibit such as the global stationary phase stress response factor RpoS of *E. coli* (Park, 2002). *C. jejuni* also lacks the oxidative stress defence regulators OxyR and SoxRS (Parkhill et al., 2000). Both are well-known regulators in *E. coli* that mediate resistance to superoxide and hydrogen peroxide stress. *C. jejuni* instead harbours the iron-regulated peroxidase response regulator PerR, which coordinates the expression of oxidative stress genes (Garenaux et al., 2008a; Palyada et al., 2009).

The dependence on the presence of oxygen for growth inevitably results in the exposure of important biological molecules to toxic oxygen intermediates, the reactive oxygen species (ROS). The main ROS are the superoxide anions ($O_2^{\cdot -}$), hydrogen peroxide (H_2O_2) and hydroxyl radicals (·OH), which are highly active molecules and able to damage DNA, proteins and cell membranes. ROS are formed as a natural by-product of the normal oxygen metabolism and have important roles in cell signalling and homeostasis. Besides these endogenous ROS, *Campylobacter* spp. have to cope with exogenous ROS, which are produced by neutrophils as a strategy for killing pathogenic bacteria. Also other bacteria produce peroxide, particularly lactic acid bacteria (LAB), in order to compete for the same niche.

During times of environmental stress e.g. heat exposure, ROS levels can dramatically increase and this may result in significant damage of cell structures. Therefore, *Campylobacter* spp. contain numerous pathways that facilitate the removal of the main ROS. However, *Campylobacter* spp. rely mainly on three enzymes suggesting a rather rudimentary and simple oxidative stress defence system (van Vliet et al., 2002; Flint et al., 2014). The iron-containing superoxide dismutase B (SodB) is the first line of defence during exposure to air as it converts superoxide anions into hydrogen peroxide and oxygen (Purdy and Park, 1994). *C. jejuni* possesses only a single superoxide dismutase, whereas three have been identified in *E. coli*. SodB plays an important role in *C. jejuni* resistance to oxidative stress as well as survival in food, chickens and epithelial cells (Pesci et al., 1994; Palyada et al., 2009; Flint et al., 2014). Catalase activity represents the main peroxide stress defence in *Campylobacter* spp. *C. jejuni* possesses a single catalase A (KatA) (Takata et al., 1992), whereas two are present in *E. coli*. Together with the alkyl hydroperoxide reductase C (AhpC) it catalyses the decomposition of hydrogen peroxide to water and oxygen (Baillon et al., 1999). The regulation of the three main detoxification enzymes is rather complex in *Campylobacter* spp. and summarised in **Figure 2-2**.

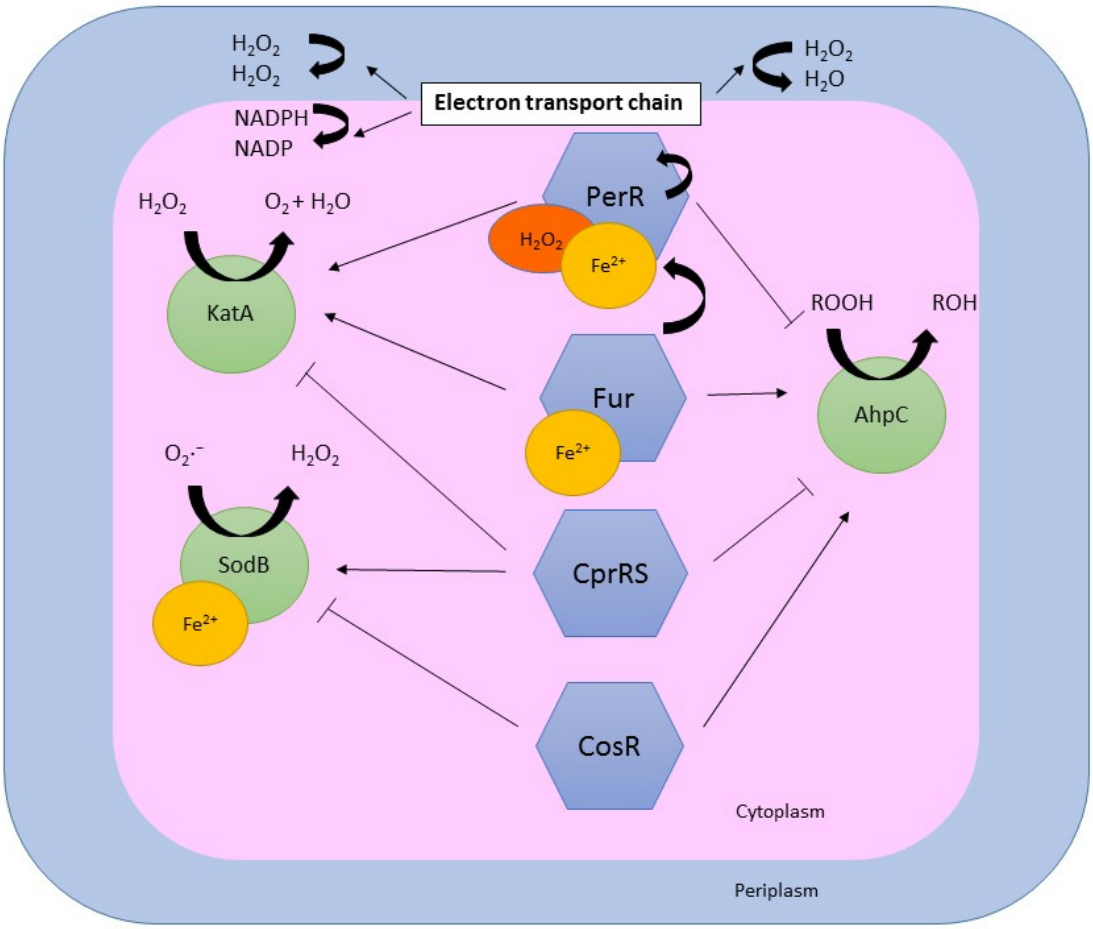

Figure 2-2: *C. jejuni* defence mechanisms against oxidative stress
(Adapted from Atack and Kelly, 2009; Hwang et al., 2011)

KatA and AhpC are negatively regulated by PerR and the inactivation of the oxidative stress regulator PerR results in constitutive expression of *kat*A and *ahp*C. In addition to PerR, the *C. jejuni* ferric uptake regulator Fur, is also involved in the direct regulation of *kat*A (van Vliet et al., 1999). *C. jejuni kat*A and *sod*B mutants have reduced ability to survive within macrophages and in human intestinal INT407 cells, respectively (Purdy et al., 1999; Day et al., 2000). In addition, a *C. coli sod*B mutant showed increased sensitivity to paraquat (a superoxide generator) exposure, suggesting a contribution of these genes to pathogenicity and oxidative stress defence (Purdy et al., 1999; Atack and Kelly, 2009; Palyada et al., 2009). While *kat*A expression is specifically induced in oxidative stress conditions by $O_2^{\cdot-}$ and H_2O_2 stress, *sod*B expression is only inducible after $O_2^{\cdot-}$ exposure (Grant and Park, 1995; Garenaux et al., 2008b). The sensor kinase CprS of the two-component regulatory system CrpRS of *C. jejuni* is important for oxidative stress resistance in *C. jejuni* as mutants lacking *cpr*S show decreased levels of SodB, but increased levels of KatA and AhpC (Svensson et al., 2009). Recently, a novel two-component regulator in *C. jejuni*, known to contribute to the viability and

various cellular processes, was identified to be involved in the oxidative stress defence in *C. jejuni*. The *Campylobacter* spp. oxidative stress regulator CosR negatively regulates *sod*B expression, but positively regulates *ahp*C (Hwang et al., 2011). Following paraquat stress the level of CosR was reduced, but not by H_2O_2. Reduced CosR levels result in increased levels of negatively regulated genes like *sod*B and lead to a decrease of positively regulated genes like *ahp*C. In contrast, PerR negatively regulates *ahp*C (van Vliet et al., 1999). Thus, PerR and CosR co-ordinately regulate *ahp*C expression in *C. jejuni*. However, little is known about the factors influencing *perR* expression. It has been demonstrated by Kim et al. (2011) that PerR is auto-regulated as PerR directly interacts with the *perR* promoter and regulates *perR* transcription. The auto-regulation of PerR is responsible for the repression of *perR* transcription by iron in *C. jejuni*. **Figure 2-3** summarises the interplay between the major oxidative defence systems in *C. jejuni*. Little is known how the oxidative stress response is regulated in *C. coli*. However, nucleotide blast analysis revealed a high degree of homology between the known stress response genes of *C. jejuni* NCTC 11168 and *C. coli* RM2228 (**Table 2-1**).

Table 2-1: Genes involved in the general/heat and oxidative stress response in *Campylobacter* spp.

Genes	Function	*C. jejuni* NCTC 11168	*C. coli* RM2228	Homology [%]
Antioxidative enzymes				
*kat*A	Catalase	Cj1385	CCO1495	89
*sod*B	Iron co-factored superoxide dismutase	Cj0169	CCO1706	92
*ahp*C	Alkyl hydroperoxide reductase	Cj0334	CCO0422	90
Oxidative stress regulators				
*per*R	Peroxidase response regulator	Cj0322	CCO0408 (furR1)	82
*cos*R	*Campylobacter* oxidative stress regulator	Cj0355c	CCO0443	82
fur	Ferric uptake regulator	Cj0400	CCO0489	86
*cpr*RS	*Campylobacter* planktonic growth regulator	Cj1226/7c	CCO1314/15	85/88
General/heat stress proteins				
*clp*B	Caseinolytic peptidase B	Cj0509c	CCO0682	85
*dna*K	Chaperone	Cj0759	CCO0813	93
*dna*J	Co-chaperone for DnaK	Cj1260c	CCO0682	85
*grp*E	Nucleotide exchange factor for DnaK	Cj0758	CCO0812	98
*gro*EL	Chaperonin	Cj1221	CCO1297	99
*gro*ES	Co-chaperonin, functional unit with GroEL	Cj1220	CCO1296	95

2.1.4.2 General and heat stress response

Damaged proteins e.g. caused by oxidative stress may not only lead to the loss of their biological function, but also tend to form stable insoluble aggregates in the cell, termed 'inclusion bodies'. Correct folding of proteins into their three-dimensional structure is of major importance for their function. Under physiological conditions, most proteins fold spontaneously without assistance into their correct and biological active form. However, some polypeptides need assistance by other proteins called chaperones. Chaperones do not only assist protein folding after biosynthesis and therefore, prevent aggregation of unfolded proteins, but also promote refolding of stress-induced unfolded or misfolded polypeptides. Chaperones are composed of several distinct classes of proteins, which are stress inducible, e.g. heat shock proteins (HSPs) (Ben-Zvi and Goloubinoff, 2001; Buchner, 2002). HSPs have been identified in *C. jejuni* including GroEL/ES, DnaJ/K, and ClpB (Konkel et al., 1998; Thies et al., 1999a; Thies et al., 1999b; Thies et al., 1999c; Stintzi, 2003; Murphy et al., 2006). Little is known how the general and heat shock response is regulated in *C. coli*, but nucleotide blast analysis revealed a high degree of homology between *C. jejuni* NCTC 11168 and *C. coli* RM2228 (**Table 2-1**).

Stringent assisted refolding of chaperone-bound proteins in an ATP-dependent manner is shown for GroEL, DnaK and ClpB. Ben-Zvi and Goloubinoff (2001) proposed an synergetic networking of all three systems that is best for refolding of stable insoluble protein aggregates. **Figure 2-3** summarises the interaction of the chaperone network for protein disaggregation and refolding in *E. coli*. ClpB is suggested to be the first to recognise and interact with the aggregates and protein binding stimulates the ATPase activity. ATP hydrolysis unfolds the denatured protein aggregates that probably help exposing new hydrophobic binding sites. The new hydrophobic exposed regions allow the binding of DnaJ and DnaK. Then, GrpE-controlled dissociation of DnaJ and DnaK from the aggregate leads to a gradual folding of the entangled polypeptide. Hydrophobic regions of the protein, > 60 kDa of size, bind to the patch of GroEL at its opening. GroES is a lid-like co-chaperonin, which binds GroEL in the presence of ATP/ADP. GroEL/ES mediated protein folding involves multiple rounds of binding, encapsulation and release of substrate protein.

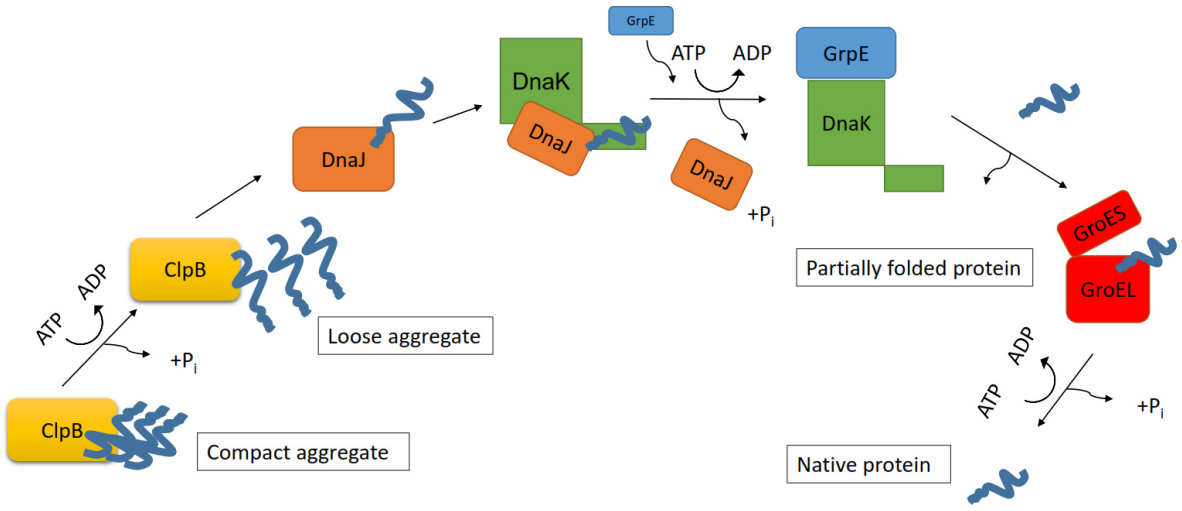

Figure 2-3: Scheme of the chaperone network for protein disaggregation and refolding in *E. coli*
(Adapted from Ben-Zvi and Goloubinoff, 2001; Calloni et al., 2012)

2.2 Prevalence of *C. coli* along the food chain

2.2.1 *C. coli* in pigs

Campylobacter spp. differ significantly in their prevalences in animal species. *C. coli* is the dominant species in pigs, whereas *C. jejuni* primarily colonises poultry (Hopkins et al., 2004; Keller et al., 2007). It is not known, whether this difference is due to host adaptation of both species or to rearing practices that favour a particular species (Payot et al., 2004). **Figure 2-4** illustrates the distribution of *Campylobacter* species in the most important food-producing animals.

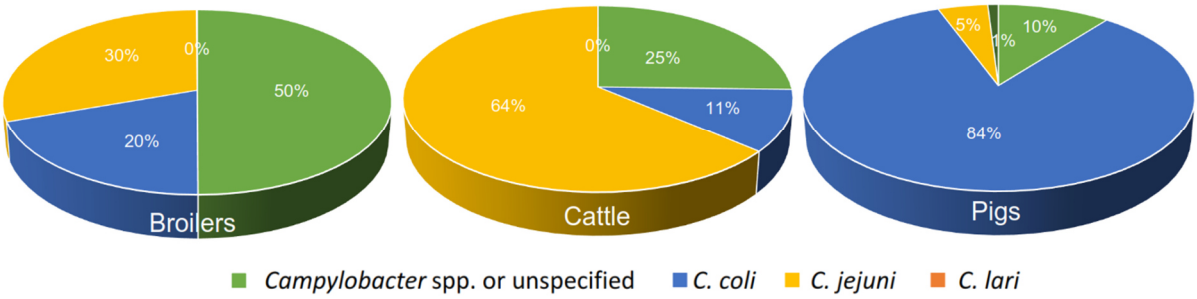

Figure 2-4: Distribution of *Campylobacter* species in the main food-producing animals in the EU in 2009
(Adopted from EFSA, 2011)

As seen in **Figure 2-4** pigs serve as main reservoir for *C. coli*. In addition, numerous studies have examined the prevalence of *Campylobacter* spp. in pig herds (**Table 2-2**).

Table 2-2: Prevalence of *C. coli* in pigs

Country	*Campylobacter* spp. prevalence [%]	Source	References
Denmark	100	47 organic raised pigs from a single farm after weaning (7–13-weeks)	Jensen et al. (2006)
France	67	53 pig farms	Denis et al. (2011)
Germany	64	15 pig farms, farrow-to-finisher herds or fattening units	Alter et al. (2005a)
	78.2	pigs at the time of slaughter	Alter et al. (2005b)
	81.2	900 faecal samples from 30 fattening pig herds	von Altrock et al. (2006)
Norway	100	97 pigs after slaughter	Nesbakken et al. (2003)
United States	57.8/100	piglets at the day-of-birth/weaned piglets and pregnant sows	Young et al. (2000)
	77	1,448 faeces samples from sows	Wright et al. (2008)

In the EU there is an increase in *Campylobacter* spp. prevalence in pigs since 2007 with up to 60% being positive. However, strong variation within the Member States could be noticed (EFSA, 2011). This increase might be explained by improved detection methods, but also due expanding pig herd sizes. It has been reported that with increased herd population there is an increased risk for *Campylobacter* spp. colonisation (Denis et al., 2011). Risk factors for the introduction of *Campylobacter* spp. to farms is still not clear, it is assumed that horizontal transmission via farmers, domestic animals, contaminated surface water, insects and other environmental sources are largely responsible for pig herd colonisation (Weijtens et al., 2000; Young et al., 2000; von Altrock et al., 2006; Guerin et al., 2007).

Only a few studies have enumerated *C. coli* excretion in pigs. However, high numbers of *C. coli* were detected in pigs ranging from 10^3 to 10^7 cfu/g faecal content (Weijtens et al., 1999; Young et al., 2000; Jensen et al., 2006; Jensen et al., 2013). The high excretion levels have been linked to be a significant survival strategy for *Campylobacter* spp. (Jones, 2001).

In summary, there is a high prevalence of *C. coli* in pigs ranging between 50% and 100% and excretion levels of up to 10^7 cfug faeces.

2.2.2 *C. coli* in food

Campylobacter spp. are highly abundant in pigs with *C. coli* as the dominating species. Thus, exposure through contaminated pork meat and products is likely to be the main source for human *C. coli* infections. In fact, Wong et al. (2007) showed that approximately half of the *Campylobacter* spp. (*C. jejuni*/*C. coli*) isolates in the pigs shared serotypes with human isolates, and also risk factor studies indicate that *Campylobacter* spp. in pork plays a potential

role in foodborne infections. Genotyping of *C. coli* isolates revealed three possible ways of contamination of the slaughter carcass surface. Genetically highly related strains were detectable on carcass surfaces of consecutively slaughtered animals. Faecal isolates and isolates from the carcass surface showed occasional high similarities. *C. coli* genotypes from tonsils and genotypes from the corresponding slaughter carcasses formed a close cluster (Alter et al., 2005a). **Table 2-3** summarises selected prevalences of *C. coli* in pork meat.

Table 2-3: Prevalence of *C. coli* in fresh pork meat

Country	Prevalence [%]	Product	Reference
Belgium	1.4–3.9	Minced pork meat	Ghafir et al. (2007)
Germany	0.3–6.4	Pork and minced meat	BfR (2011)
Ireland	5.1	Pork meat	Whyte et al. (2004)
Italy	10.3	Pork products	Pezzotti et al. (2003)
EU	0.0–6.1	Pig meat at retail	EFSA (2011)
United States	1.7	Pork meat at retail	Zhao et al. (2001)
Nepal	38.8	Dressed porcine carcass from pork shops	Ghimire et al. (2014)

There is little evidence that muscle meat itself is contaminated with *Campylobacter* spp., but numerous studies have provided data, which suggest that *Campylobacter* can be isolated from offal and much work has focused on the liver. A *Campylobacter* spp. contamination rates of 71.1% of pigs livers (42.4% *C. coli*) at retail are reported in the United Kingdom (Kramer et al., 2000). Slaughtering practices can play a significant role in contamination and can be an indicator for slaughterhouse hygiene practices that arises from ruptured intestinal contents during slaughter. In fact, in a study by Alter et al. (2005a), *C. coli* were detected in 64% of faecal samples in pigs by the time of slaughter and 21.1% were recovered on pig carcasses after the slaughter process and before the chilling period. However, the impact of chilling reduced the detection rate of *Campylobacter* spp. to 0.8%. Although the proportion of positive samples is much higher in pigs compared to fresh meat, minced pork meat is intended to be eaten raw. A *Campylobacter* infection is therefore, highly probable when contaminated with *Campylobacter* spp. as few cells are already sufficient for infection. The consumption of contaminated raw minced meat was identified as a specific risk factor for *C. coli* infection (Gillespie et al., 2002). It has been estimated that the incidence of human *Campylobacter* infections attributed to the consumption of pork meat was 2.17 cases per 100,000 inhabitants

per year in Europe just after S*almonella* and *Yersinia* with 3.37 and 2.82, respectively (Fosse et al., 2008).

2.3 Epidemiology of campylobacteriosis

2.3.1 Molecular typing

There is a variety of phenotypic and genotypic typing methods for studying *Campylobacter* spp. epidemiology and diversity. Several performance criteria are essential, in particular, typeability, reproducibility, stability, discriminatory power and typing system concordance (Nielsen et al., 2000). Phenotypic methods are based on the presence or absence of biological or metabolic activities expressed by the organism. These include metabolic activities, colony morphology, environmental tolerances and biochemical reactions (biotyping), reaction with antibodies (serotyping) and interaction with bacteriophages (phagetyping) (Eberle and Kiess, 2012). Novel typing methods are based on DNA polymorphisms. The amplified fragment length polymorphism (AFLP) is a molecular whole genome fingerprinting typing method that has a high discriminating power and is comparatively rapid (Vos et al., 1995; Lindstedt et al., 2000). It detects restriction fragment length polymorphisms by selective PCR amplification of restriction fragments from bacterial DNA and subsequent electrophoretic analysis either through autoradiography or fluorescence methodologies. This method has been adopted for epidemiological typing of *C. jejuni* and *C. coli* (Duim et al., 1999; Kokotovic and On, 1999). It is suitable for tracing infections that are restricted in time and geographical area. It allows the differentiation of *Campylobacter* on species, subspecies and even strain level and the identification of sources with high reproducibility (Duim et al., 2001; Siemer et al., 2005; Keller et al., 2007; Sheppard et al., 2009; Sheppard et al., 2010). For example, by genotyping of 276 *C. jejuni* and 87 *C. coli* strains by fluorescent AFLP (fAFLP) Hopkins et al. (2004) were able to show that *C. coli* from poultry and pig clustered separately. Due to the high variability and genetic instability of *Campylobacter* spp., other methods have to be used for long-term phylogenetic analysis (Parkhill et al., 2000; Leblanc-Maridor et al., 2011).

Multilocus sequence typing (MLST) is such a method that is based on indexing the genetic variation in housekeeping genes. Results can be easily exchanged between laboratories and collected in a central database (Dingle et al., 2001). This technique has been successfully employed for typing human, animal and environmental *C. jejuni* strains. The increasing recognition of *C. coli* as a foodborne pathogen led to the development of a novel MLST scheme (Dingle et al., 2005; Miller et al., 2006). Nucleotide sequence data for an internal fragment of each of seven housekeeping loci are usually employed. For each locus, every unique gene

fragment sequence (or MLST) allele is assigned an unique, but arbitrary number, regardless of whether allele differences have occurred as result of a single or multiple base changes. Consequently, each isolate investigated has an 'allelic profile' also called 'sequence type' (ST). Bacterial populations can be clustered according to related isolates and clusters are referred to as clonal complexes. In the case of *Campylobacter* spp. a clonal complex is defined as those isolates sharing at least four alleles with a central genotype, after which the complex is named.

Based on MLST data from 1,223 *C. coli* isolates from clinical infections, farm animals and the environment revealed a three clade division of *C. coli* (Sheppard et al., 2010; Sopwith et al., 2010). All cases of human *C. coli* infection analysed so far were caused by lineages belonging to clade 1 and 84% of STs from clade 1 belonged to the clonal complex ST-828. There were a total of 451 STs representing a high diversity within the *C. coli* population. However, numbers of STs approximately equal to the number of alleles (410) suggesting a variation of genotypes by re-assortment than de novo generation by mutation. The clonal complex ST-828 has previously been recovered from clinical cases and from agricultural sources (Dingle et al., 2005; Miller et al., 2006; Thakur et al., 2006; Thakur et al., 2009; Egger et al., 2012).

2.3.2 Transmission

Transmission of *Campylobacter* spp. is food- or waterborne and spread by the faecal-oral route. The transmission of thermophilic *Campylobacter* spp. to humans can occur indirectly and directly. Indirect transmission occurs through the consumption of raw or undercooked contaminated food of animal origin. It is estimated that about 80% of all campylobacteriosis cases are associated with contaminated food (Alter and Scherer, 2006). Particularly poultry and pork meat has been implicated in human *Campylobacter* infections (Gurtler et al., 2005; Moore et al., 2005). *Campylobacter* spp. can also be transmitted by raw or improperly pasteurized milk. In addition, *Campylobacter* spp. can also be present in seawater and thereby contaminate seafood as ingestion of raw or undercooked contaminated seafood has been reported to be a source (Wilson and Moore, 1996). Moreover, cross-contamination with ready to-eat food during the preparation in the kitchen is believed to play a key role in the transmission of *Campylobacter* spp. (Studahl and Andersson, 2000; Luber et al., 2006; de Jong et al., 2008).

Further transmission might occur by direct contact with pets and other animals. Direct animal and farm exposure including living on a farm, visiting a petting zoo etc. were also associated with illness (Studahl and Andersson, 2000; Friedman et al., 2004). The tenacity of *Campylobacter* spp. in different environments, especially water, has also to be considered for

an adequate risk assessment (Kapperud et al., 2003; Peterson, 2003; Alter et al., 2011). *Campylobacter* spp. have been frequently associated with private water supply outbreaks (Smith et al., 2006). Transmission of *Campylobacter* spp. by flies is thought to be a potential source for infection and may partly explain the increase of *Campylobacter* infections in the warmer month (Nichols, 2005). A certain percentage of human cases can be associated with foreign travel, which is why campylobacteriosis is a well-known traveller's diarrhoea in both developed and developing countries (Tam et al., 2003; Friedman et al., 2004). Campylobacteriosis usually occurs in single, sporadic cases, but it can also occur in outbreaks, when two or more people become ill from the same source (Schielke et al., 2014). However, outbreaks are commonly associated with drinking contaminated raw milk or water than person-to-person transmission (Smith et al., 2006; Janssen et al., 2008).

2.3.3 Clinical aspects

Campylobacteriosis is an inflammatory enteritis caused by ingestion of *Campylobacter* spp. The infection dose for campylobacteriosis varies between $5x10^2$ and 10^5 cells (Black et al., 1988; Tribble et al., 2010). After an incubation time of one to three days the general clinical presentation varies from mild, non-inflammatory diarrhoea to severe inflammatory, watery sometimes bloody diarrhoea often with abdominal pain, cramps and fever (Newell, 2001; Gillespie et al., 2002; Young et al., 2007). The course of infection mainly depends on the immune status of the patient and the virulence of the strain. Symptoms typically last for five to seven days and infections are mostly self-limiting. On average, 10% of the campylobacteriosis cases were reported as having been hospitalised (Schielke et al., 2014). Post-infection complications, such as reactive arthritis and acquired immune-mediated neuropathies such as Miller-Fisher and Guillain-Barré syndromes, and inflammatory bowel disease can occur. The frequency of the Guillain-Barré syndrome resulting from campylobacteriosis has been estimated as 0.1% and approximately 20% of these patients are left with some form of disability, but death is rare (Altekruse et al., 1999; Zautner et al., 2014). On the basis of the incidence estimates and a previous estimate of disease burden and costs, the European Food Safety Authority considered that the public health impact of campylobacteriosis is estimated at about 2.4 billion in the EU per year (ECDC, 2012).

It is mostly unclear why the course of infection in man and in animals differs significantly. Potential mechanisms that mediate pathogenicity include chemotaxis and motility, adhesion, ability of colonisation, invasion, epithelial translocation, intracellular survival, iron acquisition and formation of toxins (Hanel et al., 2004). The precise means, by which *Campylobacter* spp. overcome host defence factors are not known. Some studies suggest that symptoms of

enteritis are not caused by toxins, but rather a result from a local over-reaction of the intestinal innate immune system (van Putten et al., 2009). Temporary immunity against re-infection has been reported, especially in people who are frequently exposed to *Campylobacter* spp. e.g. farmers (Wagenaar et al., 2006; Janssen et al., 2008). Overall, it seems that human diarrhoea is the result of wrong bacteria in the wrong host, an unfortunate host-microbe combination, which can be considered as collateral damage (Wassenaar, 2011).

2.3.4 Incidence

Campylobacter spp. are generally regarded as one of the most common bacterial cause of gastroenteritis worldwide. The Foodborne Diseases Active Surveillance Network estimated the incidence of *Campylobacter* infection to be 2.4 million persons each year in the US in 2008. Since years campylobacteriosis constituted an ongoing public health problem for both developed and developing countries (Ruiz-Palacios, 2007). The rate of campylobacteriosis differs strongly around the world with New Zealand as the country with the highest incidence with 39/100,000 persons compared to e.g. the United States with 12.7/100,000 persons (Janssen et al., 2008). In the EU, *Campylobacter* spp. continue to be the most commonly reported gastrointestinal bacterial pathogens in humans since reporting to European Centre for Disease Prevention and Control started in 2005. Recent numbers of reported confirmed cases of human campylobacteriosis accounted for 214,268 with a notification rate of 55.49/100,000 persons in the EU in 2012 (EFSA, 2014). However, there is a high variation in reported *Campylobacter* enteritis incidences among EU countries (**Table 2-4**).

Table 2-4: Campylobacteriosis cases and incidence of cases in Europe in 2012

(adopted from EFSA, 2014)

Country	Confirmed cases	Confirmed cases/100,000 individuals
Austria	4,710	55.79
Belgium	6,607	–
Bulgaria	97	1.32
Cyprus	68	7.89
Czech Republic	18,287	174.08
Denmark	3,720	66.66
Estonia	268	20.01
Finland	4,251	78.70
France	5,079	38.89
Germany	62,504	76.54
Greece	–	–
Hungary	6,367	65.10
Ireland	2,391	52.17
Italy	774	1.27
Latvia	8	0.39
Lithuania	917	30.49
Luxembourg	581	110.70
Malta	214	51.26
Netherlands	4,248	48.83
Poland	431	1.12
Portugal	–	–
Romania	92	0.43
Slovakia	5,704	105.55
Slovenia	983	47.83
Spain	5,488	47.53
Sweden	7,901	83.32
United Kingdom	72,578	117.43
EU Total	**214,268**	**55.49**
Iceland	60	18.77
Liechtenstein	–	–
Norway	2,933	58.83
Switzerland	8,432	105.49

National surveillance data in Germany on notified *Campylobacter* infections are relatively constant with an average of ~ 60,000 annual campylobacteriosis cases with variation of 52,000 (2006) and 71,000 (2011) since notification started in 2001 (http://www3.rki.de/SurvStat;

October 2014). The latest data reveal that there were 63,645 *Campylobacter* enteritis cases reported in Germany in 2013 (http://www3.rki.de/SurvStat; October 2014). However, as the disease is self-limiting many more cases go undiagnosed or unreported. *Campylobacte*r enteritis may occur in all age groups, but clinical presentation can vary according to age. Males are more often affected than females, which might be explained by differences in kitchen and food-handling practices (Friedman et al., 2004).

There is a strong seasonality observed in human campylobacteriosis worldwide and infections occur much more frequently in the summer months than in winter (Nylen et al., 2002; Jore et al., 2010). Mechanisms behind that phenomenon are poorly understood, but seasonal variation in human behaviour/life style and variation of prevalence of *Campylobacter* spp. in their reservoirs and sources e.g. an increased incidence of *Campylobacter* poultry flock colonisation are linked to increased *Campylobacter* infections in warmer month. Thus, an increased incidence of *Campylobacter* spp. in livestock may be responsible for increased levels of carcass contamination at different times of the year.

2.3.5 Species distribution of campylobacteriosis

For epidemiological studies it is necessary to compare human strains causing infection to strains from environmental sources. At least a dozen species of *Campylobacter* have been implicated in human disease. However, *C. jejuni* and *C. coli* are the leading cause of *Campylobacter* infections in humans in developed countries. In Europe, species information was provided only for 46.3% of the reported campylobacteriosis cases with 81.1% caused by *C. jejuni*, 6.2% *C. coli* and 0.2% *C. lari* (EFSA, 2014). These data are in accordance with that of Germany in 2013 with 67% caused by *C. jejuni*, 9% by *C. coli* and 24% by *C. coli/jejuni*, not differentiated (http://www3.rki.de/SurvStat; October 2014). **Figure 2-5** summarises the species distribution of *Campylobacter* spp. attributed to *Campylobacter* infections in Germany in 2013.

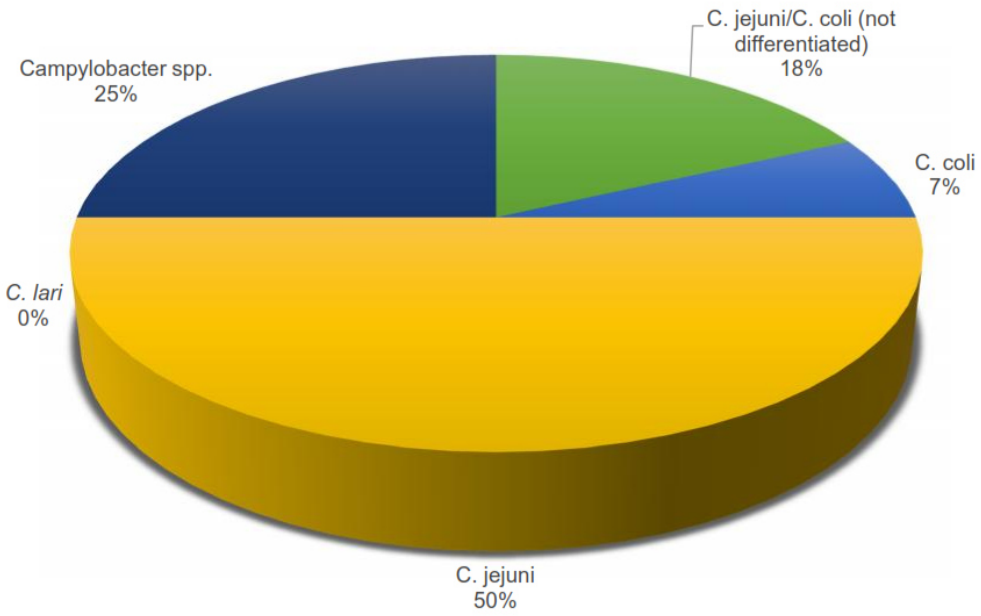

Figure 2-5: Species distribution of human campylobacteriosis cases in Germany 2013
(Source: http://www3.rki.de/SurvStat; October 2014)

In developed and developing countries often no such surveillance programs exit and most case control studies do not differentiate between *C. jejuni* and *C. coli*. Therefore, gastrointestinal diseases caused by *C. coli* infection seem to be often underestimated. Few studies have examined the prevalence of campylobacteriosis caused by *C. coli*. Gurtler et al. (2005) examined the prevalence of *C. coli* in patients with symptoms of enteric infections over a period of one year within a limited geographical area in Germany. A total of 18.6% of all *Campylobacter* spp. isolates were identified as *C. coli.* Tam et al. (2003) estimated that 7.9% of all campylobacteriosis cases in England and Wales are caused by *C. coli*, which is therefore, the second most frequently isolated species. In a study in Scotland 9.6% of all clinical isolates were identified as *C. coli,* of which 6% were attributed to pig origin (Sheppard et al., 2009). Based on MLST data, Alter et al. (2011) have estimated that up to 6% of the human infections are caused by *C. coli* from pig. A case-control study in Sweden identified the consumption of pork meat (pork with bones, loin of pork and grounded meat) as major risk factor for *Campylobacter* infections (Studahl and Andersson, 2000). In England and Wales 6% of all confirmed campylobacteriosis cases were caused by *C. coli* and patients with *C. coli* infections were more likely to having eaten special types of meats, such as meat pies, offal and paté. Pork is often the main constituent of paté (Gillespie et al., 2002).

In summary, up to 10% of human campylobacteriosis is caused by *C. coli* and infections are mainly associated with the consumption of contaminated pork meat (Gillespie et al., 2002; Gurtler et al., 2005; Rosef et al., 2009).

2.4 *C. coli* colonisation in pig

2.4.1 Digestive physiology of pigs

The primary function of the GIT is water, electrolyte and nutrient transport and absorption. Generally, the stomach functions as pre-digestive organ, as low pH, mixing of solids and liquids and peptidolytic enzymes prepare the ingested feed for digestion. The small intestine, nutrients are hydrolysed by digestive enzymes and transported across the epithelium. The small intestine can be anatomically divided into three major functionally distinct regions, the duodenum, the jejunum, and the ileum. The large intestine also consists of three functional and anatomical sections, the caecum, colon and rectum. Digestion of carbohydrates, amino acids and lipids takes place in the upper intestinal tract, while the large intestine, especially the caecum, serves as fermentation chamber of undigested carbohydrates by strict anaerobic bacteria as energy source (Lalles et al., 2007). Metabolic products by these bacteria are short-chain fatty acids, lactate and ethanol that can be further utilised by the host (Bergman, 1990). LAB and the production of lactate leads to a drop of the pH, which can act bactericide or bacteriostatic on acid sensitive bacteria e.g. *E. coli* and *Salmonella* and highlights the probiotic properties of many LAB (Cherrington et al., 1991; Pluske et al., 1998).

To perform this function, the epithelium lining the GIT is in close contact with the gastrointestinal lumen. Because the lumen is connected to the external environment and, depending on the site, has a high bacterial and antigen load, the epithelium must also prevent pathogenic agents within the gastrointestinal lumen from gaining access to internal tissues. This creates a unique challenge for the GIT to balance the requirements of forming a barrier to separate the intestinal lumen from underlying tissue, while simultaneously setting up a system for moving water, electrolytes and nutrients across the barrier. In the face of this, the epithelial cells of the gastrointestinal tract form a selectively permeable barrier that is tightly regulated. A critical component of this barrier function attributed to the intestinal epithelial cell layer is the formation of epithelial tight junctions, which participate in the polarisation into apical- and basolateral domains. Thus, the formation of tight junctions in the epithelial cell line is important for separating the mucosa from luminal components, while allowing the absorption of nutrients (Burkey et al., 2009).

2.4.2 Immune system of the gastrointestinal tract of pigs

The GIT is the first line of defence as the intestinal epithelial cells are continuously exposed to bacteria that reside in the intestinal lumen. There is a constant immunological stimulation, but yet it is not clear how the innate immune system distinguishes between commensal and

pathogenic bacteria. There are large numbers of immune cells distributed throughout the GIT. The gut-associated lymphoid tissues (GALT) are secondary lymphoid aggregates that are frequently found in the intestine in order to mount immune response. The GALT builds the main functional barrier and is influenced by microbial stimuli supporting gut-associated immunological homeostasis (Burkey et al., 2009). The Peyer´s Patches (PPs) are part of the GALT and found in the lowest portion of the small intestine. The PPs are primary lymphoid tissues and receive antigenetic signals through their interaction with microfold and dendritic cells. Microfold cells transport antigens from the intestinal lumen to the lymphocytes and are the major route for bacterial invasion. Dendritic cells and macrophages that receive antigens from M cells present them to T cells in the GALT, leading ultimately to appearance of secreting immunoglobulin (Ig) plasma cells in the mucosa. The secreted IgA are transported through the epithelial cells into the lumen where they interfere with the adhesion and invasion of bacteria (Simecka, 1998). The lamina propria together with the epithelium constitutes the mucus and is also part of the GALT. It acts as a barrier that protects internal tissues from external pathogenic microorganisms. The connective tissue of the lamina propria is very loose allowing it to be very cell rich and contains capillaries and a central lymph vessel in the small intestine, as well as lymphoid tissues. The mesenteric lymph nodes (MLNs) also belong to the immune system of the small intestine (Burkey et al., 2009; Shen, 2009). MLNs also act as a firewall to prevent live commensal intestinal bacteria from penetrating the systemic immune system.

2.4.3 Pig microbiota

There is a high similarity between the human and porcine GIT in regard to their anatomically and physiologically properties (Pogacar et al., 2010). Humans as well as pigs are monogastric omnivores that eat a combination of plant and animal matter. The gut of pigs is sterile at birth. During birth and rapidly thereafter, the piglet is exposed to vaginal, skin, and faecal microbes from the mother sow and by surrounding environmental bacteria (Mackie et al., 1999). In the early colonisation phase the microbiota of piglets has a high similarity with the sow intestinal microbiota and the initial intestinal microbiota of piglets is already established within 48h. Bacterial counts of 10^9 and 10^{10} cfu/g faeces can already be detected 12h after birth (Savage, 1977; Pluske et al., 2002). First colonisers are aerobes and facultative anaerobes that consume oxygen to provide a reduced environment for establishing an anoxic environment (Mackie et al., 1999). Facultative anaerobes and strict anaerobes follow to constitute the predominant microbiota community consisting of genera, such as *Bacteroides*, *Bifidobacterium*, *Clostridium* and *Lactobacillus*. Whilst the piglet is still suckling, the dominant bacteria within the stomach and small intestine are lactic acid bacteria like

Lactobacillus (*L.*) spp. and *Streptococcus* spp., but also coliforms e.g. *E. coli* (Pieper et al., 2008; Vahjen et al., 2010). Piglets in the commercial animal production setting experience an early and critical transition from sow´s milk onto a solid diet between 21 to 28 days of life. Birth and weaning of pigs are the most severe influences that lead to direct modification of the mammalian intestine, as nutrient supply and contact with the environment trigger a multitude of physiological and immunological responses (Lalles et al., 2007). The microbiota in piglets is subjected to drastic changes during the first weeks after weaning. These changes can dramatically alter the balance of the gastrointestinal microbiota providing an opportunity for pathogens to colonise and cause disease, poor growth performance and even death (Pluske et al., 2002). Diarrhoea in suckling and weaned piglets is one of the most important sources of large economic losses on production farms and it has generally been believed to be caused by proliferation of pathogenic bacteria, mainly *E. coli* (Melin and Wallgren, 2002). The instability of the porcine microbiota in the post-weaning period of pigs can be seen as important potential target in therapeutic and preventive interventions targeting GIT disturbances. A diverse and stable intestinal microbiota plays a vital contribution towards the health and productive performance of the host (Katouli et al., 1999; Sekirov et al., 2010).

After weaning, intestinal bacteria develop a stable and balanced 'climax community' and the adult microbiome is formed over time (Jensen-Waern et al., 1998; Lalles et al., 2007). The GIT hosts one of the most complex microbial communities with 10^{14} bacteria consisting of up to 1,000 different species (Steinhoff, 2005; Peris-Bondia et al., 2011). Densities generally increase from proximal to the distal GIT and different bacterial species belonging to almost all branches of the bacterial tree of life. However, the vast majority of intestinal bacteria cannot be cultured and therefore, most species are not studied in detail yet (Leser et al., 2002). Besides culture-based analysis, novel molecular-based techniques, such as 16S rRNA analysis or pyrosequencing, fingerprinting, DNA microarrays, quantitative real-time PCR etc. improve constantly our knowledge of the microbiome diversity (Sekirov et al., 2010).

The stomach and proximal small intestine (duodenum) contain relatively low numbers of bacteria (10^3 to 10^5 bacteria/g digesta) due to low pH and/or rapid digesta flow. In the proximal small intestine, digesta flow rate and the rate of bacterial washout exceeds the maximal growth rates of most bacterial species and the bacteria that are present typically adhere to the mucus or epithelial cell surface (Gaskins, 2001). In contrast, the distal small intestine harbours a more diverse and numerically greater population of bacteria (10^8 bacteria per/g digesta). The large intestine is the major site of microbial colonisation because of the high residence time of the digesta. The luminal contents of the colon support in excess of 400 different bacterial species with numbers as high as 10^{10} and 10^{11} culturable bacteria per g of digesta (Kelly and King,

2001). The hindgut flora is considered both diverse and stable, with the many species and strains appearing to coexist without one or few ever becoming dominant.

Bacterial groups of the porcine GIT comprise of 375 phylotypes within 13 genetic groups based on 16S rRNA analysis (Leser et al., 2002). The major bacterial groups isolated are *Bacteroides, Bifidobacterium*, *Butyrivibrio, Clostridium*, *Escherichia*, *Enterococcus*, *Eubacterium, Fusobacterium*, *Lactobacillus*, *Peptostreptococcus*, *Prevotella*, *Ruminococcus*, S*elenomonas* and *Streptococcus* (Jensen, 2001; Leser et al., 2002). Host variability in terms of diet, age, genotype, and colonisation history are parameters that influence the bacterial community in the porcine GIT (Leser et al., 2002; Dethlefsen et al., 2007). In addition to population differences there are also high variations between mucus associated microorganisms and bacteria that exist in the digesta (Pryde et al., 1999; Van den Abbeele et al., 2011). While mucosal microorganisms would be crucial for immunological priming, luminal bacteria would be important for nutrient digestion. However, Leser et al. (2002) suggests that mucosal excretion, epithelial turnover and gut motility leads to a mucosa-associated sub-population in the luminal microbiota. *Campylobacter* spp. are known to be a part of the natural microbiota of pigs (Leser et al., 2002). However, little is known about their role in the pig microbiome even though they are one major human pathogen.

2.4.4 *C. coli* colonisation in pigs

Campylobacter spp. are considered to be commensals in pigs even though single studies have shown clinical and histopathological outcomes due to *Campylobacter* infections for colostrum-deprived, neonatal piglets (Olubunmi and Taylor, 1982; Taylor, 1982; Babakhani et al., 1993). In animals, colonisation almost always has no clinical outcome indicating a lack of specific factors, e.g. receptors that are necessary for *Campylobacter* spp. to cause disease or effective immune mechanisms that are only present in animals and prevent disease. However, there is still a gap in our knowledge on corresponding host cell receptors (Backert et al., 2013).

Colonisation of *Campylobacter* spp. occurs early in life. Vertical transmission from mother to offspring appears via the faecal-oral pathway and nursery is proven to be the primary site of transmission (Weijtens et al., 1997; Young et al., 2000). Faecal samples taken from piglets right after birth were free of *Campylobacter* spp., but 41% shed *C. coli* three weeks after and 66.8% were *Campylobacter* spp. positive by the time of slaughter (Alter et al., 2005b). Soultos and Madden (2007) reported that piglets were initially contaminated with bacteria of the same genotype as those infecting their mothers. Maternal *C. coli* are the primary source of piglet infection, but different sources appear during the rearing period as new genotypes occur that largely contribute to the final microbiota (Alter et al., 2005b; Soultos and Madden, 2007).

Additionally, if one animal carries *Campylobacter* spp. it might be quickly transferred from one pig to the other and nearly all pigs remain carriers until slaughter (Weijtens et al., 1993). Faecal shedding ensures and enables transmission to the next host and molecular epidemiological methods indicate that *Campylobacter* strains circulate through the environment between domestic livestock and wild animal populations (French et al., 2005).

After passage through the acid environment present in the porcine stomach, *Campylobacter* spp. colonise competitively the mucus layer of the GIT. Chemotactic mechanisms in *Campylobacter* spp. towards mucin and more specific fucose, a constituent in mucin, are needed, since non-chemotactic mutants do not colonise animals (Takata et al., 1992). Competition for essential nutrients amongst the bacterial community in the GIT is likely to be intense and it appears that *Campylobacter* spp. have evolved mechanisms to allow them to compete successfully in this environment. Attachment to host cell receptors e.g. glycoproteins of the mucin requires bacterial expression of adhesins on their cell surface. Flagella may not only allow a corkscrew-like motility within the viscous mucin layer of the epithelial cell, but may also act as adhesins in *Campylobacter* spp. (Szymanski et al., 1995; Sylvester et al., 1996). Moreover, flagella of *C. jejuni* are also used for protein secretion, mainly via its flagella apparatus, is engulfed by intestinal cells, and can disrupt the integrity of the epithelial lining (van Putten et al., 2009). *Campylobacter* spp. were also isolated in high levels from luminal content along the entire intestine of piglets with particularly high numbers in the caecum (Weijtens et al., 1993; Jensen et al., 2013). However, there are only a few animal trials that have studied experimental *C. coli* colonisation in pigs. A first experimental infection of weaners with *C. jejuni* and *C. coli* was reported in a French study (Leblanc-Maridor et al., 2008). It could be shown that only *C. coli* were detectable at the end of the trial. Excretion rates varied between 10^3 and 10^7 cfug faeces for most of the inoculated pigs, but also for non-inoculated neighbouring pigs. Genetic variability was evidenced only in pigs inoculated with *C. coli* of porcine origin (Leblanc-Maridor et al., 2011). The only study that enumerated *C. coli* along the GIT in order to assess the reduction potential of feed additives on *Campylobacter* spp. in slaughtered pigs was reported recently (Jensen et al., 2013).

2.4.5 *C. coli* translocation within pigs

Bacterial translocation is defined as the passage of viable indigenous bacteria from the GIT to extra-gastrointestinal sites, like the MLNs, liver, spleen and blood (Berg, 1995). Three major mechanisms promote bacterial translocation: intestinal bacterial overgrowth, deficiencies in the host immune defence and increased permeability or damage of the intestinal mucosal barrier. Two main routes are considered for translocating bacteria (i) via lymphatics to MLNs

or (ii) directly via the blood to the liver and then spread systematically (Murakami et al., 2011). If the intestinal epithelial layers are not physically damaged, the enteric bacteria translocate through the epithelial cell (intracellular), subsequently the bacteria travel via the lymphatic vessels from the lamina propria to MLNs and from there to other organs. When the epithelial layers are denuded by intestinal permeability and actual mucosal damage the bacteria can spread directly via the vascular route to the liver (Berg, 1995).

Early observations in gnotobiotic mice have already suggested that *Campylobacter* spp. are enteroinvasive bacteria with a particular affinity for lymphatic organs (Fauchere et al., 1985). Invasion and translocation of *Campylobacter* spp. into porcine intestinal epithelial cells and their intracellular survival has been reported in many *in vitro* studies (Babakhani and Joens, 1993; Pogacar et al., 2010; Murphy et al., 2011). The loss of barrier function opens the way for *Campylobacter* spp. to invade and translocate the epithelial cells by either a para- or transcellular route, or both (Man, 2011; Murphy et al., 2011; Backert et al., 2013).

There are only a few studies examing the invasion and translocation of *Campylobacter* spp. to extra-gastrointestinal sites in pigs as most studies have focused on *C. jejuni* translocation (Babakhani et al., 1993; Harvey et al., 2006; Bratz et al., 2013). In a Norwegian study, 31.9% of the lymphatic tissues of slaughtered pigs were contaminated with *Campylobacte*r spp. Tonsils were contaminated with 66.7% and up to 50% of the animals showed high contamination rates of *Campylobacter* spp. for mesenteric lymph nodes, depending on the detection method (Nesbakken et al., 2003). In contrast, in a German study only 6% of the tonsils of fattening pigs at slaughter were colonised with *Campylobacter* spp. (mostly *C. coli*), while faecal excretion was high (Fredriksson-Ahomaa et al., 2009). Offal, such as the liver, which is a basic ingredient for paté and part of many menus, is discussed to be colonised by *C. coli*. An earlier study in Ireland, in which 400 pork livers were examined for the presence of *Campylobacter* spp., revealed a prevalence of 6%. Deep tissue areas were used for sampling in order to ensure no external contamination (Moore and Madden, 1998). High prevalences of *C. jejuni* are also frequently found in the spleen, liver and gall bladder of broilers (Cox et al., 2007; Cox et al., 2009). Therefore, it is discussed if *Campylobacter* spp. can also translocate to the gall bladder of pigs. In an earlier study *Campylobacter* spp. could be isolated from gall bladders of healthy pigs or pigs suffering from gastric disease with a prevalence of 13.3% and 14%, respectively (Gorgen et al., 1983). Due to the isolation of *Campylobacter* spp. in deep tissues in pig, chicken and other ruminants it is likely that these bacteria translocate between the intestine and liver via the bile duct. Interestingly, early mice models already showed a mucosal translocation of *C. coli* to MLNs in 96% (60/62) of the animals, while spleen, liver and blood remained sterile (Youssef et al., 1987). Similar observations are reported in a more recent study in mice (Bereswill et al., 2011). Overall, MLNs and extra-gastrointestinal organs

are usually sterile and bacterial translocation is mostly observed when the host immune system is compromised (Murakami et al., 2011).

It has to be mentioned that some studies do not differentiate if the *Camplyobacter* spp. occurrence at extra-gastrointestinal sites is due to real translocation events or by intestinal content spillage during evisceration. During the traditional slaughter procedure, which involves the removal of the tongue, with tonsils attached, the carcass will easily be contaminated with *Campylobacter* spp. Thus, slaughtering might be a crucial step for *Campylobacter* spp. transmission to pig carcasses.

2.5 Pre-harvest intervention measures for reducing *C. coli* in pig

Total abundance of *Campylobacter* spp. on the farm is not feasible as *Campylobacter* spp. are ubiquitously present and risk factors for the initial transmission into pig herds largely remain unknown. Currently, reduction is largely achieved by three main areas: on-farm biosecurity, hygiene during the slaughter process and hygiene during food preparation (Cody et al., 2010). Control of *Campylobacter* spp. in pork industry is presently accomplished by common cleaning and preparation practices within processing plants, yet contamination of product still occurs as evidenced by recovery of organism in supermarket retail raw meat (Baer et al., 2013). It has been shown that even cleaning and disinfection of pens and facilities did not remove all *C. coli* strains that were present within the fattening departments. However, the detection rate of *Campylobacters* spp. after cleaning and disinfection of the corresponding departments was very low with 1.6% compared to 9.2% before cleaning and disinfection. Thus, improperly cleaned units may act as a continuous source for *C. coli* infection (Alter et al., 2005b) *Campylobacter*-free breeding have also been attempted, but accounts for a high cost production and extreme hygienic practices (Weijtens et al., 2000). Preventing initial infection of *Campylobacter* spp. by these practices is a huge challenge because pigs have a long productive cycle (Horrocks et al., 2009).

Campylobacter spp. do not grow outside the host and thus, reduction of *Campylobacter* spp. at the end of the food chain is best achieved if the colonisation in live animal can be prevented or reduced (Wagenaar et al., 2008). Reducing the prevalence of *Campylobacter* spp. colonisation by pre-harvest intervention measures decreases the numbers of *Campylobacter* spp. in the following steps and may result in a low concentration or absence of *Campylobacter spp.* in pork (Wagenaar et al., 2006). Therefore, feed additives with antimicrobial properties can be given to pigs at different production stages to decrease the load of *Campylobacter* spp.

2.5.1 Feed additives as pre-harvest intervention measure

Preventive measures in primary production have a limited and unpredictable effect. So far, vaccination, competitive exclusion, bacteriophage therapy, feed additives and bacteriocins are not yet commercially available, but research is ongoing (Wagenaar et al., 2006; Wassenaar, 2011). Antibiotic treatments exist for reducing gastrointestinal concentrations of bacterial pathogens. However, because of potential residues and increasing antimicrobial resistances in humans, the use of antibiotics for pre-harvest control of *Campylobacter* spp. is undesirable (Horrocks et al., 2009). The development of 'alternatives' to antibiotic treatment to reduce the carriage of *Campylobacter* spp. in food animals, is proposed, but at present, none are available or accepted (Baer et al., 2013). The application of the antimicrobial feed additives, such as the probiotic bacterium *E. faecium* NCIMB 10415 and the trace element zinc for reducing the *Campylobacter* spp. load in pigs is discussed.

2.5.1.1 Probiotics

Probiotics have been used as feed additives for a long time with beneficial effects in livestock. Improving performance (feed intake, daily weight gain, feed conversion ratio etc.) and stabilising the health of farm animals are the two main tasks of probiotics in animal production (Simon, 2010). Currently three different groups of probiotics are authorised as feed additives in the EU: LAB, mainly *Enterococcus* spp., *Lactobacillus* spp. and *Bifidobacterium* spp., bacteria of the genus *Bacillus* and yeasts of the genus *Saccharomyces*. In pig production the central motivation of probiotic usage depends on preventing diarrhoea in the post-weaning phase, mainly by competitive exclusion of intestinal pathogens. However, the mechanisms underlying the beneficial effects of probiotics are largely unknown, but are likely to be multifactorial. Major mechanisms related to the antagonistic effect of probiotics on various microorganisms have been summarised by Bermudez-Brito et al. (2012):

- Production of antimicrobial compounds
    - Organic acids
    - Short-chain fatty acids
    - Peroxides
    - Bacteriocins
- Competition for essential nutrients pathogens require
- Competitive adherence to the mucosa and epithelium
- Strengthening of the gut epithelial barrier
- Modulation of the immune system

However, for many of the potential benefits, research is limited and application trials are not always consistent. Moreover, all effects can only be attributed to individual strains and results also depend on diverse other factors, such as the initial microbiota, immune status of the host and trial conditions (Simon, 2010).

Strains of the genus *Enterococcus* spp. are commonly used as probiotic feed additive. The strain *E. faecium* NCIMB 10415 (formerly referred to SF68) is authorised in the EU for safe use as a probiotic feed additive for pigs (Commisssion Regulation (EC) No 252/2006,). This probiotic is thought to stabilise the gut microbiota and improve animal heath welfare. Thus, improves the overall performance in production animals. *E. faecium* strains have a long and well-documented history of safe use in both humans and animals and demonstrated health benefits. The application of *E. faecium* NCIMB 10415 as a probiotic bacterium is fulfilled by certain criteria:

- Absence of acquired antibiotic resistance genes and virulence factors
- Non-pathogenic and non-toxic
- Excellent biological activity
- Rapid growth and transient colonisation in the GIT
- Produces lactic acid and bacteriocidal-like substances
- Inhibits growth of pathogenic bacteria

In pigs, a decreased incidence of diarrhoea and a lower occurrence of potentially pathogenic bacteria fed *E. faecium* NCIMB 10415 has been reported (Taras et al., 2006). Contrary, supplementation of the post-weaning diet with *E. faecium* SF68 did not affect piglet performance, but did tend to reduce serum IgG (Broom et al., 2006). Besides the effects on the growth performance on pigs, the influence of *E. faecium* supplementation on foodborne pathogens has been investigated. Studies with healthy piglets have shown that *E. faecium* has the ability to decrease the pathogenic load of *Chlamydia* and *E. coli* (Pollmann et al., 2005; Taras et al., 2006). On the other hand, increased *Salmonella* counts have been reported in weaned piglets fed *E. faecium* NCIMB 10415 (Szabo et al., 2009; Kreuzer et al., 2012). In another study, daily dietary supplementation of *E. faecium* NCIMB 10415 to healthy dogs significantly reduced *Clostridium* spp. counts, while *Campylobacter* spp. counts increased (Vahjen and Manner, 2003). Other probiotics and prebiotics were able to reduce *Campylobacter* spp., but most work has been done on *C. jejuni* in chickens. The probiotic strain *Bifidobacterium longum* PCB 133 for example decreased *C. jejuni in vitro* and *in vivo*, whereas *E. faecium* PCD 71 showed no antimicrobial activity against *Campylobacter* spp. (Santini et al., 2010). Reduced *C. jejuni* shedding in market aged broilers was reached by feeding *L. acidophilus* and *Streptococcus faecium* (Morishita et al., 1997). Moreover, *Citrobacter*

diversus, *Klebsiella pneumonia,* and *E. coli* can be effectively administered to reduce *Campylobacter* spp. colonisation in chickens (Schoeni and Wong, 1994). The bacteriocin E 50-52 produced by an *E. faecium strain* reduced *C. jejuni* by more than 100,000-fold in orally treated broilers (Svetoch et al., 2008). Probiotics of the genus *Lactobacillus* spp. and *Bifodobacterium* spp. combined with prebiotics inhibited the growth of *C. jejuni,* probably by the reduction of pH via the production of organic acids, *in vitro* (Meremae et al., 2010). Reduced levels of *Campylobacter* spp. *in vitro* was detected by co-culture experiments of *Lactobacillus* P93, but not with *Enterococcus* spp. or *E. coli* strains (Chaveerach et al., 2004). Nursery pigs fed a diet containing carbadox and copper sulfate lead to decreased faecal shedding of *Campylobacter* spp. Both agents are commonly used as growth promoters, but showed decreased feed efficiency and increased shedding of *Enterobacteriaceae,* such as *Salmonella* (Wells et al., 2010). Hence, important for researchers is the examination of the effects of feed compounds on different pathogens as well as of the animal growth performance.

2.5.1.2 Zinc

Zinc is an essential trace element for all forms of life. Over 300 enzymes and proteins are known to bind zinc, including some necessary for fundamental cell processes like RNA and DNA synthetases and transferases (Vallee and Falchuk, 1993). In agriculture, zinc was already established as essential for mammals in 1934. However, more than 20 years passed before the first descriptions of zinc deficiencies in farm animals appeared. In 1955, it was reported that zinc supplementation would cure parakeratosis in swine. For livestock under more defined conditions feed can be easily supplemented with zinc salts to prevent deficiency (Nielsen, 2012). Dietary zinc levels of 50–125 mg/kg feed meet the pig's nutrient requirement. At high dietary concentrations (> 2,000 mg ZnO/kg feet) it is well-known to reduces the incidence of diarrhoea and to increases the weight gain in newly weaned pigs (Jacela et al., 2010). However, feeding of high zinc for more than three weeks after weaning reversed these positive effects (Katouli et al., 1999). More importantly, the feeding of ZnO has been restricted in the EU to 150 mg/kg feed, mainly due to the environmental pollution (EFSA, 2014). Still, a short-term application of pharmacological ZnO concentrations is currently being considered.

For the most part, inorganic zinc sources have been utilised to meet the mineral requirements of pigs. Other forms of zinc available for supplementation in weanling diet include organically complexes, such as zinc methionine or zinc lysine (Lewis and Southern, 2000). Increased growth rates when high levels of zinc are fed in the post-weaning phase have been demonstrated with zinc provided as zinc oxide (ZnO). Other sources, such as sulphate and zinc methionine, have not consistently demonstrated positive effects.

Even though high concentrations of ZnO have been proven to promote growth by reducing post-weaning diarrhoea there is a lack of science evidence to understand the exact mode of action. The reduced incidence of diarrhoea is explained by restricting the proliferation of pathogenic bacteria in the gut (Clayton et al., 2011). The inhibition of the respiratory electron transport chain and thus, the generation of ROS has been shown to contribute to its main antimicrobial properties (Sawai et al., 1996; Sawai et al., 1998). Hydrogen peroxide production has been found to affect the antibacterial activity of ZnO (Yamamoto et al., 2001). It is also supposed that ZnO interacts with specific cell compounds of the outer membranes as gram-positive bacteria are more sensitive than gram-negative bacteria (Tayel et al., 2011). For ZnO nanoparticles (NP) the induction of oxidative stress by production of ROS has also been proven as main antimicrobial factor against many foodborne pathogens (Liu et al., 2009; Kumar et al., 2011; Tayel et al., 2011; Xie et al., 2011).

2.5.1.2.1 Effects of Zinc oxide on the porcine microbiome

In addition to the beneficial effects on pigs, increased dietary zinc directly affects the intestinal microbial community of the host. *In vivo* studies showed an altered intestinal microbiota composition in weaned piglets fed high ZnO concentrations. However, ZnO seems to have the greatest impact on LAB in particular lactobacilli, *Enterobacteriaceae* and coliform bacteria. Reduced numbers were detected for total lactobacilli, while other LAB like streptococci and *Bifidobacteria* were either increased or not affected after supplementation of high ZnO doses to weaned piglets (Hojberg et al., 2005; Broom et al., 2006; Starke et al., 2014; Vahjen et al., 2011). Contrary, Pieper et al. (2012) observed that lactic acid bacteria were not influenced by high ZnO levels, although no differentiation on some species level was performed. A strong numerical reduction was reported for strict anaerobes (Starke et al., 2014; Vahjen et al., 2010), while increased numbers of other bacteria like coliforms and enterococci were reported (Hojberg et al., 2005; Vahjen et al., 2010). In addition, species from *Proteobacteria* and *Enterobacteriaceae,* such as *Salmonella* increased numerically during the post-weaning period of piglets fed high doses of ZnO, while some dominant lactobacilli decreased (Vahjen et al., 2010). It is speculated that a numerical decrease of dominant lactobacilli may lead to increased colonisation of gram-negative enterobacteria that are more resistant against zinc ions. Moreover, this increased competition may lead to a replacement of pathogenic *E. coli*, which is known to be the main cause of PWD. Interestingly, while total numbers of lactobacilli decreased some lactobacilli species like *L. reuteri* and *L. johnsonii* were less affected by high dietary zinc supplementation (Starke et al., 2014). Thus, the antibacterial action of ZnO *in vivo* is probably not intrinsic to lactobacilli per se, but may rather be species or even strain specific.

This was also shown by Liedtke and Vahjen (2012) who observed differing *in vitro* zinc resistance among the nine *Lactobacillus* straind. The two *C. jejuni* strains tested were highly susceptible for zinc. No *C. coli* strain has been tested and thus, no prediction for *C. coli* can be made. The autors concluded that zinc resistance of commensal intestinal bacteria cannot be grouped according to their taxonomic origin, as no clear phylogenetic pattern and bacterial grouping regarding ZnO inhibition was detectable. Therefore, the antibacterial activity of ZnO in the porcine intestine cannot be generalised and is rather species-specific.

Overall, pharmacological ZnO concentrations have a massive impact on the development of the intestinal microbiota resulting in a species richness that reflects ecosystem stability. A high coliform diversity is believed to have a positive impact by promoting an active competition for colonising receptor sites of pathogenic strains and competition for nutrients (Katouli et al., 1999; Melin and Wallgren, 2002).

2.5.1.2.2 Zinc homeostasis in pigs

ZnO doses with growth promoting effects exceed the physiological need of pigs with 50–125 mg zinc/kg feed by far (commission regulation (EC) No 1334/2003). ZnO reacts in the stomach with hydrochloric acid to form soluble $ZnCl_2$ and free Zn^{2+} ions (Dintzis et al., 1995). Free intracellular zinc is limited as it is mostly bound to metallothionein in mammalian cells. This chelate complex is involved in zinc homeostasis as the intestinal and systematic level and its expression is directly affected by dietary zinc concentrations. After absorption in the small intestine, zinc is transported via the blood, bound to different transport proteins, to the liver where release from the metallothionein is controlled. Excess of zinc is toxic and overburdens the zinc metabolism resulting in accumulation mainly in bones and muscles, but also liver and kidney (Hill et al., 1983; Jensen-Waern et al., 1998). Prolonged excess of zinc has also a negative impact on pig performance as it reduced feed intake and body weight gain. Thus, supplementation of high dietary zinc concentrations should be restricted to the first two weeks post-weaning in veterinary practice (Katouli et al., 1999). Chronic effects like anaemia, malabsorption, liver and kidney damage (necrosis) may occur. Zinc homeostasis mainly depends on availability, uptake, storage and excretion. The main site of zinc homeostasis is the GIT, specifically the small intestine where absorption takes place. Homeostasis is achieved mainly by faecal excretion, while urinary zinc remains relatively constant (Poulsen and Larsen, 1995). Zinc toxicity is rare and impairs the immune system as it supresses the iron and copper uptake. Zinc deficiency is more common and can result in poor growth, retarded feed intake, abnormal skin and impaired immune functions which is summarised under the disease parakeratosis (Tucker and Salmon, 1955).

2.5.1.2.3 Zinc homeostasis in bacteria

The GIT offers a broad range of niches that differ in their zinc availability thereby enable the colonisation of different bacteria at different sites along the intestine. Most bacterial species regulate their zinc homeostasis by zinc transporter systems (Hantke, 2005). Most work has been done on *E. coli* as model organisms. Zink uptake can be achieved by low- and high-affinity zinc uptake systems. Metal ions enter the cell through specific and non-specific transporters. The low affinity zinc uptake systems are constitutively expressed, whereas the high affinity uptake systems are only expressed in times of need (Choudhury and Srivastava, 2001). At low zinc levels the high-affinity zinc uptake transporter ZnuABC facilitates the import of zinc by ATP hydrolysis (Patzer and Hantke, 1998). It is composed of a periplasmatic-binding protein ZnuA, a membrane spanning protein ZnuB, and the ATPase ZnuC. The ZnuABC system is distributed throughout nearly all bacterial species and is the most specific to zinc uptake. In the presence of zinc the zinc uptake regulator Zur represses the expression of znuABC (Patzer and Hantke, 2000). In addition to the import systems, many zinc export system in *E. coli* are described. Excessive cytoplasmic zinc is exported from the cell by the main P-type high-affinity ATPase ZntA, which is a conserved bacterial zinc efflux transporter. Other efflux systems have steadily been reported in *E. coli* (Clayton, 2011).

Campylobacter spp. must survive in zinc-deprived environments during its infection cycle. Therefore, it is assumed that zinc homeostasis plays a major role in host colonisation and *Campylobacter* spp. regulate their zinc homoeostasis by zinc transport systems as well. Orthologues of *znt*A, *zup*T and *znu*ABC have been found in *C. jejuni*, but no zur homolog (Davis et al., 2009). Mutation of the *znu*A in *C. jejuni* for example resulted in a growth deficiency in zinc limited media and a reduced colonisation ability in chickens (Davis et al., 2009). To our knowledge, zinc transporter systems based on sequence homologies have only been described for *C. jejuni* so far. However, a nucleotide BLAST search of the *C. coli* RM2228 genome revealed the presence of zinc transporter homologues to *C. jejuni* NCTC 11168 (**Table 2-5**).

Table 2-5: Zinc transport systems in *Campylobacter* spp.

Gene	Function	*C. jejuni* NCTC 11168	*C. coli* RM2228	Homology [%]
Zinc export system				
*znt*A	Zn (II)-translocating P-type ATPase	Cj1155c	CCO1228	77
Zinc import system				
*znu*A	Putative adhesion protein, ZnuABC transporter	Cj0143c	CCO1735	59
*znu*B	Membrane spanning protein, ZnuABC transporter	Cj0141c	CCO1737	83
*znu*C	ATPase, ZnuABC transporter	Cj0142c	CCO1736	83

Chapter 3: Aims and Objectives of the thesis

Campylobacter spp. are well recognised as the leading cause of bacterial foodborne diarrheal disease worldwide with *C. jejuni* and *C. coli* as the most important species. *C. coli* is highly abundant in pigs and pork meat has often been implicated as a source for human infection. Contamination of pork with *Campylobacter* spp., mainly *C. coli* occurs frequently during slaughter and approximately 10% of the pork products are contaminated with *Campylobacter* spp. However, still little is known about the colonisation ability of *C. coli* along the GIT and the translocation from the GIT to extra-gastrointestinal sites in pigs. To determine the significance of these reservoirs in contamination of pork meat during processing, it is important to know were inside the pig *Campylobacter* spp. are located. Thus, elucidating the site of colonisation is fundamental if efficacious management strategies are to be developed. Therefore, we firstly aimed to improve our knowledge about the *C. coli* colonisation and translocation ability within weaned piglets.

The high prevalence of *Campylobacter* spp. in pigs and the frequent *C. coli* contamination of pork emphasises the need for pre-harvest intervention strategies. Reducing the load of *Campylobacter* spp. in pigs may lead to a reduced surface contamination of the carcasses during processing thereby, decreasing the exposure of contaminated pork to humans. *Campylobacter* spp. do not grow outside the host and thus, reduction of *Campylobacter* spp. at the end of the food chain is best achieved if the colonisation in live animal can be reduced. At present, control of *Campylobacter* spp. in pork industry is accomplished by common cleaning and preparation practices within processing plants. However, contamination of pork meat and pork products still occurs, as evidenced by recovery of *Campylobacter* spp. at retail. The application of pre-harvest intervention measures to reduce the *C. coli* burden in pigs is currently intensively explored. Feed additives as pre-harvest intervention measure, such as probiotics and high concentration of trace elements are known to have antimicrobial properties. An altered intestinal microbiota by dietary supplementation of these additives has been demonstrated in pigs. The feeding of the probiotic *E. faecium* NCIMB 10415 and high ZnO doses of piglets is thought to decrease post-weaning diarrhoea and improves growth performance in pigs. However, the modes of action of both feed additives are still incomplete and mechanisms are not fully understood yet. The feeding of *E. faecium* NCIMB 10415 and high doses of ZnO to pigs as pre-harvest intervention measure to reduce the *Campylobacter* spp. load has not been investigated yet.

The main objective of the present thesis was therefore, to evaluate the antimicrobial properties of the probiotic strain *E. faecium* NCIMB 10415 and metal oxide ZnO against *C. coli in vitro* and *in vivo*.

In summary, the present thesis aimed to address the following objectives:

- What are the main reservoirs of *C. coli* within pigs?
- Which probiotic bacteria have antimicrobial properties against *C. coli* and what is the mode of action?
- Does the feeding of the probiotic *E. faecium* NCIMB 10415 leads to reduced *C. coli* faecal excretion rates in weaned piglets?
- Does the feeding of a high ZnO concentration reduces the *C. coli* faecal excretion in weaned piglets?
- If ZnO shows an inhibitory activity against the *C. coli*, what is the mode of action?

Chapter 4: Experimental infection of weaned piglets with *Campylobacter coli* – Excretion and translocation in a pig colonisation trial

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4.1 Abstract

Campylobacter (*C.*) is one of the most common food-borne pathogen causing bacterial enteric infections in humans. Consumption of meat and meat products that have been contaminated with *Campylobacter* are the major source of infection. Pigs are a natural reservoir of *Campylobacter* spp. with *C. coli* as the dominant species. Even though some studies focussed on transmission of *C. coli* in pig herds and the excretion in faeces, little is known about the colonisation and excretion dynamics of *C. coli* in a complex gut microbiota present in weaned piglets and the translocation to different tissues.

Therefore, an experimental trial was conducted to evaluate the colonisation and translocation ability of the porcine strain *C. coli* 5981 in weaned pigs. Thus, ten 35 days old piglets were intragastrically inoculated with strain *C. coli* 5981 ($7x10^7$ CFU/animal) encoding resistances against erythromycin and neomycin. Faecal samples were taken and *C. coli* levels were enumerated over 28 days. All piglets were naturally colonised with *C. coli* before experimental inoculation, and excretion levels ranged from 10^4 to 10^7 CFU/g faeces. However, no strain showed resistances against the additional antimicrobials used. Excretion of *C. coli* 5981 was seen for all piglets seven days after inoculation and highest counts were detectable ten days after inoculation with 10^6 CFU/g faeces.

Post-mortem, translocation and subsequent invasion of luminal *C. coli* was observed for gut tissues of the small intestine and for the gut associated lymphatic tissues, such as jejunal mesenteric lymph nodes and tonsils as well as for spleen and gall bladder.

In conclusion, this pig colonisation trial offers the opportunity to study *C. coli* colonisation in weaned piglets using the porcine strain *C. coli* 5981 without the need for gnotobiotic or specific pathogen-free animals.

Keywords

Campylobacter; colonisation; excretion; translocation; pigs

4.2 Introduction

Campylobacter (*C.*) spp. are one of the most dominant zoonotic bacterial cause of human enteritis. Infection results in clinical outcomes like diarrhoea, abdominal pain, and fever (Newell, 2001). Both species asymptomatically colonise the intestine of many farm animals but also wildlife and companion animals (Horrocks et al., 2009). Pigs are a natural reservoir of *Campylobacter* spp. (with *Campylobacter coli* predominating) with prevalence between 50%

and 100%, and excretion levels ranging from 10^2 to 10^7 colony forming units (CFU)/g faeces (Alter et al., 2005; Jensen et al., 2006). Risk factors for the introduction of *C. coli* to pig herds largely remain unclear. It is assumed that horizontal transmission via farmers, domestic animals, contaminated surface water, insects, and other environmental sources are largely responsible for introduction into herds (Guerin et al., 2007; Wassenaar, 2011). Vertical transmission from mothers to offspring may occur via the faecal-oral route (Alter et al., 2005). Although *C. coli* are responsible for only 10% of human *Campylobacter* spp. infections the impact on human health is still substantial (Wilson et al., 2008). *C. coli* can be transmitted from pigs to humans through the consumption of contaminated pork. The consumption of raw minced meat was identified as specific risk factor for *C. coli* infection (Gillespie et al., 2002).

The colonisation and excretion dynamics as well as the translocation ability into deep tissues of *C. coli* in pigs have not been thoroughly evaluated yet. A reason for this could be the high prevalence of *C. coli* in pigs. Subsequently high efforts would have to be made using gnotobiotic or specific pathogen-free (SPF) animals. However, such data are a prerequisite for assessing the risk of human *C. coli* infections derived from pork.

The objectives of this study were to investigate the (i) colonisation dynamics and (ii) excretion pattern and (iii) to describe *C. coli* translocation into different tissues by using a *C. coli* colonisation trial in weaned piglets.

4.3 Material and methods

Bacterial strains and inoculum preparation

The *C. coli* 5981 strain used in the trial was originally isolated from pig faeces in 2007. *C. coli* 5981 belongs to the ST-828 clonal complex and is a typical representative of porcine *C. coli* based on MLST analysis (Sheppard et al., 2010). In preliminary *in vitro* experiments we found that its pathogenic potential is comparable to *C. coli* ATCC 33559, as in infected human intestinal HT-29/B6 cells the epithelial integrity was disturbed by the induction of apoptosis. The strain *C. coli* 5981 was chosen due to its two additional antimicrobial resistances against erythromycin and neomycin. Preliminary tests on antimicrobial resistance patterns based on 91 porcine *C. coli* strains revealed a very low distribution (3.3%) for this particular combination. Hence, this strain is largely distinguishable from the natural *C. coli* population present in the gastrointestinal (GI) tract by its specific antimicrobial resistance pattern.

C. coli 5981 was recovered from stocks kept at -80°C by plating cryobeads (Cryobank System, Mast Diagnostica, Reinfeld, Germany) on Mueller-Hinton agar with 5% sheep blood (MHB; OXOID, Wesel, Germany) for 48 h at 37°C under microaerobic conditions (6% O_2, 7% CO_2,

80% N_2, 7% H_2) generated by the Mart Anoxomat™ system (Drachten, Netherlands). Liquid cultures were obtained by inoculation of colonies in *Brucella* broth (BB) (BD, Heidelberg, Germany) and cultivation under the same conditions for 24 h.

For pig inoculation, colonies of *C. coli* 5981 were cultivated in BB and incubated for 16 h under microaerobic conditions. 0.5 ml of this overnight (o/n) culture with an optical density of approx. 0.3 at 600 nm were inoculated in 20 ml BB and cultivated for 4 h in order to obtain a solution of approx. $7x10^7$ CFU/ml. Cell numbers were determined by performing standard plate counts according to ISO 10272-2.

Animals and experimental design of the trial

All animals (n=10) were housed and treated in accordance with the regulation of the local authority (Landesamt für Gesundheit und Soziales, Berlin; approval No. G0349/09). German Landrace piglets were received from the Institute of Animal Nutrition (Freie Universität, Berlin, Germany). Piglets were weaned for 28 days and transferred to the experimental facility where they were allocated to pens based on litter origin, gender, and weight. Animals were kept pairwise. After one week of adaption all animals were inoculated with a single dosage of approx. $7x10^7$ CFU of *C. coli* 5981 by intragastric application using a stomach feeding tube (B. Braun, Melsungen, Germany) under azaperone (1.5 mg/kg; Stresnil, Janssen-Cilag, Germany) sedation. All piglets were weighed twice a week for 28 days. 28 days post inoculation (p. i.) the piglets were sacrificed for collecting gut contents and various tissue samples. Sedation was performed by intramuscular administration of azaperone (2 mg/kg, Stresnil) with a following induction of deep anaesthesia with ketamine (25 mg/kg, Ursotamin, Serumwerke Bernburg, Bernburg, Germany), and a final overdose achieved by intravenous application of pentobarbital (200 mg/kg, Narcoren, Merial, Hallbogmoos, Germany).

Sampling of faeces and post-mortem sample preparation

Faecal samples were collected in intervals over the whole experimental period in order to monitor *C. coli* excretion and the faecal consistency. Faecal consistency was assessed using a subjective score on a five-point scale ranging from 1 to 5 (1: watery diarrhoea to 5: hard dry stool), representing one major parameter of the health status in weaned piglets. Faecal samples from all piglets were collected directly from the rectum daily for the first week and subsequently twice a week until the end of the trial. Prior experimental inoculation with *C. coli* 5981, faecal samples from mother sows (14 and 28 days post-partum) and piglets were examined for *Campylobacter* presence and tested for absence of strains exhibiting antimicrobial resistances against both erythromycin and neomycin. The use of antimicrobials as supplements to the selective media enabled the differentiation of the inoculation strain from naturally colonised *C. coli* population present in all piglets. To study the colonisation ability of

C. coli in different gut sections along the intestine and their translocation towards selected organs, tissue of the small intestine and several organs were dissected at 28 days p. i.

After euthanasia, the abdominal cavity was opened along the linea alba. Jejunal mesenteric lymph nodes (MLNs), spleen, and gall bladder were dissected prior to the collection of gut tissues of jejunum, ileum, and caecum as well as gut contents of stomach, ileum, caecum and colon. Additionally, tonsils were dissected after ventral cut through the neck tissue. Sterile instruments for sample collection were used and exchanged between each step. All sample materials were placed into sterile plastic dishes and transported immediately to the laboratory where analysis was initiated. The time between sampling and processing was accomplished within a maximum of 4 h for all samples.

Enumeration of C. coli in faeces and gut contents

To determine the *C. coli* counts, semi-quantification was performed according to ISO 10272-3. Briefly, 1 g of faecal or gut content material was diluted 1:10 in Bolton broth with Bolton selective antibiotic supplement and 5% lysed horse blood (all OXOID) in stomacher bags (Meintrup, Lähden-Holte, Germany). Samples were homogenised in BagMixer 400 (Interscience, Saint Nom, France) for 2 min at maximum speed. Serial 10-fold dilutions of up to 10^{-8} of the initial homogenate were made in selective enrichment (Bolton broth) and incubated for 48 h at 37°C in a microaerobic atmosphere. For quantification, 10 µl of each enrichment dilution was streaked on modified charcoal cefoperazonedeoxycholate agar plates (mCCDA, OXOID) with and without the addition of 30 µg/ml erythromycin (Carl Roth, Karlsruhe, Germany) and 100 µg/ml neomycin (Carl Roth) in order to distinguish the *C. coli* 5981 strain from the natural *Campylobacter* population. Plates were incubated for 48 h under conditions mentioned above. From every dilution showing bacterial growth, DNA was extracted for species verification of *C. coli* and *C. jejuni* by multiplex PCR (Wang et al., 2002).

C. coli levels were expressed as CFU per gram sample material (detection limit 10 CFU/g). Based on this method the number of *C. coli* is expressed between two log levels and lower values were used for analysis.

Enumeration of C. coli in tissues

The aseptically removed tissue samples were rinsed three times with distilled water. Before homogenisation, samples were cut aseptically into small pieces using sterile forceps and scissors. Homogenisation and isolation of bacteria was performed by pressing the tissue with a sterile syringe piston through a 70 µm sterile cell strainer (VWR, Darmstadt, Germany), and flushed with Bolton broth to create a 10-fold dilution. Cell suspensions were collected in 50 ml

tubes. Bacterial enrichment and isolation was carried out as mentioned above. Data were analysed using the median value.

DNA extraction and species identification by multiplex PCR

For isolation of DNA, bacterial colonies were scraped from plates and washed in 0.1x TE buffer (10 mMTris/HCl, pH 8.0, 1 mM EDTA). Pellets were resuspended in 5% Chelex Resin 100 (Bio-Rad, Munich, Germany). One hour incubation at 56°C was followed by 15 min at 95°C and 2 µl of the supernatants were used for multiplex PCR. Primers and PCR protocol for *C. coli* and *C. jejuni* verification are described by Wang et al. (2002).

Localisation of C. coli in small intestinal epithelium

For visualisation of *C. coli* distribution in intestinal epithelium, tissue from ileum and jejunum was immediately fixed post-mortem with 4% formaldehyde for 3 h without rinsing the luminal content in order to fix the bacteria's localisation. The tissue was stained as whole tissue mount without cutting. The fixed tissue was permeabilised with 1% TritonX-100 in PBS for 2 h at 37°C, and subsequently incubated in blocking solution (10% goat serum, 1% bovine serum albumin, 0.8% TritonX-100 in PBS) for 3 h at room temperature (RT). For detection, monoclonal mouse-anti-*Campylobacter* IgG antibody was used (1:200, Santa Cruz Biotechnology, Santa Cruz, CA, USA, Cat. no. sc-58101). Incubation with the primary antibody in blocking solution was performed o/n at 4°C. The tissue was washed four times with a prolonged washing time for 60 min each, then incubated o/n at 4°C with a secondary IgG antibody (1:500 goat-anti-mouse AlexaFluor^{488}, Invitrogen, Carlsbad, CA, USA, Cat. no. A11029) and washed again four times with blocking solution. Staining of nuclei with DAPI (4'-6-diamidino-2-phenylindole dihydrochloride) and cytoskeletal F-actin with Phalloidin-AlexaFluor^{594} (both Sigma, St. Louis, MO, USA) was performed at RT for 20 min (each 1:1000). Stainings were analysed by confocal laser-scanning microscopy (LSM, Zeiss LSM510, Jena, Germany) with Carl Zeiss LSM Image Examiner software. The primary antibody was tested prior to the study in *Campylobacter* sp.-infected cell culture and was found to sensitively detect *C. coli* and *C. jejuni*, but no other *Campylobacter* species like e.g. *C. lari*. To exclude false positive binding of the antibody to *C. jejuni* that showed sporadic occurrence, we only used samples that were tested negative for *C. jejuni*. Controls were performed with *Campylobacter*-free intestinal epithelial cells from human and porcine source (HT-29/B6 and IPEC-J2) and by adding *C. coli* and other *Campylobacter* species to the epithelia *in vitro* with subsequent LSM detection as positive control.

Genotyping of C. coli isolates by fluorescence amplified fragment length polymorphism (fAFLP) analysis

Genotyping by fAFLP analysis has a high discriminatory power to even differentiate strains of the same species (Lindstedt et al., 2000). To characterise the genetic relationships of *C. coli* isolates originating from faeces, gut contents and various tissue samples of different animals, this method was applied. Therefore, four to seven single bacterial colonies from each sample were picked and sub-cultured in BB for 24 h at 37°C under microaerobic conditions. DNA was extracted and multiplex PCR was performed for species verification as described above. Genotyping by fAFLP was carried out on a total of 47 *C. coli* isolates at which strains with identical banding patterns were excluded. The protocol and primers used are described by Duim et al. (2001). PCR fragments were separated by capillary electrophoresis (LGC Genomics, Berlin, Germany). For data analysis the band patterns ranging from 35–500 base pairs were investigated using BioNumerics version 6.1 (Applied Maths, Sint-Martens-Latem, Belgium). Patterns were normalised to the molecular mass of the internal GeneScan-500 ROX-labelled size standard (Life Technologies, Darmstadt, Germany) that was included in each sample. After normalisation, the similarities between profiles, based on peak position, were calculated using Pearson correlation coefficient with 1% curve fitting and 1% optimisation. For cluster analysis of fAFLP banding patterns, the Unweighted Pair Group Method with Arithmetic Mean (UPGMA) was used with a cluster cut-off of 90%.

4.4 Results

Clinical examination and growth performance of pigs

All animals remained in very good health conditions throughout the study. No major changes in the faecal score could be recorded. The overall faecal consistency of score 4 (well-formed and solid) remained constant for 17 days p. i., then it slightly decreased to 3.5 for four days. After that, it reached the initial score again. Piglets showed normal growth performance, achieving a body weight of 22.8±2.2 kg at the age of 63 days (28 days p. i.).

Enumeration of C. coli in faeces and excretion kinetics

In mother sows, *C. coli* could be detected for both sampling days (14 and 28 days ante partum) with levels between 10^2 and 10^7 CFU/g faeces. No growth was observed on media containing additional erythromycin and neomycin (data not shown).

Results for faecal excretion of *C. coli* 5981 counts and total *C. coli* (incl. *C. coli* 5981) counts for all piglets over a period of four weeks are shown in Fig. 4-1. Nine out of ten piglets shed

C. coli already prior to experimental infection at a mean value of approx. 10^4 CFU/g faeces but there was no detectable *C. coli* growth on erythromycin and neomycin containing media. Two animals started shedding *C. coli* 5981 one day after infection with faecal levels of 10^1 and 10^3 CFU/g, respectively. Seven days p. i. all animals excreted the inoculation strain. Highest counts were determined ten days p. i. showing a mean level of 10^6 CFU/g. Total *C. coli* (incl. *C. coli* 5981) levels ranged from 10^4 to 10^7 CFU/g faeces throughout the experiment. Hence, the *C. coli* 5981 strain was able to colonise in the natural microbiota of piglets at mean counts of $1.9x10^5$ CFU/g compared to $2.2x10^6$ CFU/g for total *Campylobacter* (Fig. 4-1).

Furthermore, very rare shedding of *C. jejuni* was seen. Only two piglets excreted *C. jejuni* of which one shed 10^3 to 10^4 CFU/g at two consecutive sampling points, whereas the second piglet excreted *C. jejuni* once with 10^3 CFU/g. However, total *C. coli* were always in the majority with up to 2 log levels higher than *C. jejuni* counts in these samples (data not shown).

Enumeration of C. coli in tissues and gut contents

High concentrations of *C. coli* were detectable in all gut contents. Lower numbers were found in tissue samples of the small intestine (jejunum, ileum and caecum). Even less *C. coli* cells were sporadically observed in gall bladder, spleen and gut-associated lymphoid tissue (GALT), i. e. jejunal MLNs, and tonsils (Fig. 4-2A and 4-2B). Highest counts of the total *C. coli* load (incl. *C. coli* 5981) were determined in the content of colon ascendens with 10^5 CFU/g, and caecum with $5.5x10^4$ CFU/g. Regarding the *C. coli* 5981 strain highest counts were observed in the caecum content with $5.5x10^4$ CFU/g and in the colon content with 10^4 CFU/g. Total *C. coli* load (incl. *C. coli* 5981) in ileum content amounted to 10^4 CFU/g, whereas cell numbers for the *C. coli* strain 5981 were two log levels lower. In the stomach content of four animals *C. coli* were detectable, three of which were positive for the inoculation strain *C. coli* 5981 (Fig. 4-2C and 4-2D). Enumeration of *C. coli* in gut tissues from jejunum, ileum, and caecum revealed lower cell counts compared to the corresponding gut contents (reduced by 1 to 3 log levels). Cell numbers included mucosa-associated as well as internalised bacteria. Low numbers of *C. coli* were present in the tonsils of two animals, whereas the inoculation strain *C. coli* 5981 was isolated from the tonsil of one animal only. *C. coli* determination in jejunal MLNs revealed the presence of *C. coli* in seven piglets, while four of these animals carried *C. coli* 5981 in jejunal MLNs at low levels. *C. coli* could also be detected in the spleen of three animals, two of which were colonised with *C. coli* 5981 at low concentrations. No *C. coli* was detectable in the bile. In one animal *C. coli* was detected in the gall bladder tissue (Fig. 4-2A and 4-2B).

Localisation of C. coli in small intestinal epithelium

To investigate the spatial distribution of *C. coli* in the intestinal mucosa, tissues were immediately fixed after killing and stained for immunofluorescence microscopy. *C. coli*-positive

signals were detected in all investigated samples. In ileum as well as jejunum, *C. coli* was mostly detected in the lumen but also attached to the epithelium at low numbers and single bacteria were found intraepithelially (Fig. 4-3). Still, no signs of epithelial damage were observed.

Genotyping of C. coli isolates by fAFLP analysis

Genetic fingerprinting was performed using fAFLP analysis on 47 *C. coli* isolates from various sample materials and animals. Isolates with >90% similarity were regarded as belonging to the same cluster. By this method, *C. coli* isolates formed multiple clusters and identical genotypes were detectable in different tissues (Fig. 4-S1). All isolates recovered from media containing erythromycin and neomycin are grouped in one cluster with the inoculation strain *C. coli* 5981. Banding patterns of *C. coli* 5981 isolates varied considerably, suggesting genomic rearrangements of the inoculation strain during passage.

4.5 Discussion

This study demonstrates the ability of the porcine strain *C. coli* 5981 to colonise in the presence of a complex gut microbiota including other *Campylobacter* strains in weaned piglets. This particular strain was tracked along the GI tract and translocation of *C. coli* towards extra-gastrointestinal sites was detected. Nine out of ten piglets shed *C. coli* prior to inoculation at a mean value of 10^4 CFU/g faeces but no growth was detectable in media containing erythromycin and neomycin, suggesting the absence of phenotypes resistant against these antimicrobials in the *Campylobacter* population. The piglet which was *C. coli*-negative before inoculation was nevertheless colonised with *C. coli* as was detected at the next sampling day. This finding might be explained by an intermittent faecal excretion of *C. coli* in pigs which has been reported previously. The heterogeneous distribution in the gut content results from the accumulation of *Campylobacter* at the bottom of the crypts in the intestinal mucosa (Leblanc-Maridor et al., 2008; Weijtens et al., 1999).

Ten 35 day old piglets were intragastrically inoculated with approx. $7x10^7$ CFU of strain *C. coli* 5981. Colonisation with this inoculation strain was observed in all piglets and resulted in faecal excretion over the whole trial period, even though intermittent shedding was occasionally observed. Faecal shedding of *C. coli* 5981 was detectable 24 h after inoculation in two piglets and seven days p. i. in all piglets. Highest *C. coli* 5981 counts in faeces were seen ten days p. i. with 10^6 CFU/g. Faecal excretion rates of the total *C. coli* population (incl. *C. coli* 5981) ranged from 10^4 to 10^7 CFU/g. These data correlate with another study using organic piglets in which *C. coli* excretion varied between 10^3 and 10^7 CFU/g faeces (Jensen et

al., 2006). Results from SPF piglets experimentally infected with a porcine *C. coli* strain and a comparable infection dosage ($5x10^7$ CFU/animal) showed similar shedding rates of 10^3 to 10^6 CFU/g faeces (Leblanc-Maridor et al., 2008). The faecal shedding rate of the inoculation strain *C. coli* 5981 remained below the shedding level of naturally colonised *C. coli* on most sampling points. Equal levels were determined at four sampling days in some animals in the middle of the trial but *C. coli* 5981 never exceeded the level of naturally colonised *C. coli*. From day 14 on, naturally colonised *C. coli* exceeded the cell number of the inoculation strain again. These observations suggest that the inoculation strain *C. coli* 5981 integrated well in the natural *C. coli* population without replacing it. Presumably there were no major competitive effects between *C. coli* 5981 and the naturally colonising *C. coli* strains.

However, one concern in using weaned piglets in contrast to gnotobiotic and to a lesser extent also SPF animals is their naturally established gut microbiota with its diversity of individual bacterial compositions. Therefore, studying the infection process of one strain in weaners must always consider the influence and dynamics of multiple genera and species.

Multiple genotypes were determined within the natural *C. coli* population in piglets by fAFLP. This concurs with other studies which have previously reported the existence of multiple *C. coli* genotypes within a pig herd or even within one pig (Soultos and Madden, 2007; Thakur and Gebreyes, 2005). All *C. coli* isolates recovered from media with antimicrobial supplementation were grouped in the same cluster as the inoculation strain, indicating an efficient separation of *C. coli* 5981 from the natural *C. coli* population. The variation in banding pattern within the *C. coli* 5981 cluster might be due to the genetic instability of porcine *C. coli* strains after *in vivo* passage (Leblanc-Maridor et al., 2011). Intra- and intergenomic recombinations, genomic rearrangements and chromosomal point mutations during passage might have led to genetic changes of the inoculation strain detectable by differences in fAFLP patterns. These genetic variations might represent adaption processes to environmental stress within the host (Ambur et al., 2009).

Another interesting finding is the detection of *C. jejuni* in the faeces of two piglets in the first half of the trial. Overall, *C. jejuni* just makes up a small part compared to the total *C. coli* population. These results are in agreement with other studies in which *C. jejuni* is found in lower numbers and reduced frequencies in pig faeces (Jensen et al., 2005). *C. jejuni* seems to co-exist with *C. coli* in pigs but presumably has a reduced colonisation potential for this host (Jensen et al., 2005 and Leblanc-Maridor et al., 2008).

Post-mortem sampling of GI content from different gut sections, such as stomach, ileum, caecum, and colon revealed increasing *C. coli* levels towards the colon. Thus, *C. coli* were more numerous in the distal than in the proximal intestinal segments suggesting a preferred

proliferation in the lower GI tract. These data are in agreement with the observation of Nesbakken et al. (2003), according to which *Campylobacter* is found most frequently in the lower intestinal tract, which generally applies to most enterobacteria (Shen, 2009). Reinfection of *Campylobacter* can be assumed due to *C. coli* detection in stomach contents, probably via the faecal-oral route.

High counts for adhesive and invasive *C. coli* were detectable in gut tissues of the small intestine (jejunum, ileum, and caecum) for naturally colonised *C. coli* and in lower quantities for *C. coli* 5981. A spatial distribution of *C. coli* in intestinal tissues could also be demonstrated by LSM. By this technique, we were able to show *C. coli* invading into the epithelium of the small intestine. That phenomenon (attachment and invasion of porcine intestinal epithelial cells) has already been described for different *Campylobacter* spp. (Murphy et al., 2011; Pogacar et al., 2010). These authors suggest that these bacteria can translocate across the intestinal epithelium either via a transcellular route and/or via a paracellular route, allowing *Campylobacter* to disseminate throughout the host. The role of M-cells in epithelial uptake of *Campylobacter* is being discussed.

Translocation of intestinal *Campylobacter* was observed by qualitative and quantitative detection of *C. coli* (incl. *C. coli* 5981) in extra-gastrointestinal sites, such as jejunal MLNs, tonsils, spleen, and gall bladder. However, low *C. coli* counts were detectable in these extra-intestinal tissues only in single animals. Little information exists about the translocation of *C. coli* from the GI tract to extra-gastrointestinal sites in pigs. Nesbakken et al. (2003) detected *C. coli* in 29.2% of jejunal MLNs in piglets at slaughter, but no enumeration was performed. In our trial, translocation of *C. coli* to jejunal MLNs occurred in seven out of ten piglets, resulting in low colonisation rates of approx. 10^1 CFU/g. Similar results were obtained for spleen samples: *C. coli* was detectable in the spleen tissue of three piglets. Comparable results were shown in a recent study by Alexandrina and Botus (2008). The authors observed that 3.2% of porcine spleens were contaminated with *Campylobacter* at the time of slaughter. Colonisation of *C. coli* in gall bladder tissue occurred in one animal at a level of 10^3 CFU/g. These findings are comparable to older data from Gorgen et al. (1983) who detected *Campylobacter* in gall bladder tissue at a rate of 13% in sick and 14% and healthy pigs. Previous studies have addressed the prevalence of *C. coli* in tonsils with contradictory results. High contamination rates (66.7%) were reported by Nesbakken et al. (2003) whereas Fredriksson-Ahomaa et al. (2009) rarely found (6%) *Campylobacter* in tonsils from fattening pigs at slaughter. In our study, the presence of *C. coli* in tonsils was only detectable in two out of ten animals at low concentrations.

4.6 Conclusion

The inoculated *C. coli* 5981strain integrated well into the natural *C. coli* population of weaned piglets. The inoculation strain could be differentiated from naturally colonising *C. coli* by its two additional antibiotic resistances. We demonstrated the invasion into intestinal epithelial cells and translocation to extra-gastrointestinal sites for naturally colonising *C. coli* and to a lesser extent for the inoculation strain *C. coli* 5981. Only low numbers of *C. coli* were detectable in extra-gastrointestinal tissue.

This pig colonisation trial using the inoculation strain *C. coli* 5981 is suitable for studying colonisation experiments without the need for gnotobiotic or SPF animals.

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Conflict of interest

The authors are solely responsible for the data and do not represent any opinion of neither the DFG nor other public or commercial entity.

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4.9 List of figures

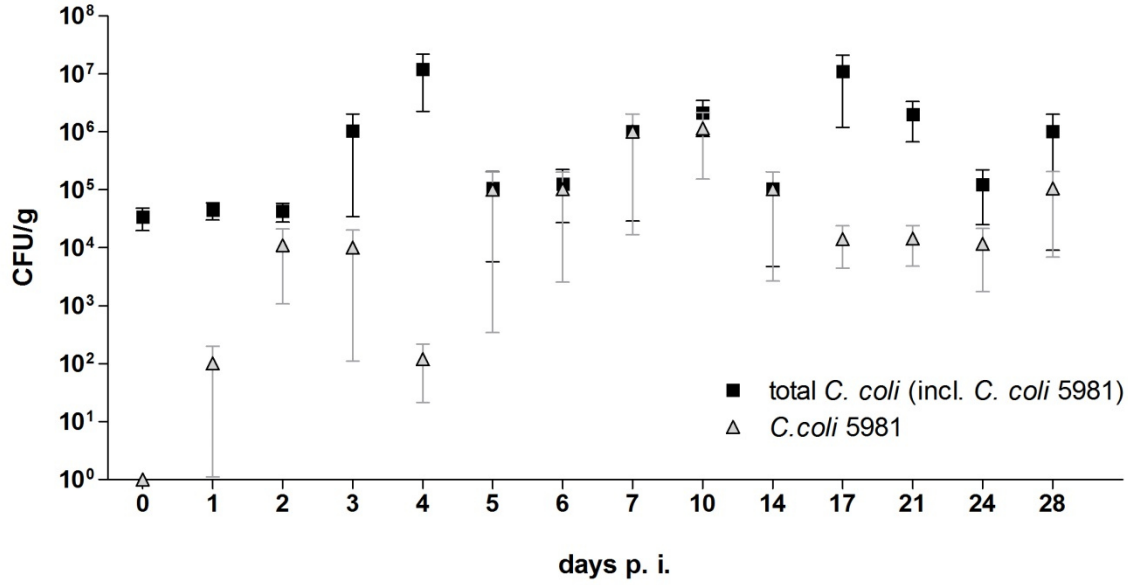

Figure 4-1: Course of faecal excretion of total *C. coli* (incl. *C. coli* 5981) and *C. coli* 5981 alone following oral inoculation of weaned piglets over 28 days

Results are expressed as CFU per gram faeces determined on selective media with and without the addition of antimicrobials for *C. coli* 5981 (triangles) and total *C. coli* (incl. *C. coli* 5981, squares) detection, respectively for 28 day post inoculation (p. i.). Error bars indicate standard errors of the means (n=10).

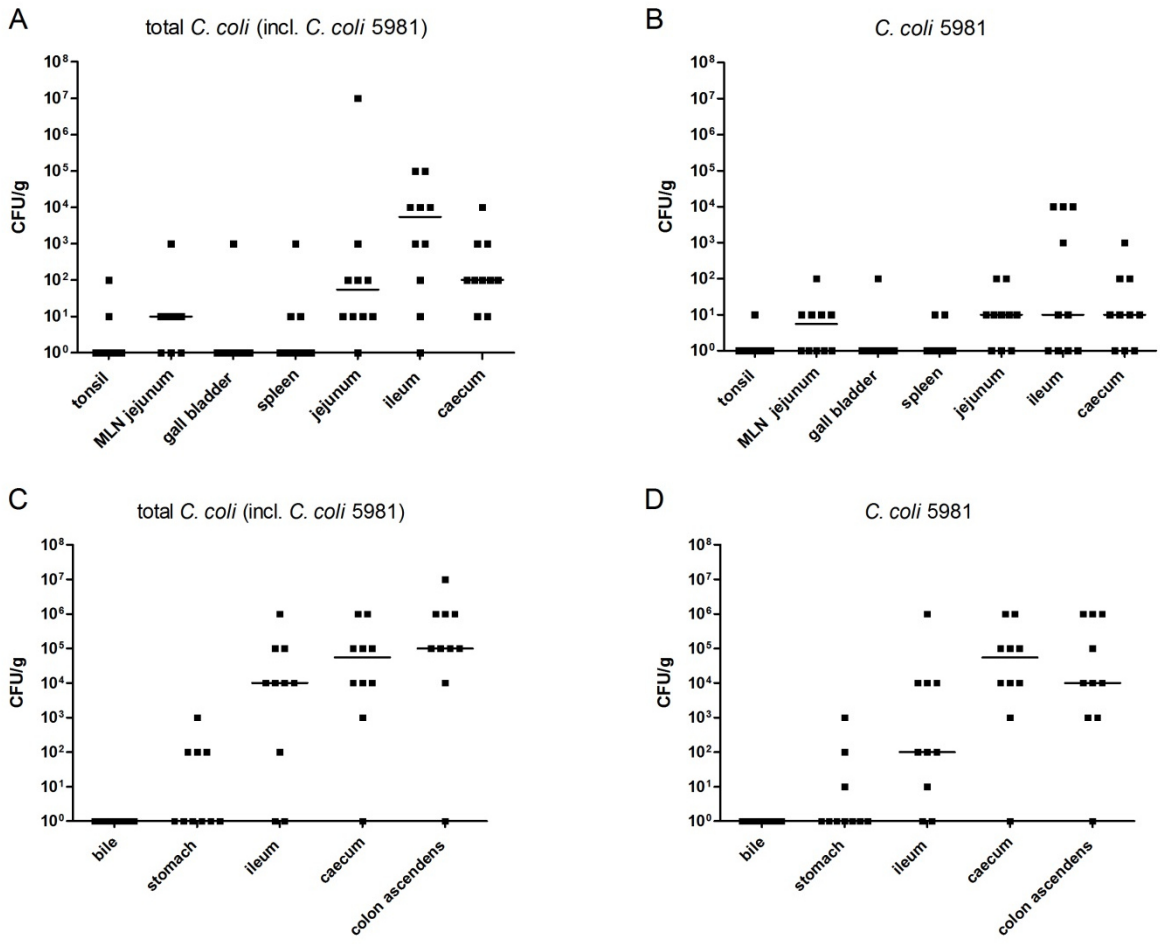

Figure 4-2: Post-mortem detection of total *C. coli* (incl. *C. coli* 5981) and *C. coli* 5981 alone in gut contents and tissue samples of weaned piglets

Results are expressed as CFU per gram sample material determined on selective media with and without the addition of antimicrobials for the inoculation strain *C. coli* 5981 and total *C. coli* (incl. *C. coli* 5981) detection, respectively. (A) Number of total *C. coli* (incl. *C. coli* 5981) in tissue samples; (B) number of *C. coli* 5981 in tissue samples; (C) number of total *C. coli* (incl. *C. coli* 5981) in gut contents and (D) number of *C. coli* 5981 in gut contents.

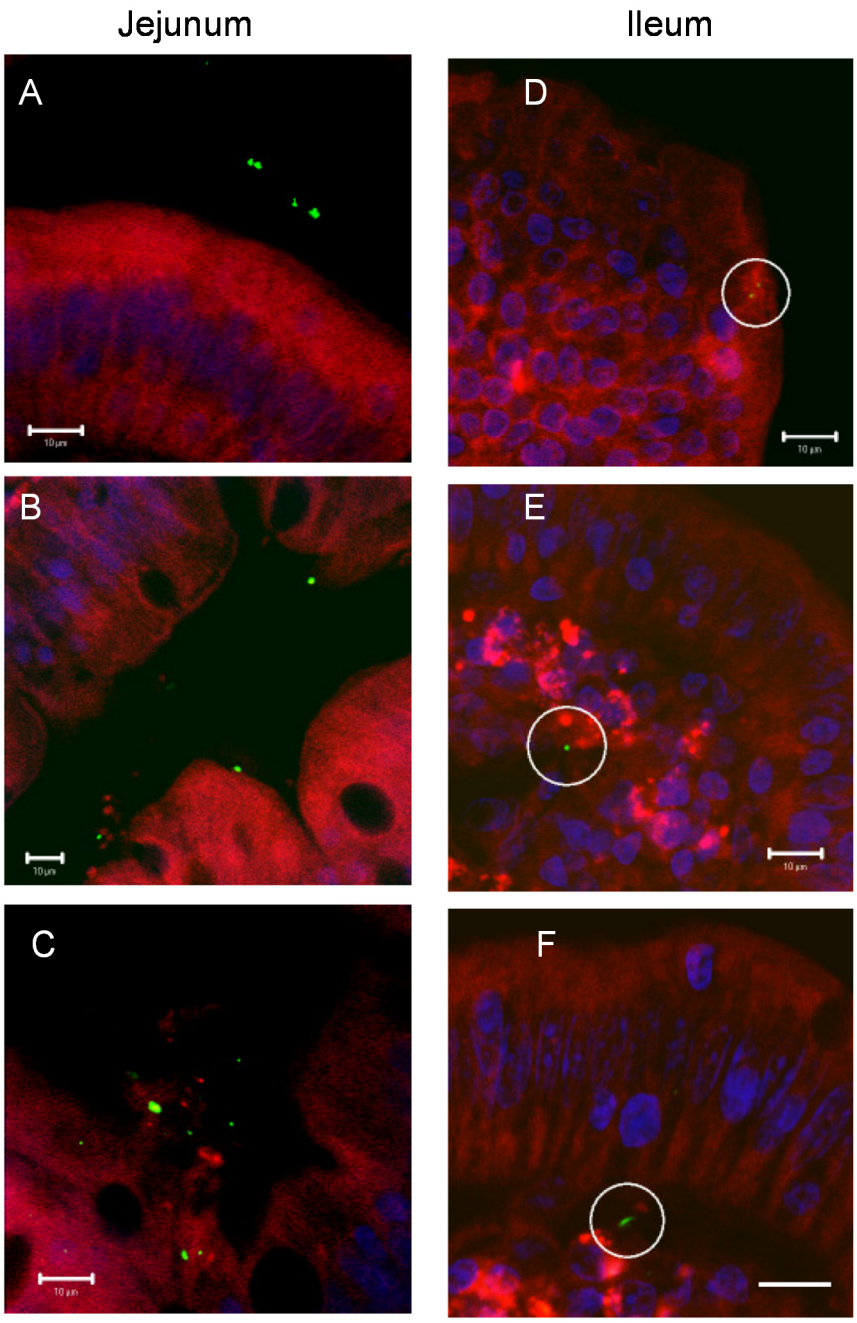

Figure 4-3: Localisation of *C. coli/C. jejuni* in small intestinal epithelium

Confocal laser-scanning microscopy revealed *C. coli/C. jejuni* to be present in luminal mucus of jejunum (A) and attached to the epithelium (B) (here as top view on neighbouring villi); as well as invaded into the epithelium (C) (villus and crypt). In ileum samples *C. coli/C. jejuni* is shown to be attached to and invaded into the epithelium (D) as well as being subepithelial localised (E and F). Immunostaining with green signal for *C. coli/C. jejuni*, nuclei are stained blue with DAPI and cytoskeletal F-actin is marked red with phalloidin. White circles indicate single *C. coli/C. jejuni*. Scale bars: 10µm.

4.10 Supporting information figures

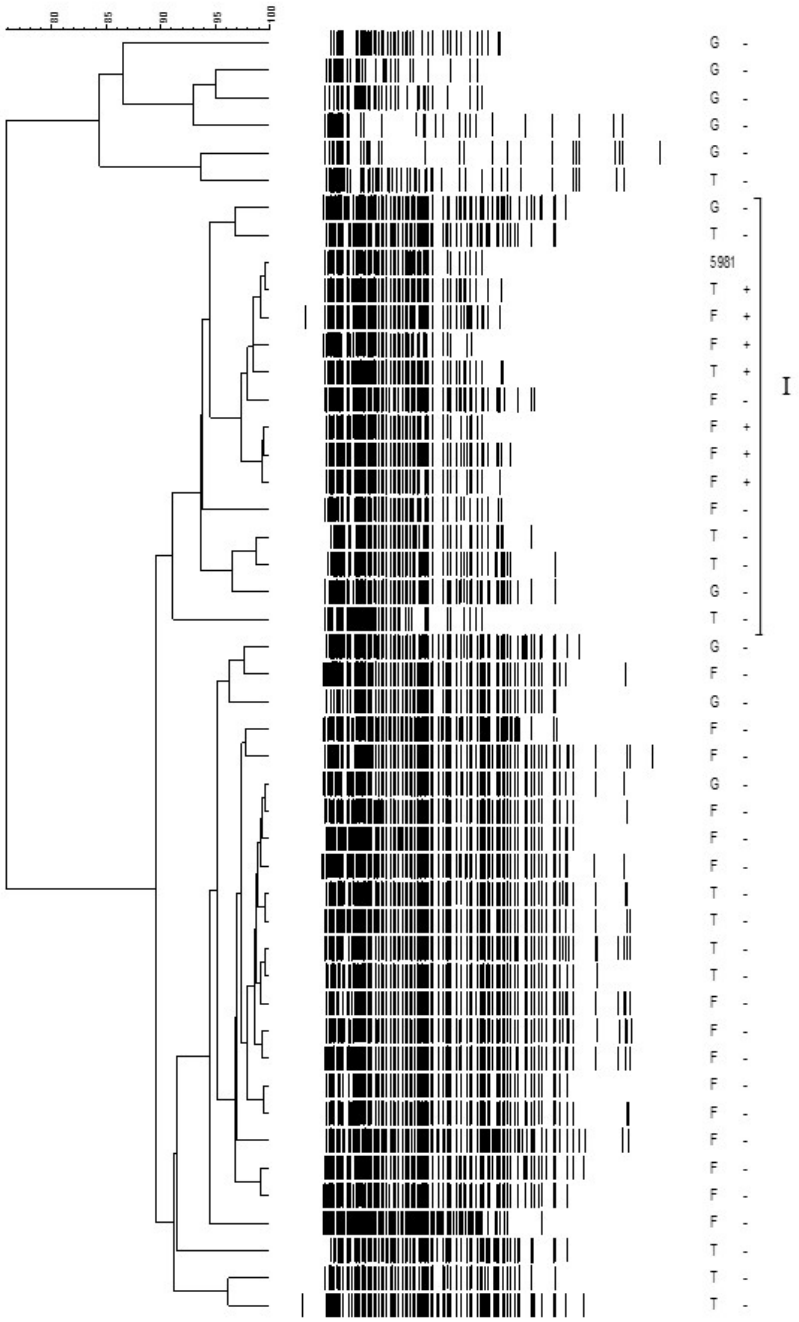

Figure 4-4: Dendrogram of selected fAFLP fingerprints of C. coli isolates

Isolates sharing more than >90% similarity are grouped in one cluster; Origin of *C. coli* isolates: F-faeces, G-gut content, T-tissue samples; - *C. coli* isolates derived from media without supplementation of erythromycin and neomycin; + *C. coli* isolates derived from media with supplementation of erythromycin and neomycin; I- *C. coli* 5981 cluster.

Chapter 5: Inhibitory effect of high-dosage zinc oxide dietary supplementation on *Campylobacter coli* excretion in weaned piglets

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5.1 Abstract

Aims: This study investigated the impact of zinc oxide (ZnO) on *Campylobacter* (*C.*) *coli* by *in vivo* and *in vitro* assays.

Methods and Results: By *in vitro* growth inhibition assays a high susceptibility of *C. coli* against ZnO could be observed. At concentrations ≥ 2.6 mM ZnO a decline in cell numbers occurred. Quantitative real-time-PCR assays demonstrated an upregulation of the main oxidative stress gene (*katA*) in response to ZnO treatment. The expression level of *katA* was increased by 5-fold after ZnO treatment. An experiment was carried out in pigs to elucidate the impact of ZnO as feed supplement on *C. coli* faecal excretion. Feeding a high-dosage ZnO concentration (3,100 mg kg^{-1}) to piglets significantly reduced the faecal excretion of *C. coli* by up to 1 log CFU g^{-1} as compared to animals receiving a low (40 mg kg^{-1}) or medium (100 mg kg^{-1}) ZnO diet.

Conclusion: *In vitro* assays showed a high susceptibility of *C. coli* against ZnO. Adding high levels of ZnO to the diet of weaned piglets reduced *C. coli* excretion significantly. There is evidence for the induction of an oxidative stress response by ZnO supplementation in *C. coli*.

Significance and Impact of Study: Supplementation of a high-dosage ZnO diet to piglets can reduce the *C. coli* load, potentially leading to a lower contamination risk of meat during slaughter.

Keywords: *C. coli*, zinc oxide, feed supplementation, excretion, pigs

5.2 Introduction

Worldwide, *Campylobacter* (*C.*) is one of the most common foodborne pathogens causing bacterial enteric infections in humans. After *C. jejuni*, *C. coli* is the second most important *Campylobacter* species for human infections. Pigs are an important reservoir for *C. coli*, with prevalences between 50 and 100 % and excretion levels ranging from 2-7 log CFU g^{-1} faeces (Alter et al. 2005; Jensen et al. 2005). Faecal contamination of meat during processing is considered to be the main route of transmission. Consumption of contaminated raw minced meat was identified as a specific risk factor for *C. coli* infection (Gillespie et al. 2002). Thus, the approach is to lower the *Campylobacter* load in slaughter animals and to subsequently reduce the faecal contamination of meat during slaughter (Rosenquist et al. 2003).

Growing pigs (up to 20 kg body weight) require approximately 100 mg Zn kg^{-1} dry matter (DM) of feed, and later approximately 80 mg Zn kg^{-1} DM. In the EU, zinc as feed supplement is usually applied as zinc oxide (ZnO). The EU permits the feeding of 150 mg Zn kg^{-1} over the

entire diet (Anonymous 2003). However, high concentrations ≥ 2,000 mg kg^{-1} of zinc (supplemented as ZnO) have been proven to promote growth by reducing post-weaning diarrhoea in commercial pig farming (Pettigrew 2006). Still, the mode of action of ZnO is not fully understood. It is suggested that ZnO provides benefit through impacts on bacterial populations in the digestive tract. The reduced incidence of diarrhoea is explained by restricting the proliferation of pathogenic bacteria in the gut (Clayton et al. 2011). Earlier studies have reported opposing results in porcine microbiota composition in pig digesta when fed with high doses of zinc. For example, Højberg et al. (2005) examined a decrease in lactic acid bacteria and lactobacilli whereas coliforms and enterococci were more numerous in newly weaned piglets. In contrast, Pieper et al. (2012) observed that lactic acid bacteria were not influenced by high ZnO levels. Janczyk et al. (2013) reported increased shedding of *Salmonellae* when high ZnO was fed and Liedtke and Vahjen (2012) showed that zinc resistance of commensal intestinal bacteria cannot be grouped according to their taxonomic origin and therefore, the antibacterial activity of ZnO in the intestine of farm animals cannot be generalized.

The purpose of this study was (i) to evaluate the inhibitory effect of ZnO on *C. coli* growth *in vitro,* (ii) to investigate the mechanisms of ZnO action on *C. coli* and (iii) to describe the impact of ZnO feed supplementation on *C. coli* excretion in weaned piglets in an animal trial.

5.3 Material and methods

Bacterial strains and growth conditions

C. coli 5981, originally isolated from pig faeces in 2007, was used for *in vitro* studies. *C. coli* 5981 belongs to the ST-828 clonal complex and is a typical representative of porcine *C. coli,* based on MLST analysis (Sheppard et al. 2010), and encodes for the virulence factors *cdt*ABC, *cad*F and *cia*B. *C. coli* 5981 was taken from stock culture (-80 °C) and grown on Mueller-Hinton agar with 5 % sheep blood (MHB; OXOID, Wesel, Germany) for 48 h at 37 °C in microaerobic conditions generated by the Mart Anoxomat system (Drachten, The Netherlands). Cells were inoculated in *Brucella* broth (BB; BD, Heidelberg, Germany) and incubated for 24 h under the same conditions.

For growth inhibition tests and for gene expression analysis, 50 µL of this broth was inoculated into 50 mL fresh BB and incubated for 16 h at 37 °C microaerobically in order to obtain cells at the late exponential phase. To determine the cell number before and after ZnO stress, dilutions of culture aliquots were plated on MHB agar and incubated for 48 h at the same conditions. Cell counts were expressed as $\log_{10}$ colony forming units (CFU) mL^{-1}.

C. coli growth inhibition by zinc oxide in vitro

A 1.3 M stock solution from analytical ZnO powder (Carl Roth, Karlsruhe, Germany) was prepared and solubility was achieved by adjusting the pH with concentrated HCl to pH 2. For the growth inhibition tests of ZnO on *C. coli* 5981, various ZnO concentrations were added to late exponential phase cultures of approx. 7 log CFU mL^{-1} (BB contains 0.52 µg zinc mL^{-1} and was therefore neglected regarding the total zinc concentration in this assay). ZnO was added to the cultures with a final concentration of 0 mM (control), 1.3 mM (100 µg mL^{-1}), 2.6 mM (200 µg mL^{-1}), 3.9 mM (300 µg mL^{-1}) and 6.5 mM (500 µg mL^{-1}). *C. coli* was incubated as mentioned above and cell count was monitored in intervals over 24 h.

Gene expression analysis of C. coli 5981 after zinc oxide exposure

The expression of selected oxidative and general stress response genes of *C. coli* was analysed. The expression of these genes was analysed after 30 min of stress induced by i) ZnO (1.3 mM), ii) paraquat (0.5 mM) (Th. Geyer, Berlin, Germany) or iii) heat (46 °C) to 9 log CFU mL^{-1} of *C. coli* 5981 in the late exponential phase (grown as mentioned above). Paraquat was used as positive control as it is known to induce oxidative stress conditions in *C. jejuni* by generating the formation of superoxide, a reactive oxygen species (ROS) (Garenaux et al. 2008; Hwang et al. 2011), while heat stress induces a different set of stress response genes (Stintzi 2003).

RNA preparation and quantitative real-time PCR analysis

Total bacterial RNA was extracted from *C. coli* 5981 suspensions containing approx. 9 log CFU ml^{-1} using the peqGOLD Bacteria RNA Kit (Peqlab, Erlangen, Germany). A DNase treatment was performed in a total volume of 40 µL containing 4 U DNase I, 40 U Ribolock, 1 x DNase buffer (all Fermentas, Leon-Rot, Germany) and 28 µL of the extracted RNA and incubated for 15 min at 37 °C. DNase was inactivated by adding 4 µL 50 mM EDTA and heating at 65 °C for 10 min. For cDNA synthesis the RevertAid Premium First Strand cDNA synthesis kit (Fermentas) was used. Reverse transcription of 1 µg total RNA was performed using random hexamer primers (Fermentas) according to manufacturer's instructions.

For gene expression analysis a set of genes involved in oxidative and general stress response was selected. Primers were designed using the Primer3 software (http://frodo.wi.mit.edu/) based on the *C. coli* RM2228 genome sequence. Primers are listed in table 5-1. Each 15 µL RT-qPCR mixture contained 2-fold SsoFast EvaGreen Supermix (Bio-Rad, Munich, Germany), specific primers (primer details are listed in Tab. 1) and 1 µL of a 1:10 dilution of cDNA as template. The amplification program started with an initial denaturation step at 95 °C for 30 s, followed by 39 cycles of 95 °C for 2 s and 46 °C for 2 s (CFX96 Real time system, Bio-Rad).

Specificity was tested by melting curve analysis *(*see Table 1 for Tm of PCR products). Gene expression was analysed with the $2^{-\Delta\Delta CT}$ method described by Livak and Schmittgen (2001) using thiC expression for normalisation. All samples, including no-RT and no-template controls were analysed in biological triplicates.

Animal trials

The animal trial was approved by the local authority (Landesamt für Gesundheit und Soziales, Berlin, Germany; approval No. G0349/09). German Landrace piglets (N = 30) were received from the Leibniz Institute for Farm Animal Biology (Dummerstorf, Germany). Prior to the study, faecal samples of mother sows were tested for *C. coli* and only piglets from *C. coli* positive mother sows were included in this study (data not shown). Piglets (naturally infected with *C. coli*) were weaned till day 28 ± 1 (possessing an initial body weight of 8.5 ± 0.9 kg). Piglets were transferred to the experimental facility where they were allocated to three treatment groups (each group, n = 10), based on litter origin, gender and weight. Animals were housed in pens, pair wise. One pen was considered an experimental unit. Feed was mixed with some water and was offered twice daily for an hour as *semi ad libitum*, to avoid refusals. Water was provided *ad libitum* via nipple drinkers. Ambient temperature was kept at 25±1°C for the first 4 weeks, and then reduced to 22±1°C, with humidity 30-55% and light regime 12 hours light, 12 hours darkness.

Pigs were fed a diet based on wheat, barley and soybean supplemented with different ZnO doses. One group received 40 mg of ZnO kg^{-1} feed, covering daily zinc requirements (low ZnO group), the second group received 100 mg ZnO kg^{-1} feed (reaching the permitted zinc concentration in the EU being 150 mg Zn kg^{-1} feed; medium ZnO group) and the third group was supplemented with 3,100 mg ZnO kg^{-1} feed (high ZnO group). The different zinc diets were fed until day 28 of the animal trial (first trial period, day 0-28). To investigate the lasting effect of the high ZnO supplementation on *C. coli* excretion, the zinc concentration of the high ZnO group was reduced to that of the medium ZnO group for another two weeks (second trial period, day 29-42). ZnO supplementation in the low and medium ZnO groups was continued according to the conditions of the first trial period. For the second trial period only five animals per group were investigated.

Sampling of faeces

Faecal samples were collected directly from the rectum. These samples were collected in intervals over the whole study period in order to monitor quantitative *C. coli* excretion and the faecal consistency. Faecal consistency was monitored daily using a subjective scoring system ranging from 1, watery diarrhoea to 5, hard dry stool (Bratz et al. 2013).

All faecal samples were placed into sterile plastic dishes and transported immediately to the laboratory where analysis was initiated. The time between sampling and processing was kept at a maximum of 4 h for all samples.

Enumeration of C. coli in faeces

To determine *C. coli* counts, semi-quantification was performed according to ISO 10272-3. Briefly, 1.5 g of faecal material was diluted in 12 mL (dilution 1:8) Bolton broth with Bolton selective antibiotic supplement and *5* % lysed horse blood (all OXOID) in stomacher bags (Meintrup, Lähden-Holte, Germany). Samples were homogenised in BagMixer 400 (Interscience, Saint Nom, France) for 2 min at maximum speed. Serial 10-fold dilutions of up to 10^{-8} of the initial homogenate were made in selective enrichment (Bolton broth) and incubated for 48 h at 37 °C in a microaerobic atmosphere. For quantification, 10 µL of each enrichment dilution was streaked on modified charcoal-cefoperazone-deoxycholate agar (mCCDA, OXOID). Plates were incubated for 48 h under conditions mentioned above. From every dilution showing bacterial growth, DNA was extracted for species verification of *C. coli* and *C. jejuni* by multiplex PCR (Wang et al. 2002). *C. coli* levels were expressed as $\log_{10}$ CFU per gram sample material (detection limit 1 CFU g^{-1}). Based on this method the number of *C. coli* is expressed between two log levels and lower values were used for analysis.

DNA extraction and species verification by PCR

For isolation of DNA, bacterial colonies were scraped from plates and washed in 0.1 x TE buffer (10 mMTris/HCl, pH 8.0, 1 mM EDTA). Pellets were resuspended in 5 % Chelex Resin 100 (Bio-Rad). One hour incubation at 56 °C was followed by 15 min at 95 °C and 2 µL of the supernatants were used for PCR. Primers and PCR protocol for *C. coli* and *C. jejuni* verification are described by Wang et al. (2002).

Analytical procedure for total zinc measurements in faeces

Faecal samples (10 g) from three animals per group were analysed for the total zinc concentration at four different time points during this study according to Pieper et al. (2012). Zinc content was determined by atomic absorption spectrometry in an AAS vario 6 spectrometer (Analytik Jena, Jena, Germany) after hydrolysis of the samples in concentrated HCl.

Statistical analysis

Calculation of statistical significance was performed with GraphPad Prism v5 (La Jolla, CA, USA) using the one-way ANOVA with Tukey's post-hoc test. Means ± standard deviation (SD) are mentioned for all variables. The effect of the different Zn doses, age and their interaction

on the pig body weight was calculated applying a general linear model with repeated measures with pen as experimental unit. Differences were considered significant at $p<0.05$.

5.4 Results

In vitro C. coli growth inhibition by zinc oxide

Growth inhibition of *C. coli* 5981 was examined in broth cultures containing various ZnO concentrations over 24 h. Significantly reduced cell counts could already be detected after 2h treatment for the ZnO groups containing ≥ 2.6 mM ZnO compared to control ($F= 31.74$; $df=29,90$; $p= 0.0001$). Without addition of ZnO the *C. coli* cell count increased within the assay to $\log_{10}$ 7.78 ± 0.26 CFU/ml while the cell count showed no changes over the whole testing period with 1.6 mM ZnO ($\log_{10}$ 6.64 ± 0.04 CFU/ml). After incubation with 2.6 mM ZnO the cell count decreased to $\log_{10}$ 1.30 ± 1.61 CFU/ml. Only sporadically colonies could be counted after 24 h exposure at 3.9 and 6.5 mM ZnO (Fig. 5-1).

Gene expression analysis of C. coli 5981 after zinc oxide exposure

To understand the mechanism of action of ZnO on *C. coli*, a set of genes involved in oxidative and general stress response was selected for gene expression analysis in *C. coli* 5981 cells after ZnO exposure (Table 5-1). As controls, an oxidative and heat stress response was generated by paraquat and a temperature upshift to 46 °C, respectively. Most apparently, the major oxidative stress gene katA was upregulated 5-fold (ZnO exposure) and 70-fold (paraquat treatment) but not by heat stress (Fig. 5-2). The expression of *sod*B and *ahp*C was not upregulated by all three stress treatments. The expression of the more generally associated stress response genes *clp*B, *gro*ES and *dna*K was slightly upregulated by ZnO treatment (2.2-fold, 2.5-fold and 2.6-fold, respectively), was unaffected after exposure to paraquat and upregulated by heat treatment (4.5-fold, 14-fold and 10-fold, respectively).

Animal health and faecal consistency

All animals remained in very good health conditions throughout the study. None of the animals developed signs of zinc deficiency or zinc intoxication. No diarrhoea occurred in any group and the faecal score remained at levels between 3 and 4 throughout the study without differences among the groups. No differences in the pig performance were detected. However, the body weight of the pigs in the low ZnO group was numerically lower (17.6 ± 0.2 kg) than in the medium (18.4 ± 0.2 kg) and high ZnO group (19.5 ± 0.1 kg) after 4 weeks of treatment (end of the first trial). At the end of the second trial, this pattern was further observed without statistical

differences (25.7 ± 0.2, 28.0 ± 0.3, 29.4 ± 0.2 kg in low, medium and high ZnO group, respectively).

Enumeration of C. coli in faeces in the three zinc oxide treatment groups

Only piglets from mother sows with confirmed *C. coli* positive faecal samples (ranging from 1 to 4 $\log_{10}$ CFU *C. coli* g^{-1}) were included in the animal trial. Quantitative data on the faecal excretion of *C. coli* in piglets of the three different ZnO treatment groups over the whole trial period (day 0-42) are summarized in Fig. 5-3. All piglets were naturally colonized with *C. coli*. Highest *C. coli* levels were detectable on the first sampling day (day 0) of the experiment. *C. coli* counts for the medium and high ZnO group reached 6.7 ± 0.82 and 6.68 ± 1.5 log CFU g^{-1} and for the low ZnO group 6.33 ± 1.23 log CFU g^{-1} faeces. From the seventh sampling day on, reduced *C. coli* levels could be observed for the high ZnO group compared to the low and medium ZnO groups with the exception of day 21 where *C. coli* counts did not show differences among the three groups. Taken all data from the first trial period together (day 0-28), a significant reduction of *C. coli* shedding of 1 log CFU g^{-1} faeces ($F= 9.14$; $df= 2,27$; $p= 0.0009$) was detectable for the high ZnO group in comparison to the low and medium ZnO group (Fig. 5-4).

To investigate the lasting effect of a reduced *C. coli* faecal excretion in the high ZnO group, the diet containing 3,100 mg ZnO kg^{-1} was lowered to the medium ZnO concentration of 100 mg ZnO kg^{-1} after 28 days for another two weeks (second trial period, day 29-42). That decrease in dietary ZnO supplementation lead to an increase of *C. coli* numbers in faeces within a few days in this group. Faecal *C. coli* levels of animals receiving an adjusted dietary ZnO supplementation equalled and even exceeded the ones of the low and medium ZnO group (Fig. 5-3).

Total zinc measurements in faeces

Faecal zinc concentrations in the three ZnO treatment groups are shown in Fig. 5-5. As zinc homeostasis in pigs is mainly regulated in the gastrointestinal tract by retention or excretion, we analysed the total zinc concentration in faeces. Therefore, samples of three animals per group on the first and last sampling day of both trial periods were taken to measure the zinc excretion.

As expected, the faecal zinc concentration was affected by the ZnO level in the feed diet (Fig. 5-5). Faecal zinc concentrations of the low ZnO group on the first day (four days after the start of zinc supplementation) was approx. 420 mg kg^{-1} faeces and increased to 860 mg kg^{-1} faeces after four weeks. It remained relatively constant at approx. 700 mg kg^{-1} zinc for the rest of the study.

Results for the medium ZnO group showed a median level of approx. 860 mg Zn kg^{-1} faeces on the first day. It decreased with time to approx. 640 mg Zn kg^{-1} faeces. On day 28 only 300 mg Zn kg^{-1} faeces were detectable in this group. Faecal zinc concentrations from piglets fed with the high ZnO diet resulted in an excessive faecal zinc concentration with up to 8,250 mg kg^{-1} after four weeks. Decreasing the diet from the high to the medium ZnO concentration in this group resulted in zinc concentrations comparable with the other two treatment groups within 4 days.

5.5 Discussion

The results of this study demonstrate the dose-dependent reduction of *C. coli* numbers by ZnO *in vitro*.

To our knowledge, no study has been published that tested the antimicrobial impact of ZnO against *C. coli* yet. Besides, recent studies concentrate on ZnO nanoparticles (NP) as their antimicrobial effect is shown to be higher compared to ZnO powder (Tayel et al. 2011). However, the research on ZnO NP focuses more on its usage as a preservative agent and disinfectant in food industry whereas ZnO powder is commonly used as feed additive in farm animals.

Based on the *in vitro* growth inhibition experiments (Fig. 5-1), we were able to demonstrate a high susceptibility of *C. coli* against zinc stress. Liedtke and Vahjen (2012) classified a broad range of intestinal bacterial species for their susceptibility to ZnO supplemented media into three categories according to their minimum inhibitory concentration (MIC). Bacteria were determined as low (18-73 µg mL^{-1}), medium (130-290 µg mL^{-1}) or highly resistant (250-580 µg mL^{-1}), with the majority of intestinal bacteria belonging to the latter group. When applying this classification scheme, *C. coli* 5981 possesses a medium zinc resistance with a MIC of < 2.6 mM (200 µg mL^{-1}). Comparable data were obtained for *C. jejuni* DSM 4688 (German Collection of Microorganisms and Cell Cultures-DSMZ) and *C. jejuni* NCTC 11168 (National Collection of Type Cultures), with MICs of 290 and 145 µg mL^{-1} ZnO, respectively (Liedtke and Vahjen 2012).

Several proteins play a role in the protection of *Campylobacter* spp. from oxidative stress. Three major proteins are involved in the inactivation of ROS (Murphy et al. 2006): Superoxide dismutase (SodB) is thought to provide the first line of defence during exposure of *C. jejuni* to air as it removes superoxide anions by their dismutation into hydrogen peroxide and oxygen (Purdy and Park 1994; Purdy et al. 1999). The peroxide stress defence protein catalase (KatA) degrades hydrogen peroxide to water and oxygen in the cytoplasm. The alkyl hydroxide

reductase (AhpC) is important in the resistance of *C. jejuni* to alkyl hydroperoxides as it can destroy toxic hydroperoxide intermediates. To describe the stress response of *C. coli* after ZnO exposure, selected genes involved in oxidative and general stress response were tested for expression changes after ZnO exposure (Fig. 5-2). We were able to show that ZnO induces a significant increase of the expression of the oxidative stress gene *kat*A. This is in agreement with the study of Xie et al. (2011), who detected an increase in *katA* expression after exposure of *C. jejuni* 81-176 to ZnO NP. In contrast to that study, *ahp*C was not upregulated in *C. coli* 5981. The expression of *sod*B was not affected by zinc oxide stress. That corresponds to the study by Xie et al. (2011). In addition, the expression of general stress response genes *gro*ES and *dna*K was upregulated under ZnO exposure. Similar results were reported in *C. jejuni* 81-176 by Xie et al. (2011). These data suggest that ZnO exposure leads -in addition to an oxidative stress response- to a general stress response in *C. jejuni*/*C. coli*.

No data exist on the impact of ZnO powder as feed additive on *Campylobacter* colonization and shedding in animals. We were able to show a reduction in *C. coli* shedding of approx. 1 log CFU g^{-1} faeces when supplementing feed with a high ZnO dose compared to excretion rates in piglets fed a low and medium ZnO dose (Fig. 5-3 and Fig. 5-4). By investigating a second trial period (Fig. 5-3) where ZnO supplementation was reduced from high (3,100 mg kg^{-1}) to medium (100 mg kg^{-1}) concentration in the high ZnO group we could demonstrate that the reduction in *C. coli* shedding at high ZnO concentrations in feed is a transient phenomenon: decreasing ZnO supplementation lead to an increase in *C. coli* shedding within a few days (Fig. 5-3). These data indicate a fast adaption of *C. coli* to new environmental zinc conditions present in the intestine of the porcine host when fed different zinc diets.

However, there was a high variation in faecal *C. coli* counts, even in the faeces of one animal at consecutive sampling points. This can be explained by the heterogeneous distribution of *C. coli* in the gut content which results in oscillating *C. coli* excretion (Weijtens et al. 1999; Leblanc Maridor et al. 2008).

A number of studies already demonstrated the positive impact of high doses of ZnO on animal health and improved weight gain in piglets (Jensen-Waern et al. 1998; Pluske et al. 2002; Pettigrew, 2006). Here, similar observation could be made. Despite no significant differences in body weight between the different ZnO groups, numerical improvement of body weight was observed with increased ZnO concentrations in the feed. These findings remained in agreement with data collected in another experiment of our group, where pigs were infected with *Salmonella* Typhimurium and fed similar ZnO levels in the diet for 6 weeks (Janczyk et al. 2013).

High ZnO doses can alter the bacterial community composition in general (Li et al. 2001) and the variety and diversity of coliforms in particular (Gielda and DiRita 2012). Nonetheless, these authors could not detect a specific effect of ZnO on the number of excreted *Escherichia* (*E.*) *coli*, enterotoxigenic *E. coli*, coliforms or *Enterococcus* spp. (Jensen-Waern et al. 1998; Katouli et al. 1999; Gielda and DiRita 2012). In contrast, Slade et al. (2011) showed that dietary ZnO supplementation (3,100 mg/kg feed) reduced shedding of enterotoxigenic *E. coli* O149 in pigs.

We analysed the zinc concentration in the faeces of the three treatment groups as zinc homeostasis is regulated mainly by faecal excretion (Poulsen and Larsen 1995). Excessive faecal zinc concentrations were detectable in the high dosage zinc dietary group whereas no major differences were observed for the other two groups (Fig. 5-5). The lack of differences in the zinc concentration in faeces between the low and medium ZnO group could be explained by either the low difference in the dietary zinc, or a heterogeneous dispersion of zinc in faecal material. Zinc was measured in fresh faecal material and differences in water concentration could have affected the measurements. Nevertheless, these data provide evidence of the minor effect of low and medium ZnO supplementation to the feed diet and explain the lack of differences in *C. coli* counts between those two groups. Reducing the high dietary ZnO dose to the medium ZnO level led to a decrease of zinc concentration in the gut within a few days. These results correspond with the observed *C. coli* excretion data where cell numbers increased within the same time period, confirming the disappearance of the inhibiting effect of excessive zinc.

Summarizing, this work provides evidence of an antimicrobial impact of ZnO against *C. coli in vitro* and *in vivo*. The application of high doses of ZnO in feed a few days prior to slaughter could be suitable to reduce the *C. coli* load in pigs at slaughter. Nonetheless, data are needed to evaluate potential side effects of high ZnO doses in feed for animal health, the zinc concentration in meat and the impact of zinc excretion into the environment with potential side-effects on soil and water bacteria.

5.6 Acknowledgement

We acknowledge Stefanie Banneke and Mechthild Ladwig (Federal Institute for Risk Assessment, Berlin) for their input during the animal trial. We thank Wilfried Vahjen (Institute of Animal Nutrition, Freie Universität Berlin) for the chemical analysis. The technical assistance of Jasmin Blume (Institute of Food Hygiene, Freie Universität Berlin) is gratefully acknowledged. The study was funded by the German Research Foundation (Deutsche Forschungsgemeinschaft, DFG) within the Collaborative Research Group (SFB,

Sonderforschungsbereich) 852/1 “Nutrition and intestinal microbiota - host interactions in the pig”.

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5.8 List of tables and figures

Table 5-1: Genes and primers used for RT-qPCR analysis

Gene function/protein encoded	Gene	Primer	Sequence 5'- 3'	Primer conc. Fw/Rev (µM)	Product size (bp)	Tm (°C)
Catalase	*kat*A	forward	TCCCAAGCGGCATTATTACT	0.3/0.3	101	76.0
		reverse	CCAACATTCTTCATCCGTGA			
Alkyl hydroperoxide reductase	*ahp*C	forward	TCAAGGGGGTATTGGTCAAG	0.3/0.3	119	77.0
		reverse	CAAGCAAGAAAGAACCACGA			
Superoxide dismutase	*sod*B	forward	GGTGGCTCATGCCAAAGTAT	0.3/0.3	118	77.5
		reverse	CTGAACCAAAAACCCCTGTC			
Chaperone	*dna*K	forward	TTACTGATGCGGTGATTACTGTGC	0.3/0.9	65	75.5
		reverse	TTCTTTCGTTGCTTTTCTTTGTGC			
Chaperone	*clp*B	forward	AGGAGTAGGAAAAACACAATCTGC	0.3/0.9	87	75.5
		reverse	TCACTCATATCAAAGCGAATCAT			
Chaperone	*gro*ES	forward	CGTAGAAGAAACCAAAACAACA	0.3/0.3	89	74.0
		reverse	TTACTGCTACTACTTCACCCATTA			
Thiamine biosynthesis protein (reference gene)	*thi*C	forward	TTCCTAGCGCTGATTTGTTTT	0.9/0.3	59	73.0
		reverse	CCAGTTTATGATGCGGTAGGATT			

Fw: forward primer; Rev: reverse primer; bp: base pairs; Tm: melting temperature of PCR product

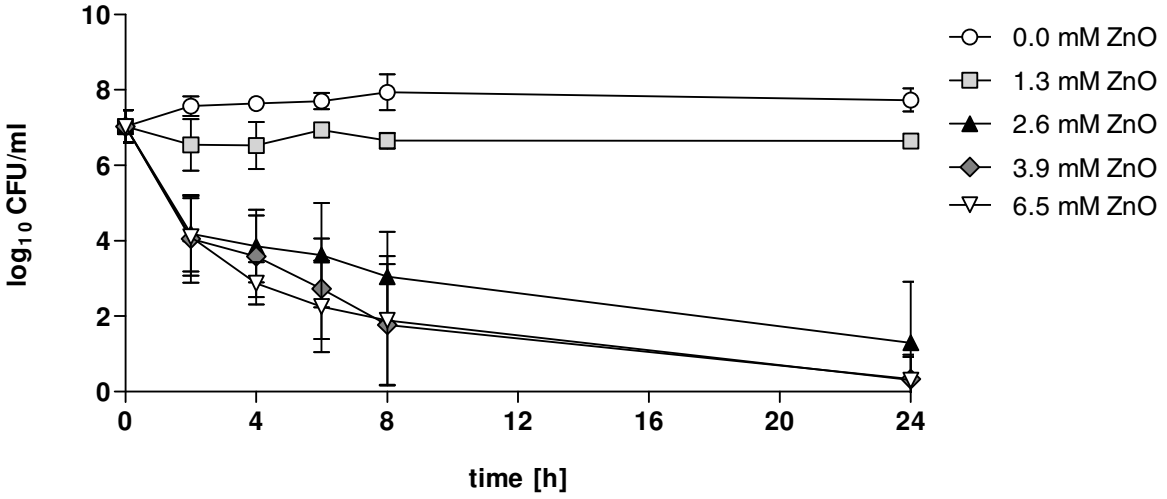

Figure 5-1: Antibacterial activities of different zinc oxide (ZnO) concentrations against *C. coli* 5981 *in vitro*
Behaviour of *C. coli* 5981 in Brucella broth in the absence of additional ZnO (circles) and in the presence of 1.3 mM ZnO (squares), 2.6 mM ZnO (dark triangle), 3.9 mM ZnO (diamonds), and 6.5 mM ZnO (bright triangle). Results are expressed as means ± standard deviation of $\log_{10}$ CFU ml^{-1} (n = 4).

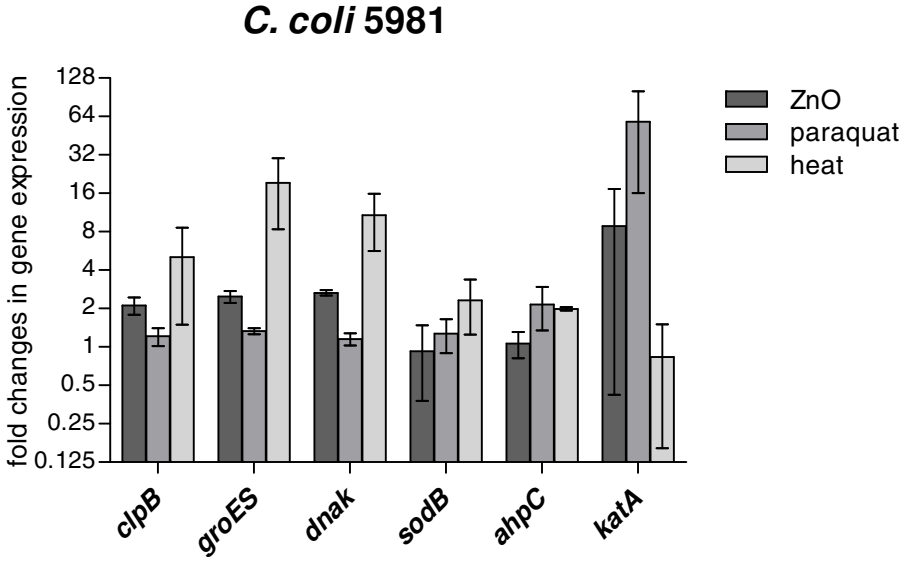

Figure 5-2: Expression changes of selected genes in *C. coli* 5981 under different stress conditions *in vitro*
C. coli 5981 cells in the late exponential phase were stressed by 1.3 mM ZnO (dark grey), by 0.5 mM paraquat (grey) or heated to 46 °C (bright grey) for 30 min. Transcripts of the selected genes were quantified by RT-qPCR, and data were analysed using the comparative critical threshold ($2^{-\Delta\Delta CT}$) method. The relative expression ratio for each gene is presented as a $\log_2$ value in the histogram. Data indicate the mean ± standard deviation (n = 3).

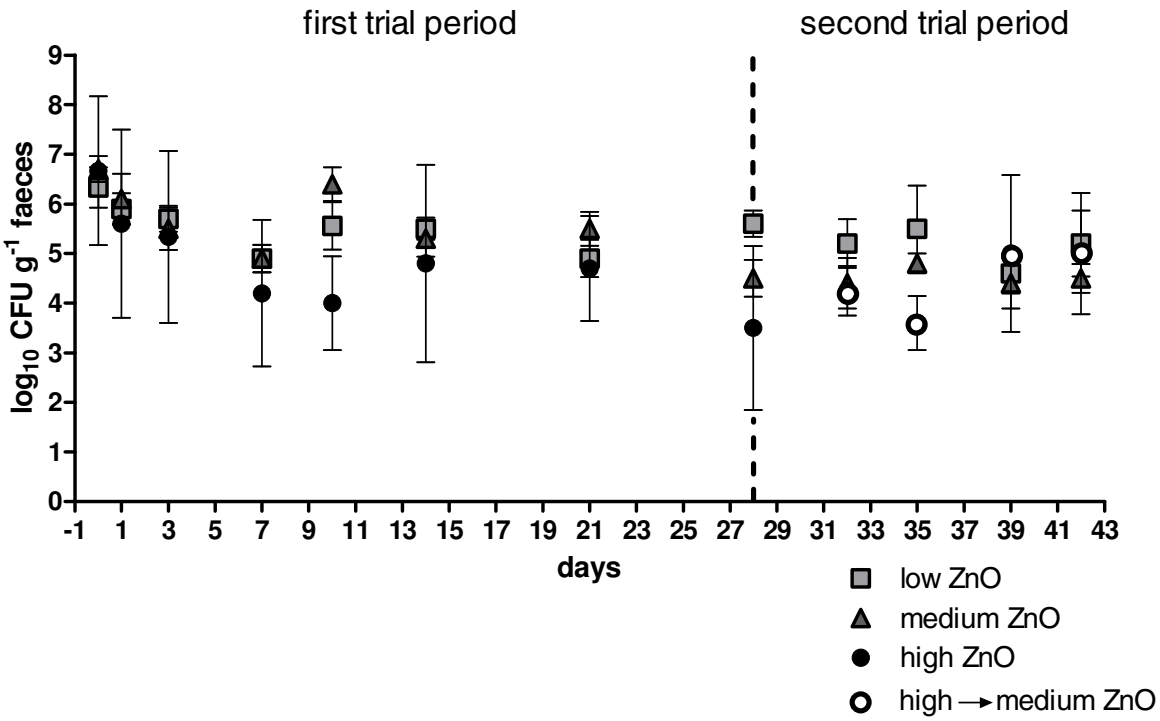

Figure 5-3: Course of faecal excretion of *C. coli* in naturally colonized weaned piglets fed three different zinc oxide concentrations from day 0-42

C. coli levels in the faeces detected by semi-quantification in the first trial period (day 0-28, n = 10) and second trial period (day 29-42, n = 5); square: low ZnO (40 mg kg^{-1}), triangle: medium ZnO (100 mg kg^{-1}) black dot: high ZnO (3,100 mg kg^{-1}), white dot: reduction from high to medium ZnO (3,100 → 100 mg kg^{-1}); ---: time point of reduction of dietary ZnO from 3,100 to 100 mg kg^{-1}; results are expressed as mean ± standard deviation of log_{10} CFU per gram faeces.

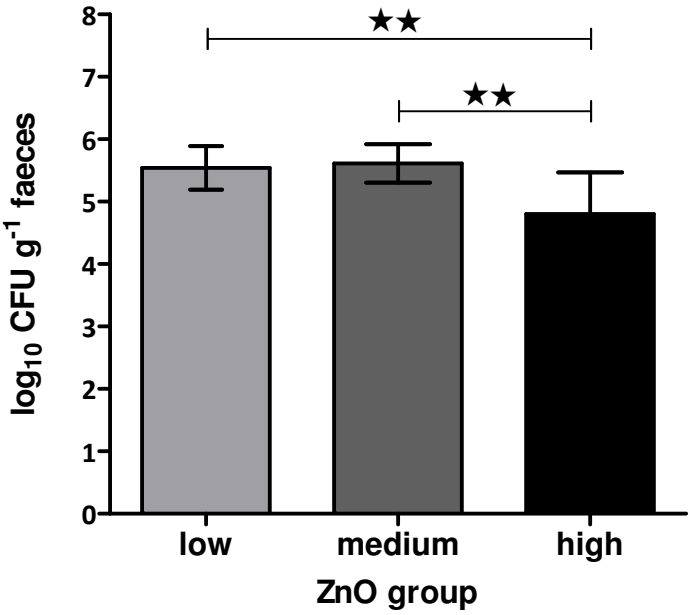

Figure 5-4: Summarized *C. coli* levels in faeces from piglets fed three different zinc oxide concentrations (first trial period: day 0-28)

Summarized *C. coli* levels in the faeces detected by semi-quantification in the first trial period (day 0-28, n = 10), results are expressed as mean ± standard deviation of $\log_{10}$ CFU per gram faeces.

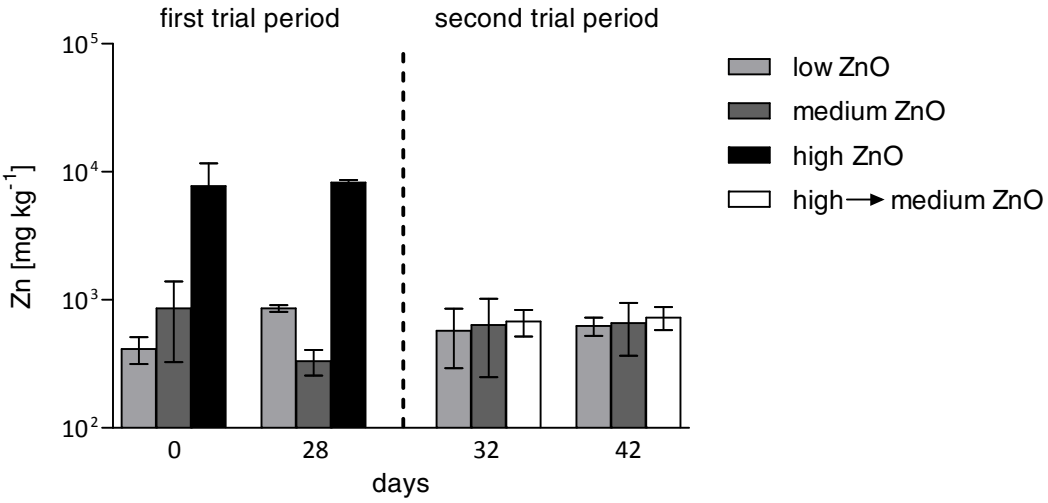

Figure 5-5: Total faecal zinc excretion by weaned piglets fed different dietary zinc oxide concentrations

Total zinc concentration was determined in faeces before and after the reduction of dietary zinc oxide from 3,100 to 100 mg kg^{-1} from the three treatment groups, results are expressed as mean ± standard deviation of mg Zn kg^{-1} (n = 3).

Chapter 6: Analysis of *in vitro* and *in vivo* effects of probiotics against *Campylobacter* spp.

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6.1 Summary

Campylobacter (*C.*) spp. are well recognised as the leading cause of bacterial foodborne diarrheal disease worldwide, with *C. jejuni* and *C. coli* as the most important species. *C. coli* is highly abundant in pigs and pork meat has often been implicated as a source for human infection. Intestinal colonisation of *C. coli* in pigs plays a role in carcass contamination during slaughter. Different pre-harvest intervention measures are proposed to reduce the *C. coli* burden in the porcine intestine. Among others, the use of probiotics to prevent or reduce the colonisation of intestinal pathogens is discussed.

One aim of this study was to screen a variety of probiotics to evaluate their inhibitory activity against *Campylobacter* spp. *in vitro*. Therefore, cell-free culture supernatants of *Lactobacillus* spp., *Bifidobacterium* spp., *Enterococcus* (*E.*) *faecium* NCIMB 10415, and *Escherichia coli* Nissle 1917 were tested against *C. jejuni* and *C. coli* by a well-diffusion agar assay. Seven out of eleven *Lactobacillus* strains showed an inhibitory activity against at least one of the three tested *Campylobacter* strains. This antagonistic activity against *Campylobacter* spp. was caused by the production of organic acids that lowered the pH. Application with pH neutralised cell-free culture supernatants abolished this inhibitory effect. Other tested strains with probiotic properties showed no inhibitory activity against any *Campylobacter* strain.

The strain *E. faecium* NCIMB 10415 was chosen to test its inhibitory activity against *C. coli in vivo*. Twenty weaned piglets were allocated into two groups, a probiotic group and a control group. The diet of the probiotic group was supplemented with *E. faecium* NCIMB 10415 (10^9 cfu/kg feed, Cylactin) since weaning, whereas the control group received no probiotic treatment. All piglets were naturally colonised with *C. coli*. The excretion load of *C. coli* was monitored for 28 days. The results indicate that dietary supplementation of *E. faecium* NCIMB 10415 did not significantly affect *C. coli* excretion levels in pigs.

In this study, *E. faecium* NCIMB 10415 showed no antagonistic activity against *C. coli in vitro* and *in vivo* and had no impact on the growth performance of weaned piglets.

Keywords: *Campylobacter* spp.*, Campylobacter coli*, probiotic, *Enterococcus faecium*, pig

6.2 Zusammenfassung

Campylobacter (*C.*) spp. sind eine der häufigsten Ursachen für bakterielle lebensmittelassoziierte Infektionen weltweit. Hierbei stellen die Spezies *C. jejuni* und *C. coli*

die wichtigsten Vertreter dar. *C. coli* dominiert im Schwein, daher wird Schweinefleisch oft als Hauptquelle für *C. coli*-Infektionen beim Menschen angesehen. Die Besiedlung des Darms mit *C. coli* beim Schwein spielt bei der Kontamination des Schweinefleisches während der Schlachtung eine entscheidende Rolle. Der Einsatz verschiedener sogenannter Pre-harvest-Interventionsmaßnahmen zur Reduktion der *C. coli*-Belastung im Schwein wird diskutiert. Unter anderem wird die Anwendung probiotischer Bakterien zur Vermeidung oder Reduktion der Besiedlung intestinaler Pathogene im Schwein derzeit in Betracht gezogen.

Ein Ziel dieser Studie war es, verschiedene Probiotika-Stämme auf ihre hemmende Wirkung gegenüber Campylobacter spp. *in vitro* zu testen. Dafür wurde die inhibierende Wirkung zellfreier Kultur-Überstände von *Lactobacillus* spp. *Bifidobacterium* spp., *Enterococcus* (*E.*) *faecium* NCIMB 10415 sowie *Escherichia coli* Nissle 1917 gegenüber *C. jejuni* und *C. coli* mittels Agar-Diffusionstest überprüft. Bei sieben von elf *Lactobacillus*-Stämmen konnte eine inhibierende Wirkung gegen mindestens einen der drei getesteten *Campylobacter*-Stämme nachgewiesen werden. Dieser antagonistische Effekt kann auf die Produktion von organischen Säuren und dem damit verminderten pH-Wert zurückgeführt werden. Der Einsatz eines pH neutralisierten zellfreien Kultur-Überstandes hob diesen inhibierenden Effekt auf. Andere getestete Stämme mit probiotischen Eigenschaften zeigten hingegen keine inhibierende Wirkung gegenüber *Campylobacter* spp.

E. faecium NCIMB 10415 wurde zur Testung seiner inhibierenden Wirkung gegenüber *C. coli* für den Einsatz den Tierversuch ausgewählt. Dafür wurden 20 abgesetzte Ferkel auf zwei Gruppen aufgeteilt, einer probiotischen Gruppe und einer Kontrollgruppe. Der probiotische Gruppe wurde bereits während der Saugphase das Probiotikum *E. faecium* NCIMB 10415 (10^9 cfu/kg Futter, Cylactin) im Futter angeboten, wohingegen bei der Kontrollgruppe keine probiotische Zugabe erfolgte. Alle Ferkel waren auf natürliche Weise mit *C. coli* besiedelt. Die Ausscheidung von *C. coli* wurde für 28 Tage kontrolliert. Die Ergebnisse zeigen, dass die Verfütterung von *E. faecium* NCIMB 10415 keinen signifikanten Einfluss auf die ausgeschiedene *C. coli*-Zellzahl der Ferkel hat.

In dieser Studie konnte keine antagonistischen Wirkung von *E. faecium* NCIMB 10415 gegenüber *C. coli in vitro* und *in vivo* gezeigt werden. Die Wachstumsleistung entwöhnter Ferkel konnte durch das Probiotikum nicht gesteigert werden.

Schlüsselwörter: *Campylobacter* spp., *Campylobacter coli*, Probiotikum, *Enterococcus faecium,* Schwein

6.3 Introduction

Campylobacter (C.) spp. are well recognised as the leading cause of bacterial foodborne diarrheal disease worldwide with *C. jejuni* and *C. coli* as the most important species for human infections. *C. coli* is highly abundant in pigs with prevalences between 50% and 100% and excretion levels of up to 10^7 cfu/g faeces (Young et al., 2000; Alter et al., 2005). It is estimated that approx. 10% of the human campylobacteriosis cases are caused by *C. coli,* mainly through the consumption of contaminated pork (Gillespie et al., 2002; Gurtler et al., 2005; Rosef et al., 2009). Slaughtering is a crucial step for *Campylobacter* spp. transmission to humans as intestinal colonisation of *C. coli* in pigs plays a major role in carcass contamination. The high prevalence of *Campylobacter* spp. in pigs and consequently pork highlights the need for strategies to control the *C. coli* colonisation in pigs. At present, complete avoidance of *Campylobacter* spp. on the farm is difficult, as risk factors for their initial transmission are still not clear (Horrocks et al., 2009; Cody et al., 2010). *Campylobacter* spp. do not grow outside the host and thus, reduction of *Campylobacter* spp. at the end of the food chain is best achieved if the colonisation on live animal can be prevented or reduced (Wagenaar et al., 2008). Different pre-harvest intervention measures are proposed to reduce the *Campylobacter* spp. load in livestock (Baer et al., 2013). The use of probiotics to prevent or reduce the intestinal *C. coli* colonisation in pigs is being discussed. The mechanism underlying their beneficial outcome is, amongst others, the antagonistic effects against pathogenic bacteria by competitive exclusion, e. g. secretion of antimicrobial substances, occupation of adhesions sites and receptors, and competition for essential nutrients (Bermudez-Brito et al., 2012). In animal production there are currently three different groups authorised as feed additives in the EU: lactic acid bacteria (LAB; mainly *Enterococcus* spp., *Lactobacillus* spp. (*L.*) and *Bifidobacterium* spp.), bacteria of the genus *Bacillus* and yeasts of the genus *Saccharomyces*. Production of substances, such as organic acids, hydrogen peroxide, fatty acids and bacteriocins by probiotics are known to enhance their ability to compete against other microbes in the gastrointestinal tract (GIT). Most research on probiotic application has been done on *C. jejuni* in chickens, but reports on their efficacy are often contradictory and inconclusive. A variety of LAB from the genus *Lactobacillus* spp., *Bifidobacteria* spp. and *Enterococcus* spp. showed inhibitory activity against *C. jejuni* strains by co-culture experiments (Santini et al., 2010). In another *in vitro* study probiotic *Lactobacillus* spp. produced lactic acid that sufficiently supressed *C. jejuni* (Neal-McKinney et al., 2012). The inhibitory effect of some probiotics against *Campylobacter* spp. was also evidenced *in vivo.* Morishita et al. (1997) reported reduced *C. jejuni* shedding in market aged broilers by feeding *L. acidophilus* and *Streptococcus faecium.* Moreover, *Lactobacillus* spp. and *Bifidobacterium* spp. competitively excluded *C. jejuni* in a mouse model (Wagner et al., 2009). In contrast, Svetoch and Stern

(2010) reviewed that they were never able to identify live bacterial isolates that would successfully compete within the GIT to control *Campylobacter* spp. Moreover, feed supplemented with *Saccharomyces boulardii* did not significantly affect caecal *Campylobacter* colonisation of experimentally challenged chickens (Line et al., 1998).

The probiotic strain *E. faecium* NCIMB 10415 is licensed as a feed additive for sows and piglets and has been demonstrated to promote growth and decrease the incidence of diarrhoea in pigs (Zeyner and Boldt, 2006). Supplementation of *E. faecium* NCIMB 10415 has been shown to modify the porcine microbiota by decreasing the pathogenic load (Pollmann et al., 2005; Taras et al., 2006). In a co-culture experiment *C. jejuni* growth was highly inhibited by *E. faecium* and feeding of a probiotic preparation including *E. faecium* has been shown to reduce the colonisation of *C. jejuni* in chickens (Ghareeb et al., 2012).

Reduction of *C. coli* in pigs by probiotics has to our knowledge not been investigated yet. Therefore, the intention of this study was first to screen a variety of probiotic bacteria for their antagonistic effect against three strains of *Campylobacter* spp. *in vitro* and second, to test if the probiotic bacterium *E. faecium* NCIMB 10415 can reduce the *C. coli* load in naturally and experimentally colonised pigs.

6.4 Material and methods

Bacterial strains and culture conditions

In total, 26 probiotic strains were used in this study (Table 6-1.). *Lactobacillus* spp., *Bifidobacterium* spp. and the *E. faecium* NCIMB 10415 strain were provided by the Institute of Microbiology and Epizootics, Freie Universität Berlin, Berlin, Germany (strains designated as IMT), while *Escherichia coli* Nissle 1917 (EcN) was isolated from Mutaflor®, a probiotic pharmaceutical.

Lactobacillus spp. and *Bifidobacterium* spp. were cultivated on de Man Rogosa and Sharpe (MRS) agar (Merck, Darmstadt, Germany). EcN and *E. faecium* NCIMB 10415 were cultivated on Luria-Bertani (LB) agar (Merck). All strains were stored at –80°C using the MAST Cryobank System (Mast Diagnostica, Reinfeld, Germany). Strains were streaked on MRS or LB agar, respectively, and incubated at 37°C for 24 h. For *Bifidobacterium* spp. anaerobic, and for *Lactobacillus* spp., EcN and *E. faecium* aerobic conditions were used. Anaerobic atmosphere was generated by the Mart Anoxomat system (Drachten, Netherlands). One colony of each strain was inoculated in MRS and LB broth respectively, and incubated under conditions mentioned above. Overnight cultures of LAB and EcN were used for the well-diffusion agar assay.

Three strains of *Campylobacter* spp. were used as target strains to test the inhibitory activity of the probiotics. The *C. jejuni* strain NCTC 11168, *C. jejuni* DSM 4688 and *C. coli* 5981 (Bratz et al., 2013b) were used. *Campylobacter* spp. were recovered from stocks kept at –80°C by plating cryobeads on Mueller-Hinton agar with 5% sheep blood (MHB; OXOID, Wesel, Germany) for 48 h at 37°C under microaerobic conditions (6% O_2, 7% CO_2, 80% N_2, 7% H_2) using the Mart Anoxomat system. Liquid cultures were obtained by inoculation of colonies in *Brucella* broth (BB) (BD, Heidelberg, Germany) and cultivation under the same conditions for 24 h.

For the animal trial, the *C. coli* 5981 strain was used as inoculation strain. It was chosen due to its antimicrobial resistances against erythromycin and neomycin. This combination has been shown to be very rarely distributed among *C. coli* isolates and enables the differentiation within naturally colonised *C. coli* strains, present in most pigs (Bratz et al., 2013a). For inoculum preparation, *C. coli* 5981 was cultured on MHB plates in microaerobic atmosphere for 48 h at 37°C. Colonies were inoculated in 3 ml BB and incubated for 16 h under the same conditions as mentioned above. From overnight cultures with an optical density of 0.3 at 600 nm 0.5 ml were inoculated in 20 ml BB and incubated for another 4 h. The cultures were further diluted in 80 ml BB in order to obtain a solution of 7 x 10^7 cfu per 5 ml. Cell numbers were determined by counting from serial dilutions.

In vitro assessment of the inhibitory activity of probiotics against Campylobacter spp. by well-diffusion agar assay

The inhibitory activity of 26 probiotic bacterial strains was studied using the well-diffusion agar assay according to Santini et al. (2010). Briefly, overnight cultures of LAB and EcN were centrifuged (15 min at 15 000 x g at 4°C). The supernatants were sterile-filtered using a 0.22 µm Millipore filter (VWR, Darmstadt, Germany). The cell-free culture supernatant (CS) were adjusted to pH 6.5 ± 0.3 with 1 N NaOH in order to obtain pH neutral cell-free culture supernatant (NCS). 500 µl overnight culture of each *Campylobacter* strain tested (~10^7 cfu/ml) was added to 20 ml Nutrient agar No. 2 (1% agar, OXOID), poured onto sterile petri dishes, and allowed to solidify. Wells of approximately 5 mm in diameter were made using a sterile metal puncher. A volume of 50 µl of CS and NCS were filled into each well. The inhibition activity of CS and NCS from probiotics was determined by the presence of clear growth inhibition zones (transparent areas around the well showing no growth of *Campylobacter* spp.). Therefore, plates were incubated for 48 h at 37°C in microaerobic atmosphere, to allow *Campylobacter* spp. growth. LB and MRS alone served as negative control. The anti-*Campylobacter* spp. activity was performed in triplicates. A clear zone defined as a transparent

area around the well showing no growth on *Campylobacter* spp. agar refers to be positive for inhibitory activity of the CS in the well-diffusion agar assay (Fig. 6-1.).

Animals, diets and experimental design of the feeding trial

All animals were housed and treated in accordance with the regulation of the local authority (Landesamt für Gesundheit und Soziales, Berlin; approval no. G0349/09). This study was performed using 20 weaned German Landrace piglets obtained from the Institute for Animal Nutrition, Freie Universität Berlin. The piglets and their mother sows were separated in two groups based on different diets. Diets were based on standard starter feed mixture. In order to obtain a heterogeneous pool of piglets, litters from at least three different sows per group were included. The probiotic group (PG) was dietary supplemented with the *E. faecium* strain NCIMB 10415, whereas the control group (CG) received no probiotic with their feed. The probiotic *E. faecium* strain NCIMB 10415 is authorised by the EU as a zootechnical additive for pigs and commercially available (Cylactin® ME10, DSM Nutritional Products Ltd, Switzerland). It was provided in a microencapsulated form and mixed to the diets of sows, suckling and weaned piglets according to the (EC) No 252/2006 recommended maximal concentration of 10^9 cfu/kg feed. *E. faecium* was provided daily to the sows of the PG three weeks before parturition until the day of weaning. Piglets were offered the respective feed with or without *E. faecium* supplementation from the age of 12 days on. Piglets were weaned at an age of 28 days and transferred to the experimental facility where they were allocated in two separate pens and kept in groups of two. The experimental diets were offered twice a day for one hour, the leftovers were collected and feed intake was recorded on dry matter basis. Drinking water was offered *ad libitum*. The pens were cleaned thoroughly twice a day. After a one week adaption period all animals were inoculated with a unique dosage of 7×10^7 cfu of the strain *C. coli* 5981 by intragastric application using a stomach feeding tube (B. Braun, Melsungen, Germany) under azaperone (1.5 mg/kg; Stresnil, Janssen-Cilag, Neuss, Germany) sedation. In a previous trial, the same strain with the same inoculum concentration has been shown to successfully colonise the GIT of weaned piglets (Bratz et al., 2013a). All piglets were weighed twice a week for 28 days.

Sampling of the faeces

After the inoculation with *C. coli* 5981, faecal samples were collected in intervals over the whole experimental period in order to monitor *Campylobacter* spp. excretion. Moreover, faecal consistency was assessed using a subjective score on a five-point scale ranging from 1 to 5 (1: liquid; 2: mushy; 3: soft; 4: solid and well formed, and 5: hard dry stool), representing one major parameter of the health status in weaned piglets. Faecal samples from piglets were taken directly from the rectum at 14 time points over 28 days. The time period between

sampling and analysis in the laboratory was not longer than 4 h for all samples. Before experimental inoculation with *C. coli* 5981, faecal samples were taken to determine the total *C. coli* load before the inoculation and verify the absence of strains exhibiting antibiotic resistances against both, erythromycin and neomycin. Post inoculation (p.i.) of the strain *C. coli* 5981, samples were taken daily for seven days and after that for every three days till the end of the study.

Enumeration of Campylobacter coli in faeces

Semi-quantification of *C. coli* levels was performed according to Bratz et al. (2013b). Briefly, 1 g of faeces was 1:10 diluted in Bolton broth with a Bolton selective antibiotic supplement and 5% lysed horse blood (all OXOID) in stomacher bags (Meintrup, Lähden-Holte, Germany). Samples were homogenised in Bagmixer 400 (Interscience, Saint-Nom-la-Bretèche, France) for 2 min at maximal speed. Serial 10-fold dilutions of up to 10^{-8} of the initial homogenates were made in selective enrichment Bolton broth and incubated for 48 h at 37°C in microaerobic atmosphere. For semi-quantification 10 µl of each dilution was plated on modified charcoal cefoperazone deoxycholate agar (mCCDA*)* plates with and without the addition of 30 mg/ml erythromycin and 100 mg/ml neomycin (Carl Roth, Karlsruhe, Germany) in order to distinguish the *C. coli* 5981 strain from the natural *Campylobacter* sp. population. Plates were incubated for further 48 h under conditions mentioned above.

C. coli levels were expressed as $\log_{10}$ cfu per gram sample material (detection limit 10 cfu/g). Based on this method the number of *C. coli* is expressed between two log levels and lower values were used for analysis.

DNA extraction and Campylobacter spp. identification by multiplex PCR

From every dilution with bacterial growth, DNA was extracted for *Campylobacter* spp. verification by multiplex PCR. Primers and PCR protocol are described elsewhere (Wang et al., 2002). However, only primers for 23S, *C. jejuni* and *C. coli* Primers were used. Therefore, cell material was scraped from plates, washed in 0.1 x TE buffer (10 mM Tris/HCl; pH 8; 1 mM EDTA) and pellets were resuspended in 5% Chelex Resin 100 (BioRad, München, Germany). One hour incubation at 56°C was followed by 15 min at 95°C and 2 µl of the supernatant were used for PCR.

Statistical analysis

Calculation of statistical significance was performed with GraphPad Prism v5 (La Jolla, CA, USA) using the nonparametric Mann-Whitney-test. Differences were considered significant at $p<0.05$.

6.5 Results

In vitro assessment of the inhibitory activity of probiotics against Campylobacter spp. by well-diffusion agar assay

Results obtained for the assessment of the antimicrobial activity of probiotics against *Campylobacter* spp. revealed that 64% (7/11) of the *Lactobacillus* strains showed antimicrobial activity against at least one of the three tested *Campylobacter* strains (Table 6-1.). Probiotic strains from other genera tested (n = 15) showed no inhibitory activity against *C. jejuni* or *C. coli.* It turned out that *C. coli* 5981 was more susceptible than the two *C. jejuni* strains. Six *Lactobacillus* strains acted antagonistically against *C. coli*, while only four and five caused a clear inhibition zone on *C. jejuni* NCTC 11168 and *C. jejuni* DSM 4688, respectively. However, when NCS or CS with a pH >4.3 were used no inhibition zones could be observed on any *Campylobacter* spp. agar. Thus, this inhibitory activity was pH-dependent as NCS completely abolished the inhibitory effect against all *Campylobacter* strains, investigated.

Influence of Enterococcus faecium NCIMB 10415 on Campylobacter coli in pigs

All animals remained in very good health conditions throughout the study. No major changes in the faecal score could be recorded in both groups. Also, no significant differences in growth performance (body weight gain and feed intake) in the PG compared to CG could be measured (data not shown).

Results for the enumeration of the inoculation strain *C. coli* 5981 alone and total *C. coli* (incl. *C. coli* 5981) in faeces of weaned pigs with or without the daily dietary supplementation of *E. faecium* NCIMB 10415 was monitored for four weeks (Fig. 6-2A. and 6-2B.). All animals excreted *C. coli* before inoculation at mean levels of 4 log cfu/g faeces in the CG and 5 log cfu/g faeces in the PG. However, none of these *C. coli* strains were resistant to erythromycin and neomycin as shown for the inoculation strain *C. coli* 5981. Three animals, two of the CG and one of the PG, started excreting *C. coli* 5981 one day p.i. at levels of 1–2 log cfu/g, respectively. *C. coli* 5981 excretion occurred much faster in the PG. Nine out of ten animals excreted the inoculation strain already four days p.i. in the PG, whereas five out of ten animals in the CG excreted *C. coli* 5981 not until the 5th day p.i. Highest counts for naturally colonised *C. coli* occurred ten days p.i in the CG and 17 days p.i. in the PG (Fig. 6-2A.). For the inoculation *C. coli* 5981 strain the highest colonisation level was reached ten days p.i. in both groups (Fig. 6-2B.).

For half of the time points examined (7/14), total *C. coli* (incl. *C. coli* 5981) levels were increased by 1–2 log levels in the PG, while equal levels were determined for the remaining sampling days with one exception on day four p.i. For the *C. coli* 5981 strain alone, the results

were more infrequent. However, for the majority of time points, *C. coli* 5981 levels were increased or were equal in the PG compared to the CG. Thus, there is a general trend for increased *C. coli* excretion levels in pigs supplemented with *E. faecium* NCIMB 10415. However, no significant differences were detectable between the groups.

6.6 Discussion

The present study was carried out to evaluate the probiotic activity of *Lactobacillus* spp., *Bifidobacterium* spp., EcN and *E. faecium* NCIMB 10415 against *Campylobacter* spp. by well-diffusion agar assays. Only *Lactobacillus* strains showed an inhibitory activity against any of the three *Campylobacter* strains. No antagonistic activity of other probiotic strains from different genera against *C. coli* or *C. jejuni* was detectable in our study. It turned out that the anti-*Campylobacter* activity of the *Lactobacillus* strains was pH-dependent. At a pH <4.3 the growth of *Campylobacter* spp. was inhibited. This inhibitory effect abolished when the supernatants of the same overnight cultures were adjusted to a neural pH of 6.5 ± 0.3. Other LAB tested in our study did either not produce enough organic acids to kill *Campylobacter* spp. or the pH maintained neutral after overnight incubation. This might be explained by different growth requirements for these bacteria to produce organic acids (Meremae et al., 2010; Hartmann et al., 2011). However, testing of growth conditions was beyond the scope of the current study. It has been stated that the growth of *Campylobacter* spp. below pH 4.9 is restricted and at pH values less than that rapidly kills this organism (Park, 2002). Suppression of *C. jejuni* growth by probiotics was reported to be caused by the low pH in liquid media (Meremae et al., 2010). It has been further reported that the production of organic acids by LAB have a strong inhibitory effect against gram-negative bacteria due to their permeabilising capacity of the bacterial outer membrane and can be considered as main antimicrobial compounds (Alakomi et al., 2000). The different inhibitory activity of probiotic genera against *Campylobacter* spp. was also observed by Chaveerach et al. (2004). In this *in vitro* study no negative effect on *Campylobacter* spp. growth was shown for *Enterococcus* spp., but for the *Lactobacillus* P93 strain. It was reported that this effect accounted not only from organic acid production, but probably also from anti-microbial peptides. However, no non-organic acid effect was the reason for the inhibitory effect in our study as the CS was heat stable and resistant to proteinase K treatment (data not shown). Some of the probiotics tested are known to produce bacteriocins that act antagonistically against intestinal pathogens (Fayol-Messaoudi et al., 2005). Bacteriocins or bacteriocin-like substances of *E. faecium* strains are highly effective against the foodborne pathogens *Salmonella*, *Helicobacter pylori* and *C. jejuni* (Kim et al., 2003; Strompfová et al., 2003; Line et al., 2008). Stern et al. (2008) reported that treatments

with viable probiotic bacteria were ineffective in reducing *C. jejuni* in chickens, while bacteriocin treatment from these corresponding bacteria substantially reduced *C. jejuni* colonisation in the live birds. Overall, despite the great importance of *in vitro* experiments in research the findings cannot be considered as valid without confirmation in animal experiments. Testing of the antagonistic activity of probiotics against *Campylobacter* spp. by culture supernatants alone cannot reflect other competitive exclusion mechanisms that are only present in the intestine of pigs.

For our *in vivo* experiment, the probiotic strain *E. faecium* NCIMB 10415 was chosen. It is a frequently used feed additive for young piglets and has been shown to decrease the occurrence of post-weaning diarrhea. Although no anti-*Campylobacter* spp. activity was detectable *in vitro* other competitive exclusion mechanisms could have led to reduced *C. coli* counts.

In pigs, *C. coli* colonisation occurs early in life. Therefore, attention was focused on an early inoculation of the GIT with the probiotic strain to establish a competitive exclusion microbiota that is able to prevent the *Campylobacter* spp. colonisation. Nevertheless, all piglets were already naturally colonised with *C. coli* before experimental inoculation with *C. coli* 5981. Neither the excretion of naturally colonised *C. coli* was reduced nor the colonisation of the inoculation strain could be prevented by the probiotic feeding in pigs. In contrast, a slight trend towards increased *C. coli* excretion levels was detectable in probiotic treated animals, although no significant differences could be observed. Increased *Campylobacter* spp. counts and enhanced adhesion of *C. jejuni* by *E. faecium* supplementation has been reported in dogs (Rinkinen et al., 2003; Vahjen and Manner, 2003*).* An increase of other gram-negative intestinal pathogens in piglets fed *E. faecium* NCIMB 10415 was also reported by Kreuzer et al. (2012). Different niches of the probiotic and *Campylobacter* spp. within the GIT of the pig might be responsible for this effect. The main habitat for LAB is the large intestine as undigested carbohydrates are fermented in this intestinal segment. LAB are able to metabolise these compounds as energy source by producing organic acids as metabolic products (Lalles et al., 2007). In contrast, *Campylobacter* spp. cannot ferment carbohydrates and their growth relies on organic acids and amino acids. Thus, it can be hypothesised that *E. faecium* NCIMB 10415 supplementation might have (i) either promoted the growth of *C. coli* as the metabolic products synthesised by *E. faecium* serve as energy and carbon source for *C. coli* or (ii) competitors of *Campylobacter* spp. were suppressed by this probiotic. In addition, although *Campylobacter* spp. prefer the colonisation of the large intestine as well, the mucus is regarded as the most likely site for *Campylobacter* spp. persistence (Takata et al., 1992; Bratz et al., 2013a). For canine intestinal mucus it has been shown that *E. faecium* strains exhibited a relatively low level of adhesion (Rinkinen et al., 2000). Thus, both bacteria seem

to occupy different niches and may not come in close contact with each other. Although it is known that *E. faecium* NCIMB 10415 produces a class IIb bacteriocin (Foulquie Moreno et al., 2003) no antagonistic activity against *C. coli* was detectable.

Studies about the effectiveness of *E. faecium* in swine are limited. In the present study no effects regarding improved body weight gain or feed intake in the *E. faecium* NCIMB 10415 treated group was evidenced. This is consistent with another study with a similar experimental set-up (Martin et al., 2012). Contrary, others reported an increased growth performance and *d*ecreased incidence of diarrhoea and post-weaning mortality after treatment with *E. faecium*, but applying regimes differed from this study (Taras et al., 2006; Zeyner and Boldt, 2006). Since this study was performed under controlled housing and hygiene conditions, all pigs remained healthy throughout the experiment and thus, no probiotics effects were observed. However, the number of animals used in the present study was not high enough to draw a final conclusion about the impact of the *E. faecium* supplementation on the growth performance in weaning piglets. Field trials with higher piglet numbers under production conditions are necessary to determine a potential impact of *E. faecium* NCIMB 10415 in piglets.

In conclusion, with this study we were able to show that *E. faecium* NCIMB 10415 showed no antagonistic activity against *C. coli in vitro* and *in vivo*.

6.7 Acknowledgement

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Conflict of interests: The authors declare that they have no conflict of interests.

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6.9 List of tables and figures

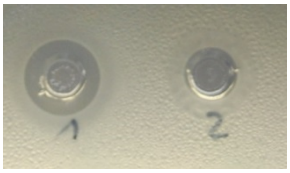

Figure 6-1: Example of an inhibition zone with the well-diffusion agar assay

Left spot shows a clear inhibition zone produced by the cell-free culture supernatant of the probiotic strain *Lactobacillus fermentum* (1) and right spot shows a turbid zone of the pH neutralised cell-free culture supernatant of the same strain (2) comparable to the negative control (MRS broth alone) with *C. coli* 5981 as target strain.

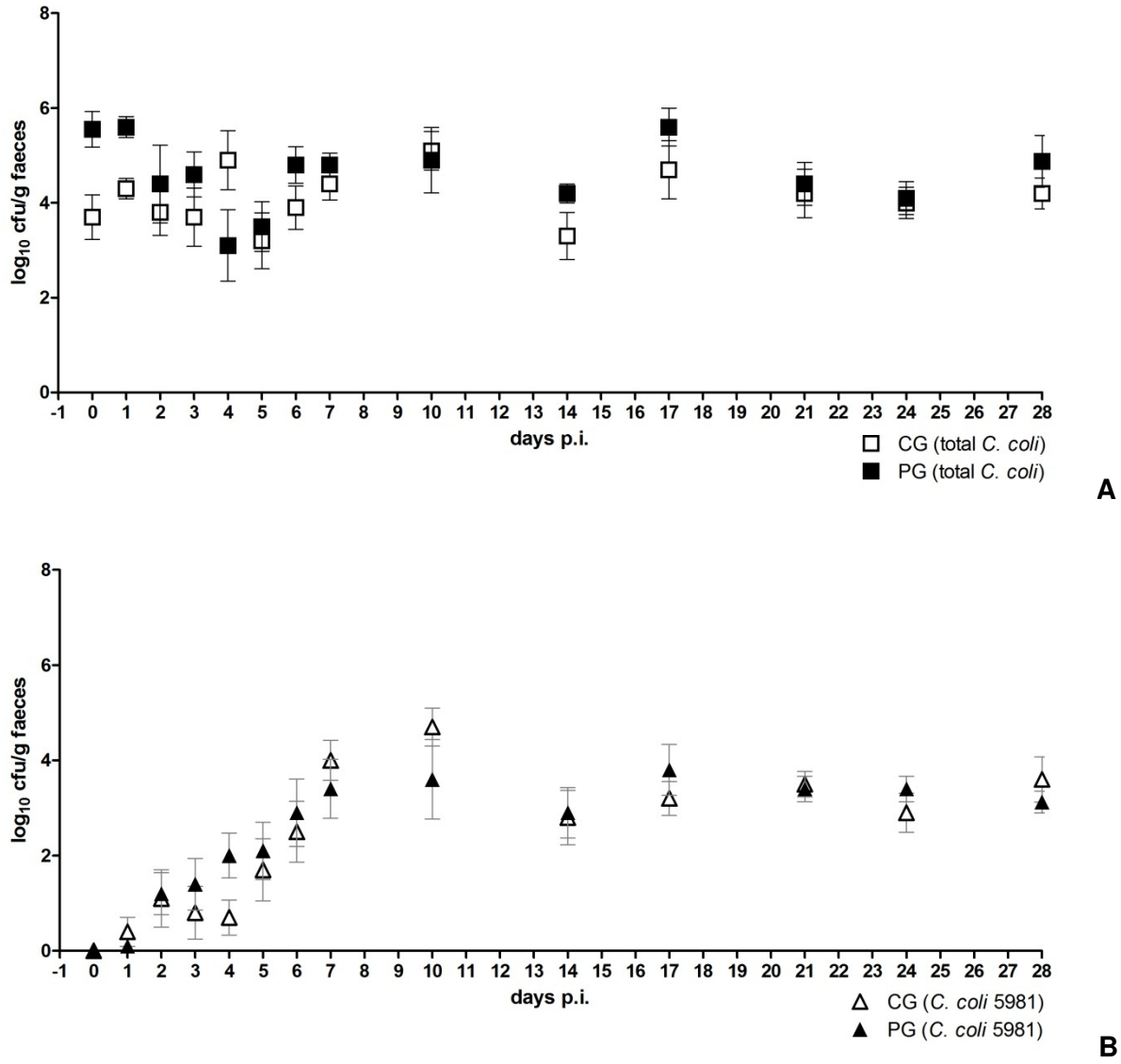

Figure 6-2: Course of faecal excretion of total *C. coli* (A) and *C. coli* 5981 alone (B) following oral inoculation with *C. coli* 5981 of weaned piglets over 28 days with or without *E. faecium* NCIMB 10415 supplementation

Results are expressed as $\log_{10}$ of colony forming units (cfu) per gram faeces determined on selective media with and without the addition of antimicrobials for *C. coli* 5981 (triangles) and total *C. coli* (incl. *C. coli* 5981, squares) detection, respectively. The probiotic treated group (PG) is represented by filled symbols and the control group (CG) by empty symbols. *C. coli* excretion was monitored at 14 time points over 28 days post inoculation (p.i.). Error bars indicate standard errors of the mean (n = 20).

Table 6-1: Inhibitory activity of supernatants of probiotic bacteria against three strains of *Campylobacter* spp. determined by well-diffusion agar assay

Strain	Strain designation	Origin	pH of CS	*C. coli* 5981		*C. jejuni* DSM 4688		*C. jejuni* NCTC 11168	
				CS	NCS	CS	NCS	CS	NCS
Enterococcus faecium	Cylactin®, NCIMB 10415	human faeces	6.4	-	n/d	-	n/d	-	n/d
Escherichia coli	Mutaflor®, Nissle 1917	human faeces	6.8	-	n/d	-	n/d	-	n/d
L. acidophilus	Danisco®, IMT 22354	yoghurt	4.4	-	-	-	-	-	-
L. brevis	IMT 22350	-	5.1	-	-	-	-	-	-
L. fermentum	ATCC 14931	fermented beets	3.9	+	-	+	-	+	-
L. garvieae	IMT 11751	-	4.7	-	-	-	-		-
L. gasseri	DSM 20077	human faeces	3.9	+	-	+	-	-	-
L. johnsonii	Nestlé®, BFE 663	yoghurt	3.9	+	-	+	-	+	-
L. paracasei subsp. *paracasei*	IMT 22353	-	4.0	+	-	+	-	+	-
L. plantarum	IMT 21742	-	3,8	+	-	-	-	-	-
L. reuteri	IMT 21493	-	4.3	-	-	+	-	-	-
L. rhamnosus	IMT 21374	yoghurt	4.0	+	-	-	-	+	-
L. thermotolerans	IMT 12012	-	4.1	+	-	-	-	-	-
B. adolescentis	DSM 20083	human faeces	6.8	-	n/d	-	n/d	-	n/d
B. angulatum	ATCC 27535	human faeces	6.6	-	n/d	-	n/d	-	n/d
B. animalis	DSM 20104	rat faeces	6.4	-	n/d	-	n/d	-	n/d
B. animalis subsp. *lactis*	DSM 10140	yoghurt	6.5	-	n/d	-	n/d	-	n/d
B. bifidum	IMT 21113	-	6.7	-	n/d	-	n/d	-	n/d
B. breve	DSM 20213	human faeces	6.6	-	n/d	-	n/d	-	n/d
B. catenulatum	ATCC 27539	human faeces	6.5	-	n/d	-	n/d	-	n/d
B. cereus	IMT 4578	-	6.3	-	n/d	-	n/d	-	n/d
B. gallicum	DSM 20093	human faeces	6.5	-	n/d	-	n/d	-	n/d
B. longum subsp. *longum*	DSM 20219	human faeces	6.8	-	n/d	-	n/d	-	n/d
B. longum subsp. *suis*	DSM 20211	pig faeces	6.8	-	n/d	-	n/d	-	n/d
B. pseudocatenulatum	ATTC 27919	human faeces	6.4	-	n/d	-	n/d	-	n/d
B. thermophilum	DSM 20210	pig faeces	6.5	-	n/d	-	n/d	-	n/d

+, clear inhibition zone; -, no inhibition zone; n/d, not done; CS, cell-free culture supernatant; NCS, pH neutral cell-free culture supernatant (pH 6.5 ±0.3); IMT: Institute for Microbiology and Epizootics, Freie Universität Berlin; ATCC, American Type Culture Collection; *BFE,* Bundesforschungsanstalt für Ernährung; DSM, Deutsche Stammlung für Mikroorganismen; NCIMB, National Collection of Industrial and Marine Bacteria; *B., Bifidobacterium* spp.*; L., Lactobacillus* spp.*;* n = 3

Chapter 7: General discussion

Background and aims

The present thesis was part of the collaborative research group (SFB) 852 'nutrition and intestinal microbiota - host interactions in the pig' that aimed to understand how feed additives, in particular the probiotic *Enterococcus faecium* (*E. faecium*) NCIMB 10415 and the trace element zinc may influence the functioning of the intestine in the pig.

Pigs are known to be frequently colonised by *C. coli* without any obvious clinical symptoms of infection. The high prevalence and excretion rate of *C. coli* in pigs cause the risk of contamination of food or food products, such as raw meat, having major consequences for human health in terms of food-borne diseases. The slaughtering process is a crucial step for the transmission of *Campylobacter* spp. to humans via the consumption of contaminated pork. Therefore, proper intervention strategies are needed to better control and prevent *C. coli* colonisation in pigs. Reducing the number of *Campylobacter* spp. in pigs at the time of slaughter would thus, help to minimise the potential carcass contamination risk at the slaughter plant and subsequently might reduce the incidence of human *C. coli* infections. Complete avoidance of *Campylobacter* spp. in pigs is difficult as they are natural inhabitants of the gut and pigs are typically colonised at a young age with high prevalences. The administration of feed additives with antimicrobial properties for the use of pre-harvest intervention against *Campylobacter* spp. in pigs has been intensively discussed (**as reviewed in Chapter 2**).

Within this present thesis, two animal trials were conducted to gain a deeper insight into the *C. coli* colonisation and the inhibitory effect of feed additives, such as zinc, applied as zinc oxide (ZnO) and the probiotic *E. faecium* NCIMB 10415 in weaned piglets (**Chapter 4, 5 & 6)**. In the first animal trial, we therefore inoculated weaned piglets with the porcine *C. coli* 5981 strain in order to study the *Campylobacter* spp. colonisation dynamic, the excretion pattern and the translocation ability to extra-gastrointestinal sites (**Chapter 4**). Furthermore, the first animal trial served to test the inhibitory effect of the probiotic strain *E. faecium* NCIMB 10415 on the inoculation strain as well as on the naturally colonised *C. coli* population (**Chapter 6**). The second animal trial aimed to assess the impact of different ZnO concentrations on *C. coli* levels in weaned piglets (**Chapter 5**).

C. coli colonisation in pig

Pigs are recognised as main reservoir for *C. coli* and thus, as a major source for human *C. coli* infections. However, little is known about the colonisation, translocation ability and excretion dynamics of *Campylobacter* spp. in pigs. To determine the significance of pigs as a reservoir in contamination of pork meat it is important to understand the ecology of *Campylobacter* spp. within pigs. Thus, elucidating the site of colonisation is fundamental if management strategies are to be developed. For example, if an efficient probiotic is to be developed, it is important to understand the microbial ecology of the intestinal tract where *Campylobacter* species reside. So far, only a few *Campylobacter* spp. colonisation studies for pigs exist and most studies have used gnotobiotic or specific pathogen-free animals (Boosinger and Powe 1988; Leblanc-Maridor et al., 2008). Therefore, we conducted a colonisation trial using the porcine strain *C. coli* 5981 as experimental inoculation strain in weaned piglets (**Chapter 4**). This strain was chosen, because it possesses a rare antibiotic resistance combination against erythromycin and neomycin, among other *C. coli* isolates. In preliminary tests, 91 porcine *C. coli* isolates were screened for their antibiotic resistance pattern. Only 3.3% of the strains showed resistances against both antimicrobials (**unpublished data**). Based on MLST analysis, *C. coli* 5981 belongs to the clonal complex ST-828. It has been showed that 94% of *C. coli* isolates tested could be grouped to the clonal complex ST-828 (Sheppard et al., 2009). Moreover, in preliminary *in vitro* experiments, we found that the pathogenic potential of *C. coli* 5981 is comparable to the reference strain *C. coli* ATCC 33559, as in infected human intestinal HT-29/B6 cells the epithelial integrity was disturbed by the induction of apoptosis. *C. coli* 5981 has further been shown to encode the virulence factors cytolethal distending toxin (*cdt*ABC), *Campylobacter invasion antigen B* (*cia*B) *and Campylobacter* adhesion to fibronectin (*cad*F) (**unpublished data**). Thus, the *C. coli* 5981 strain can be considered as a typical representative of porcine *C. coli* with the potential to cause human infections.

For the *C. coli* colonisation trial weaned piglets were used. Since nursery is the primary site for *Campylobacter* spp. transmission, it was highly supposable that these piglets were already naturally colonised with *C. coli* before the start of the study (Alter et al., 2005b; Soultos and Madden, 2007). Faeces samples were tested for *Campylobacter* spp. presence prior experimental inoculation with *C. coli* 5981. It turned out that all piglets were positive for *Campylobacter* sp., but no strain showed resistances against the additional antimicrobials used. The monitoring of the excretion dynamics of naturally colonised *C. coli* (incl. *C. coli* 5981) and *C. coli* 5981 alone could therefore, be accomplished. Colonisation with *C. coli* 5981 was evidenced for all piglets one week after inoculation and resulted in a high faecal excretion throughout the study (**Figure 4-1**). Hence, it could be concluded that the porcine *C. coli* 5981 strain is a good coloniser and integrated well in the natural *C. coli* population without major

competitive effects. However, fewer cell counts were observed for the inoculation strain when compared with the excretion levels of naturally established *C. coli* strains. The faecal excretion of *C. coli* 5981 varied between 10^4–10^6 cfu/g in all piglets with a mean of 1.9 x 10^5 cfu/g, whereas a mean excretion rate of 2.2 x 10^6 cfu/g faeces was calculated for total *C. coli.* Thus, one or more dominant strains had to be present in the GIT of the pigs. Similar shedding rates of 10^3–10^6 cfu/g faeces are reported in an experimental infection study, in which 7-week-old specific pathogen-free piglets were experimentally infected with a porcine *C. coli* strain with a comparable infection dose (Leblanc-Maridor et al., 2008). Jensen et al. (2013) enumerated the excretion levels of naturally colonised *Campylobacter* spp. in pigs before slaughter. Slightly lower levels of approx. 10^4 cfu/g faeces were detected compared to our colonisation trial. However, reduced *Campylobacter* spp. counts in aging pigs have also been reported elsewhere (Weijtens et al., 1993; Weijtens et al., 1999).

Overall, variable *C. coli* counts in subsequent faecal samples of pigs were often reported in these studies, which has also been frequently observed in own experiments. High counts could be followed by low counts and *vice versa*. Weijtens et al. (1999) summarised possible reasons for this intermittent excretion of *Campylobacter* spp. in pigs: *Campylobacter* spp. remain in niches in the animal as it has the tendency to accumulate at the bottom of the crypts in the intestinal mucosa, a niche where it can easily persist. Another reason for the sporadic excretion and the large differences between counts in successive samples could possibly be due to a heterogeneous distribution of *Campylobacter* spp. in the gut content, as they accumulate in the mucus. Thus, depending on the sample, more or less of this mucus may be present.

Besides monitoring the colonisation ability of *C. coli* 5981 and its excretion dynamic within a natural established microbiota including *C. coli*, we aimed to assess the main habitat for *Campylobacter* spp. along the entire GIT of pigs (**see Figure 4-2**). Therefore, we enumerated *C. coli* levels post-mortem in luminal contents of stomach, ileum, caecum and colon. Because of the comparable results for *C. coli* 5981 and total *C. coli*, it is referred to *C. coli* only in the following. *C. coli* were found along the entire GIT, but they primarily reside in the caecum and colon with up to 10^5 cfu/g gut content. The detection of *C. coli* in the stomach probably attributes to a reinfection by the faecal-oral route. *C. coli* were more numerous in the distal than in the proximal intestinal segments suggesting either a preferred proliferation in the lower GIT or an accumulative effect. To our knowledge, there has been only one study that enumerated *Campylobacter* spp. along the entire intestinal gut. Jensen et al. (2013) reported that the highest *Campylobacter* spp. counts were also found in the caecum and colon with similar colonisation rates as seen in our study. Only Weijtens et al. (1993) enumerated *Campylobacter* spp. in gastric, ileal and faeces samples of pigs at the slaughterhouse. The colony counts of *Campylobacter* spp. were mostly increased in the ileal contents compared to

the faeces samples, but gastric samples were mostly negative. In another analysis, high colonisation frequencies of the same gut sections as we analysed were evidenced by Nesbakken et al. (2003), but no enumeration was performed. Even though invasion and translocation of *Campylobacter* spp. into intestinal epithelial cells from a variety of *in vitro* studies is well-known (**see Chapter 2**), there are only a few studies that have investigated this subject in pigs yet. Research has mostly focussed on *C. jejuni* translocation in chicken or is based on murine model systems that are commonly used to study human *Campylobacter* infections (Newell, 2001; Cox et al., 2007; Cox et al., 2009; Bereswill et al., 2011). On this account, we aimed to determine not only the *C. coli* prevalence along the GIT, but also the invasion and translocation ability of *C. coli* to extra-gastrointestinal sites in pigs as possible reservoirs for pork contamination. Therefore, different tissues and organs were analysed for *C. coli* presence (**Figure 4-2**). Enumeration of gut tissues of jejunum, ileum and caecum revealed high *C. coli* rates with the ileum tissue as main colonisation site. In contrast, luminal *C. coli* were more numerous in colon and caecum samples. Overall, the *C. coli* loads were reduced in all examined tissues compared to the corresponding luminal gut contents. Cell numbers included mucosa-associated as well as internalised bacteria. Attachment of and invasion into the jejunal and ileal tissue of *Campylobacter* spp. was verified by laser-scanning microscopy (**Figure 4-3**). Moreover, translocation to extra-gastrointestinal sites of the GALT could be observed for jejunal MLNs, tonsils and spleens. *Campylobacter* spp. colonisation of different lymphatic organs and offal has been reported in some studies. Nesbakken et al. (2003) detected *C. coli* in 29% of jejunal MLNs and 67% in tonsils in pigl at slaughter, but no quantification was performed. In our trial, 50–70% of the jejunal MLNs and 20% of the tonsils were colonised with *C. coli*, but at low levels. In addition, very rare *C. coli* colonisation of porcine spleens were evidenced by Alexandrina and Botus (2008) and comparable results were observed in our study. In a previous study, that addressed the prevalence of *C. coli* in tonsils, *Campylobacter* spp. was detected in 6% of the samples (Fredriksson-Ahomaa et al., 2009). To our knowledge, only one study by Gorgen et al. (1983) regularly detected *Campylobacter* spp. in the gall bladder tissue of pigs. In the current study, *C. coli* were detectable in the gall bladder of one animal only. However, in the bile no *C. coli* were detectable. Moreover, no *C. coli* were recovered from liver samples (**unpublished data**). The high detection rate of *C. coli* in livers of fattening pigs and in livers at retail that are frequently reported might be due the contamination during slaughter. Interestingly, as seen in pigs, translocation of *Campylobacter* spp. to MLNs, but not to liver, spleen and blood has been evidenced in mice (Youssef et al., 1987; Bereswill et al., 2011). Contrary, in broilers high prevalences of *C. jejuni* were found in the spleen, liver and gall bladder (Cox et al., 2007; Cox et al., 2009). For *C. jejuni* in poultry it is discussed that there is internal migration between gall bladder, the bile and the

liver (Whyte et al., 2006). However, almost none of these organs have been colonised with *C. coli* in pigs in our trial. Thus, other translocation routes of *C. jejuni* in chickens and *C. coli* in pigs might exist. Bacterial translocation is promoted by intestinal bacterial overgrowth, deficiencies in host immune defences or increased permeability or damage of the intestinal mucosal barrier (**see Chapter 2**). Numerous studies have associated all three factors for translocation with the weaning process, and thus, piglets would appear to be extremely susceptible to bacterial translocation in the immediate post-weaning phase (Huang et al., 1999; Broom et al., 2006). Before experimental inoculation of piglets with *C. coli* 5981, an adaptation period of one week after weaning was given in order to minimise stress-related factors and to enable a somewhat stabilised porcine microbiota. Additionally, no clinical outcome in any of the pigs after inoculation with *C. coli* 5981 was observed. It is reported that in healthy humans and animals bacterial translocation is rare as they are cleared by the immune system. MLNs and extra-gastrointestinal organs are usually sterile and incidence of bacterial translocation is observed when the host immune system is compromised (Murakami et al., 2011). This might explain the low invasion rate of *C. coli* to extra-gastrointestinal tissues in our study in contrast to those that examined *Campylobacter* spp. colonisation in fattening pigs under commercial pig farming conditions (**see Chapter 2 & 4)**. In our colonisation trial, there were good sanitary and housing conditions, which could be reflected by a stable faecal score, normal growth performance and no clinical symptoms throughout the study. Although the translocation route was beyond the scope of this study, it can be assumed that *C. coli* translocated intracellularly through the epithelial cells and subsequently via the lymphatic vessels from the lamina propria to MLNs and from there to other organs and not directly via the vascular route to the liver. This hypothesis can be backed up by *C. coli* detection in lymphatic organs, such as the MLNs, tonsil and spleen. Moreover, no signs of epithelial barrier damage was observed by laser-scanning microscopy, although internalisation of *Campylobacter* spp. was observed.

In order to determine the genetic diversity of the naturally colonised *C. coli* population, fAFLP was performed of isolates from faeces, gut contents and various tissue samples of different piglets. Multiple clusters of *C. coli* isolates were determined by this method (**Figure 4-4**). Thus, the results demonstrate that there is a considerable genotypic diversity within a small group of animals over a short interval. This is in agreement with other investigations that have previously reported the existence of multiple *C. coli* genotypes within a single pig or within a pig herd (Thakur and Gebreyes, 2005; Soultos and Madden, 2007). Genotyping of porcine *C. coli* isolates has been undertaken with a wide range of techniques and all have evidenced a high genetic diversity (Weijtens et al., 1999; Alter et al., 2005a; Jensen et al., 2006; Malakauskas et al., 2006; Thakur et al., 2006; Madden et al., 2007). Because we only randomly picked *C. coli* isolates with a relative small sample size, no conclusion regarding dominating strains within

the naturally established *C. coli* population could have been made. Moore et al. (2002) assumed that a pig population is largely composed of persistent and dominant types, with a smaller number of hyper-variable subtypes. In the current study, some *C. coli* isolates from one cluster originated from various sample sites. Thus, identical genotypes were detectable in different tissues. These findings indicate that *C. coli* genotypes do not have preferred colonisation sites, at least in our study. In contrast, Madden et al. (2007) reported that 55% of the *C. coli* genotypes were unique to rectal samples compared with samples obtained from ileum contents of pigs. They concluded that sampling of a single site in the gut will recover only part of this population. However, ileal samples, but not rectal samples, were enriched in broth and this method was seen to yield less species diversity than direct plating of rectal swabs (Madden et al., 2000). In our study, 47 different *C. coli* isolates were analysed, whereas 200 *C. coli* isolates were typed by Madden et al. (2007). Still, relatively small sample sizes were used in both studies when the high diversity of *C. coli* genotypes within pigs were taken into account. It has to be mentioned that different typing methods were applied in both studies, which must be considered while interpreting the results. Moreover, the incubation time and temperature during enrichment seems to have a profound impact on the detection of the *Campylobacter* spp. genotype diversity. Scates et al. (2003) reported that the incubation at a single temperature of either 37 °C or 42 °C revealed a remarkable loss of *C. jejuni* genotypes. In addition, the body temperature of pigs varies between 38–39.5 °C and the incubation time during enrichment was 37 °C. Thus, we assume that some genotypes may have been repressed by the enrichment step at a single temperature and even more *C. coli* genotypes might have been present in the intestine of the pigs.

Another important finding of our study was that all isolates recovered from media containing erythromycin and neomycin were grouped in one cluster together with the original inoculation strain *C. coli* 5981. By this method, efficient strain separation could thus, be achieved. This result further verifies the successful differentiation of the inoculation strain from the natural *C. coli* population by the additional antibiotics used in the media. However, banding patterns of *C. coli* 5981 isolates varied to a certain extent. Genomic instability of *Campylobacter* spp. during *in vivo* passage has been stated (Leblanc-Maridor et al., 2011). Intra- and intergenomic recombinations, genomic rearrangements and chromosomal point mutations during *in vivo* passage might have led to genetic changes of the inoculation strain detectable by differences in fAFLP patterns. Moreover, other mechanisms, such as horizontal gene transfer via natural transformation within and between bacterial populations, may have also occurred. Genetic exchange has been observed for *C. jejuni in vitro,* but also during infection in chickens (de Boer et al., 2002). These genetic variations might represent adaptation processes to a new environment within the host (Ambur et al., 2009).

This leads to the overall conclusion that the inoculation strain *C. coli* 5981 has been proven to be an excellent coloniser in an already established microbial community including multiple *C. coli* genotypes in weaned piglets. Thus, this strain can be used for further *C. coli* colonisation studies even in naturally colonised pigs. However, the host-specific microbiota, age, diet and environment are factors that influence the colonisation of *Campylobacter* spp. and are somewhat unpredictable and have to be considered for future experiments. Moreover, our observations show that the entire GIT, but also lymphatic organs constitute big reservoirs for *C. coli* and thus, serve for potential carcass cross-contamination during slaughter. The traditional slaughter procedure involves the removal of the tongue, with tonsils attached, which can easily lead to carcass contamination with *Campylobacter* spp. It is assumed that tonsils, which harbour *C. coli*, are responsible for the spread via rinse water during the slaughter process (Altrock et al., 2013). Our finding, together with data from the current literature, emphasises the importance of hygienic precaution and a need for *Campylobacter* spp. intervention strategies to reduce *Campylobacter* spp. levels before slaughter (Malakauskas et al., 2006).

Influence of zinc oxide on *C. coli* in pigs

Feed additives with antimicrobial properties as pre-harvest intervention measure to decrease the load of *Campylobacter* spp. in pigs at different production stages, are currently intensively explored (Baer et al., 2013). A variety of feed additives, such as oral vaccination, bacteriophage therapy, competitive exclusion bacteria, bacteriocins and the use of metal oxides to reduce the *C. coli* burden in the porcine intestine are not yet commercially available, but research is ongoing (Wagenaar et al., 2006; Wassenaar, 2011). The feeding of the trace element zinc, usually applied as ZnO, is used for decades in pig farming with well documented beneficial effects (Poulsen, 1989; Jensen-Waern et al., 1998; Katouli et al., 1999; Hill et al., 2001). Dietary supplementation of ZnO above 2,000 mg/kg feed to weaned piglets results in e.g. reduced PWD and increased weight gain during the critical post-weaning phase (**reviewed in Chapter 2**). These beneficial effects on the pig performance are partly attributed to an altered intestinal microbiota (Hojberg et al., 2005). Influences on the porcine microbiota in weaned piglets fed high ZnO doses have been reported in many studies. However, these reports are often contradictory and inconclusive (Pieper et al., 2012; Vahjen et al., 2010; Starke et al., 2014; Hojberg et al., 2005). Liedke and Vahjen (2012) screened a broad range of intestinal bacterial species for their susceptibility to ZnO. The results of this study showed that the resistance of intestinal bacteria against ZnO is species-specific and the antibacterial effect of zinc cannot be assigned to a specific bacterial group. This might be the reason for at least

some of the contradictory results reported on certain bacterial communities in pigs fed a high ZnO concentration. To our knowledge, there is no study that tested the antimicrobial activity of ZnO against *C. coli* in pigs yet. The main objective of the thesis therefore was to analyse the inhibitory effect of high ZnO doses against *C. coli* (**Chapter 5**).

Firstly, a *Campylobacter* spp. colonisation model was established, which allowed a consistent and significant *C. coli* growth response to different ZnO treatments. Therefore, only weaned piglets that were naturally colonised with *C. coli* were used for the study. Additionally, a one-week adaptation period was performed to minimise variability from post-weaning growth depression. The results from this study demonstrate that the dietary supplementation of a high ZnO concentration led to a significant reduction of *C. coli* excretion levels in pigs within one week (**Figure 5-3**). The *C. coli* levels remained reduced compared with the control groups throughout the first trial period (day 0–28). A reduction of one log cfu/g faeces ($p = 0.001$) was detectable in pigs fed 3,100 mg ZnO/kg feed compared to pigs receiving 40 mg ZnO/kg feed and 100 mg ZnO/kg feed, respectively. No major differences of the *C. coli* excretion levels between the low and the medium ZnO group were detectable. The mean *C. coli* level in the high ZnO group was 10^5 cfu/g faeces, whereas 10^6 cfu/g faeces were detected in the other two groups (**Figure 5-4**). However, as seen in our *C. coli* colonisation trial, a strong variation in *C. coli* counts on subsequent sampling days was detectable.

The lasting effect of the reduced *C. coli* excretion levels in pigs fed a high ZnO concentration was examined in a second trial period (day 29–42). The zinc concentration of the high dietary ZnO group was reduced to that of the medium ZnO group for another two weeks (**Figure 5-3**). Interestingly, the *C. coli* levels in this group increased again to that of the other two groups within a few days. The GIT is thought to be the main site for zinc homeostasis in pigs. Therefore, the faecal zinc concentrations in the first and in the second trial period in all groups has been analyses in all feeding groups (**Figure 5-5**). As expected, excess feeding of zinc has led to tremendous faecal zinc excretion. In the first trial period, the faecal zinc concentration in the high ZnO feeding group was increased 10-fold compared to the low and medium ZnO-treated groups. A linear increase in the zinc excretion with increasing dietary ZnO was also observed by Buff et al. (2005). With a decreased dietary ZnO level in the second trial period, the faecal zinc concentration was considerably reduced and reached comparable levels as in the two control groups. Here again, no major differences in the faecal zinc concentrations between the low and the medium ZnO group were found throughout the trial. This probably results from the low differences in the dietary ZnO levels (40 mg ZnO/kg feed vs. 100 mg ZnO/kg feed). This effect was also reflected by similar *C. coli* excretion levels in both treatment groups. Interestingly, the differences in the faecal zinc concentrations in the first and second trial period thus, before and after the adjusted ZnO diet, were also seen by an altered *C. coli*

excretion level. The decreased dietary ZnO level led to a reduced faecal zinc excretion levels, which resulted in increased *C. coli* counts and *vice versa*. Thus, we were able to conclude that the observed reduced *C. coli* excretion load is a transient phenomenon and only lasts as long as the ZnO concentration remains high.

As mentioned above, feeding of pharmacological ZnO concentrations to weaned piglets has often been demonstrated to have a positive impact on the growth performance. Here, similar observations could be made. Despite no significant differences in body weight gain between the different ZnO treatment groups, numerical improvement of body weight gain was observed with increased ZnO concentrations in the feed. These findings are in agreement with data collected in another experiment within the collaborative research group, in which pigs were infected with *Salmonella* Typhimurium and dietary supplemented with similar ZnO levels for six weeks (Janczyk et al., 2013). To elicit a performance or health response to feed additives, the experimental conditions are important. It has often been postulated that growth promotional compounds become more effective as the environmental and nutritional challenges confronting the animal are exacerbated (Broom et al., 2006). Although controlled laboratory conditions were used in our experiments, similar results were seen for piglets reared under commercial conditions. Broom et al. (2006) observed numerical advantages regarding the body weight gain and feed intake in response to high dietary ZnO as well. In our study, all animals remained healthy and no diarrhoeal disease occurred in any group. However, a one-week adaptation period after weaning was performed in order to minimise stress-related factors during the post-weaning phase. Thus, a reduced incidence of PWD due to a high dietary ZnO dosage could not have been determined and was beyond the scope of the current study.

Although the dietary supplementation of 3,100 mg ZnO/kg feed significantly reduced the faecal *C. coli* levels in pigs; yet other parameters still have to be examined before an application in feed/agricultural industry can be recommended. For example, a successful reduction of *Campylobacter* spp. in nursery pigs fed a diet containing carbadox and copper sulfate was demonstrated, but resulted in increased shedding of *Enterobacteriaceae*, such as *Salmonella* and in a decreased feed efficiency (Wells et al., 2010). In this study the impact of high ZnO dosages on other pathogens has not been investigated. Nevertheless, numerous studies have examined the impact of pharmacological doses of ZnO on the porcine microbiota. Despite the reported contrary results, a species richness and an intestinal ecosystem stability in pigs fed high concentrations of ZnO was demonstrated in almost all studies during a short-term application (Katouli et al., 1999). The application of a pharmacological ZnO concentration for more than two weeks even reversed the positive effects e.g. reduced feed intake and lower body weight gain (Katouli et al., 1999; Buff et al., 2005; Janczyk et al., 2013). Moreover, an

accumulation of zinc by long-term supplementation in bone tissues and organs, such as the liver, kidney and pancreas was reported (Martin et al., 2013). Hence, a hampered regulation of the zinc homeostasis is suggested to cause an impaired performance during longer supply. More importantly, the feeding of ZnO has been restricted to 150 mg/kg feed in the EU, mainly due to environmental pollution issues (Jondreville et al., 2003). Pig manure is typically spread on cropland as fertilizer. Feeding at far higher levels by 10- 20 times than authorised results in a large amount of zinc in the manure that can result in accumulation of zinc in the soil with antimicrobial effects on beneficial soil and water bacteria (Pluske et al., 2002; Pettigrew, 2006). Bacteria have developed various mechanisms to overcome the toxicity of metals (Choudhury and Srivastava, 2001). Furthermore, metal resistance and antibiotic resistance are often found to reside together, particularly on mobile genetic elements, so that co-selection can occur (Baker-Austin et al., 2006; Bednorz et al., 2013). Decreased susceptibility and resistance to zinc chloride has already been observed in some bacteria in livestock (Aarestrup and Hasman, 2004).

To avoid major nutritional alterations in pigs, and to minimise the environmental pollution and costs, it is important to keep the period of high ZnO feeding to a minimum. Subsequently, the application of pharmacological ZnO concentrations during the entire rearing period, in order to keep the *C. coli* levels low, is not feasible and desirable. Because *C. coli* reduction has been demonstrated to occur already within a few days by high ZnO feeding, a supplementation shortly before slaughter may be more favourable. Although the usage of pharmacological ZnO levels in the EU is restricted, a short-term supplementation of pharmacological concentration of ZnO to the diet of pigs as a prophylactic or therapeutic agent, is currently being considered. Our results, coupled with the findings of earlier studies, lead to the conclusion of a repetitive feeding of high ZnO concentrations during the rearing period. We suggest the feeding of high ZnO for two weeks after weaning, which is sufficient to reduce post-weaning growth depression and one week before slaughter in order to reduce the *C. coli* load in pigs. However, the antimicrobial effect of high ZnO against *C. coli* in pigs, at the time of slaughter, has not been studied yet. Different zinc requirements for piglets and for fattening pigs (140–150 mg ZnO/kg feed vs. 90–150 mg ZnO/kg feed) exist and must be considered. The efficacy of *C. coli* reduction by pharmacological levels of ZnO in pigs before slaughter must be further evaluated. Another important fact that needs to be investigated is the impact of high ZnO supplementation on meat quality.

In conclusion, reduction of *C. coli* in weaned piglets occurs already within one week, but the lowering effect abolishes when the dietary zinc concentration is adjusted to the physiological requirement. Therefore, short-term feeding of high ZnO concentrations to reduce the *C. coli* load in pigs just before slaughter seems to be very promising.

The mode of action of ZnO against *C. coli*

The inhibitory activity of high dietary ZnO doses against *C. coli* could be demonstrated in weaned piglets. However, until now there is no valid explanation concerning the mode of action of the antimicrobial properties of ZnO. ZnO becomes unstable in solution and when H_2O_2 is produced, the Zn^{2+} ion concentration is increased as a result of ZnO decomposition (Domenech and Prieto, 1986). However, zinc oxide is highly insoluble in water, but displays higher solubility at acidic conditions. Due to low pH in the stomach, ZnO solubility is increased after feed intake and rather high percentages of soluble Zn^{2+} ions can be observed in pig digesta (Dintzis et al., 1995). As a consequence, free Zn^{2+} ions may reach the small intestine and act bactericidal. The antibacterial effect of heavy metals in general is attributed to the free ion, but it is unknown if ZnO itself has an effect on the bacterial growth (Silver, 1996). Therefore, the antibacterial effect of ZnO itself was tested on *C. coli* growth *in vitro* (**Chapter 5**). The growth inhibition of *C. coli* 5981 was examined in broth cultures containing various ZnO concentrations. At concentrations ≥ 2.6 mM ZnO a decline in cell numbers of *C. coli* 5981 already occurred (**Figure 5-1)**. Thus, a high susceptibility of *C. coli* against zinc stress could be demonstrated. Liedtke and Vahjen (2012) classified a broad range of intestinal bacterial species for their susceptibility to ZnO supplemented media into three categories according to their minimum inhibition concentration (MIC). Bacteria were determined as low (18–73 µg ZnO/ml), medium (130–290 µg ZnO/ml) or highly resistant (250–580 µg ZnO/ml), with the majority of intestinal bacteria belonging to the latter group. When applying this classification scheme, the tested strain *C. coli* 5981 possesses a medium zinc resistance with a MIC of < 2.6 mM ZnO, which corresponds to 200 µg ZnO/ml. Comparable data were obtained by Liedtke and Vahjen (2012) for *C. jejuni* strains (145–290 µg ZnO/ml). When using ZnO nanoparticles (NP), a higher susceptibility of *C. jejuni* towards zinc was demonstrated (Xie et al., 2011). The MIC of the *C. jejuni* strains tested ranged between 25–50 µg ZnO NP/ml. Thus, not only the MIC value is important to determine the antimicrobial impact, but also the form, in which it is applied. The antimicrobial properties of NP have been shown to be much stronger than that of ZnO powder (Tayel et al., 2011). The larger surface to volume ratio is thought to provide a more efficient antibacterial activity (ZnO powder: ~5 mm vs. ZnO NP: ≤ 50 nm). However, the research on ZnO NP focuses more on its usage as a preservative agent and disinfectant in food industry, whereas ZnO powder is commonly used as feed additive in farm animals (Tayel et al., 2011; Xie et al., 2011).

In the present thesis, the inhibitory effect of ZnO against *C. coli* has been demonstrated *in vivo* and *in vitro*. The growth of *C. coli* could be significantly reduced after exposure of ≥ 2.6 mM ZnO in liquid media (**Figure 5-1)**. Moreover, the feeding of 3,100 mg ZnO/kg feed to pigs reduced the excretion of *C. coli* by 10-fold (**Figure 5-3 & Figure 5-4**). However, the process underlining

its antimicrobial effect is not clear yet. To make a better use of ZnO in pig production, it is necessary to understand the mechanism of action of ZnO against bacteria. Several studies have proposed that ZnO powder and ZnO NP increases the production of ROS, mainly H_2O_2 and singlet oxygen, which has been proven to be the main antimicrobial factor of ZnO (Yamamoto, 2001; Liu et al., 2009; Raghupathi et al., 2011; Xie et al., 2011). Zhang et al. (2007) suggested that chemical interactions between H_2O_2 and bacteria were the dominant mechanism for the antibacterial activity. ZnO is also believed to interact with specific bacterial cell compounds of the outer membrane (Tayel et al., 2011). It has further been reported that ZnO induces ROS generation by activation of the NADPH-oxidase of the electron transport chain (Kumar et al., 2011). ROS are highly active molecules and at high concentrations able to damage DNA, proteins and cell membranes, eventually leading to cell death. To address the molecular basis of ZnO action on *C. coli,* a set of genes involved in the general and oxidative stress response was selected for gene expression studies (**Figure 5-2**). Quantitative PCR analysis revealed a significant up-regulation of the *kat*A expression after ZnO exposure. KatA is the main peroxide stress defence protein in *Campylobacter* spp. The expression of *kat*A has been shown to be specifically inducible by $O_2{\cdot}-$ and H_2O_2 stress, whereas the superoxide dismutase B (*sod*B) expression is only inducible after $O_2{\cdot}-$ exposure (**see literature review**). However, while an up-regulation of the *kat*A expression after ZnO stress could be observed, no differential gene expression could be demonstrated for *sod*B. Thus, based on our findings and on those of Yamamoto et al. (2001), we conclude that ZnO induces the production of ROS, in particular H_2O_2, which leads to an up-regulation of the *kat*A expression. This agrees with the observation by Park (1999), demonstrating that the expression of *kat*A in *C. coli* could be induced after oxidative stress. An increase in the *kat*A expression, but not in the *sod*B expression was also observed by Xie et al. (2011) after ZnO NP treatment. In the presence of paraquat, a strong oxidising agent, the expression of *kat*A was up-regulated by far more as evidenced by ZnO stress, whereas the expression of *sod*B and *ahp*C was also unaffected. Similar results have been demonstrated for *C. jejuni* by Garenaux et al. (2008b). Interestingly, in *E. coli*, an enhanced superoxide dismutase activity did not lead to increased resistance to superoxide stress, but rather in increased susceptibility due to H_2O_2 accumulation (Scott et al., 1987). In another study, the expression of *ahp*C was increased by 7-fold after ZnO NP exposure, while in our study no significant expression difference for this gene could be observed (Xie et al., 2011). As ZnO NP seem to have a greater antibacterial impact than ZnO powder, the oxidative stress in our study may have not been strong enough to induce the expression of *ahp*C. A stronger antimicrobial impact of ZnO NP than ZnO powder on the oxidative stress response was also evidenced by quantitative differences in the *kat*A expression in both studies (52-fold vs. 5-fold). *Campylobacter* spp. only

possess three main detoxification enzymes to remove ROS. However, their regulation is rather complex. The regulators CosR, CrpRS and PerR co-ordinately regulate the expression of *kat*A, *sod*B and *ahp*C. These oxidative stress defence genes are controlled by all these regulons and seem to have overlapping and compensatory functions in detoxifying oxidants.

Another important outcome of this study was that the ZnO treatment leads to an increased expression of *clp*B, *gro*ES and *dna*K genes that are involved in the general and heat stress response. While the expression of these genes was unaffected by paraquat treatment, an up-regulation of the three genes was observed by heat stress (**Figure 5-2**). These findings led to the conclusion that ZnO triggers not only the oxidative, but also the general stress response in *C. coli*. Although *Campylobacter* spp. lack the general stress response sigma factor, there seems to be an overlap between the heat shock and the oxidative stress response in this pathogen. It has been shown that the DnaJ/K system is strongly up-regulated by heat stress and toxic chemicals, particularly heavy metals (Sharma et al., 2008). Moreover, oxidants have a broad impact on genes involved in a variety of biological pathways including classical oxidative stress defence systems, but also HSPs, DNA repair, metabolism, fatty acid biosynthesis and multidrug efflux pumps (Palyda et al., 2009).

A further important observation was demonstrated for the expression profiles of zinc influx and efflux transport systems in *C. coli* by Blume (2013). The expression of the ATPase high-affinity zinc uptake system *znu*ABC and the zinc efflux transporter *znt*A gene was almost unaffected after ZnO exposure. It has been demonstrated that the *znu*ABC transporter in *C. jejuni* is essential for growth in low-zinc environments and that this transporter is required for *Campylobacter* spp. survival in chickens in the presence of a normal microbiota (Davis et al., 2009; Gielda and DiRita, 2012). In contrast, in our study an excess of zinc was present in the media, thus, there was no need for the synthesis of a high affinity zinc transport system. Contrary, the ZntA efflux system is only expressed in the presence of excessive cytoplasmatic zinc (Clayton et al., 2011; Wang et al., 2012). Therefore, it can be hypothesised that (i) there was no increased transport of zinc into the cell, (ii) the zinc homeostasis was disturbed by the excess zinc concentration or (iii) other transport mechanisms might have been used. For the gene expression analysis, a concentration of 1.3 mM ZnO was applied. At this concentration only a slight reduction of *C. coli* cell counts was evidenced (**Figure 5-1**). Hence, is seems unlikely that this moderate zinc concentration overburdens the zinc homeostasis in *C. coli*. Therefore, it is more likely that the generation of ROS, particular H_2O_2, by ZnO, takes place at the cell surface rather than an uptake of zinc into the cell where it accumulates. Unlike the negatively charged hydroxyl radicals and superoxides, hydrogen peroxide is an uncharged molecule and able to penetrate into the bacterial cells. This hypothesis can be backed up by the observed up-regulation of the *kat*A expression, which indicates an enhanced need for the

removal of H_2O_2 molecules by ZnO stress. Thus, it would be interesting to analyse the expression profiles of the three main antioxidative enzymes after peroxide treatment.

Diverse pathogens deal with oxidative stress in distinct ways. There are many variations in the battery of enzymes bacteria can express to counteract ROS and differences in the mechanisms, by which expression of these enzymes are regulated. *Campylobacter* spp. lack some important oxidative stress response genes, such as *sox*RS and *oxy*R and the stationary-phase stress response gene *rpo*S, and are therefore extremely sensitive to oxidative stress as well as to other environmental stress factors. Moreover, bacterial transport systems for heavy metal resistances have been shown to differ between bacterial species (Silver, 1996; Nies, 2003). It seems that *Campylobacter* spp. are more susceptible to ZnO stress than other gram-negative foodborne pathogens like *Salmonella enterica* serovar Enteritidis and *E. coli* (Xie et. al., 2011). Therefore, the antimicrobial action of ZnO against *Campylobacter* spp. seems extremely effective.

It would be interesting to know, which other kind of mechanisms of zinc action are responsible for its antimicrobial activity against *C. coli.* The interaction of ZnO with cell membranes is also assumed (Liu et al, 2009; Tayel et al. 2011). The toxic effect of zinc is also attributed to competitive interaction between zinc and other metal-binding sites. PerR appears to be the major regulator of oxidative stress responses in *Campylobacter* spp. in a manner that both depend on and are independent of the presence of iron and/or H_2O_2 (Palyda et al., 2009). Thus, the expression of *kat*A and *ahp*C is iron-responsive and iron and zinc compete for the same binding sites and transporters. More mechanistic studies on the effects of zinc action on *C. coli* before practical application are needed.

Taken together, these results demonstrate that ZnO has a strong antimicrobial activity against *C. coli.* This is at least partly due to the production of ROS, in particular H_2O_2, presumably at the cell surface, which is then able to diffuse into the cell thereby, triggering the oxidative and general stress response.

Influence of *E. faecium* NCIMB 10415 on *C. coli* in pigs

Another promising pre-harvest intervention measure to reduce the gastrointestinal colonisation of *C. coli* in pigs is via the administration of selected probiotic bacteria. In the Eu there are currently three different groups authorised as feed additives in animal production; lactic acid bacteria (LAB), mainly *Enterococcus* spp., *Lactobacillus* spp. and *Bifidobacterium* spp., bacteria of the genus *Bacillus*, and yeast of the genus *Saccharomyces*. The prime mode of action of probiotics is to restore and/or maintain a beneficial intestinal microbial community in

order to improve the growth performance and the natural defence against pathogenic bacteria (Fuller, 1989; Simon, 2005; Baer et al., 2013). The mechanisms of probiotic action include, amongst others, the competition with pathogens for binding sites (competitive exclusion), competition for available nutrients and production of antimicrobial compounds e.g. organic acids (**see literature review**). To date, only a few studies have shown a possible role of probiotics in preventing the shedding of *Campylobacter* spp. on the primary production level, even though *in vitro* studies reported a strong antimicrobial activity of several probiotic strains against this pathogen (Morishita et al., 1997; Chaveerach et al., 2004; Santini et al., 2010). Species of the genus *Enterococcus* are regarded as normal components of the intestinal microbiota of pigs (Devriese et al., 1994). Many strains of *E. faecium* exist and some of which have been documented with health beneficial effects. A beneficial strain of *E. faecium*, designated as NCIMB 10415, has been used as a probiotic for many years in humans, farm and companion animals (Franz et al., 2003). In pigs, the dietary supplementation with *E. faecium* NCIMB 10415 has been shown to improve the performance during the post-weaning period (Zeyner and Boldt, 2006). Growth-promoting effects have been attributed to improved body weight gain and decreased incidence of diarrhoea, mainly by reducing the gastrointestinal concentration of bacterial pathogens (Pollmann et al., 2005; Taras et al., 2006). A modulation of the intestinal microbiota composition by *E. faecium* NCIMB 10415 feeding has been documented (Vahjen et al., 2007). The potential use of probiotics, in particular *E. faecium* NCIMB 10415, as a strategy to reduce the *Campylobacter* spp. level in pigs, has to our knowledge, not previously been reported, but feeding of probiotic preparations including *E. faecium* have been shown to reduce the colonisation of *C. jejuni* in chickens (Willis and Reid, 2008; Ghareeb et al., 2012). Therefore, within the framework of the *C. coli* colonisation trial (**see Chapter 4 & 6**), dietary supplementation of the commercially available probiotic strain *E. faecium* NCIMB 10415 (Cylactin®) was tested as feed additive for its antagonistic activity against *C. coli* in pigs. The competitive exclusion mechanisms of probiotics are a prophylactic measure that aim to increase the resistance of pathogen colonisation. In pigs, *C. coli* colonisation occurs early in life. In order to prevent the colonisation of *C. coli*, it is important to establish a microbiota for competitive exclusion as early as possible, since displacing established species later might be less effective (Horrocks et al., 2009). Hence, *E. faecium* NCIMB 10415 was already applied to the sows during the gestation period until the day of weaning and offered to the piglets during weaning. Nevertheless, it turned out that all piglets were naturally colonised with *C. coli* before experimental inoculation with *C. coli* 5981. Thus, we were able to test if the probiotic bacterium *E. faecium* NCIMB 10415 could reduce the *C. coli* load in naturally and experimentally colonised pigs. The results demonstrated that *E. faecium* NCIMB 10415 supplementation did not affect the *C. coli* excretion levels in pigs.

Neither the excretion levels of naturally colonised *C. coli* could be reduced nor could the colonisation of the inoculation strain *C. coli* 5981 be prevented by the probiotic feeding of pigs (**Figure 6-2A & 6-2B**). In addition, a trend towards increased *C. coli* excretion levels was detectable in the probiotic treated animals, although no significant differences could be observed. In dogs, increased *Campylobacter* spp. counts and enhanced adhesion of *C. jejuni* by *E. faecium* NCIMB 10415 supplementation has been reported (Rinkinen et al., 2003; Vahjen and Manner, 2003). It is important to note that in animals *Campylobacter* spp. colonisation almost always has no clinical outcome, but enhanced *Campylobacter* spp. colonisation in farm and companion animals is a potential risk for promoting human infection. Further, an increase of other gram-negative intestinal pathogens in piglets fed *E. faecium* NCIMB 10415 was reported by Kreuzer et al. (2012). In an *in vitro* study, no inhibitory effect of the 56 tested *Enterococcus* isolates against any of the ten *C. jejuni/C. coli* strains has been shown (Chaveerach et al., 2004). Reasons for the absence of a negative impact of *E. faecium* NCIMB 10415 on the *C. coli* growth might be due to the occupation of different niches within the GIT and the utilisation of other nutritional compounds of both bacteria. The main habitat for LAB is the large intestine as undigested carbohydrates are mainly fermented in this intestinal segment. LAB are able to metabolise these compounds as energy source by producing organic acids as metabolic products (Lalles et al., 2007). Although the main colonisation site of *C. coli* is the large intestine as well (**see Chapter 4),** they are unable to ferment sugars and growth relies on organic and amino acids. It can be hypothesised that *E. faecium* NCIMB 10415 supplementation might have even promoted the growth of *C. coli* as their metabolic products serve as energy and carbon source for *Campylobacter* spp. In addition, *Campylobacter* spp. preferentially reside in the mucus layer, whereas *E. faecium* seems to exhibit a relatively low level of adhesion to the intestinal mucus membrane lining the GIT (Takata et al., 1992; Rinkinen et al., 2000). Thus, both bacterial groups seem to occupy different niches and may not come in close contact with each other. Although the production of a class IIb bacteriocin by *E. faecium* NCIMB 10415 is known, no antagonistic activity against *C. coli* was detectable (Foulquie Moreno et al., 2003). In a review by Svetoch and Stern (2010) it has been summarised that it was not possible to control *Campylobacter* spp. colonisation in birds with live bacterial isolates, but with bacteriocins produced by the same bacteria. However, no antagonistic activity of *E. faecium* NCIMB 10415 by the production of either organic acid or bacteriocins against *Campylobacter* spp. could be observed *in vitro* (**Table 6-1**).

Reports on the efficacy of *E. faecium* NCIMB 10415 feeding on the growth performance of pigs are somewhat limited and often contradictory and inconclusive. In the present study, no effects regarding improved body weight gain or feed intake in the *E. faecium* NCIMB 10415 treated group was evidenced. This is consistent with other studies, in which *E. faecium*

supplementation did not affect the growth of piglets (Pollmann et al., 1980; Broom et al., 2006; Chen et al., 2006; Martin et al., 2012). On the other hand, other researchers reported an increased growth performance, decreased incidence of diarrhoea and post-weaning mortality (Maeng et al., 1989; Taras et al., 2006, Zeyner and Boldt, 2006). However, differences of probiotic responses have been shown to be related to age and to the husbandry conditions (Broom et al., 2006; Chen et al., 2006). Since controlled housing conditions were used in our experiment and all pigs remained healthy throughout the experiment, probiotics cannot contribute to even healthier animals. It has often been postulated that growth promotional compounds become more effective as the environmental and nutritional challenges confronting the animal become worse. Thus, the prevailing conditions in the present study may not have been severe enough to analyse the influence of *E. faecium* NCIMB 10415 on pig performance. Moreover, the number of animals used in the present study was not high enough to draw a final conclusion about the impact of *E. faecium* feeding on the growth performance of weaned piglets. Field trials with higher animal numbers reared under production conditions are necessary to determine possible beneficial effects of *E. faecium* on piglets. Moreover, the beneficial effects of probiotics are dependent on the number of live bacteria that transiently colonise the GI tract. Probiotics must be able to survive the low pH and digestive enzymes of the proximal section of the GIT. Even though *E. faecium* NCIMB 10415 was applied in a microencapsulated form, the number of cells may not have been sufficient for probiotic effects. Although enumeration of excreted *E. faecium* was not performed in our study, it has been reported that the *E. faecium* NCIMB 10415 concentration did not exceed 10^5 to 10^6 cfu/g digesta (Macha et al., 2004; Simon, 2010). An interesting description regarding the mode of action of probiotics was given by Abbott et al. (2004), who concluded that even when probiotics seem to work, we know too little about the normal gut ecosystem to understand why. Oelschlaeger (2010) mentioned that it has to be taken into account the low number of probiotic bacteria administered with a daily dose of 10^9–10^{10} compared to 10^{14} bacteria residing in the colon, it is to some extent surprising that there are effects observable at all.

In this study, we were able to show that *E. faecium* NCIMB 10415 feeding has no significant impact on the excreted *C. coli* cell numbers in weaned piglets. Therefore, we conclude that *E. faecium* NCIMB 10415 is not a suitable probiotic bacterium to reduce or prevent the colonisation of *C. coli* in pigs. Competitive exclusion by intestinal bacteria is based on a bacterium-to-bacterium interaction mediated by competition for available nutrients, mucosal adhesion sites and secretion of antimicrobial compounds. For the effective application of a probiotic to competitively exclude pathogenic bacteria, it is important to understand its microbial ecology and physiology. More research is needed in finding new probiotic strains that

are capable of colonising the gut of pigs with the final aim of reducing the load of intestinal *Campylobacter* spp. at the farm level.

Conclusion and Perspectives

The present thesis demonstrates that the entire GIT as well as lymphatic organs of pigs constitute substantial reservoirs for potential *Campylobacter* spp. carcass cross-contamination during slaughter. This implies the importance of hygienic precaution measures to reduce the *C. coli* load in pigs. In the present work two feed additives were tested as pre-harvest intervention strategy to reduce the *C. coli* load in weaned piglets. The feed of weaned pigs was either supplemented with the inorganic trace element zinc, applied as zinc oxide, or the probiotic strain *E. faecium* NCIMB 10415 for four weeks. The following conclusions were drawn from our studies: Faecal *C. coli* counts were not markedly affected by the *E. faecium* NCIMB 10415 treatment, but the excretion of *C. coli* was significantly reduced by 10-fold in piglets fed high ZnO levels compared to piglets that received a low and medium ZnO diet. A short-term dietary supplementation of pharmacological ZnO concentrations to pigs before slaughter seems to be a promising tool, which might lead to a lower pathogen contamination risk at slaughter and this may help to enhance the food safety of pork for human consumption. However, there are still many hurdles to overcome the *Campylobacter* spp. colonisation in pigs and probably several strategies will have to be combined to efficiently eradicate this human pathogen from pig herds.

Chapter 8: Summary/Zusammenfassung

Summary

Title of the PhD thesis: Effects of antimicrobial feed additives as pre-harvest intervention measure to reduce *Campylobacter coli* in pigs

The main objective of the present thesis was to investigate the impact of the dietary supplementation of the probiotic *Enterococcus faecium* (*E. faecium*) NCIMB 10415 and different zinc oxide (ZnO) concentrations on the *Campylobacter* (*C.*) *coli* burden in pigs (**Chapter 3**).

Chapter 2 provides a detailed literature review that emphasises the importance of pigs as a reservoir for *Campylobacter* spp. and thus, for possible human *Campylobacter* infections. Moreover, this chapter introduces possible pre-harvest intervention strategies to reduce the *C. coli* load in pigs for improved veterinary public health: *Campylobacter* spp. are a major cause of foodborne diarrhoeal disease in humans worldwide. Thermophilic *Campylobacter* spp. have their natural reservoir in warm-blooded animals with *C. coli* as the dominant species in pigs. The prevalence of *C. coli* in pig herds varies between 50 and 100% with excretion rates of up to 10^7 cfu/g faeces. Several studies showed that up to 10% of pork meat and pork products at retail are contaminated with *Campylobacter* spp. Moreover, *C. coli* accounts for 5–10% of the human *Campylobacter* infections. Thus, during the slaughter process, *Campylobacter* spp. can be transmitted to the carcass and subsequently to humans through the consumption of contaminated pork. Hence, there is an urgent need for proper intervention measures to reduce human *C. coli* infections. As *Campylobacter* spp. do not grow outside the host, a decreased contamination of *Campylobacter* spp. of the final product is best achieved if the colonisation in live animal can be prevented or reduced. The administration of antimicrobial feed additives as pre-harvest intervention measure are currently being intensively explored.

In the first step of the presented thesis, a *Campylobacter* spp. colonisation model in pigs was established. It enabled the examination of the *C. coli* colonisation of different sites within the pig gut which might serve as risk factors for the carcass contamination during slaughter (**Chapter 4**). Therefore, pigs were experimentally inoculated with the porcine *C. coli* 5981 strain. Then, the *Campylobacter* spp. excretion dynamics, the *C. coli* colonisation rates within different gut sections along the gastrointestinal tract and the translocation to extra-gastrointestinal sites was studied. Based on this colonisation model, the impact of the dietary supplementation of different ZnO concentrations and the probiotic *E. faecium* NCIMB 10415 to reduce the faecal *C. coli* load was tested in weaned piglets (**Chapter 5 & 6**). The diet of

weaned piglets was supplemented with three levels of ZnO (40, 100 and 3,100 mg ZnO/kg feed) and with two levels of *E. faecium* NCIMB 10415 (0 and 10^9 cfu/kg feed). In both feeding trials, all piglets were naturally colonised with *C. coli* with excretion rates of 10^3–10^8 cfu/g faeces. The results of the colonisation trial revealed that the entire gastrointestinal tract, but also gut-associated lymphatic tissues constitute big reservoirs for *C. coli* and serve as potential reservoirs for the carcass cross-contamination during slaughter. Furthermore, we were able to demonstrate that only the dietary supplementation of 3,100 mg ZnO/kg feed, but not *E. faecium*, led to significant reduced faecal *C. coli* counts in pigs. Supplementation of the post-weaning diet with this pharmacological dose significantly reduced the *C. coli* excretion level by 10-fold within one week ($p = 0.001$) compared to piglets that received a low and medium ZnO diet. The reduction of *C. coli* excretion by high ZnO has been shown to be a transient phenomenon and only lasts as long as the ZnO feeding remains high. Reducing the pharmacological concentration of 3,100 mg ZnO/kg feed to the physiological requirement of 100 mg ZnO/kg feed led to increased *C. coli* numbers within a few days again. A 10-fold increased zinc concentration in the faeces of pigs that were dietary supplemented with 3.100 mg ZnO/kg feed was measured compared to the control groups. Despite the negative impact on the *C. coli* growth in pigs fed a high ZnO concentration, several side effects must be considered: The possible elevated zinc concentration in meat and the increased zinc excretion into the environment.

By *in vitro* growth inhibition assays a high susceptibility of *C. coli* against ZnO could also be observed. At concentrations ≥ 2.6 mM ZnO a decline in cell numbers occurred. It turned out that the antimicrobial activity is caused, at least partly, by the production of reactive oxygen species. The expression of catalase A, the main peroxide stress defence protein in *Campylobacter* spp. was up-regulated by 5-fold after ZnO treatment. Besides the activation of the oxidative stress response, the general- and heat stress response was also triggered. The expression of the chaperones *clp*B, *gro*ES and *dna*K was up-regulated after ZnO exposure by 2.2-, 2.5- and 2.6-fold, respectively. In contrast, the expression of the zinc influx transporter *znu*ABC and zinc efflux transporter *znt*A were almost unaffected after ZnO stress.

In conclusion, supplementation of high dietary ZnO, but not *E. faecium* NCIMB 10415 significantly reduced the *C. coli* load in weaned piglets. A short-term dietary administration of 3,100 mg ZnO/kg feed to pigs before slaughter seems to be a promising intervention measure to reduce the *C. coli* load. This reduction might further lead to reduced *Campylobacter* spp. contamination rates of pork and subsequently to a reduced incidence of *C. coli*.

Zusammenfassung

Titel der Dissertation: Wirkung antibakterieller Futterzusatzstoffe als pre-harvest-Interventionsmaßnahme zur Reduktion von *Campylobacter coli* im Schwein

Das Hauptziel der vorliegenden Arbeit war es, den Einsatz des Probiotikums *Enterococcus faecium* (*E. faecium*) NCIMB 10415 sowie verschiedener Zinkoxid (ZnO)-Konzentrationen als Futterzusatzstoffe zur Reduktion der *Campylobacter* (*C.*) *coli*-Belastung im Schwein zu untersuchen (**Kapitel 3**).

Kapitel 2 stellt eine umfassende Literaturübersicht zur Bedeutung des Schweins als Reservoir für *Campylobacter* spp., und somit für mögliche humane *Campylobacter*-Infektionen dar. Darüber hinaus werden in diesem Kapitel sog. pre-harvest-Interventionsmaßnahmen zur Reduktion der *C. coli*-Belastung im Schwein zur Verbesserung des gesundheitlichen Verbraucherschutzes vorgestellt: *Campylobacter* spp. sind eine der Hauptursachen von humanen lebensmittelbedingten Durchfallerkrankungen weltweit. Thermophile *Campylobacter* spp. haben ihr natürliches Reservoir in warmblütigen Tieren, wobei *C. coli* im Schwein dominiert. Die Prävalenz von *C. coli* in Schweinebeständen variiert zwischen 50 und 100%, mit Ausscheidungsraten von bis zu 10^7 KbE/g Fäzes. Zahlreiche Studien zeigen, dass bis zu 10% des Schweinefleisches und der Schweinefleischprodukte im Einzelhandel mit *Campylobacter* spp. kontaminiert sind. Des Weiteren werden 5–10% aller *Campylobacter*-Infektionen durch *C. coli* verursacht. Beim Schlachtprozess können *Campylobacter* spp. auf die Karkassen übergehen und anschließend durch den Verzehr von kontaminiertem Schweinefleisch auf den Menschen übertragen werden. Aus diesem Grund besteht eine dringende Notwendigkeit, mit Hilfe geeigneter Interventionsmaßnahmen die humanen *C. coli*-Infektionen zu reduzieren. Da *Campylobacter* spp. sich nicht außerhalb des Wirts vermehren können, wird eine verringerte Kontamination des Endprodukts am besten erreicht, wenn die Kolonisation bereits im lebenden Tier vermieden oder verringert werden kann. Als pre-harvest-Interventionsmaßnahme wird die Verabreichung antibakterieller Futterzusatzstoffe derzeit intensiv erforscht.

Zu Beginn dieser Arbeit wurde ein *Campylobacter*-Kolonisationsmodell entwickelt. Dieses diente zur Untersuchung der *C. coli*-Belastung in verschiedenen Körperabschnitten des Schweins, welche während des Schlachtens als mögliche Risikofaktoren bei der Kontamination der Karkassen dienen (**Kapitel 4**). Dazu wurden die Tiere mit dem porcinen *C. coli* 5981-Stamm experimentell inokuliert. Anschließend wurden die Ausscheidungsraten, die Kolonisationsraten der verschiedenen Magen-Darm-Abschnitte sowie die

Translokationsraten von *C. coli* zu extra-intestinalem Gewebe untersucht. Auf diesem Kolonisationsmodell basierend wurde die Wirkung verschiedener ZnO-Konzentrationen sowie die des Probiotikums *E. faecium* NCIMB 10415 als Futterzusatzstoffe zur Reduktion der *C. coli*-Zellzahl in abgesetzten Ferkeln getestet (**Kapitel 5 & 6**). Dem Futter der Ferkel wurden drei ZnO-Konzentrationen (40, 100 und 3.100 mg ZnO/kg Futter) und zwei *E. faecium* NCIMB 10415-Konzentrationen (0 und 10^9 KbE/kg Futter) zugesetzt. In beiden Fütterungsversuchen waren alle entwöhnten Ferkel bereits auf natürliche Weise mit *C. coli*, mit Ausscheidungsraten von 10^3–10^8 KbE/g Fäzes, besiedelt. Die Ergebnisse dieser Studien zeigten, dass die Kolonisation des gesamten Magen-Darm-Trakts, aber auch Darm-assoziiertes lymphatisches Gewebe beträchtliche Reservoire für *C. coli*, und somit für die Kreuzkontamination der Karkassen während des Schlachtens darstellen. Darüber hinaus konnte gezeigt werden, dass nur die Verabreichung von 3.100 mg ZnO/kg Futter, nicht jedoch von *E. faecium*, zu einer signifikanten *C. coli*-Reduktion im Fäzes, führte. Die Verfütterung der pharmakologischen ZnO-Dosis reduzierte die Ausscheidung von *C. coli* innerhalb einer Woche um ein 10-faches ($p = 0{,}001$) im Vergleich zu Ferkeln, die eine niedrige und mittlere ZnO-Diät erhielten. Diese Reduktion stellte jedoch nur ein vorübergehendes Phänomen dar und hielt nur so lange an, wie die Verfütterung der hohen ZnO-Konzentration erfolgte. Die Reduktion der pharmakologischen Konzentration von 3.100 mg ZnO/kg Futter auf den physiologischen Bedarfswert von 100 mg ZnO/kg Futter, führte zu einen Anstieg der ursprünglichen *C. coli*-Zellzahl innerhalb weniger Tage. Im Vergleich zu den Kontrollgruppen wurde im Fäzes von Tieren in der Gruppe der hoher ZnO-Konzentration eine 10-fach erhöhte Zink-Exkretion gemessen. Trotz des negativen Einflusses der hohen ZnO-Konzentration auf die *C. coli*-Zellzahl müssen einige Nebenwirkungen bei der praktischen Anwendung berücksichtigt werden: Eine mögliche Erhöhung der Zink-Konzentration im Fleisch und eine erhöhte Zink-Ausscheidung in die Umwelt.

Eine starke Sensibilität von *C. coli* gegenüber ZnO konnte auch *in vitro* mit Hilfe von Wachstumsversuchen beobachtet werden. Bei Konzentrationen ≥ 2,6 mM ZnO wurden bereits verringerte *C. coli*-Zellzahlen detektiert. Die antibakterielle Aktivität konnte, zumindest teilweise, auf die Produktion von reaktiven Sauerstoffspezies zurückgeführt werden. Die Expression von Katalase A, die wichtigste Peroxidase von *Campylobacter* spp., war durch die ZnO-Behandlung um ein 5-faches erhöht. Neben der Aktivierung der oxidativen Stressantwort wurde auch die Allgemeine Stressantwort und die Hitzestress-Antwort ausgelöst. Die Expression der Chaperone *clp*B, *gro*ES und *dna*K wurde durch die ZnO-Exposition um ein 2,2-, 2,5- bzw. 2,6-faches hochreguliert. Im Gegensatz dazu konnte kein Unterschied bei der Expression des Zink-Influx-Transporters *znu*ABC und des Zink-Efflux-Transporters *znt*A nach ZnO-Stress festgestellt werden.

Zusammenfassend lässt sich sagen, dass die Verfütterung der pharmakologische ZnO-Konzentration, nicht jedoch die des Probiotikums *E. faecium* NCIMB 10415, zu einer signifikanten Reduktion der *C. coli*-Belastung im Schwein führte. Eine kurzzeitige Verfütterung von 3.100 mg ZnO/kg Futter an Schweine kurz vor der Schlachtung könnte eine vielversprechende pre-harvest-Interventionsmaßnahme zur Reduktion von *C. coli* darstellen. Diese könnte zu einer verminderten Kontaminationsrate von Schweinefleisch und somit zu einer verringerten *C. coli*-Inzidenz beim Menschen beitragen.

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Katharina Bratz