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

Analyse der In-vitro- und In-vivo-Effekte von Probiotika gegen Campylobacter spp.

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Summary

Campylobacter (C.) spp. are well recognised as the leading cause of bacterial food-borne 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* spp. 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., *C. coli*, probiotics, *Enterococcus faecium*, pig

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 im Tierversuch ausgewählt. Dafür wurden 20 abgesetzte Ferkel auf zwei Gruppen aufgeteilt, einer probiotischen Gruppe und einer Kontrollgruppe. Der probiotischen 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 antagonistische 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

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

TABLE 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 10415w	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; NCTC: National Collection of Type Cultures; *B.: Bifidobacterium* spp.; *L.: Lactobacillus* spp.; n = 3

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.

Material and Methods

Bacterial strains and culture conditions

In total, 26 probiotic strains were used in this study (Tab. 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. 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 twelve 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 x 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.

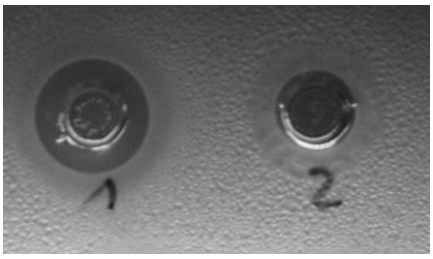

FIGURE 1: *Example of an inhibition zone with the well-diffusion agar assay. Left spot shows a clear inhibition zone produced by the cell-free culture supernatant of the probiotic strain Lactobacillus fermentum (1) and right spot shows a turbid zone of the pH neutralised cell-free culture supernatant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

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

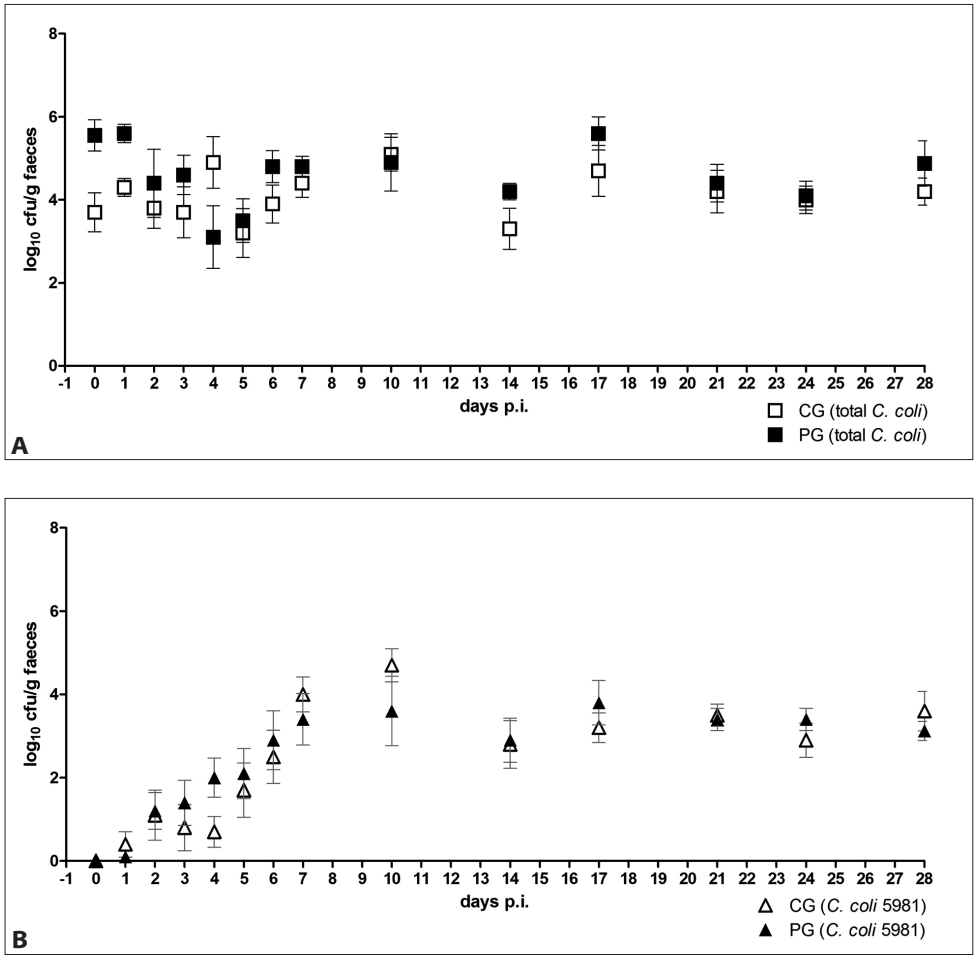

FIGURE 2: *Course of faecal excretion of total C. coli (A) and C. coli 5981 alone (B) following oral inoculation with C. coli 5981 of weaned piglets over 28 days with or without E. faecium NCIMB 10415 supplementation. Results are expressed as log_{10} of colony forming units (cfu) per gram faeces determined 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).*

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 non-parametric Mann-Whitney-test. Differences were considered significant at $p < 0.05$.

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* spp. strains (Table 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. 2A. and 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. 2A). For the inoculation *C. coli* 5981 strain the highest colonisation level was reached ten days p.i. in both groups (Fig. 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.

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 strains. 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 decreased 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.

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