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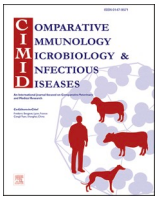

Investigation on the colonisation of *Campylobacter* strains in the pig intestine depending on available metabolites

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ABSTRACT

Campylobacter (C.) spp. represent one of the most important causes for food-borne bacterial pathogen in humans worldwide. The aim of this study was to investigate metabolic requirements of two *Campylobacter* strains of different species based on substrate utilisation (in vitro*)*. Based on these results, a correlation between the colonisation and the available substrates in different intestinal sections was recorded using an animal model. *Campylobacter coli* (ST-5777) and *C. jejuni* (ST-122) were used to inoculate 16 pigs, respectively, and one group of 16 pigs was used as control. The strains differed significantly in substrate utilisation - *C. coli* was able to metabolise various substrates (acetate, asparagine, serine, fucose, and propionate), while *C. jejuni* only utilised serine. Metabolomic analysis of intestinal content from different gut sections showed the presence of all previously tested metabolites, except for fucose. A significantly larger amount of glucose was found in the jejunum of those pigs infected with *C. coli*, while neither strain utilised it in vitro. The analysis of the intestinal contents revealed a very low proportion of *Campylobacterales* in the total microbiome, suggesting that the small percentage of the inoculated *Campylobacter* strains in the gut microflora of the animals is too low to cause differences between the control and infected groups in the composition of the metabolome. Nevertheless, knowledge of specific nutritional requirements of the pathogens combined with proof of different metabolites in the intestinal segments may provide clues about the site of colonisation in the host and improve our understanding of this zoonotic germ.

1. Introduction

Campylobacter (C.) jejuni and *C. coli,* which both belong to the ε-Proteobacteria [1], caused the majority of food-borne illnesses in Germany in 2019 [2], and represent major food-borne pathogens in many developing and industrialised countries [3–5]. The main symptoms are diarrhoea and enteritis [6], which are usually self-limiting. However, about 10 % of the patients develop severe courses [7]. The majority of human illnesses are caused by *C. jejuni* (73%) via undercooked poultry meat, while *C. coli* (10 %) is mainly transmitted via pork [2]. *Campylobacter* spp., especially *C. coli*, could be detected in up to 100 % of pig herds in Germany [8] and is considered a commensal here and also in many other farm and domestic animals [9]. Previous studies showed that different *Campylobacter* strains have different colonisation potentials. Some are host-specific, while other strains can colonise many different hosts [10,11]. The exact pathogenesis for host adaptation is still unclear. One approach to understanding which physiological characteristics enable bacteria to occupy different microbiological niches and adapt to changing environmental conditions is to examine the metabolic pathways of specific substrates [12]. The correlation between growth factor, nutritional requirements, and available substrates in the guts of different species may provide an explanation for the

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outcome of an infection.

There are several reports on the metabolism of different substrates by *Campylobacter* strains, especially *C. jejuni* [13,14]. These showed that most *C. jejuni* cannot utilise glucose and therefore have to rely on free amino and keto acids from the intestinal digesta for growth [15]. In addition, serine seems to be necessary for colonisation of the intestine [16,17]. A high variability in the metabolic properties of *C. coli* strains was described in previous studies. While some strains are unable to metabolise glucose [18], as described for *C. jejuni*, a sub-set of *C. coli* isolates use glucose as a growth substrate [19]. Wagley et al. [20] also describe the ability of *C. coli* to utilise fucose as an important fitness factor for colonisation in the pig intestine. Hofreuter et al. concluded that the capacity to utilise specific nutrients enables *Campylobacter* strains to colonise specific tissues [14]. According to the results of a previous study, the two different strains seemed to differ in the colonisation site of the porcine gut, with *C. jejuni* appearing to prefer the anterior intestinal tract, while *C. coli* colonised the caecum and colon [21]. In addition, animal species-specific differences in the proportion of *Campylobacter* spp. in the total microbiome were reported. While the proportion was rather low in pigs (1 %) [22], higher proportions were observed in poultry (10 %) [23].

The reason for different host preferences is still not clear. In order to understand the colonisation behaviour of *Campylobacter* spp., the colonisation sites of the two different *Campylobacter* strains (*C. coli*, *C. jejuni*) in the porcine intestine and the influence of the metabolome on colonisation in different intestinal sections were investigated in the presented study. Findings were related to in vitro substrate utilisation of both strains. Additionally, the proportion of *Campylobacterales* in the total microflora of the intestinal sections was determined.

2. Material and methods

2.1. Campylobacter strains and inoculum preparation

Two *Campylobacter* strains, *C. coli* ST 5777 and *C. jejuni* ST 122, originally isolated from poultry were selected for both in vitro and in vivo experiments. The sequence types (ST) were previously associated with outbreaks of gastrointestinal disease in humans [24]. Both strains had been successfully used in a previous animal infection study [21]. *Campylobacter coli* ST-5777 expressed resistance against nalidixic acid and *C. jejuni* ST 122 against streptomycin, enabling to differentiate between them during culturing.

For inoculation, nutrient broth (nutrient broth no. 2, CM0067, OXOID/ Thermo Fisher Scientific, Inc., Waltham, MA, USA) was inoculated with bacterial colonies of the respective *Campylobacter* strains and incubated in a microaerophilic atmosphere (5 % O_2, 10 % CO_2, and 85 % N_2) with Thermo Scientific™ Oxoid™ CampyGen™ (Thermo Fisher Scientific, Inc., Waltham, Massachusetts, USA) at 37.4 °C for 48 h. An infectious dose of 10^8 colony-forming units (cfu) per animal in 10 mL broth was targeted. The actual infectious dose was determined subsequently by a serial decimal dilution series.

2.2. Animal infection model

A total of 48 (23 male, 25 female) piglets of a cross breed ((Danish Landrace x German Large White) x German Landrace) were used for the experiment. They were conceived by Caesarean section to exclude environmental *Campylobacter* colonisation, and reared in isolation units. The pigs were housed and handled in accordance with the German Animal Welfare Act, which meets the criteria of the German Research Foundation and the EU Directive 2010/63/EU for animal experiments. CulinaMilk® (H. Bröring GmbH & Co. KG, Dinklage, Germany) was used as milk replacer. After weaning in the fifth week of life, the pigs were fed twice daily with a conventional rearing diet (Primo Pro, gran., 30 g/kg body weight, Deuka GmbH & Co. KG, Düsseldorf, Germany; see also Table 1) and had access to drinking water ad libitum. The piglets were acquired and raised *Campylobacter* free as previously described [25]. They underwent daily monitoring, including evaluation of the general health conditions and rectal measurement of body temperature. Before inoculation, rectal swabs were taken on a weekly basis to confirm a *Campylobacter*-negative status. At the age of seven weeks, the piglets were randomly divided into three groups (Table 2).

Table 1
Composition of the feed used in this study (Primo Pro, gran., Deuka GmbH & Co. KG.).

Ingredients	Proportion in feed (%)
Crude protein	17.0
Crude fat	3
Crude fibre	4
Crude ash	4.5
Calcium	0.75
Phosphorus	0.5
Natrium	0.2
Lysine	1.2
Methionine	0.36
Metabolic energy (megajoule kg^{-1})	13.40

For inoculation, the animals were anaesthetised with azaperone (2 mg/kg body weight Stresnil®, ElancoTM, Elanco Inc., Greenfield, IN, USA) and ketamine (20 mg/kg body weight Ketamin®, CP-Pharma®, CP-Pharma Handelsgesellschaft GmbH, Burgdorf, Germany), and inoculated via a stomach tube (CH 12, B. Braun Melsungen AG, Melsungen, Germany) as previously described [25] (Table 2). Hereafter, the control group is referred to as Group 0, the group infected with *C. coli* as Group 1, and the group infected with *C. jejuni* as Group 2.

Rectal swabs were taken on a weekly basis to identify the animals excreting the respective *Campylobacter* strains and to prove the negative status of the control animals. Four weeks after inoculation, all pigs (average body weight of 27.8 +/- 6.4 kg) were stunned and exsanguinated via throat cut before performing necropsy.

2.3. Sampling and cultivation of Campylobacter strains from faeces and intestinal content

Digesta samples were taken from defined locations of the intestinal segments. Specimens were taken from the middle of the duodenum and ileum, from the third metre of the jejunum, and from the tip of the caecum and the conical colon. For the samples from the duodenum and ileum, the difficulty arose that there was only a small amount of content in single pigs. Therefore, no quantitative determination of the duodenum was carried out and a smaller number of samples from the ileum were investigated. To detect and quantify *Campylobacter*, faeces and ingesta samples were incubated on *Campylobacter* selective charcoal cefoperazone deoxycholate agar (CCDA) plates (Thermo Fisher Scientific, Inc., Waltham, MA, USA) in a microaerobic atmosphere (5 % O_2, 10 % CO_2, and 85 % N_2) with Thermo Fisher Scientific™ Oxoid™ CampyGen™ (Thermo Fisher Scientific, Inc., Waltham, Massachusetts, USA) at 37.4 °C for 48 h. To distinguish between the strains after inoculation, CCDA was supplemented with the corresponding antibiotics (nalidixic acid or streptomycin; detailed ingredients can be found in Tables S1–S3). Additionally, Preston broth was used to enrich samples at

Table 2
Infection scheme of the animal groups.

Group	Number of Piglets	Inoculation	Inoculation Dose/10 mL (cfu)
0	16	10 mL nutrient broth	0
1	16	10 mL nutrient broth with *C. coli* ST 5777	8.5×10^8
2	16	10 mL nutrient broth with *C. jejuni* ST 122	5×10^9

37.5 °C in a microaerobic atmosphere for 48 h prior to incubation on CCDA plates for the detecting low levels of *Campylobacter*. To quantify the amount of *Campylobacter* within a sample or the inoculum, a serial decimal dilution was carried out using phosphate buffered saline (PBS) as a diluent. Each dilution step was spread onto CCDA plates in duplicates and incubated as described above. After 48 h, colonies were counted and the concentration (cfu/mL) was calculated according to the standard protocol (ISO/TS 10272–2:2006). In addition, randomly selected rectal swabs and ingesta samples from Group 1 and 2 were spread on the CCDA agar enriched with the other antibiotic in order to exclude cross-contamination.

2.4. Metabolomic analysis of the intestinal content

2.4.1. Preparation of intestinal content

For the metabolome analysis, intestinal contents were taken from the jejunum, ileum, caecum, and colon during dissection. Since the ileum of individual pigs had too little content, it was not possible to examine ileal samples from these animals. The ingesta samples were frozen at − 80 °C and stored for further processing. Before shipment, the samples were thawed on ice and 300 mg of each sample was poured into a 2 mL SafeSeal Reagent Vial (SARSTEDT AG & Co. KG, Nümbrecht, Germany, REF 72.695.400). Subsequently, 1 mL of distilled water was added, the samples were mixed on a vortex mixer for 30 s and then centrifuged (10,000× g, 0 °C, 10 min). The supernatant was removed, filter sterilised through a Filtropur S (SARSTEDT AG & Co. KG, Nümbrecht, Germany, No. 83.1826.001) into an Eppendorf reaction vessel, and stored at − 80 °C until shipment to the Institute of Biochemistry, University of Greifswald, Germany for analysis.

2.4.2. ^{1}H NMR (nuclear magnetic resonance) spectroscopic

^{1}H NMR analysis and quantification were performed as previously described, with minor modifications [26]. In brief, 400 μL of samples were mixed with 200 μL of 0.2 mol/L sodium hydrogen phosphate buffer solution, made up with 30 % D_2O (Euriso-Top, St-Aubin Cedex, France) and containing 1.74 mmol/L 3-trimethylsilyl-(2,2,3,3-D4)− 1-propionic acid (TSP) (Sigma-Aldrich, Inc., St. Louis, MO, USA) and centrifuged at 13,000 rpm and 4 °C. Analyses of the supernatants were performed in 5 mm glass tubes (103.5 mm length, Bruker Biospin GmbH, Rheinstetten, Germany) at 300 K. The Bruker AVANCE-NEO 600 NMR spectrometer equipped with a SampleJet autosampler and a 5 mm QCI cryo probe was operated by TOPSPIN 4.0.6 software (Bruker Biospin GmbH, Rheinstetten, Germany). AMIX Viewer 3.9.15 software (Bruker Biospin GmbH, Rheinstetten, Germany) was used for metabolite identification and quantification. Spectra were aligned by calibration of the signal of TSP to 0.0 ppm. Identification of metabolites was made by comparison of signals to spectra of pure compounds from an in-house library. Integrals of metabolite peaks were compared to the integral of the ERETIC signal which was generated by using external calibration with the ERETIC quantification tool based on PULCON (Wider and Dreier 2006) for absolute quantification. The underground removal tool of AMIX was used to reduce background signals before quantification. In this investigation, short-chain fatty acids were detected. The resulting metabolite concentrations were adjusted to the average dry matter content of the intestinal section and subsequently recorded as mmol/kg DM.

2.4.3. Gas chromatography-mass spectrometry (GC-MS)

Internal standard solution consisting of stable isotopic labelled compounds (see Supplementary Table S4) was added to 100 μL of each sample. Samples were frozen at − 80 °C and lyophilized. Derivatization of dry samples for GC-MS analysis was done as previously described [27]. A GC system (7890B, Agilent) with an autosampler, an injector (model G4513A), and a coupled mass selective detector (model 5977B MSD) (Agilent) were used for measurements. Metabolites were analyzed using a SIM acquisition method. GC-MS parameters were used as follows: the injection volume of 0.5 μL was split 1:25. The oven programme started with an initial temperature held at 70 °C for 2 min and continued with a heating rate of 10 °C/min up to 150 °C and 20 °C/min up to 325 °C held for 7 min. Mass spectra were acquired after a solvent delay of 5.8 min [28]. All other parameters of GC were set as previously described [27].

The quantification of metabolites was performed using MassHunter Quantitative Analysis B.08.00 (Agilent). Peak areas were normalized to peak areas of internal standard compounds. Absolute concentrations of metabolites were determined using a calibration from 0.1 to 500 nmol/sample. Relative quantification was performed for metabolites with concentrations below and above the calibration range. All metabolite concentrations were related to the amount of extracted sample material. In the gas chromatography investigations, amino acids and carbohydrates were detected.

2.5. Characterisation of the microbiota by next-gene sequencing of the variable V3/V4 region of the 16S rRNA genes with regard to the order Campylobacterales

For the microbiota analysis, intestinal contents were taken from the jejunum, ileum, caecum, and colon during dissection. The samples were frozen at − 80 °C and stored for further processing. The intestinal contents of each sample were homogenised with zirconia beads (BioSpec Products) in a MagNALyzer (Roche Diagnostics). After homogenisation, DNA was extracted using the QIAamp DNA Stool Mini Kit in accordance with the manufacturer's instructions (Qiagen). The DNA concentration was determined spectrophotometrically, and the DNA was stored at − 20 °C until use. PCR amplification over the V3/V4 region of the eubacterial 16 S rRNA genes, DNA clean-up, and MiSeq nextgen sequencing were performed as previously described [29]. The fastq files generated after nextgen sequencing were uploaded to Qiime software (Qiime 2010). The quality trimming criteria were set to a value of 19, and no mismatches were allowed in the MID sequences. The reverse reads were trimmed to a length of 250 bp and the forward and reverse sequences were merged. In a subsequent step, the chimeric sequences were predicted by the Slayer algorithm and excluded from further analysis. The resulting sequences were then classified using RDP Seqmatch, with an OTU (operational taxonomic units) discrimination score of 97 %, followed by UniFrac analysis. The results were given as a percentage of the total microbiota.

2.6. Determination of Campylobacter-growth using different substrates

2.6.1. Substrate solution preparation

The protocol was based on Hofreuter et al. [14] and was adapted according to Peh et al. [30]. The following substances were used: asparagine, aspartate, fucose, glutamine, glucose, butyrate, propionate, and serine. Substrate stock solutions (for exact amounts, see Table S5) of 200 mM concentration were prepared in Dulbecco's Modified Eagle Medium (GibcoTM DMEM, Thermo Fisher Scientific, Inc., Waltham, Massachusetts, USA). The stock solution was diluted with DMEM and supplemented with Fe^{2+} in the form of Iron(II)-ascorbate to produce substrate solutions with final concentrations of 40 mM. Iron ascorbate was added to the substrate solutions because it has the advantage of protecting the bacteria from oxidative stress during the substrate experiments. In this way, growth can be better guaranteed under the given environmental conditions [31]. Until use, the solutions were stored at 8 °C for a maximum of four weeks.

2.7. In vitro investigations on substrate utilisation

Bacterial strains were stored in cryotubes (Carl Roth GmbH + Co. KG, Karlsruhe, Germany) at − 80 °C, and cultivated by plating them onto Columbia agar plates supplemented with sheep blood (Oxoid Deutschland GmbH, Wesel, Germany). After incubation for 48 h at 42 ± 1 °C under microaerobic conditions, cultures were streaked out on fresh

Columbia blood agar plates and incubated overnight. Bacterial colonies were then suspended in sodium chloride and adjusted to a turbidity in accordance with McFarland standard 5. A 1.5 mL volume of this suspension was added to an Erlenmeyer flask containing 25 mL of pre-heated brain heart infusion (BHI) broth (Carl Roth GmbH & Co. KG, Karlsruhe, Germany), and incubated on a shaking platform (130 rpm) for three hours at 42 ± 1 °C under microaerobic conditions. Subsequently, this culture was diluted using BHI until a turbidity equivalent to a McFarland standard 0.5 was achieved, corresponding to approximately 1.5×10^8 colony-forming units / mL (cfu / mL). This bacterial solution was further diluted 1:100 using BHI to acquire the desired bacterial suspension for the in vitro experiments.

A volume of 200 µL of the bacterial suspension was added into the wells of an F-shaped bottom 48-well microtiter plate (Sarstedt AG Co. KG, Nümbrecht, Germany) containing 200 µL substrate solution in the test wells, 200 µL BHI in the wells for the positive controls, and 200 µL DMEM in the wells for the negative controls. Each positive and negative control as well as the samples were loaded in duplicate and the experiment was performed three times in total (Fig. S1).

The microtiter plate was then placed directly into a Tecan Spark automatic microplate reader (Tecan Austria GmbH, Grödig, Austria) with an integrated gas control module. The plate was stirred and incubated for 24 h at 42 ± 1 °C under microaerobic conditions. The optical density (OD) at 600 nm (OD_{600}) was automatically measured every 60 min and recorded in an Excel file.

2.8. Data analysis and statistics

Statistical analysis was performed using SAS Enterprise Guide software (version 7.1, SAS Institute Inc., Cary, NC, USA). First, residuals were calculated for all dependent variables, tested with the Shapiro-Wilk test for normality, and additionally assessed visually for normal distribution patterns. When the data were not or could not be assumed as normally distributed, non-parametric tests were used. For this purpose, the Kruskal-Wallis test was initially run using ranks. If $p < 0.05$, individual pairwise comparisons were performed using Wilcoxon's two-sample tests with Bonferroni correction ($p < 0.016$). The results of the tecan trial were analysed with a one-way ANOVA and the post-hoc Dunnett's t-test using SAS v. 7.1 (SAS Inst. Inc., Cary, NC, USA). The differences were assumed to be statistically significant if $p \leq 0.05$.

3. Results

3.1. Clinical course and excretion of the inoculated strains after infection

The general condition of the animals remained unchanged during the experimental period. Changes in faecal consistency or increased internal body temperature did not occur. Faecal samples of the control group (Group 0) remained *Campylobacter* negative throughout the trial. The inoculated strains could be re-isolated from faecal samples two days after infection (p. inf.), and all pigs excreted the respective strains at day 7. The excretion rate decreased slightly in Group 2 during the following sampling days (Table 3). Cross infection with the other *Campylobacter* strain did not occur. During dissection, the intestinal tract, liver or spleen did not show any gross lesions.

Table 3
Re-isolation rate (%) of inoculated *Campylobacter* strains from faecal samples of piglets taken over a period of four weeks (Group 1: *C. coli* ST5777: n = 16; Group 2: *C. jejuni* ST122: n = 16).

Time of sampling	Re-isolation rate in faecal swaps (%)	
Days p. inf.	**Group 1**	**Group 2**
2	87.5	28.6
7	100	100
14	100	91.6
21	100	69.3

3.2. Distribution and number (cfu/mL) of the inoculated Campylobacter strains in the intestine

During dissection, the inoculated strains were re-isolated from the ileum, caecum, and colon within both groups of infected pigs. In Group 1, the respective *C. coli* strain was not detected in the duodenum, while 31.3 % of the jejunal samples (n = 5) were positive. In Group 2, the inoculated *C. jejuni* strain was isolated from 31.3 % of the duodenal samples (n = 5), and half of the pigs harboured the strain (n = 8) in the jejunum (Table 4).

The number of colony-forming units (cfu) in the intestinal sections did not differ statistically significantly between the two groups (Table 5). However, in both groups, the proportion of *Campylobacter* spp. in the ileum and caecum was obviously higher than in the jejunum.

3.3. The metabolome in different intestinal sections

3.3.1. ^{1}H NMR spectroscopy

For the amount of acetate, butyrate, and propionate in the ingesta of the intestinal sections (Table 6), no statistically significant differences were found between the control group (Group 0) and the infected groups (Group 1 and Group 2). No short chain fatty acids (SCFA) were detected in the jejunum.

3.3.2. GC-MS

For asparagine, serine, and glutamine, no statistically significant differences in their quantity were found between the groups ($p > 0.05$; Table 7). Fucose was not detected in the pig intestine. The amount of glucose did not differ statistically significantly between the groups in the ileum, caecum, and colon ($p > 0.05$, Table 8), while the jejunum in the pigs of Group 2 contained statistically significantly higher amounts of glucose than those in Group 1 ($p = 0.0035$; Fig. S2).

3.4. Characterisation of the microbiota with regard to the order Campylobacterales

Since the abundance of *Campylobacterales* was less than 1 % in any gut section of all investigated pigs, the order level was considered sufficient to describe the low proportion of *Campylobacter* spp. in the porcine microbiome. The highest proportion was found in the ileum in Group 2 (0.39 %). There was a statistically significant difference in the jejunum and ileum between Group 0 and 2, with Group 2 having a significantly higher abundance of *Campylobacterales* (Jejunum $p = 0.0084$; Ileum $p = 0.0020$). Although it cannot be statistically proven, the proportion of *Campylobacterales* decreased along the intestinal tract (Table 9).

3.5. Utilisation of different substrates by tested C. coli and C. jejuni strains

For evaluation, the maximal optical density (max. OD) was

Table 4
Recovery of the inoculated *Campylobacter* strains from different intestinal sections after dissection in the twelfth week of life. Group 1 (n = 16): *C. coli* ST 5777, Group 2 (n = 16): *C. jejuni* ST 122.

Intestinal section	*Campylobacter* re-isolation rate	
	C. coli ST 5777 (Group 1) (%)	*C. jejuni* ST 122 (Group 2) (%)
Duodenum	0	31.3
Jejunum	31.3	50
Ileum	100	100
Caecum	100	100
Colon	100	100

Table 5
Counts of the inoculated *Campylobacter* strains (cfu/mL) in jejunum, ileum, and caecum after dissection in the twelfth week of life. Group 1 (n = 16): *C. coli* ST 5777, Group 2 (n = 16): *C. jejuni* ST 122.

Intestinal section	*Campylobacter* counts (cfu/mL)	
	C. coli ST 5777 (Group 1)	*C. jejuni* ST 122 (Group 2)
Jejunum	8.66×10^2	4.96×10^2
Ileum	1.84×10^5	7.94×10^4
Caecum	2.50×10^5	4.16×10^4

determined for each of the tested substrates in three runs. As a negative control, the OD_{600} was determined from the measurements with glucose. The results of growth experiments in brain heart infusion (BHI) served as a positive control to show that the strains can grow in the given environment. Maximal OD_{600} in BHI differed statistically significantly from the negative controls ($p < 0.05$), which confirms that all strains were able to grow in the given experimental conditions. The OD_{600}-values determined for all substances used were compared with the negative control. Statistically significant differences ($p < 0.05$) indicated growth with this substances, respectively.

The *C. coli* strain (ST-5777) utilised fucose, acetate, asparagine, propionate, and serine, while the strain could not utilise glutamine and butyrate (Fig. 2). On the other hand, the *C. jejuni* ST122 strain utilised only serine, while it did not show any growth following supplementation of glutamine, butyrate, fucose, acetate, asparagine, and propionate (Fig. 1).

4. Discussion

Campylobacteriosis is the leading food-borne disease in humans, with *C. jejuni* infections predominating. Enteritis with mild symptoms up to bloody diarrhoea has already been widely described [32]. Among the MLST genotypes of the strains used here, the *C. coli* ST5777 was isolated mainly in association with enteritis, while the *C. jejuni* ST122 also caused a case of Guillain-Barré syndrome [24], an autoimmune disorder of peripheral nerves [33]. In general, animals are symptomless carriers of *Campylobacter* spp. [9]. Poultry is primarily colonised by *C. jejuni*, whereas the porcine gut is considered to be the natural environment of *C. coli*. Nevertheless, *C. jejuni* might also colonise pigs, and *C. coli* can be isolated from poultry [34,35]. The reason for those host preferences still remains unclear, but metabolic differences might be a reason for colonising different tissues [14].

The aim of the presented study was to compare the site of colonisation of two *Campylobacter* strains (*C. jejuni* ST122 and *C. coli* ST5777) in the gut of growing pigs using an infection model, and to relate metabolic requirements by investigating the metabolome and the use of different substrates in vitro. Quantitative and qualitative cultural detection of *Campylobacter* strains was complemented by microbiome determination in the different intestinal sections of the pigs to obtain an overview of the proportion of *Campylobacterales* in total porcine microbiota.

The two strains used in the porcine infection model had originally

Table 8
Glucose content in the intestinal sections. The values are given as relative quantification area metabolite per area internal standard (Group 0, control: n = 16, Group 1, *C. coli*: n = 16, Group 2, *C. jejuni*: n = 16). The number of ileum samples is lower than the number of investigated pigs because of the lack of content for some of the animals. (Group 0, control: n = 10, Group 1, *C. coli*: n = 10, Group 2, *C. jejuni*: n = 14). Standard deviations are given in table S8.

Intestinal section	Group 0	Group 1	Group 2
Jejunum	10.65^{ab}	5.14^{b}	24.58^{a}
Ileum	1.01	0.49	0.79
Caecum	0.62	0.56	0.83
Colon	0.32	0.28	0.35

[a, b] - if different symbols are present, these groups significantly differed in glucose content

Table 9
Abundance of *Campylobacterales* in the microbiota of the intestinal sections of the tested animals at the order level. The values are given as mean values with standard deviation (Group 0, control: n = 16, Group 1, *C. coli*: n = 16, Group 2, *C. jejuni*: n = 16).

Intestinal segment	Group 0 (%)	Group 1 (%)	Group 2 (%)
Jejunum	0.0013 + /- 0.0049*	0.0599 + /- 0.1465	0.0555 + /- 0.1169*
Ileum	0.0004 + /- 0.0016*	0.0125 + /- 0.0252	0.3896 + /- 0.9349*
Caecum	0	0.0046 + /- 0.0165	0.0193 + /- 0.0719
Colon	0	0.0018 + /- 0.0071	0.0011 + /- 0.0041

* - statistically significant difference between the marked values

Table 6
Acetate, butyrate and propionate concentration in the digesta of ileum, caecum, and colon. The values are given as mean values in mmol/kg intestinal content (Group 0, control: n = 16, Group 1, *C. coli*: n = 16, Group 2, *C. jejuni*: n = 16). The number of ileum samples is lower than the number of investigated pigs because of the lack of content for some of the animals. (Group 0, control: n = 10, Group 1, *C. coli*: n = 10, Group 2, *C. jejuni*: n = 14). Standard deviations are given in table S6.

SCFA	Group 0 (mmol/kg digesta)			Group 1 (mmol/kg digesta)			Group 2 (mmol/kg digesta)		
	Ileum	Caecum	Colon	Ileum	Caecum	Colon	Ileum	Caecum	Colon
Acetate	4.35	38.42	32.97	10.98	34.36	29.14	7.31	43.94	30.99
Butyrate	0.14	8.35	7.19	2.30	7.08	6.06	0.83	7.29	6.61
Propionate	0.01	3.13	0.52	2.86	3.21	0.55	0.37	3.51	0.61

Table 7
Content of asparagine (Asp), serine (Ser), and glutamine (Glu) along intestinal tract. The values are given as mean values in mmol/kg intestinal content (Group 0, control: n = 16, Group 1, *C. coli*: n = 16, Group 2, *C. jejuni*: n = 16). The number of ileal samples is lower than the number of investigated pigs because of the lack of content of some of the animals. (Group 0, control: n = 10, Group 1, *C. coli*: n = 10, Group 2, *C. jejuni*: n = 14). Standard deviations are given in table S7.

Intestinal section	Group 0 (mmol/kg digesta)			Group 1 (mmol/kg digesta)			Group 2 (mmol/kg digesta)		
	Asp	Ser	Glu	Asp	Ser	Glu	Asp	Ser	Glu
Jejunum	0.45	0.63	0.78	0.50	0.75	0.58	0.30	0.45	0.58
Ileum	0.74	1.61	1.15	0.75	1.67	1.06	0.50	1.01	0.80
Caecum	0.06	0.13	0.20	0.05	0.14	0.20	0.05	0.14	0.15
Colon	0.09	0.21	0.21	0.09	0.20	0.18	0.09	0.23	0.16

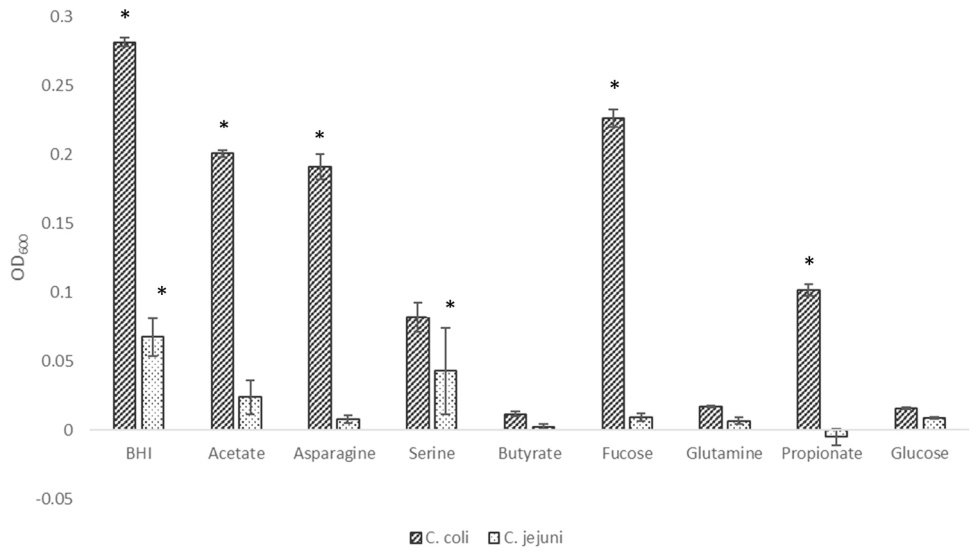

Fig. 1. Ability of *C. jejuni* ST122 and *C. coli* ST5777 to utilise acetate, asparagine, serine, butyrate, fucose, glutamine, propionate, and glucose. Values are the mean + /- SD maximal optical density reached after 24 h of growth in DMEM supplemented with the indicated compounds (20 mM) of three independent experimental measurements. Asterisks denote statistically significant ($p < 0.05$) growth differences in comparison to growth in medium with glucose.

been isolated from poultry. Since the internal body temperature is higher in poultry than in pigs [36,37], it can be assumed that the strains are better adapted to higher temperatures. Nevertheless, both strains were capable to colonise pigs, which was already previously demonstrated [25], and grew at 37 °C, which was demonstrated in the in vitro examinations.

In order to assess the influence of the inoculated *Campylobacter* strains on the metabolome, the proportion of these strains in the microbiota must be taken into account. For this purpose, the order level of *Campylobacter* spp., the *Campylobacterales*, was examined in detail in different intestinal sections. Since the relative frequency of *Campylobacterales* in the intestinal sections of the infected animals reached a maximum 0.39 % (ileum), no further differentiation was made at lower taxonomic levels. The order *Campylobacterales* includes the families *Arcobacteraceae*, *Campylobacteraceae*, *Helicobacteraceae*, and *Hydrogenimonadaceae* [38]. Accordingly, in the *Campylobacter*-negative Group 0, the associated bacterial genomes were found in the intestine. Many more bacterial strains are included in the order *Campylobacterales*, e.g., *Helicobacter* spp. [39]. Since the control group always tested negative for *Campylobacter* strains in the regular microbiological examinations, the authors assume that other bacterial strains from the order *Campylobacterales* were detected in the microbiome. Generally, the proportion of *Campylobacter* spp. in the microbiota of the investigated gut sections was quite low and was therefore only mentioned at order level, in contrast to, for example, poultry, where at the level of the genus, up to 9 % of the microbiota consists of *C. jejuni* [23].

The authors therefore assume that the influence of the inoculated strains on the metabolome is too small to be detected by means of the present investigations. Nevertheless, the *Campylobacter* strains might derive colonisation advantages of the predominant metabolome in different intestinal sections. The proportion of *Campylobacterales* seemed to decrease along the intestinal axis, which would indicate better colonisation conditions in the anterior intestinal tract. Though the detection rate of the two strains revealed a lower proportion of colonised jejunal sections, with *C. jejuni* detected in 50 % of the samples and *C. coli* in 31.3 %, thereof, in contrast, the subsequent intestinal sections were all positive for the respective strain. In the quantitative microbiological investigations, the counts of the infected strains in the intestinal sections increased in distal parts of the intestine. This observation is in contrast to a previous study where *C. jejuni* was mainly found in the small intestinal sections [21]. Hendrixson et al. also found an uneven distribution of *C. coli* and *C. jejuni* in the intestine of poultry, with *C. jejuni* in the highest concentration in the caecum, while *C. coli* was found in slightly higher concentrations in the ileum and caecum [17].

The examination of the metabolites in the different intestinal segments revealed only minor differences between the control and experimental groups. There were no differences for acetate, butyrate, propionate, asparagine, serine, fucose and glutamine. Only for glucose, a statistically significant difference was found between the contents of the jejunum in Group 1 and 2. It is suspected that the number of samples was too small to describe the high variation in glucose content in the jejunum (ranging between 0.76 and 37.71). No such high fluctuations were observed in the other intestinal sections. As both strains were found in similar amounts in the contents of the jejunum, neither strain had an advantage in colonising due to the glucose content. A possible explanation for this difference could also lie in the feeding diet. However, since all test animals received the same feed in the same quantity, this is rather unlikely. A special property of *C. jejuni* and *C. coli* is their non-glycolytic nature [16,40], although Vegge et al. found that *C. jejuni* subsp. doylei is able to utilise glucose [41]. The glucose utilisation has also been demonstrated in some *C. coli* strains [19]. Catabolism occurs via the Entner-Doudoroff pathway [19,41] and gives the strains the opportunity to occupy a new ecological niche. However, our experimental strains did not exhibit this characteristic.

Serine was utilised for growth by both, *C. coli* and *C. jejuni*. This was already described for other *C. jejuni* and *C. coli* strains [42]. The utilisation of serine is mediated by the L-serine dehydratase SdaA and the serine transporter SdaC [16,42]. Serine was present in all intestinal sections, which could help the strain colonise. The serine content in the ileum was higher (1.67 mmol/kg digesta) than in the caecum (0.14 mmol/kg digesta), but there was no difference in the counts of both inoculated *Campylobacter* strains between the intestinal sections. Nevertheless, the availability of serine in all intestinal segments could support colonisation by the *Campylobacter* strains used, especially *C. jejuni*, since this strain cannot metabolise the other substrates tested.

Acetate, asparagine, fucose, and propionate were only used by *C. coli*. Although *C. jejuni* (ST122) did not catabolise acetate, other studies described *C. jejuni* strains that can do so [13,31,43,44]. Acetate is excreted during the logarithmic growth phase of *C. jejuni*. Subsequently, it is assumed that an "acetate switch" enables the bacterium to take up acetate and use it for growth. The mechanism of molecular transport for this is unknown [45]. This suggests that our experiment may not be

suitable for exploring *C. jejuni* acetate consumption, as it does not extend beyond the logarithmic growth phase. Furthermore, acetate metabolism in *C. coli* appears to be different from this "switch" as described for *C. jejuni*, as it is used directly [45]. For propionate, there are genes present in the genome of *C. coli* strains that encode propionate-CoA ligase and 2-methylcitrate synthetase. These genes were absent in the genome sequence of *C. jejuni* [20]. Therefore propionate is assumed not to be metabolised. This was confirmed by our experiments. The utilisation of asparagine is dependent on the AnsB allele (Cj0029), coding for the enzyme asparaginase [14], which in turn converts asparagine into aspartate. This gene has also been detected in other bacteria such as *Pseudomonas putida* [46]. Also, the *C. jejuni* strain NCTC11168 investigated by Hofreuter et al. was able to utilise asparagine [14]. Our *C. jejuni* strain seemed to lack this attribute, whereas it appeared to be present in *C. coli*.

Both, acetate and propionate could not be detected in the metabolome analysis of the jejunum, which might explain the low detection rate of *C. coli* in this segment. Asparagine, which was also utilised by the *C. coli* strain, however, could be detected in higher concentrations in the anterior intestinal segments than in the posterior intestinal tract, but seemed not to contribute to a significant growth advantage for *C. coli*.

Wagley et al. [20] investigated the growth of various *C. coli* and *C. jejuni* strains in the presence of fucose. Only two of the 13 studied *C. jejuni* strains (32799 and Cj1) were able to metabolise fucose, while both *C. coli* strains (K3 and K7) showed increased growth with fucose supplemented nutrient broth. It was assumed that the presence of fucose favours the colonisation of *C. coli* in pigs, as both strains were isolated from pigs. In contrast to Wagley et al. [20], Stahl et al. [47] described that *C. jejuni* NCTC 11168 utilises fucose. Here, the gene cj0486 was identified as homologous to a fucose permease gene in *Escherichia coli* [48], allowing the use of L-fucose as a growth substrate. Fucose could not be detected in the digesta samples of the experimental animals. On the one hand, this could be due to the special motherless rearing of the pigs, which naturally differentiates the metabolome and microbiome from conventional pigs. On the other hand, the feed could also be the trigger. In other studies on the metabolome of pigs, fucose was detected in the large intestine [49].

Glutamine and butyrate were not catabolised by any of our strains. Hofreuter et al. found that the gene cju06 encodes a γ-glutamyltranspeptidase [14]. This enzyme can hydrolyse glutamine to glutamate, which the bacteria can use for growth. The gene was already described in *Helicobacter pylori* [50] and was also detected in a *C. jejuni* strain [51]. Our strains seem to lack this gene, as there was no increase in OD_{600} compared to the control during the in vitro experiment. Butyrate also does not seem to be required for growth. Little is known about the processes involved in the uptake of butyrate into the bacteria. However, there are existing studies proving the protective effect of butyrate on intestinal cells [52]. Here, the substance helps the undifferentiated intestinal cells to differentiate, thus preventing *C. jejuni* from entering the intestinal cells.

5. Conclusion

There is a wide variance in the metabolism of *C. coli* and *C. jejuni*. Therefore, no conclusion can be drawn from the characteristics of one strain to the behaviour of another strain. The small proportion of *Campylobacterales* in the total microbiome in our study allowed only a few tentative conclusions to be drawn from the metabolome regarding the colonisation behaviour of the two strains. Thus, the absence of propionate and acetate in the jejunum may have negatively influenced the colonisation of *C. coli*. However, a supply of different substrates in the metabolome can lead to preferential use of single substances from *Campylobacter* strains, although in principle others can also be metabolised. An adaption of *Campylobacter* spp. to the nutrient supply is therefore suspected. Further studies should be carried out on the essential substrates *Campylobacter* spp. need for metabolisation to improve the knowledge about environmental requirements and thus the prerequisites for colonisation in order to understand the interaction of *Campylobacter* spp. and the host.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

The data presented in this study are available in Supplementary Material. Additional data are available on reasonable request from the corresponding author.

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Institutional Review Board Statement

The study was conducted in accordance with the guidelines of the Declaration of Helsinki. Ethical authorization was granted following notification to the national veterinary authorities (33.9–42502-04–16/2132).

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.cimid.2022.101865.

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