Institut of Meat Hygiene, Meat Technology and Food Science, University of Veterinary Medicine Vienna, Austria

**Changes within the intestinal flora of broilers by colonisation with Campylobacter jejuni**

**Veränderungen der Darmflora des Geflügels durch Besiedelung mit Campylobacter jejuni**

Dmitri Sofka, Agathe Pfeifer, Barbara Gleiß, Peter Paulsen, Friederike Hilbert

In most European countries human campylobacteriosis is the most important bacterial zoonotic foodborne infection. Chicken meat is considered the main source of infection. Since most strategies assessed so far, in reducing Campylobacter colonization in chickens or in the reduction of human disease, have not been very effective, new knowledge regarding Campylobacter's interaction with the host is needed. In this study we analysed fecal and cecal samples of five chicken flocks of different Austrian farms for the occurrence of *C. jejuni* and *C. coli*, and analysed the intestinal microflora by PCR-SSCP, cultural detection of lactic acid bacteria, Enterococci, Staphylococci, Enterobacteriaceae, *E. coli*, and total aerobic colony counts. Furthermore ten chicken samples of cecal content of a flock during colonization with *Campylobacter* spp. was analysed by high throughput sequencing. With all three methods used we could detect a change within the microbiota caused by *C. jejuni*. Enumeration of different bacteria was significantly lower in fecal samples positive for *C. jejuni*, pointing out that a higher water content and thus, a preliminary stage of diarrhea might appear during Campylobacter colonization. By PCR-SSCP analysis the microbiota composition differed between colonized and non-colonized chicken fecal samples. This could also be detected in community analysis by high throughput sequencing, but this difference was only a tendency and not statistically significant. It can be concluded that *C. jejuni* is interacting with the intestinal microflora in their respective hosts and hence, this has to be taken into account when providing new strategies to combat Campylobacter colonization and disease.

**Keywords:** *Campylobacter jejuni*, microbial community, PCR-SSCP, sequencing, chicken

Introduction

Campylobacter (C.) infections in humans are still on the rise in industrialized, emerging and developing nations worldwide (Lévesque et al., 2013). Chicken and chicken meat are considered the main source of human infection (EFSA, 2010). Strategies to limit Campylobacter colonization in chickens can considerably lower the risk of human disease (Boysen et al., 2013). C. jejuni gets into the chicken stables by different means. Most often a flock colonizes between the third and the seventh week of fattening by contaminated working tools, cages, clothes, shoes or coats but Campylobacter may also be transferred by insects or rodents (Newell et al., 2011). The transfer between stables at the same farm is rather the rule than the exception. Most often a flock gets colonized when it is thinned for slaughter (part of the chickens are harvested for slaughter and the remaining chickens of the flock are fattened for a longer time) for the first time. Thinning is used to respond to consumer’s needs – requests for whole chicken as well as chicken parts – and to stay to animal welfare regulations in regards to square meter per weight. With contaminated cages, chicken harvesters or bird catchers the remaining birds are colonized within a few days (Allen et al., 2008).

Campylobacter spp. are zoonotic pathogens, that can be found in the intestinal tract of most animal species. Despite that, specific species and genotypes predominate in different hosts (Wilson et al., 2008). The organism, particularly C. jejuni, is able to colonize various hosts. It can cause symptoms in humans and can colonize the lower intestinal tract of chickens without clinical signs of disease (Janssen et al., 2008). But likewise it colonizes the duodenum in cows without symptoms but may cause diarrhoe in calves (Klein et al., 2013). One specific feature of C. jejuni has been shown by Bereswill et al. (2011): Colonization resistance in mice is dependent on the intestinal microbiota. More precisely C. jejuni can colonize and even cause disease symptoms in mice with a completely eradicated or with a human like intestinal microflora. Thus, C. jejuni’s colonization ability and its aptitude to cause disease symptoms are dependent on the microflora of the specific host. Very recently Dicksved et al. (2014) demonstrated that colonization resistance in humans is attributed to a specific intestinal microflora but C. jejuni colonization leads to a change in the intestinal microbiota in humans as well.

During colonization of the chicken’s ceca C. jejuni can reach levels of 10^9 colony forming units (cfu) of bacteria/g in the cecal content (Shane, 1992). Therefore, a shift in the intestinal microbiota by C. jejuni infection is not surprising. Recently, a study revealed, by high throughpout sequencing analysis, that the microbiota in chickens comprises four enterotypes and that Campylobacter spp. was observed to a lower extent in one of the four enterotypes (Kaakoush et al., 2014). Nevertheless flock and age of the birds contribute to the different flora composition.

In this study we used different methods like PCR-SSCP (Polymerase chain reaction single strand confirmation polymorphism) analysis and high throughput sequencing analysis of the 16S RNA gene as well as cultural methods to define the microbiota of fecal and cecal content of five flocks of different Austrian farms in correlation to C. jejuni colonization. Results revealed both, a specific microbiota, and a lower content of culturable microorganisms for Campylobacter colonized chickens.

Material and Methods

Sample collection

Samples were collected in Austria in the years 2003 to 2006 and in the year 2013. At all four different seasons (spring, summer, autumn, and winter) one flock was analysed. Fecal samples were drawn in fattening week 6 and 7 (sample number for each flock n = 36, in total n = 144). Ceca content was collected at slaughter (n = 8 for each flock, in total 32). One additional flock was sampled (cecals samples n = 31) in autumn in the year 2013 and ten samples of this flock were subjected to community analysis using high throughput sequencing. All flocks were kept under conventional husbandry conditions. Fractionated slaughter (the flock was slaughtered in two parts; interval: a week) was used.

PCR-SSCP (Polymerase chain reaction single strand confirmation polymorphism)

DNA-Isolation

0.5–1 g of sample was extracted using the QiAamp® Stool Mini Kit (Qiagen Hilden, Germany) according to manufactures advice. Most samples were analyzed immediately at arrival; transportation to the laboratories was performed under cooling conditions at 3°C.

PCR


Denaturation

25 µl of the PCR product and 16 µl denaturation buffer (95% formamide, 10 mM NaOH, 0.25% bromophenol...
blue and 0.25% xylene cyanol) were incubated at 95°C for 2 min and immediately set on ice (10 min) (Ott et al., 2004). The total volume of 41 µl per sample was loaded on PAG’s slots.

**Polyacrylamide gel electrophoresis**

A 10% PAG using 1XTBE (Tris-Borate-EDTA) buffer, Acrylamide-Bis solution (37.5:1) (Serva, Heidelberg, Germany), APS (Ammoniumpersulfat, Serva), TEMED (N,N,N’,N’-Tetramethylethylenediamine Sigma, St. Louis, USA) was casted between two glass plates (0.5 mm thick). Polymerization was carried out over night. The gel was used immediately or cooled at 7°C until use (max. 3 days of storage). The electrophoresis was carried out in 2XTBE buffer on a horizontal electrophoresis chamber (Bio Phoresis, BIORAD) under running conditions: 200V/const. for 700V/h (Power supply: Model 3000XG, BIORAD). The samples were denatured immediately before they were loaded on the gel. For visualization silver staining of PAGs was performed according to Heukeshoven and Dernik (1985). Cluster analysis was done using the Bio-Rad FingerprintingTM II version 3.0.

**Isolation of bacteria by cultural methods**

Each individual fecal sample and cecal content was serially diluted 1:10 in sterile physiological sodium chloride solution. A 10-fold dilution series from 10^-2 to 10^-10 was spread onto specific agar plates. Coli-ID-Agar plates (Bio Merieux, Marcy l’Etoile, France) were used to enumerate *E. coli* and were incubated for 24 h at 42°C. Typical colonies were counted. A random sample of these colonies was biochemically verified as *E. coli* by using the Api-20E (Bio Merieux, Marcy l’Etoile, France).

For the detection of Lactobacilli afore mentioned serial dilutions were incubated on MRS-Agar-plates (Merck, Darmstadt, Germany) for 72 h at 30°C under aerobic conditions and enumerated. *Staphylococci* were analysed on Baird-Parker Agar (Merck, Darmstadt) supplemented with egg yolk emulsion (Merck, Darmstadt, Germany) at 37°C for 48 h under aerobic conditions. Serial dilutions for the detection of *Enterococci* were plated on Chromocult-Enterococci-Agar (Merck, Darmstadt, Germany) and aerobically incubated for 24 h at 42°C. Typical colonies were enumerated. *Enterobacteriaceae* were analysed on VRBD-Agar (Merck, Darmstadt, Germany) according to manufacturer’s advice after aerobic incubation at 37°C for 24 h. Total aerobic colony counts were enumerated using serial dilutions 10^-4 to 10^-10 on Plate Count Agar (Merck, Darmstadt, Germany) incubated at 30°C under aerobic conditions and 48 h.

Thermophilic *Campylobacter* spp. were analysed according to ISO-10272-2 (2002). In brief: 15 g of fecal or cecal samples were pre-incubated in Bolton Broth (Oxoid, Basingstoke, United Kingdom) at 42°C for 48 h under microaerophilic conditions (10% CO₂, 5% O₂ and 85% N₂). The samples were then plated on two selective agars, modified CCDA (Oxoid, Basingstoke, United Kingdom) and CampyFoodAgar (Bio Merieux, Marcy l’Etoile, France) and incubated at 42°C for 48h under microaerobic conditions. Additionally, all fecal samples were directly streaked onto above mentioned selective agars (without prior enrichment). Typical colony morphology, aerobic incubation and a PCR (Linton et al., 1997) was used for identification and species differentiation between *C. jejuni* and *C. coli* as described.

**High throughput sequencing and data analysis**

Cecal samples for high throughput sequencing was isolated using a combination of mechanical and enzymatical lysis. Briefly, 175 mg cecal samples were homogenised in MagnaLyser Bead tubes using the MagnaLyser Instrument (Roche Diagnostics, Mannheim, Germany) according to standard procedures. Bacterial DNA was isolated with the MagNA Pure LC DNA Isolation Kit III (Bacteria, Fungi) in a MagNA Pure LC 2.0 Instrument (Roche Diagnostics, Mannheim, Germany) according to manufacturer’s instructions adding 100 µg lysozyme (Carl Roth GmbH, Karlsruhe, Germany) per 100 µl sample. High throughput sequencing was performed at the core facility molecular biology at the ZMF center of medical research Graz, Austria based on the 16S RNA gene variable region V1–2, 75 ng of total genomic DNA was amplified with the FLX 454 one way read (Lib-L kit, Primer A, Primer B, Roche 454 Life Science, Branford, CT, USA). Fusion primer P27-5’AGAGTTTGATCCTGCGCTCAG3’ and R357-5’CTGCTGCCTCGTCAAG3’ (Baker et al., 2003; Watanabe et al., 2001). Each PCR reaction was of 50 µl containing 1 x Fast Start High Fidelity Buffer (Roche Diagnostics, Mannheim, Germany), 2.5 U High Fidelity Enzyme (Roche Diagnostics, Mannheim, Germany), 200 µM dNTPs (Roche Diagnostics, Mannheim, Germany), 0.4 µM barcoded primers (Eurofins MWG, Ebersberg, Germany), PCR-grade water (Roche Diagnostics, Mannheim, Germany) and 75 ng DNA. PCR products were purified according to standard procedures and amplicon concentrations were determined using the Quant-iT™ PicoGreen® dsDNA Assay Kit (Life Technologies, CA, USA) according to manufacturer’s instructions. After quantification the barcode labeled amplicons were pooled equimolar and analyzed on a 2100 Bio Analyzer (Agilent Technologies, Waldbronn, Germany) using a DNA 7500 kit. Emulsion PCR with the GS Titanium MV emPCR Kit and method (Lib-L) (Roche 454 Life Science, Branford, CT, USA) was performed according to the manufacturer’s instructions. Sequencing of the pool was performed using the GS FLX Titanium Sequencing Kit XLR70 (Roche 454 Life Science, Branford, CT, USA) according to the manufacturer’s instructions.

**Data analysis and statistical analysis**

Raw sequence data generated by the Roche GS FLX sequencer was de-noised with Acacia 1.52 and processed for initial quality filtering with Qiime 1.7.0 (read length between 200 and 600, no ambiguous bases, no primer mismatches, minimum quality score of 25 and maximal number of homopolymers 6). For the assessment of operational taxonomic units, reads were clustered with uclust allowing for 3% sequence distance (97% sequence identity threshold). Representative sequences from each OTU cluster were aligned with PyNAST and taxonomy was assigned by using the RDP classifier with confidence score of 0.8. To remove PCR artifacts we used Chimera-Slayer for a chimera check on the aligned representative sequences. Phylogenetic trees constructed with FastTree based on the 16S RNA gene variable region V1–2. Phylogenetic distances were calculated with FastTree. Species richness and diversity, including estimation indices, were calculated using the R statistical Software platform supplemented by the community ecology packages Vegan and BiodiversityR. In the beta diversity analysis, data normalization was carried out by downsampling (rarefaction) to the
smallest sample size (2950) of the OTU table. Principal coordinate analysis on the normalized data was performed by Qime.

The relation of the presence and absence of C. jejuni and the detection of the amount of culturable microorganisms was assessed by statistical analyses, in which results from enumeration were converted into their decadic logarithms. Data below the limit of detection (i.e. 2 log cfu/g) were set to 1 log cfu/g. The ANOVA analyses considered the presence of Campylobacter as dependent factors, and number of flock (8,9,10,11), sampling date (day of fattening), total aerobic count, lactic acid bacteria, Staphylococci, Enterococci, Enterobacteriaceae and E. coli as independent factors. Discrimination between means was done with LSD test. Statistical significance was established at p < 0.05, unless indicated otherwise.

Results

Detection of C. jejuni in chicken fecal and cecal content
Of the four flocks three became colonized with Campylobacter and one remained free of thermophilic Campylobacter spp., respectively. Fecal samples were taken at the 6th and 7th week of fattening. Two of the positive flocks were negative for cultural detection of thermophilic Campylobacter spp. until the 7th week, one flock was positive in week six and seven. The 5th flock (sampled in 2013) became positive during the 7th week, samples were taken at slaughter. Slaughter was performed at the end of the 7th week. Thus, some samples were identified as positive, some as negative and with different quantitative amounts of Campylobacter in the ceca content.

Of these cecal samples four were defined as cultural positive for C. jejuni, one positive for C. coli and five samples defined as C. jejuni and C. coli free by cultural isolation. These ten samples were subjected to microbiome analyses using the 16S RNA genes and high throughput sequencing.

Clusters of C. jejuni positive samples using PCR-SSCP microbial community analysis
We analysed the total fecal flora by amplification of the 16S RNA genes of the whole bacterial flora and analysed these single DNA strands by polyacrylamide gel electrophoresis (PCR-SSCP). Using the PCR-SSCP cluster analysis, differences were identified at flock level and an age specific pattern could be identified as well (data not shown). No seasonal cluster was identified. Despite these strong clusters seen for age and flock, a cluster of Campylobacter positive fecal samples was identified (Fig. 1). Figure 1 shows the cluster analysis of a PCR-SSCP gel with fecal samples of naturally Campylobacter colonized and non-colonized chickens. Fecal samples of Campylobacter colonized chickens cluster together versus not colonized, regardless of the flock and age specific pattern.

Statistical significant lower amount of culturable microorganisms in C. jejuni positive samples
By cultural methods enumeration of total aerobic colony count, lactic acid bacteria, Staphylococci, Enterococci, Enterobacteriaceae and E. coli was performed in all fecal samples. Statistical analysis revealed that in C. jejuni positive samples significantly lower cfu/g fecal content were detectable than in C. jejuni negative samples. Figure 2 shows the difference in a logarithmical order of magnitude.

Microbiome analysis using high throughput sequencing during C. jejuni flock colonization
The microbial diversity in the ceca of five negative and five Campylobacter positive (four C. jejuni and one C. coli positive sample) was assessed. Figure 3A shows the overall proportion of the most important phyla. According to the sequence data the cecal microbiome consists of most common phyla of gut bacteria (Firmicutes, Proteobacteria, Bacteroidetes). The amount of these phyla in different samples analysed vary substantially: Firmicutes 22–81%, Proteobacteria 5–64% and Bacteroidetes 1–30% By looking at Campylobacter negative compared to positive samples (Fig. 3B) no statistically significant difference can be detected. On the phyla level a tendency was seen for higher amounts of Firmicutes in Campylobacter negative samples (average 62%) to Campylobacter positive ones (average 36.6%). Whereas, for Proteobacteria (average of 44.6% and 21.3% respectively) and Bacteroidetes (average of 15.4% and 6.5% respectively) a higher proportion in Campylobacter positive samples are found. A tendency, regarding higher phyla diversity, can be featured on the genus level with an average of 55 detected genera in Campylobacter negative samples and 47 detectable genera in positive ones. By using a 3D PCoA Plot analysis (Fig. 4) Campylobacter status can clearly be assessed. Interestingly the sample positive for C. coli clusters with...
and H5 harbor Helicobacter spp. Sample H23 represents the C. coli positive sample. Samples H3 and H5 were Campylobacter negative, dots are Campylobacter positive samples.

**FIGURE 3:** Microbial diversity. A: All ten samples were analysed using high throughput sequencing. Microbial diversity is shown in graphical codes. B: Samples divided into Campylobacter positive (including the C. coli positive sample) and negative samples.

**FIGURE 4:** 3D PCoA Plot with Bray-Curtis distance. Campylobacter status. Samples with a black filled diamond are Campylobacter positive, dots are Campylobacter positive samples. Sample H23 represents the C. coli positive sample. Samples H3 and H5 harbor Helicobacter spp.

negative Campylobacter samples rather than with the C. jejuni positive ceca microbiome. Two samples harbouring Helicobacter species (one of which identified as C. jejuni positive one as negative) cluster tightly together.

**Discussion**

Campylobacter colonization in chickens has reached a high level in most European countries despite various efforts to reduce flock colonization. Chicken flocks are often colonized in the last weeks before slaughter and Campylobacter counts in the cecal and fecal content are particularly high. This leads to high contamination rates of chicken carcasses and chicken meat being processed through the automated slaughter process (Ellerbroek et al., 2010; Hue et al., 2010). Risk assessment analyses have shown that reduction in Campylobacter intestinal colony counts as well as a reduction in colonized chickens may serve well in a reduction of human Campylobacteriosis (Boysen et al., 2013). Recent works show that Campylobacter closely interacts with other microorganisms (Dickseved et al., 2014; Haag et al., 2012, Hilbert et al., 2010; Kaakoush et al., 2014). The intestinal host microbiota is important for Campylobacter colonization or colonization resistance in mice, humans and chickens equally well (Beereswill et al., 2011; Dickseved et al., 2014; Kaakoush et al., 2014). Campylobacter colonization leads to a change in the intestinal microbiota (Haag et al., 2012). Thus, strategies to enhance colonization resistance or reduce colonization in chickens may include shaping the chicken microbiota. So far most experiments using pro- or prebiotics to influence the chicken gut microbiota have not been effective (Neal-McKinney et al., 2012; Santini et al., 2010). Consequently data on the chicken microbiota are deemed necessary to provide new and effective approaches to fight Campylobacter colonization. By using different experimental approaches we studied the fecal and cecal microbiota of chickens in regards to the Campylobacter status. With all three methods used (conventional enumeration of colony counts, PCR-SSCP and high throughput sequencing) the fecal or cecal microbiota in Campylobacter colonized and Campylobacter free chickens differed. Cultural colony counts of lactic acid bacteria, Staphyloccoci, Enterococci, Enterobacteriaceae, E. coli, total aerobic colony counts were found to be significantly higher in Campylobacter negative samples. Recent studies showed that even though no visible signs of clinical disease are seen in chickens when colonized with Campylobacter, colonization can lead to epithelial lesions and an inflammatory immune response with effects on the gut mucosa and will finally result in diarrhea (Humphrey et al., 2014; Awad et al., 2014). Our results on colony counts could be a sign of diarrhea. We report here colony counts in cfu/g and thus, a higher water content in Campylobacter positive samples could be responsible for the significant reduction of cultural colony counts per weight. But despite the microbial quantity, the composition of the microbiota of chickens analysed by PCR-SSCP differs between Campylobacter positive and negative samples as seen in a cluster analysis. This has recently been shown by other authors but not in as many different flocks and not in two different age groups (Kaakoush et al., 2014). Most interesting is that these findings are consistent with new data on high throughput sequencing of this study and others (Kaakoush et al., 2014). Kaakoush and co-workers (2014) defined four groups based on enterotypes: enterotype 1 dominated by Firmicutes, enterotype 2 by Firmicutes and Proteobacteria, enterotype 3 Firmicutes and Actinobacter and enterotype 4 with Firmicutes and Bacteroidetes. Their results show that particularly in enterotype 3 Campylobacter were not found as often as in the other enterotypes. In our study we were able to find samples that were dominated by Firmicutes and samples could be identified as enterotype 2 and 4 but enterotype 3 was only found in one of our samples (15.4% Acinetobacter). This sample was culture negative.
and according to sequencing data harboured no Campylobacter spp. From all genera associated with C. jejuni by Kaakoush and coworkers (2014) in our samples only the Ruminococcaceae were found to be linked to Campylobacter colonized samples. This might be due to the relatively low numbers of samples we used in our study. Haag and coworkers (2012) saw a shift due to C. jejuni induced Colitis in mice, regarding the microbiota composition. E. coli, Enterobacteriaceae, Bacteroidales, Bifidobacteria, Lactobacillus and Clostridium changed in quantity. In our study we found as well a change in quantity of Firmicutes, Proteobacteria and Bacteroides at the phyla level but we were not able to break this difference down to genus level for these bacteria. Our sample harbouring C. coli clustered with Campylobacter free samples, so we suggest that the effect of C. coli on the microbial composition may be much lower than the one seen for C. jejuni. Additionally, effects of other microorganisms like Helicobacter spp. as seen in our study, may have even more impact on the microbiota composition in chickens. Taken together it can be concluded that Campylobacter and closely related microorganisms are interacting with the intestinal microflora in their respective hosts.

Acknowledgement

This work was in part funded by the European Project “Poultryflorgut” under FP6 Specific Targeted Research Project Contract Nr 007076 and the EU funded project “CamChain”, under FP7-KBBE EMIDA ERA-NET. We thank S. Trajanoski and I. Klymiuk for community sequence data analysis.

Conflict of interest: There are no protected financial, personal or professional interests in a product, service or company that could take influence on the issues provided in the manuscript.

References


Address for correspondence:
Ao. Univ. Prof. Dr. Friederike Hilbert
Institute of Meat Hygiene, Meat Technology and Food Science
University of Veterinary Medicine Vienna
Veterinaerplatz 1
1210 Vienna
Austria
friederike.hilbert@vetmeduni.ac.at