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Genetic diversity of thermophilic *Campylobacter* originating from the poultry chain

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ABBREVIATIONS

BHI	Brain Heart Infusion
bp	base pair
C.	Campylobacter
CC	clonal complex
CFU	colony forming unit
CDT	cytolethal distending toxin
°C	degree Celsius
DNA	deoxyribonucleic acid
DEPC	Diethylpyrocarbonate
E. coli	Escherichia coli
ECDC	European Centre for Disease Prevention and Control
EFSA	European Food Safety Authority
e. g.	exempli gratia
g	gram
GBS	Guillain-Barré-Syndrom
h	hour
HACCP	Hazard Analysis and Critical Control Point
IBD	Inflammatory bowel disease
ISO	International Organization for Standardization
MOMP	Major outer membrane protein
µg	microgram
µl	microliter
µm	micrometer
MFS	Miller Fischer Syndrom
ml	milliliter
min	minute
MDR	multidrug resistance
MLST	Multilocus sequence typing
No.	Outer membrane vesicle
OMV	Number
%	percent

PCR	Polymerase Chain Reaction
PHC	Process hygiene criteria
PFGE	Pulse-field gel electrophoresis
sec	second
STEC	Shiga toxin-producing *E. coli*
SDC	sodium deoxycholate

spp.	*species pluralis*
SLD	Spotty liver disease
ST	Sequence type
TIFF	Tagged Image File Format
Tlps	Transducer like proteins
TSA	Tryptic soy agar
T3SS	Type III secretion system
CDC	US Center for Disease Control and Prevention
VBNC	Viable but nonculturable
WHO	World Health Organization

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1. INTRODUCTION

1.1. BACKGROUND INFORMATION

Thermophilic *Campylobacter (C.)* spp. has outnumbered *Salmonella* several years ago and emerged as the most commonly reported zoonotic agent causing gastrointestinal infections in humans (European Food Safety Authority [EFSA] and European Centre for Disease Prevention and Control [ECDC], 2019). Although new species of *Campylobacter* have recently been discovered, clear majority of the human cases of campylobacteriosis are caused by *C. jejuni* and, to a lesser extent, by *C. coli*, *C. lari and C. upsaliensis* (PATRICK et al., 2018; TRESSE et al., 2018). The dominant source of *Campylobacter* infection in humans is foodborne, in particular poultry meat and poultry meat products. This correlates with the high prevalence of colonization with thermophilic *Campylobacter* in chickens, though contamination level of poultry meat sold at retail can vary depending on pre- and post-harvest factors (OSIMANI et al., 2017). Other sources of *Campylobacter* infection and transmission are consumption of unpasteurized milk, contaminated drinking water and direct contact with infected pets and other animals (EFSA and ECDC, 2019; FRIEDMAN et al., 2004).

Despite the identification of some of the risk factors, the incidence and prevalence of campylobacteriosis have increased in the past decade in both- developed and developing countries. Endemic regions are Africa, Asia and the Middle East, although the incidence has increased in North America, Europe and Australia (KAAKOUSH et al., 2015). The disease follows seasonal patterns, but underlying mechanisms have not yet been fully understood. In addition to significant impact on ongoing public health and economic burdens associated with this disease, high rates of ciprofloxacin and tetracycline resistance in *Campylobacter* spp. (POST et al., 2017) and increasing number of multidrug-resistant (MDR) strains (GARCÍA-FERNÁNDEZ et al., 2018) have been reported. This raises extra concerns about future effectiveness of antibiotic treatment and further underline the importance of developing new mitigation strategies.

1.2. *CAMPYLOBACTER* SPECIES CHARACTERISTICS AND RESERVOIRS

1.2.1. Characteristic

The taxonomy of the *Campylobacteraceae* has evolved extensively since its beginnings in 1963 and presently includes the genera *Campylobacter* (45 species and 16 subspecies), *Arcobacter* (24 species), and *Sulfurospirillum* (8 species) (http://www.bacterio.net/campylobacter.htm; accessed on: 01-06-2020). Most frequently reported species in association with human diseases are *C. jejuni* (subspecies *jejuni*) and *C. coli*. Other species such as *C. lari* and *C. upsaliensis* have also been isolated from patients with diarrhoeal disease but are reported less frequently (EFSA and ECDC, 2019).

Campylobacter spp. are Gram-negative, non-spore forming microorganisms with typically spiral or curved rod appearance and the size of the cell of approximately 0.2-0.8 by 0.5 to 5 µm. Upon exposure to environmental stress, such as various oxygen situations, osmotic imbalance, change in temperature or in older cultures, cells may vary in their shape and change to coccoid form. Individual strains may be able to grow in microaerobic, anaerobic and/or aerobic conditions, with optimal growth at temperature ranging from 37 °C to 42 °C and within microaerophilic atmosphere (5 % O_2, 10 % CO_2 and 85 % N_2). Most species are motile by using one to two unsheathed polar flagella, except *C. gracilis* which is immotile, and *C. showae* with multiple flagella. Oxidase activity is present in all species except for *C. gracilis* and some strains of *C. showae* (DEBRUYNE et al., 2008). Most *Campylobacter* species do not use carbohydrates as a source of energy and rely on amino acids and intermediates of the citric acid cycle for growth. However, *C. jejuni* NCTC 11168 was reported to possess loci associated with L-fucose metabolism (MURAOKA and ZHANG, 2011), and *C. coli* was shown to be able to transport and metabolise glucose (VORWERK et al., 2015). *Campylobacter* species can reduce nitrate, though the ability to reduce nitrite is reserved to catalase-negative species which are also more oxygen sensitive (BUTZLER, 2018). *Campylobacter spp.* have a relatively small genome size at about 1.6-1.7 Mbps, rich in adenine and thymine, and low guanin-cytosine content at only about 30 % (PEARSON et al., 2007).

1.2.2. Reservoir

Campylobacter spp. are ubiquitous in the environment and can be free living, commensal or pathogenic. Species have been isolated from soil, dust, water and mucosal surfaces of the gastrointestinal and reproductive tracts of humans and several birds and mammals (BULL et al., 2006; HÄNNINEN et al., 1998; PALMER et al., 1983).

Host association is common, but not exclusive. Poultry is the primary reservoir of the thermophilic *C. jejuni subsp. jejuni* which seems to have adapt to colonize the intestinal mucosa and cecum of birds whose body temperature is 42° C. The colonisation of the ceca by *C. jejuni* occurs within 24 hours upon infection (COWARD et al., 2008), and on a large scale with recovered amount of 10^4 to 10^8 cfu/g (BEERY et al., 1988). Long time *C. jejuni* has been held for a harmless commensale in infected poultry, however, recent findings show that birds develop inflammatory response and its intensity varies between the breeds (HUMPHREY et al., 2014). There is also correlation established between newly discovered *C. hepaticus* and spotty liver disease (SLD) in chickens, characterized by multifocal liver lesions, mortality and drop in egg production (VAN et al., 2016).

C*. hyointestinalis, C. mucosalis* and *C. coli* are frequent isolates found in pigs. *C. upsaliensis* and *C. helveticus* are found in intestinal tracts of cats and dogs. *C. fetus subsp. fetus* colonizes the intestinal tracts of sheep and cattle, and occasionally humans and turtles. *C. fetus subsp. venerealis* specifically colonizes the vagina of venereally infected cows and the prepuce of bulls (LASTOVICA et al., 2014). *C. lari* group is often allied with coastal environments und watersheds (MILLER et al., 2014).

In the last two decades, genetic analysis of *Campylobacter* isolates by multilocus sequence typing (MLST) and antigene gene sequencing also demonstrated, that despite of the frequent horizontal gene transfer, *Campylobacter* populations are highly structured, with distinct genotypes associated with sources (SHEPPARD et al., 2012). Whilst isolates of *C. jejuni* assigned to the ST-21 complex are widely spread, others, such as the ST-61 complex, seem to have a more restricted distribution and are overrepresented in cattle populations. In contrast, complex strains ST-45, ST-952, and ST-677 were isolated predominantly from wild birds, wild rabbits and environmental water (KWAN et al., 2008). In case of *C. jejuni*, genetic attribution studies have estimated that clonal complexes associated with chickens can account for as much as 80 % of human infection (SHEPPARD et al., 2009).

1.3. *CAMPYLOPBACTER* VIRULENCE POTENTIAL

1.3.1. Adaptation and virulence factors

Despite their fragile appearance, *Campylobacter* carry complex multifactorial systems for motility, chemotaxis, adherence, invasion and multidrug resistance which allow them to adapt to variable niche environment. The ability to enter viable but non-culturable (VBNC) state and to form biofilms,

as well as aerotolerance of some strains, are other strategies to enhance the survival of the bacteria during stressful conditions (BOLTON, 2015).

1.3.2. Motility

Campylobacter motility through the mucus layer is essential for approaching, attaching and invading the intestinal epithelial cells (YOUNG et al., 2007).

Movement of the bacteria is driven by flagellum, which consists of two main structural components: the hook basal body complex and the extracellular filament. In addition, a number of nonstructural components are required for flagellar assembly and function (CHEVANCE et al., 2008). *Campylobacter* motility is supported by the helical shape of its cell and it also increases in mucus (ALM et al., 1993, CHABAN et al., 2018). *C. jejun*i motor torque and likely continuous high energy consumption are consistent with its habitat of the animal gut, where nutrient availability is high, but the environment is highly viscous (CHABAN et al., 2018).

Although the basic architecture of flagella is highly conserved, flagellar structure itself varies across bacterial species. Cryo-electron tomography studies have shown that flagella from members of the *Epsilonproteobacteria* which include *Campylobacter* species, are among the most divergent, exhibiting several features that most likely correspond to novel flagellar components (BEEBY et al., 2016; CHEN et al., 2011; GAO et al., 2014;). Observation of ~12 stator complexes in many proteobacteria, yet ~17 in ε-proteobacteria suggest a "quantum leap" evolutionary event (CHABAN et al., 2018). Understanding the pathways and meaning of these differences is of great significance, as little is known how these changes are regulated at molecular level. The flagellum is not only responsible for motility, but it also plays important role in pathogenesis, including host cell adhesion, biofilm formation and as a virulence factor secretion system (DASTI et al., 2010; SVENSSON et al., 2014). In recent study DNA supercoiling has been shown to affect the expression of genes involved in flagellar gene, implicating DNA supercoiling as a key regulator of motility during in vivo colonisation (SHORTT et al., 2016).

Development of a *Campylobacter* flagellar subunit vaccine is of considerable interest (POLY et al., 2018).

1.3.3. Chemotaxis

Campylobacter motility is controlled by the complex chemosensory system which is based on environmental conditions and allows the bacteria to swim toward attractants, and away from

repellants (LERTSETHTAKARN et al., 2011). Disruption of this system affects the pathogen's virulence (MATILLA et al., 2017), and can reduce *Campylobacter* ability to colonize and cause disease (CHANG and MILLER, 2006, YAO et al., 1997). Chemotaxis was shown to be essential for *C. jejuni* strains to competitively colonize the chicken gastrointestinal tract (THIBODEAU et al., 2015).

Among attracting factors are glycoprotein mucin, the principal constituent of mucus, and mucin constituent L-fucose. Amino acids such as aspartate, cysteine, serine, glutamate, and the salts of the organic acids -citrate, fumarate, α-ketoglutarate, malate, pyruvate and succinate, also serve as chemoattractants (HUGDAHL et al., 1988). It has been showed that *C. jejuni* exhibits chemotaxis to bile (cattle, human and mouse) and in particular to its major component- sodium deoxycholate (SDC), which induces virulence gene expression (LI et al., 2014a).

Chemotactic signals in bacteria are detected by transmembrane chemoreceptors that are referred to as Transducer Like Proteins (Tlps). The response towards different stimuli depends on bacteria's chemoreceptor repertoire, as well as their sensitivity and specificity toward a chemoeffector (FALKE and HAZELBAUER, 2001). Compared to the 5 chemoreceptors in *E. coli* and 4 in its close relative: *Helicobacter pylori*, *C. jejuni* species possess at least 10 chemoreceptors designated as Tlps, and 2 aerotaxis receptors Aer1-2 (MARCHANT et al., 2002). The presence of these adaptable chemoreceptors and signaling proteins suggests that the chemotactic response in *C. jejuni* must be highly sensitive (CHA et al., 2018).

1.3.4. Adhesion

Adherence of bacteria to host epithelial cells is mediated by adhesins expressed on the pathogen's surface and is determinative step in bacterial infection (JIN et al., 2001). Previous work has revealed that *C. jejuni* isolates recovered from individuals with fever and diarrhea adhered to cultured cells in greater numbers than isolates recovered from asymptomatic individuals (FAUCHERE et al., 1986). *Campylobacter* does not possess fimbriae like *E. coli* or *Salmonella*, but a number of constitutively synthesized proteins have been proposed to act as adhesins, and over 40 genetic factors have been reported to contribute to adhesion and invasion (BAIG et al., 2014). The precise relevance and influence of each of these factors remains not fully understood.

Among known *C. jejuni* adhesins are CadF, FlpA, CapA and PorA (MOMP) (FLANAGAN et al., 2009). CadF and FlpA are most established and are believed to act together (EUCKER and KONKEL, 2012). CadF mediates *Campylobacter* adhesion to fibronectin, a glycoprotein found in gastrointestinal epithelial cells. CadF has been shown necessary for in vivo colonisation of the chickens (ZIPRIN et al.,

1999). Moreover, in CadF mutants, significant reduction of the internalisation of the human intestinal epithelial cells has also been observed (KRAUSE-GRUSZCZYNSKA et al., 2007). FlpA is a second potential fibronectin binding protein and appears to have even a more significant role, as the numbers of the flpA mutants binding to human gastrointestinal cells were reduced compared to the cadF mutants (HUSSEIN, 2018).

There are conflicting reports towards the role of *Campylobacter* adhesion protein A (CapA). Whereas in one study reduced adherence and complete inability to colonize are suggested (ASHGAR et al., 2007), another one points out that it does not influence chicken colonisation (FLANAGAN et al., 2009).

Furthermore, the *capA* gene is not present in all *C. jejuni* isolates, so it is likely nonessential, and no obvious role has been attributed to CapB (FLANAGAN et al., 2009). Interestingly, a novel autotransporter- CapC, has recently been discovered, and it is now been postulated to serve as an important virulence factor (MEHAT et al., 2018). Environmental concentrations of bile also affect *C. jejuni* adhesive characteristics through outer membrane vesicles (OMV) (TAHERI et al., 2018). Several other *C. jejuni* proteins which may have a function in early stages of cellular infection include the major outer membrane protein MOMP (MOSER et al., 1997), aspartate/glutamate binding protein Peb1A (LEON-KEMPIS et al., 2006) and the surface exposed lipoprotein JlpA (JIN et al., 2001). Recent study by Freitag (FREITAG et al., 2017) also considers the flagellar tip protein FliD as an early attachment factor.

1.3.5. Invasion

Campylobacter lacks the classical Type III secretion system (T3SS) that would allow it to directly inject the effector protein into the host cell. Instead, homologous flagellar components seem to play major role in pathogenicity, as mutations in these components have been proved to have reduced invasive ability (KONKEL et al., 2004). Moreover, various studies showed that protein secreted from the flagellum affect colonization in both- poultry and humans (KONKEL et al., 1999; ZIPRIN et al., 2001). The first one described-CiaB, was reported to be important for adherence (KONKEL et al., 1999). CiaC is required for invasion of INT-407 cells (CHRISTENSEN et al., 2009; KONKEL et al., 2004). CiaD activates MAP kinase signaling pathways and is required for the development of disease (SAMUELSON et al., 2013). CiaI has been reported to be involved in intracellular survival (BUELOW et al., 2011). Among other proteins affecting invasion are FlaC, FspaA, VirK and HtrA. FlaC is involved in binding to epithelial cells and is essential for colonisation (SONG et al., 2004). FspaA plays a role in

apoptosis (POLY et al., 2007). VirK may have a role in protection against antimicrobial proteins (NOVIK et al., 2009). HtrA (high temperature requirement A) contributes to cell binding and invasion, probably by assisting in properly folding the adhesins (BAEK et al., 2011).

1.4. MULTIDRUG RESISTANCE

First line antibiotics in European countries are macrolides (azithromycin or clarithromycin). Second-line therapy is chinolones. Rising worldwide resistance to antimicrobials is, however, of a great concern (TANG et al., 2017).

Over the years, to counteract the selection pressure from antimicrobial agents used in veterinary and human medicine, *Campylobacter* has developed various mechanisms for antibiotic resistance. In 2017 World Health Organisation (WHO) listed *Campylobacter* on a global priority list of antibiotic-resistant bacteriam (https://www.who.int/medicines/publications/global-priority-list-antibiotic-resistant-bacteria/en/; accessed on: 20-05-2020).

Campylobacter can modificate or inactivate antibiotics or their targets, restrict the access of antibiotics through reducing membrane permeability and extruse antibiotic by efflux pumps. Some of these mechanisms work explicit against specific class of antimicrobials, while others generate multidrug resistance (SHEN et al., 2018).

Moreover, new strategies in *Campylobacter* continuously emerge. Examples include presence of genes of Gram-positive origin, conferring high level of resistance to macrolides, such as *ermB* (WANG et al., 2014). Conjugative plasmids containing *tetO* spread tetracycline resistance in *Campylobacter* (PéREZ-BOTO et al., 2014). Novel plasmid borne *cfr*-like gene conferring resistance to phenicols (TANG et al., 2017) and a unique variant of efflux pump CmeABC (RE-CmeABC) that shows enhanced function in multidrug resistance and is associated with exceedingly high-level resistance to fluoroquinolones (YAO et al., 2016). These newly emerged resistance mechanisms are horizontally transferable and greatly facilitate the adaptation of *Campylobacter* in the food-producing environments (SHEN et al., 2018).

1.5. BIOFILM, VBNC STATE AND AEROTOLERANCE

Campylobacter spp. has been found in a wide range of environments, and posses various tolerance mechanisms that enable its survival under the harsh conditions such as cold, low nutrition milieu or atmospheric condition (MURPHY et al., 2006). The ability to cope with oxygen tension despite its

microaerophilic nature seems to be one of the most important features of this bacterium, as hyper-aerotolerant (HAT) *C. jejuni* strains were reported to be highly prevalent in a retail poultry meat (OH et al., 2015). HAT strains also had a higher prevalence of genes implicated in human infection than aerosensitive strains (OH et al., 2017). Among mechanisms that allow *Campylobacter* persistence in aerobic environment are biofilm formation and ability to switch from its physiological form to VBNC state. Both have been reported to increase under aerobic conditions (REUTE et al., 2010; OH et al., 2015). VBNC state can also be induced through prolonged exposure to water (BRONOWSKI et al. 2014; LI et al. 2014b). Biofilm makes it possible for *Campylobacter* to survive in water for up to 3 weeks and more (LEHTOLA et al., 2006). Interestingly, *Campylobacter* exhibited prolonged survival when co-cultured with *Pseudomonas spp.* (HILBERT et al., 2010).

1.6. CAMPYLOBACTERIOSIS

Since notification of campylobacteriosis is mandatory in most EU member states, the European Food Safety Authority provides summary reports on trends and sources of zoonoses. With 246,571 confirmed cases in 2018 and an average EU notification rate of 64.1 cases per 100,000 population, *Campylobacter* was still the most commonly reported gastrointestinal bacterial pathogen in humans in the EU, followed by salmonellosis, yersiniosis, STEC infections and listeriosis. The countries with the highest notification rates per 100, 000 capita were the Czech Republic, (215.8), Slovakia (153.2) and Luxembourg (103.8) (EFSA and ECDC, 2019).

Confirmed cases likely represent only the tip of the iceberg due to underreporting. In the last 5 years there has been no significant increase or decrease of the EU notification rate of campylobacteriosis. Despite the high number of cases, fatality rate is low (0.03%). However, the cost of disease to public health systems and to lost productivity in the EU is estimated by EFSA to be around EUR 2.4 billion a year.

Person to person transmission is not very common, but possible. Interestingly, strong risk factor for campylobacteriosis is international travel (DOMINGUES et al., 2012). Exposure in early life may lead to the development of protective immunity, which may explain why in developing countries where *Campylobacter* is endemic, usually only children show clinical signs (RAO et al., 2001).

Clinical manifestation of campylobacteriosis in humans are symptoms of acute gastroenteritis, consisting of a prodromal stadium with non-specific symptoms (fever, headache, myalgia, arthralgia), followed by massive watery or bloody diarrhea. In average, the disease lasts for one week if the patient is immunocompetent and does not require antibiotic treatment. Especially in developing

countries *C. jejuni* infections are common in very young children (< 5 years), causing watery diarrhea (RAO et al., 2001). Rare complications are septicemia, meningitis, reactive arthritis (PETERSON et al., 1994), Guillain-Barre/Miller-Fisher syndrome (MISHU et al., 1993). Moreover, there is an association with irritable bowel syndrome and inflammatory bowel disease (GRADEL et al., 2009). Recent studies look for potential correlation between *C. jejuni* and colorectal tumorigenesis (HE et al, 2019). Some of the species (*C. jejuni, C. coli, C. fetus* and *C. upsaliensis*) have been reported to cause abortion in humans and animals (SIMOR et al., 1986).

The infectious dose for human infection has been reported to be as low as 500 CFU, though the sample size in this study was very small with n = 1 (ROBINSON, 1981). However, other studies were also able to confirm the illness after ingesting even lower number of 360 CFU (HARA-KUDO & TAKATORI, 2011). Mathematical modelling suggested that an intermediate dose of 9×10^4 CFU/ml has the highest ratio of illness to infection (MEDEMA et al., 1996). A Scientific Opinion of the Panel on Biological Hazards (BIOHAZ Panel, 2011 & 2020) assessed that the handling, preparation and consumption of broiler meat may account for 20 % to 30 % of human cases of campylobacteriosis, while 50 % to 80 % may be attributed to the chicken reservoir as a whole. Drinking water, especially when untreated, can present an infection threat. Outbreaks that occur can often be traced to contaminated water supply (BARTHOLOMEW et al., 2014; SMITH et al., 2006). Other sources of infection include direct contact with pets and other animals and food items such as red meat or milk (FRIEDMAN et al., 2004). Fly born transmission has also been postulated (HALD et al., 2004; EKDAHL et al., 2005).

Diagnosis of human infection is generally based on culture from human stool samples and both culture and non-culture dependent methods (PCR and ELISA) are used for confirmation https://www.rki.de/DE/Content/Infekt/EpidBull/Merkblaetter/Ratgeber_Campylobacter.html; accessed on: 15-07-2020)

1.6.1. Complications

Serious complications are associated with *Campylobacter* infection, many of which have a worse prognosis than the acute infection itself. One of them is Guillain-Barré syndrome (GBS), and a less common subtype- Miller Fisher Syndrome (MFS). GBS is an autoimmune disorder, in which the immune system mistakenly attacks the peripheral nerves and damages their myelin insulation. GBS typically occurs after an infection in which the immune response generates antibodies that cross-react with gangliosides at nerve membranes (VAN DEN BERG et al., 2014). It is estimated that

Campylobacter infection is responsible for about 30 % of all GBS. Despite the advances in the management of GBS the mortality rate is at about 5 %, and approximately 20 % of patients require prolonged intensive care (YUKI et al., 2012).

Reactive arthritis is another complication following 1 % to 5 % of campylobacteriosis cases. Predominant syndrome is a sterile joint inflammation, with predilection for joints of the lower extremity, however, small joints and tendons may also be involved. Young adults are the most commonly affected group (POPE et al., 2007). Symptoms begin approximately 1 month following infection, vary in their strength from mild oligo-arthralgia to disabling polyarthritis and resolve usually within a year, although in some patients this condition may persist for up to 5 years (BATZ et al., 2013).

Case reports of pericarditis and myopericarditis have been increasingly reported as complications following infection with *Campylobacter*, mainly with *C. jejuni* and C. *fetus* (KAAKOUSH et al., 2015).

Most of the reported cases of myocarditis or myopericarditis were of young males with benign outcome. However, these conditions can lead to arrhythmia, dilated cardiomyopathy and cardiovascular collapse, and *Campylobacter* as an assumed cause is strongly underreported. Understanding the underlying mechanism and identifying the etiology of myopericarditis as bacterial one could ensure better treatment such as with antibiotics in addition to the cardiac medications (SPAPEN et al., 2015).

Multiple other gastroenterological manifestations are associated with *Campylobacter* including inflammatory bowel disease (IBD) (GRADEL et al., 2009) and esophageal diseases (DI PILATO et al., 2016). Recent research also demonstrated protumorigenic effect of *C. jejuni* in the colon and proved the carcinogenic potential of its cytolethal distending toxin (CDT) *in vivo* (HE et al., 2019).

1.6.2. Legislation

All food business operators have a legal responsibility to produce safe food (Regulation 178/2002). This is ensured by implementation of management system based on Hazard Analysis and Critical Control Point (HACCP) and good hygiene practices (GHP). Regulation 852/2004 lays down hygiene requirements for all foodstuffs, while Regulation 853/2004 lays down more specific hygiene requirements for foods of animal origin.

Regulation 2073/2005 lays down microbiological criteria for various combinations of food commodities and microorganisms, their toxins or metabolites. *Campylobacter spp*. has not been

included in its original version, however, in august 2017 the regulation 2073/2005 has been adopted to include the food category «Carcass of broilers» for the control of *Campylobacte*r, using the analytical reference method EN ISO 10272-2 (Commission Regulation 2017/1495). Proposed process hygiene criteria (PHC) in force since January 2018 are depicted in Table 1.

Table 1. Process hygiene criteria for *Campylobacter spp.*

Food category	**Micro-organisms**	**Sampling plan**		**Limits**		**Analytical reference method**	**Stage where the criterion applies**	**Action in case of unsatisfactory results**
		N	**c**	**m**	**M**			
broiler carcass	*Campylobacter spp.*	50 (5)	c = 20 From 1.1.2020 c =15; From 1.1.2025 c = 10	1 000 cfu/g		EN ISO 10272-2	Carcases after chilling	Improvements in slaughter hygiene, review of process controls, of animals' origin and of the biosecurity measures in the farms of origin'

Source: Commission Regulation (EU) 2017/1495 of 23 August 2017 amending Regulation (EC) No 2073/2005 as regards *Campylobacter* in broiler carcasses.

The same neck skin samples used for testing compliance with the process hygiene criterion set for *Salmonella* in poultry carcasses may be used for the *Campylobacter* analyses. Furthermore, under certain circumstances e.g. satisfactory results obtained for 52 consecutive weeks, sampling frequency may be reduced or adjusted to seasonal variations. The aim of the new regulation is to keep *Campylobacter* in broiler carcasses under control to reduce the number of human campylobacteriosis cases linked to the consumption of poultry.

1.6.3. Control strategies

Effective and commonly applicable solutions for the eradication of *Campylobacter* along the food chain are still missing. According to a survey carried out by EFSA at slaughterhouse level across 26 EU countries and two other countries in Europe in 2008, broiler carcasses were contaminated at an average of 71.2 % (EFSA, 2010). Because of the shift away from antibiotic supplementation various alternative control measures have been employed both-at the farm and at processing levels to control pathogen load. While prevention of intestinal spoilage, logistic slaughter and chemical decontamination of meat and skin are recommended to reduce contamination during processing, limited information is available on how to counteract *Campylobacter* colonization in preharvest

poultry production. Thus, integrated approaches are required considering that newly hatched chicks are pathogen free and, in most flocks, colonization occurs within 2-3 weeks of hatching, birds typically remain colonized for life and the prevalence at the farm level can reach up to 100 %. To date, three general strategies have been proposed as pre-harvest measures: reduction of environmental exposure through biosecurity measures, increasing host resistance to reduce carriage in the gut (e.g. genetic selection strategies, competitive exclusion, immunization) and the use of antimicrobial alternatives to reduce or even eliminate the pathogen load (e.g. bacteriophage and bacteriocin application) (SIBANDA et al., 2018; SORO et al., 2020).

1.7. STUDY OBJECTIVES

Thermophilic *Campylobacter spp.* (*C. jejuni*, *C. coli*, *C. lari* and *C. upsaliensis*) are the most commonly reported zoonotic agents causing gastrointestinal infections (bloody diarrhea) in humans. Complications are septicemia, meningitis, cholecystitis and Guillain–Barré/Miller Fisher syndrome. *Campylobacter* sources are raw and undercooked poultry meat, unpasteurized milk and contaminated surface water.

Within the project "Austrian Competence Centre for Feed and Food Quality, Safety and Innovation FFoQSI" collaboration in the Non-K Project Area was initialized to collaborate with the poultry industry and private diagnostic laboratories with focus on "the prevention of thermophilic *Campylobacter* contamination on poultry meat".

A side project and the goal of this diploma thesis was established with an aim to confirm isolated *Campylobacter* species via PCR as well to perform molecular subtyping of *C. jejuni* and *C. coli* isolated at farm and slaughterhouse level (from feces, intestinal content and environment) (expected number of isolates n=200). Therefore, within the scope of the present study, presumptive *Campylobacter spp*. Isolates provided by the external laboratory HYGIENICUM® GmbH Institut für Lebensmittelsicherheit und Hygiene, were microspically prescreened, differentiated by Catalase and Oxidase reaction and PCR confirmed (WANG et al., 2002) at the Institute of Milk Hygiene. An isolate collection was established for further subtyping purposes. All isolates were cultivated on Tryptic soy agar under the microaerophilic conditions (42° C, 1-2 days) and introduced in the pulsed field gelectrophoresis (PFGE) including the restriction enzyme *SmaI*. Furthermore, *Campylobacter* isolates were submitted to multi-locus sequence typing (MLST) for genotype comparison to previous studies and estimation of *Campylobacter* global spread or local clonality (SCHALLEGGER et al., 2016).

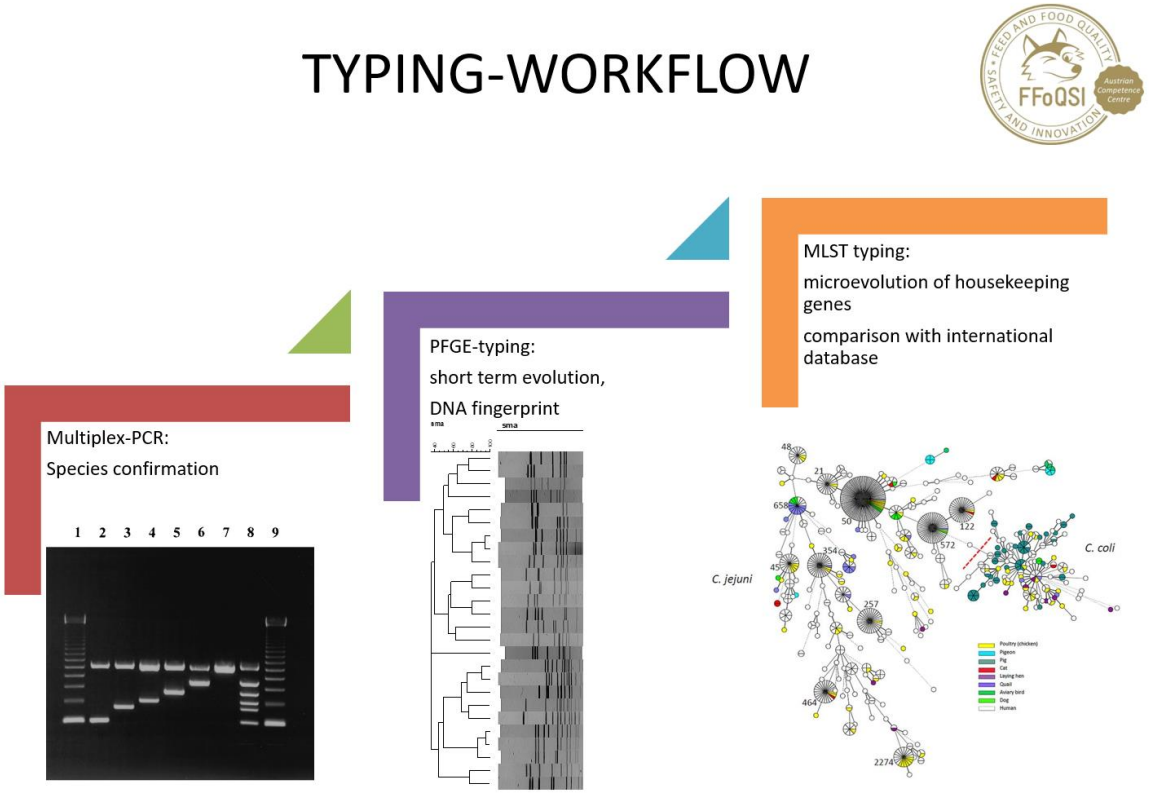


Figure 1. *Campylobacter* detection and sequence typing workflow. The process is composed of 3 steps: a) multiplex PCR confirmation, b) PFGE- comparison of isolate specific DNA band patterns-genetic fingerprint, c) investigation of allelic profiles (MLST) and comparison with international database.

Practical applications

C. jejuni and *C. coli* are the leading causes of bacterial gastroenteritis in humans worldwide. The EU notification rate of human campylobacteriosis was 64.1 per 100,000 population in 2018 (EFSA zoonosis report, 2019). Monitoring of thermophilic *Campylobacter* present in poultry is still a goal to be fulfilled by the EU member states. The espected typing results from the diploma thesis will be accomplished with antibiotic resistance data to estimate the risk of resistance among poultry associated isolates.

The study will give an insight into the genetic diversity of thermophilic *Campylobacter* species in different Austria regions in order to help the industry to track down the pathways of contamination, so that the protective measures can be taken, as well as it will establish a database for further research in order to obtain a better understanding of different mechanism in antimicrobial drug resistance.

2. MATERIALS & METHODS

Details about equipment and consumables applied in this study are provided in Appendix Table 1.

2.1. *CAMPYLOBACTER* ISOLATE COLLECTION

A total of 334 isolates suspicious for *Campylobacter* were provided by the cooperation partner HYGIENICUM® GmbH Institut für Lebensmittelsicherheit und Hygiene. The received *Campylobacter* isolates were cultured on Tryptic soy agar (TSAY) plus 0.6 % yeast extract (Biokar Diagnostics, Beauvais Cedex, France) for 24–48 h at 42° C under microaerobic conditions (5 % CO_2, 10 % O_2, 85 % N_2 gas, Linde Gas GmbH, Stadl Paura, Austria). Grown colonies were examined macroscopically for contamination presence and tested for catalase (Sigma-Aldrich Corp., St. Louis, MO, USA) and oxidase activity (Oxidase 50 AMP, bioMerieux, Marcy-l'Étoile, France) according to the manufacturer instructions. Finally, the *Campylobacter* isolates were cryopreserved in cryomedium consisting of Brain heart infusion-broth (BHI, Oxoid Ltd., Hampshire, United Kingdom), 60 % glycerol (Sigma-Aldrich Corp.) and defibrinated horse blood (Oxoid Ltd.) and stored at -80°C until further analysis.

2.2. DNA ISOLATION WITH CHELEX® 100 RESIN

The DNA extraction protocol was adapted from WALSH et al (1991) utilizing Chelex-100® Resin (Bio-Rad Laboratories Inc., Hercules, CA, USA). DNA extraction was performed on *Campylobacter* overnight cultures previously incubated on TSAY (42 °C, microaerobic conditions). With inoculation loop collected bacterial material was suspended in 100 µl 0.01M TrisHCl buffer pH 7.4 (Sigma-Aldrich Corp.), shortly vortexed and in the next step 400 µl Chelex-100® Resin solution was added to suspension. The suspension was shortly mixed and incubated for 10 min at 100° C. In the next step, the mixture was centrifuged for 5 sec at 15 000 x g. Finally, 100 µl of supernatant was transferred into 1.5 ml sterile Eppendorf (Eppendorf AG, Hamburg, Germany) tubes. The DNA extracted by described procedure was directly used as a template for PCR respective MLST or stored at -20° C until further processing.

2.3. *CAMPYLOBACTER* MULTIPLEX PCR AND ELECTROPHORESIS

Presumptive *Campylobacter* isolates were confirmed by multiplex PCR according to method established by WANG et al. (2002), targeting five genes specific for the species *C. jejuni, C. coli, C. lari, C. fetus* and *C. upsaliensis* as well as the 23S rRNA gene present in all *Campylobacter spp*. The primer sequences used in the multiplex PCR approach are depicted in Table 2. For the PCR reaction DSM 24156 *C. coli,* DSM 24189 *C. jejuni, DSM 5365 C. upsaliensis and DSM 5361 C. fetus* served as a

positive control. Two types of negative controls were included per each multiplex PCR run, the negative control for the DNA extraction procedure and the PCR run (non-template control=NTC). For negative DNA extraction control, no bacterial material was added into the solutions during the DNA extraction procedure. Sterile processed water incubated with 0.1% diethyl pyrocarbonate (DEPC; Thermo Fisher Scientific Inc.) was used instead of adding DNA into the PCR master mix. Each multiplex PCR tube consisted of PCR buffer, $MgCl_2$, primer, deoxynucleoside triphosphate (dNTP) and Taq polymerase concentration as shown in Table 3.

Table 2. *Campylobacter* primers for species confirmation.

Primer	Sequence (5'-3')	Species	Target	Amplicon size (bp)
CJF	ACTTCTTTATTGCTTGCTGC	*Campylobacter jejuni*	*hipO*	323
CJR	GCCACAACAAGTAAAGAAGC			
CCF	GTAAAACCAAAGCTTATCGTG	*Campylobacter coli*	*glyA*	126
CCR	TCCAGCAATGTGTGCAATG			
CLF	TAGAGAGATAGCAAAAGAGA	*Campylobacter lari*	*glyA*	251
CLR	TACACATAATAATCCCACCC			
CUF	AATTGAAACTCTTGCTATCC	*Campylobacter upsaliensis*	*glyA*	204
CUR	TCATACATTTTACCCGAGCT			
CFF	GCAAATATAAATGTAAGCGGAGAG	*Campylobacter fetus*	*sapB2*	435
CFR	TGCAGCGGCCCCACCTAT			
23SF	TATACCGGTAAGGAGTGCTGGAG	*Campylobacter spp.*	*23S rRNA gene*	650
23SR	ATCAATTAACCTTCGAGCACCG			

Source: Wang et al., 2002.

DNA amplification was performed using Thermocycler VWR Doppio (VWR, Vienna, Austria). The PCR parameters for multiplex PCR are depicted in Table 3.

PCR amplicons were visualized by gel electrophoresis on 1.5 % agarose gel. For this purpose, 1.5 g agarose (PeqLab, Erlangen, Germany) were added to 100 ml of 1x Tris-borate-EDTA (TBE) buffer (Carl Roth GmbH + Co. KG, Karlsruhe, Deutschland) (89 mM Tris base, 89 mM boric acid, and 2 mM EDTA pH 8) and heated for about 2 min. in the microwave until completely dissolved. The solution was cooled down to about 50° C and 2 µl of DNA binding dye, PeqGreen (PeqLab), were added for DNA visualization under the UV-light. Subsequently agarose was poured into a gel tray with the well comb in place and let to solidify for about 30 min. Once solidified, gel was placed in electrophoresis unit (Bio-Rad Laboratories Inc.) that was previously filled with 1x TBE buffer. Sample loading buffer (Bio-Rad Laboratories Inc.) was mixed with 10 µl of the PCR product and pipetted into the wells. The electrophoresis conditions were 120 V for 30 minutes. The recorded gel images gels were stored electronically as .tiff files using GelDoc 2000 (Bio-Rad Laboratories Inc.).

Table 3. Mastermix Composition for *Campylobacter* multiplex-PCR.

Mastermix	**final conc.**		**stock conc.**		**1x in µL**
DEPC water					11
10x PCR buffer	1x				2.5
MgCl2	2	mM	50	mM	1
CJF (hipO)	500	nM	50000	nM	0.25
CJR (hipO)	500	nM	50000	nM	0.25
CLF (glyA)	500	nM	50000	nM	0.25
CLR (glyA)	500	nM	50000	nM	0.25
CCF (glyA)	1000	nM	50000	nM	0.5
CCR (glyA)	1000	nM	50000	nM	0.5
CUF (glyA)	2000	nM	50000	nM	1
CUR (glyA)	2000	nM	50000	nM	1
23SF	200	nM	50000	nM	0.1
23SR	200	nM	50000	nM	0.1
dNTP`s	200	µM	5000	mM	1
Taq pol (Plat.)	1.5	U	5	U/µl	0.3
Mastermix					20
Template					5
Reaction volume					25

Source: Wang et al., 2002.

PCR conditions:	**Temperature**	**Duration**	**Number of cycles**
Initial denaturation	94°C	2 min	
Denaturation	94°C	30sec	
Annealing	59°C	30sec	30
Elongation	72°C	30sec	
Final elongation	72°C	7 min	
Storage	4°C	∞	

Source: Wang et al., 2002.

2.4. PULSED-FIELD GEL ELECTROPHORESIS (PFGE)

Pulsed-field gel electrophoresis (PFGE) is a molecular fingerprinting technique by which genomic DNA is isolated from the bacteria to produce a DNA fingerprint for a bacterial isolate (https://www.cdc.gov/pulsenet/pdf/campylobacter-pfge-protocol-508c.pdf; accessed: 18-03-2020). The isolated DNA is digested with restriction enzymes and the digestion products are loaded on an agarose gel, separated according to DNA fragment size by applying an electric field of alternating

polarity and allowing approximate measurement of fragment length. The fragments are resolved into a pattern based on molecular size resulting in DNA fingerprint with specific pattern that enables the investigation and comparison of relatedness between the bacterial strains.

The PFGE method can be divided into 6 main steps, bacteria suspension preparation, agarose plug preparation, cell lysis, restriction enzyme digestion, electrophoresis and data analysis.

In the current study genotyping of *Campylobacter* isolates was performed according to a standard operating procedure for Pulsnet PFGE for *Campylobacter jejuni*, provided by Centres for disease control and prevention (CDC), (https://www.cdc.gov/pulsenet/pdf/campylobacter-pfge-protocol-508c.pdf; accessed: 18-03-2020). The *Campylobacter* isolates used for the PFGE were cultivated under microaerophlic conditions on TSAY agar at 42° C for 24 h. The grown material was suspended in 1x PBS pH 7.4 (0.137 M NaCl, 0.0027 M KCl, 0.01 M Na_2HPO_4, 0.0018 M KH_2PO_4) and a final OD_{600} of 1.6 (Photometer, Shimadzu Europa GmbH, Duisburg, Germany) was set in the suspension. In the next step 1 % SeaKem Gold agarose (Lonza Group AG, Basel, Switzerland) in 10x Tris-EDTA (TE) buffer pH 8 (10 mM Tris/HCl pH 8, 1 mM EDTA pH 8) was prepared and kept in waterbath at 56° C until further use. Into each cell suspension 20 µl of Proteinase K (20 mg/ml stock) was added and mixed gently. For casting agarosis plugs 400 µl of 1 % agarose was added into suspension and pipetted up and down three times. Without introducing bubbles, the mixture was immediately transferred into reusable plug molds and kept at RT for 20 min to solidify. For cell lysis, the plugs were transferred into 5 ml of cell lysis buffer (50 mM Tris/HCl pH 8, 50 mM EDTA pH 8.0 and 1% N-Lauroylsarcosine) supplemented with 25 µl of Proteinase K (0.1 mg/mL final conc.) and incubated over night at 54° C under agitation (~120 rpm). On the following day, the agarose plugs were wased once with ddH_2O for 10 min at 54° C and three times in 10 ml 10x TE buffer pH 8 under the same conditions. The plugs were stored in 2 ml tubes in 10x TE buffer pH 8 at 4° C until further processing. The restriction digest was was performed with *Sma*I restriction enzyme (Thermo Fisher Scientific, Waltham, USA). The agarose plugs were cut into small peaces and equilibrated in pre-restriction incubation step for 10 min at RT in mixture composed of 10 µl of 10x Tango restriction buffer and 90 µl sterile ddH_2O water per agarose slice. In the next step the the agarose slices were incubated at for 4 h at 25° C in 100 µl restriction buffer consisting of of 89.6 µl of sterile ddH_2O, 10 µl 10x Tango restriction buffer and 2 µl restriction enzyme per agarose slice. Upon the restriction digestion the enzyme/buffer mixture was removed, and each slice was washed for 10 min at RT in 0.5x Tris-borate EDTA buffer (TBE) (45 mM Tris, 45 mM boric acid, 1 mM EDTA pH 8). Restricted plug slices were were loaded onto comb and carefully, without introducing bubbles, immersed into 1 % SeaKem Gold agarose in 0.5x TBE buffer.

The gel was let to solidify at room temperature for minimum 30 min. In the following step the comb was removed, and the gel was placed into the black frame in electrophoresis chamber that was filled with 0.5x TBE buffer. The electrophoresis conditions consisted of an initial switch time of 5 s and a final switch time of 55 s, gradient of 6 V/cm and an included angle of 120. After the electrophoresis run of the 22.5 h was completed, gels were stained with ethidium bromide solution (Sigma Aldrich Corp.), and the band pattern was observed under UV illumination. Patterns were digitally photographed with Gel Doc 2000 (Bio-Rad Laboratories, Inc.) and saved as Tagged Image File Format (TIFF).

The unweighted pair group method using arithmetic averages and the Dice correlation coefficient were applied with a position tolerance of 1.5 %. PFGE types with less than three band difference were considered as closely related, according to Tenover et al. (1995).

2.5. MULTILOCUS SEQUENCE TYPING (MLST)

The technique relies on data of nucleotid sequences of 450-500 bp internal fragments of *C. jejuni* and *C. coli* house-keeping genes, and it provides important information about the nucleotide divergence of the core genome, the clonal origin, the recombination rate and the phylogenetic relationship among strains. The main advantage of this method is that it gives unambiguous data that are reproducible among laboratories.

In this study *C. jejuni*, and *C. coli isolates* were submitted to amplification of the seven housekeeping genes *aspA* (aspartate ammonialyase), *glnA* (glutamine synthetase), *gltA* (citrate synthase), *glyA* (serine hydroxymethyltransferase), *pgm* (phosphoglyceromutase), *tkt* (transketolase) and *uncA* (ATP synthase alpha subunit) (DINGLE et al., 2001; JOLLEY et al., 2018). The primer used for PCR amplification and sequencing, as well as PCR conditions were applying as described in *Campylobacter* MLST protocol on homepage of *Campylobacter* MLST database (https://pubmlst.org/campylobacter/; accessed on:18-04-2020). The amplified PCR products were transferred in 96 well plates and submitted for nucleotid sequencing to LGC Genomics Ltd (https://shop.lgcgenomics.com/; accessed on:18-04-2020). The sequencing results were compared with the *Campylobacter* MLST sequence and isolate database (https://pubmlst.org/campylobacter/; accessed on: 18-04-2020).

3. RESULTS

A total of 334 presumptive *Campylobacter* isolated from broiler caecal/intestinal samples were kindly provided by project partner HYGIENICUM® GmbH Institut für Lebensmittelsicherheit und Hygiene. In the first prescreening steps which included morphological identification, Gram staining, oxidase and catalase activity tests, we were able to confirm 262 isolates as *Campylobacter* species. Other 72 isolates showed either no-growth or *Bacillus, Staphylococcus* or *Pseudomonas* spp. colony morphology. A total of 244 *Campylobacter* isolates were confirmed by PCR method (n=207/244; 84.8 % *C. jejuni*; n=37/244; 15.2% *C. coli*) and cultivable for further subtyping.

The *Campylobacter* spp. isolates were assigned to abattoirs (A-E), associated broiler farms (A1-E4) and districts (Table 4).

Table 4. *Campylobacter* species included in this study and their sample association.

Abattoir	Farm	District	Species (n)	Species (n)
A	A1	Amstetten	CJE (4)	
A	A2	Wolfsberg	CJE (13)	CCO (1)
B	B1	Hartberg-Fürstenfeld	CJE (11)	
B	B2	Hartberg-Fürstenfeld	CJE (12)	
B	B3	Graz-Umgebung	CJE (20)	
B	B4	Südoststeiermark	CJE (21)	
B	B5	Südoststeiermark	CJE (8)	
B	B6	Südoststeiermark	CJE (13)	
B	B7	Südoststeiermark	CJE (10)	
B	B8	Südoststeiermark	CJE (6)	
C	C1	Amstetten	CJE (7)	
C	C2	Südoststeiermark		CCO (17)
C	C3	Südoststeiermark	CJE (16)	
C	C4	Südoststeiermark	CJE (1)	CCO (5)
D	D1	Deutschlandsberg		CCO (3)
D	D2	Deutschlandsberg	CJE (20)	CCO (1)
D	D3	Südoststeiermark	CJE (7)	
D	D4	Südoststeiermark		CCO (10)
E	E1	Amstetten	CJE (6)	
E	E2	Urfahr-Umgebung	CJE (10)	
E	E3	Braunau am Inn	CJE (17)	
E	E4	Freistadt	CJE (5)	
Total			207	37

Abbreviations: A-E, abattoir code, A1-E4, broiler farm code; CJE, *C. jejuni*, CCO, *C. coli*.

All analyzed *Campylobacter* spp. isolates orginated from 22 broiler farms associated to five abattoirs in four Austrian federal states and nine districts (Table 4). *C. jejuni* was the dominant species with n=207 confirmed isolates in 19 flocks and distributed as follows: 101 *C. jejuni* isolates in abattoir B, 38 isolates in abattoir E, 27 isolates in abattoir D, 24 in abattoir C and 17 in abattoir A.

C. coli (n=37) isolates were confirmed in six farms and three associated abattoirs, with the following distribution: abattoir C 22 isolates, abattoir D 14 isolates and abattoir A one isolate (Table 4).

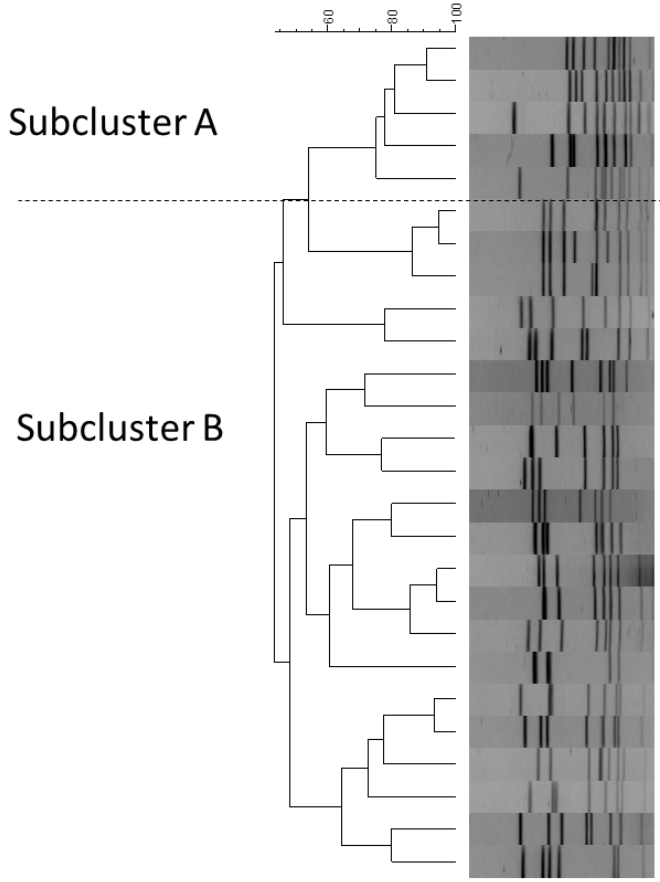
Both *C. jejuni* and *C. coli* species were detected on farms A2, C4 and D2 whereas farms C3, D1 and D4 harboured exclusively *C. coli*. The broiler flock with the highest amount of *Campylobacter* was B3 and C3 for *C. jejuni* (n=20 and n=16), C2 and E3 for *C. coli* (each n=17) and D2 for *C. jejuni* and *C. coli* (n=21) (Table 4).

The PFGE subtyping resulted in 21 *C. jejuni* and five *C. coli* fingerprints (restriction enzyme *Sma*I) (Figure 2). *C. coli* and *C. jejuni* clustered in separate subclusters (75 % similarity; subcluster A & B).

The majority of *Campylobacter* PFGE types (4 *C. coli* and 15 *C. jejuni* PFGE profiles) were specific for each broiler farm. In three broiler farms located in Upper Austria (E2, E3 and E4) two *C. jejuni* PFGE types each were isolated in parallel (Table 5). The same situation was observed for broiler farm B2 (district Hartberg-Fürstenfeld), B4 and C3 (both district Südoststeiermark). In broiler farm D3 (district Südoststeiermark) even three distinct *C. jejuni* PFGE profiles were isolated (CJE12, CJE13 and CJE14).

C. coli and *C. jejuni* mixed populations were observed for caecal samples from broiler farm A2 (CJE9, CCO2; district Wolfsberg-federal state Carinthia), D2 (CJE12, CJE13, CJE20 and CCO3; district Deutschlandsberg) and C4 (CJE 10, CCO4; district Südoststeiermark).

Interestingly, PFGE analysis revealed that some *Campylobacter* genotypes were shared between different broiler farms in the same federal state (Figure 2; Table 5). *C. coli* profile CCO3 was shared between two broiler farms (D1, D2) in the district of Deutschlandsberg (federal state Styria). Both farms are broiler fatteners for slaughterhouse D. *C. jejuni* PFGE profile CJE10 was detected at broiler farm B1and C4 (federal state Styria, Hartberg-Fürstenfeld, Südoststeiermark). *C. jejuni* PFGE profile CJE12 and CE13 were detected in three broiler farms (B8, D2 and D3) in the district of Deutschlandsberg and Südoststeiermark (federal state Styria), supplying broilers to slaughterhouse B and D. In addition, *C. jejuni* PFGE profile CJE15 and CE18 were detected in different federal states (C3 federal state Styria and E2 Upper Austria). The same was observed for *C. jejuni* PFGE type CJE 11, which was isolated from broiler caecal samples assigned to farm B2 (Styria, Hartberg-Fürstenfeld) and C1 (Lower Austria, Amstetten).



PFGE profile (*SmaI*)	ST	CC	Abbatoir	Farm	District
CCO3	854	ST-828	D	D1, D2	Deutschlandsberg
CCO4	825	ST-828	C	C4	Südoststeiermark
CCO5	1142	ST-828	D	D4	Südoststeiermark
CCO1	860	ST-828	C	C2	Südoststeiermark
CCO2	887	ST-828	A	A2	Wolfsberg
CJE13	824	ST-257	B, D	B8, D2, D3	Deutschlandsberg, Südoststeiermark
CJE4	824	ST-257	B	B5	Südoststeiermark
CJE3	824	ST-257	B	B5	Südoststeiermark
CJE15	2066	ST-52	C, E	C3, E2	Südoststeiermark, Urfahr-Umgebung
CJE16	400	ST-353	E	E4	Freistadt
CJE12	1911		D	D2, D3	Deutschlandsberg, Südoststeiermark
CJE21	2254	ST-257	E	E4	Freistadt
CJE17	354	ST-354	B	B7	Südoststeiermark
CJE19	354	ST-354	A	A1	Amstetten
CJE8	51	ST-443	E	E3	Braunau am Inn
CJE1	227	ST-206	B	B4	Südoststeiermark
CJE20	239		D	D2	Deutschlandsberg
CJE7	2304		B	B3	Graz-Umgebung
CJE18	400	ST-353	C, E	C3, E2	Südoststeiermark, Urfahr-Umgebung
CJE5	NEW	ST-206	B	B6	Südoststeiermark
CJE10	NEW	ST-464	B, C	B1, C4	Hartberg-Fürstenfeld, Südoststeiermark
CJE2	464	ST-464	E	E1	Amstetten
CJE14	824	ST-257	D	D3	Südoststeiermark
CJE11	446	ST-446	B, C	B2, C1	Hartberg-Fürstenfeld, Amstetten
CJE9	400	ST-353	A	A2	Wolfsberg
CJE6	7000		B	B4	Südoststeiermark

Figure 2. UPGMA-Unweighted Pair Group Method with Arithmetic mean cluster analysis based on *Campylobacter* spp. PFGE fingerprint patterns (*Sma*I). Similarity between patterns was estimated by using Dice correlation coefficient with a position tolerance of 1.5 %. PFGE types with less than three band difference were considered as closely related. Abbreviations: PFGE, pulsed-field gel electrophoresis; ST, sequence type; CC, clonal complex; A-E, abattoir code; A1-E4, broiler farm code.

The MLST typing resulted in 16 *C. jejuni* and five *C. coli* sequence types (STs). The discriminary power of *C. jejuni* MLST was lower in comparison to PFGE typing (21 PFGE fingerprints) (Table 5).

Three *C. jejuni* isolates were assigned to new STs. Thereof, two *C. jejuni* isolates shared the same ST (CC-464 complex) with the new allelic profile *aspA* (24), *glnA* (2), *gltA* (2), *glyA* (2), *pgm* (560), *tkt* (3) and *uncA* (1) and PFGE profile CE10 (farm B1 and C4).

Additionally, one novel *C. jejuni* ST indicated the new allelic profile *aspA* (6), *glnA* (30), *gltA* (5), *glyA* (2), *pgm* (2), *tkt* (1) and *uncA* (5) and PFGE profile CE5 (farm B6), which could be assigned to ST206-complex.

The most frequently occurring *C. jejuni* ST824 (ST-257 complex) was discovered in Styrian fattening farms delivering broilers to slaughterhouse B and D. The second most prevalent *C. jejuni* ST400 (ST-353 complex) was isolated from broiler farms located in Upper Austria, Styria and Carinthia which deliver broilers to slaughterhouse E, C and A. Gentoypes occurring at broiler farms located in different federal states were: *C. jejuni* ST267 (ST-283 complex), ST354 (ST-354 complex), ST446 (ST-446 complex) and ST2066 (ST-52 complex). A local *C. jejuni* genotype ST1911 was shared between broiler farms located in Styria and both delivering broilers to abbatoir D. All *C. coli* STs were assigned to ST-828 complex and isolated from broiler farms in Styria and Carinthia (abbatoir A, C and D).

C. coli ST854 was isolated from broiler farm D1 and D2, both supplying broilers to slaughterhouse D.

The highest genotypical diversity was observed in the district of Südoststeiermark covering 12 *Campylobacter* genotypes (three *C. coli* and nine *C. jejuni* STs). The second most relevant diversity among *Campylobacter* genotypes was identified in the district of Deutschlandsberg (one *C. coli* and three *C. jejuni* STs).

The highest *Campylobacter* genotype diversity was delivered to slaughterhouse B (9 distinct *C. jejuni* STs), C (4 distinct *C. jejuni* and 2 distinct *C. coli* STs) and E (6 distinct *C. jejuni* STs) (Table 5).

Table 5. Genetic diversity of *Campylobacter* based on PFGE profiles, sequence types and clonal complexes obtained in this study.

Abattoir	Farm	District	Species	*PFGE profile (SmaI)*	ST	CC	*aspA*	*glnA*	*gltA*	*glyA*	*pgm*	*tkt*	*uncA*
E	E1	Amstetten	*C. jejuni*	CJE2	464	ST-464	24	2	2	2	10	3	1
C	C1	Amstetten	*C. jejuni*	CJE11	446	ST-446	47	55	5	10	11	68	8
A	A1	Amstetten	*C. jejuni*	CJE19	354	ST-354	8	10	2	2	11	77	6
E	E2	Urfahr-Umgebung	*C. jejuni*	CJE15	2066	ST-52	9	10	5	10	22	3	6
E	E2	Urfahr-Umgebung	*C. jejuni*	CJE18	400	ST-353	8	17	5	2	10	59	6
E	E3	Braunau am Inn	*C. jejuni*	NT	267	ST-283	4	7	40	4	42	51	1
E	E3	Braunau am Inn	*C. jejuni*	CJE8	51	ST-443	7	17	2	15	23	3	12
E	E4	Freistadt	*C. jejuni*	CJE21	2254	ST-257	8	2	4	62	4	5	6
E	E4	Freistadt	*C. jejuni*	CJE16	400	ST-353	8	17	5	2	10	59	6
B	B1	Hartberg-Fürstenfeld	*C. jejuni*	CJE10	NEW	ST-464	24	2	2	2	560	3	1
B	B2	Hartberg-Fürstenfeld	*C. jejuni*	CJE11	446	ST-446	47	55	5	10	11	68	8
B	B2	Hartberg-Fürstenfeld	*C. jejuni*	NT	267	ST-283	4	7	10	4	42	51	1
B	B3	Graz-Umgebung	*C. jejuni*	CJE7	2304		2	4	5	25	11	3	5
D	D1	Deutschlandsberg	*C. coli*	CCO3	854	ST-828	33	38	30	82	104	43	17
D	D2	Deutschlandsberg	*C. jejuni*	CJE12	1911		7	84	5	10	119	178	26
D	D2	Deutschlandsberg	*C. coli*	CCO3	854	ST-828	33	38	30	82	104	43	17
D	D2	Deutschlandsberg	*C. jejuni*	CJE13	824	ST-257	9	2	2	2	11	5	6
D	D2	Deutschlandsberg	*C. jejuni*	CJE20	239	ST-21	2	1	5	3	2	1	6

Abbreviations: A-E, abattoir code; A1-E4, broiler farm code; CJE, *C. jejuni*, CCO, *C. coli,* PFGE, pulsed-field gel elektrophoresis; ST, sequence type; CC, clonal complex; *aspA,* aspartate ammonialyase; *glnA*, glutamine synthetase; *gltA*, citrate synthase; *glyA*, serine hydroxymethyltransferase; *pgm,* phosphoglyceromutase; *tkt,* transketolase; *uncA*, ATP synthase alpha subunit.

Table 5 continued: Genetic diversity of *Campylobacter* based on PFGE profiles, sequence types and clonal complexes obtained in this study.

Abattoir	Farm	District	Species	*PFGE profile (SmaI)*	ST	CC	*aspA*	*glnA*	*gltA*	*glyA*	*pgm*	*tkt*	*uncA*
B	B4	Südoststeiermark	*C. jejuni*	CJE1	227	ST-206	2	4	5	2	2	1	5
B	B4	Südoststeiermark	*C. jejuni*	CJE6	7000		7	21	2	62	525	48	1
B	B5	Südoststeiermark	*C. jejuni*	CJE3	824	ST-257	9	2	2	2	11	5	6
B	B5	Südoststeiermark	*C. jejuni*	CJE4	824	ST-257	9	2	2	2	11	5	6
B	B6	Südoststeiermark	*C. jejuni*	CJE5	NEW	ST-206	6	30	5	2	2	1	5
B	B7	Südoststeiermark	*C. jejuni*	CJE17	354	ST-354	8	10	2	2	11	12	6
B	B8	Südoststeiermark	*C. jejuni*	CJE13	824	ST-257	9	2	2	2	11	5	6
C	C2	Südoststeiermark	*C. coli*	CCO1	860	ST-828	33	39	30	79	113	47	17
C	C3	Südoststeiermark	*C. jejuni*	CJE15	2066	ST-52	9	10	5	10	22	3	6
C	C3	Südoststeiermark	*C. jejuni*	CJE18	400	ST-353	8	17	5	2	10	59	6
C	C4	Südoststeiermark	*C. coli*	CCO4	825	ST-828	33	39	30	82	113	47	17
C	C4	Südoststeiermark	*C. jejuni*	CJE10	NEW	ST-464	24	2	2	2	560	3	1
D	D3	Südoststeiermark	*C. jejuni*	CJE12	1911		7	84	5	10	119	178	26
D	D3	Südoststeiermark	*C. jejuni*	CJE13	824	ST-257	9	2	2	2	11	5	6
D	D3	Südoststeiermark	*C. jejuni*	CJE14	824	ST-257	9	2	2	2	11	5	6
D	D4	Südoststeiermark	*C. coli*	CCO5	1142	ST-828	256	153	30	82	104	43	36
A	A2	Wolfsberg	*C. jejuni*	CJE9	400	ST-353	8	17	2	2	10	59	6
A	A2	Wolfsberg	*C. coli*	CCO2	887	ST-828	33	38	30	82	104	85	68

Abbreviations: A-E, abattoir code; A1-E4, broiler farm code; CJE, *C. jejuni*, CCO, *C. coli,* PFGE, pulsed-field gel elektrophoresis; ST, sequence type; CC, clonal complex; *aspA,* aspartate ammonialyase; *glnA*, glutamine synthetase; *gltA*, citrate synthase; *glyA*, serine hydroxymethyltransferase; *pgm,* phosphoglyceromutase; *tkt,* transketolase; *uncA*, ATP synthase alpha subunit.

The molecular typing of *Campylobacter* isolates allows conclusions to be drawn about the source of entry of *C. jejuni* and *C. coli* into the poultry production chain. A database research for *Campylobacter* sequence types (STs) at the PubMLST homepage is depicted in Figure 3. The search concentrated on STs isolated in this study to identify the global spread and niche attribution. STs very common in the database are *C. coli* ST854 and ST825 and *C. jejuni* ST400, ST464, ST51, ST354 and ST267. The latter *C. coli* STs are present in a high diversity of niches (humans, poultry, ruminants, pigs, wild birds and environment). The global spread *C. jejuni* ST400, ST464, ST51, ST354 and ST267 are also present in a high diversity of niches except for the pig niche.

Some *C. jejuni* and *C. coli* genotypes seem to be present in the fattening farms over a longer period of time. The following global relevant *C. jejuni* ST267, ST354, ST400 and *C. coli* ST 854 were also detectable at several sampling times in our study. Furthermore, *C. jejuni* ST 446, ST824 and ST2066 were highly present during several sampling events in our study. Actually, *C. jejuni* ST446 and ST2066 are rather restricted to the human and poultry niche, whereas ST824 can also found in isolates from the pig niche. *C. coli* isolates were less heterogeneous in composition (all sequence type ST-828 complex) compared to the *C. jejuni* population. Genotyping is a major contribution to the elucidation of persistent *Campylobacter* strains and support of intervention measures.

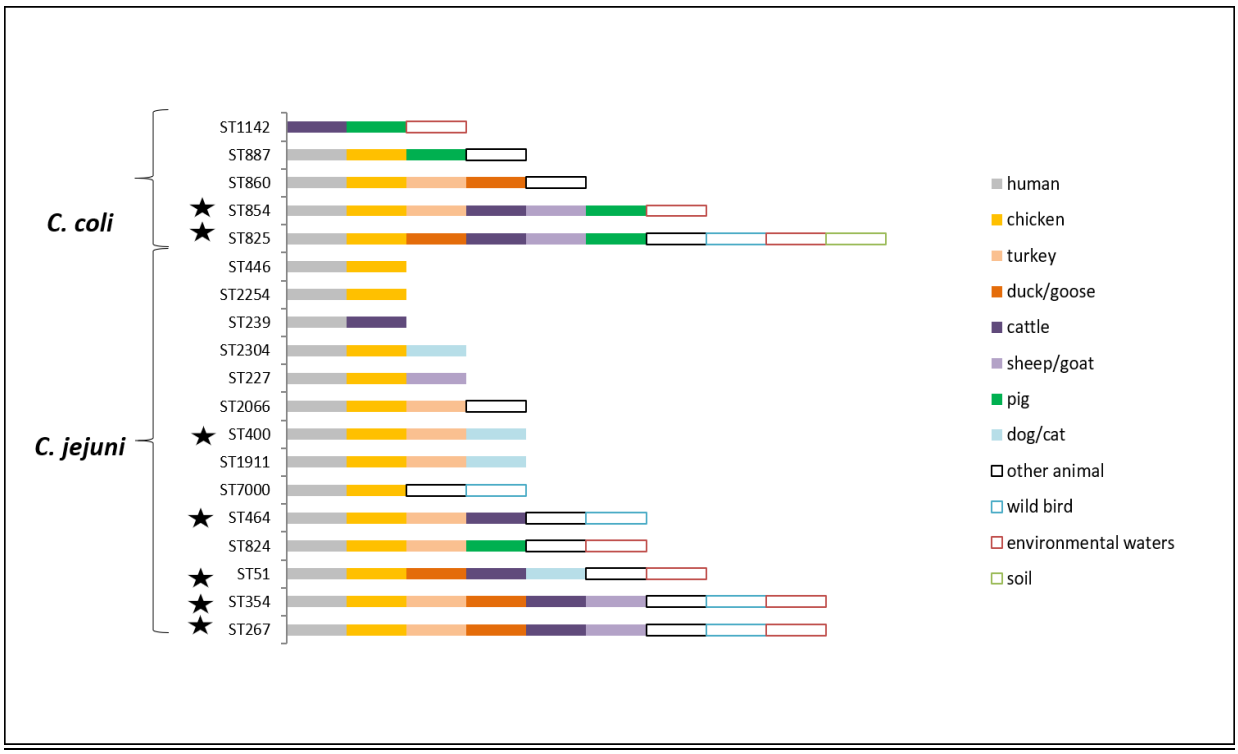


Figure 3. *Campylobacter* sequence types (STs) isolated from broiler caecal samples- source attribution. Source: https://pubmlst.org/campylobacter/; data basis n=5546 related sequences). Very common STs in the international MLST-database are marked with an asterisk.

Table 6. *Campylobacter* sequence types (STs) isolated in this study in comparison with the PubMLST database.

SPECIES	**CLONAL COMPLEX**	**ST**	**PFGE types**	**Abattoir**	**Farm**	**Federal state (Austria)**	**ISOLATES (n) in MLST database**	**Global distributed**
Campylobacter jejuni	ST-443 complex	**51**	**CJE8**	E	E3	UA	1285	yes
	ST-354 complex	**354**	**CE17, CE19**	A, E	A1, B7	LA, Styr.	1197	yes
	ST-206 complex	**227**	**CJE1**	B	B4	Styr.	129	yes
	ST-464 complex	**464**	**CJE2**	E	E1	LA	595	yes
	ST-283 complex	**267**	**NT**	B, E	B2, E3	Styr., UA	457	yes
	ST-353 complex	**400**	**CJE9, CJE18**	A, C, E	A2, C3, E2, E4	Car., Styr., UA	250	yes
	ST-257 complex	**2254**	**CJE21**	E	E4	UA	182	yes
		824	**CJE3, CJE4, CJE13, CJE14**	B, D	B5, B8, D2, D3	Styr., UA	157	yes
	ST-446 complex	**446**	**CJE11**	B, C	B2, C1	Styr., LA	25	no
	ST-52 complex	**2066**	**CJE15**	C, E	C3, E2	Styr., UA	24	no
		1911	**CJE12**	D	D2, D3	Styr.	25	no
	Singleton	**2304**	**CJE7**	B	B3	Styr.	19	no
	Singleton	**7000**	**CJE6**	B	B4	Styr.	10	no
	ST-21 complex	**239**	**CJE20**	D	D2	Styr.	3	no
	ST-206	**NEW**	**CJE5**	B	B6	Styr.	0	no
	ST-464	**NEW**	**CJE10**	B, C	B1, C4	Styr.	0	no
Campylobacter coli	ST-828 complex	**825**	**CCO4**	C	C4	Styr.	607	yes
		854	**CCO3**	D	D1, D3	Styr.	400	yes
		860	**CCO1**	C	C2	Styr.	119	yes
		887	**CCO2**	A	A2	Car.	45	no
		1142	**CCO5**	D	D4	Styr.	17	no

Source: https://pubmlst.org/campylobacter/; data basis n=5546 related sequences. Abbreviations: ST, sequence type; PFGE, pulsed-field gel elektrophoresis; CJE, *C. jejuni*; CCO, *C. coli*; NT, not typeable; A-E, abattoir code; A1-E4, broiler farm code; Federal districts: UA, Upper Austria, LA, Lower Austria, Styr., Styria, Car., Carinthia; MLST, multilocus sequence typing.

The global distribution of *Campylobacter* isolated in this study was determined by comparing the datset to the PubMLST database (Table 6). *C. jejuni* STs were global spread (n=8/16), as well as locally relevant (n=8/16). *C coli* STs were also distributed globally (n=3/5). *C. jejuni* and *C. coli* STs prevalent in more than one broiler farm were attributed to global STs (ST354, ST400, ST267, ST824 and ST854) as well as local STs (ST446, ST2066 and ST1911, STNEW (ST-464 complex).

4. DISCUSSION and CONCLUSION

In this study we concentrated on the prevalence of *Campylobacter* genotypes present at the primary production of broilers. The *Campylobacter* isolates were collected to estimate the contamination level of broiler farms located in four federal states of Austria (Styria, Upper and Lower Austria, Carinthia).

Poultry is frequently colonized by *C. jejuni* and most human infections, approximately 90%, originate from this livestock niche. About 10% of Campylobacteriosis are associated to *C. coli,* which seem to be lesser prevalent at broiler farms (BABACAN et al., 2020; EFSA and ECDC, 2019).

This is concordance with our findings where 84.8% and 15.2% of isolates were confirmed as *C. jejuni* and *C. coli*, respectively. *C. coli* were rather restricted to certain districts of Carinthia (Wolfsberg) and Styria (Südoststeiermark, Deutschlandsberg). Broiler farms (A2, C1-3, D1-4) harboring *C. coli* were delivering broilers to slaughterhouse A, C and D located in Carinthia and Styria. It is highly questionable whether *C. coli* is introduced via a local contamination route along the chain or originally via parent farms, hatcheries, special breeds or neighbouring farm animals (BABACAN et al., 2020; FROST et al., 2020). We speculate that there is a direct influence of the high pig density in districts and *C. coli*. WIECZOREK et al. (2020) observed similar in Poland at slaughterhouse level, where *C. coli* was predominat over *C. jejuni*. Another explanation might be that *C. coli* strains can better adapt to the slaughterhouse environment due to aerotolerance or co-selection of multi-drug resistance and resistance to environmental stress (GUK et al., 2019; O'KANE and CONNERTON, 2017). Some authors reported that certain *Campylobacter* genotypes were identified within the same farm longitudinally, suggesting environmental conditions able to support their persistence (IANETTI et al., 2020).

Certain prevalent *C. jejuni* STs within the CC-21 complex, which are often involved in human infections, were showed aerotolerance in parallel to a higher peracetic acid and cold tolerance (OH et al., 2019). KIATSOMPHOB et al. (2019) tested 70 *C. jejuni* strains from different sources for their

aerotolerance and reported that almost all were aerotolerant. Interestingly, hyper-aerotolerant *C. jejuni* were more prevalent among broiler and cattle isolates than in isolates from humans. SOPWITH et al. (2008) identified *C. jejuni* ST-45 most prevalent in surface waters. The authors concluded that ST-45 is more adapted to survival outside a host, making it a key driver of transmission between livestock, environment, and humans.

As reported also by other authors, *C. jejuni* genotypes were more heterogenous composed in comparison to *C. coli,* as *21 C. jejuni* PFGE types and 15 STs and only five *C. coli* PFGE types or STs were identified (SCHALLEGGER et al., 2016; VIDAL et al., 2016).

The genetic diversity of *C. jejuni* isolates at farm level should be considered when designing studies to understand *Campylobacter* populations in broiler production and the impact of biosecurity interventions (VIDAL et al., 2016). SCHALLEGGER et al. (2016) investigated the occurrence of *Campylobacter* in Austrian broiler flocks located in Upper Austria and Styria in 2015. The genotypes were highly diverse and flock related. In total, ten relevant *C. jejuni* STs were differentiated and five are also know to be distributed globally (ST51, ST50, ST1073, ST824 and ST881). Two *C. jejuni* genotypes were also identified in this study: ST446 (CC446) in a broiler farm in Upper Austria and ST824 (CC257) in a Styrian broiler farm (SCHALLEGGER et al., 2016). Actually, *C. jejuni* ST446 and ST824 were present in broiler farms located in Styria, Upper Austria and Lower Austria (Table 4). ST824 is the only *C. jejuni* genotype with relation to pig husbandry. *C. jejuni* ST446 has a strong link to the human interface. This leads us to the conclusion that the latter STs are relevant and recurrent in Austrian broiler farms.

Some of the STs detected in this study are also present among cattle isolates and isolates from wild birds and environment (PubMLST database): *C. coli* ST825, ST854 and ST1142 (all ST-828 complex) as well as *C. jejuni* ST51, ST239 (ST-21 complex), ST267, ST354 and ST464 (Figure 3).

Generalists as *C. jejuni* and *C. coli* assigned to ST-21, ST-45 and ST-828 clonal complexes, appear to have broad host ranges. DEARLOVE et al. (2016) deduced 89% of clinical cases to a chicken source, 10% to cattle and 1% to pig. Common strains of *C. jejuni* and *C. coli* causing very often human infections are adapted to a generalist lifestyle and allow a fast transmission between different hosts. These generalistic *Campylobacter* strains should be identified in a monitoring at primary production and compared to human strains on a regularly base to apply a targeted risk assessment. In the case of identification of *Campylobacter* genotypes often involved in human infections, biosecurity measures should be adapted at farm and slaughterhouse level.

There are numerous strategies to reduce thermophilic *Campylobacter* within the herd and on poultry carcasses. Currently, on- farm control options, apart from genetics of chicks (robustness), are based mainly on biosecurity and hygiene measures. Vertical transmission of *Campylobacter spp*. from hatcheries to commercial flocks is assumed to play a negligible role and the protective measures aim to avoid horizontal introduction into the herd (CALLICOTT et al., 2006; SIBANDA et al., 2018).

In 2020 the EFSA Biohaz Panel evaluated the effectiveness of 20 *Campylobacter* control strategies at primary production. Following biosecurity measures were identified to lower the risk for *Campylobacter* transmission: hygienic barriers at the entrance and separate equipment for each broiler house, absence of husbandry in close proximity, restricted personnel access and regular advanced trained staff, addition of disinfectants to drinking water, effective cleaning and disinfection between downtimes, discontinued thinning, feed and water additives, bacteriophages and vaccination (EFSA Panel on Biological Hazards -BIOHAZ, 2020).

Finally, the methods of sampling and isolation play a crucial role in epidemiological approaches. Microbiological surveillance supports us to gain insight distribution and circulation of food-borne pathogens along the poultry chain and to develop intervention strategies. Past evidence figured out that isolation and enrichment methods for *Campylobacter* may lead to the differential recovery of genotypes, impede our ability to identify a mixed species sample and may cause a potentially biased prevalence estimation (HETMAN et al., 2020; SCHALLEGGER et al., 2016). Selecting multiple *Campylobacter* colonies from direct plating and enrichment is important to identify predominant genotypes circulating in broiler flocks and at slaughterhouse level during multiple seasons.

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7. EXTENDED SUMMARY

Campylobacter is a leading cause of bacterial gastroenteritis worldwide. Although the infection is usually self-limited, complications following campylobacteriosis can be quite severe and include among others Guillian Barré Syndrom, arthritis and septicemia. The consumption of undercooked poultry meat is regarded to be the main source of infections.

Campylobacter intervention strategies are focused on the primary production integrating a high diversity of biosecurity measures as restricted personnel access, hygienic barriers at the entrance, addition of disinfectants to drinking water, effective cleaning and disinfection between downtimes, discontinued thinning, feed and water additives e.g. effective microorganisms and again bacteriophage application and vaccination.

This study was conducted to define and evaluate the genetic profiles of thermophilic *Campylobacter* species, isolated from caecal broiler samples, which can be related to the presence at broiler farms (n=22) and associated slaughterhouses (n=5). Establishing a database of genomic variants allows tracking the possible pathways of spreading, with the goal of improving the preventing measures and interventions. Furthermore, the obtained data will help future research to understand and explore *Campylobacter* mechanism in antibiotic drug resistance and aerotolerance.

We performed PCR confirmation on 244 *Campylobacter* isolates, which confirmed *C. jejuni* as the dominant species (84.8 % *C. jejuni* and 15.2% *C. coli*). Subtyping resulted in a heterogenous *C. jejuni* and genetically more uniform *C. coli* population (all ST-828 complex). In detail, 21 and 5 and 16 and 5 *C. jejuni* and *C. coli* pulsed-field gelelectrophoresis (PFGE) profiles and multi-locus sequence types (MLST) were identified. *C. jejuni* ST267, ST354, ST400, ST2066, ST446, ST824 and *C. coli* ST854 were detected in caecal samples at several sampling times indicating a local and in same cases global relevance.

8. ZUSAMMENFASSUNG

Campylobacter ist weltweit eine führende Ursache der bakteriellen Gastroenteritis. Obwohl die Infektion in der Regel abklingen, können die Komplikationen nach einer Campylobacteriose recht schwerwiegend sein und u.a. Guillian Barré Syndrom, Arthritis und Septikämie umfassen. Der Verzehr nicht ausreichend erhitztem Geflügelfleisch gilt als die Hauptquelle von Campylobacteriose.

Campylobacter Interventionsstrategien konzentrieren sich auf die Primärproduktion und integrieren eine große Vielfalt von Biosicherheitsmaßnahmen, wie eingeschränkten Zugang für das Personal, hygienische Barrieren am Eingang, Zugabe von Desinfektionsmitteln zum Trinkwasser, wirksame Reinigung und Desinfektion zwischen den Leerstehzeiten, Einstellung des „Rausfangens“ und Zurücklassen eines Teils der Herde, Futter- und Wasserzusätze wie z.B. wirksame Mikroorganismen und neuerdings Bakteriophagenapplikation und Impfung.

Diese Studie wurde durchgeführt, um die genetischen Profile thermophiler *Campylobacter*-Arten zu definieren und zu bewerten, die aus Zäkumproben von Masthähnchen isoliert wurden und die mit dem Vorkommen in Masthähnchenbetrieben (n=22) und zugehörigen Schlachthöfen (n=5) in Verbindung gebracht werden können. Der Aufbau einer Datenbank mit genomischen Varianten ermöglicht es, die potentiellen Wege der Ausbreitung zu verfolgen, mit dem Ziel, die Präventionsmaßnahmen und Interventionen zu verbessern. Darüber hinaus werden die gewonnenen Daten der zukünftigen Forschung helfen, den *Campylobacter*-Mechanismus bei Antibiotikaresistenz und Aerotoleranz zu verstehen und zu erforschen.

Wir führten eine PCR-Bestätigung an 244 *Campylobacter* Isolaten durch, die *C. jejuni* als dominante Spezies bestätigte (84,8 % *C. jejuni* und 15,2 % *C. coli*). Die Subtypisierung führte zu einer heterogenen *C. jejuni* und einer genetisch eher einheitlicheren *C. coli* Population (alle ST-828 complex). Im Einzelnen wurden 21 und 5 und 16 und 5 *C. jejuni* und *C. coli* Pulsfeld-Gelelektrophorese (PFGE) Profile und Multi-Locus-Sequenztypen (MLST) identifiziert. *C. jejuni* ST267, ST354, ST400, ST2066, ST446, ST824 und *C. coli* ST854 wurden in Zäkumproben zu mehreren Probenahmezeitpunkten nachgewiesen, was auf eine lokale und in denselben Fällen auf eine globale Relevanz hinweist.

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9. APPENDIX

Table 1. Equipment and materials.

I. Equipment	**Manufacturer**
Anaerobic jar	VWR, laboratory equipment, Pennsylvania, USA
Balance	Sartorius AG, Göttingen, Deutschland
Centrifuge 5424	Eppendorf AG, Hamburg, Germany
Chef DR III system	Bio-Rad Laboratories Inc., Hercules, USA
Elektrophorese-Unit	Bio-Rad Laboratories Inc., Hercules, USA
Freezer -20°C	Liebherr International AG, Bulle Schweiz
Freezer -80°C	Sanyo Electric Co. Ltd, Hamburg, Germany
GelDoc 2000 UV-Imaging System	Bio-Rad Laboratories Inc., Hercules, USA
Incubator 42°C	Ehret, Emmendingen, Deutschland
Photometer	Shimadzu Europa GmbH, Duisburg, Germany
Pipette 1-10 µl	Eppendorf AG, Hamburg, Germany
Pipette 10-100 µl	Eppendorf AG, Hamburg, Germany
Pipette 100-1000 µl	Eppendorf AG, Hamburg, Germany
Shaking waterbath	GFL, Burgwedel, Germany
Thermocycler	VWR, laboratory equipment, Pennsylvania, USA
Thermomixer compact	Eppendorf AG, Hamburg, Germany
Vortexer	VWR, laboratory equipment, Pennsylvania, USA
II. Materials	
Cotton tipped applicators	L & R GmbH, Rengsdorf, Germany
Cryogenic vials 2,0	Biologix Group Ltd, Shandong China
Eppendorf tubes 1,5ml	Eppendorf AG, Hamburg, Germany
Eppendorf tubes 2,0ml	Eppendorf AG, Hamburg, Germany
Falcon tubes 16ml	VWR, laboratory equipment, Pennsylvania, USA
Inoculating loops 1µl	Sarstedt, Nümbrecht, Deutschland
Inoculating loops 10µl	Sarstedt, Nümbrecht, Deutschland
Parafilm	Bemis Company Inc., Neenah, USA
Petri dishes	Sterilin Ltd., Newport, UK
Plug molds	Bio-Rad Laboratories Inc., Hercules, USA
Safe-Lock-Tubes 0,5 ml	Eppendorf AG, Hamburg, Germany
SafeSeal SurPhob Pipettenspitzen 1-1250 µl	Biozym Scientific GmbH, Hessisch Oldedorf, Deutschland

Table 1 continued: Equipment and materials.

III. Chemicals	Manufacturer
100bp DNA ladder	MBI Fermentas, St. Leon-Rot, Germany
10x PCR Buffer (-MgCl2)	Invitrogen - Thermo Fisher Scientific Inc., Waltham USA
1xTBE Puffer	Carl Roth GmbH+Co. KG, Karlsruhe, Germany
10x TBE-Puffer	Carl Roth GmbH+Co. KG, Karlsruhe, Germany
Agarose	Peqlab, Erlangen, Germany
Brain-Heart-Infusion-Broth	Oxoid Limited, Hampshire, UK
Catalase	Sigma Aldrich Corp., St. Louis, MO, USA
Chelex 100® Resin	Bio-Rad Laboratories GmbH, Mernes-la-Coquette, France
Defibrinated horse blood	Oxoid Limited, Hampshire, UK
DEPC treated water (Aqua dest.)	Thermo Fisher Scientific Inc., Waltham, USA
dNTP Mix 20mM	Thermo Fisher Scientific Inc., Waltham, USA
EDTA	Sigma Aldrich Corp., St. Louis, MO, USA
Glycerol	Sigma Aldrich Corp., St. Louis, MO, USA
KpnI enzyme	Thermo Fisher Scientific Inc., Waltham, USA
MgCl2 50 mM	Invitrogen - Thermo Fisher Scientific Inc., Waltham USA
Mixed CO2, O2, N2 gas	Linde Gas GmbH, Stadl Paura, Austria
Oxidase 50 AMP	bioMerieux, Marcy-l'Étoile, France
peqGOLD Universal Agarose	Peqlab, Erlangen, Deutschland
peqGreen	Peqlab, Erlangen, Deutschland
Platinum ® Taq DNA Polymerase	Invitrogen - Thermo Fisher Scientific Inc., Waltham USA
Primer	Microsynth AG, Balgach, Switzerland
Proteinase K	Roche Diagnostics GmbH, Vienna, Austria
Sample Loading Buffer (SLB)	Institut für Milchhygiene, VUW, Vienna, Austria
SeaKem Gold® agarose	Lonza Ag, Switzerland
SmaI Enzyme #ER0663	Thermo Fisher Scientific Inc., Waltham, USA
Tango buffer	Thermo Fisher Scientific Inc., Waltham, USA
Tris HCl	Sigma Aldrich Corp., St. Louis, MO, USA
Trishydroxymethylaminomethan	Sigma Aldrich Corp., St. Louis, MO, USA
Tryptic soy agar	Biokart Diagnostics, Beauvais Cedex, France