Eesti Maaülikool
Estonian University of Life Sciences

CAMPYLOBACTER SPP. IN FRESH BROILER CHICKEN MEAT AND PIG CAECAL SAMPLES IN ESTONIA

KAMPÜLOBAKTERID VÄRSKES KANABROILERILIHAS JA SEA UMBSOOLESISALDISES EESTIS

TRIIN TEDERSOO

A Thesis
for applying for the degree of Doctor of Philosophy
in Veterinary Science

Väitekiri
filosoofiadoktori kraadi taotlemiseks
loomaarstiteaduse erialal

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For the world you are someone, but for someone you are the world.
/Erich Fried/

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LIST OF ORIGINAL PUBLICATIONS

The present thesis consists of the following publications referred to by Roman numerals in the text. The publications have been reproduced with kind permission from the publishers.

I **Tedersoo T.**, Roasto M., Mäesaar M., Kisand V., Ivanova M., Meremäe K. (2022). The prevalence, counts, and MLST genotypes of *Campylobacter* in poultry meat and genomic comparison with clinical isolates. *Poultry Science*, 101:101703. doi: 10.1016/j.psj.2022.101703.

II **Tedersoo T.**, Roasto M., Mäesaar M., Häkkinen L., Kisand V., Ivanova M., Valli M.H., Meremäe K. (2022). Antibiotic resistance in *Campylobacter* spp. isolated from broiler chicken meat and human patients in Estonia. *Microorganisms*, 10:1067. doi: 10.3390/microorganisms10051067.

III **Tedersoo T.**, Roasto M., Mäesaar M., Fredriksson-Ahomaa M., Meremäe K. (2023). Antimicrobial resistance of *Campylobacter coli* isolated from caecal samples of fattening pigs at slaughter. *Microorganisms*, 11:1540. doi:10.3390/microorganisms11061540.

The contribution of the authors to the original research articles

Paper	Original idea and structure of the paper	Data collection, sample analysis	Data analysis	Preparation of manuscript
I	MR, **TT**, KM	**TT**, MR, VV, MM, KM, MI	**TT**, MR, MM, KM	All authors
II	MR, **TT**	**TT**, MR, KM, MM, MHV, MI, LH	**TT**, MR, MM, MHV	All authors
III	**TT**, MR	**TT**, MR, KM, MFA	**TT**, MR, MM	All authors

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ABBREVIATIONS

AMR	**a**nti**m**icrobial **r**esistance
CFU	**c**olony-**f**orming **u**nit
CIA	**c**ritically **i**mportant **a**ntimicrobial
COVID-19	**co**rona**vi**rus **d**isease 20**19**
DALY	**d**isability-**a**djusted **l**ife **y**ears
DNA	**d**eoxyribo**n**ucleic **a**cid
ECDC	**E**uropean **C**entre for **D**isease Prevention and **C**ontrol
EFSA	**E**uropean **F**ood **S**afety **A**uthority
EU	**E**uropean **U**nion
EUCAST	**E**uropean **C**ommittee on **A**ntimicrobial **S**usceptibility **T**esting
EURL-AR	**E**uropean **U**nion **R**eference **L**aboratory for **A**ntimicrobial **R**esistance
LABRIS	National Center for **Lab**oratory Research and **Ris**k Assessment
MIC	**m**inimal **i**nhibitory **c**oncentrations
MLST	**m**ulti**l**ocus **s**equence **t**yping
MDR	**m**ulti**d**rug-**r**esistance
PFGE	**p**ulsed-**f**ield **g**el **e**lectrophoresis
ST	**s**equence **t**ype
WHO	**W**orld **H**ealth **O**rganization
WGS	**w**hole-**g**enome **s**equencing

1. INTRODUCTION

According to the World Health Organization (WHO) (2012), the European Food Safety Authority (EFSA) and the European Centre for Disease Prevention and Control (ECDC) (2021a), campylobacteriosis is the most often reported zoonosis in the European Union (EU) since 2005. A total of 127,840 confirmed cases of human campylobacteriosis were reported in 2021, with a notification rate of 41.1 per 100,000 people in the EU (EFSA and ECDC, 2021a). The illness burden of *Campylobacter* human infection in the EU is 0.35 million disability-adjusted life years (DALYs) annually with a total cost of 2.4 billion euros, and the estimated global illness burden of *Campylobacter* is more than 3.7 million DALYs (Kirk *et al.*, 2015). According to the EU One Health 2021 Zoonoses Report (EFSA and ECDC, 2022), 185 confirmed cases of human campylobacteriosis were reported in 2021 in Estonia, with a notification rate of 13.9 per 100,000 inhabitants. In Estonia, the notification rate for human campylobacteriosis is approximately three-fold lower than the average for the EU. The number of human campylobacteriosis may be even higher because infectious diseases are underestimated or underreported (Gibbons *et al.*, 2014). The majority of *Campylobacter* spp. infections are mild and self-limiting. However, it can result in severe systemic illness or mortality in children, older adults, and immunocompromised individuals (WHO, 2020). Campylobacteriosis is a notifiable disease and a significant concern for both public health and the food industry (Tumbarski, 2019).

According to EFSA (2018), *Campylobacter jejuni* and *Campylobacter coli* are the primary *Campylobacter* species, causing 80 and 10% of human infections, respectively. Poultry, especially broiler chicken meat, is considered the principal source of human campylobacteriosis; however, other food sources can also cause human campylobacteriosis (Mäesaar *et al.*, 2020; EFSA and ECDC, 2021a; CDC, 2023). It is critical to apply efficient biosecurity measures at the farm level to avoid the spread of contamination from the outside environment to farms (Whiley *et al.,* 2013). Contamination can also be transmitted through transportation; for instance, through contaminated transportation crates or within a slaughterhouse environment (Rasschaert *et al.*, 2020). Contamination of the meat chain can lead to human *Campylobacter*-infections (Rosner *et al.*, 2017). According to Rosner *et al.* (2017), campylobacteriosis

often occurs because of the consumption of undercooked meat or contaminated food. According to Skarp *et al.* (2016), the entire broiler chicken processing chain plays a significant role in the transmission of *Campylobacter* from farms to forks.

Various studies have shown that *C. jejuni* is a dominant poultry species (Meremäe *et al.*, 2010; Korsak *et al.*, 2015; Rossler *et al.*, 2019; Yushina *et al.*, 2020). Pigs are the main reservoir of *C. coli* (Haruna *et al.*, 2013; Kempf *et al.*, 2017), indicating that contaminated pork can potentially pose a risk to human health in the context of *C. coli* infections. Several studies have identified a potential link between pigs and human strains (Avrain *et al.*, 2004; Milnes *et al.*, 2008; Meistere *et al.*, 2019).

Severe cases of campylobacteriosis often require therapy with first-line antimicrobials, such as fluoroquinolones (ciprofloxacin) and macrolides (erythromycin) (Moore *et al.*, 2006; Alfredson and Korolik, 2007; Blaser and Engberg, 2008; Luangtongkum *et al.*, 2009; Geissler *et al.*, 2017; Dai *et al.*, 2020). Over time, *Campylobacter* has acquired resistance to antimicrobials that are critical for the treatment of human infections (Du *et al.*, 2018; EFSA and ECDC, 2018; Dai *et al.*, 2020). According to Wieczorek and Osek (2013), it is a high risk of multidrug-resistant (MDR) *Campylobacter* spp. transmission from animals to humans. MDR is described as resistance to three or more unrelated antimicrobials (Magiorakos *et al.*, 2012). The increasing antimicrobial resistance (AMR) observed in thermophilic *Campylobacter* spp. has significant implications for public health and, therefore necessitates greater attention (Collignon *et al.*, 2016). Intensive animal husbandry is associated with the use of antimicrobials. Food-producing animals frequently harbour resistant microorganisms that can potentially be transmitted to humans through contaminated food (FAO, 2021). Antimicrobial resistant *C. coli* is a growing public health concern (Gillespie *et al.*, 2002; Di Donato *et al.*, 2020).

This thesis provides information on the distribution of genotypes among the *C. jejuni* and *C. coli* isolates from Baltic broiler chicken meat and Estonian human patients with a focus on describing potential associations between the isolated genotypes of different origins. In addition, this is the first overview of the relationships between genotypic and phenotypic antibiotic resistance in *C. jejuni* and *C. coli* isolates originating from Estonian retail and patients with severe enteric infections. Furthermore,

the pig-related *Campylobacter* spp. AMR information is reported for the first time in Estonia.

The aims of this thesis are (**I**) to determine the prevalence, counts and the link between broiler chicken meat and human *Campylobacter* isolates in Estonia, and (**II**) to determine the resistance of thermophilic *Campylobacter* spp. originating from broiler chicken meat and Estonian human patients, and (**III**) to analyse the occurrence and AMR of *Campylobacter spp.* isolates originating from caecal samples of fattening pigs in Estonia.

2. REVIEW OF THE LITERATURE

2.1. Overview of *Campylobacter* spp.

2.1.1. History

Campylobacter was first documented by Theodor Escherich in 1886 and identified as a non-culturable spiral-shaped bacterium isolated from the intestines of deceased children diagnosed with diarrhoea and infected with *Cholera infantum* (Vandamme *et al.*, 2010; Epps *et al.*, 2013). According to Skirrow (2006), the first Vibrio-like bacterium (*Campylobacter*) was isolated in 1906 from the uteri of aborted sheep. In 1912, *Vibrio fetus* was isolated (Smith and Taylor, 1919), and 15 years later, *Vibrio jejuni* was discovered (Jones *et al.,* 1931). According to Doyle (1948), *Vibrio coli* was first isolated in 1944 from pigs with diarrhoea. In 1963, these microorganisms were classified as *Campylobacter* to differentiate them from *Vibrio* spp. (On, 2001). Since 1909, *Campylobacter* spp. have been known to cause diseases in animals, and since 1980, they have been recognised as a cause of human diseases (Silva *et al.*, 2011).

2.1.2. Taxonomy

Campylobacter spp. belong to the genus *Campylobacter*, phylum *Proteobacteria*, class *Epsilonproteobacteria*, order *Campylobacterales*, and family *Campylobacteraceae* (Ammar *et al.*, 2021). According to Silva *et al.* (2018), the taxonomy of the genus *Campylobacter* continues to vary because of the reclassification of species into different genera or the discovery of new species. The genus *Campylobacter* comprises 39 species and 16 subspecies, of which *C. jejuni, C. coli*, *C. lari*, *C. fetus, C. upsaliensis, C. curvus,* and *C. concisus* are considered pathogenic (Ammar *et al.*, 2021).

2.1.3. Reservoirs and transmission routes

Broiler chicken meat is considered the primary source of human campylobacteriosis infections; however, other sources, such as raw milk, pork, and untreated water, can also cause campylobacteriosis in humans (Mäesaar *et al.*, 2020; EFSA and ECDC, 2021a; CDC, 2023). *C. jejuni* is the most important species within the genus *Campylobacter* and is a

leading cause of bacterial gastroenteritis in humans (Skarp *et al.*, 2016; Costa and Iraola, 2019). According to Whiley *et al.* (2013), wild animals, especially birds, play an important role in spreading *Campylobacter* spp. between animals and humans. Therefore, applying high-level biosecurity measures at the farm level is highly crucial to prevent the spread of contamination. The infection spreads rapidly once *Campylobacter* enters a broiler chicken farm (Sibanda *et al.,* 2018), and *Campylobacter* colonisation can escalate to as high as 10^9 colony-forming units (CFU) per gram (Rosenquist *et al.*, 2006). According to Nastasijevic *et al.* (2020), poultry excretes substantial quantities of *Campylobacter* (10^4–10^{7-9} CFU/g) into the environment.

Campylobacter is present in the intestinal tract of infected food-producing animals; therefore, there is a risk of contaminating meat and its products with *Campylobacter* during the slaughtering and subsequent processing steps (Fosse *et al.*, 2009; Facciolà *et al.*, 2017). Furthermore, the processing and handling of contaminated raw chicken and its products by consumers may lead to cross-contamination at the home kitchen level during post-marketing stages. Cross-contamination of prepared food and raw chicken can occur because of improper consumer hygiene practices such as washing raw chicken in water, which can contaminate food preparation surfaces, utensils, and other ready-to-eat foods (Luber *et al.*, 2006; Ammar *et al.*, 2021). Campylobacteriosis usually occurs sporadically; however, *Campylobacter* outbreaks have been traced back to the source of the contamination (Luber *et al.*, 2006). *C. jejuni* and *C. coli* case-control studies and outbreak investigations have identified the handling of raw chicken as a critical risk factor for human *Campylobacter* infections (Allerberger *et al.*, 2003; Kapperud *et al.,* 2003; Friedman *et al.,* 2004; Lindmark *et al.,* 2004). The complex task of attributing and tracing the initial source of infection is challenging because of the high genetic diversity and wide range of hosts of *Campylobacter* spp. (Skarp *et al.*, 2016; Gölz *et al.*, 2018).

2.2. *Campylobacter* spp. infections

2.2.1. Campylobacteriosis

Campylobacteriosis is a bacterial disease caused by the thermophilic *Campylobacter* species (CDC, 2023). According to Debruyne *et al.* (2008) and Llarena *et al.* (2015), up to 90% of the outbreaks and sporadic

instances of campylobacteriosis in humans are caused by *C. jejuni* subsp. *jejuni* and *C. coli*. Human *Campylobacter* infection is the most frequent zoonotic bacterial foodborne disease, and in rare cases, it can cause death in immunocompromised patients who have been diagnosed with cancer, acquired immunodeficiency syndrome, and liver disease (EFSA, 2017; Bhunia, 2018; Garcia-Sanchez *et al.*, 2018a). According to the EFSA and ECDC (2022), 26 (0.03%) deaths due to campylobacteriosis in confirmed human cases and six (0.6%) deaths among foodborne outbreak cases in the EU were reported in 2021. Antimicrobial therapy is usually not necessary because diarrhoea associated with campylobacteriosis is often self-limiting (Bhunia, 2018). The incubation period for *Campylobacter* infection is two to five days, and the infection is more common among children younger than five years, males, and older adults more than 65 years of age (CDC, 2023). *Campylobacter* gastroenteritis is characterised by high body temperature, vomiting, weight loss, abdominal pain/cramps, headache, as well as acute watery and occasional bloody diarrhoea (Skarp *et al.*, 2016; Bhunia, 2018). In addition, *Campylobacter* can cause inflammatory bowel disease, endocarditis, pneumonia, septicaemia, and post-infectious diseases, including Miller-Fisher and Guillain-Barre syndrome (Igwaran and Okoh, 2019). Antimicrobial treatment is necessary when severe systemic or persistent infections occur (Johnson *et al.*, 2017; Bhunia, 2018).

2.2.2. *Campylobacter* infections in the European Union

Before the coronavirus disease 2019 (COVID-19) outbreak in the year 2019, 220,683 campylobacteriosis cases in the EU were reported. There were 127,840 confirmed human campylobacteriosis cases in the EU in 2021, with a notification rate of 41.1 cases per 100,000 individuals (EFSA and ECDC, 2022). In 2021, a slight increase (2.1%) was observed in comparison to the previous year. According to the EU One Health 2021 Zoonoses Report, 10,469 (23.2%) individuals were hospitalised and 26 deaths were reported, with a case fatality rate of 0.03% (EFSA and ECDC, 2022). Campylobacteriosis cases in 2021 were predominantly caused by *C. jejuni* (88.4%), followed by *C. coli* (10.1%), *C. fetus* (0.2%), *C. upsaliensis* (0.1%), and *C. lari* (0.1%)(EFSA and ECDC, 2022). From 2007 to 2021, campylobacteriosis was the most frequently reported foodborne gastrointestinal infection in humans in the EU (EFSA and ECDC, 2022).

2.2.3. *Campylobacter* infections in Estonia

In 2021, there were 185 confirmed campylobacteriosis cases in Estonia, with a notification rate of 13.9 cases per 100,000 people, which was lower than the average rate in the EU (41.1) (EFSA and ECDC, 2022). Compared to 2020, the number of confirmed cases in Estonia dropped by 30.2% (EFSA and ECDC, 2022), which may have been due to the COVID-19 pandemic.

Campylobacteriosis cases were registered in all counties in Estonia except Võrumaa and Valgamaa. The highest notification rates per 100,000 inhabitants were reported in Pärnu (32.5), Järvamaa (26.5), and Ida-Virumaa (22.6) (Terviseamet, 2021). In total, 94 (50.8%) patients were hospitalised. *C. jejuni* caused infections in 44.3% of the cases, while in 49.2% of the cases, the species was not determined. In 11 cases, a link to food could be presumed, including chicken meat in four cases and other foods in seven cases. There were 51.3% campylobacteriosis cases in children aged one to 14 years, 21.1% in individuals aged 29 to 39 years, and 14.6% in older age groups (Terviseamet, 2021). Among them, 50.3% were men and 49.7% were women. Seven cases were presumed to have been acquired from Egypt, Italy, Greece, Sweden, Turkey, and Russia (Terviseamet, 2021). AMR was reported in 214 isolates of which seven were MDR *C. coli*. Resistance to ciprofloxacin and tetracyclin was reported in 87.8 and 49.7% of the isolates, respectively, while 11.7% of the isolates were sensitive to all the antimicrobial agents (Terviseamet, 2021).

2.3. *Campylobacter* spp. prevalence

2.3.1. *Campylobacter* spp. in broiler chicken meat

Poultry meat is the primary source of campylobacteriosis in humans. Broiler meat handling, preparation, and consumption are associated with 20–30% of human infections, whereas 50–80% can be traced back to the chicken reservoir (Di Giannatale *et al.*, 2019). Most human campylobacteriosis cases are caused by *C. jejuni* (Burnham and Hendrixson, 2018). According to Deng *et al.* (2020), poultry is one of the main sources of human *Campylobacter* infections. According to Asmai *et al.* (2020), *Campylobacter* spp. prevalence ranged from 2 (Estonia) to 100% (Luxembourg), with a mean prevalence of 71.2%. The incidence

of broiler batches with *Campylobacter* contamination in member states was reported to be 57.1% on average (EFSA and ECDC 2021a). The following table (Table 1) reflects the examples of *Campylobacter* prevalence at retail stores.

Table 1. *Campylobacter* prevalence in European broiler chicken meat at retail stores

Country	Study period	Prevalence (%)	Reference
France	2009	76.0	Guyard-Nicodème *et al.*, 2015
Estonia	2012	14.8	Mäesaar *et al.*, 2014
Latvia	2008–2016	50.2	Meistere *et al.*, 2019
Lithuania	2009	46.5	Bunevičienė *et al.*, 2010
Finland	2013	11.0	Skarp *et al.*, 2016
Italy	2013	18.6	Nobile *et al.*, 2013
Germany	2011–2014	34.0–54.0	Rosner *et al.,* 2017
Austria	2013	71.0	EFSA, 2015

2.3.2. *Campylobacter* spp. in pigs and pork

Compared to broiler chicken meat, contamination of pork with *Campylobacter* and the role of pork in the development of *Campylobacter* enteritis in humans have not been well studied. Pig-derived *C. coli* have been linked to *Campylobacter* infections in humans (Mossong *et al.*, 2016; Rosner *et al.*, 2017). Pigs are the primary reservoirs of *C. coli* (Fosse *et al.*, 2009; Yushina *et al.*, 2020). A high *Campylobacter* prevalence in pigs has been observed in many European countries, with *Campylobacter* prevalence ranging from 0 to 92.7% (Pezzotti *et al.*, 2003; Avrain *et al.*, 2004; Tadesse *et al.*, 2011; EFSA and ECDC, 2015). A study in Latvia between 2008 and 2016 reported that the highest prevalence of *Campylobacter* in pigs was 83.3%, with 91.2% of these *Campylobacter* isolates being *C. coli* (Meistere *et al.*, 2019). *C. coli* was more prevalent in pig carcasses (50.4%) than in faeces (32.9%), according to a study conducted on Italian pigs at slaughterhouses (Di Donato *et al.*, 2020). *C. coli* prevalence in slaughtered pigs in the Czech Republic and Denmark was reported to be as high as 92.8 and 90.1%, respectively (Boes *et al.*, 2005; Steinhauserova *et al.*, 2005).

Campylobacter occurrence in pork is lower than that in poultry meat (Korsak *et al.*, 2015), which may be due to the longer slaughter time, cooling of carcasses, and drying of the meat surface (Narvaez-Bravo *et al.*, 2017). The study conducted by Andrzejewska *et al.* (2019) in Northern Poland, found that 10.9% of pork samples were *Campylobacter*-

positive. Other studies on the prevalence of *Campylobacter* spp. in retail pork have reported low levels (0.5–2%) of *Campylobacter* contamination (Zhao *et al.*, 2001; Hong *et al.*, 2007; Noormohamed and Fakhr, 2013; Lapierre *et al.*, 2016). In a study conducted in Canada by Narvaez-Bravo *et al.* (2017), pork samples tested negative for *Campylobacter*.

2.4. Antimicrobial resistance of *Campylobacter* spp.

The uncontrolled use of antimicrobials in human treatment and their excessive use in animal production are the main causes of bacterial resistance (Silva *et al.*, 2018). The average antimicrobial consumption by food-producing animals was estimated to be 93,309 tons of active ingredients in 2017, and the global consumption of antimicrobials by the livestock industry is expected to grow by 11.5% by 2030 (Tiseo *et al.*, 2020). According to the sales of veterinary antimicrobial agents in 31 European countries reported in 2021, antimicrobial consumption in food animals was 84.4 mg per population correction unit (5,219.6 tons) (EMA, 2022).

In the EU countries according to the European Parliament and Council Regulation 1831/2003/EC on additives for use in animal nutrition, the use of antibiotic based growth promoters has been prohibited since 1st of January 2006. Antimicrobials are still widely used in food-producing animals for treatment and infection prevention, leading to the selection of resistant bacteria with the potential to spread to humans (Stapleton *et al.*, 2010; Elhadidy *et al.*, 2018).

According to the WHO, AMR is one of the most important threats to public health (Xiong *et al.*, 2018). Severe cases of campylobacteriosis are often treated with tetracyclines, macrolides or fluoroquinolones, but because of the *C. jejuni* and *C. coli* increasing resistance against these antimicrobials, the treatment of infections might be compromised (Bolton, 2015). Therefore, the increase in MDR *C. coli* and *C. jejuni* isolates is alarming (Wieczorek *et al.*, 2019; Neogi *et al.*, 2020).

Campylobacter exhibits high genomic plasticity, which supports the emergence of resistant mutants (Parkhill *et al.*, 2000). Four types of AMR mechanisms of *Campylobacter* are known: (1) mutation-mediated, modification of the antibiotic target and/or its expression; (2) antibiotic's inability to reach its target (expression of outer membrane protein); (3)

multidrug efflux pumps related; (4) inactivation or modification of the antibiotic (β-lactamase production) (Iovine, 2013). Mechanisms that cause fluoroquinolone resistance include deactivation of the fluoroquinolone target and fluoroquinolone efflux (Luo *et al.*, 2003; Ge *et al.*, 2005; Yan *et al.*, 2006). Quinolone and fluoroquinolone resistance in *Campylobacter* is mainly caused by a Thr-86-Ile mutation in gyrase (*gyrA*) (Iovine, 2013). Target modification, efflux, and altered membrane permeability are the three main mechanisms underlying macrolide resistance (Tang *et al.*, 2020). The mechanisms of tetracycline resistance in *Campylobacter* involve alterations in the ribosomal targets of tetracycline and efflux (Roberts, 2005; Iovine, 2013). Tetracycline resistance is primarily caused by the ribosomal protection protein encoded by the *tetO* gene, which is transported on plasmids or carried on chromosomes (Chopra and Roberts, 2001; Gibreel *et al.*, 2004). Aminoglycoside (streptomycin, gentamicin) resistance is mainly caused by *aad9, aadE, aadE-Cc* and *aph(3')-IIIa* resistance genes (Fabre *et al.*, 2018; Cobo-Díaz *et al.*, 2021; Guernier-Cambert *et al.*, 2021; Ocejo *et al.*, 2021).

2.4.1. The antimicrobial resistance of *Campylobacter* spp. isolated from poultry meat

According to data in the EU Summary Report on Antimicrobial Resistance in Zoonotic and Indicator Bacteria from Humans, Animals, and Food in 2020/2021, human and animal-origin *C. jejuni* and *C. coli* showed high to extremely high levels of resistance to fluoroquinolones (EFSA and ECDC, 2023). Fluoroquinolones are classified as critically important antimicrobials (CIA) for treating *Campylobacter* infections in humans (EFSA and ECDC, 2023). Garcia-Sanchez *et al.* (2020) reported a correlation between the use of fluoroquinolones in poultry and an increase in resistance among chicken and human-origin *Campylobacter* isolates.

In 2021, the highest levels of resistance in *C. jejuni* and *C. coli* broiler isolates were to ciprofloxacin, nalidixic acid (overall 52–100%), and tetracycline (overall 42.9–100%). The resistance to gentamicin in *C. coli* broiler isolates and resistance to erythromycin in *C. jejuni* broiler isolates was observed in low levels, 1.0–2.6% and 0.6–12.5%, respectively. Higher proportions of resistance were observed in *C. coli* broiler isolates to erythromycin (0.6–40.0%) (EFSA and ECDC, 2023). Antimicrobial use in poultry in Denmark is low because the use of carbapenems, third-

and fourth-generation cephalosporins, fluoroquinolones, and colistin is restricted (DANMAP, 2020). In Norway, 90.8% of *C. jejuni* broiler isolates were susceptible to all the tested antimicrobials (Simonsen *et al.*, 2021). Mäesaar *et al.* (2016) found that 87.5% of Latvian and 84.8% of Lithuanian *Campylobacter* isolates originating from broiler chicken meat were resistant to fluoroquinolones.

2.4.2. The antimicrobial resistance of *Campylobacter* spp. isolated from pigs

Directive 2003/99/EC and Commission Implementing Decision (EC) 2020/1729 were established to monitor AMR in *C. coli* isolates from caecal samples of fattening pigs.

Doxycycline, tiamulin, and amoxicillin are the most commonly used antimicrobials in pigs and cattle in Estonia in 2022 (Estonia State Agency of Medicines, 2023). Little is known about AMR of *Campylobacter* spp. in pigs in Estonia. Meistere *et al.* (2019) found high streptomycin resistance (54.9%) in *C. coli* isolates obtained from pigs in Latvia. An Italian study (Pezzotti *et al.*, 2003) identified high rates of tetracycline (76.6%) and streptomycin (89.4%) resistance among *C. coli* isolates from pigs. *C. coli* resistance to ciprofloxacin has been frequently (51.7%) reported (EFSA and ECDC, 2023) in isolates from fattening pigs. Low to extremely high levels of ciprofloxacin resistance, ranging from 6.9 to 78.2%, have been observed in different countries. Tetracycline resistance in *C. coli* pig isolates in the reporting countries ranged from 0.0 to 86.5%. Interestingly, member states with the highest prevalence of erythromycin-resistant *C. coli* from fattening pigs in 2021 also showed the highest level of erythromycin-resistant *C. coli* from humans, indicating that pigs may serve as a source of erythromycin-resistant *C. coli* in humans (EFSA and ECDC, 2023). Different studies have reported fluoroquinolone resistance among pig *Campylobacter* isolates from Latvia (53.5%) (Meistere *et al.*, 2019), Poland (57.1%) (Wieczorek and Osek, 2013) and Finland (18.3 and 34.0%, respectively) (Finnish Food Safety Authority, 2016; Finnish Food Authority 2021). Di Donato *et al.* (2020) recently reported that *C. coli* isolated from pigs exhibited extremely high resistance to quinolones (74.7%) and fluoroquinolones (70.1%). According to the EFSA and ECDC (2023), 9.7% of MDR *C. coli* isolates have been reported from fattening pigs.

2.4.3. The antimicrobial resistance of *Campylobacter* spp. isolated from humans

Human *C. jejuni* and *C. coli* isolates were found to have high resistance to fluoroquinolones and extremely high levels of ciprofloxacin resistance (ranging from 22.2 to 100%) in the EU in 2021 (EFSA and ECDC, 2023). Ciprofloxacin resistance in *C. jejuni* isolated from humans increased from 2013 to 2021 in 12 reporting countries, while erythromycin resistance decreased in seven countries (EFSA and ECDC, 2023). The EFSA and ECDC (2023) reported extremely high levels (70.3%) of tetracycline resistance in *C. coli* human isolates. According to the report, human *C. coli* isolates in Estonia had the second-highest level of combined resistance to ciprofloxacin and erythromycin (25.9%) in the EU, followed by Portugal (EFSA and ECDC, 2023). Multidrug resistance was generally low (1%) in *C. jejuni* human isolates. Compared with *C. jejuni*, it was noticeably higher in *C. coli* human isolates (9.9%) (EFSA and ECDC, 2023).

2.5. Subtyping of *Campylobacter* spp.

Subtyping or characterisation of bacteria beyond the species level is essential in epidemiological investigations to determine transmission routes and identify potential sources of infection (Hansson *et al*., 2018). Subtyping of *Campylobacter* spp. has traditionally been performed using conventional methods based on phenotypic characteristics (Hansson *et al*., 2018). The development and availability of more discriminatory and accurate molecular methods have led to a paradigm shift and have increasingly replaced historically used subtyping approaches (Llarena *et al*., 2017). Numerous techniques for molecular subtyping based on the analysis of bacterial deoxyribonucleic acid (DNA) (Sabat *et al*., 2013) have been described, including ribotyping (Owen *et al*., 1990), amplified fragment length polymorphisms (Kokotovic and On, 1999), pulsed-field gel electrophoresis (PFGE) (Ribot *et al*., 2001), and multilocus sequence typing (MLST) (Dingle *et al*., 2001).

For a long time, the "gold standard" for genotyping *Campylobacter* was PFGE, for which standardised methods and protocols were developed (Neoh *et al*., 2019). While PFGE subtyping is based on the variability of DNA restriction banding patterns (Tenover *et al*., 1995), MLST utilises a more systematic curation of seven housekeeping gene (*asp*, *gln*, *glt*, *gly*,

pgm, *tkt*, *unc*) fragments based on their nucleotide differences (Dingle *et al.*, 2001). However, compared with whole-genome sequencing (WGS), both methods are inferior because they provide limited information about bacterial genomes (Kovac *et al.*, 2017).

WGS analyses using next-generation sequencing have been proven to be more discriminatory and informative (Llarena *et al.*, 2017) and are therefore increasingly used in epidemiological studies, including AMR studies (Pendleton *et al.*, 2013; Revez *et al.*, 2014; Neoh *et al.*, 2019). The integration of WGS has been successfully used for public health surveillance and epidemiological studies of several foodborne pathogens, such as *Campylobacter*, *Salmonella*, *Listeria*, and shigatoxin-producing *Escherichia coli* (Joensen *et al.*, 2014; Lassen *et al.*, 2016; Inns *et al.*, 2017; Lahti *et al.*, 2017; Llarena *et al.*, 2017). WGS analyses of *C. jejuni*/*C. coli* have enhanced outbreak detection and source tracing, thereby preventing human infections (Kovanen *et al.*, 2016; Joensen *et al.*, 2020; Arning *et al.*, 2021).

There are multiple different WGS data analysis methods for *C. jejuni* and *C. coli* surveillance and epidemic investigations, such as high-quality single nucleotide polymorphisms, as well as core and whole-genome MLST; however, *in silico* seven-gene MLST analysis based on WGS data is actively used as a first-line method to differentiate outbreak-associated and sporadic *Campylobacter* isolates (Dingle *et al.*, 2001; Gardner *et al.*, 2011; Kwan *et al.*, 2014, Jolley *et al.*, 2018; Mäesaar *et al.*, 2018).

2.6. *Campylobacter* spp. related legislation

For monitoring and controlling *Campylobacter* spp., legislation established in the EU must be followed.
The monitoring of zoonoses and zoonotic agents in the EU is performed per the harmonised procedures presented in Zoonoses Directive 2003/99/EC.
The Commission Implementing Decision (EU) 2013/652 is on the monitoring and reporting of AMR in zoonotic and commensal bacteria.
Commission Regulation (EC) No 2073/2005 establishes processing hygiene criteria regarding *Campylobacter*.

The most efficient way to reduce the prevalence of *Campylobacter* spp. in the EU broiler chicken meat chain is to implement efficient control

measures at primary production and in slaughterhouses, and to raise consumer awareness (EFSA BIOHAZ Panel, 2020; Nastasijevic *et al.*, 2020). As poultry meat has a high risk of cross-contamination with *Campylobacter* at the slaughter level, legislation establishing specific process hygiene criteria for broiler carcasses is applicable at the processing and retail levels. The two categories of microbiological criteria outlined in Commission Regulation (EC) No. 2073/2005 dated 15 November 2005 on microbiological criteria for foodstuffs pertain to process hygiene and food safety. Food safety criterion applies to products or foodstuffs that are marketed or are available in the market, and process hygiene criteria indicate acceptable operation of the production process. According to Commission Regulation (EC) No. 2073/2005 dated 15 November 2005 on microbiological criteria for foodstuffs, a process hygiene criterion is established, where *Campylobacter* spp. counts of broiler carcasses (neck skin) after chilling must not exceed 1,000 CFU/g. This prevents poultry meat with more than 1,000 CFU/g (carcasses/neck skin) from reaching the market (EFSA and ECDC, 2022). Food business operators that do not comply with this restriction are required to take corrective action, which includes good manufacturing practices and hazard analysis, as well as critical control points-based food safety management procedure assessment and their verification (EFSA and ECDC, 2022). On 1 January 2020, a more stringent sampling plan and process hygiene criteria were introduced. Under these criteria, a maximum of 15 samples ($n = 50$, $c = 15$) is allowed for *Campylobacter* spp. to exceed the limit of 1,000 CFU/g. Starting from 1 January 2025 this requirement will become more stringent, with a sampling plan of $n = 50$ and $c = 10$ (Nastasijevic *et al.*, 2020; Zwietering *et al.*, 2023).

3. AIMS OF THE STUDY

The general aim of this study was to determine the prevalence and AMR of thermophilic *Campylobacter* spp. in broiler chicken meat isolated from retail stores and fattening pigs at slaughter.

The specific aims of the work were outlined as follows:

To determine the prevalence and counts of *Campylobacter* spp. in fresh broiler chicken meat of Estonian, Latvian, and Lithuanian origin at the Estonian retail outlets. Additionally, to investigate the genetic relatedness of *Campylobacter* spp. isolated from broiler chicken meat at the retail level in Estonia and Estonian human patients (**I**).

To determine the antimicrobial susceptibility of *Campylobacter* spp. isolated from fresh broiler chicken meat originating from Baltic countries sold in Estonian retail stores, as well as human clinical isolates obtained from patients with *Campylobacter* enteritis in Estonia (**II**).

To determine the occurrence of resistance of *Campylobacter* spp. from the caecal samples of fattening pigs at slaughter (**III**).

4. MATERIALS AND METHODS

4.1. Materials

4.1.1. Poultry meat isolates (I, II)

Between September 2018 and October 2019, 429 company-packaged fresh broiler chicken meat products were collected monthly from Estonian food retail outlets. Altogether, there were 163, 133, and 133 samples of Estonian, Latvian, and Lithuanian origins, respectively. The samples were brought to the laboratory in a portable cooler that was maintained at a temperature ranging between +2 and +6 °C. *Campylobacter* spp. detection and enumeration in broiler chicken meat samples were performed according to ISO 10272-1:2017 and ISO 10272-2:2017. Of 429 broiler chicken meat samples, 141 *Campylobacter* isolates were obtained. Using MLST, 25 broiler chicken meat isolates were sequenced and genotyped to determine the genetic similarities between Estonian, Latvian, and Lithuanian broiler chicken meat and *Campylobacter* spp. isolates from Estonian human patients. All studies were performed in the laboratory of the Estonian University of Life Sciences Chair of Veterinary Biomedicine and Food Hygiene.

4.1.2. Clinical isolates (I, II)

In cooperation with Estonian hospitals, 18 *C. jejuni* and two *C. coli* isolates associated with human *Campylobacter* infections in Estonia were acquired for sequence typing (**I**).

This study included 15 isolates from ambulatory and hospitalised patients from Estonian hospitals (**II**).

4.1.3. Fattening pig isolates (III)

Sampling was carried out by competent authority officials within the National Monitoring Program following the EU Directive 2003/99/EC and the Commission Implementing Decision (EU) 2013/652 on the monitoring and reporting of AMR in zoonotic and commensal bacteria. At the slaughterhouses, samples were collected over five years, in 2015,

2017, and 2019. *Campylobacter* spp. direct and enrichment culture methods were used according to ISO 10272-1:2018 standard. A total of 229 fattening pig caecal samples were examined at the National Center for Laboratory Research and Risk Assessment (LABRIS), where isolation and antibiotic susceptibility tests of *Campylobacter* spp. were performed.

4.2. Methods

4.2.1. Isolation and identification (I, II, III)

Campylobacter spp. were isolated and identified according to the ISO 10272-1:2017 guidelines. In brief, 10 g of skin from broiler chicken meat samples were added to 90 mL of Preston enrichment broth and incubated for 24 h under microaerobic conditions at 41.5 ± 0.5 °C. Subsequently, 10 µL of the enrichment broth was plated onto modified charcoal cefoperazone deoxycholate (mCCD) agar medium (Oxoid Ltd., Basingstoke, United Kingdom) and incubated for 48 h at 41.5 ± 0.5 °C under microaerobic conditions. Colonies typical of *Campylobacter* on mCCD agar plates were streaked on Columbia blood agar (Oxoid Ltd.) and incubation was carried out for 24 h at 41.5 ± 0.5 °C. Gram staining, motility analysis, as well as oxidase and catalase assays, were performed as further confirmation tests. For further studies, *Campylobacter* spp. isolates were stored in glycerol broth at −80 °C (**I**, **II**).

Campylobacter spp. were isolated and identified according to the ISO 10272-1:2018 guidelines. Briefly, 10 g of pig intestinal contents from the caecum and 90 mL of Bolton broth (Oxoid Ltd.) were homogenized for a minute in a Stomacher 400 Circulator (Heidolph Instruments GmbH & Co. KG; Schwabach, Germany). After that, the samples were incubated for four to six hours under microaerobic conditions at 37 °C, and subsequently at 41.5 ± 0.5 °C for 44 ± 4 h. Next, 10 µL of the enrichment broth was inoculated onto mCCD agar (Oxoid Ltd.) and incubated for 44 ± 4 h at 41.5 ± 0.5 °C under microaerobic conditions. After the incubation, colonies typical of *Campylobacter* were streaked on Columbia blood agar (Oxoid Ltd.) plates and incubated in microaerobic conditions using anaerobic jars with CampGen™ reagents (Oxoid Ltd.) for 24 h at 41.5 ± 0.5 °C. Colonies that were Gram-negative, motile, oxidase-positive, and those that did not grow in aerobic conditions at 41.5 ± 0.5 °C as well as at 25 °C were determined to be *Campylobacter* spp. The results for 10 g of sample were expressed as either detected or

undetected. Cryotubes (Technical Service Consultants Ltd., Lancashire, United Kingdom) were used to store *Campylobacter* strains at −82 °C (**III**).

4.2.2. Enumeration (I)

Campylobacter spp. were enumerated according to the ISO 10272-2:2017 guidelines. Briefly, 10 g of the sample was placed into 90 mL buffered peptone water and stomached for 60 s. Subsequently, 0.1 mL of this substance that was diluted 10-fold was streaked onto two mCCD agar (Oxoid Ltd.) plates and incubated in microaerobic conditions at 41.5 ± 0.5 °C for 48 h. By following, typical *Campylobacter* colonies were sub-cultured on Columbia blood agar (Oxoid Ltd.). *Campylobacter* colonies were confirmed using Gram staining, motility analysis, as well as oxidase and catalase assays.

4.2.3. Antimicrobial resistance (II, III)

Based on the manufacturer's instructions, the minimal inhibitory concentrations (MIC) for nalidixic acid, ciprofloxacin, tetracycline, streptomycin, erythromycin, and gentamicin were determined using the broth microdilution method with the EUCAMP2 panel (TREK Diagnostic Systems Ltd., East Grinstead, United Kingdom). Briefly, the *Campylobacter* isolates were cultured on Columbia blood agar (Oxoid Ltd.) and incubated for 44 ± 4 h at 41.5 ± 0.5°C under microaerobic conditions. One μL loopful of bacterial material was carried to 2 mL of saline solution, achieving an estimated inoculum density of 10^8 CFU/mL. Subsequently, 50 μL of the inoculum was transferred to 10 mL of cation-adjusted Mueller–Hinton broth supplemented with 5% laked horse blood (Oxoid Ltd.). After this, 100 μL of bacterial suspension was transferred into microtiter plates, which were then incubated for 44 ± 4 h at 35 ± 2 °C with CampGenTM reagents (Oxoid Ltd.) under microaerobic conditions in anaerobic jars. The lowest concentration that completely inhibited *Campylobacter* growth was identified as the MIC. Bacterial suspension (10 μL) was carried to Columbia agar (Oxoid Ltd.) and incubated for 40−48 h at 37 °C under microaerobic conditions to assess the purity of bacterial suspension. A colony count per plate that ranged from 50 to 250 was accepted.

According to the European Commission's Implementing Decision (EC) 2013/652 on the monitoring and reporting of AMR in zoonotic and commensal bacteria, the cut-off values suggested by the European Committee on Antimicrobial Susceptibility Testing (EUCAST) were utilised for *C. jejuni* and *C. coli*. The epidemiological MIC cut-off values for *C. jejuni* were: erythromycin > 4 μg/mL, ciprofloxacin > 0.5 μg/mL, tetracycline > 1 μg/mL, streptomycin > 4 μg/mL, nalidixic acid > 16 μg/mL or gentamicin > 2 μg/mL.

Erythromycin > 8 μg/mL, ciprofloxacin > 0.5 μg/mL, tetracycline > 2 μg/mL, streptomycin > 4 μg/mL, nalidixic acid > 16 μg/mL and gentamicin > 2 μg/mL, were the cut-off values for *C. coli*.

Quality control was performed following the guidelines and instructions of the EU Reference Laboratory for Antimicrobial Resistance (EURL-AR). The strains used for the quality control were *C. jejuni* ATCC 33560 and *C. coli* EURL-AR strain 2012-70-443-2. Antimicrobial susceptibility testing was performed in LABRIS.

4.2.4. Genotyping, antimicrobial resistance genes and point mutations analyses (I)

The GeneJET Genomic DNA Purification Kit (Thermo Fisher Scientific) was used to extract DNA for WGS and genotyping. The Illumina Nextera XT library preparation kit was used to create sequencing libraries according to the manufacturer's instructions (Illumina Inc., San Diego, CA). Sequencing was performed on an Illumina NextSeq500 System with a high-output kit in the paired-end 2 × 151 bp mode. The following procedures were followed for library preparation and sequencing at the Institute of Genomics Core Facility, University of Tartu, Estonia. Using FastQC v0.11.9 (Andrews, 2010), the quality of the raw readings was evaluated. Trimmomatic v0.39 was used to trim the reads with the default parameters for paired-end reads (Bolger *et al.*, 2014). SPAdes v3.14.1, in single-cell mode and k-mer sizes of 21, 33, 55, and 77 were used to assemble the reads (Bankevich *et al.*, 2012). The *C. jejuni/coli* MLST database (pubMLST) was used to assign sequence types (ST) and clonal complexes to all the assembled *Campylobacter* spp. genomes (n = 55) obtained from broiler chicken meat (n = 35) and human clinical cases (n = 20) (Jolley and Maiden, 2010).

Within the subset of isolates, AMR genes and point mutations of *C. jejuni* (n = 27) and *C. coli* (n = 8) isolates that had previously been identified as resistant using the MIC test were analysed. In the present study, only genotypic resistance mechanisms matching to phenotypic AMR were found and reported, susceptible campylobacters were excluded from the analysis. With the exception of options "plus" and organism "*Campylobacter*", AMRFinderPlus v3.10.23 with database v2021-12-21.1 was used in accordance with the default settings (Feldgarden *et al.*, 2019, Feldgarden *et al.*, 2021). Detected resistance genes with coverage of less than 80% were excluded from the analysis.

4.2.5. Statistical analyses (I, II, III)

The results were recorded, and statistical analyses were conducted using Microsoft Excel 2010 software (Microsoft Corporation; Redmond, WA, USA) and R v4.2.3 (R Core Team, 2021; **I**, **II**, **III**). The Clopper-Pearson (exact) technique was used to determine the Binomial Probability confidence intervals (CI) at a 95% confidence level for determining the prevalence and counts of *Campylobacter* in fresh broiler meat products of different origins (**I**). The prevalence of *Campylobacter* spp. in fresh broiler chicken meat and samples of different origins, as well as the AMR of *Campylobacter* spp. in fresh broiler chicken meat from different origins, were compared using the Chi-square test (Chi-Square Test Calculator, 2021;). Statistical significance was set at $P < 0.05$. A logistic regression model was used, followed by pairwise multiple comparisons with Tukey correction to determine (1) the differences in the proportions of *Campylobacter*-positive pig caecal samples between the studied years, (2) the differences in the proportion of total AMR between 2015, 2017, and 2019, and (3) differences in total AMR between specific antimicrobials in all the years combined (**III**). Statistical significance was set at $P < 0.05$. A one-sample proportion test with continuity correction was used to calculate the 95% CI (**III**).

5. RESULTS

5.1. *Campylobacter* spp. prevalence and counts in poultry meat (I)

During 2018–2019, 32.9% of broiler chicken meat samples were observed to be contaminated with *Campylobacter* spp. (Table 2). Among these, 1.8% (95% CI 0−5.3) fresh broiler chicken meat samples from Estonia, 36.8% (95% CI 28.6−45.6) from Latvia, and 66.9% (95% CI 58.2−74.8) from Lithuania tested positive for *Campylobacter* spp. The prevalence of *Campylobacter* spp. in fresh broiler chicken meat was significantly associated ($P < 0.05$) with the country of origin.

Table 2. *Campylobacter* spp. in fresh broiler chicken meat in Estonian retail stores during 2018−2019

Country of origin	Number of samples	Positive samples		
		Number	Percentage	CI 95%
Estonia	163	3	1.8	0–5.3
Latvia	133	49	36.8	28.6–45.6
Lithuania	133	89	66.9	58.2–74.8
Total	**429**	**141**	**32.9**	**28.4–37.5**

Table 3 shows the distribution of *Campylobacter* counts among the 141 broiler chicken meat samples. Among *Campylobacter*-positive samples, 14.5% (n = 62) had bacterial counts below 100 CFU/g. One (0.8%) Latvian and 27 (20.3%) Lithuanian-origin fresh broiler chicken meat samples were exceeding the process hygiene criteria (1,000 CFU/g).

Table 3. *Campylobacter* spp. enumeration data from fresh broiler chicken meat from different countries of origin available in Estonian retail stores during 2018−2019

Country of origin	Enumeration (CFU/g)				
	0*	< 100**	100−499	500−1,000	> 1,000
Estonia	160	2	1	0	0
Latvia	84	39	6	3	1
Lithuania	44	21	27	14	27
Total	**288**	**62**	**34**	**17**	**28**

*Negative detection and negative enumeration

**Negative enumeration and positive detection, threshold

CFU - colony-forming unit

The highest *Campylobacter* count found in samples of Latvian origin samples was 1,500 CFU/g. The high *Campylobacter* counts in the Lithuanian samples ranged from 1,000 to 5,000 CFU/g. Fresh broiler chicken meat products of Estonian origin exhibited significantly ($P < 0.05 < 0.00001$) lower *Campylobacter* counts than those of Latvian and Lithuanian origin. One fresh broiler chicken meat sample of Estonian origin was the only one found to possess *Campylobacter* spp. at 2.3 $\log_{10}$CFU/g. Two positive samples of Estonian origin had counts below the quantification threshold (100 CFU/g) and were excluded from the calculation of concentration averages.

5.2. Genotyping of poultry meat and human origin *C. jejuni* and *C. coli* isolates (I)

Twenty *Campylobacter* spp. isolates from human patients in Estonia and 35 *Campylobacter* spp. isolated from broiler chicken meat of Estonian (n = 1), Latvian (n = 10), and Lithuanian (n = 24) origins were sequenced and genotyped using MLST. Table 4 shows the sequencing types (STs) distribution of *Campylobacter* spp. isolates originating from broiler chicken meat and humans in Estonia.

Twenty-two STs from 55 *Campylobacter* spp. isolates were identified. Four STs were common to human and broiler chicken meat, nine STs were found exclusively in humans, and 10 STs were associated with broiler chicken meat. In the context of *C. jejuni* (n = 45), ST2229 (n = 7; 16%), ST19 (n = 4; 9%), ST122 (n = 4; 9%), ST464 (n = 4; 9%), ST9882 (n = 4; 9%), ST354 (n = 3; 7%), ST572 (n = 3; 7%), and ST7355 (n = 3; 7%) were the most frequently isolated STs. In the context of *C. coli,* ST832 (n = 4; 40%) and ST872 (n = 4; 40%) were the most prevalent genotypes (n = 10).

Table 4. Distribution of *Campylobacter jejuni* and *Campylobacter coli* sequencing types in the broiler chicken meat from the Baltic countries and human patients from Estonia

Source*	EST	LV	LT	H
*Campylobacter jejuni/ coli*** sequence types	10997 (1)	356 (1)	19 (4)	22 (2)
		832* (2)	354 (3)	50 (2)
		2229 (7)	614 (2)	353 (1)
			832* (2)	429 (1)
			872* (4)	572 (3)
			6461 (1)	824 (1)
			122 (2)	**122 (2)**
			464 (2)	**464 (2)**
			7355 (1)	**7355 (2)**
			9882 (3)	**9882 (1)**
				1595* (1)
				1624* (1)
				11001 (1)
No. of isolates	**1**	**10**	**24**	**20**

*Source: EST, Estonia; LV, Latvia; LT, Lithuania; H, human patients

***C. coli* multilocus sequence types

Bold indicates common *C. jejuni* sequence types found in broiler chicken meat and human patients

Only one Estonian origin sample contained *C. jejuni* ST10997. ST356 and ST2229 were only present in *C. jejuni* samples originating from Latvia. In the context of samples of Lithuanian origin, ST19, ST354, ST614, ST6461, ST122, ST464, ST7355, and ST9882 were exclusively present in *C. jejuni*, while only ST872 was present in *C. coli*. ST832 was common in the samples of Latvian- and Lithuanian-origin *C. coli* isolates. ST122, ST464, ST7355, and ST9882 were detected in fresh broiler chicken meat

and human *Campylobacter* isolates. No common genotypes were observed for isolates of Estonian, Latvian, or Lithuanian origins.

Twenty *Campylobacter* isolates from human patients were identified to be either *C. jejuni* (n = 18) or *C. coli* (n = 2). Thirteen STs were identified, with the most common STs for *C. jejuni* being ST572 (n = 3; 15%), ST22 (n = 2; 10%), ST50 (n = 2; 10%), ST122 (n = 2; 10%), ST464 (n = 2; 10%), and ST7355 (n = 2; 10%). Furthermore, for *C. coli,* the most common STs were ST1595 (n = 1; 5%) and ST1624 (n = 1; 5%).

The 35 broiler chicken meat isolates were identified to be either *C. jejuni* (n = 27) or *C. coli* (n = 8). The most common STs for *C. jejuni* were ST2229 (n = 7; 26%), and ST19 (n = 4; 15%), while the most common STs for *C. coli* were ST832 (n = 4; 50%) and ST872 (n = 4; 50%).

5.3. Antimicrobial resistance of *C. jejuni* and *C. coli* originating from poultry meat and human patients (II)

Nalidixic acid and ciprofloxacin resistance in *Campylobacter* strains were highest (55 strains, 90.2% each), followed by tetracycline (35 strains, 57.4%), streptomycin (26 strains, 42.6%), and erythromycin (4 strains, 6.6%). All the strains were sensitive to gentamicin (Table 5).

Table 5. Antimicrobial resistance profile of *Campylobacter jejuni* and *Campylobacter coli* isolates of different origins

	Resistant *Campylobacter* spp. isolates (%)				
Antimicrobial	**Estonia (n = 4)**	**Latvia (n = 16)**	**Lithuania (n = 26)**	**Human (n = 15)**	**Total (n = 61)**
Nalidixic acid	0 (0)	16 (100)	26 (100)	13 (86.7)	55 (90.2)
Ciprofloxacin	0 (0)	16 (100)	26 (100)	13 (86.7)	55 (90.2)
Tetracycline	0 (0)	3 (18.8)	20 (76.9)	12 (80.0)	35 (57.4)
Streptomycin	0 (0)	11 (68.8)	11 (42.3)	4 (26.7)	26 (42.6)
Erythromycin	0 (0)	1 (6.3)	3 (11.5)	0 (0)	4 (6.6)
Gentamicin	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
Multidrug-resistant*	0 (0)	2 (12.5)	11 (42.3)	4 (26.7)	17 (27.9)

*Resistance to three or more unrelated antimicrobials

Six (9.8%) isolates, four from broiler chicken meat of Estonian origin and two from human *Campylobacter* strains were sensitive to all the tested antimicrobials. There were significant differences ($P < 0.05$) in resistance to nalidixic acid, ciprofloxacin, and tetracycline among Baltic origin

Campylobacter isolates of fresh broiler chicken meat. Estonian, Latvian, and Lithuanian *Campylobacter* strains isolated from broiler chicken meat did not differ in their susceptibility to streptomycin, erythromycin, and gentamycin. In total, 55 isolates (90.2%) were resistant to one or more antimicrobials. There were four (7.3%) and two (3.6%) sensitive isolates among broiler chicken meat and human clinical isolates, respectively. *Campylobacter* isolates from broiler chicken meat of Latvian and Lithuanian origin exhibited considerably higher AMR to one or more antimicrobials ($P < 0.05$) than isolates of Estonian origin. *Campylobacter* isolates from Estonian broiler chicken meat exhibited a significantly reduced ($P < 0.05$) fluoroquinolone resistance than those from Latvian and Lithuanian broiler chicken meat and Estonian human patients. Differences between human clinical isolates and broiler chicken meat isolates of Latvian ($P = 0.13$) and Lithuanian ($P = 0.06$) origins were not statistically significant. Of the isolates, 27.9% ($n = 17$) were MDR, of which 18.0% ($n = 11$) were of Lithuanian broiler chicken meat origin, 3.3% ($n = 2$) were of Latvian broiler chicken meat origin, and 6.6% ($n = 4$) were from Estonian patients. All isolates from broiler chicken meat of Latvian and Lithuanian origin were resistant to fluoroquinolones.

Ciprofloxacin/nalidixic acid/tetracycline resistance was the most common AMR pattern among *Campylobacter* isolates (32.7%). Other prevalent resistance patterns included ciprofloxacin/nalidixic acid/tetracycline/streptomycin (25.5%) and ciprofloxacin/nalidixic acid/streptomycin (16.4%).

Phenotype-related genotypic resistance determinants were identified in 28 *C. jejuni* and seven *C. coli* isolates. Genotypic AMR mechanisms corresponded to the aminoglycoside, macrolide, quinolone, and tetracycline phenotypic resistance identified via the MIC test. In the context of streptomycin resistance, *aadE, aadE-Cc,* and *aph(3')-IIIa*, were detected. The *tetO* gene was detected in the context of tetracycline resistance. Two point mutations in *23S* and *gyrA* (Thr-86-Ile) associated with erythromycin and quinolone resistance were also detected.

5.4. Antimicrobial resistance of *C. coli* in pig caecal samples (III)

Among 229 pig caecal samples, 119 (52.0%) were *Campylobacter*-positive. In total, 93.3% of isolates ($n = 111$) were resistant to at least one antimicrobial. Furthermore, 55 (46.2%), 38 (32.0 %), and 18 (15.1

%) were resistant to one, at least two, and three or more unrelated antimicrobials, respectively (Table 6). Only eight (6.7%) isolates were sensitive to all tested antimicrobials.

Table 6. Antimicrobial resistance of *Campylobacter coli* isolated from pig caecal samples

Year	No. of isolates	Resistant to				Multidrug-resistant (%)*	Sensitive (%)
		one	two	three	four		
2015	33	17	7	4	1	5 (15.2)	4 (12.1)
2017	20	10	6	3	0	3 (15.0)	1 (5.0)
2019	66	28	25	10	0	10 (15.2)	3 (4.5)
Total	**119**	**55**	**38**	**17**	**1**	**18 (15.1)**	**8 (6.7)**

*Resistance to three or more unrelated antimicrobials.

In 2015, 2017, and 2019, the proportion of resistant isolates was extremely high, at 87.9, 95.0, and 95.5%, respectively. The resistance pattern of *C. coli* isolates to the studied antimicrobials is shown in Table 7.

Table 7. Antimicrobial resistance pattern of *Campylobacter coli* isolates to studied antimicrobials

Antimicrobial	Resistant isolates/%			
	2015 (n = 33)	2017 (n = 20)	2019 (n = 66)	All (n = 119)*
Erythromycin	1/3.0	0/0.0	0/0.0	1/0.8[a]
Ciprofloxacin	9/27.3	6/30.0	26/39.4	41/34.5[b]
Tetracycline	12/36.4	8/40.0	34/51.5	54/45.4[b,c]
Gentamicin	1/3.0	0/0.0	0/0.0	1/0.8[a]
Nalidixic acid	8/24.2	4/20.0	26/39.4	38/31.9[b,c]
Streptomycin	24/72.7	17/85.0	48/72.7	89/74.8[d]

*Superscripts with the same letter did not differ significantly ($P > 0.05$) from each other

Among the isolates streptomycin (74.8%) and tetracycline (45.4%) resistance was predominant, followed by ciprofloxacin (34.4%) and nalidixic acid (31.9%). All the isolates obtained from 2017 and 2019 were susceptible to erythromycin and gentamicin. There were no statistically significant variations in AMR over the years. However, fluoroquinolone and tetracycline resistance increased slightly during the study period. The ciprofloxacin resistance rates were 27.3, 30.0, and 39.4% in 2015, 2017, and 2019, respectively. Tetracycline resistance ranged from 36.4 to 51.5% during the study period. The proportion of AMR to different antimicrobials exhibited statistically significant differences ($P < 0.05$), except for erythromycin, gentamicin, ciprofloxacin, tetracycline/

nalidixic acid, nalidixic acid, and tetracycline. Resistance to streptomycin was substantially higher than that to other antimicrobials and remained high throughout the study period, with the highest proportion in 2017 (85.0%). Streptomycin resistance was the most common among *C. coli* isolates (33.6%), followed by resistance to tetracycline/streptomycin (17.6%) and ciprofloxacin/tetracycline/ nalidixic acid/streptomycin (13.4%).

The highest MIC values for *C. coli* isolates were observed for streptomycin (54.6%), nalidixic acid (20.2%), tetracycline (17.7%), and ciprofloxacin (5.9%). A total of 16 (13.4%, 95% CI 8.1−21.2) MDR isolates showed a ciprofloxacin/tetracycline/nalidixic acid/ streptomycin phenotypic pattern.

6. DISCUSSION

6.1. *Campylobacter* spp. in poultry meat (I, II)

In the present study, the prevalence of *Campylobacter* spp. in fresh broiler chicken meat products of Estonian origin was significantly lower ($P < 0.05$) in comparison to Latvian and Lithuanian fresh broiler chicken meat products, which aligns with a previous study conducted in Estonia (Mäesaar *et al.*, 2014). According to Bunevičienė *et al.* (2010), *Campylobacter* prevalence in Lithuanian fresh broiler chicken meat was 46.5% in 2009. Furthermore, Kovalenko *et al.* (2013) reported high levels of *Campylobacter* in broiler chicken meat production in Latvia, with 92.5% of broiler chicken intestine samples, 60.8% of neck skin samples, and 56.3% of broiler chicken carcasses testing positive for *Campylobacter* spp. A comparison of the studies conducted in Estonia in the period between 2000 and 2019 (Roasto *et al.*, 2005; Meremäe *et al.*, 2010; Mäesaar *et al.*, 2014), it can be deduced that the proportion of *Campylobacter*-positive fresh broiler chicken meat samples of Estonian origin decreased from 15.8% in the earliest study (Roasto *et al.*, 2005) to 1.8% in the present study. According to these studies, the *Campylobacter* prevalence in fresh broiler chicken meat products at Estonian retail outlets of Latvian and Lithuanian origin increased since 2012, from 25.8 to 36.8% and from 10.6 to 66.9%, respectively. This indicates that even in geographically close countries, the prevalence of *Campylobacter* spp. in broiler chicken meat can be significantly different, which may be attributed to various factors including differences in the food safety assurance systems employed by companies "from farm to meat industry".

The proportion of *Campylobacter*-positive poultry meat at the retail level varies throughout Europe, ranging from 73.3% in the United Kingdom (Jorgensen *et al.*, 2015) to 11 and 12% in Finland and Denmark, respectively (Skarp *et al.*, 2016). High levels of *Campylobacter* contamination in fresh retail poultry meat have also been found in Austria (71%), France (76%), Spain (70%), Slovenia (54%), Poland (50%), and Italy (34.1%) (Skarp *et al.*, 2016; Stella *et al.*, 2017). The average proportion of *Campylobacter*-positive fresh broiler chicken meat in the EU was 38.6% in 2018 and 29.6% in 2019 (EFSA and ECDC, 2021b). Compared to other European countries, the prevalence of *Campylobacter* in Estonia is low. The fact that strict biosecurity and self-control measures are

implemented at the farm, slaughterhouse, and meat industry levels, as well as risk assessment-based control measures implemented at all stages of production, may have contributed to this decrease in *Campylobacter*-positive sample proportions of fresh broiler chicken meat of Estonian origin. Insufficient biosafety measures at farm and slaughterhouse levels may cause the spread of *Campylobacter* at the retail level (Perez-Arnedo and Gonzalez-Fandos, 2019). *Campylobacter* has been isolated from farm surroundings, and if biosecurity measures are insufficient, *Campylobacter* may be introduced into the farm (Cody *et al*., 2010). The transmission of *Campylobacter* from farm to fork involves many different stages of the poultry meat production chain, including primary production at rearing farms, transportation to the slaughterhouse, the slaughtering process, processing of chicken meat products, retailing, handling, and consumption of chicken meat (Skarp *et al*., 2016). Unlike Lithuania and Latvia, all the broiler chicken slaughterhouses and farms in Estonia are owned by a single meat company that uses integrated food safety management systems at every stage of broiler chicken meat production.

When consuming *Campylobacter*-contaminated undercooked broiler meat, *Campylobacter* infections are likely to occur (de Boer and Hahné, 1990). As few as 500 *Campylobacter* cells, according to Keener *et al.* (2004), can cause *Campylobacter* infections in humans. According to Tribble *et al.* (2010) and Kirkpatrick *et al.* (2013), the lowest documented infectious dose that causes clinical gastrointestinal symptoms ranges from 10^2 to 10^5 CFU.

Commission Regulation (EC) No. 2073/2005 on microbiological criteria for foodstuffs does not determine the food safety criteria for *Campylobacter* spp.; however, a process hygiene criterion was established for *Campylobacter* spp. in broiler carcasses in 2017. This regulation establishes a limit of 1,000 CFU/g of *Campylobacter* for poultry carcasses and neck skin as sampling material.

In the present study, the count of *Campylobacter* spp. exceeded 1,000 CFU/g in 6.5% of fresh broiler chicken meat samples at the Estonian retail level. Most (20.3%) of these samples were of Lithuanian origin, and only 0.8% of the Latvian-origin samples exceeded a concentration of 1,000 CFU/g. In all samples of Estonian origin, the *Campylobacter* count was low, and the proportion of positive samples was significantly low (1.8%). It can be argued that retail-level fresh broiler chicken meat

samples differ from neck skin samples collected at the slaughterhouse level. However, retail-level samples are closer to consumers, therefore meat contaminated with high concentrations of *Campylobacter* poses a significant public health concern.

In the present study, fresh broiler chicken meat products of Estonian origin had significantly ($P < 0.05$) lower *Campylobacter* counts than those of Latvian and Lithuanian origin. Therefore, compared to broiler chicken meat from Estonian producers, imported products may carry a higher risk of human *Campylobacter* infections. In addition, a study conducted by Mäesaar *et al.* (2014) found that Estonian broiler chicken meat samples exhibited significantly lower *Campylobacter* prevalence and numbers than samples originating from Latvia and Lithuania. Many studies have concluded that there is a greater risk of campylobacteriosis if the prevalence and numbers of *Campylobacter* spp. in fresh broiler chicken meat are high at the retail level because it is likely to cross-contaminate home kitchens, especially food preparation surfaces and processed food products (Meremäe *et al.*, 2010; Roasto *et al.*, 2010; Cardoso *et al.*, 2021).

According to many studies, the number of cases of human campylobacteriosis rises during the summertime, and the prevalence of *Campylobacter* in poultry is also higher during warm seasons (Bunevičienė *et al.*, 2010; Meremäe *et al.*, 2010; Kovalenko *et al.*, 2013; Mäesaar *et al.*, 2014; Jaakola *et al.*, 2015; Nastasijevic *et al.*, 2020). *Campylobacter* spp. and temperature have a strong positive association, as reported by Djennad *et al.* (2019). Additionally, because of higher temperatures, *Campylobacter* spp. are more prevalent during summer and early autumn. However, we did not detect any seasonal differences in the proportions of *Campylobacter*-positive fresh broiler chicken meat samples. The proportion of *Campylobacter*-positive samples of Lithuanian origin was consistently high throughout the study period. The EFSA (2018) has also reported a minor increase in human campylobacteriosis cases during the winter, indicating *Campylobacter* contamination in food during the cold months. According to Newell and Fearnley (2003), differences in the seasonality of *Campylobacter* colonisation in broilers can be influenced by the presence of vectors, the survival mechanisms of *Campylobacter* spp., and farm management, including sanitation efficacy.

In silico MLST was used to determine the genotypes of *C. jejuni* isolated from broiler chicken meat and clinical samples (Table 3). Genotyping

revealed four shared STs (ST122, ST464, ST7355, and ST9882) out of the 22 STs established in broiler chicken meat and human isolates. All *C. jejuni* broiler chicken meat isolates that shared STs with humans exhibited a Lithuanian origin. According to Mäesaar *et al.* (2018), ST353 was the most common genotype in human clinical *C. jejuni* isolates, while ST5, ST45, and ST50 were the most prevalent in broiler chicken meat isolates. However, in the present study, these genotypes were rarely detected or were completely absent. In our study, the most prevalent *C. jejuni* genotypes were ST2229 and ST19. Although we detected ST122 and other common genotypes (ST7355 and ST9882) in both human samples and broiler chicken meat products of Lithuanian origin, previous studies by Aksomaitiene *et al.* (2019) and Meistere *et al.* (2019) did not detect these genotypes in Lithuanian and Latvian samples, respectively. Nevertheless, Wieczorek *et al.* (2020) reported *C. jejuni* genotype ST122 in chicken meat and human samples originating from Poland. Similar to our study, *C. jejuni* genotype ST464 was identified in Lithuanian broiler products (Aksomaitiene *et al.*, 2019). Genotype ST464 has also been identified in Latvian and Lithuanian patients (Aksomaitiene *et al.*, 2019; Meistere *et al.*, 2019). Similarly, in a Polish study, ST464 was identified as a common genotype in both humans and chickens (Wieczorek *et al.*, 2020). In this study, genotype ST464 was also one of the shared STs detected in broiler chicken meat and clinical samples, suggesting that imported poultry products could be a possible source and reservoir of human *Campylobacter* ST464 infections in Estonia.

6.2. Antimicrobial resistance of *Campylobacter* spp. in poultry meat and clinical isolates (II)

The present study revealed a significant level of AMR in *Campylobacter* spp. isolated from broiler chicken meat of Lithuanian and Latvian origin, as well as clinical isolates of Estonian origin. Antimicrobial-resistant microorganisms are a major public health concern (WHO, 2015). Antimicrobial use has been linked to the rapid spread of AMR worldwide (O'Neill, 2016). *Campylobacter* is becoming increasingly resistant to CIAs, including fluoroquinolones and quinolones (Conesa *et al.*, 2022). According to EU Regulation No. 1831/2003, the use of antimicrobials as growth promoters in food-producing animals has been prohibited since 2006. However, in poultry, antimicrobials are still widely used, leading to the selection of resistant strains that may spread to humans (Stapleton *et al.,* 2010; Elhadidy *et al.*, 2018). In Scandinavian countries,

such as Denmark, CIAs, including carbapenems, third- and fourth-generation cephalosporins, fluoroquinolones, and colistin, are restricted. In Denmark, cephalosporins and colistin are not used in poultry production, and the use of fluoroquinolones is almost nil (DANMAP, 2020). Also, in Sweden, the use of antimicrobials in poultry production is low (Björkman *et al.*, 2021) and the proportion of resistant bacteria isolated from animals is also low (EMA, 2019; Swedres-Svarm, 2019). According to the FINRES-Vet 2020 report (Finnish Food Authority, 2021), AMR among *Campylobacter* spp. isolates from broilers has decreased in 2020 compared to previous years. Additionally, quinolone resistance has decreased, and resistance to tetracycline, erythromycin, gentamicin, and streptomycin is low, which is consistent with the trend towards reducing the use of antibiotics in farm animals in Finland. According to Simonsen *et al.* (2021), the AMR (9.2%) among *C. jejuni* isolates from broilers in Norway is significantly low.

In the EU, ciprofloxacin resistance in human *Campylobacter* isolates was extremely high in 2019, for *C. jejuni* and *C. coli* it was 61.5 and 61.2%, respectively (EFSA and ECDC, 2021b). In the present study, 86.7% of human *Campylobacter* strains were resistant to ciprofloxacin and nalidixic acid which was approximately 20% higher than that previously reported in Estonia by Mäesaar *et al.* (2016). In a study conducted in Lithuania, Aksomaitiene *et al.* (2019) reported that 88.1% of *C. jejuni* isolates from human clinical cases were resistant to ciprofloxacin. A high proportion (100%) of Latvian and Lithuanian-origin *Campylobacter* isolates from fresh broiler chicken meat were resistant to these antimicrobials. This finding aligns with the high fluoroquinolone resistance observed in Latvian (87.5%) and Lithuanian (84.8%) *Campylobacter* isolates originating from broiler chicken meat in a previous Estonian study conducted by Mäesaar *et al.* (2016), which may potentially indicate the widespread use of these antimicrobials in broiler chicken production. It has been proven that using the synthetic fluoroquinolone, enrofloxacin, to treat infections in chickens causes *Campylobacter* spp. to develop fluoroquinolone resistance (Endtz *et al.*, 1991). Aksomaitiene *et al.* (2019) reported that all *C. jejuni* isolates from broiler products from Lithuanian retail were ciprofloxacin-resistant, which is consistent with the findings of the present study. According to Kovalenko *et al.* (2014) and Meistere *et al.* (2019), isolates of Latvian origin exhibit extremely high (88–100%) resistance to fluoroquinolones and quinolones. The mechanisms responsible for quinolone resistance in broiler meat and human *Campylobacter* isolates

(n = 35) were determined to involve point mutations (Thr-86-Ile) in the *gyrA* gene (Cobo-Díaz *et al.*, 2021). In addition to the high fluoroquinolone resistance among *Campylobacter* isolates from broiler chicken meat, a high proportion (76.9%) of tetracycline-resistant Lithuanian broiler chicken meat isolates was found in the present study, which was considerably higher than that reported in a previous study conducted by Mäesaar *et al.* (2016) (19.6%). In comparison, the proportion of tetracycline-resistant human isolates in the present study was 80.0%, which is similar to that found in Lithuanian broiler chicken meat isolates, and twice as high as that reported in a study conducted in 2011–2013 (Mäesaar *et al.*, 2016). The resistance of *Campylobacter* spp. to tetracycline in broilers is also high in Portugal and Spain, ranging from 90 to 100% (Torralbo *et al.*, 2015; Garcia-Sanchez *et al.*, 2018b). Tetracycline resistance among *Campylobacter* spp. in broilers in Ireland has increased by 10% since the early 2000s (Lynch *et al.*, 2020). In comparison, in Estonia, in the last decade, resistance to tetracycline has increased by 35.7% (Mäesaar *et al.*, 2016). This increase is attributed exclusively to Lithuanian and Latvian *Campylobacter* isolates obtained from broiler chicken meat in Estonian retail stores. In the present study, phenotypically tetracycline-resistant *Campylobacter* isolates (n = 23) harboured the *tetO* gene. According to the EFSA and ECDC (2021b), the proportions of *C. jejuni* and *C. coli* resistant to tetracycline were 47.2 and 66.9%, respectively. There was also high streptomycin resistance among Latvian and Lithuanian broiler chicken meat isolates, 68.8 and 42.3% respectively. In the present study, 26.7% of human patient's origin *Campylobacter* isolates were resistant to streptomycin. This is much higher than those reported by Mäesaar *et al.* (2016). Overall, the high resistance against the tested antibiotics found in the present study indicates that the use of antibiotics is not well managed at the broiler chicken farm level. Resistance to erythromycin among Latvian and Lithuanian broiler chicken meat isolates increased from 1.6% in 2012 (Mäesaar *et al.*, 2016) to 9.5% in the present study. According to the EFSA and ECDC (2021b), low levels of erythromycin resistance have been observed in *C. jejuni* and *C. coli* (1.5 and 12.9%, respectively). The highest *C. coli* erythromycin resistance was observed in Portugal (73.1%). Furthermore, all human isolates in our study were susceptible to gentamicin and erythromycin. Notably, all Estonian-origin broiler chicken meat isolates (n = 4) were sensitive to all tested antimicrobials.

It can be concluded that the approach of the Nordic countries towards reducing antibiotic usage and restricting the use of CIAs, such as carbapenems, third- and fourth-generation cephalosporins, fluoroquinolones, and colistin, should be monitored. However, this is possible only when strict biosecurity and self-control measures are implemented to prevent the spread of pathogenic microorganisms at the farm level. The extremely low prevalence and counts of *Campylobacter*, as well as low AMR among *Campylobacter* strains isolated from fresh broiler chicken meat of Estonian origin, may be linked to the application of a vertically integrated management system and strict biosecurity and biosafety measures at all levels of broiler chicken production.

6.3. Antimicrobial resistance of *C. coli* isolated from pigs at slaughter (III)

There is no previously published information on the prevalence and AMR of *Campylobacter* in pigs in Estonia. Human *Campylobacter* infections have also been linked to pig-derived *C. coli* (Mossong *et al.*, 2016; Rosner *et al.*, 2017). *C. coli* in pig intestines have the potential to contaminate the entire meat processing chain if hygiene measures are insufficient (Sheppard *et al.*, 2011; Mughini Gras *et al.*, 2012). Additionally, the presence of antimicrobial-resistant *Campylobacter* spp. in pork could potentially affect public health. The percentage of *C. coli* isolates among human campylobacteriosis cases in Estonia between 2015 and 2019 ranged from 6.0 to 13.4%, with the highest proportion (13.4%) observed in 2018, which was related to three *C. coli* outbreaks (Terviseamet, 2023). It is important to highlight that between 2015 and 2019 in Estonia, human clinical isolates of *C. coli* exhibited an MDR profile that was eight-fold greater than that of *C. jejuni* (Terviseamet, 2023).

The present study gathered five-year data on the AMR of *C. coli* isolated from pigs at the Estonian slaughterhouse level. It was found that 52% of the samples tested were positive for *Campylobacter*. In comparison with our findings, a higher prevalence of *Campylobacter* spp. has been detected in pigs in Latvia and Italy (83.3 and 63.5%, respectively) (Pezzotti *et al.*, 2003; Meistere *et al.*, 2019). Similar to previous studies, all the isolates in our study were identified as *C. coli* (Di Donato *et al.*, 2020; Agbankpe *et al.*, 2022). Additionally, research conducted in slaughterhouses in Denmark and Latvia found that *C. coli* presence accounted for over 90% of pig *Campylobacter* isolates (Boes *et al.*, 2005; Meistere *et al.*, 2019). In

the literature, there are extremely few examples of *Campylobacter*-free pig herds or those with very low *Campylobacter* prevalence. Specifically, only pathogen-free pig herds were observed to be *Campylobacter*-negative (Kolstoe *et al.*, 2014). An earlier study conducted by Lindblad *et al.* (2007) reported that the prevalence of *Campylobacter* in pigs in Swedish slaughterhouses was significantly low (1.0%).

To monitor AMR in *C. coli* in fattening pigs at slaughterhouses, the Commission Implementing Decision (EC) 2020/1729 was established according to Directive 2003/99/EC for the monitoring of zoonoses and zoonotic agents. In the Commission Implementing Decision 2013/652/EU, erythromycin, ciprofloxacin, tetracycline, gentamicin, nalidixic acid, and streptomycin were the antimicrobials used for monitoring *C. coli* resistance; however, in 2020, it was repealed by Decision 2020/1729/EU, in which streptomycin and nalidixic acid were replaced by chloramphenicol and ertapenem. The antimicrobials listed in Directive (EC) No 2003/99 were used in this study because the present study included the isolates collected from 2015 to 2019 at the pig slaughterhouse level. According to Directive (EC) No 2003/99 the EUCAST thresholds for resistance were used.

According to the Estonia State Agency of Medicines (2023), doxycycline, tiamulin, and amoxicillin were the most commonly used antimicrobials in pigs and cattle in 2022 in Estonia. In addition, tetracyclines, penicillins, and pleuromutilins represented the highest-selling antimicrobial classes in veterinary medicine in 2022, accounting for 25.5, 22.3, and 18.4% of sales, respectively (Estonia State Agency of Medicines, 2023).

In this study, a significantly high proportion of resistant *C. coli* isolates were detected, with particularly high resistance to streptomycin, tetracycline, and fluoroquinolones (74.8, 45.4, and 34.4%, respectively). Similarly, a Latvian study reported extremely high numbers of streptomycin-resistant *C. coli* isolates in pig caecal samples (Meistere *et al.*, 2019). In addition, an Italian study found *C. coli* isolated from pigs were highly resistant to tetracycline and streptomycin (Pezzotti *et al.*, 2003). Lower *C. coli* resistance to these antimicrobials has been reported in Finland (Finnish Food Safety Authority, 2016) and Denmark (Aarestrup *et al.*, 1997). In Denmark, the use of tetracycline in pigs has decreased since 2009, and the use of CIAs has been phased out (DANMAP, 2020).

The first-line treatment for invasive human infections is fluoroquinolones such as ciprofloxacin (WHO, 2019), which means that *Campylobacter* resistance to fluoroquinolones requires special attention. In the present study, resistance to nalidixic acid (quinolones) and ciprofloxacin (fluoroquinolones) was 31.9 and 34.4%, respectively. According to the EU Summary Report on Antimicrobial Resistance in 2020–2021, ciprofloxacin resistance was 65.8 and 69.6% in *C. coli* human isolates, respectively (EFSA and ECDC, 2023). Portugal and Estonia reported the highest levels (100%) of ciprofloxacin resistance among *C. coli* human isolates. Additionally, ciprofloxacin resistance in food-producing animals ranged between 41.7 and 80.4%. *C. coli* isolated from fattening pigs exhibited a 51.7% ciprofloxacin resistance in 2021. In 2021, *C. coli* isolated from humans demonstrated a significantly high proportion of tetracycline resistance (70.3%). In human *C. coli* isolates from Estonia, the combined resistance to ciprofloxacin and erythromycin was 25.9%, which was the second-highest level among EU-reporting countries after Portugal (EFSA and ECDC, 2023).

Different percentages of fluoroquinolone resistance among pig *Campylobacter* isolates have been observed in various studies, including 18.3 and 34.0% in Finland, 53.5% in Latvia, and 57.1% in Poland (Wieczorek and Osek, 2013; Finnish Food Safety Authority, 2016; Meistere *et al.*, 2019; Finnish Food Authority, 2022). *C. coli* from pigs were observed to be significantly resistant to both quinolones (74.7%) and fluoroquinolones (70.1%) in a recent Italian study (Di Donato *et al.*, 2020).

MDR zoonotic microorganisms are one of the most important public health concerns and require monitoring and mitigation actions (ECDC *et al.*, 2017; Catalano *et al.*, 2022). MDR among *C. coli* is an alarming trend, especially because pork is one of the most highly consumed meats in many countries worldwide (Pascoe *et al.*, 2017; Mourkas *et al.*, 2019; Marotta *et al.*, 2020; Kuus *et al.*, 2021; Finnish Food Authority, 2022). The proportion of MDR isolates in the present study was 15.1%, which is higher than that reported by the EFSA and ECDC (2023), in which 9.7% of *C. coli* isolates from fattening pigs were MDR. The high percentage of MDR *C. coli* isolates suggests the overuse of antibiotics at the pig farm level (Tang *et al.*, 2020).

In this study, the lowest resistance was observed against erythromycin (0.8%), which was categorised as CIA (WHO, 2019). Finland and Latvia have reported similar findings (Finnish Food Safety Authority, 2016; Meistere *et al.*, 2019). Recently, the Finnish Food Authority (2022) reported that erythromycin resistance is rare among *C. coli* isolates from Finnish pigs.

Strict biosecurity measures must be implemented along with appropriate farm and veterinary practices to prevent the emergence and spread of MDR microorganisms and reduce the use of antimicrobials in food-producing animals (Economou and Gousia, 2015; Kahn *et al.*, 2019; Huber *et al.*, 2022). Optimal nutrition, clean water, and suitable living conditions are important for reducing the use of antimicrobials and the risk of AMR (Albernaz-Gonçalves *et al.*, 2022). To prevent the spread of AMR, the WHO organised a global action plan to restrict the use of antimicrobials in food-producing animals and promote the responsible use of antimicrobials (WHO, 2015). The World Organization for Animal Health Standards on Antimicrobial Resistance and Use of Antimicrobials supported the WHO-organised Global Action Plan (OIE, 2016). Two important EU regulations supporting the ethical use of antimicrobials in animals were enforced at the beginning of 2022. Regulation (EU) 2019/6 places restrictions on certain antimicrobial agents and encourages the use of alternatives, thus strengthening the monitoring of antimicrobial use in animals to prevent the development and spread of AMR. Regulation (EU) 2019/4 states that good husbandry, biosecurity, and management practices should never be replaced by medical treatment, particularly with antimicrobials. According to Charlier *et al.* (2022), research and innovation must be encouraged to create novel antimicrobials, vaccines, and alternative treatments to reduce the use of antimicrobials.

Owing to the high number of *C. coli*-positive caecal samples and high AMR among *C. coli* isolates, contaminated pork should be regarded as a public health risk in Estonia. As no such research has been conducted, source attribution studies must be performed to determine the primary causes of *C. coli* infections in humans. In addition, research on pig farm risk factors and consumer education efforts to prevent *Campylobacter* infections are required.

7. CONCLUSIONS

According to the present study, samples of Estonian origin exhibited significantly lower *Campylobacter* prevalence and numbers in fresh broiler chicken meat in comparison to samples of Latvian and Lithuanian origin sold at Estonian retail stores. Compared to fresh broiler chicken meat produced in Estonia, imported meat is more likely to cause campylobacteriosis in humans. In the present study, high genetic diversity was observed among *Campylobacter* isolates from fresh broiler chicken meat. Genotyping has revealed links between campylobacteriosis in Estonia and imported fresh broiler chicken meat (**I**).

The study revealed that 90.2% of the *Campylobacter* strains isolated from broiler chicken meat in 2018–2019 were resistant to one or more antimicrobials, and 27.9% of the isolates were MDR. *Campylobacter* isolates from fresh broiler chicken meat of Estonian origin were sensitive to all the tested antimicrobials, whereas 100% of the Latvian and Lithuanian *Campylobacter* isolates from broiler chicken meat were resistant to one or more antimicrobials. However, 86.7% of Estonian human strains were resistant to one or more antimicrobials. The AMR profile of Lithuanian broiler chicken meat isolates overlapped with those isolated from humans in Estonia. In conclusion, Lithuanian and Latvian broiler chicken meat sold in Estonian retail stores was contaminated with highly resistant *Campylobacter* spp. Additionally, *Campylobacter* isolates of human origin exhibited a significantly high level of resistance. This implies that eating *Campylobacter*-contaminated broiler chicken meat could pose a potential risk to Estonians. Resistant bacteria in food can reach consumers and pose health risks. Hence, it is crucial to follow common policies and implement standard practices for antimicrobial use to reduce *Campylobacter* resistance (**II**).

A total of 15.1 campylobacteriosis cases per 100,000 people were reported in Estonia in the year 2022, with approximately 10% of human campylobacteriosis infections linked to *C. coli*. This study revealed high occurrence of *C. coli* in caecal samples of fattening pigs in Estonia. Also, the proportion of antimicrobial resistant isolates was extremely high. Therefore, pork consumption poses a potential public health risk in Estonia. Because of the high resistance among *C. coli*, it is necessary to restrict the use of CIA in the treatment of pigs and to enhance the monitoring and control of antimicrobial usage in farm animals (**III**).

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9. SUMMARY IN ESTONIAN

Kampülobakterid värskes kanabroilerilihas ja sea umbsoolesisaldises Eestis

Sissejuhatus

Alates aastast 2005 on kampülobakterenteriit Euroopa Liidus enim diagnoosituid toidutekkeline bakteriaalne gastroenteriit (WHO, 2012; EFSA ja ECDC, 2021a). Euroopa Toiduohutusameti ja Haiguste Ennetamise ja Tõrje Euroopa Keskuse andmetel registreeriti 2021. aastal Euroopa Liidus kampülobakterioosi haigestumine 127 840 korral, mis on 41,1 haigusjuhtumit 100 000 inimese kohta (EFSA ja ECDC, 2021a). Eestis registreeriti 2021. aastal 185 kampülobakterenteriidi juhtumit, mis on 13,9 haigusjuhtumit 100 000 inimese kohta (Terviseamet, 2023).

Peamised kampülobakteri liigid on *C. jejuni* ja *C. coli*, mis põhjustavad vastavalt 80% ja 10% inimese kampülobakterioosi juhtumitest (EFSA, 2018). Inimese kampülobakterioosi suurimaks nakkusallikaks peetakse linnuliha, eriti kanabroileriliha, kuid ka muid toiduallikaid, nagu toorpiim, sealiha ja töötlemata vesi (Mäesaar jt, 2020; EFSA ja ECDC, 2021a; CDC, 2023).

Väitekirja eesmärgid

Uuringu peaeesmärk oli analüüsida termofiilsete kampülobakterite levimust ja antibiootikumiresistentsust Eesti jaemüügis müüdavas kanabroilerilihas ja tapamajas nuumsigade umbsoolesisaldise proovides.

Väitekirja eesmärgid:

Selgitada välja termofiilsete kampülobakterite levimus, arvukus Eesti, Läti ja Leedu päritolu värskes kanabroilerilihas, ning seonduvad genotüübid, et määrata kindlaks lindudelt pärinevate kampülobakterite tüvede seos inimese kampülobakterenteriidi juhtumitega Eestis (**I**).

Määrata Eesti jaemüügis müüdavast kanabroilerilihast ja inimestelt isoleeritud kampülobakterite antibiootikumiresistentsus (**II**).

Määrata nuumsigade umbsoolesisaldise proovidest termofiilsete kampülobakterite esinemus ja resistentsus antibiootikumide suhtes (**III**).

Kampülobakterite levimus, arvukus ja genotüübid kanabroilerilihas (I)

Kampülobakterite levimuse ja arvukuse määramisel kasutati standarditel ISO 10272-1:2017 ja ISO 10272-2:2017 põhinevaid meetodeid. Analüüside tulemusel isoleeriti kampülobaktereid kokku 141-st (32,9%) värskest kanabroileriliha proovist, mis koguti jaemüügist. Eesti päritolu broileriliha proovidest (n = 163) oli *Campylobacter*-positiivseid proove kolm (1,8%). Läti päritolu proovidest (n = 133) oli positiivseid proove 49 (36,8%). Leedu päritolu broileriliha proovidest (n = 133) oli positiivseid proove 89 (66,9%).

Leedu toodetest 20,3% sisaldas kampülobaktereid ≥ 1000 PMÜ/g, mis ületab seadusandlusega kehtestatud protsessi hügieenikriteeriumit, ja Läti toodete puhul oli see näitaja 0,8%. Eesti päritolu proovid eelmainitud kriteeriumit ei ületanud. Kahes Eesti päritolu proovis, mis osutusid *Campylobacter*-positiivseks, oli kampülobakterite kontsentratsioon alla arvukuse määramise piiri (100 PMÜ/g).

Kampülobakterite isolaatide täisgenoomsel sekveneerimisel (*whole-genome sequencing*, WGS) ekstraheeriti desoksüribonukleiinhape (*deoxyribonucleic acid,* DNA) kasutades GeneJET Genomic DNA Purification Kit (ThermoFisher Scientific, Waltham, MA). Sekveneerimise andmetega raamatukogude tegemisel kasutati Illumina Nextera XT ettevalmistuskomplekti vastavalt tootja protokollile (Illumina Inc., San Diego, CA). Viiekümne viie testitud kampülobakteri isolaadi analüüsil tuvastati 22 sekveneerimistüüpi (*sequence type*, ST). *C. jejuni* (n = 45) sagedasemad ST-d olid ST2229 (n = 7; 16%), ST19 (n = 4; 9%), ST122 (n = 4; 9%), ST464 (n = 4; 9%), ST9882 (n = 4; 9%), ST354 (n = 3; 7%), ST572 (n = 3; 7%) ja ST7355 (n = 3; 7%). *C. coli* (n = 10) sagedasemad ST-d olid ST832 (n = 4; 40%) ja ST872 (n = 4; 40%). Inimese ja kanabroileriliha ühised ST-d olid ST122, ST464, ST7355 ja ST9882. Kõik nimetatud genotüübid seondusid vaid Leedu päritolu kanabroilerilihase isoleeritud *Campylobacter* spp. isolaatidega.

Kanabroileriliha proovidest isoleeritud kampülobakterite antibiootikumiresistentsus (II)

Kampülobakterite tuvastamine ja isoleerimine viidi läbi vastavalt standardile ISO 10272-1:2017. Kampülobakterite isolaatide antibiootikumitundlikkuse fenotüübiliseks määramiseks teostati minimaalse inhibeeriva kontsentratsiooni (MIC) määramine kasutades EUCAMP2 paneeli (TREK Diagnostic Systems Ltd., East Grinstead, Ühendkuningriik). Lisaks fenotüübilisele resistentsuse määramisele tehti ka isolaatide WGS ja bioinformaatiline analüüs resistentsusmehhanismide määramiseks.

Eesti jaemüügis kogutud proovidest isoleeritud kampülobakteri tüvedel määrati antibiootikumiresistentsus. Valimi moodustasid neli Eesti, 16 Läti ja 26 Leedu päritolu *Campylobacter* spp. isolaati. Täiendavalt kaasati valimisse inimestelt isoleeritud 12 *Campylobacter jejuni* ja kolm *Campylobacter coli* isolaati.

Uuriti kampülobakterite resistentsust nalidiksiinhappe, tsiprofloksatsiini, tetratsükliini, streptomütsiini, erütromütsiini ja gentamütsiini suhtes.

Eesti päritolu broileriliha isolaadid osutusid kõik tundlikuks iga testitud antimikroobse aine suhtes. Kõik Läti ja Leedu päritolu kanabroilerilihast isoleeritud kampülobakterid ning enamik (86,7%) inimestelt isoleeritud kampülobakteritest olid resistentsed ühe või mitme antibiootikumi suhtes.

Kampülobakterite resistentsus oli suurim nalidiksiinhappe ja tsiprofloksatsiini suhtes (võrdselt 90,2%). Tetratsükliini, streptomütsiini ja erütromütsiini suhtes osutusid resistentseteks vastavalt 57,4%, 42,6% ja 6,6% uuritud tüvedest. Kõik tüved olid aga tundlikud gentamütsiini suhtes.

Multiresistentsust ehk resistentsust kolme või enama eri gruppidesse kuuluva antimikroobse aine suhtes esines kokku 26,2%-l isolaatidest, neist 10 (38,5%) olid Leedu, kaks (12,5%) Läti päritolu kanabroilerilihast ja neli (26,7%) inimestelt isoleeritud *Campylobacter* spp. isolaati. Multiresistentsetele kampülobakterite tüvedele oli iseloomulik üheaegne resistentsus nalidiksiinhappe ja tsiprofloksatsiini ehk kinoloonide suhtes.

WGS-i teel saadud andmete analüüsi kaasati 35 *Campylobacter* spp. isolaati geneetiliste resistentsusmehhanismide määramiseks. Kõigil minimaalse inhibeeriva kontsentratsiooni (*minimal inhibitory concentration*, MIC) meetodiga resistentseks osutunud isolaatidel leiti vastavad genotüübilised antibiootikumiresistentsuse mehhanismid. Tuvastati *aadE*, *aadE-Cc*, *aph(3')-IIIa* geenid, mis on seotud streptomütsiiniresistentsusega. Tetratsükliiniresistentsuse korral tuvastati *tetO* geenid. Erütromütsiini ja kinoloonide suhtes oli resistentsus punktmutatsioonidest vastavalt *23S* ja *gyrA* geenides.

Nuumsigade umbsooleproovidest isoleeritud *C. coli* antibiootikumiresistentsus (III)

Kampülobakterite isoleerimine viidi läbi vastavalt standardile ISO 10272-1:2018. Antibiootikumitundlikkuse fenotüübiliseks määramiseks kasutati MIC määramist kasutades EUCAMP2 paneeli (TREK Diagnostic Systems Ltd., East Grinstead, Ühendkuningriik). Aastatel 2015, 2017 ja 2019 nuumsigade umbsooleproovidest 119 (52,0%) olid *Campylobacter*-positiivsed ja kõik tuvastatud kampülobakterid osutusid liigiliselt *C. coli*'deks. Enamik (93,3%) isolaatidest olid resistentsed ühe või mitme antibiootikumi suhtes. Kõige enam esines resistentsust streptomütsiini (74,8%) ja tetratsükliini (45,4%) suhtes, millele järgnes resistentsus tsiprofloksatsiini (34,4%) ja nalidiksiinhappe (31,9%) suhtes. 2017. ja 2019. aastal osutusid kõik kampülobakterite isolaadid erütromütsiini ja gentamütsiini suhtes tundlikuks. Multiresistentsust esines 15,1%-l isolaatidest.

Järeldused

Eesti päritolu kanabroilerilihas esines kampülobaktereid vaid kolmes proovis. Eestis jaemüügis müüdav Läti ja Leedu värske kanabroileriliha oli kampülobakteritega saastunud. Kampülobakterite kontsentratsioon Läti päritolu toodetes oli madal, kuid Leedust pärit värsket kanabroileriliha iseloomustas kogu uuringu vältel kampülobakterite kõrge levimus ja kõrge kontsentratsioon. Käesolevas uuringus täheldati värskest kanabroilerilihast isoleeritud kampülobakterite hulgas suurt geneetilist mitmekesisust. Inimestelt ja kanabroilerilihast tuvastatud ühised kampülobakterite sekveneerimistüübid kattusid vaid importtoodetega, mistõttu võivad esineda seosed imporditud kanabroileriliha tarbimise ja inimeste kampülobakterioosi juhtumite vahel Eestis.

Et hoida kampülobakterioosi haigestumise risk võimalikult madal, tuleb saastunud imporditud kanabroileriliha toodetele enam tähelepanu pöörata (**I**).

Uuringu kohaselt oli aastatel 2018–2019 kanabroilerilihast isoleeritud *Campylobacter* spp. isolaatidest 27,9% multiresistentsed ja 90,2% resistentsed ühe või mitme antibiootikumi suhtes. Erinevalt Eesti kanabroilerilihast isoleeritud kampülobakterite tüvedest osutusid Leedu ja Läti päritolu isolaadid testitud antibiootikumide suhtes resistentseks. Leedust pärit kanabroileriliha isolaatide AMR-profiil sarnanes Eesti inimeste isolaatide omaga. Sellest võib järeldada, et kampülobakteriga saastunud imporditud kanabroileriliha söömine võib kujutada endast potentsiaalset ohtu rahva tervisele, sest selle saastumis- ja resistentsusmäär on kõrge. Antibiootikumidele resistentsed bakterid võivad jõuda toiduga tarbijateni ja ohustada nende tervist. *Campylobacter* spp. antimikroobse resistentsuse vähendamiseks on oluline järgida ravijuhiseid nii humaan- kui ka veterinaarmeditsiinis (**II**).

Nuumsigade umbsooleproovide analüüsimise tulemusel selgus, et sead on sageli resistentsete kampülobakterite kandjad, mistõttu võib sealiha tarbimine põhjustada ohtu rahva tervisele. *C. coli* isolaatide kõrge antimikroobse resistentsuse tõttu tuleb piirata kriitiliselt olulisi antimikroobseid aineid sigade raviskeemides ning ennetada loomade haigestumist. (**III**).

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I

ORIGINAL PUBLICATIONS

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MICROBIOLOGY AND FOOD SAFETY

The prevalence, counts, and MLST genotypes of *Campylobacter* in poultry meat and genomic comparison with clinical isolates

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ABSTRACT Since 2005 campylobacteriosis has been the most commonly reported gastrointestinal infection in humans in the European Union with more than 200,000 cases annually. Also *Campylobacter* is one of the most frequent cause of food-borne outbreaks with 319 outbreaks reported to EFSA, involving 1,254 cases of disease and 125 hospitalizations in EU in 2019. Importantly poultry meat is one of the most common source for the sporadic *Campylobacter* infections and for strong-evidence campylobacteriosis food-borne outbreaks in EU.

In present study, 429 fresh broiler chicken meat samples of Estonian, Latvian, and Lithuanian origin were collected from Estonian retail level and analyzed on a monthly basis between September 2018 and October 2019. *Campylobacter* spp. were isolated in 141 (32.9%) of 429 broiler chicken meat samples. Altogether 3 (1.8%), 49 (36.8%), and 89 (66.9%) of Estonian, Latvian, and Lithuanian origin broiler chicken meat samples were positive for *Campylobacter* spp. Among *Campylobacter*-positive samples, 62 (14.5%) contained *Campylobacter* spp. below 100 CFU/g and in 28 (6.5%) samples the count of *Campylobacter* spp. exceeded 1,000 CFU/g. A high prevalence of *Campylobacter* spp. in fresh broiler chicken meat of Lithuanian and Latvian origin in Estonian retail was observed. Additionally, 22 different multilocus sequence types were identified among 55 genotyped isolates of broiler chicken meat and human origin, of which 45 were *Campylobacter jejuni* (***C. jejuni***) and 10 were *Campylobacter coli* (***C. coli***). The most prevalent multilocus sequence types among *C. jejuni* was ST2229 and among *C. coli* ST832, ST872. *C. jejuni* genotypes found in both broiler chicken meat and human origin samples were ST122, ST464, ST7355, and ST9882, which indicates that imported fresh broiler chicken meat is likely the cause of human campylobacteriosis in Estonia.

Key words: *Campylobacter* spp., prevalence, counts, MLST sequence types, fresh broiler chicken meat

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INTRODUCTION

Campylobacter is the most common cause of human bacterial gastroenteritis in the world and campylobacteriosis is commonly reported zoonosis in humans in European Union (**EU**) since 2005 (World Health Organization, 2013; European Food Safety Authority EFSA and European Centre for Disease Prevention and Control ECDC 2021). According to European Food Safety Authority (**EFSA**), there were 220,682 confirmed human cases in EU in 2019, with notification rate of 59.7 per 100,000 population on average. In Estonia, 348 confirmed cases of human campylobacteriosis were registered in 2019, with a notification rate of 26.4 per 100,000 inhabitant (Estonian Health, 2021). The human campylobacteriosis notification rate in Estonia is 2.3 times lower than the average in European Union. However, the true numbers of human campylobacteriosis may be higher than officially reported, because infectious diseases are often underestimated, under-ascertained, or underreported (Gibbons et al., 2014). Campylobacteriosis is a notifiable infectious disease and an important problem for the public health and food industry (Tumbarski, 2019). The two main *Campylobacter* species causing a disease in humans are *C. jejuni* and *C. coli* causing approximately 80% and 10% of campylobacteriosis cases (EFSA, 2018). Different studies have demonstrated that *C. jejuni* is the predominant species in poultry (Meremäe et al, 2010; Korsak et al., 2015;

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Rossler et al., 2019; Yushina et al., 2020). According to Mäesaar et al. (2020) poultry is the main source of *C. jejuni* human infections in the Baltic States. The source of human campylobacteriosis is primarily considered to be poultry, especially broiler chicken meat, but also other food sources such as raw milk, pork and untreated water (Mäesaar et al., 2020; European Food Safety Authority EFSA and European Centre for Disease Prevention and Control ECDC 2021). *Campylobacter* infection occurs often due to consumption of undercooked chicken meat, or other food cross-contaminated during preparation process (Rosner et al., 2017). Entire processing chain of broiler chicken meat has a major importance of transmitting *Campylobacter* from farm to fork (Skarp et al., 2016).

In many countries the seasonal peak in the number of human campylobacteriosis cases and in *Campylobacter* prevalence in poultry is in summer months (Bunevičienė et al., 2010; Meremäe et al., 2010; Kovalenko et al., 2013; Mäesaar et al., 2014; Jaakola et al., 2015; Nastasijevic et al., 2020). However according to EFSA (2018), there is also a small but distinct winter peak that has been apparent in the human campylobacteriosis cases in the past few years.

The aim of this study was to determine the prevalence, counts and genetic relatedness of *Campylobacter* spp. isolated from broiler chicken meat at retail level of Estonia and from Estonian human patients.

MATERIALS AND METHODS

Sample Collection

Altogether 429 fresh broiler chicken meat samples were collected on a monthly basis between September 2018 and October 2019 at Estonian retail level. The collection included 163, 133, and 133 fresh broiler chicken meat samples of Estonian, Latvian, and Lithuanian origin. Company packaged fresh broiler chicken meat products, mostly legs and half-legs, were obtained from the biggest food retail outlets representing the biggest broiler chicken meat sales turnover in Estonia. The samples were transported to the laboratory in a portable cooler kept at +2° to +6°C. All the analyses were carried out in the laboratory of the Chair of Food Hygiene and Veterinary Public Health of the Estonian University of Life Sciences.

Additionally, in collaboration with the Estonian hospitals, 18 *C. jejuni* and 2 *C. coli* isolates related with human *Campylobacter* infections in Estonia were obtained during the study period in 2018−2019 for sequence typing.

Campylobacter *spp. Isolation and Identification*

Campylobacter spp. detection and enumeration from broiler chicken meat samples was performed according to the ISO 10272−1:2017 and ISO 10272-2:2017. For *Campylobacter* spp. detection, 10 g of skin aseptically taken from broiler chicken meat samples was transferred into 90 mL Preston enrichment broth and incubated in a microaerobic conditions at 41.5 ± 0.5°C for 24 h. Then, 10 μL loopful of Preston enrichment material was inoculated onto mCCD agar medium (Oxoid; Basingstoke, Hampshire, UK). For *Campylobacter* spp. enumeration, 10 g of skin aseptically taken from broiler chicken meat samples was placed into a sterile plastic bag and 90 mL buffered Peptone water was added. The samples were stomached for 60 s. Then, 0.1 mL of 10-fold dilution material was taken and carried onto the surface of 2 mCCD (Oxoid) agar plates. All plates were incubated in microaerobic conditions at 41.5°C ± 0.5°C for 48 h. Typical *Campylobacter* colonies on mCCDA plates were streaked on Columbia blood agar (Oxoid Ltd;), which were incubated for 24 h at 41.5°C ± 0.5°C. Additional confirmation tests included Gram staining, motility analysis, oxidase, and catalase tests. Isolates confirmed as *Campylobacter* spp. were stored at −80°C in glycerol broth (20% [vol/vol] glycerol in 1% [wt/vol] proteose peptone) for further studies.

Whole-Genome Sequencing and Genotyping

DNA was extracted using GeneJET Genomic DNA Purification Kit (ThermoFisher Scientific, Waltham, MA). The sequencing libraries were prepared with Illumina Nextera XT library preparation kit, according to manufacturer's protocol (Illumina Inc., San Diego, CA). Libraries were pooled in equimolar concentrations and the sequencing was carried out on an Illumina NextSeq500 System using high output kit in paired end 2 × 151 bp mode. The library preparation and sequencing were conducted as follows in the Institute of Genomics Core Facility, University of Tartu, Estonia.

Quality of the raw reads was checked using FastQC v0.11.9 (http://www.bioinformatics.babraham.ac.uk/projects/fastqc). Reads were trimmed using Trimmomatic v0.39 with default parameters for paired-end reads (Bolger et al., 2014). The reads were assembled using SPAdes v3.14.1 with single-cell mode and k-mer sizes 21, 33, 55, 77 (Bankevich et al., 2012). All assembled *Campylobacter* spp. genomes (n = 55) obtained from broiler chicken meat (n = 35) and human clinical cases (n = 20) were deposited and STs and CCs were assigned using the *Campylobacter jejuni/coli* multilocus sequence typing (**MLST**) database (pubMLST) (Supplementary Table 1; Jolley and Maiden, 2010).

Full minimum spanning tree (**MST**) of MLST allelic differences of 55 *C. jejuni* and *C. coli* isolates was constructed using goeBURST algorithm (Francisco et al., 2009) as implemented in PHYLOViZ v2.0 (Nascimento et al., 2017).

Statistical Analysis

Binomial Probability Confidence Interval (CI) at 95% confidence level in the prevalence and counts of the

Campylobacter in the poultry meat products of different origin was calculated using the Clopper-Pearson (exact) method using R (R Core Team, 2021). Chi-square test was used to test for statistically significant associations between prevalence of *Campylobacter* spp. in fresh broiler chicken meat and sample origin (https://www.socscistatistics.com/tests/chisquare2/default2.aspx). Results were considered statistically significant for P values of ≤ 0.05.

RESULTS AND DISCUSSION

Prevalence and Counts of Campylobacter *spp*

Campylobacter spp. was isolated in 141 (32.9%) of 429 broiler chicken meat samples in 2018-2019 (Table 1). In total, 3 (1.8%, CI_{95} 0−5.3%) of Estonian origin, 49 (36.8%, CI_{95} 28.6−45.6%) of Latvian origin and 89 (66.9%, CI_{95} 58.2%−74.8%) of Lithuanian origin fresh broiler chicken meat samples were positive for *Campylobacter* spp. The associations between prevalence of *Campylobacter* spp. in fresh broiler chicken meat and country of origin was statistically significant ($P < 0.00001$). Study conducted by Mäesaar et al. (2014) in 2012, found that the prevalence of *Campylobacter* spp. in Estonian origin fresh broiler chicken meat products was significantly lower ($P < 0.001$) than in Latvian and Lithuanian fresh broiler chicken meat products. Bunevičienė et al. (2010) determined a high *Campylobacter* prevalence (46.5% of positive samples) in the Lithuanian fresh broiler chicken meat in 2009. Additionally, high proportion of *Campylobacter* contamination of broiler chicken meat at slaughterhouse and retail level was reported in Latvia by Kovalenko et al. (2013). Our studies have shown that the contamination of fresh chicken broiler meat of Estonian origin with *Campylobacter* spp. has decreased year by year. Contrary, fresh broiler chicken meat of Latvian and Lithuanian origin is often contaminated with *Campylobacter* spp. at the Estonian retail level (Table 2). In European countries the proportion of *Campylobacter*-positive poultry meat at retail level has been different, 73.3% in the UK (Jorgensen et al., 2015) and much lower in Finland and Denmark, respectively 11 and 12% (Skarp et al., 2016). High proportion of *Campylobacter* contaminated poultry meat at retail has been also reported in Austria (71), France (76), Spain (70), Slovenia (54), Poland (50), and Italy (34.1%) (Skarp et al., 2016; Stella et al., 2017). According to the EU zoonoses report the average occurrence of *Campylobacter* in the fresh broiler meat in EU was 38.6 and 29.6%, respectively in year 2018 and 2019 (European Food Safety Authority EFSA and European Centre for Disease Prevention and Control ECDC 2021). This indicates that compared to many other European countries the prevalence of *Campylobacter* in Estonia is very low. Changes over time in the prevalence and counts of *Campylobacter* spp. in fresh chicken meat samples of Estonian retail is shown in the Table 2. A comparison of our previous studies reveals the decrease of *Campylobacter* spp. prevalence in fresh broiler chicken meat samples of Estonian origin from 15.8 to 1.8%. Since 2012, the prevalence of Latvian and Lithuanian *Campylobacter* spp. in fresh broiler chicken meat has increased, from 25.8 to 36.8% and from 10.6 to 66.9%, respectively. One possible explanation to the decreasing trend in *Campylobacter* prevalence of fresh broiler chicken meat of Estonian origin is that strict biosecurity and self-control measures at farm, slaughterhouse and meat industry level are applied, also risk assessment based control measures are implemented at all stages of production. It is known that the spread of *Campylobacter* at retail level could be related to the low effectiveness of biosecurity measures applied on farm and slaughterhouse level. *Campylobacter* is widespread in nature and there are many sources of infection through which *Campylobacter* is brought to the farm if biosecurity measures are not effective enough. Different stages in the poultry production for example, primary production at rearing farms, transport, slaughtering process, processing of chicken meat products, retailing, handling, and consumption of chicken meat, play an important role in the transmission of *Campylobacter* from farm to fork (Skarp et al., 2016). The same authors point out that the *Campylobacter* contamination and colonization at farm level reflect the *Campylobacter* contamination of carcasses and poultry meat. Several authors have emphasized the critical importance of defeathering and evisceration in poultry processing (Saleha et al., 1998; Sasaki et al., 2013; Gruntar et al., 2015).

A *C. jejuni* survival study in poultry processing plant environment revealed that some *C. jejuni* genotypes might survive the cleaning and disinfection procedures (García-Sánchez et al., 2017).

Present study was not aimed to seek for possible reasons for the higher prevalence of *Campylobacter* spp.

Table 1. Prevalence of *Campylobacter* spp. in fresh broiler chicken meat samples of Estonian, Latvian, and Lithuanian origin collected at the Estonian retail level in 2018−2019.

		No. of positive samples (%)		
Country of origin	No. of samples	Detectionmethod	Enumerationmethod[1]	CI 95% ofpositive %[2]
Estonia	163	3 (1.8)	0 (0)	0−5.3
Latvia	133	49 (36.8)	11 (8.3)	28.6−45.6
Lithuania	133	89 (66.9)	70 (52.3)	58.2−74.8
Total	429	141 (32.9)	81 (18.9)	28.4−37.5

[1]Samples with both positive detection and positive enumeration result, the threshold of 100 CFU/g.
[2]Confidence interval is given for detection method results.

Table 2. *Campylobacter* prevalence and counts in retail broiler chicken meat from different studies performed in Estonia in period 2000−2019.

Period	Method	Country of origin			Reference
		Estonia	Latvia	Lithuania	
2000−2002	Detection	44/279 (15.8)	NS	NS	Roasto et al., 2005
2002−2007	Detection	163/1,320 (12.3)	NS	NS	Meremäe et al., 2010
2012	Detection	22/149 (14.8)	8/31 (25.8)	19/180 (10.6)	Mäesaar et al., 2014
	Enumeration	2.8	3.4	3.2	
2018−2019	Detection	3/163 (1.8)	49/133 (36.8)	89/133 (66.9)	Present study
	Enumeration	2.3*	2.5	2.8	

Detection: No. of positive broiler chicken meat samples/No. of samples (positive %).
Enumeration: samples with both positive detection and positive enumeration result, Mean $\log_{10}$CFU/g.
*Only one sample contained 2.3 $\log_{10}$CFU/g. NS, no samples.

among Latvian and Lithuanian origin fresh poultry meat at Estonian retail compare to Estonian products. Differently to Lithuania and Latvia, the only broiler chicken slaughterhouse and all broiler chicken farms in Estonia are belonging to one international meat company which has integrated food safety management systems at all production stages from feed production to final meat products.

According to Commission Regulation (**EC**) No 2073/2005 on microbiological criteria for foodstuffs no food safety criteria has been established for *Campylobacter* spp. However, a very important change took place in 2017, when a process hygiene criterion was introduced for *Campylobacter* spp. on broiler carcasses. According to the regulation the limit of 1,000 CFU/g of *Campylobacter* spp. applies with the priority to the whole poultry carcases with the neck skin as sampling material. The distribution of *Campylobacter* counts of 141 broiler chicken meat samples is shown in Table 3. Among *Campylobacter*-positive samples, 62 (14.5%) contained *Campylobacter* spp. below 100 CFU/g. More than 1,000 CFU/g were determined from one (0.8%) Latvian and from 27 (20.3%) Lithuanian origin fresh broiler chicken meat samples. The highest count of *Campylobacter* (1,500 CFU/g) in Latvian origin samples was detected in February 2019. Among *Campylobacter*-positive samples of Lithuanian origin, the high counts of *Campylobacter*, ranging from 1,000 to 5,000 CFU/g, occurred throughout the year from October 2018 to August 2019. Several authors have reported the high prevalence of *Campylobacter* spp. at retail and the presence of heavily contaminated ($>10^4$ CFU/g) broiler meat and meat preparations (Humphrey et al., 2007; Suzuki and Yamamoto, 2009). *Campylobacter* infections are likely to occur when eating undercooked broiler meat or cross-contaminated food (de Boer and Hahné, 1990).

Table 3. *Campylobacter* enumeration data from fresh broiler chicken meat collected at Estonian retail level in 2018−2019.

Origin	*Campylobacter* counts (CFU/g)				
	0*	<100**	100−499	500−1,000	>1,000
Estonia	160 (98.2)	2 (1.2)	1 (0.6)	0 (0)	0 (0)
Latvia	84 (63.2)	39 (29.3)	6 (4.5)	3 (2.3)	1 (0.8)
Lithuania	44 (33.1)	21 (15.8)	27 (20.3)	14 (10.5)	27 (20.3)
Total	288 (67.1)	62 (14.5)	34 (25.6)	17 (4.0)	28 (6.5)

Number of samples (percentage).
*Negative detection and negative enumeration.
**Negative enumeration and positive detection, the threshold.

Present study showed that *Campylobacter* counts in Estonian origin fresh broiler chicken meat were significantly ($P < 0.00001$) lower than in fresh broiler chicken meat products from Latvian and Lithuanian origin. It indicates that imported broiler chicken meat products may pose higher risk for human *Campylobacter* infections than Estonian broiler chicken meat. Also our previous study in 2012 found that *Campylobacter* counts in fresh broiler chicken meat was significantly lower ($P < 0.001$) in samples of Estonian origin compared to these originating from Latvia and Lithuania, which could pose the higher human campylobacteriosis risk (Mäesaar et al., 2014). High prevalence together with high counts of *Campylobacter* spp. in fresh chicken meat sold at retail level carries the campylobacteriosis risk, because it gives higher probability for *Campylobacter* cross-contamination at home kitchen level (Meremäe et al., 2010; Roasto et al., 2010). According to Keener et al. (2004) as few as 500 *Campylobacter* cells can cause an infection in human.

Campylobacter studies performed in Estonia show the decrease of *Campylobacter* spp. counts in fresh broiler chicken meat samples of Estonian, Latvian, and Lithuanian origin. In present study only one Estonian origin fresh chicken meat sample contained *Campylobacter* spp. 2.3 $\log_{10}$CFU/g. Other 2 chicken meat samples of Estonian origin that were *Campylobacter*-positive had *Campylobacter* counts below the quantification limit (100 CFU/g), and were not included in the calculation of the concentration averages.

Many studies have reported a seasonal peak in the number of human campylobacteriosis cases and higher *Campylobacter* prevalence in poultry during the summer months (Bunevičienė et al., 2010; Meremäe et al., 2010; Kovalenko et al., 2013; Mäesaar et al., 2014; Jaakola et al., 2015; Nastasijevic et al., 2020). According to Djennad et al. (2019), it was found that 33.3% of expected human campylobacteriosis cases are temperature dependent in England and Wales. Kovanen et al. (2014) found that the seasonal peak for *Campylobacter* human infections in summer months is related to different summer activities such as barbecuing, drinking well water, swimming in natural waters, and being more contact with animals and soil. Also,

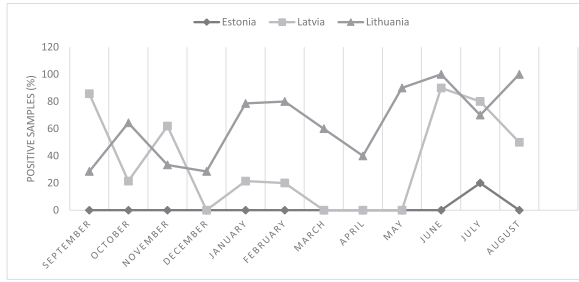

Figure 1. The proportion of *Campylobacter* spp. positive fresh broiler chicken meat samples of Estonian, Latvian, and Lithuanian origin in September 2018 to August 2019.

differences in seasonality of *Campylobacter* colonization in broilers can be affected by the farm management, the presence of different vectors and the survival mechanisms of *Campylobacter* spp. in the environment and in the host (Newell and Fearnley, 2003).

An earlier Estonian study by Mäesaar et al. (2014) found seasonal variation in the proportions of *Campylobacter*-positive fresh broiler chicken meat samples with a seasonal peak in the warm summer months. In present study the only positive samples (n = 3) among Estonian products were found in July. However, a distinct seasonal difference in *Campylobacter* contamination of broiler chicken meat samples at Estonian retail level was not found in present study (Figure 1). The proportion of *Campylobacter* spp. positive fresh broiler chicken meat samples of Lithuanian origin was consistently high during the whole study period. Similarly EFSA (2018) has reported a small but distinct winter peak for human campylobacteriosis in the past few years indicating the presence of *Campylobacter* contamination of food also in the cold months.

Sequencing Analyses

Altogether 55 *Campylobacter* isolates were sequenced and genotyped using for MLST to determine the genetic similarities of Estonian, Latvian, and Lithuanian broiler chicken meat origin *Campylobacter* spp. with isolates from Estonian human patients.

The STs distribution among *Campylobacter* spp. isolated from fresh broiler chicken meat and from humans is shown in Table 4. Among 55 *Campylobacter* spp. isolates, 22 sequence types were determined. Nine sequence types were only found in human, 4 ST-s were found both for human and broiler chicken meat isolates, and the remaining ten ST-s were related only with broiler chicken meat.

The most commonly isolated sequence types for *C. jejuni* (n = 45) were ST2229 (n = 7; 16%), ST19 (n = 4; 9%), ST122 (n = 4; 9%), ST464 (n = 4; 9%), ST9882 (n = 4; 9%), ST354 (n = 3; 7%), ST572 (n = 3; 7%), and ST7355 (n = 3; 7%). Most prevalent genotypes for *C. coli* (n = 10) were ST832 (n = 4; 40%), ST872 (n = 4; 40%).

C. jejuni ST10997 was only present in one Estonian origin of sample. ST356 and ST2229 were present in Latvian origin of *C. jejuni* samples. ST19, ST354, ST614, ST6461, ST122, ST464, ST7355, and ST9882 were present in Lithuanian origin of *C. jejuni* and ST872 in *C. coli* samples. ST832 was detected in samples of both Latvian and Lithuanian origin *C. coli* isolates. In our study genotypes ST122, ST464, ST7355, and ST9882 were obtained from both fresh chicken meat and from human *Campylobacter* isolates (Figure 2). However, no common genotypes for isolates originating from all 3 countries were found in present study.

Mäesaar et al. (2018) observed ST353 as the most prevalent genotype from human clinical *C. jejuni* isolates and ST5, ST45, and ST50 as the most prevalent

Table 4. Distribution of sequence types among the *Campylobacter jejuni* and *Campylobacter coli* isolates from Baltic broiler chicken meat and Estonian human patients.

Source**	Sequence type, ST												Total No. of isolates
EST	10,997$^{(1)}$												1
LV	356$^{(1)}$	832*$^{(2)}$	2,229$^{(7)}$										10
LT	19$^{(4)}$	354$^{(3)}$	614$^{(2)}$	832*$^{(2)}$	872*$^{(4)}$	6,461$^{(1)}$	**122$^{(2)}$**	**464$^{(2)}$**	**7,355$^{(1)}$**	**9,882$^{(3)}$**			24
H	22$^{(2)}$	50$^{(2)}$	353$^{(1)}$	429$^{(1)}$	572$^{(3)}$	824$^{(1)}$	**122$^{(2)}$**	**464$^{(2)}$**	**7,355$^{(2)}$**	**9,882$^{(1)}$**	1,595*$^{(1)}$	1,624*$^{(1)}$	11,001$^{(1)}$	20

Bold is indicating *Campylobacter* spp. sequence types of broiler chicken meat origin also found from Estonian human patients. $^{(No)}$Number of *C. jejuni* or *C. coli** strains.
C. coli MLST genotypes.
**Source of isolates: EST; Estonia; LV, Latvia; LT, Lithuania; H, human.

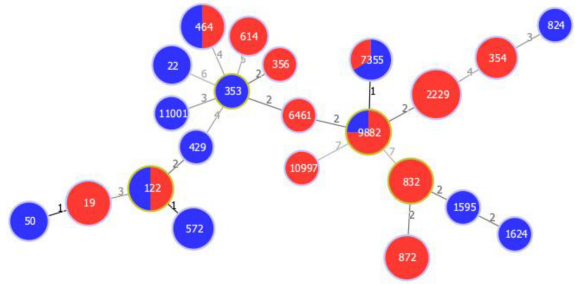

Figure 2. Full minimum spanning tree (MST) of 55 *Campylobacter jejuni* and *Campylobacter coli* MLST allelic profiles. Nodes are named after STs and color-coded according to isolate sources: chicken (red) and human (blue). Links are labelled with number of allelic differences.

STs from poultry chicken meat isolates. In current study *Campylobacter* spp. isolates from human patients (n = 20) were assigned to 2 species *C. jejuni* (n = 18) and *C. coli* (n = 2). In combined 13 different STs were detected, where the most prevalent STs were *C. jejuni* ST572 (n = 3; 15%), ST22 (n = 2; 10%), ST50 (n = 2; 10%), ST122 (n = 2; 10%), ST464 (n = 2; 10%), and ST7355 (n = 2; 10%). The isolates from broiler chicken meat (n = 35) were similarly assigned to 2 species 27 *C. jejuni* and 8 *C. coli*. Additionally, 13 different STs were assigned and the most prevalent STs for *C. jejuni* were ST2229 (n = 7; 26%), ST19 (n = 4; 15%) and for *C. coli* ST832 (n = 4; 50%) and ST872 (n = 4; 50%). Very few persistent *Campylobacter* genotypes were observed between mentioned studies performed in Estonia.

Meistere et al. (2019) found genotype ST464 to be present in one *C. jejuni* human isolate in their *Campylobacter* species prevalence study conducted in Latvia. According to Aksomaitiene et al. (2019) the majority of *C. jejuni* strains from broiler products in Lithuania were assigned to genotype ST464. Similarly in the Polish study the ST464 was one of the most common genotype detected both from chickens and humans (Wieczorek et al., 2020). In present study the genotype ST464 was also present both in human and chicken meat *Campylobacter* isolates, which indicates poultry as a potential reservoir and source of human *Campylobacter* infections.

Further research is needed to study other possible sources (pig, cattle, sheep, and pet animal) of *Campylobacter* human infections in Estonia. Limited sample size regarding genotyping analyses should be taken into account while interpreting the results.

CONCLUSIONS

Present study found that *Campylobacter* prevalence and counts in fresh broiler chicken meat was significantly lower in samples of Estonian origin compared to these originating from Latvia and Lithuania and sold at Estonian retail level. Imported fresh broiler chicken meat carries higher human campylobacteriosis risk in Estonia compare to fresh broiler chicken meat produced in Estonia. High genotype diversity among *Campylobacter* isolates from fresh retail chicken meat in Estonia was found. Only isolates originating from Lithuanian broiler chicken meat products were overlapping with isolates obtained from human patients of Estonia. Genotyping indicated associations between imported fresh broiler chicken products with campylobacteriosis cases in Estonia. *Campylobacter* counts in Estonian and imported products decreased compared with earlier study periods. Over time significant decrease in the prevalence and concentration of *Campylobacter* in Estonian broiler chicken meat indicates the possibility to reduce *Campylobacter* contamination by application of effective biosafety and other control measures within entire meat production chain.

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DISCLOSURES

The authors declare that there is no conflict of interest.

SUPPLEMENTARY MATERIALS

Supplementary material associated with this article can be found in the online version at doi:10.1016/j.psj.2022.101703.

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Article

Antibiotic Resistance in *Campylobacter* spp. Isolated from Broiler Chicken Meat and Human Patients in Estonia

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Abstract: Poultry meat is considered the most important source of *Campylobacter* spp. Because of rising antimicrobial resistance in *Campylobacter* spp., this study investigated the antimicrobial resistance of *Campylobacter* isolates from fresh broiler chicken meat originating from the Baltic countries sold in Estonian retail settings. Additionally, human clinical isolates obtained from patients with *Campylobacter* enteritis in Estonia were analysed. The aim of this study was to investigate the susceptibility of *Campylobacter* spp. to nalidixic acid, ciprofloxacin, tetracycline, streptomycin, erythromycin and gentamicin. The broth microdilution method with the EUCAMP2 panel was used for MIC determination and antimicrobial mechanisms were analysed using WGS data. A total of 46 *Campylobacter* strains were analysed, of which 26 (42.6%) originated from Lithuanian, 16 (26.2%) from Latvian, and 4 (6.6%) from Estonian fresh broiler chicken meat. In addition, 15 (24.6%) *Campylobacter* strains of patients with campylobacteriosis were tested. The antimicrobial resistance patterns of *Campylobacter* spp. isolated from fresh broiler chicken meat samples of Estonian, Latvian and Lithuanian origin collected in Estonian retail, and from patients with *Campylobacter* enteric infections, were determined. A total of 46 (75%) of the isolates tested were *C. jejuni* and 15 (25%) were *C. coli*. *Campylobacter* resistance was highest to nalidixic acid (90.2% of strains) and ciprofloxacin (90.2%), followed by tetracycline (57.4%), streptomycin (42.6%) and erythromycin (6.6%). All strains were sensitive to gentamicin. Additionally, antimicrobial resistance genes and point mutations were detected in 27 *C. jejuni* and 8 *C. coli* isolates previously assigned as resistant with the phenotypic method. A high antibiotic resistance of *Campylobacter* spp. in Lithuanian- and Latvian-origin broiler chicken meat and Estonian clinical isolates was found. Similar antibiotic resistance patterns were found for broiler chicken meat and human *Campylobacter* isolates.

Keywords: *Campylobacter* spp.; antibiotic resistance; multidrug resistance; fresh broiler chicken meat; resistance genes

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1. Introduction

Campylobacter is small spiral Gram-negative bacterial pathogen and the main agent of campylobacteriosis, which is the most common zoonotic disease in the EU transmitted to humans directly from animals, or through the foodborne route [1,2]. Recent source attribution analysis has revealed the prevalent role of broiler chickens as the cause of human *Campylobacter* infections in the Baltic countries [3]. Broilers and broiler chicken meat are the most important sources of *Campylobacter* infections in humans [4–6]. Disease caused by *Campylobacter* spp. is generally mild and self-limited. Nevertheless, it can cause severe systematic infection in children/elderly people and humans with immunosuppression, and also—in very rare cases—Guillan-Barré syndrome [7,8]. Such occasions often

require therapy with first-line antibiotics such as fluoroquinolones, e.g., ciprofloxacin, and macrolides, e.g., erythromycin [9–14]. Over time, *Campylobacter* has acquired resistance to these antibiotics which are considered critically important for the treatment of *Campylobacter* infections [11,15,16]. The increasing resistance of thermophilic *Campylobacter* spp. to antibiotics could lead to detrimental effects on public health [2,17–19]; therefore, the World Health Organization has classified many of these antimicrobials as critically important for human medicine [20].

Previous studies [21,22] have identified different levels of *Campylobacter*-contaminated poultry meat in the Baltic states, and the proportion of antimicrobial-resistant *Campylobacter* spp. strains in poultry meat has been found to be very high in both Lithuania and Latvia [23,24]. This is affecting public health and needs to be addressed.

This study aimed to determine the proportions of antimicrobial-resistant *Campylobacter* strains from fresh broiler chicken meat of Estonian, Latvian and Lithuanian origin at the Estonian retail level, and among strains of human clinical infections. Additionally, resistance pheno- and genotypes were determined and compared. The results of this work will make it possible to assess the trends in antimicrobial resistance over a long period and determine related public health risks.

2. Materials and Methods

2.1. Campylobacter Isolates

In total, 61 *Campylobacter* isolates were studied, of which 46 (75%) were *C. jejuni* and 15 (25%) *C. coli*. From the broiler chicken meat isolates, 34 (73.9%) were *C. jejuni* and 12 were (26.1%) *C. coli*. Among the clinical isolates, 12 (80%) and 3 (20%) were *C. jejuni* and *C. coli*, respectively. All isolates were obtained from a previous study on *Campylobacter* spp. in Estonia [25]. The samples consisted of all *Campylobacter* strains isolated from Estonian (n = 4), Latvian (n = 16) and Lithuanian (n = 26) fresh broiler chicken meat products in Estonian retail from our previous study [25]. Additionally, isolates from human patients (n = 15) originating from ambulatory and hospitalized patients from north and north-eastern Estonia were obtained from East-Tallinn Central Hospital and Rakvere Hospital were included. *Campylobacter* detection from broiler chicken meat samples was performed according to ISO 10272-1:2017 [26], as described by Tedersoo et al. [25]. In brief, for *Campylobacter* spp. detection, 10 g of broiler chicken meat sample was transferred into 90 mL Preston enrichment broth and incubated in microaerobic conditions at 41.5 ± 0.5 °C for 24 h. Then, a 10 µ loopful of Preston enrichment broth was inoculated onto mCCD agar medium (Oxoid Ltd.; Basingstoke, Hampshire, UK). All plates were incubated in microaerobic conditions at 41.5 °C ± 0. 5 °C for 48 h. Typical *Campylobacter* colonies on mCCDA plates were streaked on Columbia blood agar (Oxoid Ltd.; Basingstoke, Hampshire, UK), which were incubated for 24 h at 41.5 °C ± 0.5 °C. Additional confirmation tests included Gram staining, motility analysis, and oxidase and catalase tests were performed.

2.2. Antimicrobial Susceptibility Testing

Minimal inhibitory concentration (MIC) values for nalidixic acid, ciprofloxacin, tetracycline, streptomycin, erythromycin and gentamicin were determined by using the broth microdilution method with the EUCAMP2 panel (TREK diagnostic Systems Ltd., East Grinstead, UK) in accordance with the manufacturer's protocols.

The cut-off values recommended by the European Committee on Antimicrobial Susceptibility Testing were used for *C. jejuni* and *C. coli* in accordance with the European Commission implementing decision 2013/652/EU on the monitoring and the reporting of antimicrobial resistance in zoonotic and commensal bacteria. *C. jejuni* was assigned resistant when MIC values equated to: erythromycin > 4 µg/mL, ciprofloxacin > 0.5 µg/mL, tetracycline > 1 µg/mL, streptomycin > 4 µg/mL, nalidixic acid > 16 µg/mL or gentamicin > 2 µg/mL. *C. coli* was assigned resistant when MIC values equated to: erythromycin > 8 µg/mL, ciprofloxacin > 0.5 µg/mL, tetracycline > 2 µg/mL, streptomycin > 4 µg/mL, nalidixic acid > 16 µg/mL or gentamicin > 2 µg/mL.

Analyses were performed in the Veterinary and Food Laboratory of Estonia, which is also the national reference laboratory.

2.3. Whole-Genome Sequencing and Analysis of Resistant Genes

Molecular analysis, whole-genome sequencing and bioinformatics were performed as described by Tedersoo et al. [25]. In brief, the sequencing was carried out on an Illumina NextSeq500 System (Illumina, Inc.; San Diego, CA, USA) using the NextSeq 500/550 High Output Kit v2.5 (300 Cycles) in paired-end 2 × 151 bp mode. All genome sequences were submitted to the *C. jejuni*/*C. coli* multilocus sequence typing (MLST) database [27].

Antimicrobial resistance genes and point mutations of *C. jejuni* (n = 27) and *C. coli* (n = 8) isolates previously assigned as resistant with the MIC test were detected in the subset of isolates. MIC-sensitive campylobacters were not included in the analysis and only genotypic resistance mechanisms corresponding to phenotypic AMR were identified and reported in present study. AMRFinderPlus v3.10.23 with database v2021-12-21.1 (downloaded 3 March 2022) was used according to the default settings, except for the organism "*Campylobacter*" and the "plus" options [28,29]. Genes with coverage of less than 80% were not included in the analysis.

2.4. Statistical Analysis

MS Excel 2010 software (Microsoft Corporation; Redmond, WA, USA) was used to record the results. The Chi-squared test was used to test for statistically significant associations between the antimicrobial resistance of *Campylobacter* spp. in fresh broiler chicken meat from different sources. The results were considered statistically significant for p values of ≤ 0.05.

3. Results

The results showed that a total of six (9.8%) isolates were sensitive to all the tested antibiotics: four isolates from Estonian-origin chicken meat and two human *Campylobacter* strains. *Campylobacter* isolates of broiler chicken meat origin showed the highest resistance to quinolones, tetracycline and streptomycin. In addition, clinical *Campylobacter* isolates were found to be most resistant against the same antibiotics. All *Campylobacter* strains were sensitive to gentamicin (Table 1). Significant differences ($p < 0.001$) were found in nalidixic acid, ciprofloxacin and tetracycline resistance among Estonian versus Latvian and Lithuanian *Campylobacter* isolates from fresh broiler chicken meat. The Estonian broiler-chicken-meat-origin *Campylobacter* isolates were significantly ($p < 0.001$) less resistant to fluoroquinolones than those strains which originated from Latvian and Lithuanian broiler chicken meat and Estonian human patients. There were no differences detected in streptomycin, erythromycin or gentamicin resistance between *Campylobacter* broiler chicken meat isolates of Estonian versus Latvian and Lithuanian origin. Resistance in the human isolates and broiler chicken meat isolates of Latvian ($p = 0.13$) and Lithuanian ($p = 0.06$) origin did not differ significantly. A total of 55 (90.2%) isolates were resistant to one or more antibiotics: 10 (16.4%) were resistant to one antibiotic, 28 (45.9%) were resistant to at least two antibiotics not belonging to the same group of antimicrobials (fluoroqinolones and quinolone (ciprofloxacin, nalidixic acid), macrolides (erythromycin), tetracyclines (tetracycline) and aminoglycosides (streptomycin, gentamicin)), and 17 (27.9%) isolates were resistant to three or more antibiotics not belonging to the same group. The proportion of isolates resistant to *C. jejuni* and *C. coli* was 87% and 100%, respectively. Antimicrobial resistance to one or more antimicrobial was significantly higher ($p < 0.001$) in the *Campylobacter* isolates from the broiler chicken meat of Latvian and Lithuanian origin compared to that of Estonian origin. It was found that 27.9% of isolates were multidrug-resistant, of which 11 isolates (18.0%) were of Lithuanian and 2 (3.3%) of Latvian broiler chicken meat origin, and 4 (6.6%) were from Estonian human patients. Multidrug resistance was defined as resistance to three or more antibiotics not belonging to the same group.

All Latvian and Lithuanian isolates originating from broiler chicken meat were resistant to fluoroquinolones.

Table 1. Resistance of *C. jejuni* and *C. coli* isolates of different origins to antibiotics.

Antibiotic	Resistant *Campylobacter* spp. Number of Isolates Depending on Origin/Total Isolates Tested (%)			
	Estonia	Latvia	Lithuania	Human
Nalidixic acid	0/4 (0)	16/16 (100)	26/26 (100)	13/15 (86.7)
Ciprofloxacin	0/4 (0)	16/16 (100)	26/26 (100)	13/15 (86.7)
Tetracycline	0/4 (0)	3/16 (18.8)	20/26 (76.9)	12/15 (80.0)
Streptomycin	0/4 (0)	11/16 (68.8)	11/26 (42.3)	4/15 (26.7)
Erythromycin	0/4 (0)	1/16 (6.3)	3/26 (11.5)	0/15 (0)
Gentamicin	0/4 (0)	0/16 (0)	0/26 (0)	0/15 (0)

The resistance phenotypes of *Campylobacter* isolates are presented in Table 2. The most prevalent antimicrobial resistance pattern was Cip/Nal/Tet, with 55.3% and 21.7% in human and chicken meat isolates, respectively. Other common resistance phenotypes were Cip/Nal/Tet/Str and Cip/Nal/Str.

Table 2. *Campylobacter*-resistant phenotypes.

Antibiotic Resistance Phenotype [a,b]	*Campylobacter* spp. Number of Strains (%)			
	Estonia (n = 4)	Latvia (n = 16)	Lithuania (n = 26)	Human (n = 15)
Cip/Nal/Tet/Str/Ery	-	-	3 (11.5)	-
Cip/Nal/Tet/Str	-	2 (12.5)	8 (30.8)	4 (26.7)
Cip/Nal/Tet	-	1 (6.2)	9 (34.6)	8 (53.3)
Cip/Nal/Str	-	9 (56.3)	-	-
Cip/Nal/Ery	-	1 (6.2)	-	-
Cip/Nal	-	3 (18.8)	6 (23.1)	1 (6.7)
Resistant to one or more antibiotics	0 (0)	16 (100)	26 (100)	13 (86.7)
Susceptible to all antibiotics	4 (100)	-	-	2 (13.3)
Multidrug resistant [c]	0 (0)	2 (12.5)	10 (38.5)	4 (26.7)
Total number of tested isolates	4 (100)	16 (100)	26 (100)	15 (100)

[a] Tested antibiotics: NAL—nalidixic acid; Cip—ciprofloxacin; TET—tetracycline; STR—streptomycin; ERY—erythromycin; GEN—gentamicin. [b] The number of resistant isolates was 55. The phenotypes of the antibiotic-resistant isolates were calculated based on 55 isolates. [c] Multidrug resistant is defined as strain resistant to three or more unrelated (not belonging to the same class of antibiotics) antimicrobials.

MIC values of *Campylobacter* are shown in Table 3. Very high minimum inhibitory concentrations were found for four erythromycin-resistant, 26 ciprofloxacin-resistant, 31 tetracycline-resistant, 51 nalidixic acid-resistant and 26 streptomycin-resistant *Campylobacter* isolates with MIC values $\geq$ 128 µg/mL, $\geq$16 g/mL, $\geq$64 µg/mL, $\geq$64 µg/mL and $\geq$16 µg/mL, respectively.

Altogether, 28 *C. jejuni* and 7 *C. coli* isolates, of which 29 were of broiler chicken meat origin and 6 were of human origin, previously assigned as resistant with the MIC test, were sequenced and all their antimicrobial resistance genes and point mutations are presented in Table 4. In total, 29 *Campylobacter* isolates from broiler chicken meat and 6 *Campylobacter* isolates of human origin showed resistance to quinolones, and all contained a point mutation T86I in the *gyrA* gene. The genotypic antibiotic resistance against tetracyclines (*tetO*) was 62% and 83% in broiler chicken meat (n = 18) and human isolates (n = 5), respectively. A total of 52% of broiler chicken meat isolates (n = 15) showed resistance against aminoglycosides and macrolides. The resistance against macrolides in human isolates was 50% (n = 3).

Table 3. The minimum inhibitory concentrations of *C. jejuni* and *C. coli* isolates (n = 61).

No. of Isolates	AA [d]	No. of Isolates with MIC Value (μg/mL) of [a]										
		0.12	**0.25**	**0.5**	**1**	**2**	**4**	**8**	**16**	**32**	**64**	**128**
46 [b]	ERY	-	-	-	40	2	-	-	-	-	-	4 [(4)]
	CIP	4	-	-	-	1	3	17	21 [(8)]	-	-	-
	TET	-	-	23	-	-	-	-	1	2	20 [(16)]	-
	GEN	7	13	20	6	-	-	-	-	-	-	-
	NAL	-	-	-	-	1	-	-	-	3	39 [(23)]	-
	STR	-	-	6	4	9	3	-	22 [(20)]	-	-	-
15 [c]	ERY	-	-	-	15	-	-	-	-	-	-	-
	CIP	2	-	-	-	-	1	7	5 [(2)]	-	-	-
	TET	-	-	3	-	-	-	-	-	1	11 [(6)]	-
	GEN	2	2	7	3	1	-	-	-	-	-	-
	NAL	-	-	-	-	-	1	1	-	1	12 [(6)]	-
	STR	-	-	-	2	5	4	-	4 [(3)]	-	-	-

[(no)] Number of *C. jejuni* strains with MIC values exceeding the EUCAMP2 maximum concentration range. [a] MIC values for isolates were evaluated according to manufacturer's instructions (National Veterinary Institute, Uppsala, Sweden). Solid–vertical lines indicate break points between sensitive and resistant isolates for *C. jejuni*, and dashed–vertical lines for *C. coli*, if different from *C. jejuni*. [b] Estonian-, Lithuanian- and Latvian-origin broiler chicken meat sampled from Estonian retail in 2018–2019. [c] *C. jejuni* and *C. coli* strains of human origin isolated in 2018–2019 in Estonia. [d] Antimicrobial agents: NAL—nalidixic acid; Cip—ciprofloxacin; TET—tetracycline; STR—streptomycin; ERY—erythromycin; GEN—gentamicin.

Table 4. Comparison of *C. jejuni* and *C. coli* phenotypic and genotypic antibiotic resistance, including mechanisms, patterns, sources and origin.

Antibiotic (Class)	Phenotype/Genotype (n/n)	Mechanism (n)	Pattern (n) [a]	Source (n)	Country (n: j/c) [b]
Streptomycin (Aminoglycosides)	16/16	*aadE* (5) [c]	CIP/NAL/TET/**STR** (5)	Chicken (5)	Lithuania (5: 4j/1c)
		aadE-Cc (3)	CIP/NAL/TET/**STR**/ERY (3)	Chicken (3)	Lithuania (3: 3c)
			CIP/NAL/**STR** (5)	Chicken (5)	Latvia (5: 5j)
		aph(3')-IIIa (8)	CIP/NAL/TET/**STR** (3)	Chicken (2)	Latvia (1: 1j) Lithuania (1: 1j)
				Human (1)	Estonia (1: 1j)
Erythromycin (Macrolides) [d]	4/4	*23S* A2075G (4)	CIP/NAL/TET/STR/**ERY** (3)	Chicken (3)	Lithuania (3: 3c)
			CIP/NAL/**ERY** (1)	Chicken (1)	Latvia (1: 1c)
Ciprofloxacin/ Nalidixic acid (Quinolones)	35/35	*gyrA* T86I (35)	**CIP**/**NAL**/TET (12)	Chicken (8)	Lithuania (7: 6j/1c) Latvia (1: 1j)
				Human (4)	Estonia (4: 3j/1c)
			CIP/**NAL**/TET/STR (8)	Chicken (7)	Lithuania (6: 5j/1c) Latvia (1: 1j)
				Human (1)	Estonia (1: 1j)
			CIP/**NAL** (6)	Chicken (5)	Lithuania (4: 4j) Latvia (1: 1c)
				Human (1)	Estonia (1: 1j)
			CIP/**NAL**/STR (5)	Chicken (5)	Latvia (5: 5j)
			CIP/**NAL**/TET/STR/ERY (3)	Chicken (3)	Lithuania (3: 3c)
			CIP/**NAL**/ERY (1)	Chicken (1)	Latvia (1: 1c)
Tetracycline (Tetracyclines)	23/23	*tetO* (23)	CIP/NAL/**TET** (12)	Chicken (8)	Lithuania (7: 6j/1c) Latvia (1: 1j)
				Human (4)	Estonian (4: 3j/1c)
			CIP/NAL/**TET**/STR (8)	Chicken (7)	Lithuania (6: 5j/1c) Latvia (1: 1j)
				Human (1)	Estonia (1: 1j)
			CIP/NAL/**TET**/STR/ERY (3)	Chicken (3)	Lithuania (3: 3j)

[a] Tested antibiotics: NAL—nalidixic acid; CIP—ciprofloxacin; TET—tetracycline; STR—streptomycin; ERY—erythromycin. Bold indicates concurrence between genotypic and phenotypic resistance. [b] j—*C. jejuni*; c—*C. coli*. [c] One isolate also had the aad9 gene. [d] *50S* L22 (A103V) modification was detected in 14 erythromycin MIC-sensitive isolates.

4. Discussion

As stated in the European Union Regulation No. 1831/2003, antimicrobials as growth promoters in food animal production have been banned since 2006 [30]. Antimicrobials are still used intensively in poultry for therapy and infection prophylaxis, which has caused the spread of resistant strains to humans [31,32]. However, some countries are showing positive trends, for example, antimicrobial use in poultry in Scandinavian countries is generally low. Denmark has declared carbapenems, third- and fourth-generation cephalosporins, fluoroquinolones and colistin as 'critically important', and the use of these antimicrobials is restricted [33]. Based on the report of DANMAP [33], cephalosporins and colistin are not used in Danish poultry production and the use of fluoroquinolones is close to zero.

The situation in Swedish broiler production is very good since the use of antibiotics is infrequent [34]. Consequently, the prevalence of resistant bacteria isolated from animals in Sweden is low [35,36].

FINRES-Vet [37] reports that the occurrence of antibiotic-resistant *Campylobacter* spp. from broilers has been at a low level. Compared to previous years, in 2020, the proportion of quinolone-resistant isolates dropped and the resistance to tetracycline, erythromycin, gentamicin or streptomycin remained low.

The annual NORM-VET 2020 report showed improvements of antimicrobial resistance in Norway [38]. As stated in this report, the prevalence of antimicrobial resistance among *C. jejuni* isolates from broilers is low; 90.8% of isolates were susceptible to all tested antimicrobials. Although the isolates were commonly resistant to quinolones and streptomycins, there were no multidrug-resistant isolates detected [38].

The rapid spread of antimicrobial resistance has been identified across the world and it is associated with the use of antimicrobials [39]. According to the European Food Safety Authority and the European Centre for Disease Prevention and Control (EFSA and ECDC) [40], in 2019, ciprofloxacin resistance in human *Campylobacter* isolates was high to extremely high (at the EU level it was 61.5% and 61.2% for *C. jejuni* and *C. coli*, respectively). The resistance to erythromycin was low (1.5% and 12.9% for *C. jejuni* and *C. coli*, respectively). *C. coli* erythromycin resistance was extremely high in Portugal (73.1%). The tetracycline resistance proportions were 47.2% and 66.9% for *C. jejuni* and *C. coli*, respectively [40]. According to EFSA and ECDC [40], the resistance to gentamycin in 2019 was low. In China, the prevalence of resistance in *Campylobacter* from human patients to ciprofloxacin, tetracycline and nalidixic acid is very high (89.7%, 74.6% and 69.0%, respectively), due to the extensive use of these antimicrobials without prescription [41]. In Ireland, compared to the early 2000s, tetracycline resistance among *Campylobacter* spp. in broilers has risen by approximately 10% [42]. In Portugal and Spain, *Campylobacter* spp. resistance to tetracycline in broilers is high: between 90 and 100% [43,44]. In a study conducted in Lithuania, Aksomaitiene et al. [23] found that *C. jejuni* isolates from human clinical cases were most frequently resistant to ciprofloxacin (88.1%), but all human isolates were sensitive to gentamicin and erythromycin. In our study, all the Estonian-origin broiler chicken meat *Campylobacter* isolates (n = 4) were sensitive to all of the studied antimicrobials. The small number of isolates was related to the very low *Campylobacter* prevalence (1.8% from 163 samples) in Estonian-origin broiler chicken meat [25]. The most frequently observed resistance (86.7%) of human strains was against ciprofloxacin and nalidixic acid. This high antimicrobial resistance among human strains probably indicates that ciprofloxacin and nalidixic acid would not be suitable for human *Campylobacter* infection treatment. In Estonia, the first choice of antibiotic treatment for human patients with severe *Campylobacter* infection is azithromycin, followed by ciprofloxacin as the alternative choice. In the present study, the proportions of *Campylobacter* isolates from fresh broiler chicken meat that were resistant to ciprofloxacin and erythromycin, all of Latvian and Lithuanian origin, were 91.3% and 8.7%, respectively. According to the EFSA and ECDC [40], in 2019, the highest levels of resistance in broiler meat were for nalidixic acid and ciprofloxacin (64–90%), and also for tetracycline (43–53%). A previous Estonian study by Mäesaar et al. [45] found similarly high proportions of fluoroquinolone resistance among Latvian (87.5%) and

Lithuanian (84.8%) *Campylobacter* isolates originating from broiler chicken meat. In the present study, fluoroquinolone resistance of Latvian and Lithuanian isolates originating from broiler chicken meat was 100%, which probably reflects the wide use of these antibiotics in poultry production in these countries. The use of synthetic fluoroquinolone (enrofloxacin) for the treatment of respiratory and gastrointestinal infections in poultry has been shown to induce fluoroquinolone resistance in *Campylobacter* spp. [46]. Similarly to the results of the present study, Aksomaitiene et al. [23] found that all *C. jejuni* isolates from broiler products from Lithuanian retail settings were resistant to ciprofloxacin. Meistere et al. [24] reported that Latvia has one of the highest proportions of fluoroquinolone resistance among *C. jejuni* (97.5%) in broilers. Furthermore, Kovalenko et al. [47] found a high proportion of *Campylobacter* isolates from Latvian broilers resistant to fluoroquinolones (100%), ciprofloxacin (100%), nalidixic acid (87.9%) and streptomycin (39.6%). In the present study, fluoroquinolone resistance among human isolates was 86.7%, and 91.3% in broiler meat. Mäesaar et al. [45] found resistance to fluoroquinolones to be higher for humans (67.9%) than for broilers (60.2%). Multidrug-resistant strains were co-resistant to nalidixic acid and ciprofloxacin. Several studies in Canada and the USA have reported *Campylobacter* spp. ciprofloxacin resistance in up to 47% of *Campylobacter* strains [18,48,49]. In addition to high fluoroquinolone resistance among broiler chicken meat isolates, the present study observed high tetracycline resistance among Lithuanian broiler chicken meat isolates (76.9%) and high streptomycin resistance among Latvian and Lithuanian broiler chicken meat isolates: 68.8% and 42.3%, respectively. Tetracycline resistance among human isolates was 80.0%, which matched with the tetracycline resistance found among Lithuanian broiler chicken meat isolates (76.9%). In Table 4, the phenotypic resistance pattern and related genotypic mechanisms (gene, point mutation) are shown. All phenotypic resistance found for aminoglycosides, macrolides, quinolones and tetracyclines determined with the MIC test had corresponding genotypic antibiotic resistance mechanisms. In previous studies, *aad9*, *aadE*, *aadE-Cc* and *aph(3′)-IIIa* resistance genes [50–53] associated with aminoglycoside resistance were detected from all isolates with corresponding phenotypic resistance. For tetracycline resistance only, the *tetO* [54] gene was detected from isolates showing phenotypic resistance to tetracycline in the MIC test. The latter has also been found in several other studies [54]. Two point mutations in *23S* (A2075G) and *gyrA* (T86I) genes associated with erythromycin and quinolone resistance [52,55] in campylobacters were also found in our study. In addition, a previous study conducted in Estonia found *gyrA* (T86I) mutation in quinolone-resistant *C. jejuni* ST5 isolates [56]. Additionally, 50*S* ribosomal protein L22 modification (A103V) [31] was detected in 14 isolates with no corresponding phenotypic erythromycin (macrolide) resistance found. The majority of isolates with matching geno- and phenotypic resistance had high MIC, and often exceeded the maximum concentration ranges.

High *Campylobacter* resistance in chicken meat can be a key risk factor for the treatment of severe human campylobacteriosis cases in Estonia. The high proportions of resistance and similar antimicrobial pheno- and genotypes found from imported broiler chicken meat products and for Estonian human clinical isolates indicate that the consumption of imported broiler chicken meat might pose the risk of *Campylobacter* to the Estonian population.

The application of a vertically integrated management system and strict biosecurity and biosafety measures at all levels of broiler chicken production may be the reason for the very low *Campylobacter* prevalence and counts as well as low antimicrobial resistance among *Campylobacter* strains isolated from the Estonian-origin fresh broiler chicken meat.

5. Conclusions

Among the *Campylobacter* strains isolated from broiler meat in 2018–2019, a total of 90.2% were resistant to one or more kind of antibiotics. Multidrug resistance was found in 27.9% of isolates. *Campylobacter* isolates from Estonian fresh chicken meat were sensitive to all of the tested antibiotics. Isolates of Latvian and Lithuanian origin were 100% resistant to one or more of the antibiotics, and 86.7% of the Estonian human strains were resistant to

one or more of the antibiotics. There was high antibiotic resistance in *Campylobacter* spp. in Lithuanian and Latvian isolates from fresh broiler chicken meat in the Estonian retail market. There was also a high antibiotic resistance in *Campylobacter* spp. of human origin. This suggests that broiler chicken meat poses a potential risk to humans as it is well known that broiler chicken meat is a main source of human campylobacteriosis. To minimize the emergence of *Campylobacter* resistance, it is crucially important to follow common policies and implement good practices on antimicrobial usage at the farm level. Resistant bacteria in the food production chain can easily reach the consumer and pose a serious risk to public health.

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Antimicrobial Resistance of *Campylobacter coli* Isolated from Caecal Samples of Fattening Pigs at Slaughter

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Abstract: Pigs are known as the main *Campylobacter coli* reservoirs. Campylobacteriosis, the most commonly reported gastrointestinal disease in humans, is mainly caused by the consumption of poultry meat, and little is known about the role of pork. Pigs are often associated with *C. coli*, including antimicrobial-resistant isolates. Therefore, the entire pork production chain must be considered as an important source of antimicrobial-resistant *C. coli*. This study aimed to determine the antimicrobial resistance of *Campylobacter* spp. isolated from caecal samples of fattening pigs at the Estonian slaughterhouse level over a five-year period. The proportion of *Campylobacter*-positive caecal samples was 52%. All *Campylobacter* isolates were identified as *C. coli*. A high proportion of the isolates were resistant to most of the studied antimicrobials. The resistance to streptomycin, tetracycline, ciprofloxacin and nalidixic acid was 74.8%, 54.4%, 34.4% and 31.9%, respectively. In addition, a high proportion (15.1%) of the isolates were multidrug-resistant and, in total, 93.3% were resistant to at least one antimicrobial.

Keywords: *Campylobacter coli*; antimicrobial resistance; pigs; caecal samples; slaughterhouse

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1. Introduction

In the European Union (EU), there are approximately 246,000 cases of *Campylobacter* infections per year, mainly due to contaminated food [1]. In general, *Campylobacter* infection is self-limiting, but in the case of immunodeficiency, it can cause very serious health complications, even death [2]. Poultry meat is the main source of human campylobacteriosis, but pork is also considered an important source of *Campylobacter coli*-related human cases [3,4]. The transmission of *Campylobacter* primarily occurs through farm animals and may further contaminate the entire food production chain [5,6]. Pigs are the main reservoir of *C. coli* [7–10] and contaminated pork can pose a health risk for humans. Different studies have found a possible link between human and pig strains [9]. There is also a risk of multidrug-resistant (MDR) *Campylobacter* spreading from animals to humans [11]. MDR is defined as the antimicrobial resistance of microorganisms to three or more unrelated antimicrobials [12]. As pigs are usually subclinically infected with *Campylobacter* spp., there is a high contamination possibility of carcasses during the slaughter process [13]. Intensive farming is associated with the use of antimicrobials, and food-producing animals are often carriers of antimicrobial-resistant (AMR) microorganisms, which can be transmitted to humans via contaminated food [14]. Several studies have raised the importance of AMR *C. coli* as an emergent problem in humans [10,15]. Macrolides and fluoroquinolones are the first-choice antimicrobials in campylobacteriosis treatment [16]. According to EFSA and ECDC [17], AMR *Campylobacter* is very common, especially to fluoroquinolones, which are

defined as critically important antimicrobials for humans; therefore, their veterinary use should be avoided.

The aim of the study was to determine the occurrence of resistance of *Campylobacter* spp. isolated from caecal samples of fattening pigs at slaughter.

2. Materials and Methods

2.1. Campylobacter Isolates and Antimicrobial Susceptibility Testing

The sampling was carried out by the veterinary officials of the Agriculture and Food Board (AFB) within the Estonian National Monitoring Programme, according to European Union (EU) Directive 2003/99/EC [18] and the Commission's implementing decision of 12 November 2013 [19], on the monitoring and reporting of antimicrobial resistance in zoonotic and commensal bacteria. At slaughterhouses, a total of 229 caecal samples representing all Estonian fattening pig farms (one per year) were analysed. Within a five-year period, 87, 68 and 74 samples were collected in 2015, 2017 and 2019, respectively. Direct and enrichment culture methods were used in accordance with instructions laid out in the ISO 10272-1:2018 standard [20]. *Campylobacter* spp. isolation and antimicrobial susceptibility testing were performed at the National Centre for Laboratory Research and Risk Assessment (LABRIS). According to the method, 10 g of pig intestinal contents from the caecum was aseptically taken and placed into a sterile plastic bag, where 90 mL of Bolton broth (Oxoid Ltd.; Hampshire, United Kingdom) was added, and homogenized for one minute in a Stomacher 400 Circulator (Heidolph Instruments GmbH & Co. KG; Schwabach, Germany). The samples were then incubated under microaerobic conditions at 37 °C for 4–6 h, followed by 41.5 ± 0.5 °C for 44 ± 4 h. Further on, 10 µL of the enrichment broth was plated on mCCDA agar (Oxoid Ltd.) and incubated under microaerobic conditions at 41.5 ± 0.5 °C for 44 ± 4 h. Typical *Campylobacter* colonies on mCCDA were streaked on Columbia blood agar (Oxoid Ltd.) plates and then incubated under microaerobic conditions in anaerobic jars with CampGen^{TM} reagents (Oxoid Ltd.) at 41.5 ± 0.5 °C for 24 h. *Campylobacter* was confirmed when colonies were Gram-negative, motile, oxidase-positive and did not grow in aerobic conditions at 41.5 ± 0.5 °C and at 25 °C. *Campylobacter* isolates were stored at −82 °C in cryotubes (Technical Service Consultants Ltd.; Lancashire, UK).

The minimal inhibitory concentration (MIC) values of the isolates were determined against erythromycin, ciprofloxacin, tetracycline, streptomycin, nalidixic acid and gentamicin according to the European Committee on Antimicrobial Susceptibility Testing (EUCAST) recommendations for a EUCAMP2 plate (TREK Diagnostic Systems Ltd.; East Grinstead, UK). Briefly, the *Campylobacter* isolates were first cultured on Columbia blood agar (Oxoid Ltd.) and incubated at 41.5 ± 0.5 °C for 44 ± 4 h at microaerobic conditions. A loopful (1 µL) of bacterial growth was transferred to the test tube containing 2 mL of physiological solution, achieving an estimated inoculum density of 10^8 CFU/mL. After that, 50 µL of the inoculum was pipetted into the test tube, which contained 10 mL of cation-adjusted Mueller–Hinton broth with 5% of laked horse blood (CAMHB+LHB) (Oxoid Ltd.). Finally, 100 µL of bacterial suspension was transferred into microtiter plates, which were incubated at 35 ± 2 °C under microaerobic conditions in anaerobic jars with CampGen^{TM} reagents (Oxoid Ltd.) for 44 ± 4 h. The MIC was determined as the lowest concentration that completely inhibited campylobacter growth. The purity control of the bacterial suspension was also performed by plating 10 µL of suspension on Columbia agar (Oxoid Ltd.) and incubating it at 37 °C under microaerobic conditions for 40–48 h. Colony counts from 50 to 250 per plate were accepted. The cut-off values recommended by the EUCAST were used for *Campylobacter coli* in accordance with the European Commission implementing decision 2013/652/EU [19] on the monitoring and reporting of antimicrobial resistance in zoonotic and commensal bacteria. The cut-off values for *C. coli* were erythromycin >8 µg/mL, ciprofloxacin >0.5 µg/mL, tetracycline >2 µg/mL, streptomycin >4 µg/mL, nalidixic acid >16 µg/mL and gentamicin >2 µg/mL. The quality control was performed according to the EU Reference Laboratory for Antimicrobial Resistance (EURL-AR) instructions and

protocols. The strains used for the quality control of *Campylobacter* MIC analyses were *C. coli* EURL-AR strain 2012-70-443-2 and *C. jejuni* ATCC 33560.

2.2. Species Identification

A multiplex PCR assay was used for the identification and differentiation of Campylobacter species according to the standard operating procedure of the LABRIS. Genomic DNA was extracted according to the manufacturer's instructions from a loopful of bacterial growth from the blood agar using the RTP® Bacterial DNA Mini Kit (STRATEC Molecular GmbH, Berlin, Germany). DNA amplification was conducted with an Eppendorf Mastercycler® ep gradient (Eppendorf AG, Hamburg, Germany), with the following thermal cycling conditions: 95 °C for 6 min, followed by 30 cycles of 95 °C for 30 s, 59 °C for 30 s and 72 °C for 30 s, and a final extension at 72 °C for 7 min. The amplified PCR products were separated in 1.5% agarose gel at 125 V for 55 min, stained in ethidium bromide (Sigma-Aldrich Chemie GmbH, Taufkirchen, Germany) solution and visualized with a ChemiDoc™ XRS+ system (Bio-Rad Laboratories Inc., Hercules, CA, USA).

2.3. Statistical Analysis

Microsoft Excel 2010 software (Microsoft Corporation; Redmond, WA, USA) and R v4.2.3 [21] were used to record the results and for statistical analyses, respectively. A logistic regression model, followed by pairwise multiple comparisons with Tukey correction, was used to determine (1) the differences in proportions of *Campylobacter* positive caecal samples between the studied years; (2) the differences in total antimicrobial resistance proportion between 2015, 2017 and 2019; and (3) differences in total antimicrobial resistance between specific antimicrobials in all the years combined. The results were considered statistically significant at $p < 0.05$. Confidence intervals (95% CI) were calculated using a 1-sample proportion test with continuity correction.

3. Results

Campylobacter spp. positive caecal samples of fattening pigs at the slaughterhouse level in 2015, 2017 and 2019 are shown in Table 1. The proportion of *Campylobacter*-positive caecal samples in 2019 was significantly different ($p < 0.001$) compared to previous years (Table 1). In total, 52% of the caecal samples were *Campylobacter*-positive in the studied period.

Table 1. *Campylobacter coli* in caecal samples of fattening pigs at the slaughterhouse level [1].

Year	No. of Samples [2]	No. of Positive Samples (%)	95% CI [3]
2015	87	33 (37.9)	27.9–49.0
2017	68	20 (29.4)	19.3–41.9
2019	74	66 (89.2)	79.3–94.9
Total	229	119 (52.0)	45.3–58.6

[1] National monitoring programme. [2] One sample per farm per year. [3] 95% confidence interval.

The results of antimicrobial susceptibility testing showed that only eight (6.7%) isolates were sensitive to all the tested antimicrobials (Table 2). A total of 111 (93.3%) isolates were resistant to one or more antimicrobials: 55 (46.2%) isolates were resistant to one antimicrobial, 38 (32.0%) were resistant to at least two antimicrobials, and 18 (15.1%) were resistant to three or more antimicrobials not belonging to the same group. The proportion of resistant isolates was extremely high in all the studied years. The resistance to one or more antimicrobials was 87.9%, 95.0% and 95.5% in the years 2015, 2017 and 2019, respectively.

Table 2. The number of resistant and susceptible *Campylobacter coli* isolates from caecal samples of fattening pigs at the slaughterhouse level.

Year	No. of Isolates	Antimicrobial-Resistant Isolates (No.)				Multidrug-Resistant [1]	Susceptible to All Antimicrobials (%)
		Resistant to One	Resistant to Two	Resistant to Three	Resistant to Four		
2015	33	17	7	4	1	5	4 (12.1)
2017	20	10	6	3	-	3	1 (5.0)
2019	66	28	25	10	-	10	3 (4.5)
Total	119	55	38	17	1	18	8 (6.7)

[1] Number of isolates resistant to three or more unrelated antimicrobials.

The resistance of *C. coli* isolates is presented in Table 3. A very high proportion of isolates were resistant to streptomycin (74.8%) and tetracycline (45.4%), followed by ciprofloxacin (34.4%) and nalidixic acid (31.9%). All isolates found in 2017 and 2019 were sensitive to erythromycin and gentamicin. No statistical differences in the AMR of specific antimicrobials between different years were found; however, the resistance to fluoroquinolones and tetracycline increased slightly in the study period. In 2015, the resistance to ciprofloxacin was 27.3%, followed by 30.0% and 39.4% in 2017 and 2019, respectively. During the same period, tetracycline resistance ranged from 36.4% to 51.5%. Throughout the study period, the resistance to streptomycin remained extremely high with the highest proportion (85.0%) in the year 2017. Statistically significant differences ($p < 0.05$) in the proportion of AMR between specific antimicrobials were found, except for erythromycin and gentamicin, ciprofloxacin and tetracycline/nalidixic acid, and nalidixic acid and tetracycline. Resistance against streptomycin was significantly higher compared to the other five antimicrobials tested in our study (Table 3).

Table 3. Resistance of *Campylobacter coli* isolates to studied antimicrobials.

Antimicrobial	Number of Resistant Isolates/All Isolates Tested (%)			
	2015	2017	2019	All [1]
Erythromycin	1/33 (3.0)	0/20 (0)	0/66 (0)	1/119 (0.8) [a]
Ciprofloxacin	9/33 (27.3)	6/20 (30.0)	26/66 (39.4)	41/119 (34.4) [b]
Tetracycline	12/33 (36.4)	8/20 (40.0)	34/66 (51.5)	54/119 (45.4) [b,c]
Gentamicin	1/33 (3.0)	0/20 (0)	0/66 (0)	1/119 (0.8) [a]
Nalidixic acid	8/33 (24.2)	4/20 (20.0)	26/66 (39.4)	38/119 (31.9) [b,c]
Streptomycin	24/33 (72.7)	17/20 (85.0)	48/66 (72.7)	89/119 (74.8) [d]

[1] Superscripts with the same letter did not differ significantly ($p > 0.05$) from each other.

In total, 33.6% of *C. coli* isolates were resistant only to streptomycin, 17.6% to a combination of tetracycline/streptomycin and 13.4% to ciprofloxacin/tetracycline/nalidixic acid/streptomycin. The detected resistance phenotypes are presented in Table 4.

The highest MIC values were exceeded for 54.6%, 20.2%, 17.7% and 5.9% of *C. coli* isolates against streptomycin, nalidixic acid, tetracycline and ciprofloxacin, respectively. Conversely, 99.2% of the isolates were sensitive to erythromycin and gentamicin (Table 5). In total, 93.3% of *C. coli* isolates were resistant to at least one antimicrobial and 15.1% of the isolates were MDR. A total of 16 (13.4% [95% CI: 8.1–21.2]) MDR isolates demonstrated a phenotypic combination of ciprofloxacin/tetracycline/nalidixic acid/streptomycin (Table 4).

Table 4. Antimicrobial resistance patterns of *Campylobacter coli* isolated from caecal samples of fattening pigs.

Antimicrobial Resistance Phenotype [1]	Number of *C. coli* Isolates (%)			Number of All Isolates (%) [2]
	2015	2017	2019	
Ery/Cip/Tet/Nal/Str	1 (3.0)	0 (0)	0 (0)	1 (0.8)
Cip/Tet/Nal/Str	3 (9.1)	3 (15.0)	10 (15.2)	16 (13.4)
Cip/Tet/Nal	2 (6.1)	0 (0)	5 (7.6)	7 (5.9)
Cip/Nal/Str	1 (3.0)	2 (10.0)	6 (9.1)	9 (7.6)
Cip/Tet/Str	1 (3.0)	0 (0)	0 (0)	1 (0.8)
Cip/Nal	1 (3.0)	0 (0)	5 (7.6)	6 (5.0)
Tet/Str	4 (12.1)	3 (15.0)	14 (21.2)	21 (17.6)
Cip/Tet	0 (0)	1 (5.0)	0 (0)	1 (0.8)
Cip/Str	0 (0)	1 (5.0)	0 (0)	1 (0.8)
Tet	1 (3.0)	1 (5.0)	5 (7.6)	7 (5.9)
Gen	1 (3.0)	0 (0)	0 (0)	1 (0.8)
Str	14 (42.4)	8 (40.0)	18 (27.3)	40 (33.6)
Resistant to one or more antimicrobials	29 (87.9)	19 (95.0)	63 (95.5)	111 (93.3)
Susceptible to all antimicrobials	4 (12.1)	1 (5.0)	3 (4.5)	8 (6.7)
Total	33 (100)	20 (100)	66 (100)	119 (100)

[1] Tested antimicrobials: Ery, Erythromycin; Cip, Ciprofloxacin; Tet, Tetracycline; Nal, Nalidixic acid; Str, Streptomycin; Gen, Gentamicin. [2] The number of resistant isolates in total was 111. Phenotypes of antimicrobial-resistant isolates have been calculated based on 119 isolates.

Table 5. The minimum inhibitory concentrations (MIC) of *Campylobacter coli* isolates.

No. of Isolates	Antimicrobial [1]	No. of Isolates with MIC Value (µg/mL)										
		0.12	0.25	0.5	1	2	4	8	16	32	64	128
119	Ery	-	-	-	113	3	2	-	-	-	-	1 (1)
	Cip	58	17	4	1	4	-	13	22 (7)	-	-	-
	Tet	20	-	40	5	-	1	1	1	4	47 (21)	-
	Gen	10	4	19	60	25	-	-	1 (1)	-	-	-
	Nal	-	-	-	3	4	12	44	18	7	31 (24)	
	Str	-	-	-	-	2	28	20	69 (65)			

(no) The number of *C. coli* isolates with MIC values exceeding the EUCAMP2 maximum concentration. [1] Tested antimicrobials: Ery, Erythromycin; Cip, Ciprofloxacin; Tet, Tetracycline; Nal, Nalidixic acid; Str, Streptomycin; Gen, Gentamicin. Solid vertical lines indicate breakpoints between sensitive and resistant isolates. The cut-off values: Nal > 16 µg/mL; Cip > 0.5 µg/mL; Tet > 2 µg/mL; Str > 4 µg/mL; Ery > 8 µg/mL; Gen > 2 µg/mL.

4. Discussion

C. coli originating from pigs has been associated with human *Campylobacter* infections [22,23]. Pork is the most consumed meat in Estonia with 42 kg per inhabitant in 2021 [24]; therefore, the presence of AMR *Campylobacter* in pork has a potential public health impact. Among foodborne pathogens, the prevalence and AMR of *Campylobacter* in poultry [25,26] and the prevalence of *Salmonella* in the Estonian pork production chain [27] are well studied, but there are no studies on the prevalence and AMR of *Campylobacter* in fattening pigs in Estonia.

In this study, we have gathered the information on the AMR of *C. coli* isolated from fattening pigs at the Estonian slaughterhouse level within a five-year period. We found that 52% of the caecal samples of fattening pigs collected at the slaughterhouse level were positive for *Campylobacter*. However, compared to the proportions of positive samples, Pezzotti et al. [28] in Italy and Meistere et al. [9] in Latvia found higher *Campylobacter* spp. prevalence in rectal swabs and caecal samples, respectively, 63.5% and 83.3%. In the present study, all isolates detected from caecal samples were identified as *C. coli*, which is consistent with other similar studies [8,10,29]. Additionally, slaughterhouse studies in

Latvia and Denmark revealed that ~90% of the pig-origin isolates were *C. coli* [9,30]. In addition, Wieczorek and Osek [11] in Poland found that *Campylobacter* isolated from pig carcasses were mostly *C. coli*.

C. coli in the intestinal tracts of pigs can easily cause contamination in the entire pork processing chain if there is a lack of effective biosafety and other control measures [5,6]. There are few examples of very low *Campylobacter* prevalence or even of *Campylobacter*-free pig herds available in the literature. In Norway, specific-pathogen-free (SPF) pig herds were studied, and it was found that the intervention of *Campylobacter* at the herd level is possible because some SPF herds tested *Campylobacter* negative both in autumn and summer [31]. Lindblad et al. [32] demonstrated a very low (1.0%) prevalence of *Campylobacter* in pig carcasses in Swedish slaughterhouses.

AMR is a global public concern because resistant pathogens might compromise the treatment of infections and may even result in the death of animals and humans. Unfortunately, the number of *Campylobacter* isolates resistant to critically important antimicrobials (CIAs), especially fluoroquinolones, is increasing [33]. Initially, the monitoring of AMR in *C. coli* isolates from caecal samples gathered at slaughter from fattening pigs based on the Member State decision to test *C. coli* was laid down by the Commission Implementing Decision 2013/652/EU [19], following Directive 2003/99/EC [18], on the monitoring of zoonoses and zoonotic agents. The antimicrobials included for resistance monitoring of *C. coli* isolates were erythromycin, ciprofloxacin, tetracycline, gentamicin, nalidixic acid and streptomycin. In 2020, the Commission Implementing Decision 2020/1729/EU [34] repealed Decision 2013/652/EU [19]. Due to the amended Decision, nalidixic acid and streptomycin were removed, and chloramphenicol, together with ertapenem, was added to the list of antimicrobials included in the AMR monitoring of *C. coli* from fattening pigs. As requested by the EU legislation, EUCAST thresholds for resistance need to be followed [19,34]. In this study, the antimicrobials given in Implementing Decision 2013/652/EU [19] were used because it was applicable for the period 2015–2019. In recent years, AMR monitoring for pig-origin *C. coli* isolates has not been carried out in Estonia.

The present study found a very high proportion of resistant *C. coli* isolates throughout the study period in pig caecal samples at slaughter. According to the Estonian Agency of Medicines [35], the most used antimicrobials in pigs and cattle in 2022 were doxycycline, tiamulin and amoxicillin. Of the overall sale proportion, the most sold antimicrobial classes used for veterinary purposes in Estonia in 2022 were tetracyclines, penicillins and pleuromutilins, respectively, 25.5%, 22.3% and 18.4% [35].

In the present study, a high proportion of isolates was resistant to streptomycin (74.8%), tetracycline (45.4%) and fluoroquinolones (34.4%). Similarly, a Latvian study [9] found an extremely high proportion of streptomycin-resistant *C. coli* isolated from pig caecal samples. In addition, in Italy, Pezzotti et al. [28] found that *C. coli* isolates of pig origin were highly resistant to tetracycline and streptomycin. A lower resistance against these antimicrobials was observed in Finland [36] and Denmark [37]. Since 2009, the use of CIAs in Danish pig herds has been phased out and the use of tetracyclines in pigs has decreased significantly [38].

Fluoroquinolones (e.g., ciprofloxacin) are categorised as CIA [39] and are used as a first-line treatment for invasive human infections. Therefore, the resistance to fluoroquinolones among *Campylobacter* isolates needs special attention. According to the European Union Summary Report [17], the *C. coli* resistance to ciprofloxacin was high to extremely high (>70%) in human isolates and very high to extremely high in isolates from fattening pigs (51.7%) [17]. In research studies, different levels of fluoroquinolone resistance among pig *Campylobacter* isolates, e.g., in Finland 18.3% and 34.0% [36,40], in Latvia 53.5% [9] and in Poland 57.1% [11], have been found. In a recent Italian study [10], *C. coli* isolates of pig origin were extremely resistant both to quinolones (74.7%) and fluoroquinolones (70.1%). In our study, the resistance to quinolones (nalidixic acid) and fluoroquinolones (ciprofloxacin) was 31.9% and 34.4%, respectively.

According to the World Health Organization's (WHO's) medically important antimicrobials list [39], erythromycin is categorized as CIA. In the present study, the resistance against erythromycin was very low (0.8%) with only one resistant isolate. Similar findings have been reported from our neighbouring countries Finland and Latvia [9,36]. A study conducted in Finland showed that tylosin treatment in pigs caused an increase in *C. coli* resistance to erythromycin [41]. However, according to Finnish AMR monitoring, erythromycin resistance in *C. coli* isolates from Finnish pigs occurs very rarely [40].

MDR among zoonotic isolates needs special attention because they are known as the most serious global public health threats of this century [42,43]. In the present study, 15.1% of *C. coli* isolates were MDR, which was defined as the resistance of the isolate to three or more unrelated antimicrobials [12]. MDR is an alarming trend among *C. coli* [16,40,44,45]. In the EU, a total of 9.7% of *C. coli* isolates from fattening pigs were reported as being MDR [17]. The high proportion of MDR *C. coli* isolates indicates an overuse of antimicrobials in animal husbandry [46]. To decrease the use of antimicrobials in food-producing animals and minimize the emergence and spread of MDR bacteria, the implementation of good farm and veterinary practices and application of strict biosecurity measures are necessary [47–49]. Additionally, according to Albernaz-Gonçalves et al. [50], the use of antimicrobials and the risk of AMR can be reduced by providing good nutrition, clean water and appropriate living conditions. The WHO has coordinated a global action plan to limit the use of antimicrobials in food-producing animals and to promote responsible use practices to reduce the development of AMR [51]. The World Organisation for Animal Health (OIE) has developed standards on AMR and the use of antimicrobials that also support the global action plan [52]. At the beginning of the year 2022, two important EU regulations came into force promoting the responsible use of antimicrobials in animals. The Veterinary Medicinal Products Regulation (EU) 2019/6 [53] imposes restrictions on the use of certain antimicrobials and promotes the use of alternatives. This regulation also strengthens the monitoring of antimicrobial use in animals, aiming to prevent the development and spread of AMR. In addition, the Regulation (EU) 2019/4 [54] on the manufacture, placement on the market and use of medicated feed states that "Medicinal treatments, especially with antimicrobials, should never replace good husbandry, biosecurity and management practices". To reduce the use of antimicrobials, there is a need to encourage research and innovation to develop new antimicrobials, vaccines and alternative treatments [55]. According to the reports of the Estonian Health Board, the proportion of *C. coli* isolates among human campylobacteriosis cases in Estonia in 2015–2019 was in the range of 6.0–13.4% [56]. In the mentioned period, the year 2018 had the highest proportion (13.4% [95% CI: 10.3–17.2]) of *C. coli* infections. This was related to three *C. coli* outbreaks with nine cases combined. It is important to note that during 2015–2019, the MDR among *C. coli* was eight times higher than in *C. jejuni* among human clinical isolates in Estonia [56]. Between 2014 and 2021, *Campylobacter* was the most common human intestinal pathogen, with *C. jejuni* accounting for approximately 90% of cases and *C. coli* for less than 10% [57]. According to the European Union Summary Report on Antimicrobial Resistance in zoonotic and indicator bacteria from humans, animals and food in 2020–2021 [17], the average level of ciprofloxacin resistance in the EU was 65.8% and 69.6% for *C. coli* human isolates in the years 2020 and 2021, respectively. The highest levels of resistance (100%) to ciprofloxacin in *C. coli* human isolates were reported for Portugal and Estonia. In addition, the resistance to ciprofloxacin in food-producing animals was estimated to be high to extremely high, ranging from 41.7% to 80.4% in the EU. *C. coli* isolates of fattening pigs exhibited 51.7% resistance to ciprofloxacin in the reported countries in 2021. In addition, an extremely high level (70.3%) of resistance to tetracycline was detected in *C. coli* isolates from humans in 2021. The levels of combined resistance to both ciprofloxacin and erythromycin, which are considered critically important for the treatment of campylobacteriosis, was 25.9% in human *C. coli* isolates in Estonia. This was the second highest level of this particular combined resistance after Portugal at the EU level in 2021 [17]. In summary, due to the high proportion of *C. coli*-positive caecal samples and the high resistance of *C. coli* isolates

to studied antimicrobials, the consumption of contaminated pork should be considered a public health risk in Estonia. There is a need to perform source attribution studies to identify the main sources of *C. coli* human infections because no studies have been performed yet on the topic. In addition, investigating risk factors on pig farms and conducting awareness campaigns for consumers on how to avoid *Campylobacter* infection are needed.

5. Conclusions

In Estonia, approximately 10% of human campylobacteriosis cases are related to *C. coli* and in the year 2022 there were 15.1 campylobacteriosis cases per 100,000 inhabitants. The current study found that pigs are reservoirs of AMR *C. coli*; therefore, the consumption of contaminated pork is a potential public health risk. The thorough monitoring and control of the use of antimicrobials at the farm level are extremely important. Due to the high proportion of resistance, there is a need to limit the use of critically and highly important antimicrobials such as fluoroquinolones and macrolides in the treatment of pigs and to improve the monitoring and control of antimicrobial use in farm animals.

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Institutions and positions

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Education

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1996–2000	Põlva Gymnasium

Fields of research

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Research projects

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2023–…	Riigi Laboriuuringute ja Riskihindamise Keskus (LABRIS), peaspetsialist, riskihindamise osakond (1,00)
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Teadustöö põhisuunad

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CERCS KLASSIFIKAATOR: B680 Rahvatervishoid, epidemioloogia;

Teadusprojektid

2022–2026	"Looduslike bioaktiivsete ainete toimete mehhanismide uurimine loomsetes toitudes" (PRG1441), Mati Roasto, Eesti Maaülikool, veterinaarmeditsiini ja loomakasvatuse instituut, veterinaarse biomeditsiini ja toiduhügieeni õppetool

LIST OF PUBLICATIONS

1.1. Scholarly articles indexed by Web of Science Citation Index Expanded, Social Sciences Citation Index, Arts & Humanities Citation Index and/or indexed by Scopus (excluding chapters in books)

Tedersoo T., Roasto M., Mäesaar M., Fredriksson-Ahomaa M., Meremäe K. (2023). Antimicrobial resistance of *Campylobacter coli* isolated from caecal samples of fattening pigs at slaughter. *Microorganisms*, 11:1540. doi:10.3390/microorganisms11061540.

Tedersoo T., Roasto M., Mäesaar M., Kisand V., Ivanova M., Meremäe K. (2022). The prevalence, counts, and MLST genotypes of *Campylobacter* in poultry meat and genomic comparison with clinical isolates. *Poultry Science*, 101:101703. doi: 10.1016/j.psj.2022.101703.

Tedersoo T., Roasto M., Mäesaar M., Häkkinen L., Kisand V., Ivanova M., Valli M.H., Meremäe K. (2022). Antibiotic resistance in *Campylobacter* spp. isolated from broiler chicken meat and human patients in Estonia. *Microorganisms*, 10:1067. doi: 10.3390/microorganisms10051067.

3.4. Articles/presentations published in conference proceedings not listed in Section 3.1

Tedersoo T., Roasto M., Meremäe K. (2021). The prevalence and antimicrobial resistance of *Campylobacter* spp. isolated from fresh broiler chicken meat sold at Estonian retail. *One Health EJP Annual Scientific Meeting 2021*. 9–11 June 2021, Copenhagen, Denmark. OHEJP ASM2021. 1−2.

Tedersoo T., Roasto M., Meremäe K. (2021). The prevalence and antimicrobial resistance of *Campylobacter* spp. isolated from fresh broiler chicken meat at Estonian retail level. *I International Scientific and Practical Internet Conference "Problems and Achievements of Modern Biotechnology"*. March 25, 2021. Ministry of Health of Ukraine and National University of Pharmacy, Department of Biotechnology. National University of Pharmacy of Ukraine. 1−2.

Tedersoo T., Roasto M., Meremäe K. (2020). *Campylobacter* spp. in fresh broiler chicken meat at Estonian retail level. *44th Conference for Students of Agriculture and Veterinary Medicine with International Participation, Proceesings Book*, 1: 44th Conference for Students of Agriculture and Veterinary Medicine. 15.12.2020, University of Novi Sad, Faculty of Agriculture. 71−74.

3.5. Articles/presentations published in local conference proceedings

Tedersoo T., Roasto M., Sõgel J., Meremäe K. (2021). Kampülobakterid liha tootmise ahelas [*Campylobacter* spp. in the meat production chain]. Konverentsi "Terve loom ja tervislik toit 2021" artiklite kogumik, Eesti Maaülikool, 53−61.

6.7. Other creative activities

Tedersoo T., Roasto M., Meremäe K. (2021). Termofiilsed kampülobakterid. 1−1.

VIIS VIIMAST KAITSMIST

SILVA VILUMETS
KAHJURITE JÄTKUSUUTLIKUD TÕRJESTRATEEGIAD RAPSI INTEGREERITUD TAIMEKAITSES
SUSTAINABLE APPROACHES TO OILSEED RAPE PEST CONTROL: STEPS TOWARDS TO INTEGRATED PEST MANAGEMENT
Professor Eve Veromann
9. aprill 2024

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Professor Ülo Niinemets, dotsent Eve Runno-Paurson, Eve Kaurilind
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MITMEASTMELINE SALVESTUSSTRATEEGIA TAASTUVENERGIA OSAKAALU SUURENDAMISEKS TARBIJA JUURES
MULTIPLE STAGE STORAGE STRATEGY FOR THE INCREASING OF RENEWABLE FRACTION OF THE CONSUMER
Professor Andres Annuk, professor Emmanuel Karapidakis
19. aprill 2024

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ASSESSMENT OF DEVELOPMENTAL POTENTIAL OF IN VITRO PRODUCED DAIRY CATTLE EMBRYOS BY INVASIVE AND NON-INVASIVE METHODS
Professor Ülle Jaakma, professor Sulev Kõks
2. mai 2024

AGE KÄRSSIN
EESTI METSLOOMADEL ESINEVAD *TRICHINELLA* LIIGID
TRICHINELLA SPECIES IN WILD ANIMALS IN ESTONIA
Teadur Brian Lassen, teadur Pikka Sirkku Jokelainen
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