ESTONIAN UNIVERSITY OF LIFE SCIENCES

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ANTIMICROBIAL SENSITIVITY OF *CAMPYLOBACTER* SPP. ISOLATED FROM FRESH BROILER CHICKEN MEAT OF ESTONIAN RETAIL LEVEL

EESTIS MÜÜDAVAS VÄRSKES KANABROILERILIHAS ISOLEERITUD *CAMPYLOBACTER* SPP. TUNDLIKKUS ANTIMIKROOBSETE ÜHENDITE SUHTES

Final Thesis

Curriculum in Veterinary Medicine

Supervisors: Professor Mati Roasto

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LÜHIKOKKUVÕTE

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Termofiilsed kampülobakterid põhjustavad Euroopa Liidus enim bakteriaalseid sooleinfektsioone inimestel ning kanabroileriliha on *Campylobacter jejuni* kõige olulisem kampülobakterioosi allikas. Käesoleva uuringu eesmärk oli määrata Eesti jaemüügi tasandil ostetud värskest Eesti, Läti ja Leedu päritolu kanabroilerilihast ning Eesti inimestelt isoleeritud *Campylobacter* spp. antimikroobne resistentsus. Minimaalsed inhibeerivad kontsentratsioonid määrati kasutades EUCAMP2 meetodit. Kampülobakterite isolaate testiti erütromütsiini, tsiprofloksatsiini, tetratsükliini, streptomütsiini, gentamütsiini ja nalidiksiinhappe suhtes. Väga kõrge (100%) resistentsus fluorokinoloonidele määrati Eesti jaemüügi tasandil Leedu ja Läti päritolu *Campylobacter* isolaatidel, mis pärinesid värskest broilerilihast. Kliiniliste (inimese) isolaatide hulgas oli resistentsus fluorokinoloonide suhtes 86,7%. Kõrge resistentsus tetratsükliini suhtes määrati Leedu päritolu broileri kanalihal ja Eesti inimestelt pärinevatelt kampülobakterite isolaatidel, vastavalt 76,9% ja 80,0%. Streptomütsiiniresistentsete tüvede osakaal oli kõrge Läti ja Leedu päritolu broileriliha kampülobakterite isolaatide puhul, vastavalt 68,8% ja 42,3%. Multiresistentsete isolaatide proportsioon uuritud kampülobakterite isolaatide hulgas oli 26,2%. Kõik isolaadid olid tundlikud gentamütsiini suhtes. Erütromütsiini suhtes oli resistentsus madal ning kõik uuritud Eesti broileri kanaliha isolaadid olid tundlikud kõikide uuritud antibiootikumide suhtes. Leedu ja Läti päritolu kanabroilerilihast isoleeritud *Campylobacter* isolaatide ja kliiniliste (inimestelt isoleeritud) tüvede kõrge resistentsus uuritud antibiootikumide suhtes kujutab rahvatervise probleemi. Antimikroobse resistentsuse vähendamiseks peab antibiootikumide kasutamise kontroll broilerilihafarmi tasandil olema tõhusam ning antibiootikume ei tohi kasutada ennetavas ja metafülaktilises ravis, samuti kasvu soodustamise eesmärgil.			
Märksõnad: *Campylobacter* spp., antibiootikumidele resistentsus, värske kanabroileri liha			

SUMMARY

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Campylobacter spp. are causing most of the human bacterial enteric infections in European Union, and broiler chicken meat is the most important source of *Campylobacter jejuni*. Present study aimed to determine the antimicrobial resistance of *Campylobacter* spp. isolated from fresh broiler chicken meat of Estonian, Latvian and Lithuanian origin purchased at Estonian retail level, and among human *Campylobacter* isolates. Minimal inhibitory concentrations for tested antimicrobials were determined by using the EUCAMP2 method. *Campylobacter* isolates were tested against erythromycin, ciprofloxacin, tetracycline, streptomycin, gentamicin and nalidixic acid.

Very high resistance (100%) to fluoroquinolones was determined for Lithuanian and Latvian origin *Campylobacter* isolates originated from fresh broiler chicken meat at Estonian retail level. Among clinical (human) isolates the resistance to fluoroquinolones was 86.7%. High resistance to tetracycline was determined for Lithuanian origin broiler chicken meat and Estonian human isolates, respectively 76.9% and 80.0%. The proportion of streptomycin resistant strains was high for Latvian and Lithuanian origin broiler chicken meat *Campylobacter* isolates, respectively 68.8% and 42.3%. The overall proportion of multidrug resistance among studied *Campylobacter* isolates was 26.2%. All isolates were sensitive to gentamicin. Low resistance to erythromycin was found. All studied Estonian broiler chicken meat isolates were sensitive to all studied antimicrobials. High resistance among Lithuanian and Latvian origin broiler chicken meat *Campylobacter* isolates, and among clinical isolates is public health concern. To reduce antimicrobial resistance, the control of the use of antibiotics at broiler chicken meat farm level should be more effective, and antibiotics cannot be used in preventative and metaphylactic treatments, also in growth promotion purposes.

Keywords: *Campylobacter* spp., antibiotic resistance, fresh broiler chicken meat

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LIST OF ABBREVIATIONS

AMR	antimicrobial resistance
CAMHB	Cation-Adjusted Mueller–Hinton Broth
CFU	Colony Forming Unit
COVID-19	Coronavirus disease 2019
EC	European Commission
EFSA	European Food Safety Authority
EU	European Union
EUCAST	European Committee on Antimicrobial Susceptibility Testing
FDA	Food and Drug Administration
MIC	minimal inhibitory concentration
PSI	Czekanowski proportional similarity index
THL	Terveyden ja hyvinvoinnin laitos (Finnish Institute for Health and Welfare)
WHO	World Health Organization

INTRODUCTION

Campylobacteriosis is the most reported gastrointestinal disease in humans in the European Union (EU) and has been so since 2005 (EFSA and ECDC, 2019). In 2019, the number of confirmed cases of human campylobacteriosis was 220,682 corresponding to an EU notification rate of 59.7 per 100,000 population. The trend for campylobacteriosis in humans remained stable during 2014-2019 (European Food Safety Authority EFSA and ECDC, 2021).

In Estonia, 265 confirmed cases of human campylobacteriosis were reported in 2020, with a notification rate of 20.0 per 100,000 inhabitants (Terviseamet, 2021). The number of disease cases decreased in 2020 compared to the year 2019, but this may also be due to the effect of coronavirus disease 2019 (COVID-19). In year 2019, a total of 348 campylobacteriosis cases were reported by the Health Board of Estonia (Terviseamet, 2021).

Studies have indicated that broiler chicken meat is the major, but not the only, source of *Campylobacter* infection in the human population (Di Giannatale *et al*., 2014; González-Hein *et al*., 2013; Mäesaar *et al*., 2020). Fluoroquinolones are one of the most commonly used classes of antibiotics, and ciprofloxacin is the most widely used fluoroquinolone in the world (Lillenberg *et al*., 2010). Latvian study by Kovalenko *et al*. (2014) demonstrated extremely high resistance (almost 100%) to ciprofloxacin and nalidixic acid. Foodborne pathogenic microorganisms resistant to antimicrobials are a serious concern, as they often compromise the effective treatment of human foodborne infections (Walsh and Fanning, 2008). It is well known that the use of antibiotics for preventive or prophylactic purposes or in therapy may select resistant bacteria and resistant strains, also pathogenic microorganisms may reflect the antimicrobial usage history over longer time periods (Smith *et al*., 2007).

The aim of the thesis is to determine the antimicrobial resistance of *Campylobacter* spp. isolated from fresh broiler chicken meat of Estonian, Latvian, and Lithuanian origin purchased at Estonian retail level.

1. REVIEW OF THE LITERATURE

1.1. General description of *Campylobacter* spp.

Bacteria that belong to the genus *Campylobacter spp.* are gram-negative, spiral, non-spore forming rods. They form spherical or coccoid bodies in older cultures. Cell measurements are between 0.2 to 0.9 microns wide and 0.5 to 5.0 microns long (Epps *et al*., 2013). The genus *Campylobacter* consist of 32 officially described species and 9 subspecies (Costa and Iraola, 2019). They have single polar flagellum, bipolar flagella, or no flagellum, depending on the species. They are chemoorganotrophs, which means that they get their energy sources from amino acids or tricarboxylic acid cycle intermediates. Most of the species of campylobacteria grow under microaerophilic conditions and have respiratory type of metabolism. However, there are also several species (*C. concisus, C. curvus, C. rectus*) that prefer anaerobic conditions for their growth. Most species of *Campylobacter* pathogenic to humans grow best in an atmosphere containing approximately 10% CO_2 and approximately 5% of O_2. Temperature range allowing growth for *Campylobacter* is relatively narrow, maximum temperature being 46 °C and minimum 30 °C. According to growth temperature ranges these bacteria are called as thermophilic microorganisms (Humphrey *et al*., 2007). *Campylobacter* spp. will not grow below pH of 4.9 and above pH 9.0, and grow optimally at pH 6.5-7.5 (Silva *et al*., 2011).

Campylobacter are inactivated with frozen storage at -15 °C in three days, however the freezing does not free the food from other pathogens. *Campylobacter jejuni* is the major cause of gastroenteritis worldwide. There are some other *Campylobacter* species that cause gastroenteritis in humans, like *C. coli*, *C. helveticus* and *C. hyointestinalis* (Kaakoush *et al*., 2015). Most common causes of gastroenteritis in humans are *C. jejuni* and *C. coli* (Olkkola *et al*., 2016; Skarp *et al*., 2016; Woźniak-Biel *et al*., 2018).

Poultry are a natural host for *Campylobacter* and broilers are often colonised, most commonly with *C. jejuni* (Shane, 1992). Poultry is known to be the main reservoir for human campylobacteriosis and is responsible for estimated 80% of infection cases. Transmission to humans is most often associated with handling and consumption of poultry that is contaminated during slaughter and carcass processing (Bolton, 2015). At slaughterhouse level *Campylobacter* contamination of carcasses is mainly due to faecal contamination (García-Sánchez *et al*., 2018). *Campylobacter* spp. are considered to be a part of the normal microbiota of the gastrointestinal tract in numerous domestic animals and birds, such as commercial broiler chicken, which are considered to be mainly asymptomatic carriers (García-Sánchez *et al*., 2018). Evidence suggests that even low numbers of *Campylobacter* contamination in poultry drinking water may be a significant risk factor in colonisation of whole flocks (Snelling *et al*., 2005).

1.2. Antimicrobial resistance as global problem

The antimicrobial resistance (AMR) is noted by World Health Organisation (WHO) as one of the top three threats to public health (Xiong *et al*., 2018). Antimicrobials which include antibiotics, antivirals, antifungals and antiparasitics, are medicine that are used to treat infections in humans, animals, and plants (WHO, 2020a). The AMR is of the same extent as global warming (Ferri *et al*., 2017). Darwinian selection is the process that drives microorganism trough antimicrobial selection pressure, exchange their fitness by acquiring and expressing resistance genes and then share those genes with other bacteria. So, the use and overuse of antimicrobials is the driving force of the resistance phenomenon. When antimicrobial gets the resistance gene, it can be transmitted from animals to humans and vice versa. Therefore, it is important to understand the One Health principles, which generally means that the health of people is closely connected to the health of animals, plants, and environment around us, and the antimicrobial resistance can be transmitted by all of these vehicles (McEwen and Collignon, 2018).

We are already in situation where it has been reported that the pigs had gram-negative microorganism resistant to the last resort antibiotic, colistin. Because of this, there is hardly any treatment option left. Several outbreaks are reported, for example in 2014 multiresistant *Salmonella* Heidelberg in the USA which was linked to one supplier. In some regions of world, the prophylactic use of antimicrobials is still existing, especially in large-scale broiler chicken farms. There is a risk that although the antibiotics used in animals are not used in human, there still may be the option that pathogens share the drug efflux pumps which cause AMR. Organism that expresses efflux pumps can be resistant for many of antimicrobial compounds, including those used in healthcare. Sick animals certainly need treatment, but it is needed to reconsider the use of antimicrobials as growth-enhancers and prophylactic use of antibiotics (Venter *et al*., 2017).

1.2.1. One Health

World Health Organisation lists that One Health areas of work are food safety, control of zoonotic diseases, laboratory services, neglected tropical diseases, environmental health, and antimicrobial resistance (WHO, 2021).

One Health is a concept where different stakeholders across local, national, and global layer work together to achieve the best health to people, environment, and animals. It is a paradigm where interdisciplinary collaboration is introduced and encouraged. It is becoming clear that the entire health system needs to adopt One Health concept as recent threats by AMR and zoonotic diseases emerge (Ryu *et al*., 2017).

One Health is considering all the components that may lead to, or increase, the threat of the disease. These diseases are particularly zoonoses. Components may be environmental and ecological/wildlife, but they may also be domestic animal and human factors. Public health, animal management, ecological approaches and veterinary medicine are the best ways to control or prevent zoonoses. One problem is zoonoses that do not cause disease symptoms in their animal host, but are able to infect people e.g., *Campylobacter* spp. One Health approach means reducing the infection carriage in poultry (by farmers and veterinarians), lower meat contamination (by meat companies and veterinary public health workers), applying the preventative measures in kitchen (by consumers) with proper cooking and hygiene (Cunningham *et al*., 2017).

AMR is in global agenda with collaboration across various countries and sectors. There are national action plans, but more decisive actions are needed both nationally and internationally. Efforts should be addressed to low-income and middle-income countries, where international research co-operations can build the capacity (Wernli *et al*., 2020).

1.2.2. History of antimicrobials used for farm animals

Penicillin was first used to treat human diseases, and was extensively used during the World War II, but was also used by veterinarians to treat mastitis in cows. As early as 1946, improvement of chicks' growth after use of streptomycin was documented. This was the first finding in growing series of evidence: swine and chicken treated with chlortetracycline, similar positive effect of other antimicrobials for growth of chicken, swine and cattle was witnessed. In 1951, the Food and Drug Administration (FDA) in the USA approved the use of antimicrobials as a growth promoter in animals (Xiong *et al*., 2018).

In European countries the growth promoter effect of antibiotics was discovered in the 1940s. Animals were fed with dried mycelia of *Streptomyces aureofaciens* containing chlortetracycline residues which improved their growth. Antibiotic action as a growth promoter is related to interactions with intestinal microbial population. Sweden was first country that banned the use of antimicrobials for non-therapeutic purposes, between 1986 and 1988. First ban was for growth promoter and second for prophylaxis. Denmark, the Netherlands, United Kingdom, and other EU countries followed. These countries banned the use of all essential antibiotics as prophylactic agents in 2011. Despite these developments, it is estimated that over 60% of all antibiotics produced are used in livestock production, including poultry (Agyare *et al*., 2018).
In early 1990s enrofloxacin was introduced into animal production in Asia and in Europe. At the same time, fluoroquinolone resistance started to increase among *Campylobacter* spp. isolated from humans. Similar phenomenon was observed in the United Kingdom and USA, after fluoroquinolones were approved for use in veterinary medicine. Many countries where the use of fluoroquinolones is minimal in animal production, the incidence of fluoroquinolone resistant strains is in moderate or at low level. For example, in Australia, Finland, and Sweden the resistance against fluoroquinolones is low (Wieczorek and Osek, 2013).
Unfortunately, antibiotics are used also for other purposes than therapeutic treatment, e.g. are used for prophylactic purposes (i.e., they are used in disease prevention); metaphylactic purposes (i.e., treating whole group of animals where one or more animals show disease symptoms), and they are used for growth promotion of production animals (Roskam *et al*., 2019).

1.3. Antimicrobial resistance of *Campylobacter* spp. in poultry including broiler chicken meat production

Most humans suffering campylobacteriosis get well without therapeutic intervention other than fluid and electrolyte replacement. More severe cases are often treated with macrolide, tetracycline or (fluoro)quinolone antibiotics, but by the increasing resistance to these antibiotics in *C. jejuni* and *C. coli*, the efficacy of such treatments is currently compromised (Bolton, 2015). There is a clear positive association between the use of fluoroquinolones in poultry production and in increase of resistance among chicken and human origin *Campylobacter* isolates (García-Sánchez *et al*., 2020).

Antimicrobial resistance in bacteria originated from food of animal origin has become a major problem in public health for both developing and developed countries in recent years. “Intrinsic resistance in *C. jejuni* and *C. coli* has been described against penicillin and most of the cephalosporin’s as well as trimethoprim, sulfamethoxazole, rifampicin and vancomycin” (Wieczorek and Osek, 2013).

“The antimicrobial resistance mechanisms can be grouped into three categories: (I) antimicrobial reduction inside the bacteria; (II) antimicrobial inactivation and (III) modification of the target on which the antimicrobial acts” (Florez-Cuadrado *et al*., 2018). With first category, the bacteria control the amount of antimicrobial that is inside either preventing the entry or expelling it outdoors. In second category, the bacteria have capability to modify or degrade the antimicrobial. Third category mechanism works by modifying the molecular target of antimicrobial action trough mutations or methylation, so that it is not acting against this bacterium. Shortly, all these mechanisms are determined by specific mutations or by the expression of antimicrobial resistance genes (Florez-Cuadrado *et al*., 2018).

1.3.1. Antimicrobial resistance of *Campylobacter* spp. in Nordic countries

In Nordic countries 64,034 cases of *Campylobacter* spp. were reported between 2000 and 2015. Final models for *Campylobacter* and climate showed that when temperature was increasing significantly in the summer months when there was also precipitation and number of heavy precipitation events in the previous week, the number of cases increased. In addition, if there was only heat wave in summer without precipitation or precipitation in the winter, lower number of campylobacter cases were reported. Generally, this supports a theory that cases that have not been proven to be from a poultry source could be transmitted from the environment sources (Kuhn *et al*., 2020).

Consumption of veterinary antimicrobials in food-production animals in Finland is low and it has been decreasing even further in recent years. The antimicrobial resistance situation in bacteria from animals and food has stayed relatively good. Among *Salmonella* and *Campylobacter* isolated from Finnish food-production animals the resistance levels were mainly low. Antimicrobial resistance has been monitored since 2003 systematically. *C. jejuni* resistance levels have been quite stable until year 2013 and isolates that are resistant have been detected mainly at a low level. In years 2014-2018 there were two peaks in quinolone resistance. Tetracycline resistance also peaked at the same time. No or low resistance was detected to erythromycin, gentamicin and streptomycin for the whole surveillance period (Finnish Food Authority *et al*., 2019).

Iceland's main concern is quinolone resistance (Thorsteinsdottir *et al*., 2010).

In Norway case-control studies have shown that most common source for campylobacteriosis is drinking untreated water. Eating or preparing poultry and barbeque meals have also been identified as a risk factor (Norwegian Veterinary Institute, 2019). The prevalence of antimicrobial resistance among *C. jejuni* isolates from Norwegian broilers is low. Main group of isolates were susceptible to all microbial agents (93.0%). There were 86 isolates. Resistance to two antimicrobial agents was detected (quinolones) in 3.5% and resistance to three antimicrobial agents was detected (quinolones and tetracycline or streptomycin) in 3.5% of isolates. Ciprofloxacin and nalidixic acid were the most frequently identified determinants and they belong to antimicrobial group quinolones. European perspective, ongoing quinolone resistant in *C. jejuni* is quite low in Norwegian broilers compared to EFSA reports in some other countries, for example Latvia. Nordic countries have the lowest antimicrobial resistance levels (Norwegian Veterinary Institute, 2019).

1.3.2. Antimicrobial resistance of *Campylobacter* spp. in Baltic countries

Previous studies performed in Baltic countries between 2010 and 2013 found that the resistance levels of *Campylobacter spp*. in Estonia were lowest with 12.3% of proportion of *Campylobacter* resistant strains, compared with resistance levels in Lithuanian (46.5%) and Latvian (56.3%) broiler chicken meat origin campylobacters, respectively. Authors concluded that differences in resistance levels among *Campylobacter* isolates originating from broiler chicken meat of different Baltic countries are probably associated with different farm management systems of broiler chickens. Authors also concluded that lower *Campylobacter* resistance in Estonia is due to the restrictive use of antimicrobials since year 2006 when antimicrobials are not allowed to be used as feed additives (Mäesaar *et al*., 2016).

1.4. Legislation associated with the use of antimicrobials in broiler chicken production.

The aims of EU legislation are to ensure a high level of protection of human health and to decrease incidence of zoonosis in humans, as well the protection of animal health and welfare, plant health and environment. According to Commission Regulation (EC) No 178/2002, food cannot be placed on the market when it is unsafe. Unsafe means that it is injurious to health or unfit for human consumption.

Commission Regulation (EC) No 178/2002 lays down general food law which says that food must be safe for human consumption, and that responsibility to assure food safety lies on food producers. According to Commission Regulation (EC) No 2073/2005 of 15 November 2005 on microbiological criteria for foodstuffs, microbiological criteria for foodstuff must be followed by food business operators.

By the Regulation (EC) No 1831/2003 of 22 September 2003 "on additives for use in animal nutrition" it is established that since January 2006 the use of all antimicrobial feed additives are banned within the EU in order to reduce the numbers of resistant bacteria in farm animal. Article 5, section 4 of this regulation constitutes that "Antibiotics, other than coccidiostats or histomomostats, shall not be authorized as feed additives".

In year 2018 the European Parliament and Council approved new regulations: Regulation (EU) 2019/4 of 11 December 2018 on the manufacture, placing on the market and use of medicated feed. In this Regulation it is written that "prevention of disease is better than cure. Medicinal treatments, especially with antimicrobials, should never replace good husbandry, bio-security and management practices".

Regulation (EU) 2019/6 of 11 December 2018 on veterinary medicinal products. This Regulation aims "to reduce the administrative burden, enhance the internal market and increase the availability of veterinary medicinal products, while guaranteeing the highest level of public and animal health and environmental protection". In Regulation (EU) 2019/6 it is written that "This Regulation should set high standards of quality, safety and efficacy for veterinary medicinal products in order to meet common concerns as regards the protection of public and animal health and of the environment. At the same time, this Regulation should harmonise the rules for the authorisation of veterinary medicinal products and the placing of them on the Union market".

Generally, these regulations ban the use of antibiotics in preventative and metaphylactic treatments, also in growth promotion, and will add the possibility to reserve certain antibiotics for human use only. Regulations went into force at the end of January 2022.

1.5. Prevention and control of *Campylobacter* antimicrobial resistance

There are many ways to control the antimicrobial resistance in poultry production. The focus in prevention is to know why *Campylobacter* is spreading and how to stop that. “Multiple risk factors have been associated with an increased proportion of *Campylobacter* positive flocks.” (García-Sánchez *et al*., 2020) Factors that have been identified in this study were: season, presence of other animals in the farm or in close proximity, presence of rodents, vectors such as flies, flock size, several flock houses in the same farm, thinning or depopulation practices, drinking water distribution and the administration of antibiotics (García-Sánchez *et al*., 2020). Human campylobacteriosis cases are mainly obtained via the foodborne route, so successful control of the disease requires both aspects of the animal reservoir and human host (Dai *et al*., 2020).

EFSA announced control options on farm level that have higher probability to have more than 10% effect, and these are: vaccination, feed and water additives, discontinued thinning, employing few and well trained staff, avoiding drinkers that allow standing water, addition of disinfectants to drinking water, hygienic anterooms at broiler house entrance, designed tools per broiler house, reduced slaughter age, no animals in close proximity of the broiler houses, bacteriophages, effective cleaning and disinfection, selective breeding, rodent control, fly nets and keeping insects out. There were some challenges among these control options. For example, with vaccination, there is no effective vaccine yet and feed and water additives have conflicting evidence regarding the effectiveness of additives. In addition, when adding disinfectants to water, it is not clear if it is effective as an individual biosecurity measure. Bacteriophages use, as currently forecasted, has limited practicality (EFSA, 2020).

Vertical transmission does not appear to be an important risk factor. Recent articles reported no evidence of vertical transmission of *Campylobacter spp.* when hatching eggs to commercial flocks (EFSA, 2020).

Many alternative strategies rather than antibiotics to deal with *Campylobacter* problem have been proposed. These strategies are for example: the use of prebiotics, probiotics, fatty acids, bacteriocins, bacteriophages, immunization, and antibiotic adjuvants. Sadly, none of these strategies are as effective as antibiotics against *Campylobacter* (Dai *et al*., 2020). Some of these new strategies are promising, like antibiotic adjuvants that are not themselves antibacterial but when combined with antibiotics can be improving the utility of existing antibiotic. Also promising strategies are N-glycan based vaccine, bacterin and phage therapy. The N-glucan vaccine has been showing promising results for preventing *Campylobacter* colonization in both layer chickens and broiler chickens. The vaccine is made of a conserved glycan, so it is expected to provide broad protection against different *C. jejuni* strains. More information and field trials are needed to prove the efficacity of the N-glycan vaccine.

Bacteriocins are small peptides that are in bacterial origin. This has antibacterial activities because they can disrupt the bacterial membrane. Bacteriocins have been given promising results in trials (Dai *et al*., 2020). There was drastic reduction of *C. jejuni* colonization in chicken compared with untreated group (Stern *et al*., 2006). Bacteriophage therapy is efficient because bacteriophages are bacterial viruses. They can infect and lyse bacterial cells. There have been promising results in trials with bacteriophages (Dai *et al*., 2020).

On consumer level, preventing the spreading of campylobacteriosis can be stopped by proper cooking and good kitchen hygiene (WHO, 2020b).

2. AIMS OF THE STUDY

The aim of the study is to determine the antimicrobial resistance of *Campylobacter jejuni* and *Campylobacter coli* isolated from fresh broiler chicken meat of Estonian, Latvian, and Lithuanian origin purchased at Estonian retail level, and among human clinical isolates of *Campylobacter jejuni* and *Campylobacter coli*.

3. MATERIALS AND METHODS

3.1. Sample material

The samples were collected from the biggest Estonian supermarket retail outlets in Tartu where meat is sold for domestic consumption. Estonia, Latvia, Lithuania represents the most common origins of the broiler chicken meat available in the Estonian retail market. A total of 429 fresh broiler chicken meat samples were collected on a monthly basis between September 2018 and October 2019. Altogether 163 samples were of Estonian origin and the number of Latvian and Lithuanian origin fresh broiler chicken samples was 133 for both countries separately. Related with positive samples, altogether 46 broiler chicken meat origin isolates were selected to perform the minimal inhibitory concentration (MIC) testing. Isolation of the campylobacters was performed in microbiology laboratory of the Chair of Food Hygiene and Veterinary Public Health of the Estonian University of Life Sciences. MIC testing was performed in Veterinary and Food Laboratory in Tartu. *Campylobacter* spp. human isolates (n = 15) of the present study were obtained in co-operation with Central Laboratory of East-Tallinn Central Hospital.

3.2. Minimal inhibitory concentration testing

Campylobacter isolates were tested against erythromycin, ciprofloxacin, tetracycline, streptomycin, gentamicin and nalidixic acid for MICs via the EUCAMP2 panel (TREK diagnostic Systems Ltd., East Grinstead, United Kingdom) as described by the manufacturer. Briefly, the *Campylobacter* isolates were first cultured on Brucella blood agar (Oxoid; Basingstoke, Hampshire, England) and incubated at 41.5 ± 0.5 ºC for 48 h. A loopful (1µl) of bacterial growth was transferred to 10 ml of cation adjusted Mueller-Hinton (CAMHB) broth (Oxoid; Basingstoke, Hampshire, England) that contained 5% of laked horse blood (Oxoid, Basingstoke, Hampshire, England) and incubated at 37 ± 1 ºC under microaerobic conditions for 20 ± 2 hours to achieve a level of approximation 108 CFU/ml. After incubation, the bacterial suspension was diluted to 106 CFU/ml such that 100 µl of bacterial suspension was inoculated into the 10 ml of cation adjusted Mueller-Hinton broth containing the laked horse blood. Next, 100 µl of bacterial suspension was transferred into each well of microtiter plates. The plates were incubated at 37 ± 1 ºC for 40-48 h in microaerobic conditions. The MIC was determined as the lowest concentration that completely inhibited visible growth of the campylobacters in accordance with the instructions provided by the test manufacturer. Control of the purity of the bacterial suspension was performed by plating 10 µl of bacterial suspension on Brucella agar and incubating at 37 °C for 40-48 h in microaerobic conditions. The density of the bacterial suspension was controlled by transferring 100 µl of the final bacterial suspension into 10 ml of Ringer's solution, mixing well, and then plating 50 µl of bacterial suspension on Brucella agar and incubating at 37 °C for 40-48 h in microaerobic conditions. Colony counts from 50 to 250 per plate were accepted. *C. jejuni* ATCC 33560 was used as a control strain.

Epidemiological cut-off values for *C. jejuni* as recommended by EUCAST (2019) were used to evaluate susceptibility. *C. jejuni* was considered to be resistant when the MIC values were as follows: erythromycin >4 µg/ml, ciprofloxacin >0.5 µg/ml, tetracycline >1 µg/ml, streptomycin >4 µg/ml, nalidixic acid >16 µg/ml and gentamicin >2 µg/ml.

Epidemiological cut-off values for *C. coli* as recommended by EUCAST (2019) were used to evaluate susceptibility. *C. coli* was considered to be resistant when the MIC values were as follows: 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.

3.3. Statistical analysis

The Czekanowski proportional similarity index (PSI) was calculated using Excel (Microsoft Corporation, Washington, USA) to compare AMR profiles from Latvian, Lithuanian broiler chicken meat and Estonian clinical (human) *Campylobacter* spp. isolates (Rosef *et al*., 1985). PSI values range from 1 (for identical frequency distribution) to 0 (distribution with no common AMR profiles).

4. RESULTS

MIC tests were made to find antimicrobial resistance for the following antibiotics: erythromycin, ciprofloxacin, tetracycline, streptomycin, gentamicin and nalidixic acid. Samples were taken from Lithuanian, Latvian, and Estonian fresh broiler chicken meat at Estonian retail level, additionally clinical *Campylobacter* isolates related with human infection cases were analysed. The results showed that overall resistance to ciprofloxacin was 90.2% (Table 2) and the same proportion of the isolates were resistant to nalidixic acid. Overall resistance against erythromycin was 6.6%. Resistance to tetracycline was 57.4% and to streptomycin 42.6%. All *Campylobacter* isolates of the present study were sensitive against gentamicin. High proportion (26.2%, n=16) of the *Campylobacter* isolates were multiresistant (resistant to 3 or more unrelated antibiotics).

Among Latvian broiler chicken meat origin *Campylobacter* isolates there was 100.0% of resistance to ciprofloxacin and nalidixic acid. Furthermore, Lithuanian broiler chicken meat origin *Campylobacter* isolates were 100.0% resistant to ciprofloxacin and nalidixic acid. Because of very low *Campylobacter* occurrence in Estonian origin fresh broiler chicken meat, only four isolates were found, and they were all sensitive to studied antimicrobials. Among Estonian human origin *Campylobacter* isolates, the resistance to ciprofloxacin was 86.7% and to tetracycline 80.0%; to nalidixic acid 86.7% and to streptomycin 26.7%. Four (26.7%) human origin *Campylobacter* isolates were multiresistant to studied antimicrobials (Table 2). Very high resistance (76.9%) to tetracycline was found for Lithuanian broiler chicken meat origin *Campylobacter* isolates, and for Latvian origin isolates it was lower, 18.8%. Figure 1 illustrates the resistance against different antibiotics between Latvian, Lithuanian, and Estonian broiler chicken meat origin, and Estonian human origin *Campylobacter* isolates.

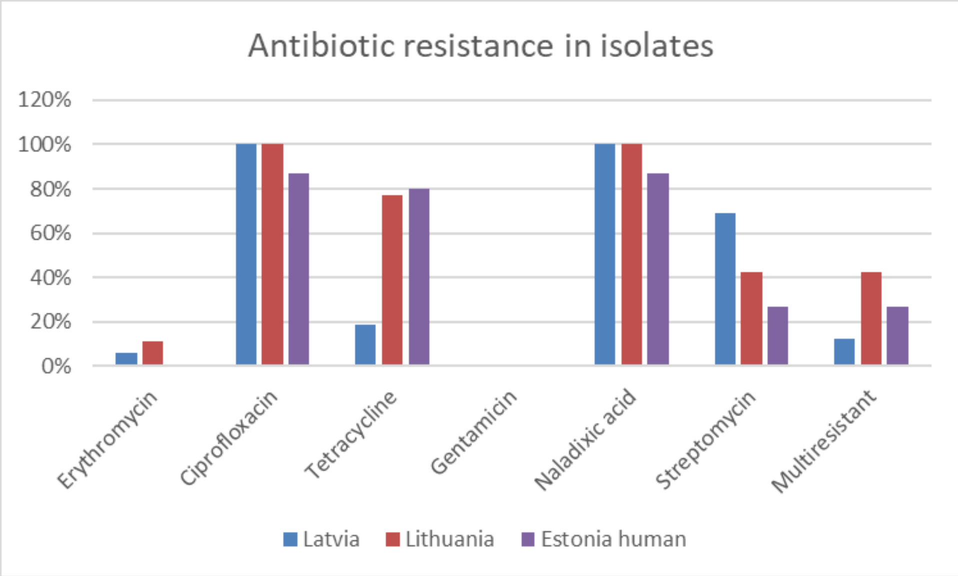

Figure 1. Antibiotic resistance percentage among broiler chicken isolates of Latvian and Lithuanian origin and Estonian human isolates

Table 1 shows the distribution of the MIC values for the 46 broiler chicken meat *Campylobacter* isolates and for 15 human *Campylobacter* isolates obtained from human campylobacteriosis cases. Resistant for one antibiotic were eight *C. jejuni* and two *C. coli* isolates. All the *C. coli* isolates were resistant to at least one antimicrobial. In total six *C. jejuni* isolates were sensitive to all antimicrobials. Resistant to two antimicrobials were 22 *C. jejuni* isolates from *C. jejuni* and six *C. coli* isolates. Proportion of *C. jejuni* and *C. coli* isolates in present study was 75% and 25%, respectively. All *C. coli* isolates were resistant to nalidixic acid and ciprofloxacin. Almost all (87%) *C. jejuni* isolates were resistant to nalidixic acid and ciprofloxacin. *C. jejuni* was sensitive to erythromycin and gentamycin. Four *C. coli* isolates from total of 15 were resistant against erythromycin. Resistant against three unrelated antimicrobials for *C. jejuni* and *C. coli* was 22 % and 47 %, respectively.

Most (80%) of the *Campylobacter* human were *C. jejuni* and others were determined to be *C. coli*.

Table 1. Minimal inhibitory concentrations[a] for *Campylobacter jejuni* and *Campylobacter coli* isolates from broiler chicken meat[b] and human origin[c]

No. of isolates	**AA[d]**	**No. of isolates with MIC value (µg/ml) of**										
		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 *Campylobacter* strains with MIC values exceeding the maximum concentration range of EUCAMP2 panel
[a]MIC values for isolates were evaluated according to manufacturer instructions (TREK diagnostic Systems Ltd., East Grinstead, United Kingdom)
Solid vertical lines indicate cut-off values (breakpoints between sensitive and resistant strains) 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 at Estonian retail level 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

In table 2, the antimicrobial resistance data of 61 *Campylobacter* isolates is presented. Because of very low *Campylobacter* occurrence in Estonian origin fresh broiler chicken meat only 4 isolates were obtained, and all of these were sensitive to all studied antimicrobials. Out of 15 Estonian human *Campylobacter* isolates, only two were sensitive to all antimicrobials. In total 13 Estonian human *Campylobacter* isolates were resistant to nalidixic acid and ciprofloxacin. All Estonian human isolates were sensitive to erythromycin and gentamicin. There was resistance against tetracycline (80%) and streptomycin (26.7%) among Estonian human isolates. For Lithuanian and Latvian broiler chicken meat isolates, there was 100% resistance to one or more antibiotics. Two (12.5%) Latvian isolates were multidrug resistant and multidrug resistance was observed for 10 (38.5%) Lithuanian broiler chicken meat origin *Campylobacter* isolates (38.5%). Gentamicin was the only antibiotic to which all the isolates were sensitive. In total, 16 (26.2%) isolates were multidrug resistant and 55 (90.2%) of isolates were resistant to one or more antibiotics.

Table 2. The antibiotic resistance of *Campylobacter* spp. isolated from fresh broiler chicken meat at Estonian retail in 2018-2019

Antibiotic	No. of resistant isolates (%)				
	Estonia n=4	Latvia n=16	Lithuania n=26	Human, EST 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)
Sensitive to all six	4 (100)	0 (0)	0 (0)	2 (13.3)	6 (9.8%)
Resistant to one or more	0 (0)	16 (100)	26 (100)	13 (86.7)	55 (90.2)
Multidrug resistant	0 (0)	2 (12.5)	10 (38.5)	4 (26.7)	16 (26.2%)

In table 3 antimicrobial resistance phenotypes are presented and phenotype proportions are calculated using only resistant isolates. In present study three (7.1% from resistant isolates) broiler chicken meat origin *Campylobacter* isolates were simultaneously resistant against four unrelated antimicrobials, which means multiresistance. Most commonly broiler chicken meat origin *Campylobacter* isolates were resistant to ciprofloxacin, nalidixic acid, tetracycline and streptomycin (23.8%). These strains are multiresistant as they were resistant against three unrelated antimicrobials. The same proportion of the *Campylobacter* isolates were resistant to ciprofloxacin, nalidixic acid and tetracycline. Second largest antimicrobial resistance phenotype group was containing *Campylobacter* isolates which were simultaneously resistant against ciprofloxacin and Nalidixic acid and same proportion (21.4%) were simultaneously resistant against ciprofloxacin, nalidixic acid and streptomycin.

Human clinical samples were most commonly (61.5%) simultaneously resistant to ciprofloxacin, nalidixic acid and tetracycline. Second largest (30.8%) resistance phenotype group was aforementioned antibiotics plus streptomycin. One isolate (7.7%) was resistant to both fluoroquinolones (nalidixic acid and ciprofloxacin).

Resistance against one or more antibiotics was found for 42 (91.3%) broiler chicken *Campylobacter* isolates and for 13 (86.7%) human *Campylobacter* isolates. Only four (8.7%) broiler chicken meat isolates and two (13.3%) human *Campylobacter* isolates were sensitive against all antibiotics.

Table 3. Antimicrobial resistance phenotypes among *Campylobacter jejuni* and *Campylobacter coli* isolates from broiler chicken meat* and human clinical samples

Antimicrobial resistance phenotype[a]	Broiler chicken meat (n=46)		Human clinical samples (n=15)	
	No	Proportion**, %	No	Proportion**, %
CI/NA/TC/SM/EM	3	7.1	-	-
CI/NA/TC/SM	10	23.8	4	30.8
CI/NA/TC	10	23.8	8	61.5
CI/NA/SM	9	21.4	-	-
CI/NA/EM	1	2.4	-	-
CI/NA	9	21.4	1	7.7
Resistant to one or more antimicrobials	42	91.3	13	86.7
Sensitive for all antimicrobials	4	8.7	2	13.3

*All countries included (Estonia, Latvia, Lithuania)

**Antimicrobial resistance phenotype proportions are calculated using only resistant isolates

[a]Antimicrobial agents: EM, Erythromycin; CI, Ciprofloxacin; TC, Tetracycline; SM, Streptomycin; GM, Gentamicin; NA, Nalidixic acid

-, Not detected

In table 4 the resistance phenotypes of country of origin can be observed. In both Latvia and Lithuania, resistance to one or more antimicrobials was 100% which means that all the *Campylobacter* isolates were resistant at least to one antimicrobial. Among human origin *Campylobacter* isolates 86.7% were resistant to one or more antimicrobials. Estonian broiler chicken isolates were the only ones that were not resistant at all.

Those isolates which had simultaneous resistance against four unrelated antimicrobials were all Lithuanian broiler chicken meat origin. In addition, simultaneous resistance against three unrelated antimicrobials was at highest proportion (30.8%) for Lithuanian origin *Campylobacter* strains. Interestingly, the same proportion (30.8%) resistance phenotype with same antimicrobials was found for Estonian human origin *Campylobacter* isolates.

From table 4 we can also see that simultaneous resistance against fluoroquinolones and tetracycline was in high proportion related with human *Campylobacter* isolates and lesser for broiler chicken *Campylobacter* isolates.

Table 4. Antimicrobial resistance phenotypes among *Campylobacter* spp. by country of origin

Antimicrobial resistance phenotype[a]	No. of isolates (%)				No. of isolates
	EST	LV	LT	HUMAN	
CI/NA/TC/SM/EM	-	-	3 (11.5)	-	3
CI/NA/TC/SM	-	2 (12.5)	8 (30.8)	4 (30.8)	14
CI/NA/TC	-	1 (6.2)	9 (34.6)	8 (61.5)	18
CI/NA/SM	-	9 (56.3)	-	-	9
CI/NA/EM	-	1 (6.2)	-	-	1
CI/NA	-	3 (18.8)	6 (23.1)	1 (7.7)	10
Resistant to one or more antimicrobials	-	16 (100)	26 (100)	13 (86.7)	55 (90.2)

EST, Estonia; LV, Latvia, LT, Lithuania

[a]Antimicrobial agents: EM, Erythromycin; CI, Ciprofloxacin; TC, Tetracycline; SM, Streptomycin; GM, Gentamicin; NA, Nalidixic acid

-, Not detected

According to PSI calculations it was found that AMR profiles of human clinical *Campylobacter* isolates were most similar to Lithuanian broiler chicken meat origin isolates (PSI = 0.68), followed by Latvian isolates (PSI = 0.25). PSI calculation was not done for Estonian broiler chicken meat isolates because all isolates were sensitive to all antimicrobials.

5. DISCUSSION

In this study the *Campylobacter* spp. isolates was studied for antimicrobial resistance and related phenotypes. The study showed that resistance is crossing the borders of the countries, because imported meat is sold in Estonian retail. It is very important that every country is keeping the use of antimicrobials as low as possible. Resistance is growing when antimicrobials are used widely and in wrong way. One Health is an important concept in terms of antimicrobial resistance, because everything in nature is related and antimicrobial resistance can spread from farm animals to human and *vice versa*, but also from farm animals to wild animals etc. One study found that there is also resistance towards antimicrobials in wild birds that carry *Campylobacter*. Additional argument towards everything being interconnected is that antimicrobial resistance is also spreading within wild animals that have not been treated with antimicrobials (Aksomaitiene *et al*., 2019).

In the present study we found high number of isolated *Campylobacter* strains with minimal inhibitory concentration (MIC) values exceeding the EUCAMP2 maximum concentration ranges. This is especially true for fluoroquinolones and tetracycline, and unfortunately both for broiler chicken meat and human origin *Campylobacter* isolates. Also, previous Estonian studies (Mäesaar *et al*., 2016; Praakle-Amin *et al*., 2007) showed *Campylobacter* spp. resistance against fluoroquinolones. Antimicrobial resistance has been decreasing during the years for *Campylobacter* strains isolated from Estonian broiler chicken meat products. Previously it was at high level, but present study result indicated that human origin *Campylobacter* antimicrobial resistance patterns are more similar with *Campylobacter* spp. resistance patterns found in isolates of Lithuanian origin broiler chicken meat sold in Estonia.

In Finland, antimicrobial resistance programme FINRES-Vet shows that quinolone resistance of chicken origin *C. jejuni* has significantly increased for unknown reason from 0% in 2013 to 25% in 2014 (Tuominen, 2016). Another report shows that fluoroquinolone resistance is high for human isolates as fluoroquinolone resistance was 70% in 2019 and 41% in 2020. Most of the *Campylobacter* infections come from abroad (THL, 2021).

In present study, the MIC values of chicken meat isolates are similar to those of Estonian human origin *Campylobacter* isolates. This may indicate that chicken meat is the main source of human *Campylobacter* infection. Recent Estonian source attribution study (Mäesaar *et al*., 2020) found that poultry is the main source of *Campylobacter jejuni* infections followed by cattle and wild bird. Furthermore, in present study similar antimicrobial resistance phenotype profiles and proportions of resistance are indicating the broiler chicken meat as the probable source of human *Campylobacter* infections.

Present study found that there can be higher risk to obtain antibiotic resistance *Campylobacter* isolates from Latvian and Lithuanian origin broiler chicken meat. Also, studies performed in Latvia (Meistere *et al*., 2019) and Lithuania (Aksomaitiene *et al*., 2019) have found high proportion of *Campylobacter* isolates resistant against antimicrobials including multiresistance. In present study all Latvian and Lithuanian *Campylobacter* isolates were resistant to one or more antibiotics and human *Campylobacter* isolates were 86.7% resistant to one or more antibiotics.

It is important to find a way to prevent antimicrobial resistance from spreading. Antimicrobials must be used more carefully.

Positive aspect of the present study is that Estonian origin *Campylobacter* isolates were all sensitive to all studied antimicrobials. This is probably indicating that antibiotics are not used in preventative and metaphylactic treatments nor in growth promotion. This is also in accordance with legislation associated with the use of antimicrobials in broiler chicken production.

In WHO they list critically important antimicrobials for human medicine. Fluoroquinolones are in this list. This is one of the reasons why countries need to work together and provide stricter legislation for use of antimicrobials in animal production. This is needed to assure that soon we would not run out of treatment possibilities for some diseases because of antimicrobial resistance. For treating serious *Salmonella* spp., *E. coli* and other pathogenic microorganisms related infections, fluoroquinolones are one of the few treatments available (WHO, 2019).

The complete susceptibility to targeted antimicrobial classes (ciprofloxacin, nalidixic acid, erythromycin, gentamicin, tetracycline) was detected in a report of EFSA. Among poultry isolates complete susceptibility was noted slightly higher levels in *C. jejuni* isolates compared to *C. coli*. *C. jejuni* which is major cause of gastroenteritis in humans. In EFSA report (EFSA, 2021) market difference between countries could be detected with percentages of complete susceptibility. For example, in *C. jejuni* complete susceptibility ranges from <5% in Cyprus, Latvia, Portugal and Lithuania to >70% in Finland, Sweden, Iceland and Norway. The multidrug resistance of *C. coli* (6 member states) was 8.0% in 2018 and for *C. jejuni* it was 1.3% in 2018 among 25 member states. The results have been taken in different scale. For *C. jejuni* sample size was 3519 isolates and *C. coli* there were only 339 isolates, so the results are not directly comparable. In addition, the resistance to one or two antimicrobial classes for *C. coli* was 85% and for *C. jejuni* 77.4%. According to EFSA (2021) only 21.3% of isolates were susceptible for all antimicrobials among *C. jejuni* isolates and 7.1% of *C. coli* isolates. Present study found high resistance of *Campylobacter* spp. which is important public health risk for broiler chicken meat consumers. Approach that prevention is better than treatment is very important to follow as well as high level of biosecurity at broiler chicken farm level to prevent AMR and spread of campylobacteria.

6. CONCLUSIONS

Present study found that all Estonian broiler chicken meat origin *Campylobacter* isolates were sensitive against all studied antimicrobials. Unfortunately, all Lithuanian and Latvian broiler chicken meat origin *Campylobacter* isolates were resistant to fluoroquinolones. Furthermore, it was found that among Lithuanian and Latvian origin isolates there was high resistance to tetracycline and streptomycin. Human origin *Campylobacter* isolates had similar resistance to imported broiler chicken meat origin isolates. Multiresistance was observed for 26.2% of human and broiler chicken meat origin *Campylobacter* isolates. This study showed how resistance is spreading over countries' borders. High resistance of Lithuanian and Latvian origin *Campylobacter* isolates needs further attention and corrective measures in these countries. The results of the *Campylobacter* contamination including antimicrobial resistance studies performed in Estonia indicate that the problems caused by the inappropriate use of antimicrobials extend beyond the country in which a food originates; therefore, both domestic and international interventions and agreements are required to implement common policies on antimicrobial usage and to minimize the emergence of *Campylobacter* resistance. There is need to control *Campylobacter* prevalence and the usage of antimicrobials at broiler chicken farm level more efficiently. There is need to reduce the non-essential use of antibiotics.

SUMMARY

Fresh broiler chicken meat is the most important source of *Campylobacter* spp. which causes campylobacteriosis, mostly with enteric infection symptoms in human. In the Nordic countries such as Finland, Sweden, and Norway one of the most important sources for human campylobacteriosis beside of poultry meat is also not properly treated ground water, but in Baltic countries poultry is the main source of *Campylobacter* spp. infections followed by cattle and wild bird. This study shows that the antibiotic treatment might be compromised, because *Campylobacter* spp. has become resistant to certain antibiotics. This resistance differs in different countries probably depending on their attitude towards antimicrobial usage in animal production. Situation in country doesn't stay in the countries' borders because meat is not only sold domestically but also often exported to other countries. This study showed how resistance is spreading over countries' borders. High resistance of Latvian and Lithuanian origin *Campylobacter* isolates needs further attention and corrective actions at farm level. Low *Campylobacter* prevalence of Estonian origin broiler chicken meat together with finding that the few isolated strains were all sensitive against all studied antimicrobials is showing that there is possible to achieve good results with well-designed vertically integrated management systems what is applied in Estonian large-scale broiler chicken meat production company.

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APPENDICES

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Mati Roasto	09.05.2022
Supervisor's name and signature	Date
Kadrin Meremäe	09.05.2022
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