Eesti Maaülikool
Estonian University of Life Sciences

CAMPYLOBACTER SPP. AND *LISTERIA MONOCYTOGENES* IN POULTRY PRODUCTS IN ESTONIA

CAMPYLOBACTER SPP. JA *LISTERIA MONOCYTOGENES* LINNULIHATOODETES EESTIS

KRISTI PRAAKLE

A Thesis
for applying for the degree of Doctor of Philosophy
in Veterinary Medicine and Food Science
(Food Hygiene and Food Quality)

Väitekiri
filosoofiadoktori kraadi taotlemiseks
veterinaarmeditsiini ja toiduteaduse
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CONTENTS

LIST OF ORIGINAL PUBLICATIONS

The thesis is a summary of the following three original publications (I-III), which are referred to by Roman numerals in the text. The papers are reproduced by kind permission of the publishers.

I **Praakle-Amin, K.**, Roasto, M., Korkeala, H., Hänninen, M.-L. 2007. PFGE genotyping and antimicrobial susceptibility of *Campylobacter* in retail poultry meat in Estonia. *International Journal of Food Microbiology*, 114, 105-112.

II Mäesaar, M., **Praakle, K.**, Meremäe, K., Kramarenko, T., Sõgel, J., Viltrop, A., Muutra, K., Kovalenko, K., Matt, D., Hörman, A., Hänninen, M.-L., Roasto, M. 2014. Prevalence and counts of *Campylobacter* spp. in poultry meat at retail level in Estonia. *Food Control*, 44, 72-77.

III **Praakle-Amin, K.**, Hänninen, M.-L., Korkeala, H. 2006. Prevalence and genetic characterization of *Listeria monocytogenes* in retail broiler meat in Estonia. *Journal of Food Protection,* 69, 436-440.

The contribution of the authors to the papers

Publication	Original idea and study design	Sample collection and analysis	Data analysis and interpretation	Manuscript preparation
I	**KP**, MLH	**KP**, MR	**KP**, MR, MLH, HK	**KP**, MR, MLH
II	MR, **KP**, MM	MM, MR, **KP**, KM, KAM	MM, **KP**, KM, AH, DM, JS, MLH, KK, TK, AV, MR	MM, **KP**, MLH, MR
III	**KP,** HK	**KP**	**KP**, HK, MLH	**KP**, HK

AH – Ari Hörman, AV – Arvo Viltrop, DM – Darja Matt, HK – Hannu Korkeala, JS – Jelena Sõgel, KAM – Kaisa Muutra, KK – Kaspars Kovalenko, KM – Kadrin Meremäe, **KP – Kristi Praakle**, MLH – Marja-Liisa Hänninen, MM – Mihkel Mäesaar, MR – Mati Roasto, TK – Toomas Kramarenko

ABBREVIATIONS

AFLP	Amplified fragment length polymorphism
ALOA	Agar Listeria according to Ottaviani and Agosti
a_w	Water activity
CAMP	Christine, Atkins, MunchPetersen test
CFU	Colony-forming unit
CLSI	Clinical and Laboratory Standards Institute
DNA	Deoxyribonucleic acid
EFSA	European Foof Safety Authorithy
ELISA	Enzyme-linked immunosorbent assay
EUCAST	European Committee on Antimicrobial Susceptibility Testing
FDA	Food and Drug Administration
IDF	International Dairy Federation
ISO	International Organization of Standardization
MAP	Modified atmosphere packaging
mCCDA	Modified Charcoal Cefoperazone Deoxycholate Agar
MH	Mueller-Hinton
MIC	Minimal inhibitory concentration
MLST	Multilocus sequence typing
NASBA	Nucleic acid sequence-based amplification
NMKL	Nordic Committee on Food Analysis
PALCAM	Selective plating agar containing polymyxin B, acriflavin, lithium chloride, ceftazidime, aesculin and mannitol
PCR	Polymerase chain reaction
PFGE	Pulsed-field gel electrophoresis
RAPD	Random amplification of polymorfic DNA
RTE	Ready-to-eat
TSA	Tryptic soy agar
TSB	Trypticase soy broth
TSYEA	Tryptone soy yeast extract agar
WGS	Whole genome sequencing

1. INTRODUCTION

Consumption of poultry meat has increased and will continue to increase globally. Poultry meat consumption increased by almost 126% between 1990 and 2009, from 40.173 thousand tonnes a year to 90.664 thousand tonnes a year. Also *per capita* consumption increased by almost 77% between 1990 and 2009, from 7.7 kg to 13.6 kg (Henchion *et al*., 2014). In Estonia, the poultry meat consumption in 2013 was 30.6 thousand tonnes, and presently stands at about 23 kg *per capita* (Statistics Estonia, 2014).

Campylobacteriosis, caused by thermotolerant *Campylobacter* species, is continued to be one of the most commonly reported zoonosis in humans in the European Union (EU), and *Campylobacter* is recognized as the most common causative agents of bacterial gastroenteritis in developed world (Altekruse *et al*., 1999; Rautelin and Hänninen, 2000; Wesley, 2009; EFSA, 2015). In 2013, there were 214,779 confirmed human campylobacteriosis cases, an average of 64.8 confirmed cases of campylobacteriosis per 100,000 of the EU population (EFSA, 2015). According to estimations, the actual number of cases is believed to be around nine million each year, and the total costs of campylobacteriosis to public health systems and to lost productivity in the EU is estimated to be around 2.4 billion euros a year (EFSA, 2011a).

Campylobacter may colonize the intestines of clinically healthy poultry. Therefore, after fecal contaminaton of poultry carcasses at slaughterhouse level *Campylobacter* may also be a contaminant of the poultry products at retail level. Slaughterhouse studies have shown that the main source of contamination poultry carcasses by *Campylobacter* species is the intestinal content (Wedderkopp *et al*., 2000; Newell *et al*., 2001; Berrang *et al*., 2004). The cross-contamination of the poultry carcasses may occur at scalding, evisceration and water-chilling stages which transfer the *Campylobacter* contamination to the retail level (Hue *et al*., 2010; Kudirkienė *et al*., 2011). According to the surveillance monitoring in EU member states in 2013, the level of *Campylobacter* positive broiler chicken flocks at farm and slaughterhouse level varied widely, from 0% (Italy) to 100% (Germany), and the proportion of *Campylobacter* positive fresh broiler meat samples at retail level from 0% (Czech Republic and Italy) to 74% (Luxembourg) (EFSA, 2015). In a worldwide literature survey Suzuki and Yamamoto (2009) summarized that 58% of retail poultry, on the

average, are contaminated with *Campylobacter*. Furthermore, several epidemiological case-control studies have established that ingesting undercooked poultry products significantly increase the risk for acquisition of foodborne campylobacteriosis (Eberhart-Phillips *et al*., 1997; Kramer *et al*., 2000; Studahl and Andersson, 2000; Neimann *et al*., 2003; Schönberg-Norio *et al*., 2004). Therefore, the control of *Campylobacter* in poultry and poultry meat is a major public health strategy in the prevention of human campylobacteriosis (EFSA, 2011a).

In recent years increasing numbers of antimicrobial resistant *Campylobacter* isolates have been observed. Antimicrobial resistance has emerged among *Campylobacter* mainly as a consequence of the wide use of the antimicrobial agents, especially fluoroquinolones, macrolides, and tetracyclines in food animal production, and also in human medicine (Endtz *et al*., 1991; Piddock *et al*., 2000; Aarestrup and Engberg, 2001; Engberg *et al*., 2001; Moore *et al*., 2006; Alfredson and Korolik, 2007; Roasto *et al*., 2007; Rozynek *et al*., 2008; Chen *et al*., 2010; Wieczorek *et al*., 2013; EFSA, 2014b; Fraqueza *et al*., 2014; Di Giannatale *et al*., 2014). Pathogens resistant to antimicrobials are of serious concern because they might compromise the effective treatment of infections in animals and humans (EFSA, 2014b).

The consumption of foods contaminated with *Listeria monocytogenes* can result in listeriosis, an uncommon disease, but with the high fatality rate (Crim *et al*., 2014; EFSA, 2015). Listeriosis can be life-threatening to the young children, elderly, pregnant women and persons with weakened immune systems (Schlech and Acheson, 2000; Vázquez-Boland *et al*., 2001; Swaminathan and Gerner-Smidt, 2007; Bennion *et al*., 2008; Goulet *et al*., 2012; Hernandez-Milian and Payeras-Cifre, 2014). In 2013, 1,763 confirmed human cases of listeriosis were reported in EU which is 0.44 cases per 100,000 population. The EU case-fatality rate was 15.6%, and a total of 191 deaths due to listeriosis were reported (EFSA, 2015). In the United States (USA), *L. monocytogenes* accounts for about 2500 cases, 2289 hospitalizations, and 449 deaths each year, and the mortality rate (ca. 28%) remains the highest of all foodborne pathogens (Wesley, 2009).

Different foods, including poultry products, have been associated with listeriosis (Ojeniyi *et al*., 2000; Levine *et al*., 2001; Miettinen *et al*., 2001; Gudbjörnsdóttir *et al*., 2004; Cartwright *et al*., 2013). Healthy birds may shed *L. monocytogenes* in fecal material asymptomatically (Skovgaard and

Morgen, 1988). Poultry meat becomes contaminated during slaughter and processing, therefore, the prevention of poultry product contamination with *L. monocytogenes* is of major importance (Ojeniyi *et al*., 1996; Miettinen *et al*., 2001; Lundén *et al*., 2003; Rørvik *et al*., 2003).

The main goals of the present thesis were to determine the prevalence and counts of *Campylobacter* spp. and the prevalence of *L. monocytogenes* in Estonian and imported raw poultry meat, to serotype and pulsed-field gel electrophoresis (PFGE) genotype both *Campylobacter* and *L. monocytogenes* isolates, as well as to determine the antimicrobial susceptibility of the *Campylobacter* isolates.

2. REVIEW OF THE LITERATURE

2.1. *Campylobacter* spp. and campylobacteriosis

2.1.1. The genus *Campylobacter*

The genus *Campylobacter* was proposed by Sebald and Véron (1963) and belongs to the family *Campylobacteriaceae*. There are a total of 23 species and 6 subspecies validly described in this family (Nachamkin, 1999; Vandamme, 2000; On, 2001; Euzéby, 2006; Fitzgerald and Nachamkin, 2007; Humphrey *et al*., 2007; Debruyne *et al*., 2008; Lastovica and Allos, 2008; Silva *et al*., 2011; Zhou *et al*., 2013). Mostly *Campylobacter jejuni* and *C. coli* are causing infections in human. But also other species e.g. *C. lari, C. fetus, C. concisus, C. hyointestinalis, C. sputorum, C. ureolyticus, C. rectus, C. gracilis, and C. upsialiensis* have been recognized being able to cause infections in human (Tam *et al*., 2003; Ryan, 2004; Snelling *et al*., 2005; Blaser and Engberg, 2008; Lastovica and Allos, 2008; Man, 2011; EFSA, 2014a).

Campylobacter species are Gram-negative nonsporeforming rods, 0.2 to 0.8 μm wide and 0.5 to 5 μm long, curved, spiral or S-shaped cells. They generally have a single polar unsheathed flagellum at one or both ends of the cells, and are motile with a characteristic corkscrew-like motion (Snelling *et al*., 2005; Debruyne *et al*., 2008; Kay *et al*., 2011).

C. jejuni and *C. coli* can colonize the intestinal tract of many domestic and wild animals and birds. *Campylobacter* have also been isolated from humans, foods, and natural waters (Hörman *et al*., 2004; Whyte *et al*., 2004; Boes *et al*., 2005; Humphrey *et al*., 2007; Waldenström *et al*., 2007; Young *et al*., 2007; Acke *et al*., 2011; Silva *et al*., 2011; EFSA, 2014a).

2.1.2. Growth and survival conditions of *Campylobacter* spp.

Campylobacter spp. are microaerophilic, growing best in an atmosphere containing approximately 5% O_2, 10% CO_2, and 85% N_2 (Nachamkin, 1999; Garénaux *et al*., 2008). Some *Campylobacter* species may also grow under aerobic or anaerobic conditions (Vandamme, 2000). The optimum growth temperature for *C. jejuni* and *C. coli* is 42 °C, and they do not grow at temperatures below 30 °C (Lee *et al*., 1998; Humphrey *et al*., 2007, Silva *et al*., 2011). These characteristics reduce the ability of *Campylobacter* to

multiply outside of an animal host, in carcasses or meat during processing or storage (Park, 2002).

Campylobacter are sensitive to freezing and drying (Altekruse *et al*., 1999, Sampers *et al*., 2010). The death rate of *Campylobacter* is dependent on temperature. They die more rapidly on dry surface at room temperature than at refrigeration conditions (Humphrey *et al*., 2007; Silva *et al*., 2011). They can survive at refrigeration temperatures (4 °C) and in meat stored frozen (at -18 to -22 °C) for several weeks (Bhaduri and Cottrell, 2004; Murphy *et al*., 2006; El-Shibiny *et al*., 2009; Oyarzabal *et al*., 2010; Sampers *et al*., 2010). *Campylobacter* does not grow in environments with water activity (a_w) lower than 0.987 (optimal $a_{w=}$0.997), and in concentrations of sodium chloride greater than 2%. The optimum pH for their growth is 6.5-7.5 (Park, 2002; Silva *et al*., 2011).

2.1.3. Isolation and identification

For the isolation and identification of *Campylobacter* the selective media containing one or more oxygen scavengers (lysed or defibrinated blood, charcoal, ferrous iron, pyruvate, etc.) and/or selective agents (particularly antibiotics) or nonselective agar media followed by the characterization on Gram staining, typical morphology and on biochemical tests (e.g. indoxyl acetate hydrolysis, hippurate hydrolysis) for the differentiation of thermophilic *Campylobacter* species is used (Corry *et al*., 1995; Humphrey *et al*., 2007; Silva *et al*., 2011; On, 2013). Current conventional methods for detection of *Campylobacter* in foods involve a pre-enrichment in a liquid medium (e.g. Bolton broth, *Campylobacter* enrichment broth, Preston broth) before plating onto selective media (e.g. Preston agar, mCCDA – modified Charchoal Cefoperazone Deoxycholate Agar, Butzler agar) (Baylis *et al*., 2000; Silva *et al*., 2011). To supress the growth of competing organism antibiotics such as cefoperazone, cycloheximide, trimethoprim, rifampicin, vancomycin, and polymyxin B are added to enrichment media and selective agars (Corry *et al*., 1995). Most fequently for detection, isolation and enumeration of *Campylobacter* standard methods (ISO, 2006a, 2006b) are used. Bolton broth is used for the enrichment step and the suspension is incubated at 37 °C in a microaerophilic atmosphere for 4-6 h, followed by 41.5 °C for 40-48 h and plating on selective mCCDA and another agar medium (Silva *et al*., 2011).

Nowadays species level identification of *Campylobacter* is performed by the use of the polymerase chain reaction (PCR) methods. A variety of target genes e.g. 16S rRNA, 23S rRNA, *mapA*, *ceuE*, *hipO*, *glyA*, *bipA* for PCR identification of *Campylobacter* species are used (Linton *et al*., 1996; On and Jordan, 2003; Lund *et al*., 2004; Mateo *et al*., 2005; Garcia-Gil, 2013). For example, a colony multiplex PCR assay can be used for the identification and differentiation of *C. jejuni*, *C. coli*, *C. lari*, *C. upsaliensis*, and *C. fetus* subsp. *fetus* as described by Wang *et al.* (2002). According to this method the following target genes are used: *hipO* and 23S rRNA from *C. jejuni*; *glyA* from each of *C. coli*, *C. lari*, and *C. upsaliensis*; and *sapB2* from *C. fetus* subsp. *fetus*. In species identification PCR tests are favorable because biochemical tests may give variable results for different strains that belong to the same species (Siemer *et al*., 2005).

2.1.4. Subtyping

There are multiple typing methods available used for epidemiological studies, bacterial source tracking or determining the genetic diversity of pathogens isolated from different food and non-food sources. Formerly, serotyping was used for typing *C. jejuni* and *C. coli* isolates (Frost *et al*., 1998; Rautelin and Hänninen, 1999; Wassenaar and Newell, 2000). Two serotyping schemes have been developed for *Campylobacter* subtyping: the Penner scheme (Penner and Hennessy, 1980) based on heat-stabile antigens, and the Lior scheme (Lior *et al*., 1982) based on heat-labile antigens. However, there are many disadvantages in the use of serotyping methods e.g. these are technically demanding, time consuming, the availability of sera is limited, there are high numbers of untypeable strains, and the discriminative ability is not good enough (Rautelin and Hänninen, 1999; Maher *et al*., 2006).

Molecular typing methods have replaced serotyping for more than ten years. The molecular typing methods available are based on analysis of the bacterial DNA digested with specific restriction enzymes, on PCR amplification of particular genetic targets, or on the identification of DNA sequence polymorphisms (Foley *et al*., 2009; Sabat *et al*., 2013). Further on, whole genome sequencing (WGS) using next generation sequencing technologies are more extensively used because of the wide use in epidemiology. Analysis of WGS data will provide a much better understanding of pathogens, their evolution, phylogeny and its virulence (Bryant *et al*., 2012; Pendleton *et al*., 2013; Revez *et al*., 2014a). As WGS

data and bioinformatics tools for analysis become more accessible, more accurate geospatial information on the origin of strains and investigating outbreak isolates will become available (Parkhill and Wren, 2011). Recently, WGS was applied to study a milk-borne outbreak isolates of *C. jejuni* and the criteria useful in the assessment of the relatedness of the isolates was developed (Revez *et al*., 2014b).

Sensitive subtyping of *Campylobacter* isolates remains an important requirement for epidemiological studies, especially for tracing the sources and routes of potential transmission to humans, to identify and monitor both temporally and spatially associated strains with important phenotypic characteristics, to develop strategies to control organisms within the food chain, and because of some other epidemiological reasons (de Boer *et al*., 2000; Nielsen *et al*., 2000; Wassenaar and Newell, 2000; Lienau *et al*., 2007; Behringer *et al*., 2011; Ahmed *et al*., 2012; Taboada *et al*., 2013).

A widely used method for molecular typing of *C. jejuni* and *C. coli* is PFGE which is still known as an "golden standard" even while some other subtyping methods including WGS have been proved to be more discriminatory (Gibson *et al*., 1997; Hänninen *et al*., 2000; Ribot *et al*., 2001; Swaminathan *et al*., 2001; Kärenlampi *et al*., 2003; Foley *et al*., 2009; Thakur *et al*., 2009; Taboada *et al*., 2013). Furthermore, PFGE is widely used because of standardized protocols and equipment in molecular subtyping network e.g. PulseNet (Swaminathan *et al*., 2001), and the PFGE equipment and methodological skills to perform this typing exist in most laboratories. PFGE is based on gel electrophoresis of restriction digested genomic DNA, and it appers to be a highly discriminatory method especially when used with the two restriction enzymes, *Sma*I and *Sac*II/*Kpn*I (Gibson *et al*., 1997; Hänninen *et al*., 2003; Michaud *et al*., 2001).

Multilocus sequence typing (MLST) is the method used nowadays in population studies as well as for source tracing using mathematical modelling. MLST is unambiguos, versatile, discriminatory, and has capability of detecting mixed cultures of *Campylobacter*, genetic exchange, and recombination between *Campylobacter* species (Miller *et al*., 2005b; Dingle *et al*., 2008; de Haan *et al*., 2013). MLST data is based of sequencing data of seven conserved loci, and MLST data is more often produced by WGS (Kovanen *et al*., 2014).

2.1.5. Antimicrobial resistance of *Campylobacter* spp.

In recent years increasing numbers of resistant *Campylobacter* isolates have been observed. Antimicrobial resistance has emerged among *Campylobacter* mainly as a consequence of the wide use of the antimicrobials, especially fluoroquinolones, macrolides, and tetracyclines in food animal production, and also in human medicine (Aarestrup and Engberg, 2001; Hakanen *et al.*, 2003; Moore *et al.*, 2006; Alfredson and Korolik, 2007; Roasto *et al.*, 2007; EFSA, 2014b; Fraqueza *et al.*, 2014; Kovalenko *et al.*, 2014). Generally, human *Campylobacter* infections are self-limiting and majority of patients require no antimicrobial treatment. However, severe, prolonged or systemic infections do require treatment. Macrolides (e.g. erythromycin) are the first-choice of drug, and fluoroquinolones (e.g. ciprofloxacin) are often the second-choice of drug recommended for the treatment of human campylobacteriosis (Allos, 2001; Engberg *et al.*, 2001; Zhao *et al.*, 2010). Various European countries have implemented national monitoring programs to assess susceptibility to antibiotics among *Campylobacter*, and a wide variety in the distribution of resistant *C. jejuni* isolates among different countries have been shown (de Jong *et al.*, 2012; EFSA, 2014b).

Standardized susceptibility testing methods have been developed for *C. jejuni* and *C. coli*. The common methods for antimicrobial susceptibility testing are the minimal inhibitory concentration (MIC) tests performed as agar dilutions or broth microdilutions. Additionally, disk diffusion methods might be used which also include the commercial E-test® (McDermott *et al.*, 2004, 2005; CLSI, 2007). The commercial broth microdilution method includes the VetMic® testing system (Ge *et al.*, 2013). The protocols have been published as the standards of the Clinical and Laboratory Standards Institute (CLSI, 2014) in the USA and the European Committee on Antimicrobial Susceptibility Testing (EUCAST, 2014) in Europe.

Epidemiological cut-off values are based on wild-type of *Campylobacter* isolates and clinical breakpoint values are derived from data of human *Campylobacter* isolates. According to EUCAST there are some differences in epidemiological cut-off values for *C. jejuni* and *C. coli*. The difference is for erythromycin and tetracycline where lower MIC values for resistance are given to *C. jejuni* (EURL-AR, 2012). CLSI breakpoints for *Campylobacter* are available for ciprofloxacin, doxycycline, erythromycin, and tetracycline (CLSI, 2011).

2.1.6. Campylobacteriosis

Campylobacteriosis, caused mostly by *C. jejuni* and *C. coli*, is continuously the most commonly reported zoonosis in humans in the EU, and *Campylobacter* is recognized as the most common causative agents of bacterial gastroenteritis in the world (Altekruse *et al*., 1999; Rautelin and Hänninen, 2000; Wesley, 2009; EFSA, 2015). In 2013, there were 214,779 confirmed human campylobacteriosis cases with an average of 64.8 confirmed cases of campylobacteriosis per 100,000 of the EU population (EFSA, 2015). According to the data of Estonian Health Board (Estonian Health Board, 2015) the average notification rate of human campylobacteriosis cases in Estonia was 22.15 per 100,000 inhabitants from 2011 to 2014.

The species mostly associated with human infection are *C. jejuni* followed by *C. coli* and *C. lari*, but other *Campylobacter* species are also known to cause infections in humans (EFSA, 2014a). Most typically, infection with *C. jejuni* results in an acute, self-limited gastrointestinal illness characterized by diarrhea, fever, and abdominal cramps. In most patients, the diarrhea is either loose and watery or grossly bloody. Local complications of *Campylobacter* infections occur as a result of direct spread from the gastrointestinal tract and can include cholecystitis, pancreatitis, peritonitis, and massive gastrointestinal hemorrhage. Extraintestinal manifestations of *Campylobacter* infection are quite rare and may include meningitis, endocarditis, septic arthritis, osteomyelitis, and neonatal sepsis (Allos, 2001; Pires, 2014). In some cases *Campylobacter* infection may cause Guillain-Barré and Miller Fisher syndrome that can lead to serious health issues and in very seldom cases even death (Fica *et al*., 2011; Kuwabara, 2011).

The risk factors for human *Campylobacter* infections are considered to be handling or eating poultry meat, eating raw or undercooked meat, drinking unpasteurized milk or untreated water, swimming in natural waters, contact with domestic animals, and travelling (Rodrigues *et al*., 2001; Kapperud *et al*., 2003; Neimann *et al*., 2003; Ekdahl and Andersson, 2004; Friedman *et al*., 2004; Schönberg-Norio *et al*., 2004; Wingstrand *et al*., 2006; Heuvelink *et al*., 2009; Ricotta *et al*., 2014). The European Food Safety Authorithy (EFSA) scientific report concluded that the handling, preparation and consumption of broiler meat may account for 20% to 30% of all human campylobacteriosis cases, and 50% to 80% of human

Campylobacter infections may be attributed to the chicken reservoir as a whole (EFSA, 2011a). Therefore, the control of *Campylobacter* in poultry and poultry meat is a major public health strategy in the prevention of human campylobacteriosis.

2.2. *Listeria monocytogenes* and listeriosis

2.2.1. The genus *Listeria* and species *L. monocytogenes*

L. monocytogenes was found for the first time in 1911 in rabbits in Sweden (Hülphers, 1911). The bacterium was described by Murray *et al.* (1926) in rabbits and guinea-pigs in research laboratories, who named it *Bacterium monocytogenes*. In 1940 Pirie changed the genus name to *Listeria* (Pirie, 1940). The genus *Listeria* belongs to the family *Listeriaceae* and currently includes the species *L. monocytogenes*, *L. ivanovii*, *L. grayi*, *L. innocua*, *L. welshimeri*, *L. seeligeri*, *L. marthii*, *L. rocourtiae*, *L. weihenstephanensis*, *L. fleischmannii*, *L. floridensis*, *L. aquatica*, *L. cornellensis*, *L. riparia*, and *L. grandensis* (Rocourt and Buchrieser, 2007; McLauchin *et al.*, 2009; Graves *et al.*, 2010; Leclercq *et al.*, 2010; Beretsch *et al.*, 2013; Lang Halter *et al.*, 2013; den Bakker *et al.*, 2014). Of these, *L. monocytogenes* and *L. ivanovii* are pathogenic. *L. monocytogenes* can cause disease both in humans and animals, *L. ivanovii* is principally an animal pathogen being particulary associated with listeriosis in ruminants (Low and Donachie, 1997; Vázquez-Boland *et al.*, 2001; Guillet *et al.*, 2010; EFSA, 2014a). The remaining species are regarded as nonpathogenic.

L. monocytogenes is a small (0.4–0.5 μm in diameter and 1–2 μm in length), Gram-positive rod with rounded ends, and a facultative anaerobe with no capsule. *L. monocytogenes* is catalase-positive, oxidase-negative, and motile at 20 to 25 °C due to peritrichous flagella (Farber and Peterkin, 1991; Rocourt and Buchrieser, 2007; McLauclin and Rees, 2009).

2.2.2. Ecology and growth conditions of *L. monocytogenes*

L. monocytogenes is widespread in the environment including plant material, soil, water, sewage, and has been found in human, animal, and poultry feces (Skovgaard and Morgen, 1988; Farber and Peterkin, 1991; Low and Donachie, 1997).

L. monocytogenes, a psychrotrophic bacterium, is able to grow at low temperatures and can survive freezing. The growth temperatures range from <0 to 45 °C, but the optimal growth temperature is between 30 to 37 °C (Junttila *et al.*, 1988; Walker *et al.*, 1990; McLauchlin and Rees, 2009). *L. monocytogenes* can survive or grow in range of pH values between 4.3 and 9.6, at salt concentrations of up to 14% (Farber *et al.*, 1989, Parish and Higgins, 1989; Walker *et al.*, 1990; Low and Donachie, 1997), and at low water activity (a_w 0.90) (Nolan *et al.*, 1992; Marth, 1993). *L. monocytogenes* is a facultative anaerobe, and is able to grow in modified atmosphere packaging (MAP) and vacuum packaging conditions (Lungu *et al.*, 2009). Not all the strains of *L. monocytogenes* are similar, because they have differences in adaptation to environments, resistance to adverse conditions, and virulence. Elimination of the *Listeria* from the food chain is very hard to achieve, but contamination needs to be minimized and growth to high numbers prevented (Hellström, 2011).

2.2.3. Isolation and identification

L. monocytogenes is an important foodborne pathogen, and is widely tested in food, environmental and clinical samples. For the isolation and identification of *L. monocytogenes* the conventional culture methods based on selective enrichment and plating on selective media followed by the characterization of *Listeria* spp. based on colony morphology, sugar fermentation and haemolytic properties are involved (Farber and Peterkin, 1991; Bille *et al.*, 1992; Gasanov *et al.*, 2005; Donnelly and Nyachuba, 2007; Jadhav *et al.*, 2012). Several international standard methods e.g. from the Food and Drug Administration (FDA, 2003), the International Dairy Federation (IDF, 1995) and the International Organization for Standardization (ISO, 1996a, 1996b) are available for detection of *L. monocytogenes* in foods. In these methods, the Gram-positive, catalase-positive, oxidase-negative rods with tumbling motility at 25 °C, showing a narrow β-haemolysis on blood-media, fermenting of rhamnose but not xylose, and CAMP (Christine, Atkins, MunchPetersen test) positive with *Staphylococcus aureus* and negative with *Rhodococcus equi* are verified as *L. monocytogenes* (Farber and Peterkin, 1991; McLauclin and Rees, 2009*)*. To identify *Listeria* isolates, commercial biochemical test such as API *Listeria* is used (Bille *et al.*, 1992).

Currently in Estonia for the detection and isolation of *L. monocytogenes* EVS-EN ISO 11290-1:2000/A1:2004 method is used. Examination for *L.*

monocytogenes includes a primary and secondary enrichment. Samples are incubated in half Fraser broth at 30 °C for 24 h. After incubation, 0.1 ml is transferred to the tube containing 10 ml of full-strength Fraser broth, and incubated at 37 °C for 48 h. After that, the half- and full-strength Fraser broths are plated-out on ALOA and PALCAM agar. Selective agar plates are incubated at 37 °C for 24 to 48 h. Typical colonies (n = 5) presumed to be *Listeria* spp. are streaked from both agars onto the tryptone soya yeast extract agar (TSYEA), and plates are incubated at 37 °C for 24 h. The confirmation tests are performed using the pure culture obtained from TSYEA. Isolates that are catalase-positive, Gram-positive and with characteristic tumbling motility are inoculated on 5% sheep blood agar plates to determine the haemolytic reaction, respectively β-haemolysis for *L. monocytogenes*. For following confirmation carbohydrate utilization and CAMP tests are performed. All confirmed *L. monocytogenes* isolates are usually stored at -80 °C in bacterial protect tubes.

Conventional methods are still widely used, especially in national food and veterinary laboratories. Additionally, enzyme-linked immunosorbent assay (ELISA) can be used. However, the current trend is towards the use of DNA-based methods e.g. PCR, DNA hybridization, and nucleic acid sequence-based amplification (NASBA) (Gasanov *et al.*, 2005; Liu, 2006).

2.2.4. Subtyping

To study *L. monocytogenes* isolates both conventional and molecular methods can be used. Serotyping, a convetional phenothyping method, has been a classical tool in subtyping of *L. monocytgenes* (Wagner and Allerberger, 2003). Strains of *L. monocytogenes* are divided into serotypes based on somatic (O) and flagellar (H) antigens: 1/2a, 1/2b, 1/2c, 3a, 3b, 3c, 4a, 4ab, 4b, 4c, 4d, 4e and 7 (Seeliger and Höhne, 1979, Schönberg *et al.*, 1996). The majority (95%) of human listeriosis cases are caused by serotypes 1/2a, 1/2b and 4b, and food commonly harbours serotypes 1/2a and 1/2c (Vázquez-Boland *et al.*, 2001; Graves *et al.*, 2007). Traditional analysis of *L. monocytogenes* by serotyping is time consuming, laborius, and the reproducibility of serotyping is not always satisfactory (Doumith *et al.*, 2004; Graves *et al.*, 2007). PCR-based serotyping assays, generally multiplex PCR, have been developed to identify *L. monocytogenes* serogrups rapidly and efficiently (Doumith *et al.*, 2004; Jadhav *et al.*, 2012; Lambertz *et al.*, 2013; Salazar *et al.*, 2015).

Serotyping alone is of limited value in epidemiological investigations (Farber and Peterkin, 1991; Graves *et al*., 2007), and therefore, molecular typing methods have been used to subtype the strains of *L. monocytogenes* (Wiedmann, 2002; Gasanov *et al*., 2005; Liu, 2006; Jadhav *et al*., 2012). Subtyping methods can be based on use of restrictions enzymes e.g. PFGE, ribotyping, amplified fragment length polymorphism (AFLP), and the use of PCR e.g. random amplification of polymorphic DNA (RAPD), MLST.

PFGE is found to be a highly reproducible, discriminatory, effective molecular typing method, and has been used successfully to characterize *L. monocytogenes* in different studies (Buchrieser *et al*., 1993; Ojeniyi *et al*., 1996; Lyytikäinen *et al*., 2000; Miettinen *et al*., 2001; Autio *et al*., 2002; Lundén *et al*., 2003; Rørvik *et al*., 2003; Lukinmaa *et al*., 2004; Liu, 2006; Bērziņš *et al*., 2007). PFGE is standardized and used in networks e.g. PulseNet in USA for public health and food laboratories to routinely subtype foodborne pathogenic bacteria and rapidly detect foodborne disease clusters which may have the common source (Martin *et al*., 2006; Graves *et al*., 2007).

Ribotyping is based on the restriction of total genomic DNA followed by electrophoretic separation of the fragments obtained. This method has been used in epidemiological and *L. monocytogenes* transmission studies (Wiedmann, 2002; Aarinsalo *et al*., 2003; Klaeboe *et al*., 2006; Graves *et al*., 2007; Matloob and Griffiths, 2014).

The AFLP method is based on the selective PCR amplification of restriction fragments from a total digest of genomic DNA (Vos *et al*., 1995). The method has been used for characterization of *L. monocytogenes* strains and for identification of *Listeria* species (Autio *et al*., 2003; Keto-Timonen *et al*., 2003; Fonnesbech Vogel *et al*., 2004; Graves *et al*., 2007; Lomonaco *et al*., 2011).

RAPD is based on the amplification of random DNA segments with single primers of arbitrary nucleotide sequence (Williams *et al*., 1990; Lawrence *et al*., 1993), and has been used in epidemiological, listeriosis outbreak, and food industry studies (Destro *et al*., 1996; Fonnesbech Vogel *et al*., 2004; Martinez *et al*., 2003; Graves *et al*., 2007).

In MLST, DNA sequencing of multiple genes or gene fragments is used to differentiate bacterial subtypes and to determine the genetic relatedness of isolates (Maiden *et al*., 1998; Wiedmann, 2002; Graves *et al*., 2007). MLST has been shown to be highly discriminatory for *L. monocytogenes* (Salcedo *et al*., 2003). It has been used to characterize large collection of isolates (e.g. in phylogenetics studies), in epidemiological studies, and for studies on the molecular evolution (Cai *et al*., 2002; Revazishvili *et al*., 2004; Ragon *et al*., 2008; Knabel *et al*., 2012; Haase *et al*., 2014).

WGS is used to subtype *L monocytogenes* isolates and can be used in epidemiological investigations (Hyytiä-Trees *et al*., 2007; Gilmour *et al*., 2010; Chen *et al*., 2011; Cao *et al*., 2015). Currently, high-throughput genome sequencing technologies are largely used as a research tool, and increasingly introduced in the clinics (van El *et al*., 2013).

2.2.5. Listeriosis

Human listeriosis cases are almost exclusively caused by *L. monocytogenes*, and the bacterium has emerged as one of the most important foodborne pathogen (Schlech and Acheson, 2000; Liu, 2006; Crim *et al*., 2014; EFSA, 2015). The incidence of listeriosis is low (<1 case per 100,000 inhabitants), but the mortality rate of the infection is high, 20 to 30% (Vázquez-Boland *et al*., 2001; Painter and Slutsker, 2007; Bennion *et al*., 2008; Wesley, 2009; Hernandez-Milian and Payeras-Cifre, 2014). In 2013, 1763 confirmed human cases of listeriosis, 0.44 cases per 100,000 population, were reported in EU which is an 8.6% increase compared with year 2012. The EU case-fatality rate was 15.6%, and a total of 191 deaths due to listeriosis were reported in 2013 (EFSA, 2015). In the USA, annually *L. monocytogenes* accounts for about 2500 cases, 2289 hospitalizations, 449 deaths, and the mortality rate (ca. 28%) remains the highest of all foodborne pathogens (Wesley, 2009). According to the data of Estonian Health Board (2015) the average notification rate of human listeriosis cases in Estonia was 0.15 per 100,000 inhabitants from 2011 to 2014. In 2014 in Estonia one *L. monocytogenes* caused meningitis infection, but no death cases, was reported.

Listeriosis can occur as sporadic disease or as outbreak (Swaminathan and Gerner-Smidt, 2007). Three serotypes, 1/2a, 1/2b, 4b, are associated with the majority (95%) of human listeriosis cases, and food commonly harbours serotypes 1/2a and 1/2c (Farber and Peterkin, 1991; Rocourt and

Bille, 1997; Vázquez-Boland *et al*., 2001; Graves *et al*., 2007). In Estonia, according to the study of Kramarenko *et al*. (2013) the most prevalent *L. monocytogenes* serotype in different foods was 1/2a (74%), followed by 1/2b (7%), 1/2c (7%), 4b (8%), and 4d (4%). However, there are no reports about human origin *L. monocytogenes* sero- and sequencetypes in association with human listeriosis cases in Estonia.

L. monocytogenes can cause both invasive and non-invasive infections. The invasive listeriosis causes infections of the central nervous system and bacteremia in persons of high risk group e.g. elderly and very young people, those with immunocompromising conditions and pregnant women (Rocourt and Bille, 1997; Schlech and Acheson, 2000; Vázquez-Boland *et al*., 2001; Norton and Braden, 2007; Swaminathan and Gerner-Smidt, 2007; Bennion *et al*., 2008; Goulet *et al*., 2012; EFSA, 2014a; Hernandez-Milian and Payeras-Cifre, 2014). The symptoms of the non-invasive listeriosis are associated with gastrointestinal illness (Schlech and Acheson, 2000).

The infectious dose for listeriosis remains unclear, but according to epidemiological data it is suspected to be high, as the contamination level in foods responsible for listeriosis cases are typically more than 10^4 per gram of food (Ooi and Lorber, 2005). According to the Maijala *et al*. (2001) infection may also be caused by a prolonged daily consumption of contaminated food containing 10^1 to 10^5 CFU/g of *L. monocytogenes* bacteria.

2.3. *Campylobacter* spp. and *L. monocytogenes* in foods

2.3.1. Prevalence of *Campylobacter* spp. in foods

Campylobacter have been isolated from many types of foods including raw milk, beef, pork, lamb, poultry, seafood, and salads (Wilson and Moore, 1996; Eberhart-Phillips *et al*., 1997; Jacobs-Reitsma, 2000; Studahl and Andersson, 2000; Kapperud *et al*., 2003; Neimann *et al*., 2003; Humphrey *et al*., 2007; Heuvelink *et al*., 2009; Suzuki and Yamamoto, 2009; Wijnands *et al*., 2014). Many food producing animal and poultry species carry *Campylobacter* in their intestines, and foods can be contaminated during processing (Humphrey *et al*., 2007). However, most cases of foodborne campylobacteriosis are associated with handling raw poultry, eating raw or undercooked poultry meat, or cross-contamination of raw to cooked

foods (Butzler and Oosterom, 1991; Eberhart-Phillips *et al*., 1997; Tauxe *et al*., 1997; Studahl and Andersson, 2000; Kramer *et al*., 2000; Nadeau *et al*., 2002; Neimann *et al*., 2003; Humphrey *et al*., 2007; Suzuki and Yamamoto, 2009; EFSA, 2014a). Drinking unpasteurized milk has been associated with several milk-borne campylobacteriosis outbreaks (Schildt *et al*., 2006; Heuvelink *et al*., 2009).

In 2013, the prevalence of *Campylobacter* in fresh broiler meat samples varied widely in EU, from 0% (Czech Republic and Italy) to 74% (Luxembourg) (EFSA, 2015). An EU wide baseline study showed the average *Campylobacter* prevalence for broiler carcasses to be about 76% (EFSA, 2010). In a worldwide literature survey Suzuki and Yamamoto (2009) summarized that 58% of retail poultry, on the average, are contaminated with *Campylobacter*. The prevalence of *Campylobacter* in raw poultry meat in different countries is shown in Table 1.

Table 1. Prevalence of *Campylobacter* in raw poultry meat in different countries

Country or region	Prevalence (%)	Level	Reference
Argentine	93	Retail	López *et al.*, 2003
Barbados	58	Retail	Workman *et al.*, 2005
Bulgaria	76	Retail	Stoyanchev *et al.*, 2007
Cameroon	93	Slaughter	Garin *et al.*, 2012
China	2	Retail	Wang *et al.*, 2013
Denmark	24	Slaughter	Hald *et al.*, 2000
Estonia	11	Retail	Roasto *et al.*, 2011
Iceland	62	Slaughter	Stern *et al.*, 2003
Iran	63	Retail	Taremi *et al.*, 2006
Ireland	50	Retail	Whyte *et al.*, 2004
Japan	59	Retail	Suzuki and Yamamoto, 2009
Korea	68	Retail	Han *et al.*, 2007
Latvia	56	Retail	Kovalenko *et al.*, 2013
Lithuania	47	Retail	Bunevičienė *et al.*, 2010
New Caledonia	97	Slaughter	Garin *et al.*, 2012
North America	64	Retail	Suzuki and Yamamoto, 2009
Turkey	83	Retail	Savaşçi and Özdemir, 2006
USA	72	Retail	Suzuki and Yamamoto, 2009
Vietnam	15	Slaughter	Garin *et al.*, 2012
Western Europe	56	Retail	Suzuki and Yamamoto, 2009

2.3.2. Prevalence of *L. monocytogenes* in foods

L. monocytogenes has been found in different types of raw and processed foods including meat (MacGowan *et al.*, 1994; Nørrung *et al.*, 1999; Uyttendaele *et al.*, 1999b; Gudbjörnsdóttir *et al.*, 2004; Farber *et al.*, 2007; Bērziņs *et al.*, 2009; Kramarenko *et al.*, 2013), dairy (Greenwood *et al.*, 1991; MacGovan *et al.*, 1994; Miettinen *et al.*, 1999; Nørrung *et al.*, 1999; Waak *et al.*, 2002; Meyer-Broseta *et al.*, 2003; Lewis *et al.*, 2006; Kramarenko *et al.*, 2013; Ruusunen *et al.*, 2013), fish (MacGovan *et al.*, 1994; Rørvik *et al.*, 1995; Lyhs *et al.*, 1998; Johansson *et al.*, 1999; Nørrung *et al.*, 1999; Gudbjörnsdóttir *et al.*, 2004; Markkula *et al.*, 2005; Miettinen and Wirtanen, 2005; Uyttendaele *et al.*, 2009; González *et al.*, 2013; Kramarenko *et al.*, 2013) and poultry (Table 2) products.

L. monocytogenes has been regularly found in raw poultry products and the prevalence being as high as 63% (MacGowan *et al*., 1994). Different *L. monocytogenes* contamination studies have been performed which reported that poultry meat becomes contaminated during slaughter and processing (Ojeniyi *et al*., 1996; Miettinen *et al*., 2001; Lundén *et al*., 2003; Rørvik *et al*., 2003). In food processing plants *L. monocytogenes* can contaminate a variety of processed foods (Lyytikäinen *et al*., 2000; Chasseignaux *et al*., 2001; Autio *et al*., 2002; Lundén *et al*., 2003; Gudbjörnsdóttir *et al*., 2004; Miettinen and Wirtanen, 2006), and the prevalence in processed poultry products being as high as 25% (Uyttendaele *et al*., 1999a).

Ready-to-eat (RTE) meat products with a long shelf-life are associated with the high risk of transmission of *L. monocytogenes*. High prevalence and numbers of *L. monocytogenes* have been linked to certain food items, such as soft cheeses, blue mould cheeses, smoked fish, paté, deli-meats, unpasteurized milk, fermented raw-meat sausages, non-re-heated frankfurters, hot dogs and deli-salads (Swaminathan and Gerner-Smidt, 2007; Wagner *et al*., 2007; Uyttendaele *et al*., 2009; Allerberger and Wagner, 2010). Additionally, many RTE foods have extended shelf-life which provides time for *L. monocytogenes* growth to high numbers (Gandhi and Chikindas, 2007). *L. monocytogenes* prevalence in various Estonian RTE food products has been described by Kramarenko *et al.* (2013) who found that cold-smoked fish products were contaminated with *L. monocytogenes* in high proportions (33%).

Table 2. Prevalence of *Listeria monocytogenes* in poultry products in different countries

Product	Prevalence (%)	Country	Reference
Raw poultry meat	60	United Kingdom	Pini and Gilbert, 1988
	47	Denmark	Skovgaard and Morgen, 1988
	61	Norway	Rørvik and Yndestad, 1991
	59	United Kingdom	Lawrence and Gilmour, 1994
	63	United Kingdom	MacGowan *et al.*,1994
	27	Belgium, France	Uyttendaele *et al.*, 1997
	30	Belgium	Uyttendaele *et al.*, 1999a
	32	Spain	Capita *et al.*, 2001
	62	Finland	Miettinen *et al.*, 2001
	41	Portugal	Antunes *et al.*, 2002
	51	Norway	Rørvik *et al.*, 2003
	18	Northern Ireland	Soultos *et al.*, 2003
	17	Nordic countries	Gudbjörnsdóttir *et al.*, 2004
	36	Spain	Vitas *et al.*, 2004
	38	Greece	Sakaridis *et al.*, 2011
	34	Canada	Cook *et al.*, 2012
	20	Malaysia	Goh *et al.*, 2012
	17	Japan	Sasaki *et al.*, 2014
Processed poultry products	13	New Zealand	Hudson *et al.*, 1992
	9	Denmark	Ojeniyi *et al.*, 1996
	13	Belgium, France	Uyttendaele *et al.*, 1997
	25	Belgium	Uyttendaele *et al.*, 1999a
	7	Denmark	Ojeniyi *et al.*, 2000
	2	USA	Levine *et al.*, 2001
	20	Spain	Cabedo *et al.*, 2008

3. AIMS OF THE STUDY

The aims of the present study were:

1. To determine the prevalence (**I, II**) and counts (CFU/g) (**II**) of *Campylobacter* spp., and prevalence of *L. monocytogenes* (**III**) in Estonian and imported raw poultry meat to get information on *Campylobacter* spp. and *L. monocytogenes* contamination in raw poultry meat in Estonia.

2. To serotype and pulsed-field gel electrophoresis (PFGE) genotype both *Campylobacter* spp. **(I)** and *L. monocytogenes* **(III)** isolates to understand the genetic diversity of pathogen isolates originating from raw poultry meat in Estonia.

3. To determine the antimicrobial susceptibility of the *Campylobacter* spp. isolates (**I**) to clarify the resistance patterns and multiresistance among *Campylobacter* isolated in Estonia.

4. MATERIALS AND METHODS

4.1. Sample collection (I, II, III)

4.1.1. *Campylobacter* spp. (I, II)

Study performed in 2002–2003 (I)

Altogether, 580 raw broiler chicken and 30 turkey meat samples from retail stores in Estonia were obtained between January 2002 and December 2003. Of broiler chicken samples 396 were of Estonian origin, and 20, 78, 18, 10, 21, 12 and 25 were imported to Estonia from Belgium, Denmark, Finland, Germany, Hungary, Sweden and USA, respectively. All turkey sampels were imported from Hungary. Products of Estonian origin were fresh, and those of foreign origin were frozen. The fresh meat samples were sold both packaged and unpackaged, and the frozen samples were all sold unpackaged. During transportation to the laboratory, the samples were kept cool in portable insulated boxes by ice packs, and were stored at 4 °C until analysis.

Study performed in 2012 (II)

This study included two surveys (first and second survey), and a total of 600 poultry meat samples at retail level in Estonia were studied from January to December 2012.

First survey

The first survey was organized by the Estonian Veterinary and Food Board, and included poultry meat sampling from retail outlets throughout Estonia. Altogether, 380 poultry meat samples were collected. Of these samples the majority was broiler chicken meat (78%), and the remaining part (22%) consisted of turkey, laying hen and duck meat. Of the collected poultry meat samples 39% were of Estonian, 8% of Latvian and 47% of Lithuanian origin, and 6% of the samples were imported from other European countries (Belgium, Finland, Germany, Hungary and Poland). The collected samples were from a range of poultry meat categories including fresh meat (58%), meat preparations (25%), whole carcasses

(12%), and minced meat (5%). Meat samples were transported to the laboratory within sampling day in a portable cooler at a temperature of 4-6 °C, and microbiological analyses began on the same day.

Second survey

The second survey was designed by our researchgroup at the Department of Food Hygiene the Estonian University of Life Sciences to estimate the prevalence and counts of *Campylobacter* spp. in high contamination-risk category products such as fresh broiler chicken meat containing skin (drumsticks, wings and breasts). In total, 220 meat samples were collected from Estonian supermarket chain retail outlets within the 12 months. Of the collected broiler chicken meat samples 54% were of Estonian, 9% of Latvian and 37% of Lithuanian origin. Estonian and Lithuanian products were available for purchase in all 12 months, and Latvian products from September to December 2012. Only company-packaged fresh broiler chicken meat was sampled in order to exclude the possibility of *Campylobacter* cross-contamination during storage. Meat samples were transported to the laboratory within sampling day in a portable cooler at a temperature of 4-6 °C, and microbiological analyses began immediately on arrival of samples.

4.1.2. *L. monocytogenes* (III)

A total of 240 raw broiler chicken legs (120 of Estonian and 120 of foreign origin) from 12 retail stores in the two largest cities (Tallinn and Tartu) of Estonia were studied from January to December 2002. All samples of Estonian origin were from one of the country's main producers of poultry products. Of these, 104 were obtained from stores that sold only products of the main producer, and 16 were obtained from stores that also sold poultry products from other countries. Of the samples of foreign origin, 60, 18, 21, 12 and 9 were imported from Denmark, Finland, Hungary, Sweden and USA, respectively. Products of Estonian origin were fresh, and those of foreign origin were frozen. The fresh meat samples were sold both packaged and unpackaged, and the frozen samples were all sold unpackaged. During transportation to the laboratory, the samples were kept cool in portable insulated boxes by ice packs, and were stored at 4 ºC until analysis.

4.2. Isolation (I, II, III) and enumeration (II)

4.2.1. *Campylobacter* spp. (I, II)

Study performed in 2002–2003 (I)

The isolation of *Campylobacter* was carried out in two laboratories. The Department of Food Hygiene and Environmental Health at University of Helsinki analysed altogether 290 samples using the following method. One hundred milliliters of peptone (0.1%)–saline (0.85%) solution was added to the whole sample (broiler leg) in a plastic bag, and the sample was massaged by hand for 1 minute. Twenty milliliters of the suspension was added into 80 ml of *Campylobacter* enrichment broth (Lab M, Bury, Lancashire, UK), and enriched at 37 ºC for 24 h and 48 h under microaerobic conditions (5% O_2, 10% CO_2, 85% N_2). Microaerobic conditions were produced in jars by using Oxoid gas-generating kits according to the manufacturer instructions (Oxoid, Basingstoke, Hampshire, UK).

The Estonian Veterinary and Food Laboratory analysed 320 of the samples for *Campylobacter* using the method of the Nordic Committee on Food Analysis (NMKL, 1990). Briefly, 250 ml of Preston enrichment broth (Oxoid, Basingstoke, Hampshire, UK) was added to a 25 g of sample (minced meat or skin and muscle of breast, carcass, thigh, wing), and the sample was stomached for 60 s. Incubation was carried out at 42 ± 0.5 ºC for 24 h under microaerobic conditions.

In both methods, after 24 h and 48 h incubation a loopful of the enrichment broth was plated on mCCDA (Oxoid, Basingstoke, Hampshire, UK), and examined for typical growth after 48 h. Typical grayish, campylobacter-like colonies growing on mCCDA plates were streaked on Brucella blood agar (Oxoid, Basingstoke, Hampshire, UK), and confirmed by Gram staining, motility analysis, oxidase and catalase test as campylobacters. The isolate from each positive sample was identified as *C. jejuni* as being positive or *C. coli* as being negative in hippurate hydrolysis test. Additionally, an indoxyl acetate hydrolysis test was performed for hippurate negative isolates, and the isolates negative in this test were regarded as *Campylobacter* spp. After the original isolation, the strains were stored at -70 °C in glycerol broth (15% [vol/vol] glycerol in 1% [wt/vol] proteose peptone).

Study performed in 2012 (II)

The isolation of *Campylobacter* was carried out in two laboratories, and for *Campylobacter* detection the same methodology was applied.

First survey

All analyses in first survey were performed at the Estonian Veterinary and Food Laboratory. *Campylobacter* detection was carried out according to the method described in ISO 10272-1:2006 (ISO, 2006a). The detection of *Campylobacter* was made primarily from the skin material, if available, and secondly from meat, depending on the sample type (e.g. skinless poultry meat fillets). To 10 g of skin or meat in a sterile plastic bag 90 ml of Bolton broth (Oxoid, Basingstoke, Hampshire, UK) was added, and samples were processed for one minute in a stomacher. Samples were incubated at 37 °C for 4 h to 6 h followed at 41.5 ± 0.5 ºC for 44 ± 4 h under microaerobic conditions. Microaerobic conditions were produced in anaerobic jars and by using CampyGen™ reagents (Oxoid, Basingstoke, Hampshire, UK). After enrichment, 10 µl of the enrichment broth was plated onto mCCDA agar (Oxoid, Basingstoke, Hampshire, UK), and incubated at 41.5 ± 0.5 ºC for 48 h under microaerobic conditions. Typical *Campylobacter* colonies on mCCDA plates were streaked onto Columbia blood agar (Oxoid, Basingstoke, Hampshire, UK) plates, and incubated at 41.5 ± 0.5 ºC for 24 h in microaerobic conditions. The bacteria isolated from poultry meat that showed typical growth characteristics on mCCDA, were Gram-negative, had corkscrew-like darting motility, were oxidase-positive and had no growth at 41.5 ± 0.5 ºC in aerobic conditions with growth at 25 °C in microaerobic conditions, were considered to be *Campylobacter* spp. After isolation, the randomly selected strains were stored at -82 °C in glycerol broth (20% [vol/vol] glycerol in 1% [wt/vol] proteose peptone).

Second survey

All analyses in the second survey were performed at the Department of Food Hygiene at the Estonian University of Life Sciences. The main difference compared to the first survey was that only broiler chicken meat skin material was used, and both *Campylobacter* detection and enumeration methods were applied. The detection of *Campylobacter* from fresh broiler chicken meat samples was carried out according to the ISO 10272-1:2006

(ISO, 2006a) method described in the first survey. For identification and differentiation of *Campylobacter* isolates conventional multiplex PCR assay was used as described by Wang *et al.* (2002).

Enumeration was carried out according to the method described in ISO 10272-2:2006 (ISO, 2006b). In brief, 0.1 ml of 10^{-1} and 10^{-2} broiler chicken meat skin dilutions were streaked onto mCCDA agar (Oxoid, Basingstoke, Hampshire, UK), and incubated at 41.5 ± 0.5 ºC for 44 to 48 h. Randomly selected five presumptive *Campylobacter* colonies were further subcultured on Columbia blood agar (Oxoid, Basingstoke, Hampshire, UK), and later identified by microscopic examination, Gram staining, and biochemical tests.

After isolation, the randomly selected strains were stored at -82 °C in glycerol broth (20% [vol/vol] glycerol in 1% [wt/vol] proteose peptone).

4.2.2. *L. monocytogenes* (III)

Microbiological analyses of *L. monocytogenes* were started within 24 h of sample collection. One hundred millilitres of peptone (0.1%)–saline (0.85%) solution was added to the whole broiler leg in the plastic bag, and the broiler leg was massaged by hand for 1 minute. Twenty-five millilitres of this peptone-saline solution was used for the enrichment procedure. The isolation of *L. monocytogenes* was carried out by a two-step enrichment method according to the recommendations of the ISO 11290-1 (ISO, 1996a), with the use of half-Fraser and Fraser broth (Oxoid, Basingstoke, Hampshire, England). Both enrichment broths were plated on PALCAM agar (Oxoid, Basingstoke, Hampshire, UK) and *L. monocytogenes* blood agar (Lab M, Bury, Lancashire, UK) as suggested by Johansson (1998). Five typical colonies from each selective plate were streaked on blood agar, and five β-haemolytic colonies were confirmed by catalase reaction, Gram staining, and biochemical identification using the API *Listeria* test (bio Mérieux, Marcy-l'Etoile, France).

4.3. Serotyping (I, III)

4.3.1. *Campylobacter* spp. (I)

A total of 54 *C. jejuni* isolates (chosen arbitrarily) were serotyped using commercial *Campylobacter* antisera according to the manufacturer instructions (Denka Seiken, Tokyo, Japan). Before the serotyping test, the isolates were cultured on Brucella blood agar (Oxoid, Basingstoke, Hampshire, UK) plates at 37 °C for 48 h in microaerobic conditions.

4.3.2. *L. monocytogenes* (III)

One to eight representative isolates from each PFGE type were selected for serotyping, resulting in a total of 71 *L. monocytogenes* isolates. Serotyping was performed with commercial *Listeria* antisera according to the instructions given by the manufacturer (Denka Seiken, Tokyo, Japan), with some modifications. For detection of the O-antigen, the cells were cultured on Trypticase soy agar (TSA; Difco, Becton Dickinson) plates. Detection of the flagellar H-antigens (A, B, C, and D) was performed at 25 °C in TSA tubes.

4.4. PFGE genotyping (I, III)

4.4.1. *Campylobacter* spp. (I)

PFGE typing was performed for 70 *Campylobacter* isolates, representing one isolate from each positive sample. *In situ* DNA was isolated and characterized by PFGE (Gibson *et al.*, 1994; Hänninen *et al.*, 1998). The DNA was digested with *Sma*I or *Kpn*I (New England Biolabs, Beverly, Mass.) (20 U per sample), and the restriction fragments were separated with ramped pulses of 1 to 30 s and 1 to 25 s for 19 h, respectively.

The computer software program BioNumerics 3.5 (Applied Maths, Sint-Martens-Latem, Belgium) was used for numerical analysis of *Sma*I and *Kpn*I macrorestriction patterns Similarity analysis was carried out using the Dice coefficient (position tolerance, 1.0%). The dendrogram was constructed using the unweighted pair-group method with arithmetic averages.

4.4.2. *L. monocytogenes* (III)

Altogether, 169 *L. monocytogenes* isolates were obtained for PFGE typing and represented one isolate from each positive sample. Cultures for DNA isolation were grown overnight in Trypticase soy broth (TSB; Difco, Becton Dickinson, Sparks, Md.) at 37 ºC. In situ DNA was isolated and digested with the restriction enzyme *Asc*I (New England Biolabs, Beverly, Mass.) in agarose plugs, and was then characterized by PFGE as described by Autio *et al.* (2002) with the use of pronase (Roche Diagnostics GmbH, Mannheim, Germany) instead of proteinase K.

Numerical analysis of *Asc*I macrorestriction patterns was performed by the computer software program BioNumerics 3.5 (Applied Maths, Sint-Martens-Latem, Belgium). Similarity analysis was carried out by use the Dice coefficient (position tolerance, 1.0%). The clustering and construction of the dendrogram were performed by the unweighted pair-group method with arithmetic averages.

4.5. Antimicrobial susceptibility testing of *Campylobacter* spp. (I)

All *Campylobacter* isolates were tested by the disc diffusion method against ampicillin (25 μg), ciprofloxacin (5 μg), erythromycin (15 μg), gentamicin (10 μg), nalidixic acid (30 μg), and tetracycline (10 μg) (Oxoid, Basingstoke, Hampshire, UK), and by the Epsilometer test (E-test) (AB Biodisk, Solna, Sweden) against ampicillin, ciprofloxacin, erythromycin, and tetracycline.

Campylobacter isolates were first grown on blood agar plates and were transferred in 5 ml of Mueller-Hinton (MH) broth (Oxoid, Basingstoke, Hampshire, UK), and incubated at 37 °C for 24 h under microaerobic conditions. Inoculum from the MH broth was diluted and a turbidity equivalent of a 0.5 McFarland standard was adjusted in physiological peptone-saline water. The growth suspension was spread on the MH blood agar plates (Oxoid, Basingstoke, Hampshire, UK; supplemented with 7% horse blood), and the disks or E-test strips containing antimicrobial compounds were laid on the plates. The plates were incubated at 37 °C for 24 h in microaerobic conditions. The diameter of the growth inhibition zone was measured according to the CLSI (2004). MIC values were determined by E-test according to the instructions given by the manufacturer (AB Biodisk).

The following zone diameter (mm) and MIC breakpoints for resistance were applied: ampicillin ≤ 13 mm and MIC ≥ 32 μg/ml, ciprofloxacin ≤ 26 mm and MIC ≥ 4 μg/ml, erythromycin ≤ 26 mm and MIC ≥ 32 μg/ml, gentamicin ≤ 12 mm, nalidixic acid ≤ 26 mm, and tetracycline ≤ 31 mm and MIC ≥ 16 μg/ml (DANMAP, 2004; CLSI, 2004).

4.6. Statistical analysis (II, III)

4.6.1. *Campylobacter* spp. (II)

All individual results were recorded using MS Excel 2010 software (Microsoft Corporation, Redmond, Wash.), and statistical analysis was performed with the Statistical Package R in order to determine if there were statistically significant differences at 95% and 99% confidence levels in the prevalence and counts of *Campylobacter* in the broiler chicken meat of different origin in the second survey using the Kruskal-Wallis rank sum test and chi-square test. Additionally, with the same tests seasonal variation in *Campylobacter* contamination was analysed in order to elucidate differences in prevalences between different sampling months.

4.6.2. *L. monocytogenes* (III)

The prevalence data were analyzed statistically by chi-square test.

5. RESULTS

5.1. Prevalence (I, II, III) and counts (II) of *Campylobacter* spp. and *L. monocytogenes*

5.1.1. *Campylobacter* spp. in retail poultry meat in Estonia (I, II)

Study performed in 2002–2003 (I)

Of the 580 raw broiler chicken and 30 turkey meat samples purchased from retail stores in Estonia, 8% and 73%, respectively, were positive for *Campylobacter* spp. Altogether, 48 broiler chicken (8 Danish, 36 Estonian, 1 Finnish, and 3 USA origin) and 22 turkey (Hungarian origin) *Campylobacter* isolates were obtained. Of these isolates, 91% were identified as *C. jejuni*, 6% *C. coli*, and 3% *Campylobacter* spp.

Study performed in 2012 (II)

First survey

Of the 380 poultry meat samples purchased from retail stores in Estonia 13% were positive for *Campylobacter* (Table 3). The proportion of *Campylobacter* contamination in poultry meat samples was 15% for Estonian, 27% for Latvian, 11% for Lithuanian products, and 0% for poultry products originating from other European countries. Within the tested meat categories highest *Campylobacter* contamination was found in minced meat (24%) followed by fresh meat (13%), whole carcasses (11%), and meat preparations (10%). The contamination of fresh broiler chicken meat of Estonian, Latvian and Lithuanian origin was 17%, 23% and 9%, respectively.

Table 3. *Campylobacter* in various categories of poultry meat in retail market in Estonia in 2012[1]

Country of origin	No. of positive samples/No. of all samples (positive %)					
	Fresh meat	Whole carcass	Minced meat	Meat preparations	Total	95% CI[2]
Estonia	10/56 (18)	1/20 (5)	4/11 (36)	7/60 (12)	22/147 (15)	10%-22%
Latvia	5/22 (23)	3/6 (50)	NS	0/2 (0)	8/30 (27)	13%-46%
Lithuania	14/125 (11)	1/18 (6)	1/10 (10)	3/26 (11)	19/179 (11)	7%-16%
Other[3]	0/16 (0)	NS	NS	0/8 (0)	0/24 (0)	0%-17%
Total	29/219 (13)	5/44 (11)	5/21 (24)	10/96 (10)	49/380 (13)	10%-17%

[1] Survey conducted by the Estonian Veterinary and Food Board

[2] Confidence interval

[3] Belgium, Finland, Germany, Hungary, Poland

NS, no samples available

Second survey

The prevalence of *Campylobacter* in 220 fresh broiler chicken meat samples (drumsticks, wings and breasts containing skin) obtained from retail stores in Estonia was 35% (Table 4). Among Estonian, Latvian and Lithuanian origin broiler chicken meat samples the proportions of *Campylobacter* positive products were 20%, 60% and 50%, respectively. Of the *Campylobacter* isolates, 89% were identified as *C. jejuni*, 8% as *C. coli*, and 3% as *Campylobacter* spp.

Tabel 4. *Campylobacter* detection in fresh broiler chicken meat in retail market in Estonia in 2012[1]

Country of origin	No of all samples	No. of positive samples (positive %)	95% CI[2]
Estonia	118	24 (20)	14%-29%
Latvia	20	12 (60)	39%-78%
Lithuania	82	41 (50)	39%-61%
Total	220	77 (35)	29%-42%

[1] Survey conducted by the Estonian University of Life Sciences

[2] Confidence interval

The results of the *Campylobacter* enumeration on fresh broiler chicken meat were categorised as follows: <100 CFU/g; 100-499 CFU/g; 500-1000 CFU/g and >1000 CFU/g (Table 5).

Table 5. *Campylobacter* enumeration data in fresh broiler chicken meat in 2012

Country of origin	*Campylobacter* counts (CFU/g)				
	0[1]	<100[2]	100-499	500-1000	>1000
Estonia	94 (80)	13 (11)	7 (6)	2 (2)	2 (2)
Latvia	8 (40)	2 (10)	1 (5)	2 (10)	7 (35)
Lithuania	41 (50)	7 (9)	12 (15)	10 (12)	12 (15)
Total	143 (65)	22 (10)	20 (9)	14 (6)	21 (9)

[1] Negative detection and negative enumeration

[2] Negative enumeration and positive detection, the quantification limit

Number of samples (%)

Enumeration results, in the case of positive results from enumeration analyses, showed that the overall arithmetic *Campylobacter* CFU mean was 3.2 $\log_{10}$ CFU/g of product (Table 6) with the highest mean contamination loads in Latvian products and the lowest in those from Estonian, 3.4 $\log_{10}$ CFU/g and 2.8 $\log_{10}$ CFU/g, respectively. The mean contamination load for Lithuanian origin broiler chicken products was 3.2 $\log_{10}$ CFU/g. Among Estonian, Latvian and Lithuanian origin products, with positive enumeration results, a contamination level of above 1000 *Campylobacter* CFU/g was found in 2%, 35% and 15% of samples, respectively.

Tabel 6. *Campylobacter* enumeration results in fresh broiler chicken meat in the retail market in Estonia in 2012[1]

Country of origin	Average $\log_{10}$ CFU/g (count per g)	Median $\log_{10}$ CFU/g (count per g)	95% CI[2] CFU/g
Estonia	2.8 (660)	2.5 (300)	190-1120
Latvia	3.4 (2600)	3.3 (1800)	919-4261
Lithuania	3.2 (1600)	2.9 (800)	372-2814
Total	3.2 (1600)	3.0 (900)	782-2390

[1] Samples with positive enumeration results

[2] Confidence interval, only samples positive in enumeration analyses

Significant differences for *Campylobacter* prevalence and counts between Estonian and Lithuanian ($p < 0.001$) and between Estonian and Latvian

($p < 0.001$) fresh poultry products were found. Estonian fresh poultry meat products had significantly ($p < 0.001$) lower *Campylobacter* prevalence and counts compare to Lithuanian and Latvian poultry products sold at Estonian retail. No statistical difference was found for *Campylobacter* contamination, both counts and prevalence, between Latvian and Lithuanian origin products.

Both surveys

The monthly variation in prevalence of *Campylobacter* in Estonian fresh broiler chicken meat from January to December in 2012 were 0%, 0%, 0%, 0%, 33%, 0%, 17%, 75%, 42%, 22%, 16% and 17%. There was a seasonal variation in the proportions of *Campylobacter* positive samples with a seasonal peak in the warm months of July, August and September ($p < 0.001$).

5.1.2. *L. monocytogenes* in retail broiler meat in Estonia (III)

Of the 240 raw broiler legs purchased from retail stores in Estonia, 70% were positive for *L. monocytogenes*. The prevalence in broiler legs of Estonian origin varied from 33% to 100% and, in legs of foreign origin, from 22% to 83% from various stores. The prevalence of *L. monocytogenes* in broiler legs of Estonian origin (88%) was significantly higher than in broiler legs of foreign origin (53%) ($p < 0.001$). Of the broiler legs bought from stores selling only products of the predominant Estonian poultry meat plant 89% were positive for *L. monocytogenes*. The broiler legs of Estonian origin purchased in Tartu had a significantly higher contamination level than those purchased in Tallinn, 100% and 83%, respectively ($p < 0.05$).

5.2. Serotype distribution and genetic characterization of *Campylobacter* spp. (I) and *L. monocytogenes* (III) in retail poultry meat in Estonia

5.2.1. Serotype distribution of *Campylobacter* spp. (I)

Eleven serotypes were obtained from 54 *C. jejuni* isolates. Of the isolates, 22% (12/54) were nontypeable. The most common serotypes O:1,44; O:21, and O:55 accounted for 28%, 13%, and 13% of the isolates. The isolates from chicken meat ($n = 37$) included ten serotypes, and the frequent serotypes were O:1,44 (32%) and O:21 (19%). The isolates from

turkey meat (n = 17) belonged to three serotypes: O:55 (29%), O:1,44 (18%), and O:18 (12%).

5.2.2. Serotype distribution of *L. monocytogenes* (III)

A total of 71 isolates (one to eight representative isolates from each PFGE type) were serotyped, and three different serotypes were obtained: 1/2a, 1/2b, and 4b. All broiler legs of Estonian origin and most broiler legs of foreign origin had serotype 1/2a (92%). Isolates of serotype 1/2b (7%) were of Danish, Finnish, and Hungarian origin, and isolate of serotype 4b (1%) was of Hungarian origin.

5.2.3. PFGE genotypes of *Campylobacter* spp. (I)

The PFGE genotyping of 70 *Campylobacter* isolates yielded 29 *Sma*I and 34 *Kpn*I PFGE types. The DNA of five isolates was not digested by *Sma*I. Combination of the macrorestriction patterns resulted in 37 PFGE types. Of these, 33 PFGE types were from *C. jejuni* (91%), two from *C. coli* (6%), and two from *Campylobacter* spp. (3%) isolates.

5.2.4. PFGE genotypes of *L. monocytogenes* (III)

The characterization of *L. monocytogenes* isolates recovered from broiler legs of Estonian (n = 106) and foreign (n = 63) origin yielded 22 and 24 PFGE types, respectively. Combining these PFGE types, 35 different types were obtained. Of these 35 PFGE types, eleven came only from isolates of broiler legs of Estonian origin, four of Danish origin, two of Finnish origin, and four of Hungarian origin. Fourteen PFGE types came from isolates that originated from more than one country. In some cases, the same PFGE types were recovered from broiler legs that had originated from different countries but that had been obtained from the same stores. In several cases, the same PFGE types were detected in samples of Estonian origin that came from different stores over the course of several months (during five to ten different months).

5.3. Antimicrobial susceptibility of *Campylobacter* spp. (I)

In the disc diffusion method, resistance to ciprofloxacin, nalidixic acid, tetracycline, ampicillin, and erythromycin occurred in 66%, 66%,

44%, 34%, and 14% of the *Campylobacter* isolates (n = 70). Resistance to ciprofloxacin, nalidixic acid, tetracycline, ampicillin, and erythromycin occurred in 44%, 44%, 22%, 19%, and 17% of the Estonian isolates (n = 36) and in 88%, 88%, 68%, 50%, and 12% of the imported isolates (n = 34), respectively. All isolates were susceptible to gentamicin.

Resistance to ciprofloxacin, nalidixic acid, tetracycline, ampicillin, and erythromycin occurred in 50%, 50%, 27%, 23%, and 14% of the chicken isolates (n = 48). Two *C. coli* isolates from chicken showed resistance to ampicillin, ciprofloxacin, and nalidixic acid. One isolate of *Campylobacter* spp. from chicken was resistant to ampicillin, and the other isolate to ciprofloxacin and nalidixic acid. Of the turkey isolates (n = 22) all were resistant to ciprofloxacin and nalidixic acid, 82% to tetracycline, and 59% to ampicillin.

Resistance occurred in 57 isolates (81%) out of 70 tested to at least one of the antimicrobials. Fifteen isolates (21%) were resistant to one, 30 isolates (43%) to two, and 12 isolates (17%) to three antimicrobial agents. The resistance of *Campylobacter* isolates to two antimicrobials showed a combination of ampicillin and ciprofloxacin (9%), ampicillin and erythromycin (4%), and ciprofloxacin and tetracycline (30%). The resistance of isolates to three antimicrobials showed a combination of ampicillin, ciprofloxacin, and erythromycin (4%), and ampicillin, ciprofloxacin, and tetracycline (13%). The highest level of resistance recorded was to ciprofloxacin (66%) followed by tetracycline (44%), ampicillin (34%), and erythromycin (14%).

6. DISCUSSION

6.1. Prevalence (I, II, III) and counts (II) of *Campylobacter* spp. and *L. monocytogenes*

6.1.1. *Campylobacter* spp. in retail poultry meat in Estonia (I, II)

Study performed in 2002–2003 (I)

In the beginning of 2000s consumption of poultry meat has increased in Estonia, and broiler chicken meat was imported to Estonia from different countries such as Belgium, Denmark, Finland, Germany, Hungary, Sweden, and USA. Products of forein origin were sold at retail level as forzen, and own local production was sold as fresh. We found that the prevalence of *Campylobacter* on fresh broiler chicken meat of Estonian origin was 9% what is generally lower as described in other studies from other countries (Suzuki and Yamamoto, 2009). Imported frozen poultry meat at retail level showed higher *Campylobacter* contamination (in total 16%, in turkey meat 73%) than that of Estonian origin even when the freezing is known to decrease the counts of *Campylobacter* by 1 to 2 log units (Sandberg *et al*., 2005; Ritz *et al*., 2007; González and Hänninen, 2012). The contamination of poultry meat can occur during slaughter process at the plant (Newell *et al*., 2001; Atanassova *et al*., 2007; Reich *et al*., 2008). Furthermore, *Campylobacter* contamination may occur in different sections of the entire food chain (Wagenaar *et al*., 2006; Kudirkienė *et al*., 2011). However, the cross-contamination at retail market cannot be excluded, because majority of the products were sold unpackaged. *Campylobacter* positive fresh and frozen poultry meat may be a source of cross-contamination in home kitchen during food preparation if proper hygienic handling is not followed, and increase significantly the risk for acquisition of foodborne campylobacteriosis (Kapperud *et al*., 1992; Dominguez *et al*., 2002; Neimann *et al*., 2003; Atanassova *et al*., 2007). Registration of human campylobacteriosis cases in 2002 (114 cases) and 2003 (98 cases) was low in Estonia (Estonian Health Board, 2015) either suggesting minor effect on human infections, or most probably reflecting underregistration of cases.

Study performed in 2012 (II)

Both surveys

Similarly as in the study performed in Estonia in 2002–2003 differences in various origin poultry meat contamination with *Campylobacter* spp. were evident also in both surveys in 2012. In the present study poultry meat produced in Estonia and Lithuania was less often *Campylobacter* positive (17% and 23%, respectively) than that produced in Latvia (40%). However, the contamination levels varied depending of the types of samples studied. The studies in other Baltic countries showed high *Campylobacter* occurrence. The mean proportion of *Campylobacter* positive broiler chicken carcasses at Latvian retail level was about 56% (Kovalenko *et al*., 2013). Bunevičienė *et al*. (2010) showed that fresh broiler chicken meat products (drumsticks and wings) at retail level in Lithuania were contaminated with *Campylobacter* at up to 47%. These studies report similar contamination proportions in the second survey reported here, where *Campylobacter* contamination in Latvian and Lithuanian fresh broiler chicken meat was 60% and 50%, respectively.

Poultry meat contamination with *Campylobacter* spp. in the first and second surveys was 13% and 35%, respectively. This difference can be explained by differences in the sampling methods. In the second survey only skin material from drumsticks, wings and breasts was analysed. The first survey also included fillets and other fresh broiler meat products without skin material. *Campylobacter* may colonize the intestines of poultry, and the cross-contamination of the poultry carcasses may occur during slaughter process, mostly during scalding and most evidently skin. Later washing stages only partly remove fecal material from carcass surfaces. An additional suface contamination of the skin may occur during a poorly executed evisceration process at slaughter while the caecal material can be transferred onto carcasses (Allen *et al*., 2007; Reich *et al*., 2008). Therefore, the most heavily contaminated part of the carcass is the skin (Jeffrey *et al*., 2001; Davis and Conner, 2007; Seliwiorstow *et al*., 2015). Removal of skin will decrease *Campylobacter* counts. A study by Katzav *et al*. (2008) showed the occurrence of *Campylobacter* in chicken slices and barbecue sticks to be about 9%, in chicken breast fillets about 5%, and in chicken products with skin and bone about 30%. The results of the present study showed high *Campylobacter* prevalence in fresh broiler chicken meat skin

samples. Within company-packaged fresh broiler chicken meat sold at Estonian retail level the majority (about 70%) are drumsticks, wings and breasts where the skin material is included.

According to an EU-wide baseline survey in 2008 (EFSA, 2011b) the *Campylobacter* prevalence for broiler chicken batches in Estonia was only 2.0% which was the lowest among the EU countries on that time. According to the first and second surveys of the present study *Campylobacter* prevalence in fresh broiler chicken meat of Estonian origin was 17% and 20%, respectively. Differences compared to the prevalence found in the EU-baseline study are also probably related to the different sampling methods and sampling year. For the baseline study broiler chicken carcasses were collected and neck skin samples taken at the laboratory for *Campylobacter* prevalence, instead of company-packaged broiler fresh meat samples (drumsticks, wings, breasts) that were used in the surveys presented here. Neck skin is one of the most positive and the highest contaminated carcass site (Baré *et al*., 2013). However, Jørgensen *et al*. (2002) reported that *Campylobacter* spp. in raw chicken was more frequently isolated from samples containing carcass-rinse and carcass-rinse plus whole skin samples in comparison with those containing neck-skin only.

It was found that *Campylobacter* strains isolated from broiler chicken meat at Estonian retail level and originating either from Estonian, Latvian and Lithuanian producers the majority of the strains were *C. jejuni* (89%). However, 24% of Lithuanian meat samples contained *C. coli*. It reflects some differences between Baltic countries, but no deep conclusions may be done because the number of Latvian *Campylobacter* isolates was small, and previous Estonian studies have identified 25% of all selected *Campylobacter* isolates as *C. coli*, all these were isolated from Estonian origin broiler chicken meat products (Roasto *et al*., 2005).

An EU baseline survey reported that *Campylobacter* counts on broiler carcasses of Estonian origin were <10 CFU/g in 98% of positive cases (EFSA, 2010). In the second survey of present study higher *Campylobacter* contamination levels for Estonian broiler chicken products were found. Especially high counts (CFU > 500/g) represented 45% of the samples among Latvian and 27% among Lithunian products while only 4% of Estonian products had this level of contamination. A Lithuanian study (Bunevičienė *et al*., 2010) reported lower *Campylobacter* counts (mean 2.0 $\log_{10}$ CFU/g) in broiler

chicken meat. Counts of *Campylobacter* on carcasses play role in the risk assessment of broiler meat in the acquisition of campylobacteriosis. It has been estimated that decreasing counts a public health risk reduction > 50% or > 90% could be achieved if all broiler batches would comply with microbiological criteria with a critical limit of 1000 or 500 CFU/g of neck and breast skin, respectively, while 15% and 45% of all tested batches would not comply with these criteria (EFSA, 2011a).

There was a seasonal variation in prevalence of *Campylobacter* in Estonian fresh broiler chicken meat. The *Campylobacter* contamination increased in spring, remained high during July, August and September, and decreased at the end of autumn. June in 2012 was atypically rainy and cold in Estonia, which may be a possible reason for the sudden decrease in *Campylobacter* positive broiler chicken meat samples. A similar seasonal variation has been shown in the Northen European countries (Jore *et al*., 2010). Further on, a distinct seasonality in human campylobacteriosis cases have been also shown by previous studies in Europa (Rautelin and Hänninen, 2000; Horrocks *et al*., 2009) and in New Zealand (Brieseman, 1990). Reports on human campylobacteriosis cases in Estonia have shown that most human *Campylobacter* infections occurred during warm summer months (Meremäe *et al*., 2010), the season when the highest *Campylobacter* prevalence of the poultry products at Estonian retail level were found in the present study.

6.1.2. *L. monocytogenes* in retail broiler meat in Estonia (III)

Raw broiler legs obtained from retail stores in Estonia showed a high level of contamination with *L. monocytogenes* (70%). The prevalence of *L. monocytogenes* in broiler legs of Estonian origin in general and in broiler legs obtained from stores selling only products of the predominant Estonian poultry meat plant was higher (88% and 89%, respectively) than that reported by Genigeorgis *et al.* (1989) (16%) or Miettinen *et al.* (2001) (68%) in broiler legs. Furthermore, the broiler legs of Estonian origin bought in Tartu were all (100%) contaminated by *L. monocytogenes*. In study by Kramarenko *et al.* (2013) it was found that the prevalence of *L. monocytogenes* in broiler chicken meat was only 1% during the years 2008 to 2010 in Estonia.

The high prevalence in broiler legs at the Estonian retail level could be because of contamination that may have occurred during processing at the plant. All broiler legs of Estonian origin came from one processing

plant. However, cross-contamination of retail poultry products cannot be excluded. Broiler legs were sold unpackaged from the same counter, and cross-contamination in the stores sold products that originated from various countries is possible. It is recognized that the presence of *L. monocytogenes* in raw foods cannot be completely eliminated, but through the application of effective hygienic measures it is possible to reduce *L. monocytogenes* occurrence and level in food products (Roasto *et al*., 2010).

6.2. Serotype distribution and genetic characterization of *Campylobacter* spp. (I) and *L. monocytogenes* (III) in retail poultry meat in Estonia

6.2.1. Serotype distribution of *Campylobacter* spp. (I)

Our study showed high serotype diversity among *Campylobacter* isolates from raw retail poultry meat in Estonia reflecting most probable the diverse origin of the samples from five different countries and that their hosts were chicken and/or turkey. Nine of the eleven *C. jejuni* serotypes obtained were common in poultry products of Estonian origin, and five in those imported to Estonia. The serotype distribution, however, did not show association with the origin of the sample. The most common serotypes were O:1,44; O:21, and O:55, accounting for 54% of the isolates. Serotype distribution differences occurred for chicken and turkey isolates. The chicken isolates had two common serotypes (O:1,44 and O:21) out of ten, whereas turkey isolates belonged to only three different serotypes (O:1,44; O:18 and O:55). In the Danish (Nielsen and Nielsen, 1999) and New Zealand studies (Devane *et al*., 2005), the serotype O:1,44 was also one of the most common in poultry products, and this serotype seems to have global distribution among strains isolated from human *Campylobacter* infections (Nielsen *et al*., 1997; Vierikko *et al*., 2004; Devane *et al*., 2005; Miller e*t al*., 2005a). The most frequently isolated serotype in chicken meat in New Zealand was O:21 (Devane *et al*., 2005), the second most common serotype in our study. The presence of serotypes O:2, O:4-complex, and O:12, common to both chickens and human patients (Fricker and Park, 1989; Hudson *et al*., 1999; Perko-Mäkelä *et al*., 1999; Petersen *et al*., 2001; Saito *et al*., 2005), occurred in only 13% of the isolates studied.

Serotyping of *C. jejuni* showed that 22% of the isolates were nontypeable, and seven of the nontypeable isolates originated from turkey meat

imported from Hungary. By using the same commercial serotyping set as in our study, Rautelin and Hänninen (1999) found 14% of the isolates, and in a Danish study, using their own antisera, 16% of the isolates remained nontypeable (Nielsen and Nielsen, 1999) revealing the need to improve the present serotyping methods. One reason for nontypeability is the low production of capsular antigens responsible for the serotype specificity of *C. jejuni*, another reason could be new serotypes not accounted for in the present test (Jacobs-Reitsma *et al.*, 1995). More recently, PFGE and especially MLST typing have been replaced serotyping. Genetic lineages corresponding certain MLST types are often associated with certain serotypes indicating common evolution of capsule structures which determine serotype of *C. jejuni* (Dingle *et al.*, 2001; Manning *et al.*, 2003).

6.2.2. Serotype distribution of *L. monocytogenes* (III)

Serotype 1/2a was predominant in Estonian poultry products. The same serotype was also predominant in raw chicken from Portugal (Guerra *et al.*, 2001). In the USA and Spain, serotype 1/2b (Bailey *et al.*, 1989; Vitas *et al.*, 2004) and, in Finland, serotype 1/2c (Miettinen *et al.*, 2001) have been the most common serotypes found in poultry meat.

6.2.3. PFGE genotypes of *Campylobacter* spp. (I)

This study showed high PFGE genotype diversity among *Campylobacter* isolates from raw retail poultry meat in Estonia. The genotyping of the 70 *Campylobacter* isolates showed *Kpn*I to be more discriminatory, yielding 34 PFGE types compared to 29 obtained by *Sma*I. Furthermore, the DNA of five strains was not digested by *Sma*I. The genotypes of the isolates from the poultry products of different countries were not overlapping, except two *Sma*I PFGE types (isolates from Estonia and Hungary and isolates from Estonia and USA), and one *Kpn*I PFGE type (isolates from Estonia and USA). Our results, as well as the data from several previous studies (Gibson *et al.*, 1994; Hänninen *et al.*, 1998; Wassenaar and Newell, 2000), however, emphasize the utility of two restriction enzymes, such as *Sma*I *and Kpn*I, in PFGE typing studies of *Campylobacter*. In our study the majority of the isolates sharing a similar PFGE genotype originated from one country. The association of genotypes with country of origin requires further studies using a larger collection of isolates, however. We

found several serotypes within one PFGE type and within one serotype several PFGE types. For example, one PFGE type contained the serotypes O:11, O:55, and nontypeable isolates, and the common serotypes of our study, O:1,44; O:21, and O:55, contained up to 12, three, and two different PFGE types, respectively.

6.2.4. PFGE genotypes of *L. monocytogenes* (III)

Thirty-five PFGE types were presented by 169 *L. monocytogenes* strains. These data suggest that the *L. monocytogenes* strains recovered from the broiler legs showed wide genetic diversity. The PFGE types recovered from the broiler legs of Estonian and Hungarian origin were obtained from stores that sold only products from these countries and were possibly associated with the producing country. Strains that shared the same PFGE types (14 PFGE types) were identified among isolates of broiler legs that originated from different countries. Three predominant PFGE types possessed by 38% of all strains were recovered from broiler legs originating from Denmark, Estonia, Hungary, and USA. Because the broiler legs had been sold unpackaged and from the same counter, one of the reasons for the same PFGE type could be cross-contamination in the stores that sold products that had originated from various countries. For example, in three stores, the same PFGE types were detected in broiler legs of both Estonian and foreign origin, and in one store, the strains from broiler legs of Danish and Swedish origin shared the same PFGE types. Recovery of strains that shared the same PFGE types from different stores obtained during the course of several months suggests a wide temporal distribution of many of the *L. monocytogenes* strains isolated in broiler legs. Because the broiler legs of Estonian origin came from one processing plant, the PFGE types are likely associated with contamination during processing (Miettinen *et al.*, 2001, Ojeniyi *et al.*, 1996, Rørvik *et al.*, 2003). The occurrence of the same PFGE types in broiler legs of Estonian origin during the course of several months indicates that these strains are persistent (Lundén *et al.*, 2003). Furthermore, *L. monocytogenes* from broiler legs can contaminate retail counters and cause cross-contamination of other raw foods if hygienic procedures are inadequate. This emphasizes the need for strict hygienic conditions during processing and at retail level to prevent cross-contamination.

6.3. Antimicrobial susceptibility of *Campylobacter* spp. (I)

An important finding of our study was the recognition of a high number (81%) of *Campylobacter* isolates with increased antimicrobial resistance. Antimicrobial resistance level was especially high to ciprofloxacin (44 isolates MIC ≥ 32 μg/ml), tetracycline (23 isolates MIC ≥ 256 μg/ml), and ampicillin (22 isolates MIC ≥ 256 μg/ml). The resistance to antimicrobials, except erythromycin, was higher in isolates from imported poultry products than in those originating from Estonia. The *Campylobacter* isolates from turkey meat had a higher resistance to ampicillin, ciprofloxacin, nalidixic acid, and tetracycline than those from chicken meat. All isolates resistant or susceptible by the disk diffusion method showed the same results by E-test.

Ciprofloxacin resistance was high among isolates from both imported (88% of the isolates) and domestic products (44% of the isolates). Furthermore, 100% of the turkey and 50% of the broiler isolates showed resistance to ciprofloxacin. All isolates with resistance to ciprofloxacin were also resistant to nalidixic acid. A study in Spain (Sáenz *et al*., 2000) showed very high prevalence 98% of ciprofloxacin resistance in *Campylobacter* isolates from broiler intestinal samples. The study by Endtz *et al*. (1991) showed a link for the first time between veterinary fluoroquinolone use and increasing fluoroquinolone resistance in poultry and human isolates of *Campylobacter*. Later studies have confirmed their results (Smith *et al*., 2000; Engberg *et al*., 2001). Enrofloxacin, a fluoroquinolone group antimicrobial, is accepted for therapeutic use in poultry in Estonia (Estonian State Agency of Medicines, 2015), possibly explaining the high level of resistance detected among Estonian isolates.

Different studies typically find tetracycline resistance among poultry isolates. Ledergerber *et al*. (2003) reported a much lower (12%) tetracycline resistance, but Ge *et al*. (2003) found a higher resistance (82%) among poultry than in our study (44%). Neverthless, we found a higher resistance for turkey isolates (82%) than in the Belgian study (37%) (Van Looveren *et al*., 2001). Tetracycline is also accepted for therapeutic use in poultry in Estonia (Estonian State Agency of Medicines, 2015).

Ampicillin is a widely used antimicrobial in veterinary medicine, and amoxicillin is accepted for therapeutic use in veterinary medicine in

Estonia (Estonian State Agency of Medicines, 2015). Resistance to ampicillin in broiler isolates, 23%, was at a similar level, and resistance in turkey isolates, 59%, was higher than found in the Belgian study, 24% and 33%, respectively (Van Looveren *et al.*, 2001). However, ampicillin is not recommended for the treatment of *Campylobacter* infections due to the high incidence of resistance to this drug among human isolates (Navarro *et al.*, 1993).

Campylobacter isolates displayed the lowest resistance frequency against erythromycin (14%). All resistant isolates were *C. jejuni* and they were either from Danish or Estonian chicken products. All turkey isolates were susceptible to erythromycin. Belgium, Ireland, and Switzerland (Fallon *et al.*, 2003; Ledergerber *et al.*, 2003; Van Looveren *et al.*, 2001) also reported a low erythromycin resistance. Erythromycin is considered as a first line choice for the treatment of *C. jejuni* infections, and low resistance among retail meat isolates supports this common policy of antimicrobial use. Additionally, similar to Ge *et al.* (2003) and Van Looveren *et al.* (2001), none of the chicken and turkey isolates showed resistance to gentamycin.

We found a high level (60%) of multidrug (two or three antimicrobial agents) resistant isolates. Fallon *et al.* (2003) found 30% of the isolates resistant to two or more antimicrobials. In our study, 69% of isolates consisted of two or three antimicrobials originated from poultry products imported from Denmark or Hungary. All turkey isolates were resistant to two (59%) or three (41%) antimicrobials. Multiresistant isolates consisted of a combination of ampicillin, ciprofloxacin, erythromycin, or tetracycline.

In general, the PFGE genotypes and antimicrobial susceptibility profiles correlated, except for one PFGE type. Seven isolates of this PFGE type showed resistance to ampicillin, ciprofloxacin, and tetracycline, whereas four isolates were resistant to ciprofloxacin and tetracycline but sensitive to ampicillin. All of these eleven isolates were from turkey meat originating from Hungary and obtained during the same time period. These results may indicate that in a multiresistant bacterial population with identical genotype, the resistance patterns may be different.

Since campylobacteriosis is transmitted particularly via foods of animal origin, the presence of antimicrobial resistant *Campylobacter* isolates in raw

meat has important public health implications. In order to confirm the real resistance situation, further investigations on resistance patterns in *Campylobacter* along the whole poultry production chain are necessary. In Estonia, a national monitoring program for antibiotic resistance, including both human and food *Campylobacter* isolates, is needed. Presently there is very weak connection between antibiotic resistance programs of zoonotic disease agents on veterinary medicine and human medicine level. Also there is no clear information about antibiotics usage at poultry farm level. The prophylactic use of antibiotics on poultry farm level is strictly banned in Estonia.

7. CONCLUSIONS

- In the study performed in 2002–2003 the prevalence of *Campylobacter* was higher in imported poultry meat products (16%) than in those originating from Estonia (9%). The highest prevalence was detected for imported turkey meat (73%). **(I)**
- In the study performed in 2012 the overall *Campylobacter* prevalence (21%) in poultry meat was higher compare to study period in 2002–2003 (11%). The highest prevalence of *Campylobacter* was in fresh broiler chicken meat of Latvian origin (40%), followed by Lithuanian (28%) and Estonian (19%) origin. The mean CFU/g of *Campylobacter* in fresh broiler chicken meat in *Campylobacter* positive samples was 3.20 $\log_{10}$ CFU/g and median 3.0 $\log_{10}$ CFU/g which shows high *Campylobacter* counts and indicates increased public health risk to acquire *Campylobacter* infection from broiler chicken meat. The seasonal peak for *Campylobacter* contamination of broiler chicken meat was in the warm summer months – July, August and September. (**II**)
- The prevalence of *L. monocytogenes* in raw broiler legs purchased from retail stores in Estonia was 70%. The prevalence of *L. monocytogenes* in broiler legs of Estonian origin (88%) was significantly higher than in broiler legs of foreign origin (53%) ($p < 0.001$). (**III**)
- Penner serotyping of 54 *C. jejuni* isolates revealed 11 different serotypes (22% of the isolates were nontypeable) what showed high serotype diversity among *Campylobacter* isolates from raw retail poultry meat in Estonia. The most common serotypes O:1,44; O:21, and O:55 accounted for 28%, 13%, and 13% of the isolates, respectively. Genotypic characterization of *Campylobacter* isolates ($n = 70$) by PFGE genotyping using restriction enzymes *Sma*I and *Kpn*I yielded 29 and 34 PFGE types, respectively, revealing high diversity among isolates. (**I**)
- Seventy-one *L. monocytogenes* isolates were serotyped, and three serotypes (1/2a, 1/2b, 4b) were obtained. Majority of the isolates studied had serotype 1/2a (92%). Altogether, 169 *L. monocytogenes* isolates (106 Estonain and 63 foreign) were characterized by PFGE genotyping using restriction enzyme *Asc*I. The isolates showed a wide genetic diversity, with 35 different PFGE types being obtained. (**III**)

- High level of resistance to ciprofloxacin (66%), nalidixic acid (66%), tetracycline (44%), ampicillin (34%), and erythromycin (14%) were detected among the 70 *Campylobacter* isolates in 2002–2003. The simultaneous resistance to two or three antimicrobial agents occurred in 60% of the isolates. The *Campylobacter* isolates from turkey meat had higher resistance to ampicillin, ciprofloxacin, nalidixic acid, and tetracycline than those from broiler chicken meat. None of the broiler chicken isolates were resistant to gentamicin, and no turkey isolates to erythromycin or gentamicin. (**I**)

For conclusion, to achieve EU targets in public health risk reduction appropriate control measures should be applied at all broiler chicken meat production stages. The problems caused by *Campylobacter* spp. and *L. monocytogenes* extend beyond the country in which a poultry meat originates, therefore, both domestic and international agreements are required to implement common policies on contamination reduction and antimicrobial usage to minimize the emergence of target foodborne pathogens. There is need for more systematic foodborne pathogens isolates collection and molecular typing to determine the contamination routes, and verify foodborne outbreak cases in Estonia. Co-operation at veterinary and human medicine level is needed for proper epidemiological studies in Estonia.

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SUMMARY IN ESTONIAN

Campylobacter spp. ja *Listeria monocytogenes* linnulihatoodetes Eestis

Sissejuhatus

Termofiilsed kampülobakterid on paljudes maades ühed põhilisi inimese bakteriaalse mao-peensoolepõletiku põhjustajaid ja Euroopa Liidu (EL) riikides on kampülobakterioos jätkuvalt kõige sagedamini esinev zoonoos. Aastal 2013 registreeriti EL-is 214 779 termofiilsetest kampülobakteritest põhjustatud haigusjuhtu, mis teeb 100 000 inimese kohta keskmiselt 64,8 ametlikult registreeritud haigusjuhtu. Euroopa Toiduohutusameti (inglise keeles: *European Food Safety Authority*, EFSA) andmetel on EL-is hinnanguliselt 9 miljonit kampülobakterioosi juhtu aastas ja sellest haigusest tulenevalt on rahvatervisele tekitatud kahju ligikaudu 2,4 miljardit eurot aastas.

Listerioos on nakkushaigus, mille kutsub esile bakter *Listeria monocytogenes* ja mis ohustab eelkõige vastsündinuid, vanureid, rasedaid ja immuunpuudulikkusega inimesi. Listerioosi esinemissagedus on küll madal, kuid haigestunute suremus on kõrge. Aastal 2013 registreeriti Euroopa Toiduohutusameti andmetel EL-is 1763 listerioosi juhtu, mis teeb 0,44 juhtu 100 000 inimese kohta. Haiguse tõttu suri 191 inimest, mis tähendab, et registreeritud haigestunute seas oli surmavus 15,6%.

Haigusjuhtude põhjuseks on bakteritega *Campylobacter* spp. või *L. monocytogenes* saastunud toiduainete, sh linnuliha, tarbimine. Linnud võivad olla nii kampülobakterite kui ka listeeriate asümptomaatilised kandjad. Peamiselt saastub linnuliha fekaalse saastumise teel lindude algtöötlemisel tapamajas või ristsaastumise teel linnuliha edaspidise töötlemise käigus.

Käesoleva väitekirja peamised eesmärgid olid:

1. Määrata *Campylobacter* spp. levimus (**I, II**) ja arvukus (PMÜ/g) (**II**) ning *L. monocytogenes*'e levimus (**III**) Eestis toodetud ja Eestisse imporditud toores linnulihas, et selgitada linnuliha potentsiaalset tähtsust nakkuse ülekandumises inimesele.
2. Serotüpiseerida ja genotüpiseerida pulseeriva välja geelelektroforeesi (inglise keeles: *pulsed-field gel electrophoresis*, PFGE) meetodil toorest

linnulihast isoleeritud kampülobakteri (*Campylobacter* spp.) **(I)** ja listeeria (*L. monocytogenes*) **(III)** tüved geneetilise mitmekesisuse mõistmiseks.

3. Määrata *Campylobacter* spp. isolaatide tundlikkus antibiootikumide suhtes (**I**), et välja selgitada resistentsuse kombinatsioonid ja multiresistentsete tüvede esinemine.

Doktoritöös kasutati Rahvusvahelise Standardiseerimise Organisatsiooni ja Põhjamaade Toidu Analüüsimetoodika Komitee uurimismeetodeid, mille alusel tuvastati ja loendati *Campylobacter* spp. ning tuvastati *L. monocytogenes*. Serotüpiseerimine tehti vastavalt tootjapoolsetele juhenditele, kasutades spetsiifilisi antiseerumeid (Denka Seiken, Tokio, Jaapan). Genotüpiseerimisel kasutati Helsingi Ülikooli toiduhügieeni ja keskkonnatervise osakonnas välja töötatud juhendeid. Tundlikkus antibiootikumide suhtes määrati diskdifusiooni meetodil ja E-testi abil (Epsilometer test; AB Biodisk, Solna, Rootsi).

Campylobacter spp. uuringud aastatel 2002–2003 (I)

Kokku uuriti 580 (396 Eesti päritolu ja 184 Belgiast, Saksamaalt, Soomest, Taanist, Ungarist ja USA-st imporditud) toore kanabroileriliha ja 30 (Ungarist imporditud) toore kalkuniliha proovi, mis koguti Eesti jaemüügikauplustest. Eesti päritolu linnuliha oli värske ja seda müüdi nii pakendatud kui ka pakendamata kujul, importlinnuliha oli külmutatud ja seda müüdi pakendamata kujul.

Kanabroileriliha proovidest osutus 8% ja kalkuniliha proovidest 73% kampülobakteriga (*Campylobacter* spp.) saastunuks. Eesti päritolu ja imporditud linnuliha proovidest osutus saastunuks vastavalt 9% ja 16%. Ühtekokku isoleeriti 70 kampülobakteri tüve (kanabroilerilihast 48 ja kalkunilihast 22), millest 64 olid *C. jejuni*, 4 *C. coli* ja 2 *Campylobacter* spp.

54-st *C. jejuni* tüvest (valitud juhuslikult) tuvastati 11 serotüüpi, 22% (12/54) isolaatidest osutusid mitte serotüpiseeritavateks. Peamised serotüübid olid O:1,44; O:21 ja O:55 ning nendesse kuulusid vastavalt 28%, 13% ja 13% isolaatidest. Kanabroilerilihast tuvastati kõige rohkem *C. jejuni* tüvesid (n = 37), mis kuulusid serotüüpidesse O:1,44 (32%) ja O:21 (19%). Kalkunilihast isoleeritud *C. jejuni* tüved (n = 17) jaotusid kolme serotüüpi: O:55 (29%), O:1,44 (18%) ja O:18 (12%).

Kokku genotüpiseeriti PFGE meetodil 70 kampülobakteriisolaati, mille tulemusel saadi 29 *Sma*I ja 34 *Kpn*I tüüpi. Seega, bakteriraku ensüüm *Kpn*I omas suuremat genotüpiseerimise võimet, kusjuures viie kampülobakteri isolaadi DNA ei lõhustunudki *Sma*I ensüümi kasutades. 29 *Sma*I ja 34 *Kpn*I tüüpide makrorestriktsiooni kombinatsioonil saadi 37 tüüpi, millest 33 tüüpi koosnesid *C. jejuni* (91%), 2 *C. coli* (6%) ja 2 *Campylobacter* spp. (3%) isolaatidest. Üldiselt võib öelda, et erinevate riikide toodetest isoleeritud kampülobakterite tüved ei kattunud genotüübiliselt koosluselt üksteisega, välja arvatud *Sma*I tüüp 1 (nii Eesti kui ka Ungari tüved) ja tüüp 10 (nii Eesti kui ka USA tüved) ning *Kpn*I tüüp 22 (nii Eesti kui ka USA tüved). Enamik tüvedest, mis kuulusid samasse genotüüpi, pärinesid ühe ja sama riigi linnulihatoodetest. Uuringutega leiti, et ühte genotüüpi kuulunud kampülobakterite tüved kuulusid sageli erinevatesse serotüüpidesse ja ühte serotüüpi kuulunud tüved kuulusid sageli erinevatesse genotüüpidesse.

Kõigil isoleeritud kampülobakterite tüvedel (n = 70) määrati antibiootikumide tundlikkus tsiprofloksatsiini, nalidiksiinhappe, tetratsükliini, ampitsilliini, erütromütsiini ja gentamütsiini suhtes ning resistentsete tüvede osakaal oli vastavalt 66%, 66%, 44%, 34%, 14% ja 0%. Eesti päritolu kampülobakteri isolaatide (n = 36) resistentsus tsiprofloksatsiini, nalidiksiinhappe, tetratsükliini, ampitsilliini ja erütromütsiini suhtes oli vastavalt 44%, 44%, 22%, 19% ja 17% ning importlihast isoleeritud tüvede (n = 34) resistentsus vastavalt 88%, 88%, 68%, 50% ja 12%. Vähemalt ühe antibiootikumi suhtes olid resistentsed 57 isolaati (81%). Täpsemalt: 15 isolaati (21%) olid resistentsed ühele antibiootikumile, 30 isolaati (43%) kahele ja 12 isolaati (17%) kolmele antibiootikumile. Kahe antibiootikumi suhtes resistentsete kampülobakteri isolaatide resistentsuse kombinatsioonid olid järgmised: ampitsilliinile ja tsiprofloksatsiinile (9%), ampitsilliinile ja erütromütsiinile (4%) ning tsiprofloksatsiinile ja tetratsükliinile (30%). Kolme antibiootikumi suhtes resistentsete kampülobakteri isolaatide resistentsuse kombinatsioonid olid järgmised: ampitsilliinile, tsiprofloksatsiinile ja erütromütsiinile (4%) ning ampitsilliinile, tsiprofloksatsiinile ja tetratsükliinile (13%). Kõige kõrgem oli resistentsus tsiprofloksatsiini (66%) suhtes, seejärel tetratsükliini (44%), ampitsilliini (34%) ja erütromütsiini (14%) suhtes.

Campylobacter spp. uuringud aastal 2012 (II)

Aastal 2012 tehti Eestis kaks uuringut, mille raames uuriti kokku 600 linnulihaproovi. Esimese uuringu korraldas Veterinaar- ja Toiduamet ning analüüsid tegi Veterinaar- ja Toidulaboratoorium. Kokku uuriti

380 linnulihaproovi. Teine uuring tehti Eesti Maaülikooli toiduhügieeni osakonnas ja kokku uuriti 220 linnulihaproovi. Mõlema uuringu peamine eesmärk oli uurida bakteri *Campylobacter* spp. levimust värsketes linnulihatoodetes Eesti jaemüügis. Teise uuringu eesmärk oli lisaks levimusele määrata ka kampülobakterite arvukus Eesti, Läti ja Leedu päritolu värskes kanabroilerilihas.

Kampülobakterite levimus värskes kanabroilerilihas oli esimeses uuringus 13% ja teises uuringus 35%. Erinevuse põhjus võib olla see, et teises uuringus analüüsiti üksnes neid kanabroileriliha proove, mis sisaldasid nahka (koivad, tiivad ja rinnakud). Tulemustest võib järeldada, et kampülobakteriga saastunud tooteid on märkimisväärselt rohkem nahka sisaldavate toodete hulgas. Oluline on mainida, et peamiselt müüakse Eesti jaekaubanduses selliseid värskeid kanabroileriliha tooteid, mis sisaldavad nahka.

Arvukuse analüüsis loendati positiivsete proovide kampülobakterite keskmiseks 3,2 $\log_{10}$ PMÜ/g ehk 1600 bakterit 1 grammi toote kohta. Keskmine kampülobakterite arvukus oli kõrgeim Läti päritolu toodetes (2600 PMÜ/g), järgnesid Leedu (1600 PMÜ/g) ja Eesti (660 PMÜ/g) tooted.

Eesti ja Leedu päritolu linnuliha saastumist uuriti kokku 12 järjestikku kuud. Mõlema uuringu analüüsist nähtub, et Eesti jaekaubanduses müüdava linnuliha saastatus kampülobakteriga suureneb kevadel, püsib kõrge soojadel suvekuudel ja hakkab langema sügisel.

L. monocytogenes uuringud (III)

Kokku analüüsiti 240 (120 Eesti päritolu ja 120 imporditud) toorest broilerikoiba, mis koguti Tallinna ja Tartu suurematest jaemüügikauplustest ja turgudelt aastal 2002. Importtooted olid Rootsi, Soome, Taani, Ungari ja USA päritolu. Eesti päritolu linnuliha oli värske ja müüdi nii pakendatud kui ka pakendamata kujul, importlinnuliha oli külmutatud ja müüdi pakendamata kujul.

L. monocytogenes esines 70% proovides. Eesti ja imporditud linnuliha proovidest osutus positiivseks vastavalt 88% ja 53%. Bakteriga *L. monocytogenes* saastunud linnulihaproove esines rohkem Tartu kui Tallinna jaekaubanduses, vastavalt 100% ja 83%.

Kokku serotüpiseeriti 71 *L. monocytogenes* isolaati, mis kuuluvad kolme serotüüpi: 1/2a, 1/2b ja 4b. 96% isolaatidest kuulus serotüüpi 1/2a, 3% serotüüpi 1/2b ja 1% serotüüpi 4b. Serotüüp 1/2a domineeris nii Eesti kui ka imporditud linnulihas. Serotüüp 4b esines vaid Ungari päritolu toodetes.

Ühtekokku genotüpiseeriti PFGE meetodil 169 *L. monocytogenes*'e isolaati, millest 106 isoleeriti Eesti ja 63 imporditud linnulihast. Genotüpiseerimisel saadi 35 genotüüpi, millest 11 sisaldusid üksnes Eesti, 2 Soome, 4 Taani ja 4 Ungari toodetes. 14 genotüüpi olid sellised, mida leiti erinevate, ka üksteisest kaugemal paiknevate riikide toodetest. Mõnel juhul esines üks ja sama genotüüp eri maadest pärit linnuliha proovides, mis olid ostetud ühest müügikohast. See võib viidata võimalikule ristsaastumisele müügikohas. Pikema uurimisperioodi jooksul leiti Eesti päritolu linnuliha eri proovidest korduvalt sama genotüüp. See võib viidata saastumisele linnuliha käitlevas ettevõttes.

Järeldused

Eestis toodetud ja Eestisse imporditud linnuliha osutus saastunuks bakteritega *Campylobacter* spp. ja *L. monocytogenes*. Uuritud patogeenide tüpiseerimine viitas sero- ja genotüübilisele mitmekesisusele ning riigispetsiifiliste genotüüpide olemasolule. Ainult üksikud isolaadid olid sellised, mis esinesid mitme riigi linnulihas. Tuvastati kõrge isoleeritud kampülobakterite tüvede resistentsus fluorokinoloonide, tetratsükliini, ampitsilliini ja erütromütsiini suhtes.

Eestis on vaja tõhustada linnuliha kontrolli kogu toiduahela ulatuses – algtootmisest farmis kuni jaekaubanduseni. Kontrollprogrammide kasutuselevõtmisel peaks lähtuma Põhjamaade ja/või teiste EL-i riikide senisest kogemusest ja praktikast. Kontrollprogrammid peaksid keskenduma bioohutuse tagamisele linnufarmides, et vältida karjade nakatumist. Häid hügieeni- ja tootmistavasid peab rakendama kogu toiduahela ulatuses. Antibiootikumide kasutamine linnufarmides peab olema rangelt kontrollitud ja lubamatu on nende profülaktiline kasutamine. Senisest rohkem tuleks teha koostööd veterinaar- ja humaanmeditsiinis (nt bakterite *Campylobacter* spp. ja *L. monocytogenes* haiguspuhangute uurimisel), et lahendada ühiselt rahvatervisega seonduvaid probleeme.

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I

ORIGINAL PUBLICATIONS

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PFGE genotyping and antimicrobial susceptibility of *Campylobacter* in retail poultry meat in Estonia

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Abstract

In the present study, the *Campylobacter* isolates from retail poultry meat in Estonia were sero- and genotyped, and the antimicrobial susceptibility was determined. Forty-eight chicken (36 Estonian, 12 imported) and 22 turkey (imported) *Campylobacter* isolates from 580 raw broiler chicken (396 Estonian, 184 imported) and 30 turkey (imported) meat samples were studied. Of the isolates, 64 were *C. jejuni*, 4 *C. coli*, and 2 *Campylobacter* spp. Penner serotyping of 54 *C. jejuni* isolates revealed 11 different serotypes, and 22% of the isolates were nontypeable by the commercial antisera. The most common serotypes O:1,44; O:21, and O:55 accounted for 28%, 13%, and 13% of the isolates, respectively. Differences in serotype distribution were seen for chicken and turkey isolates. Genotypic characterization of all *Campylobacter* isolates ($n=70$) was performed by pulsed-field gel electrophoresis (PFGE). *Sma*I and *Kpn*I yielded 29 and 34 PFGE types, respectively, revealing high diversity among isolates. The serotype distribution did not show an association with the origin of the sample, but the majority of the isolates sharing a similar PFGE genotype originated from one country. High levels of resistance to ciprofloxacin (66%), nalidixic acid (66%), tetracycline (44%), ampicillin (34%), and erythromycin (14%) were detected among the 70 *Campylobacter* isolates. The simultaneous resistance to two or three antimicrobial agents occurred in 60% of the isolates. The *Campylobacter* isolates from turkey meat had higher resistance to ampicillin, ciprofloxacin, nalidixic acid, and tetracycline than those from chicken meat. None of the chicken isolates were resistant to gentamicin, and no turkey isolates to erythromycin or gentamicin.

Keywords: *Campylobacter*; Poultry meat; PFGE; Serotyping; Antimicrobial susceptibility

1. Introduction

Campylobacter jejuni is the most common bacterial cause of human food-borne illnesses in developed countries (Altekruse et al., 1999; Friedman et al., 2000; Rautelin and Hänninen, 2000). Several epidemiological case-control studies have established that ingesting undercooked poultry products significantly increases the risk for acquisition of food-borne campylobacteriosis (Eberhart-Phillips et al., 1997; Studahl and Andersson, 2000; Kramer et al., 2000; Neimann et al., 2003; Schönberg-Norio et al., 2004). Slaughterhouse studies have shown that the main source of contamination of *C. jejuni* poultry carcasses is their intestinal contents (Wedderkopp et al., 2000; Newell et al., 2001; Berrang et al., 2004).

Serotyping is a widely used method for typing *C. jejuni* (Rautelin and Hänninen, 1999; Wassenaar and Newell, 2000). Two serotyping schemes have been developed for campylobacter subtyping (Penner and Hennessy, 1980; Lior et al., 1982). Tracing the sources and understanding the epidemiology of *Campylobacter* is increasingly done by molecular typing (de Boer et al., 2000; Nielsen et al., 2000; Wassenaar and Newell, 2000). A widely used method for molecular typing of *C. jejuni* is pulsed-field gel electrophoresis (PFGE) (Gibson et al., 1995; Hänninen et al., 2000; Kärenlampi et al., 2003). It appears to be a highly discriminatory method especially when used with the two restriction enzymes, *Sma*I and *Sac*II/*Kpn*I (Gibson et al., 1997; Hänninen et al., 1998; Michaud et al., 2001).

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Table 1
Distribution of *Campylobacter jejuni* serotypes isolated from raw retail poultry

Serotype	No. of isolates originating from different countries[a]				
	DK	EE	FI	HU	US
O:1,44	4	6	1	3	1
O:2		2			
O:4-complex		2			
O:11		1			
O:12		3			
O:18				2	
O:21	3	4			
O:27		1			
O:38		1			
O:41	1				
O:55		2		5	
NT[b]		3		7	2
Total	8	25	1	17	3

[a] Country: DK, Denmark; EE, Estonia; FI, Finland; HU, Hungary; US, The United States.
[b] NT, nontypeable.

Erythromycin is the antimicrobial agent recommended for the treatment of human campylobacteriosis (Engberg et al., 2001). Antimicrobial resistance has emerged among *Campylobacter* mainly as a consequence of the use of antimicrobial agents, especially fluoroquinolones, macrolides, and tetracyclines in food animal production (Endtz et al., 1991; Jacobs-Reitsma, 1997; Piddock et al., 2000; Smith et al., 2000; Aarestrup and Engberg, 2001; Engberg et al., 2001).

The aims of the present study were to serotype and PFGE genotype *Campylobacter* isolates originating from raw retail poultry meat in Estonia, as well as to determine the antimicrobial susceptibility of the *Campylobacter* isolates to ampicillin, ciprofloxacin, erythromycin, gentamicin, nalidixic acid, and tetracycline.

2. Materials and methods

2.1. Isolates

We studied 48 broiler chicken (8 Danish, 36 Estonian, 1 Finnish, and 3 U.S. origin) and 22 turkey (Hungarian origin) *Campylobacter* isolates from 580 raw broiler chicken (396 Estonian, 184 imported) and 30 turkey (imported) meat samples obtained from retail stores in Estonia between January 2002 and December 2003. Of the isolates, 64 were identified as *C. jejuni*, 4 *C. coli*, and 2 *Campylobacter* spp.

The isolation of *Campylobacter* was carried out in two laboratories. The Department of Food and Environmental Hygiene, University of Helsinki analysed altogether 290 samples using the following method. One hundred milliliters of peptone (0.1%)–saline (0.85%) solution was added to the whole sample (broiler leg) in a plastic bag, and the sample was massaged by hand for 1 min. Twenty milliliters of the suspension was added into 80 ml of *Campylobacter* enrichment broth (Lab M, Bury, Lancashire, UK) and enriched at 37 °C for 24 h and 48 h under microaerobic conditions (5% O_2, 10% CO_2, 85% N_2). Microaerobic conditions were produced in jars by using Oxoid gas-generating kits according to the manufacturer's instructions (Oxoid, Basingstoke, Hampshire, UK).

The Central Veterinary and Food Laboratory in Tartu, Estonia analysed 320 of the samples for *Campylobacter* using the method of the Nordic Committee on Food Analysis (Anonymous, 1990), which includes enrichment in Preston broth. The addition of 25 g of sample (minced meat or skin and muscle of breast, carcass, thigh, wing) to 250 ml Preston enrichment broth (Oxoid) followed by the sample being stomached for 60 s. Incubation was carried out at 42±0.5 °C for 24 h under microaerobic conditions.

In both methods, after 24 h and 48 h incubation a loopful of the enrichment broth was plated on modified charcoal cefoperazone deoxycholate agar (mCCDA) (Oxoid), and examined for typical growth after 48 h. Typical grayish, campylobacter-like colonies growing on mCCDA plates were streaked on Brucella blood agar (Oxoid), and confirmed by gram staining, motility analysis, oxidase and catalase test as campylobacters. The isolate from each positive sample was identified as *C. jejuni* as being positive or *C. coli* as being negative in hippurate hydrolysis test. Additionally, an indoxyl acetate hydrolysis test was performed for hippurate negative isolates, and the isolates negative in this test were regarded as *Campylobacter* spp. After the original isolation, the strains were stored at −70 °C in glycerol broth (15% [vol/vol] glycerol in 1% [wt/vol] proteose peptone).

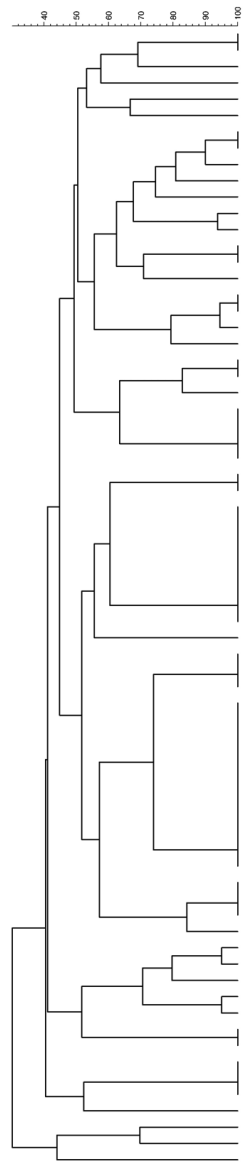
2.2. Serotyping

Arbitrarily chosen 54 *C. jejuni* isolates were serotyped using commercial *Campylobacter* antisera according to the manufacturer's instructions (Denka Seiken, Tokyo, Japan). Before the serotyping test, the isolates were cultured on Brucella blood agar (Oxoid) plates at 37 °C for 48 h in microaerobic conditions.

2.3. In situ DNA isolation and PFGE

PFGE typing was performed for 70 *Campylobacter* isolates, representing one isolate from each positive sample. As described previously, *in situ* DNA was isolated and characterized by PFGE (Gibson et al., 1994; Hänninen et al., 1998). The DNA was digested with *Sma*I or *Kpn*I (New England Biolabs, Beverly, Mass.) (20 U per sample), and the restriction fragments were separated with ramped pulses of 1 to 30 s and 1 to 25 s for 19 h, respectively.

Fig. 1. Combined dendrogram of *Sma*I and *Kpn*I macrorestriction patterns (MRP) of *Campylobacter* isolated from raw retail poultry meat in Estonia. Similarity analysis was performed using the Dice coefficient, and clustering was performed by the unweighted pair-group method with arithmetic averages (position tolerance, 1.0%). Country: DK, Denmark; EE, Estonia; FI, Finland; HU, Hungary; US, United States. Species: CC, *Campylobacter coli*; CJ, *Campylobacter jejuni*; Csp. *Campylobacter* spp. [a]ND, not digested. [b]NT, nontypeable. [c]NP, not performed.



PFGE type	MRP *SmaI*[a]	MRP *KpnI*	Product	Country	Species	Serotype[b, c]
18	12	12	turkey	HU	CJ	O:18
18	12	12	turkey	HU	CJ	O:18
17	11	11	chicken carcass	EE	CJ	O:1,44
5	4	4	chicken wing	US	CJ	NT
14	ND	33	chicken carcass	EE	Csp.	NP
6	5	5	chicken wing	US	CJ	NT
13	10	10	chicken wing	EE	CJ	O:1,44
13	10	10	chicken wing	EE	CJ	O:1,44
34	28	10	chicken leg	EE	CJ	O:1,44
33	27	27	chicken leg	EE	CJ	O:27
35	10	36	chicken leg	EE	CJ	O:1,44
28	22	22	chicken leg	EE	CJ	O:1,44
36	10	22	chicken leg	US	CJ	O:1,44
12	9	9	chicken carcass	EE	CJ	O:2
12	9	9	chicken carcass	EE	CJ	O:2
32	26	26	chicken leg	FI	CJ	O:1,44
8	6	31	chicken wing	DK	CJ	O:1,44
8	6	31	chicken wing	DK	CJ	O:1,44
7	6	6	chicken wing	DK	CJ	O:1,44
9	6	32	chicken wing	DK	CJ	O:1,44
20	14	14	turkey	HU	CJ	O:1,44
20	14	14	turkey	HU	CJ	O:1,44
21	15	15	turkey	HU	CJ	O:1,44
27	21	21	chicken wing	EE	CJ	O:4-complex
27	21	21	chicken leg	EE	CJ	O:4-complex
27	21	21	chicken leg	EE	CJ	O:38
27	21	21	chicken leg	EE	CJ	NT
11	8	8	chicken wing	EE	CC	NP
11	8	8	chicken wing	EE	CC	NP
4	3	3	chicken wing	EE	CJ	NP
4	3	3	chicken carcass	EE	CJ	NP
4	3	3	chicken wing	EE	CJ	NT
4	3	3	chicken wing	EE	CJ	O:11
4	3	3	chicken wing	EE	CJ	O:55
4	3	3	chicken wing	EE	CJ	O:55
4	3	3	chicken wing	EE	CJ	NT
4	3	3	chicken wing	EE	CJ	NP
10	7	7	chicken wing	DK	CJ	O:41
2	1	1	chicken carcass	EE	CJ	O:12
2	1	1	chicken carcass	EE	CJ	O:12
2	1	1	chicken carcass	EE	CJ	O:12
16	1	35	turkey	HU	CJ	NP
16	1	35	turkey	HU	CJ	NP
16	1	35	turkey	HU	CJ	NT
16	1	35	turkey	HU	CJ	O:55
16	1	35	turkey	HU	CJ	O:55
16	1	35	turkey	HU	CJ	NT
16	1	35	turkey	HU	CJ	O:55
16	1	35	turkey	HU	CJ	NT
16	1	35	turkey	HU	CJ	O:55
16	1	35	turkey	HU	CJ	NT
16	1	35	turkey	HU	CJ	O:55
1	ND	30	chicken carcass	EE	CJ	O:21
1	ND	30	chicken carcass	EE	CJ	O:21
1	ND	30	chicken carcass	EE	CJ	O:21
15	ND	34	chicken carcass	EE	CJ	O:21
22	16	16	turkey	HU	CJ	NT
25	19	16	turkey	HU	CJ	NT
24	18	18	turkey	HU	CJ	NP
19	13	13	turkey	HU	CJ	NP
26	20	20	turkey	HU	CJ	NP
3	2	2	chicken carcass	EE	CC	NP
3	2	2	chicken carcass	EE	CC	NP
29	23	23	chicken leg	DK	CJ	O:21
29	23	23	chicken leg	DK	CJ	O:21
29	23	23	chicken leg	DK	CJ	O:21
23	17	17	turkey	HU	CJ	NT
31	25	25	chicken leg	EE	CJ	NP
37	29	29	chicken leg	EE	CJ	NP
30	24	24	chicken leg	EE	Csp.	NP

Table 2
Distribution of *Campylobacter* PFGE genotypes from raw retail poultry with *Sma*I and *Kpn*I, according to country

Country	Number of strains	Number of PFGE genotypes		PFGE genotypes[a]	
		*Sma*I	*Kpn*I	*Sma*I	*Kpn*I
Denmark	8	3	5	6, 7, 23	6, 7, 23, 31, 32
Estonia	36[b]	14	17	1, 2, 3, 8, 9, 10, 11, 21, 22, 24, 25, 27, 28, 29	1, 2, 3, 8, 9, 10, 11, 21, 22, 24, 25, 27, 29, 30, 33, 34, 36
Finland	1	1	1	26	26
Hungary	22	10	9	1, 12, 13, 14, 15, 16, 17, 18, 19, 20	12, 13, 14, 15, 16, 17, 18, 20, 35
United States	3	3	3	4, 5, 10	4, 5, 22
Total	70	29	34		

[a] Underlined PFGE genotype has been detected in poultry that originated from more than one country.
[b] The DNA of five isolates was not digested by *Sma*I.

2.4. PFGE pattern analysis

The computer software program BioNumerics 3.5 (Applied Maths, Sint-Martens-Latem, Belgium) was used for numerical analysis of *Sma*I and *Kpn*I macrorestriction patterns. Similarity analysis was carried out using the Dice coefficient (position tolerance, 1.0%). The dendrogram was constructed using the unweighted pair-group method with arithmetic averages.

2.5. Antimicrobial susceptibility testing

All *Campylobacter* isolates were tested by the disc diffusion method against ampicillin (25 μg), ciprofloxacin (5 μg), erythromycin (15 μg), gentamicin (10 μg), nalidixic acid (30 μg), and tetracycline (10 μg) (Oxoid), and by the Epsilometer test (*E*-test) (AB Biodisk, Solna, Sweden) against ampicillin, ciprofloxacin, erythromycin, and tetracycline.

Campylobacter isolates were first grown on blood agar plates and were transferred in 5 ml of Mueller–Hinton (MH) broth (Oxoid), and incubated at 37 °C for 24 h under microaerobic conditions. Inoculum from the MH broth was diluted and a turbidity equivalent of a 0.5 McFarland standard was adjusted in physiological peptone–saline water and the growth suspension was spread on the MH blood agar plates (Oxoid, supplemented with 7% horse blood), the disks or *E*-test strips containing antimicrobial compounds were laid on the plates. The plates were incubated at 37 °C for 24 h in microaerobic conditions. The diameter of the growth inhibition zone was measured according to the CLSI (2004). MIC values were determined by *E*-test according to the instructions given by the manufacturer (AB Biodisk). *C. jejuni* 143483 was used as control strain in the antimicrobial susceptibility testing (Hakanen et al., 2002).

The following zone diameter (mm) and MIC breakpoints for resistance were applied: ampicillin ≤ 13 mm and MIC ≥ 32 μg/ml, ciprofloxacin ≤ 26 mm and MIC ≥ 4 μg/ml, erythromycin ≤ 26 mm and MIC ≥ 32 μg/ml, gentamicin ≤ 12 mm, nalidixic acid ≤ 26 mm, and tetracycline ≤ 31 mm and MIC ≥ 16 μg/ml (Anonymous, 2004; CLSI, 2004).

3. Results

3.1. Serotype distribution

Eleven serotypes were obtained from 54 *C. jejuni* isolates (Table 1, Fig. 1). Of the isolates, 22% (12/54) were nontypeable. The most common serotypes O:1,44; O:21, and O:55 accounted for 28%, 13%, and 13% of the isolates. The isolates from chicken meat ($n=37$) included ten serotypes, and the frequent serotypes were O:1,44 (32%) and O:21 (19%). The isolates from turkey meat ($n=17$) belonged to three serotypes: O:55 (29%), O:1,44 (18%), and O:18 (12%).

3.2. PFGE genotypes

The PFGE genotyping of 70 *Campylobacter* isolates yielded 29 *Sma*I and 34 *Kpn*I PFGE types (Table 2, Fig. 1). The DNA of five isolates was not digested by *Sma*I. Combination of the macrorestriction patterns resulted in 37 PFGE types (Fig. 1). Of these, 33 PFGE types were from *C. jejuni* (91%), 2 from *C. coli* (6%), and 2 from *Campylobacter* spp. isolates (3%).

3.3. Antimicrobial susceptibility

In the disc diffusion method, resistance to ciprofloxacin, nalidixic acid, tetracycline, ampicillin, and erythromycin occurred in 66%, 66%, 44%, 34%, and 14% of the *Campylobacter* isolates ($n=70$). Resistance to ciprofloxacin, nalidixic acid, tetracycline, ampicillin, and erythromycin occurred in 44%, 44%, 22%, 19%, and 17% of the Estonian isolates ($n=36$) and in 88%, 88%, 68%, 50%, and 12% of the imported isolates ($n=34$), respectively. All isolates were susceptible to gentamicin.

Resistance to ciprofloxacin, nalidixic acid, tetracycline, ampicillin, and erythromycin occurred in 50%, 50%, 27%, 23%, and 14% of the chicken isolates ($n=48$). Two *C. coli* isolates from chicken showed resistance to ampicillin, ciprofloxacin, and nalidixic acid. One isolate of *Campylobacter* spp. from chicken was resistant to ampicillin, and the other isolate to ciprofloxacin and nalidixic acid. Of the turkey isolates ($n=22$) all were resistant to ciprofloxacin and nalidixic acid, 82% to tetracycline, and 59% to ampicillin.

Resistance occurred in 57 isolates (81%) out of 70 tested to at least one of the antimicrobials (Table 3). Fifteen isolates (21%) were resistant to one, 30 isolates (43%) to two, and 12 isolates (17%) to three antimicrobial agents. The resistance of *Campylobacter* isolates to two antimicrobials showed a combination of ampicillin and ciprofloxacin (9%), ampicillin and erythromycin (4%), and ciprofloxacin and tetracycline (30%). The resistance of isolates to three antimicrobials showed a combination of ampicillin, ciprofloxacin, and erythromycin (4%), and ampicillin, ciprofloxacin, and tetracycline (13%). The highest level of resistance recorded was to ciprofloxacin (66%) followed by tetracycline (44%), ampicillin (34%), and erythromycin (14%).

4. Discussion

Our studies showed high serotype and genotype diversity among *Campylobacter* isolates from raw retail poultry meat in

Table 3
MICs for ampicillin (AM), ciprofloxacin (CI), erythromycin (ERY), and tetracycline (TC) of *Campylobacter* isolated from raw retail poultry meat in Estonia

Country of origin[a]	No. of isolates	Antimicrobial agents	No. of isolates with MIC (μg/ml)[b]												
			≤ 0.06	0.125	0.25	0.5	1	2	4	8	16	32	64	128	≥ 256
DK	8	AM				2	2				1				3
		CI					1					7			
		ERY			1	1	1	1							4
		TC	1	1			1			1			1		3
EE	36	AM				3	7	8	4	3	4	1	1		5
		CI	4	10	3	2		1				16			
		ERY			1	8	12	3	4	1	1	1			5
		TC	10	6	5	4	2	1			2	3	1		2
FI	1	AM													1
		CI			1										
		ERY							1						
		TC				1									
HU	22	AM					3	4	1	1					13
		CI								1	1	20			
		ERY		1	5	8	2	2	3	1					
		TC	2	1		1									18
US	3	AM				1				1	1				
		CI	1	1								1			
		ERY			1		1				1				
		TC		1	1						1				

The MIC values for the isolates were evaluated accordance to the Danmap (2004) and Clinical and Laboratory Standards Institute (2004). Solid vertical lines indicate breakpoints between susceptible and resistant isolates.

[a]Country: DK, Denmark; EE, Estonia; FI, Finland; HU, Hungary; US, United States.

[b]The E-test values between two-fold dilutions were rounded up to the next upper two-fold value before the categorization according to manufacturer instructions (AB Biodisk, Solna, Sweden).

Estonia. Nine of the eleven *C. jejuni* serotypes obtained were common in poultry products of Estonian origin, and five in those imported to Estonia. The serotype distribution did not show association with the origin of the sample. The most common serotypes were O:1,44; O:21, and O:55, accounting for 54% of the isolates. Serotype distribution differences

occurred for chicken and turkey isolates. The chicken isolates had two common serotypes (O:1,44 and O:21) out of ten, whereas turkey isolates belonged to only three different serotypes (O:1,44; O:18 and O:55). In the studies in Denmark (Nielsen and Nielsen, 1999) and New Zealand (Devane et al., 2005), the serotype O:1,44 was also one of the most common in poultry products, and this serotype seems to have global distribution among strains isolated from human *Campylobacter* infections (Nielsen et al., 1997; Vierikko et al., 2004; Devane et al., 2005; Miller et al., 2005). The most frequently isolated serotype in chicken meat in New Zealand was O:21 (Devane et al., 2005), the second most common serotype in our study. The presence of serotypes O:2, O:4-complex, and O:12, common to both chickens and human patients (Fricker and Park, 1989; Hudson et al., 1999; Perko-Mäkelä et al., 1999; Petersen et al., 2001; Saito et al., 2005), occurred in only 13% of the isolates studied.

Serotyping of *C. jejuni* showed that 22% of the isolates were nontypeable, and seven of the nontypeable isolates originated from turkey meat imported from Hungary. By using the same commercial serotyping set as in our study, Rautelin and Hänninen (1999) found 14% of the isolates, and in a Danish study, using their own antisera, 16% of the isolates remained nontypeable (Nielsen and Nielsen, 1999) revealing the need to improve the present serotyping methods. One reason for nontypeability is the low production of capsular antigens responsible for the serotype specificity of *C. jejuni*, another reason could be new serotypes not accounted for in the present test (Jacobs-Reitsma et al., 1995).

The genotyping of the 70 *Campylobacter* isolates showed *Kpn*I to be more discriminatory, yielding 34 PFGE types compared to 29 obtained by *Sma*I. Furthermore, the DNA of five strains was not digested by *Sma*I. The genotypes of the isolates from the poultry products of different countries were not overlapping, except *Sma*I PFGE types 1 (isolates from Estonia and Hungary) and 10 (isolates from Estonia and USA), and *Kpn*I PFGE type 22 (isolates from Estonia and USA). Our results, as well as the data from several previous studies (Gibson et al., 1994; Hänninen et al., 1998; Wassenaar and Newell, 2000), however, emphasize the utility of two restriction enzymes, such as *Sma*I and *Kpn*I, in PFGE typing studies of *Campylobacter*. In our study the majority of the isolates sharing a similar PFGE genotype originated from one country. The association of genotypes with country of origin requires further studies using a larger collection of isolates, however.

We found several serotypes within one PFGE type (Fig. 1). For example, the PFGE type 4 contained the serotypes O:11, O:55, and nontypeable isolates, and PFGE type 27 contained O:4-complex, O:38, and a nontypeable isolate. Furthermore, within one serotype, several PFGE types were found. For instance, the common serotypes of our study, O:1,44; O:21, and O:55, contained up to 12, 3, and 2 different PFGE types.

An important finding of our study was the recognition of a high number (81%) of *Campylobacter* isolates with increased antimicrobial resistance. Antimicrobial resistance level was especially high to ciprofloxacin (44 isolates MIC $\geq$ 32 μg/ml), tetracycline (23 isolates MIC $\geq$ 256 μg/ml), and ampicillin (22 isolates MIC $\geq$ 256 μg/ml). The resistance to antimicrobials, except erythromycin, was higher in isolates from imported poultry products than in those originating from Estonia. The *Campylobacter* isolates from turkey meat had a higher resistance to ampicillin, ciprofloxacin, nalidixic acid, and tetracycline than those from chicken meat. All isolates resistant or susceptible by the disk diffusion method showed the same results by *E*-test.

Ciprofloxacin resistance was high among isolates from both imported (88% of the isolates) and domestic products (44% of the isolates). Furthermore, 100% of the turkey and 50% of the broiler isolates showed resistance to ciprofloxacin. All isolates with resistance to ciprofloxacin also tested resistant to nalidixic acid. A study in Spain (Saenz et al., 2000) showed very high prevalence 98% of ciprofloxacin resistance in *Campylobacter* isolates from broiler intestinal samples. The study by Endtz et al. (1991) showed a link for the first time between veterinary fluoroquinolone use and increasing fluoroquinolone resistance in poultry and human isolates of *Campylobacter*. Later studies have confirmed their results (Smith et al., 2000; Engberg et al., 2001). Enrofloxacin and flumequine, both fluoroquinolone group antimicrobials, are accepted for poultry treatment in Estonia (Anonymous, 2005), possibly explaining the high level of resistance detected among Estonian isolates.

Different studies typically find tetracycline resistance among poultry isolates. Ledergerber et al. (2003) reported a much lower (12%) tetracycline resistance but Ge et al. (2003) found a higher resistance (82%) among poultry than in our study (44%). Nevertheless, we found a higher resistance for turkey isolates (82%) than in the Belgian study (37%) (Van Looveren et al., 2001). Tetracycline (doxycycline) is also accepted for treatment of poultry in Estonia (Anonymous, 2005).

Ampicillin is a widely used antimicrobial in veterinary medicine. Resistance to ampicillin in broiler isolates, 23%, was at a similar level, and resistance in turkey isolates, 59%, was higher than found in the Belgian study (24% and 33%, respectively, Van Looveren et al., 2001). Ampicillin is not recommended, however, for the treatment of *Campylobacter* infections due to the high incidence of resistance to this drug among human isolates (Navarro et al., 1993). Amoxicillin is accepted for use in veterinary medicine in Estonia (Anonymous, 2005).

Campylobacter isolates displayed the lowest resistance frequency against erythromycin (14%). All resistant isolates were *C. jejuni* and they were either from Danish or Estonian chicken products. All turkey isolates were susceptible to erythromycin. Belgium, Ireland, and Switzerland (Fallon et al., 2003; Ledergerber et al., 2003; Van Looveren et al., 2001) also reported a low erythromycin resistance. Erythromycin is considered as a first line choice for the treatment of *C. jejuni* infections and low resistance among retail meat isolates supports this common policy of antimicrobial use. Additionally, similar to Ge et al. (2003) and Van Looveren et al. (2001), none of the chicken and turkey isolates showed resistance to gentamicin.

In our study we found a high level (60%) of multidrug (two or three antimicrobial agents) resistant isolates. Fallon et al. (2003) found 30% of the isolates resistant to two or more

antimicrobials. In our study, 69% of isolates consisted of two or three antimicrobials originated from poultry products imported from Denmark or Hungary. All turkey isolates were resistant to two (59%) or three (41%) antimicrobials. Multiresistant isolates consisted of a combination of ampicillin, ciprofloxacin, erythromycin, or tetracycline.

In general, the PFGE genotypes and antimicrobial susceptibility profiles correlated, except for PFGE type 16. Seven isolates of this PFGE type showed resistance to ampicillin, ciprofloxacin, and tetracycline, whereas four isolates were resistant to ciprofloxacin and tetracycline but sensitive to ampicillin. All of these eleven isolates were from turkey meat originating from Hungary and obtained during the same time period. These results may indicate that in a multiresistant bacterial population with identical genotype, the resistance patterns may be different.

Since campylobacteriosis is transmitted particularly via foods of animal origin, the presence of antimicrobial resistant *Campylobacter* isolates in raw meat has important public health implications. In order to confirm the real resistance situation, further investigations on resistance patterns in *Campylobacter* along the whole poultry production chain are necessary. In Estonia, a national monitoring program for antibiotic resistance, including both human and food isolates, is needed.

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Prevalence and counts of *Campylobacter* spp. in poultry meat at retail level in Estonia

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ABSTRACT

Campylobacter contamination of poultry meat at retail level was studied in two surveys during the twelve-month period of 2012 in Estonia. The data from these surveys were combined and analyzed, partially together, in order to comprehensively estimate the prevalence and possible seasonality of *Campylobacter* in poultry and in poultry meat products in Estonia. Mostly Estonian, Lithuanian and Latvian products, representing the most typical origins of poultry products on the Estonian retail market, were sampled and analyzed in these surveys. The first survey, organized by the Estonian Veterinary and Food Board, focused on *Campylobacter* prevalence in poultry meat at retail level. The second survey, at the Estonian University of Life Sciences, focused on *Campylobacter* prevalence and counts in fresh broiler chicken meat at retail level. Additionally, broiler chicken caecal samples were collected at slaughterhouse level for the estimation of the seasonal variation of *Campylobacter* colonization. Caecal samples were collected weekly from a broiler chicken slaughterhouse belonging to a company representing over 95% of all commercial broiler production in Estonia. A total of 606 poultry meat samples at retail level and 380 broiler chicken caecal samples at slaughterhouse level were collected and analyzed. A total of 20.8% of the poultry meat and 39.2% of the caecal samples were found positive for *Campylobacter* spp. The mean number of *Campylobacters* in fresh broiler chicken meat in the positive samples was 3.20 $\log_{10}$CFU/g. A distinct seasonal variation in the *Campylobacter* contamination of broiler chicken meat was observed, which peaked during the warm summer period.

1. Introduction

Campylobacteriosis, caused by thermotolerant *Campylobacter* species, was the most commonly reported zoonosis in the European Union (EU) in 2012, with 214,268 confirmed human cases, an average of 55.49 confirmed cases of campylobacteriosis per 100,000 of the EU population (EFSA, 2014). According to EFSA scientific opinion (2011a), the actual number of all cases is estimated to be around nine million each year, and the total cost of

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campylobacteriosis to public health in the EU is estimated to be around 2.4 billion euros a year (EFSA, 2011a). *Campylobacter* spp., predominantly *Campylobacter jejuni* and *Campylobacter coli*, most commonly cause gastroenteritis in humans, but also other extra-intestinal diseases and rare cases of post-infection conditions, such as Miller-Fisher and Guillain–Barré syndrome, which can cause serious health complications (Fica et al., 2011; Kuwabara, 2011). Campylobacters may colonize the intestines of clinically healthy birds, and therefore the poultry products. Broiler chicken meat is considered to be the main source of foodborne *Campylobacter* infection (EFSA, 2013; FAO/WHO, 2001; Humphrey, O'Brien, & Madsen, 2007). In its scientific opinions, EFSA has concluded that the handling, preparation and consumption of broiler meat may account for 20%–30% of all human campylobacteriosis cases, while 50%–80% may be attributed to the chicken reservoir as a whole. Therefore, the control of *Campylobacter* in poultry and poultry meat is a major public health strategy in the prevention of human campylobacteriosis (EFSA, 2009, 2010, 2011a). Studies at slaughterhouse level have demonstrated that cross-contamination of the chicken carcasses may occur at scalding, evisceration and water-chilling stages which transfers the *Campylobacter* contamination to the retail level (Hue et al., 2010; Kudirkiene, Bunevičienė, Bruṇdsted, & Ingmer, 2011). In the United Kingdom, the joint government and industry target has been set to reduce *Campylobacter* contamination on whole chicken carcasses in slaughterhouses by 2015. More specifically, the target is based on *Campylobacter* counts, focusing on decreasing the proportion of birds in the most contaminated group i.e. >1000 CFU/g.

In 2011, the proportion of *Campylobacter* positive broiler meat samples varied widely between EU member states, from 3.2% (Austria) to 84.6% (Luxembourg), while the level of *Campylobacter* in broiler chicken flocks varied from 0% in Estonia to 92% in Slovenia (EFSA, 2013). An EU-wide baseline study showed the average *Campylobacter* prevalence for fresh broiler chicken carcasses to be 75.8% (EFSA, 2010). A worldwide literature survey has showed that about 58% of chickens are contaminated with *Campylobacter* (Suzuki & Yamamoto, 2009). Previous studies have shown differences between Estonia and the other Baltic countries for *Campylobacter* prevalence in fresh broiler chicken meat. In Estonia, *Campylobacter* prevalence in fresh chicken meat has been 15.8% from 2000 to 2002, and from 14% in 2003–2007 (Meremäe et al., 2010; Roasto, Praakle, Korkeala, Elias, & Hänninen, 2005). According to Kovalenko, Roasto, Liepinš, Mäesaar, and Hörman (2013) 56.3% of broiler chicken carcasses were *Campylobacter*-positive in Latvia. A Lithuanian study by Bunevičienė, Kudirkienė, Ramonaitė, and Malakauskas (2010) reported higher than 40% *Campylobacter* contamination for fresh broiler chicken meat. Seasonal variation of *Campylobacter* occurrence in poultry has been previously found in Estonia and Latvia. A distinct seasonal peak in summer and in early autumn was found in Estonia (Meremäe et al., 2010) while Kovalenko et al. (2013) reported an increase in *Campylobacter* contamination in Latvia in early spring; contamination remained high during the summer months and decreased at the end of autumn.

This is the first scientific notice reporting quantitative data on *Campylobacter* spp. contamination of fresh broiler chicken meat of Baltic origin sold at the retail level in Estonia.

The overall aim of this study was to determine the prevalence and seasonality of *Campylobacter* spp. contamination of poultry meat at the retail level in Estonia.

2. Materials and methods

Two surveys for *Campylobacters* in poultry and poultry meat were carried out in 2012 in Estonia. The results of these surveys were combined, taking into account the methodological and analytical characteristics of the survey protocols. In both surveys meat sampling was made at retail level in retail stores where most poultry meat is sold and purchased in Estonia for domestic consumption. Furthermore, sales proportions were taken into account while sampling. Mostly company-packaged fresh broiler chicken meat was collected at Estonian retail level. All twelve months were included, and for *Campylobacter* detection the same methodology was applied. However, the surveys differed in some aspects which are described below.

2.1. Sample collection

Of the two separate surveys, a total of 606 poultry meat samples at retail level and 380 broiler chicken caecal samples at slaughterhouse level were collected during the year 2012. Most of the collected poultry meat samples were of Estonian (44.1%), Lithuanian (43.2%) and Latvian (8.4%) origin, but some samples originated from Poland (1.7%), Germany (1.1%), Finland (0.9%), Belgium (0.3%), and Hungary (0.3%).

In contrast to the second survey, the first survey included not only broiler chicken meat samples (76,3%) but also turkey meat (18.7%), laying hen meat (4.7%) and duck meat (0.3%). The collected samples were from a range of poultry meat categories, including fresh meat (56.7%), carcasses (11.4%), minced meat (5%), meat preparations (24.9%) and heat-treated poultry meat products (2%). Furthermore, the second survey protocol included both *Campylobacter* detection and enumeration methods, and sampling also included broiler chicken caecal sampling at slaughterhouse level for estimation of seasonality.

2.1.1. First survey

The first survey was organized by the Estonian Veterinary and Food Board, and included poultry meat sampling from retail outlets from throughout Estonia. In this survey 386 poultry meat samples were collected from different categories, such as fresh meat, carcasses, minced meat and meat preparations. Broiler chicken meat samples comprised 297 of the total of 386 samples. Meat samples were transported to the laboratory within sampling day in a portable cooler at a temperature of 4–6 °C; microbiological analyses began on the same day.

2.1.2. Second survey

The second survey was designed to estimate the prevalence and counts of *Campylobacter* spp. in high contamination-risk category products, such as fresh broiler chicken meat containing skin (drumsticks, wings and breast). Samples were collected from Estonian supermarket chain retail outlets. In the second survey only Estonian (53.6%), Lithuanian (37.3) and Latvian (9.1%) originating fresh broiler chicken meat samples were collected and analyzed. The proportion of other countries than Baltic fresh broiler chicken meat in Estonian retail is very small. Fresh broiler chicken meat sales proportions were taken into account while sampling at retail level. Estonian and Lithuanian products were available for purchase in all twelve months, while Latvian fresh broiler chicken products were available in Estonian retail outlets from September to December 2012. Only company-packaged fresh broiler chicken meat was sampled, in order to exclude the possibility of *Campylobacter* cross-contamination during storage. In total, 220 fresh poultry meat samples were collected within the 12 months of the second survey. To estimate *Campylobacter* colonization seasonality, 380 caecal samples were collected weekly from the slaughterhouse, which is owned by the company representing over 95% of all commercial broiler production in Estonia. This company owns six separate farms with 62 flocks in separate housing, with

approximately 22,000 birds per flock. Broiler chicken caecal material from randomly selected flocks (three pooled samples per farm) representing all six farms was collected from June to October, which was assumed to be the seasonal peak for *Campylobacter* contamination in Estonia. Caecal samples were taken from caecum blind sacs near the cloaca of the intestines of broiler chickens, and the material was directly transferred into tubes containing 10 ml of Bolton enrichment broth (Oxoid, Basingstoke, Hampshire, England). One loopful (10 μl) of caecal material was taken per bird, and the material from five birds was pooled into a single tube. Both caecal and meat samples were transported to the laboratory within sampling day in a portable cooler, and microbiological analyses began immediately on arrival of samples.

2.2. Isolation, identification and enumeration of Campylobacter spp.

The isolation of *Campylobacter* was carried out in two laboratories.

2.2.1. First survey

All analyses in the first survey were performed at the Estonian Veterinary and Food Laboratory. *Campylobacter* detection was carried out on 386 poultry meat samples according to the method described in ISO 10272-1:2006. The detection of *Campylobacter* was made primarily from the skin material, if available, and secondly from meat, depending on the sample type e.g. skinless poultry meat fillets. According to the detection method used, 10 g of skin or meat material was removed aseptically and placed into a sterile plastic bag. The plastic bag was then filled with 90 ml of sterile Bolton broth, and samples were processed for 1 min in a stomacher and then incubated, under microaerobic conditions, at 37 °C for 4 h–6 h, followed by 44 ± 4 h at 41.5 ± 0.5 °C. After enrichment, 10 μl of the enrichment broth was plated onto mCCDA agar (Oxoid; Basingstoke, Hampshire, England) and incubated for 48 h at 41.5 ± 0.5 °C under microaerobic conditions. Typical *Campylobacter* colonies on mCCDA plates were streaked onto Columbia blood agar (Oxoid) plates, which were incubated for 24 h at 41.5 ± 0.5 °C in microaerobic conditions using anaerobic jars and CampyGen™ reagents (Oxoid). The bacteria isolated from poultry meat that showed typical growth characteristics on mCCDA: were gram-negative, had corkscrew-like darting motility, were oxidase positive and had no growth at 41.5 ± 0.5 °C in aerobic conditions, whit growth at 25 °C in microaerobic conditions, were considered to be *Campylobacter* spp.

2.2.2. Second survey

The Laboratory of Food Hygiene of the Estonian University of Life Sciences analyzed 220 fresh broiler chicken meat samples and 380 caecal samples, collected from the second survey. The main difference compared to the first survey was that only broiler chicken meat skin material was used, and both *Campylobacter* detection and enumeration methods were applied. The detection of *Campylobacter* from fresh broiler chicken meat samples was carried out according to the ISO 10272-1:2006 method described above.

Enumeration was carried out according to the method described in ISO 10272-2:2006. In brief, 0.1 ml of 10^{-1} and 10^{-2} broiler chicken meat skin dilutions were streaked onto modified CCDA agar and incubated for 44–48 h at 41.5 ± 0.5 °C. Randomly selected five presumptive *Campylobacter* colonies were further subcultured on Columbia blood agar and later identified by microscopic examination, gram staining, and biochemical tests, as described in the ISO method.

Additionally, Bolton enrichment broth tubes with pooled caecal material were held at 4–6 °C and transported to the laboratory during sampling day. On arrival the tubes were immediately transferred into an incubator, and incubated at 41.5 ± 0.5 °C for 24 h in microaerobic conditions, following which *Campylobacter* detection and verification was carried out in accordance with ISO 10272-1:2006.

Other International Standard Organization norms (6887-1, 1999; 6887-2, 2004 etc.) were also followed in sample preparation procedures in both laboratories.

After isolation, the randomly selected strains were stored at −82 °C in glycerol broth (20% [vol/vol] glycerol in 1% [wt/vol] proteose peptone).

2.3. Campylobacter species identification

Conventional multiplex PCR assay was used for identification and differentiation of *C. jejuni*, *C. coli*, *Campylobacter lari*, *Campylobacter upsaliensis*, and *Campylobacter fetus* subsp. *fetus*, as described by Wang et al. (2002).

2.4. Statistical analysis

All individual results were recorded using MS Excel 2010 software (Microsoft Corporation, Redmond, Wash.), and statistical analysis was performed with the Statistical Package R in order to determine if there were statistically significant differences at 95% and 99% confidence levels in the prevalence and counts of *Campylobacter* in the poultry products of different origin using the Kruskal–Wallis rank sum test and Chi-square test. Additionally, seasonal variation in *Campylobacter* contamination was analyzed in order to elucidate differences in prevalence's between different sampling months.

3. Results and discussion

3.1. First survey

Among 386 poultry meat samples, the mean proportion of *Campylobacter* positive samples was 12.7% (Table 1). The proportion of *Campylobacter* contamination was 25.8% for Latvian products,

Table 1
Campylobacter in various categories of poultry meat by country of the origin in the retail market in Estonia in 2012.[a]

Country of the origin	No. of positive samples/No. of all samples (positive %)					
	Fresh meat	Whole carcass	Minced meat	Meat preparations	Heat-treated	Total
Estonia	10/56(17.9)	1/20(5.0)	4/11(36.4)	7/60(11.7)	0/2(0.0)	22/149(14.8)
Lithuania	14/125(11.2)	1/18(5.6)	1/10(10.0)	3/26(11.5)	0/1(0.0)	19/180(10.6)
Latvia	5/22(22.7)	3/6(50.0)	NS	0/2(0.0)	0/1(0.0)	8/31(25.8)
Other[b]	0/16(0.0)	NS	NS	0/8(0.0)	0/2(0.0)	0/26(0.0)
In total	29/219(13.2)	5/44(11.4)	5/21(23.8)	10/96(10.4)	0/6(0.0)	49/386(12.7)

NS, No samples available.
[a] Survey conducted by the Estonian Veterinary and Food Board.
[b] Poland, Germany, Finland, Belgium, Hungary.

14.8% for Estonian products, 10.6% for Lithuanian products and 0% for poultry products from countries other than the Baltic countries. Among raw poultry products fresh broiler chicken meat of Baltic origin is mostly sold in the Estonian retail market. The contamination of fresh broiler chicken meat of Estonian, Lithuanian and Latvian origin was 17.0%, 9.1% and 22.7% respectively. Within the tested meat categories, highest *Campylobacter* contamination was found in minced meat (23.8%) followed by fresh meat (13.2%), whole carcasses (11.4%) and meat preparations (10.4%). Sampling of the heat-treated poultry meat products was not included in the first survey sampling plan. These samples ($n = 6$) were taken by official veterinarian unintentionally, and as it is expected all were *Campylobacter* negative.

3.2. Second survey

Among 220 fresh broiler chicken meat samples (drumsticks, wings and breast), the mean proportion of *Campylobacter* positive samples was 35% (Table 2). Among Estonian, Lithuanian and Latvian-origin broiler chicken meat samples the proportions of *Campylobacter* positive products were 20.3%, 50.0% and 60.0%, respectively. The number of analyzed Latvian products in this study was small because Latvian-origin fresh broiler chicken meat products were available for purchase at the Estonian retail level only from September to December in 2012. Nevertheless, similarly to these results, almost 60% of *Campylobacter* prevalence in fresh broiler chicken meat was reported in a recent Latvian study (Kovalenko et al., 2013).

According to EFSA scientific opinion (2011a) a public health risk reduction of >50% or >90% could be achieved if all broiler batches were to comply with a microbiological criterion of a critical limit of 1000 or 500 CFU/g of neck and breast skin, respectively. The results of the *Campylobacter* enumeration on fresh broiler chicken meat in this study were categorized as follows: <100 CFU/g; 100–499 CFU/g; 500–1000 CFU/g and >1000 CFU/g. An EU baseline survey reported that *Campylobacter* counts on broiler carcasses of Estonian origin were <10 CFU/g in 98% of positive cases (EFSA, 2010). In the current study higher *Campylobacter* contamination levels for Estonian broiler chicken products were found (Table 3). Enumeration results, in the case of positive results from enumeration analyses, showed that the overall arithmetic *Campylobacter* CFU mean was 3.2 $\log_{10}$CFU/g of product (Table 2) with the highest mean contamination loads in Latvian-origin products and the lowest in those from Estonia, respectively 3.4 $\log_{10}$CFU/g and 2.8 $\log_{10}$CFU/g. The mean contamination load for Lithuanian-origin broiler chicken products available at Estonian retail was 3.2 $\log_{10}$CFU/g. A previous Lithuanian study (Bunevičienė et al., 2010) reported lower *Campylobacter* counts (mean 2.0 $\log_{10}$CFU/g) in broiler chicken meat. Among Estonian, Lithuanian and Latvian-origin products, with positive enumeration results, a contamination level of above 1000 *Campylobacter* CFU/g was found in 1.7%, 14.6% and 35.0% of samples, respectively.

Table 2
Campylobacter detection and enumeration in fresh broiler chicken meat by country of origin in the retail market in Estonia in 2012.[a]

Country of the origin	No. of positive samples/No. of all samples (positive %)	Enumeration results of positive samples,[b] $\log_{10}$CFU/g	
		Mean	Median
Estonia	24/118 (20.3)	2.8	2.5
Lithuania	41/82 (50.0)	3.2	2.9
Latvia	12/20 (60.0)	3.4	3.3
Total	77/220 (35.0)	3.2	3.0

[a] Survey conducted by the Estonian University of Life Sciences.
[b] Samples with positive detection and positive enumeration result, the threshold of 100 CFU/g.

Table 3
Campylobacter enumeration data obtained from fresh broiler chicken meat in the Estonian retail market in 2012.

Origin	*Campylobacter* counts (CFU/g)				
	0[a]	<100[b]	100–499	500–1000	>1000
Estonia	94 (79.7)	13 (11.0)	7 (5.9)	2 (1.7)	2 (1.7)
Lithuania	41 (50.0)	7 (8.6)	12 (14.6)	10 (12.2)	12 (14.6)
Latvia	8 (40.0)	2 (10.0)	1 (5.0)	2 (10.0)	7 (35.0)
Total	143 (65.0)	22 (10.0)	20 (9.1)	14 (6.4)	21 (9.5)

Number of samples (percentage).
[a] Negative detection and negative enumeration.
[b] Negative enumeration and positive detection, the threshold.

3.3. Both surveys

Fresh broiler chicken meat contamination in the first and second surveys was 13.50% and 35%. This difference can be explained by differences in the sampling methods. In the second survey only skin material from drumsticks, wings and breast portions was analyzed for *Campylobacter*. The first survey also included fillets and other fresh broiler meat products without skin material. It is well-known that *Campylobacter* spp. can colonize the intestinal tract of broiler chickens at high levels, and during a poorly executed evisceration process at slaughter the caecal material can be transferred onto carcases (Allen et al., 2007; Reich, Atanassova, Haunhorst, & Klein, 2008). A Finnish study by Katzav, Isohanni, Lund, Hakkinen, and Lyhs (2008) showed the occurrence of *Campylobacter* in chicken slices and barbecue sticks to be 9.4%, in chicken breast fillets 4.7% and in chicken products with skin and bone 30.4%. The results of the present study showed that the percentage of *Campylobacter*-positive fresh broiler meat samples was higher when skin material was included. Within company-packaged fresh broiler chicken meat sold at Estonian retail level the majority (~70%) is sold as drumsticks, wings and breast portion where the skin material is included.

According to an EU-wide baseline survey (EFSA, 2011b) the *Campylobacter* prevalence for broiler chicken batches in Estonia was only 2.0%, which was the lowest among the EU-countries. According to the first and second surveys of the present study, *Campylobacter* prevalence in fresh broiler chicken meat of Estonian origin was 17.0% and 20.3%. Differences compared to the prevalence found in the EU-baseline study are also probably related to the different sampling methods. For the baseline study broiler chicken carcasses were collected and neck skin samples taken at the laboratory for *Campylobacter* prevalence, instead of company-packaged broiler fresh meat samples (drumsticks, wings, breast portions) that were used in the surveys presented here. It is not well-known how representative the neck and breast-skin are for estimating whole-carcass regions, and which sampling schemes, e.g. number of samples or time interval between sampling, are most effective. Jørgensen et al., (2002) reported that the likelihood of detecting *Campylobacter* spp. in a raw chicken appeared not to be significantly influenced by sample type, but examination of samples containing carcass rinse fluid and neck-skin detected a higher *Campylobacter* count than examination of the neck-skin sample alone. Because of non-similarities in study design, the Jørgensen et al., (2002) findings cannot explain the differences found in the currently described surveys. Nevertheless, the proportion of *Campylobacter* positive Estonian origin broiler chicken carcasses in the first survey was 5% (Table 1), which is more comparable to the EU base-line study Estonian results where the *Campylobacter*

prevalence was 2%. It is essential to note that most of the broiler chicken fresh meat in Estonia is sold as packaged drumsticks, wings and other portions instead of whole carcasses.

Campylobacter studies in other Baltic countries have shown high *Campylobacter* occurrence in Latvia, where the mean proportion of *Campylobacter* positive broiler chicken carcasses at Latvian retail level was 59.2% in 2010, as reported by Kovalenko et al. (2013). A Lithuanian study by Buneviˇcienė et al. (2010) showed that fresh broiler chicken meat (drumsticks and wings) at the retail level were contaminated at up to 46.5%, and broiler chicken carcasses at slaughterhouse level at up to 45.8%. These studies report similar contamination proportions to the second survey reported here, where *Campylobacter* contamination in Latvian and Lithuanian fresh broiler chicken meat was 60% and 50%, respectively.

The prevalence's of *Campylobacter* spp. on fresh broiler chicken meat samples of Estonian and Lithuanian origin (data available for all 12 months, both surveys combined) during the year 2012 are shown in Fig. 1. It is possible to deduce that *Campylobacter* spp. contamination increased in early spring, remained high during the summer months, and decreased at the end of autumn. June 2012 was atypically rainy and cold in the Baltic countries, which may be a possible reason for the sudden decrease in *Campylobacter* positive broiler chicken meat samples shown in Fig. 1. The seasonal variation of *Campylobacter* contamination was also studied at slaughterhouse level, where the caecal samples were taken at assumed seasonal peaks, from June to October. Among the Estonian broiler chicken farms and flocks at the slaughterhouse level studied, the overall prevalence's of *Campylobacter* in caecal material monthly from June to October were 0%, 39%, 92%, 45% and 0%, respectively. The prevalence's of *Campylobacter* in Estonian fresh broiler chicken meat (Fig. 1), in the same months, were 0%, 16.7%, 75.0%, 41.7% and 22.2%. Generally, the higher the *Campylobacter* contamination was at farm level the higher it was in broiler chicken meat samples at retail level. There was seasonal variation in the proportions of *Campylobacter* positive samples with a seasonal peak in the warm summer months of July, August and September ($p < 0.001$). A distinct seasonality in broiler chicken *Campylobacter* contamination and in human campylobacteriosis cases have been shown by previous European studies (Horrocks, Anderson, Nisbet, & Ricke, 2009; Rautelin & Hänninen, 2000) and in New Zealand (Brieseman, 1990). Reports on human campylobacteriosis cases in Estonia have shown that most *Campylobacter* human infections occurred from June to September (Meremäe et al., 2010), the season when the highest *Campylobacter* prevalence and counts of the poultry products at Estonian retail level were found in the current study. *Campylobacter* counts (second survey), between Estonian, Lithuanian and Latvian fresh broiler chicken products ($n = 220$), were compared. Significant differences for *Campylobacter* prevalence and counts between Estonian and Lithuanian ($p < 0.001$) and between Estonian and Latvian ($p < 0.001$) fresh poultry products were found. No statistical difference was found for *Campylobacter* contamination, both counts and prevalence, between Latvian and Lithuanian-origin products. Estonian fresh poultry meat products had significantly ($p < 0.001$) lower *Campylobacter* prevalence and counts compare to Lithuanian and Latvian poultry products sold at Estonian retail. The reasons for the higher *Campylobacter* contamination for Latvian and Lithuanian fresh poultry meat were not part of the study, but are probably related to differences in production and management systems at poultry farm, slaughterhouse and at meat industry levels. Data presented in Fig. 1 show a high proportion of *Campylobacter* positive Lithuanian-origin broiler chicken meat samples in December. One possible explanation for this could be the fact that the lowest number of samples was taken in December, and so the data was least reliable for this month, but the same number of samples were collected both for Estonian and Lithuanian products. Nevertheless, the proportion of *Campylobacter* positive products was 16.7% for Estonian and 60% for Lithuanian-origin fresh broiler chicken meat products.

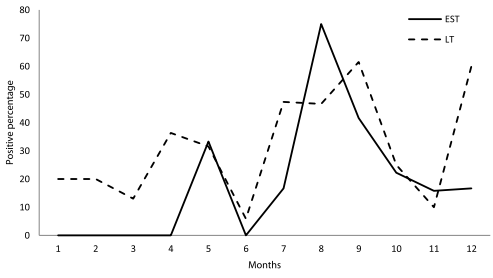

Fig. 1. Proportion of *Campylobacter* positive fresh broiler chicken meat samples in the Estonian retail market in 2012. Both surveys combined ($n = 340$); EST, Estonian origin; LT, Lithuanian origin.

Campylobacter species distribution among poultry products originating from different countries may be different. Therefore, a PCR assay for *Campylobacter* species identification, described by Wang et al. (2002), was performed which resulted in estimations of 89% *C. jejuni*, 8% *C. coli* and 3% *Campylobacter* spp. isolates, with no essential differences between countries of origin.

4. Conclusion

High numbers of *Campylobacter* on fresh broiler chicken meat of Latvian and Lithuanian origin were found in the Estonian retail market.

Campylobacter prevalence in fresh broiler chicken meat of Estonian origin was lower compared to most EU-countries, but higher than previously reported by the EFSA. The seasonal peak for *Campylobacter* contamination of poultry meat was in the summer.

To achieve EU targets in public health risk reduction in Estonia, appropriate *Campylobacter* control measures should be applied at all broiler chicken meat production stages, with a special emphasis on the warm summer months.

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Research Note

Prevalence and Genetic Characterization of *Listeria monocytogenes* in Retail Broiler Meat in Estonia

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ABSTRACT

The prevalence and genetic diversity of *Listeria monocytogenes* in raw broiler legs at the retail level in Estonia were studied. A total of 240 raw broiler legs (120 from Estonia and 120 of foreign origin, which had been imported to Estonia from Denmark, Finland, Hungary, Sweden, and the United States) from 12 retail stores in the two largest cities in Estonia (Tallinn and Tartu) were investigated from January to December 2002. Of these, 70% were positive for *L. monocytogenes*. The prevalence of *L. monocytogenes* in broiler legs of Estonian origin (88%) was significantly higher than in broiler legs of foreign origin (53%) ($P < 0.001$). Altogether, 169 (106 Estonian and 63 imported) *L. monocytogenes* isolates were characterized by pulsed-field gel electrophoresis (PFGE) typing after treatment with the restriction enzyme *Asc*I. The isolates showed a wide genetic diversity, with 35 different PFGE types obtained. Of these, 11 PFGE types came only from isolates of broiler legs of Estonian origin, 4 of Danish origin, 2 of Finnish origin, and 4 of Hungarian origin. Fourteen PFGE types came from isolates of broiler legs that originated from various countries. The strains that shared the same PFGE types from isolates of Estonian origin were recovered from broiler legs that came from different stores over the course of several months. Seventy-one *L. monocytogenes* isolates, including all PFGE types, were serotyped, and three serotypes (1/2a, 1/2b, and 4b) were obtained. Serotype 1/2a accounted for 96% of the isolates.

The consumption of foods contaminated with *Listeria monocytogenes* can result in listeriosis, an uncommon but potentially fatal disease. Listeriosis can be life threatening to elderly persons, persons with weakened immune systems, and women who are pregnant. Poultry products have been associated with listeriosis *(7–10, 14, 15, 22)*.

Healthy birds may shed *L. monocytogenes* in fecal material asymptomatically *(23)*. However, poultry meat becomes contaminated during slaughter and processing *(17, 18, 20)*. Contamination rates for *L. monocytogenes* in raw poultry products have ranged from 10 to 62% *(3, 6, 12, 17, 19, 21, 24–26)*. The prevention of poultry product contamination with *L. monocytogenes* is therefore of major importance.

Within the past few years, consumption of poultry meat has increased in Estonia and presently stands at about 22 kg per capita *(2)*. To our knowledge, no data exist about the prevalence of *L. monocytogenes* in poultry products. The goal of this study was thus to determine the prevalence of *L. monocytogenes* in raw broiler legs of Estonian and foreign origin sold on the Estonian retail market. To obtain information on the diversity of *L. monocytogenes* isolates, genotyping with pulsed-field gel electrophoresis (PFGE) was performed.

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MATERIALS AND METHODS

Samples. A total of 240 raw broiler legs (120 from Estonia and 120 of foreign origin) from 12 retail stores (supermarkets) in the two largest cities (Tallinn and Tartu) of Estonia were studied from January to December 2002. All samples of Estonian origin were from one of the country's main producers of poultry products, where broiler chickens were reared for meat and slaughtered after 6 or 7 weeks. Of these, 104 were obtained from stores that sold only products of the main producer, and 16 were obtained from stores that also sold poultry products from other countries. Of the samples of foreign origin, 60, 18, 21, 12, and 9 were imported from Denmark, Finland, Hungary, Sweden, and the United States, respectively. Products of Estonian origin were fresh, and those of foreign origin were frozen. All broiler legs had been stored unpackaged on the store counter (1 to 5°C). Each sampled broiler leg was placed in a separate sterile plastic bag. During transportation to the laboratory, the samples were kept cool in portable insulated boxes by ice packs and were stored at 4°C until analysis.

Isolation of *L. monocytogenes*. Microbiological analyses for *L. monocytogenes* were started within 24 h of sample collection. One hundred milliliters of peptone (0.1%)–saline (0.85%) solution was added to the whole broiler leg in the plastic bag, and the broiler leg was massaged by hand for 1 min. Twenty-five milliliters of this peptone–saline solution was used for the enrichment procedure. The isolation of *L. monocytogenes* was carried out by a two-step enrichment method according to the recommendations of the International Organization for Standardization, with the use of half-Fraser and Fraser broth (Oxoid, Basingstoke, Hampshire, UK) *(1)*. Both enrichment broths were plated on PALCAM agar

TABLE 1. *Prevalence of* Listeria monocytogenes *in raw broiler legs in retail stores (A–L) in Estonia*

City or store	No. of positive samples/total no. of samples (%) originating from different countries[a]						
	DK	EE	FI	HU	SE	US	Total
Tallinn							
A	NS[b]	30/38 (79)	NS	NS	NS	NS	30/38 (79)
B	NS	10/12 (83)	NS	NS	NS	NS	10/12 (83)
C	NS	2/2 (100)	NS	NS	NS	NS	2/2 (100)
D	NS	17/19 (90)	NS	NS	NS	NS	17/19 (90)
E	22/49 (45)	10/10 (100)	NS	NS	10/12 (83)	2/9 (22)	44/80 (55)
F	NS	1/3 (33)	10/18 (56)	NS	NS	NS	11/21 (52)
G	NS	NS	NS	16/21 (76)	NS	NS	16/21 (76)
Subtotal	22/49 (45)	70/84 (83)	10/18 (56)	16/21 (76)	10/12 (83)	2/9 (22)	130/193 (67)
Tartu							
H	NS	4/4 (100)	NS	NS	NS	NS	4/4 (100)
I	3/11 (27)	3/3 (100)	NS	NS	NS	NS	6/14 (43)
J	NS	6/6 (100)	NS	NS	NS	NS	6/6 (100)
K	NS	19/19 (100)	NS	NS	NS	NS	19/19 (100)
L	NS	4/4 (100)	NS	NS	NS	NS	4/4 (100)
Subtotal	3/11 (27)	36/36 (100)	NS	NS	NS	NS	39/47 (83)
Total	25/60 (42)	106/120 (88)	10/18 (56)	16/21 (76)	10/12 (83)	2/9 (22)	169/240 (70)

[a] Country: DK, Denmark; EE, Estonia; FI, Finland; HU, Hungary; SE, Sweden; US, United States.
[b] NS, no samples available.

(Oxoid) and *L. monocytogenes* blood agar (Lab M, Bury, Lancashire, UK), as suggested by Johansson *(13)*. Five typical colonies from each selective plate were streaked on blood agar, and five beta-hemolytic colonies were confirmed by catalase reaction, Gram staining, and biochemical identification with the API *Listeria* test (bioMérieux, Marcy-l'Etoile, France).

In situ DNA isolation and PFGE. Altogether, 169 *L. monocytogenes* isolates were obtained for PFGE typing and represented one isolate from each positive sample. Cultures for DNA isolation were grown overnight in Trypticase soy broth (Difco, Becton Dickinson, Sparks, Md.) at 37°C. In situ DNA was isolated and digested with the restriction enzyme *Asc*I (New England Biolabs, Beverly, Mass.) in agarose plugs and was then characterized by PFGE as described by Autio et al. *(4)* with the use of pronase (Roche Diagnostics GmbH, Mannheim, Germany) instead of proteinase K.

PFGE pattern analysis. Numerical analysis of *Asc*I macrorestriction patterns was performed by the computer software program BioNumerics 3.5 (Applied Maths, Sint-Martens-Latem, Belgium). Similarity analysis was carried out by use of the Dice coefficient (position tolerance, 1.0%). The clustering and construction of the dendrogram were performed by the unweighted pair-group method with arithmetic averages.

Serotyping. Serotyping was performed with commercial *Listeria* antisera according to the instructions given by the manufacturer (Denka Seiken, Tokyo, Japan), with some modifications. For detection of the O-antigen, the cells were cultured on Trypticase soy agar (TSA; Difco, Becton Dickinson) plates. Detection of the flagellar H-antigens (A, B, C, and D) was performed at 25°C in TSA tubes.

Statistical analysis. The prevalence data were analyzed statistically by the chi-square test.

RESULTS

Of the raw broiler legs purchased from retail stores in Estonia, 70% were positive for *L. monocytogenes* (Table 1). The prevalence in broiler legs of Estonian origin varied from 33 to 100% and, in legs of foreign origin, from 22 to 83% from various stores. The prevalence of *L. monocytogenes* in broiler legs of Estonian origin (88%) was significantly higher than in broiler legs of foreign origin (53%) ($P < 0.001$). Of the broiler legs bought from stores selling only products of the predominant Estonian poultry meat plant, 89% were positive for *L. monocytogenes*. The broiler legs of Estonian origin purchased in Tartu had a significantly higher contamination level than those purchased in Tallinn, 100 and 83%, respectively ($P < 0.05$).

The characterization of *L. monocytogenes* isolates recovered from broiler legs of Estonian ($n = 106$) and foreign ($n = 63$) origin yielded 22 and 24 PFGE types, respectively. Combining these PFGE types, 35 different types were obtained (Fig. 1). Of these PFGE types, 11 (2, 5, 6, 7, 14, 22, 25, 27, 28, 30, and 34) came only from isolates of broiler legs of Estonian origin, 4 (16, 20, 23, and 31) of Danish origin, 2 (15 and 32) of Finnish origin, and 4 (9, 10, 13, and 35) of Hungarian origin. Fourteen (1, 3, 4, 8, 11, 12, 17, 18, 19, 21, 24, 26, 29, and 33) PFGE types came from isolates that originated from more than one country. PFGE types 4, 21, and 33 were predominant, accounting for 13, 15, and 10% of the isolates, respectively. The isolates of these three PFGE types were recovered from broiler legs of Danish, Estonian, Hungarian, and U.S. origin.

In some cases, the same PFGE types were recovered from broiler legs that had originated from different countries but that had been obtained from the same stores (Table

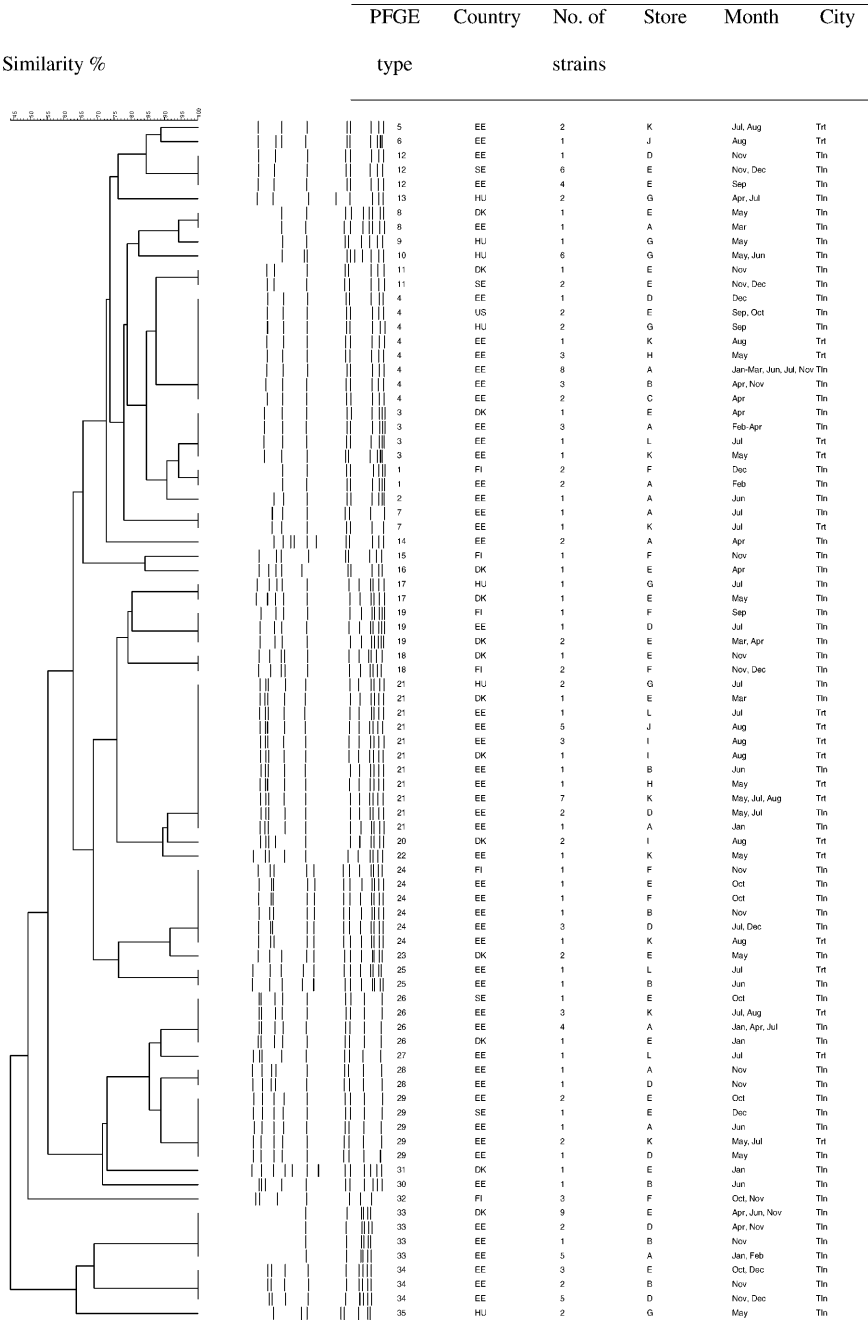

FIGURE 1. *Dendrogram of defined PFGE patterns of* Listeria monocytogenes *strains isolated from raw broiler legs in retail stores in Estonia. Similarity analysis was performed by use of the Dice coefficient, and clustering was performed by the unweighted pair-group method with arithmetic averages (position tolerance, 1.0%). Country: DK, Denmark; EE, Estonia; FI, Finland; HU, Hungary; SE, Sweden; US, United States. City: Tln, Tallinn; Trt, Tartu.*

TABLE 2. *Distributions of* Listeria monocytogenes *PFGE types and serotypes in raw broiler legs in retail stores (A–L)*

Store	No. of PFGE types	PFGE types originating from different countries[a]						No. of serotyped isolates		
		DK	EE	FI	HU	SE	US	1/2a	1/2b	4b
A	12		1, 2, 3, 4, 7, 8, 14, 21, 26, 28, 29, 33					14		
B	7		4, 21, 24, 25, 30, 33, 34					5		
C	1		4					1		
D	9		4, 12, 19, 21, 24, 28, 29, 33, 34					6		
E	17	3, 8, 11, 16, 17, 18, 19, 21, 23, 26, 31, 33	12, 24, 29, 34			11, 12, 26, 29	4	20	2	
F	6		24	1, 15, 18, 19, 24, 32				5	2	
G	7				4, 9, 10, 13, 17, 21, 35			5	1	1
H	2		4, 21							
I	2	20, 21	21					1		
J	2		6, 21					1		
K	9		3, 4, 5, 7, 21, 22, 24, 26, 29					6		
L	4		3, 21, 25, 27					1		
Total	35							65	5	1

[a] Country: DK, Denmark; EE, Estonia; FI, Finland, HU, Hungary; SE, Sweden; US, United States.

2). In store E, strains of PFGE types 12 and 29 were found in broiler legs of Estonian and Swedish origin, and strains of PFGE types 11 and 26 were found in legs of Danish and Swedish origin. Isolates of PFGE type 24 were detected in broiler legs of both Estonian and Finnish origin that were obtained from store F. Strains of PFGE type 21 were detected in store I in broiler legs of both Estonian and Danish origin.

In several cases, the same PFGE types were detected in samples of Estonian origin that came from different stores over the course of several months (Fig. 1). The strains of PFGE type 4 were obtained from broiler legs that came from various stores during 10 different months and from store A during six different months. Isolates of PFGE types 3, 21, and 24 were detected in broiler legs from various stores during five different months.

One to eight representative isolates from each PFGE type were selected for serotyping, which resulted in a total of 71 isolates. Three different serotypes were obtained: 1/2a, 1/2b, and 4b (Table 2). All broiler legs of Estonian origin and most broiler legs of foreign origin had serotype 1/2a. Isolates of serotype 1/2b were of Danish, Finnish, and Hungarian origin, and isolates of serotype 4b were of Hungarian origin.

DISCUSSION

Raw broiler legs showed a high level of contamination with *L. monocytogenes* (70%). The prevalence of *L. monocytogenes* in broiler legs of Estonian origin in general and in broiler legs obtained from stores selling only products of the predominant Estonian poultry meat plant (stores A, B, C, D, H, J, K, and L) was higher (88 and 89%, respectively) than that reported by Genigeorgis et al. *(11)* (16%) or Miettinen et al. *(17)* (68%) in broiler legs. Furthermore, the broiler legs of Estonian origin bought in Tartu were all (100%) contaminated by *L. monocytogenes*. To our knowledge, this is the first time that such a high prevalence of *L. monocytogenes* in raw broiler legs has been reported. The high prevalence in broiler legs at the Estonian retail level could be because of contamination that may have occurred during processing at the plant. However, cross-contamination of retail poultry products cannot be excluded, because products were sold unpackaged.

Thirty-five PFGE types were presented by 169 *L. monocytogenes* strains. These data suggest that the *L. monocytogenes* strains recovered from the broiler legs showed wide genetic diversity. The PFGE types recovered from the broiler legs of Estonian (2, 5, 6, 7, 14, 22, 25, 27, 28, 30, and 34) and Hungarian origin (9, 10, 13, and 35) were obtained from stores that sold only products from these countries and were possibly associated with the producing country. Strains that shared the same PFGE types (14 PFGE types) were identified among isolates of broiler legs that originated from different countries. For example, the strains of PFGE types 1, 3, 4, 8, 12, 19, 21, 24, 26, 29, and 33 were common in broiler legs of both Estonian and foreign

origin. Three predominant PFGE types (4, 21, and 33), possessed by 38% of all strains, were recovered from broiler legs of Danish, Estonian, Hungarian, and U.S. origin. The strains of PFGE types 11, 17, and 18 were of Danish, Finnish, Hungarian, and Swedish origin.

Because the broiler legs had been sold unpackaged and from the same counter, one of the reasons for the same PFGE types could be cross-contamination in the stores that sold products that had originated from various countries. In stores E, F, and I, the same PFGE types (12, 21, 24, and 29) were detected in broiler legs of both Estonian and foreign origin (Table 2). In store E, the strains from broiler legs of Danish and Swedish origin shared the same PFGE types (11 and 26).

Recovery of strains that shared the same PFGE types (3, 4, 21, and 24) from different stores obtained during the course of several months suggests a wide temporal distribution of many of the *L. monocytogenes* strains isolated in broiler legs. Because the broiler legs of Estonian origin came from one processing plant, the PFGE types are likely associated with contamination during processing *(17, 18, 20)*. The occurrence of the same PFGE types (3, 4, 21, and 24) in broiler legs of Estonian origin during the course of several months indicates that these strains are persistent *(16)*. Furthermore, *L. monocytogenes* from broiler legs can contaminate retail counters and cause cross-contamination of other raw foods if hygienic procedures are inadequate. This emphasizes the need for strict hygienic conditions during processing and at the retail level to prevent cross-contamination.

Serotype 1/2a was predominant in Estonian poultry products. The same serotype was also predominant in raw chicken from Portugal *(12)*. In the United States and Spain, serotype 1/2b *(5, 26)* and, in Finland, serotype 1/2c *(17)* have been the most common serotypes found in poultry meat.

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Scientific publications of 5 last years and up to 5 major publications from an earlier period	
1.1	Mäesaar, M., **Praakle, K.**, Meremäe, K., Kramarenko, T., Sõgel, J., Viltrop, A., Muutra, K., Kovalenko, K., Matt, D., Hörman, A., Hänninen, M.-L., Roasto, M. 2014. Prevalence and counts of *Campylobacter* spp. in poultry meat at retail level in Estonia. *Food Control*, 44, 72-77. Roasto, M., **Praakle-Amin, K.**, Meremäe, K., Tamme, T., Hörman, A., Lillenberg, M., Hänninen, M.-L. 2009. Food control and research on *Campylobacter* spp. in Estonia. *Archiv für Lebensmittelhygiene*, 60, 109-115. **Praakle-Amin, K.**, Roasto, M., Korkeala, H., Hänninen, M.-L. 2007. PFGE genotyping and antimicrobial susceptibility of *Campylobacter* in retail poultry meat in Estonia. *International Journal of Food Microbiology*, 114, 105-112. **Praakle-Amin, K.**, Hänninen, M.-L., Korkeala, H. 2006. Prevalence and genetic characterization of *Listeria monocytogenes* in retail broiler meat in Estonia. *Journal of Food Protection*, 69, 436-440. Roasto, M., **Praakle, K.**, Korkeala, H., Elias, P., Hänninen, M.-L. 2005. Prevalence of *Campylobacter* in raw chicken meat of Estonian origin. *Archiv für Lebensmittelhygiene*, 56, 61-62.
Training activities (last 5 years)	VL.1166 Basics of veterinary public health and food hygiene (in Estonian and English) VL.0135 Animal hygiene (in Estonian and English) VL.0337 Herd health and veterinary prophylaxis VL.0738 Legislation of food VL.1172 Food production chain, 1st and 2nd part VL.1173 Basics of plant food product technology VL.1174 Basics of beverage technology VL.1151 Fundamentals of the design of foodstuff technological lines VL.1158 Cooking and nutrition

In-service training

Seminar „Wooden Breast Syndrome - Properties, causes and ways to alleviate“, University of Helsinki, Finland, 11.-12.03.2015

Seminar „Farm animal welfare research, education and implementation in Central and SouthEastern Europe - how to promote the human potential“, Zagreb, Croatia, 10.–11.02.2014

Course „Fish and fishery products and production hygiene“, The Open University of Estonian University of Life Sciences, 31.01.2014

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Project „FLAVOURE“ conference „Food/Feed quality, safety and risks in agriculture“, Tallinn, Estonia, 25.-28.10.2011

Course „Art of Conducting Lectures“, Tartu University Open University Centre, Estonia, 04.-05.02.2010

Course „Public speaking“, Tartu University Open University Centre, Estonia, 26.-27.01.2010

Course „Healthy nutrition“, Olustvere School of Rural Economics and Service Industry, Estonia, 25.09.2009

International Workshop „Microbiological indices in the whole cycle of animal origin food production chain“, Rakvere, Estonia, 17.-18.09.2009

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Viimase 5 a teadus-publikatsioonid ja soovi korral kuni 5 olulisemat publikat-siooni varasemast perioodist.	
1.1	Mäesaar, M., **Praakle, K.**, Meremäe, K., Kramarenko, T., Sõgel, J., Viltrop, A., Muutra, K., Kovalenko, K., Matt, D., Hörman, A., Hänninen, M.-L., Roasto, M. 2014. Prevalence and counts of *Campylobacter* spp. in poultry meat at retail level in Estonia. *Food Control*, 44, 72-77. Roasto, M., **Praakle-Amin, K.**, Meremäe, K., Tamme, T., Hörman, A., Lillenberg, M., Hänninen, M.-L. 2009. Food control and research on *Campylobacter* spp. in Estonia. *Archiv für Lebensmittelhygiene*, 60, 109-115. **Praakle-Amin, K.**, Roasto, M., Korkeala, H., Hänninen, M.-L. 2007. PFGE genotyping and antimicrobial susceptibility of *Campylobacter* in retail poultry meat in Estonia. *International Journal of Food Microbiology*, 114, 105-112. **Praakle-Amin, K.**, Hänninen, M.-L., Korkeala, H. 2006. Prevalence and genetic characterization of *Listeria monocytogenes* in retail broiler meat in Estonia. *Journal of Food Protection*, 69, 436-440. Roasto, M., **Praakle, K.**, Korkeala, H., Elias, P., Hänninen, M.-L. 2005. Prevalence of *Campylobacter* in raw chicken meat of Estonian origin. *Archiv für Lebensmittelhygiene*, 56, 61-62.
Õppetöö (viimased 5 aastat)	VL.1166 Veterinaarse rahvatervise ja toiduhügieeni alused (eesti ja inglise keeles) VL.0135 Loomatervishoid (eesti ja inglise keeles) VL.0337 Karja tervis ja veterinaarprofülaktika VL.0738 Toiduainete seadusandluse alused VL.1172 Toidu tooteahel, 1. ja 2. osa VL.1173 Taimsete toiduainete tehnoloogia põhikursus VL.1174 Jookide tehnoloogia põhikursus VL.1151 Toiduainetööstuse tehnoloogiliste liinide projekteerimise alused VL.1158 Toidu valmistamine ja toitumine

Erialane enesetäiendamine	Seminar „Wooden Breast Syndrome - Properties, causes and ways to alleviate“, Helsingi Ülikool, Soome, 11.-12.03.2015 Seminar „Farm animal welfare research, education and implementation in Central and SouthEastern Europe - how to promote the human potential“, Zagreb, Horvaatia, 10.–11.02.2014 Kursus „Kala ja kalatoodete ohutus ning tootmishügieen“, Eesti Maaülikooli avatud ülikool, 31.01.2014 Kursus „Õppimine ja õpetamine kõrgkoolis“, Tartu Ülikool, 28.08.–21.11.2013 Seminar „Workshop for research leaders and lecturers in the field of farm animal welfare“, Bratislava, Slovakkia, 31.05.-01.06.2012 Seminar „Improving animal welfare: a practical approach“, Riia, Läti, 19.-20.04.2012 Seminar „Identifying and raising core welfare awareness“, Varssavi, Poola, 27.-29.03.2012 Kursus „Toiduhügieen“, Eesti Maaülikooli avatud ülikool, 20.01.2012 Kursus „Toiduhügieen ja -ohutus“, Olustvere Teenindus- ja Maamajanduskool, 28.-30.11.2011 Projekt „FLAVOURE“ konverents „Food/Feed quality, safety and risks in agriculture“, Tallinn, 25.-28.10.2011 Kursus „Loengupidamise kunst õppejõududele“, Tartu Ülikooli avatud ülikooli keskus, 04.-05.02.2010 Kursus „Avalik esinemine“, Tartu Ülikooli avatud ülikooli keskus, 26.-27.01.2010 Kursus „Tervislik toitumine“, Olustvere Teenindus- ja Maamajanduskool, 25.09.2009 Rahvusvaheline seminar „Microbiological indices in the whole cycle of animal origin food production chain“, Rakvere, 17.-18.09.2009 VÕTA kursus karjääri-, õpi- ja teistele nõustajatele, Tartu Ülikool, 15.-16.06.2009 SAFOODNET 3. seminar „Risk management by hygienic desing and efficient sanitation programs“, Tallinn, 04.–06.05.2009

Projekt „FLAVOURE“ kursus „Food and feed quality and safety“, Tallinn, 26.–27.03.2009

Euroopa Toiduohutusameti seminar „Join EFSA in assessing food safety risks in Europe“, Varssavi, Poola, 26.–27.11.2008

SAFOODNET 2. seminar „Microbial risk management in food processes“, Lyngby, Taani, 13.–15.10.2008

Konverents „Food Micro 2008“, Aberdeen, Šotimaa, 01.–04.09.2008

Kongress „*Clostridium botulinum* – Epidemiology, Diagnosis, Genetics, Control and Prevention“, Helsingi, Soome, 16.-19.06.2008

Enesetäiendamine Helsingi Ülikoolis RAK 2004-2006 meetme 1.1 projekti nr 1.0101.06-0480 „Eesti ülikoolide akadeemilise personali enesetäiendussüsteemi loomine“ raames, Soome, 02.-15.04.2008

Kursus „Methodology of Research“, Eesti Maaülikool, 11.-12.02.2008

Kursus „Eneseväljenduskunsti alused“, Eesti Maaülikool, 06.-08.02.2008

SELF II „Ajajuhtimise koolitus“, Tartu, 23.01.2008

„Kiirlugemise kursus“, Tartu, 16.-17.01.2008

XIII Rahvusvaheline Loomatervise kongress, ISAH–2007, Tartu, 17.–21.06.2007

Kursus „Workshop on bioethics“, Helsingi Ülikool, Soome, 30.11.2004–03.03.2005

Seminar „Microbial ecology of meat and microbiological hazards in meat industry“, Jelgava, Läti, 05.–06.05.2004

Nordic Forum seminar „Enlargement of the EU and Food Safety in Estonia“, Tartu, 12.03.2004

Kursus „Molecular typing methods in food microbiology and evaluation of the microbiological quality of water“, Helsingi Ülikool, Soome, 19.–23.01.2004

Kursus „Taxonomy and evolution of pathogens and nonpathogens“, Helsingi Ülikool, Soome, 25.–26.08.2003

Kursus „Food Microbiology and Principles of PCR-diagnostics“, Helsingi Ülikool, Soome, 03.–07.03.2003

Kursus „PCR-diagnostika toiduainete mikrobioloogias“, Helsingi Ülikool, Soome, 11.–15.02.2002

Kursus „Põllult lauale“, Helsingi Ülikool, Soome, 09.01., 14.–17.01.2002

LIST OF PUBLICATIONS

1.1. Scholarly articles indexed by Thomson Reuters Web of Science (excluding articles indexed in Thomson Reuters Conference Proceedings Citation Index) and/or published in journals indexed by ERIH (European Reference Index of the Humanities) categories INT1 and INT2 and/or indexed by Scopus (excluding chapters in books)

Mäesaar, M., **Praakle, K.**, Meremäe, K., Kramarenko, T., Sõgel, J., Viltrop, A., Muutra, K., Kovalenko, K., Matt, D., Hörman, A., Hänninen, M.-L., Roasto, M. 2014. Prevalence and counts of *Campylobacter* spp. in poultry meat at retail level in Estonia. *Food Control*, 44, 72-77.

Roasto, M., **Praakle-Amin, K.**, Meremäe, K., Tamme, T., Hörman, A., Lillenberg, M., Hänninen, M.-L. 2009. Food control and research on *Campylobacter* spp. in Estonia. *Archiv für Lebensmittelhygiene*, 60, 109-115.

Praakle-Amin, K., Roasto, M., Korkeala, H., Hänninen, M.-L. 2007. PFGE genotyping and antimicrobial susceptibility of *Campylobacter* in retail poultry meat in Estonia. *International Journal of Food Microbiology*, 114, 105-112.

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Roasto, M., **Praakle, K.**, Korkeala, H., Elias, P., Hänninen, M.-L. 2005. Prevalence of *Campylobacter* in raw chicken meat of Estonian origin. *Archiv für Lebensmittelhygiene*, 56, 61-62.

1.2. Peer-reviewed articles in other international research journals with an ISSN code and international editorial board, which are circulated internationally and open to international contributions; articles of ERIH category NAT

Roasto, M., Meremäe, K., **Praakle-Amin, K.**, Hörman, A., Elias, T., Lillenberg, M., Elias, A., Kramarenko, T., Häkkinen, L., Põltsama, P., Mäesaar, M., Elias, P., Hänninen, M.-L. 2011. Termofiilsete kampülobakterite uuringud Eestis 2000–2010. *Agraarteadus. Journal of Agricultural Science.* Akadeemilise Põllumajanduse Seltsi väljaanne, 22, 31-39.

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3.4. Articles and presentations published in the conference proceedings not listed by Thomson Reuters Conference Proceedings Citation Index

Roasto, M., **Praakle-Amin, K.**, Juhkam, K., Tamme, T., Hörman, A., Hänninen, M.-L. 2008. Food control and research on *Campylobacter* spp. in Estonia. The First International Meeting on Food Control Research, University of Helsinki, Finland, October 15-17, 2008. University of Helsinki, Finland: University of Helsinki Press, 28-29.

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3.5. Articles and presentations published in local conference proceedings

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6.7. Other creative activities

Kark, Mati; Kadastik, Meelis; Vilu, Arvo; Ainla, Alar; Taal, Rivo; Annikve, Peeter; Lenk, Andreas; Luts, Heiki; Kaldoja, Hans; Kruusmaa, Maarja; Uibopuu, Helen; Niglas, Liivo; Alumäe, Tanel; Roasto, Mati; **Praakle, Kristi**; Vurma, Allan; Järve, Marju; Reintam, Endla; Reivelt, Kaido; Sibul, Ivar; Pauklin, Mikk; Verš, Evelin; Gordon, Rauno; Hektor, Andi; Lapimaa, Triin; Kask, Andres. 2005. Pähklipurejad [Videosalvestis]

VIIS VIIMAST KAITSMIST

GARRI TRALMAN

ROD-THROUGH-PLATE FIXATOR IN THE TREATMENT OF LONG BONE FACTURES OF SMALL ANIMALS
KOMBINEERITUD METALLOOSTEOSÜNTEES PIKKADE TORULUUDE MURDUDE RAVIKS VÄIKELOOMADEL
Professor **Vladimir Andrianov**, professor **Marina Aunapuu**
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ON USING MATHCAD SOFTWARE FOR MODELLING, VISUALIZATION AND SIMULATION IN MECHANICS
MATHCAD TARKVARAPÕHINE MODELLEERIMINE, VISUALISEERIMINE JA SIMULEERIMINE MEHAANIKAS
Professor **Mati Heinloo**
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THE EFFECT OF DIFFERENT FARMING SYSTEMS ON POTATO TUBER YIELD AND QUALITY
VILJELUSSÜSTEEMIDE MÕJU KARTULI MUGULASAAGILE JA KVALITEEDILE
Dotsent **Are Selge**, teadur **Viacheslav Eremeev**, vanemteadur **Evelin Loit**
4. detsember 2015

HIIE IVANOVA

RESPONSES OF RESPIRATORY AND PHOTORESPIRATORY DECARBOXYLATIONS TO INTERNAL AND EXTERNAL FACTORS IN C_3 PLANTS
RESPIRATOORSE JA FOTORESPIRATOORSE DEKARBOKSÜÜLIMISE VASTUSED SISEMISTE JA VÄLISTE FAKTORITE TOIMELE C_3 TAIMEDES
Professor **Ülo Niinemets**, vanemteadur **Olav Keerberg**, vanemteadur **Tiit Pärnik**
8. jaanuar 2016

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SOIL PROPERTIES AFFECTED BY COVER CROPS AND FERTILIZATION IN A CROP ROTATION EXPERIMENT
VAHEKULTUURIDE JA VÄETAMISE MÕJU MULLA OMADUSTELE KÜLVIKORRAKATSES
Dotsent **Endla Reintam**, emeriitprofessor **Anne Luik**
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