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(met een samenvatting in het Nederlands)

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Contents

Chapter 1

General introduction

Zoonoses caused by bacteria commonly present in poultry are a public health threat. This chapter introduces major biological hazards and technological and managerial approaches to improve the hygiene during broiler processing.

1. Poultry meat consumption

Poultry meat is an important protein source in the human diet around the globe and it is predicted that its consumption will surpass pork consumption by 2020. In the European Union, the average consumption in 2014 was 26.8 kg/capita, varying among countries from 11.3 kg/capita in Sweden to 39 kg/capita in Portugal (Anonymous, 2015a). Poultry meat is relatively cheap compared to other meat types, it is easily accessible, has dietary characteristics demanded by consumers (e.g. low fat content) and lacks religious obstacles to consumption, all of which contribute to its global acceptance (Magdelaine et al., 2008). Its accessibility and affordability have been enhanced by intensive livestock production and high speed processing lines (up to 13,500 animals per hour). Currently a live broiler can be transferred into a food product (e.g. a fillet) within 3.5 hours (Barbut, 2014).

2. Public health hazards related to poultry meat

Poultry and poultry meat products are potential sources of biological hazards for humans in the form of harmful micro-organisms. Live animals carry these bacteria in their intestinal tract and on their exterior (Berrang et al., 2000a; Kotula and Pandya, 1995). These organisms may contaminate meat as a result of cross-contamination during processing (Berrang et al., 2001; Musgrove et al., 1997) and this creates a risk for consumers. Recently, a qualitative risk assessment was conducted in order to determine the most relevant biological hazards for public health that should be included in a modernized poultry meat inspection system (European Food Safety Authority, 2012). Risk ranking of the hazards was based on the magnitude of impact of the hazard on human health, the severity in humans of disease caused by a particular hazard, the proportion of human cases related to poultry meat, and occurrence of the hazard in poultry batches. *Campylobacter* spp., *Salmonella* spp. and ESBL producing bacteria were considered to be the most relevant hazards.

2.1 Salmonella

Salmonellosis is the second most frequently reported zoonosis in the European Union. Its symptoms in humans most frequently include self-limiting gastroenteritis with fever, diarrhoea and abdominal pain. Bloodstream infections can, however, be fatal (Rabsch et al., 2015). Worldwide, human salmonellosis is mostly caused by two

serovars, namely *Salmonella* Enteritidis, and *Salmonella* Typhimurium (European Food Safety Authority, 2015; Hendriksen et al., 2011).

According to 2013 data, in Europe there were 82,694 reported confirmed salmonellosis cases. This is a 31.8% decrease since 2008 (European Food Safety Authority, 2015). This decrease is a result of the National *Salmonella* control programs (EC regulation No 2160/2003) implemented in the European Union in order to control and monitor *Salmonella* through the food chain (European Food Safety Authority, 2015; Hugas and Beloeil, 2014). Within the food chain, poultry and eggs are important *Salmonella* reservoirs (European Food Safety Authority, 2015). Further, in broiler production *Salmonella* can be transmitted horizontally and vertically (Heyndrickx et al., 2002): vertically from infected breeding stock, and horizontally during rearing, for example via vectors such as rodents, insects, wild birds, individuals entering the farm etc., or through contaminated feed or drinking water (Heyndrickx et al., 2002; Mead, 2004).

Successful control of *Salmonella* in broilers can be achieved through a range of preventive measures that should be applied in the entire production chain, i.e. at breeding and broiler farms, during catching and transport, at the slaughterhouse, at retail and during preparation both in restaurants and by consumers (Van Immerseel et al., 2009). Control measures at the pre-harvest phase, e.g. hygiene and biosecurity as well as vaccination (Van Immerseel et al., 2009) contributed to the decrease in prevalence in broiler flocks; prevalence, according to the latest report (2013) was 3.7% in the European Union (European Food Safety Authority, 2015). Microbiological criteria for *Salmonella* in foodstuffs have been in force since 2006 ((EC) No 2073/2005) and were revised by Regulations (EC) No 1441/2007 and 1086/2011 (European Food Safety Authority, 2015). The criterion for fresh poultry carcasses requires absence of *Salmonella* in 25g of a pooled sample of neck skin ((EC) No 1086/2011). A decrease in non-compliance among the samples and tested batches was observed in the EU between 2012 and 2013, confirming the success of *Salmonella* control in poultry production (European Food Safety Authority, 2015).

2.2 Campylobacter

Campylobacteriosis has, since 2005, been the most frequently reported zoonosis in the EU (European Food Safety Authority, 2015). The usual symptoms of campylobacteriosis in humans are gastroenteritis: watery or bloody diarrhoea, abdominal pain, fever, headache and nausea. The infections are mostly self-limiting within a few days or a week. Infrequently, complications can develop, such as reactive arthritis, neurological disorders, irritable bowel syndrome, inflammatory bowel disease and Guillain-Barré syndrome (Wagenaar et al., 2015). Campylobacteriosis can also be fatal, and in 2013 in the EU there were 56 deaths attributed to this disease, 25 more than in 2012 (European Food Safety Authority, 2015).

The number of reported and confirmed campylobacteriosis cases in humans in 2013 in the European Union was 214,779 (European Food Safety Authority, 2015). The real annual number of campylobacteriosis cases in the EU in 2009 was estimated to be at 9.2 million (Havelaar et al., 2013). The cost of campylobacteriosis in the European Union has been estimated at 2.4 billion € annually (European Food Safety Authority, 2011a). In the Netherlands, the disease burden of campylobacteriosis was estimated to be one of the highest of all diseases and equal in 2011 to 3,250 DALY/year. DALY means disability-adjusted life years and it is a metric of loss of health as a result of disease, comprising of years of life in good health that are lost in the population due to disability and death (Mangen et al., 2013).

At the moment of writing this chapter, there were 33 *Campylobacter* species and 14 subspecies according to "Straininfo: http://www.straininfo.net/taxa/462" (Verslyppe et al., 2014). Thermophilic *C. jejuni* and *C. coli* are the two most frequently isolated species from poultry and humans (Wagenaar et al., 2015). Thus, in this thesis term *Campylobacter* relates to these species. Thermophilic *Campylobacter* grows between 30 and 45 °C with an optimum at 42 °C, in microaerobic atmosphere. Despite demanding growth conditions and sensitivity to environmental stress including desiccation, heat, ultra-violet radiation, atmospheric oxygen, and salinity (Wagenaar et al., 2015), *Campylobacter* may survive outside its host for as long as ten months (Inglis et al., 2010).

Campylobacter may inhabit the guts of warm-blooded animals, as this environment meets its growth requirements and especially colonizes avian species, which have body temperatures around its optimum growth temperature (Wagenaar et al., 2015). *Campylobacter* thus colonizes various types of poultry (Wagenaar et al., 2006). In broilers, prevalence differs across the various Member States in the European Union, with low rates in Nordic countries and high rates in southern countries, e.g. Spain, Italy, with an average of 71% for all EU countries according to a baseline study performed in 2008 (European Food Safety Authority, 2010a). *Campylobacter* prevalence in broiler flocks shows a seasonal peak during the summer months that is sharper in northern than in southern European countries (European Food Safety Authority, 2010a; Nylen et al., 2002). Vertical transmission of *Campylobacter* is reported to be rare if it exist at all (Callicott et al., 2006) and broiler batches become colonized mainly through horizontal transmission routes (Shanker et al., 1990). Colonized broiler chickens are asymptomatic (Dhillon et al., 2006) and *Campylobacter* spreads rapidly through the entire flock (van Gerwe et al., 2009).

According to risk assessment studies, most campylobacteriosis cases in humans (80%) can be attributed to the chicken reservoir, with handling, preparation and consumption of broiler meat accounting for one third of all cases (European Food Safety Authority, 2011a). This confirms that measures to control *Campylobacter* at primary production would be the most effective to combat campylobacteriosis. However,

currently, these measures have limitations (Wagenaar et al., 2013). Biosecurity for example is highly dependent on geographical location (European Food Safety Authority, 2008). The variation in prevalence among European countries is influenced not only by e.g. climatic factors, but also by management differences in poultry production. For example, in the 'free-range' and/or 'organic' methods popular in France and the United Kingdom, prevention of *Campylobacter* is almost impossible, since flocks have access to the environment (Cui et al., 2005; European Food Safety Authority, 2011a; Heuer et al., 2001). In any event, it is not expected in the short term that a major decrease of colonized flocks with *Campylobacter* is achievable. The alternative strategy is to reduce the numbers of *Campylobacter* on poultry meat (Havelaar et al., 2007). As there are generally more farms than slaughterhouses, focusing the control measures on removing or inactivating *Campylobacters* on carcasses during processing might be a good approach. Only a fraction of *Campylobacter* concentration present on the meat at the end of processing will reach the consumer, because this bacterium does not grow outside its host (Swart et al., 2013). Reducing the concentration on broiler meat, rather than reducing on-farm prevalence, has been reported based on risk assessment as an effective and efficient strategy to diminish the number of human campylobacteriosis (Nauta et al., 2009).

Unlike in the case of *Salmonella*, effective measures to control *Campylobacter* in broiler production have not yet been implemented in the EU. Yet strategies aiming at reducing *Campylobacter* numbers on broiler chicken carcasses would both reduce its risk and also potentially reduce other hazards, such as ESBL/AmpC producing *E. coli,* since biological hazards related to poultry originate from the intestinal tract of broilers.

2.3 ESBL/AmpC producing E. coli

Microorganisms can develop resistance to antibiotics either through mutation or through acquisition of a resistance gene, as reflected in the definition of antimicrobial resistance by the WHO (Anonymous, 2015c). In addition, microbial species can be intrinsically resistant to certain antimicrobial agents (Anonymous, 2009). Resistance implies that antimicrobials (including antibiotics, antivirals, antifungi and antiparasites) can become ineffective against such bacteria, viruses, fungi, protozoa and helminths). As a result standard treatments can become ineffective (Anonymous, 2015c). The health care costs of infections caused by antimicrobial resistant bacteria are higher, as they lead to prolonged hospitalization and higher mortality (Cosgrove, 2006; De Kraker et al., 2011; Mauldin et al., 2010; Melzer and Petersen, 2007; Tumbarello et al., 2010). It has been estimated that in 2007 there were 25,000 deaths caused by infections with selected antibiotic-resistant bacteria (i.e. MRSA, vancomycin-resistant *Enterococcus faecium*, third-generation cephalosporin resistant *E. coli* and *K. pneumoniae* and carbapenem-resistant *P. aeruginosa*) among the residents of the European Union, Iceland and Norway.

This resulted in approximately € 900 million extra health care costs (Anonymous, 2009).

Resistance can a.o. be caused by extended-spectrum beta-lactamases (ESBL), plasmid-encoded enzymes that can affect a wide spectrum of beta lactam antibiotics, including penicillins, 2nd-, 3rd- and 4th-generation cephalosporins and monobactams (e.g. aztreonam), but not the carbapenems or the cephamycins (e.g. cefoxitin) (European Food Safety Authority, 2011b). ESBL plasmids have been detected in *Enterobacteriaceae* including most commonly *Escherichia coli* and *Klebsiella pneumonia.* Resistance can also be caused by AmpC beta-lactamases (AmpC), intrinsic cephalosporinases that are found in many gram-negative bacteria on their chromosomal DNA and makes them resistant to penicillins, 2nd- and 3rd-generation cephalosporins, cefamycins (cefoxitin), but usually not to 4th-generation cephalosporins (cefepime, cefquinome) and carbapenem. AmpC genes are also found on plasmids most frequently in *Enterobacteriaceae* (except *Klebsiella* and *Salmonella)*, and in *Pseudomonas* and *Acinetobacte*r (European Food Safety Authority, 2011b).

Infections caused by ESBL/AmpC producing *E. coli* are reported worldwide (Bush, 2008; Castanheira et al., 2008; Coque et al., 2008) including in countries with low antimicrobial usage in human medicine such as the Netherlands and Denmark (Grave et al., 2010; van de Sande-Bruinsma et al., 2008). ESBL/AmpC producing *E. coli* are a concern for public health as its resistance hinders the treatment of infections, including those in the urinary tract, soft tissue and intra-abdominal area (Livermore et al., 2007; Pitout and Laupland, 2008; Rodriguez-Bano et al., 2004; Woodford et al., 2004). The presence of ESBL/AmpC in *E. coli* makes the use of 2nd-, 3rd- and 4th-generation of cephalosporins ineffective against this pathogen (Coque et al., 2008; Marchaim et al., 2010; Rodriguez-Bano et al., 2004; Rodriguez-Bano et al., 2010).

The epidemiology of beta lactam resistance in *E. coli* is changing rapidly. According to Hawkey and Jones (2009), in the 1990s the most commonly reported ESBLs genes included TEM/SHV types. These were most frequently associated with hospital acquired infections with *Klebsiella pneumoniae*, carrying variants such as TEM-1, TEM-2 and SHV-1 with increased activity towards the extended spectrum cephalosporins (Bradford, 2001). Since 2000, a shift in ESBL types in Europe has been observed and currently the most frequently reported types belong to the CTX-M family (Canton et al., 2012; Livermore et al., 2007; Rossolini et al., 2008). The infections caused by ESBL/AmpC producing *E. coli* can be also acquired outside hospitals in patients with no hospitalization history or antimicrobial treatment (Dubois et al., 2010; Friedmann et al., 2009; Pitout and Laupland, 2008; Valverde et al., 2004). Tschudin-Sutter et al. (2012) observed infrequent nosocomial transmission.

ESBL/AmpC producing *E. coli* have been detected in various habitats, including food producing animals (i.e. broilers, poultry, swine) (Blaak et al., 2011; Dierikx et al., 2010; Escudero et al., 2010; Machado et al., 2008; Randall et al., 2011; Smet et al.,

2008), retail meat (Overdevest et al., 2011; Paterson et al., 2010; Warren et al., 2008), companion animals (Dierikx et al., 2012; Ewers et al., 2012), and in the environment (i.e. surface water, wildlife, flies) (Blaak et al., 2011; 2014; Guenther et al., 2011). Among various food producing animals, the prevalence of ESBL/AmpC producing *E. coli* is highest in poultry (Anonymous, 2015b). For example, in 2014 in the Netherlands the prevalence in broiler livestock was 67%, whereas 34% in laying hens, 18% in slaughter pigs, 23% in white veal calves, 14% in rose veal calves, and 9% in dairy cows (Anonymous, 2015b).

As far as retail meat is concerned, the prevalence of ESBL/AmpC producing *E. coli* is highest in chicken meat comparing to other meat types (Anonymous, 2015b; Overdevest et al., 2011). In chicken meat, ESBL/AmpC producing *E. coli* has also been reported in meat originating from organic farming, which harbours ESBL genes and strain types similar to those in meat from conventional farming (Kola et al., 2012; Stuart et al., 2012). The prevalence of ESBL/AmpC producing *E. coli* was higher in processed chicken meat than in raw meat, which could be potentially explained by cross contamination during processing (Anonymous, 2015b).

The phenotypes, ESBL/AmpC genotypes and plasmids (mobile genetic elements) of ESBL/AmpC producing *E. coli* found in chickens and chicken meat were reported to be similar to those found in humans (Kluytmans et al., 2013; Leverstein-van Hall et al., 2011; Overdevest et al., 2011). Based on these similarities, several studies have suggested that the food chain is a potential transmission route of ESBL/AmpC producing *E. coli* to humans (Kluytmans et al., 2013; Leverstein-van Hall et al., 2011; Overdevest et al., 2011). In contrast other authors have reported differences between ESBL/AmpC producing *E. coli* isolates from poultry and human (Campos et al., 2014; Randall et al., 2011; Wu et al., 2013).

In a recent study Been et al. (2014) demonstrated by use of whole genome sequencing that pairs of human and poultry isolates, that had previously been reported as indistinguishable based on their ESBL genotypes and strain typing (Kluytmans et al., 2013; Leverstein-van Hall et al., 2011; Voets et al., 2013) were not closely related. The authors suggest that classical methods used in the previous studies, such as Multi Locus Sequence Typing do not provide sufficient resolution and therefore, cannot be used to establish a relationship between isolates from various reservoirs. Results obtained by Been et al. (2014) demonstrated no clonal transmission of ESBL/AmpC producing *E. coli* from poultry to humans. However, the authors demonstrated the presence of similar plasmids in various reservoirs, thereby confirming the contribution of plasmids to the dissemination of resistance between reservoirs. Based on these findings, they concluded that the transmission of ESBL/AmpC producing *E. coli* from poultry to human via food chain is less evident. Overall, the role of the food chain in this transfer remains unknown (Bonten and Mevius, 2015).

Despite the prevalence of ESBL/AmpC producing *E. coli* in the poultry sector being widely studied, there is currently a lack of quantitative data about ESBL/AmpC producing *E. coli* in broiler processing. Its concentrations on the final product (i.e. carcasses after chilling) are not known, nor are quantitative data at points along the processing line available. Such data could support risk assessment studies, could help to validate Process Hygiene Criteria and also help determining interventions in processing hygiene.

3. *Campylobacter* Process Hygiene Criteria

Various risk assessment models have been developed to describe the dynamics of *Campylobacter* contamination of broiler meat during processing (Hartnett et al., 2001; Nauta et al., 2007; Rosenquist et al., 2003). Despite differences in the described dynamics, all of these models have concluded that reducing *Campylobacter* concentration on broiler meat would be more effective than reducing prevalence (Nauta et al., 2009). Reduction of *Campylobacter* numbers on broiler chicken carcass has been estimated to be the most effective intervention to diminish campylobacteriosis cases in humans in the shorter term (Nauta et al., 2009). This supports the implementation of targets such as Process Hygiene Criteria (PHC) based on microbiological risk assessment, as a way to develop control measures aimed at producing carcasses with less than the established limit of microbiological contamination (European Food Safety Authority, 2012).

PHC was defined in Regulation (EC) No 2073/200516 on microbiological criteria for foodstuffs as "a criterion that indicates the acceptable functioning of the production process. The criterion sets an indicative contamination value above which corrective actions are required in order to maintain the hygiene of the process in compliance with food law". The PHC can be used to stimulate improvements in hygienic practices during processing, through validation and verification of HACCP-based processes, procedures, and implementation of hygiene control measures. Moreover PHC can be used, for example, to communicate the level that should be achieved to control a hazard (European Food Safety Authority, 2011a) and monitor the ability of slaughterhouses to reduce public health hazards (European Food Safety Authority, 2012). Exceeding the limit specified by the PHC requires implementation of the corrective actions to maintain processing hygiene.

Implementation of PHC is supported by the outcome of risk assessment, which has demonstrated that compliance of batches of fresh broiler meat to a critical limit for *Campylobacter* of 1000 CFU/gram of neck and breast skin would reduce 50% of campylobacteriosis cases, as would 90% compliance with a limit of 500 CFU/gram (European Food Safety Authority, 2011a; Nauta et al., 2012). The limit of 1000 CFU/g for neck and breast skin from carcasses sampled after chilling was however exceeded in

20% of the batches tested during a baseline survey conducted in the European Union in 2008 (European Food Safety Authority, 2010a). Achieving this limit was also a challenge for the broiler chicken slaughterhouses in the Netherlands, as demonstrated in a nationwide baseline survey (Anonymous, 2011). There, one third of tested batches exceeded 1000 CFU/g of breast skin after chilling (Swart et al., 2013).

Both European and Dutch surveys have demonstrated that *Campylobacter* numbers in slaughter batches differ widely. Some slaughterhouses are more capable than others in reducing and controlling *Campylobacter* concentrations on carcasses (European Food Safety Authority, 2010a; Swart et al., 2013). This suggests that improvements in processing hygiene are attainable. However neither study provided an explanation of differences found between batches and slaughterhouses. *Campylobacter* concentrations on carcasses at various processing steps were not investigated, neither was information gathered on processing conditions that potentially might have influenced these differences. It was not studied whether various processing steps influenced the *Campylobacter* concentration on carcasses similarly in various slaughterhouses. Also information on factors influencing the variation was lacking. The EFSA opinion (European Food Safety Authority, 2010b) recommended investigating batch and slaughterhouse related factors that lead to an increase or decrease in risk of broiler meat contamination with *Campylobacter*. Such information would help to determine what strategies enable efficient reduction of *Campylobacter* concentrations on meat.

Further, it was proposed by EFSA that the ability of slaughterhouses to reduce hazards on broiler meat such as *Campylobacter* and ESBL/AmpC producing *E. coli* could be indicated by generic *E. coli* (European Food Safety Authority, 2012). This is desirable because the quantification methods for *E. coli* are less expensive, less time consuming and independent on seasonal presence as is the case for *Campylobacter* (European Food Safety Authority, 2012; Habib et al., 2012). Correlation between *Campylobacter* and *E. coli* concentrations on broiler chicken carcasses has been studied, but results show either presence or lack of correlation (Berrang and Dickens, 2000; Duffy et al., 2014; Williams and Ebel, 2014). There is currently insufficient information to conclude whether *E. coli* and both *Campylobacter* and ESBL/AmpC producing *E. coli* concentrations indeed change through broiler processing in a similar way. More quantitative data obtained along the entire processing line is needed to understand such changes.

4. Quantification of *Campylobacter* on broiler carcasses

Quantitative data on *Campylobacter* on broiler chicken carcasses is frequently generated via the culture method (Anonymous, 2006), which consumes time and resources, limiting the number of samples that can be investigated. This was also the case in the European baseline study (European Food Safety Authority, 2010a), in which only

one sample of neck and breast skin per batch was collected to measure *Campylobacter* concentrations. A single sample provides no information about the variance in *Campylobacter* contamination within a batch, which is an important parameter for risk assessment studies (Nauta et al., 2012) and the determinations of targets in Process Hygiene Criteria.

Quantification methods based on DNA detection offer an alternative to traditional culture methods and a possibility to investigate more samples in a shorter time. However they do not provide better sensitivity comparing to culture methods and are detecting both live and dead cells, which is not informative for risk assessments. Alternative methods have been studied combining PCR with pre-treatment of samples with photoreactive dyes in order to differentiate live cells from dead cells. Those dyes include e.g. ethidium monoazide (EMA) (Nogva et al., 2003) and propidium monoazide (PMA) (Nocker et al., 2006). These dyes are able to penetrate dead cells or cells with compromised membranes, and intercalate into double-stranded nucleic acids. As a result of exposure to light, cross-linking of the DNA occurs and its PCR amplification is inhibited (Nocker et al., 2006; Nocker et al., 2009; Nocker et al., 2007). Various bacteria have been quantified by real time PCR with PMA (PMA-qPCR), including *Escherichia coli* O157:H7, *Listeria monocytogenes*, *Listeria innocua*, *Salmonella* and *Campylobacter* (Banihashemi et al., 2012; Elizaquível et al., 2012; Løvdal et al., 2011; Nocker et al., 2009; Pan and Breidt, 2007). Also, a PMA-qPCR protocol has been proposed to quantify live *C. jejuni*, *C. coli* and *C. lari* on broiler chicken carcasses after chilling (Josefsen et al., 2010). The latter method has not yet been used and validated for samples collected from carcasses at different steps along the processing line.

5. Microbiology of broiler chicken processing in a slaughterhouse

The exterior of broilers and their gastrointestinal tract are the sources of bacteria during processing (Berrang et al., 2000a; Kotula and Pandya, 1995). During processing, broiler meat may become contaminated through cross-contamination from faeces, feathers, direct contact between carcasses, indirect contamination via equipment (e.g. rubber defeathering fingers), or airborne spread via aerosols in the slaughtering area (Allen et al., 2003a; Allen et al., 2003b; Berrang et al., 2001; Buhr et al., 2003; Musgrove et al., 1997). Steps during broiler processing are illustrated in Figure 1.

Studies were conducted to determine the *Campylobacter* concentrations after processing steps including scalding, defeathering, evisceration, washing and chilling (Guerin et al., 2010). They indicated that *Campylobacter* increased during defeathering, but decreased during scalding, washing and chilling, and that evisceration had a variable effect (Berrang and Dickens, 2000; Cason et al., 1997; Izat et al., 1988; Klein et al., 2007; Paulin, 2011; Rosenquist et al., 2006; Wempe et al., 1983).

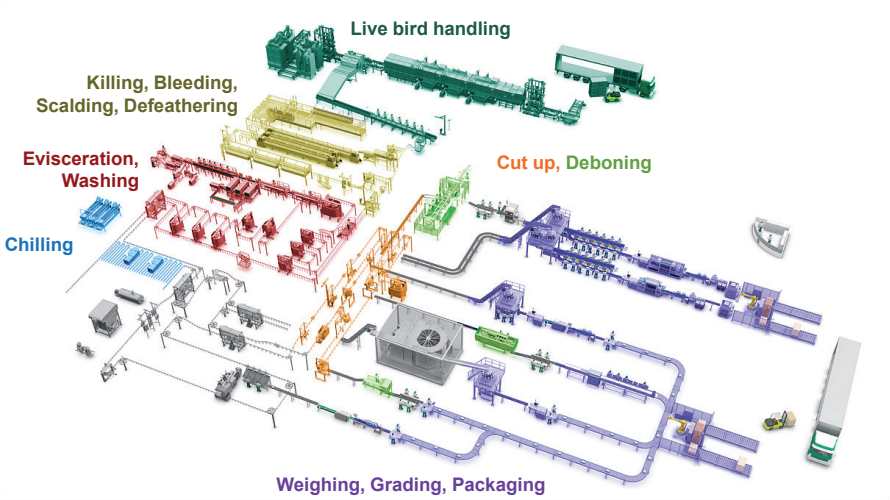

Figure 1
Processing steps in a poultry slaughterhouse. Source: Meyn Food Processing Technology B.V. "Wall to Wall Solutions".

Some of the studies analysed selected processing steps only, and others provided data along the processing line (Guerin et al., 2010). The latter were however done either in the US where the processing parameters differ from those in Europe (Berrang and Dickens, 2000), or if in Europe were performed far in the past (Oosterom et al., 1983). Thus the mechanism and magnitude of changes in *Campylobacter* concentration through processing and factors influencing these changes remain unclear (Guerin et al., 2010). It remains currently unexplained why the magnitude of changes is different between batches and between slaughterhouses.

Slaughterhouses may apply different methods, technologies and parameters during processing, all of which can influence the final product properties, including safety. These differences are driven by customers' needs at different markets and varying regulations e.g. between Europe and US (Barbut, 2014). The stunning method can be electrical (low or high voltage) or gas, scalding can be done in steam or immersion in hot water (and time and temperature can vary), while chilling can be done either by cold air, or air with water spray or by immersion in water (Mead, 2004). It has been reported that immersion scalding at a higher temperature leads to a higher reduction in *Campylobacter* concentrations on carcasses (Lehner et al., 2014; Yang et al., 2001). Also scalding temperature was reported to impact *Salmonella* concentration on carcasses after defeathering. This is because scalding at higher temperatures will cause the epidermis from the carcass to be removed during defeathering, leading to changes in skin microtopography. The lack of epidermis on the skin facilitates the attachment of *Salmonella* to the skin after defeathering (Kim et al., 1993).

Investigation of factors related to processing technology and hygiene implementation and their influence on *Campylobacter* concentration on chicken

carcasses was not included in the European *Campylobacter* baseline survey (European Food Safety Authority, 2010b). Insight into such factors and their associations with *Campylobacter* concentration changes during processing could potentially explain the observed differences in *Campylobacter* concentrations on carcasses after chilling between slaughterhouses and batches (European Food Safety Authority, 2010a).

6. Interventions to reduce *Campylobacter* on broiler meat

Interventions during broiler processing that have been investigated include both physical and chemical decontamination (European Food Safety Authority, 2011a). Studies on chemical decontamination evaluated substances such as organic acids (lactic and acetic acid) (Bolder, 2007; Burfoot and Allen, 2013), chlorine and aqueous chlorine dioxide (Bolder, 2007; Nagel et al., 2013; Northcutt et al., 2005), acidic electrolysed oxidising water (Kim et al., 2005), acidified sodium chlorite (Bashor et al., 2004; Bolder, 2007; Kere Kemp et al., 2001), peroxyacetic (peracetic) acid (Bauermeister et al., 2008; European Food Safety Authority, 2014; Nagel et al., 2013) and trisodium phosphate (Bashor et al., 2004; Whyte et al., 2001). After revising various decontamination methods, Loretz et al. (2010) concluded that chemical treatment reduced bacterial contamination by 1-2 orders of magnitude. Although in the EU decontamination is permitted (Anonymous, 2004), no specific chemical agents for decontaminating raw poultry have yet been authorized (European Food Safety Authority, 2011a). In addition, chemical decontamination is poorly accepted by consumers (MacRitchie et al., 2014).

Physical decontamination is mainly based on treatments which either decrease or increase the temperature of the carcasses or portions, or use irradiation. Studied physical treatments include hot water or steam (Berrang et al., 2000b; James et al., 2007; Whyte et al., 2003), steam ultrasound (Boysen and Rosenquist, 2009; Hansen and Larsen, 2007; Musavian et al., 2014), freezing (Georgsson et al., 2006; Sandberg et al., 2005), crust freezing (Boysen and Rosenquist, 2009; Haughton et al., 2012; James et al., 2007), irradiation (Farkas, 1998) and ultraviolet (UV) light (Corry et al., 1995; Isohanni and Lyhs, 2009). Physical decontamination also has limitations. For example steam and hot water pasteurization, although effective in reducing *Campylobacter* on chicken carcass surface by 1.3 log CFU/g of breast skin, deteriorates the appearance of the treated carcasses (Whyte et al., 2003). Application of combined treatment of steam and ultrasound has been reported to overcome the unacceptable effect on the appearance (Musavian et al., 2014). Freezing reduces effectively *Campylobacter* between 1-2 log (Georgsson et al., 2006; Sandberg et al., 2005). Implementation of freezing might be difficult because of logistic and economic reasons, especially in countries where the *Campylobacter* prevalence in flocks is high and the consumers prefer to purchase fresh meat (Havelaar et al., 2007). Irradiation is highly effective (Farkas, 1998) but is

poorly accepted by consumers (MacRitchie et al., 2014). Decontamination is considered as a supplementary measure and not a substitute to good hygiene practices, and thus EFSA recommends the development of control measures based on improvement in the equipment design and slaughter practices (European Food Safety Authority, 2011a). These recommendations do not take into account the management aspects of processing control, but like interventions related to processing technology, these may lend themselves to interventions.

Luning and Marcelis (2006) proposed a techno-managerial approach, suggesting that food quality, with safety as one of the quality attributes (Luning and Marcelis, 2009a), depends on the behaviour of the food and human systems and their interactions. Behaviour of the food systems relates to changes in food properties as a result of e.g. variability in contamination level. Behaviour of the human systems relates to variability in decisions taken (Luning and Marcelis, 2009b). The goal of food quality management is to provide a food product that complies with consumer and legal requirements or even exceeds them (Luning and Marcelis, 2007), and can incorporate both technological and managerial aspects.

In the techno-managerial approach, technological aspects are related to the dynamics of the food systems (food characteristics i.e. bacterial level in the incoming batches) and the technological conditions applied to control and predict food behaviour. Technological conditions refer to the characteristics of applied technologies (like hygienic design features of equipment and facilities) and processing parameters (Luning and Marcelis, 2007; Luning and Marcelis, 2009b). Decisions on acceptable food dynamics (e.g. setting standards and tolerances) and design and operation of facilities, processes, and equipment will affect the food product. From a technological perspective, the quality control activities focus on keeping product and processes within acceptable tolerances, with corrective actions where necessary (Luning et al., 2008). Assurance activities focus on validating and verifying the process, equipment and product performance (Luning et al., 2009).

Managerial aspects are related to human behaviour, namely variation in decision making behaviour. This variation can be influenced by the individual characteristic of people who take decisions, i.e. their perception, attitudes, choice intentions, and their administrative environment. These in turn are influenced by available information systems and procedures, the competences of people, division of tasks, responsibilities and rules (Luning and Marcelis, 2007; Luning and Marcelis, 2009b). To prevent variation in decision making behaviour, food quality management would need to ensure compliance with procedures that help food handlers to take correct decisions during food production (Luning and Marcelis, 2007). Procedures are necessary to produce products with an acceptable bacterial contamination level (Luning et al., 2008). However documentation required by HACCP is seen by processors as an obstacle (Nguyen et al., 2004) rather than

a help in fulfilling required tasks. Thus, as has been frequently observed, food handlers do not always follow prescribed hygiene practices (Baş et al., 2006; Jianu and Chiş, 2012; Walker et al., 2003). Recently, it was demonstrated that variable compliance with procedures does indeed impact product safety parameters (Sanny et al., 2011; 2013). The fact that the level of food safety may be influenced by the knowledge, attitude and practice of food handlers has been studied in various food premises (Angelillo et al., 2000; Ansari-Lari et al., 2010; Baş et al., 2006; Tokuç et al., 2009). Knowledge was defined as a body of acquired facts, attitude as a mental reaction to knowledge, and practice (behaviour) as actions taken as a result of knowledge acquisition and attitude developed (Azanza and Zamora-Luna, 2005). It was reported that the attitudes of food handlers support implementation of their knowledge in practice (Ko, 2013). Broiler processing, despite high automation (Barbut, 2014), still requires interaction of humans who control the process with the equipment. Currently there is no information about compliance of food handlers with procedures affecting processing hygiene in a broiler slaughterhouse. The managerial aspect during broiler processing has had little attention. Insight in these aspects however could explain the differences in bacterial contamination between slaughterhouses and provide a background in which to develop interventions.

Effective interventions to reduce number of *Campylobacter* on broiler meat have not been implemented so far for a variety of reasons (Wagenaar et al., 2015). One of these is the complexity of *Campylobacter* sources and transmission routes. Another is the unequal distribution of costs and benefits of implementing the interventions, where investments need to be done by the industry and the general population obtains public health benefits. Another reason is that certain cost-effective measures, including chemical decontamination and irradiation, are accepted neither by consumers nor by governments (European Food Safety Authority, 2011a; MacRitchie et al., 2014). Combating *Campylobacter* and other biological hazards in the poultry sector requires a holistic approach that includes the various stakeholders.

7. Outline of the thesis

The general aim of this thesis was to study changes in the contamination of broiler chicken carcasses with *Campylobacter*, ESBL/AmpC producing *E. coli* and generic *E. coli* through the broiler processing line, and to identify and quantify factors related to processing technology and management that might influence these changes. The purpose was to obtain background knowledge necessary for developing interventions that will reduce the bacterial contamination on carcasses during and after processing. To achieve this aim, specific research questions were studied in the following chapters.

The study described in **Chapter 2** aimed at investigating whether the concentration of viable *Campylobacter* on broiler chicken carcasses could be measured by a rapid quantification method, i.e. PMA-qPCR. To this aim, quantitative data on *C. jejuni*, *C. coli* and *C. lari* on broiler chicken carcasses after five selected processing steps in slaughterhouses obtained by PMA-qPCR were compared with results obtained by the traditional culture method.

The following study described in **Chapter 3** aimed at investigating whether the pattern of changes in *Campylobacter* concentration during processing varies within and between slaughterhouses, and how it corresponds with *E. coli.* The quantitative data on *Campylobacter* and *E. coli* concentrations on broiler chicken carcasses through five processing steps in two slaughterhouses were described. With a statistical model, it was analysed whether the changes in concentrations of both bacteria were variable between batches, slaughterhouses and organisms.

A goal of the study described in **Chapter 4** was to identify which explanatory variables were associated with increases and decreases in *Campylobacter* and *E. coli* concentration during the tested processing steps. To this end, in parallel to collection of quantitative data on bacterial concentration in the tested broiler chicken batches, data on nineteen potentially explanatory variables were collected by administering questionnaires to farmers and quality managers in slaughterhouses and by conducting measurements. Mixed effects statistical models were used to determine potential associations within each slaughterhouse.

The study described in **Chapter 5** aimed at investigating whether the concentrations of ESBL/AmpC producing *E. coli* changed through processing in a similar way as those of *Campylobacter* and *E. coli*. The quantitative data on ESBL/AmpC producing *E. coli* throughout processing in the studied slaughterhouses were described and their similarities and differences with data on *Campylobacter* and *E. coli* concentrations were analysed. In addition, it was identified whether the genotypes of ESBL/AmpC producing *E. coli* on broiler chicken carcasses corresponded with known poultry associated genotypes of *E. coli*. Moreover, it was analysed whether the distribution of genotypes varied throughout processing.

The study described in **Chapter 6** aimed at testing whether a technological intervention (i.e. the prototype equipment to brush the surface of carcasses) reduced the *E. coli* and *Enterobacteriaceae* concentration on carcasses before scalding. It was studied whether the reduction was realised on the whole carcass or on the treated areas only. In addition, it was investigated which areas of carcasses were, before scalding, most contaminated. Further, studies were done on the effect of the brushes on parameters that may directly (pH) or indirectly (dry matter content) influence the bacterial reduction in the scalding water.

The study described in **Chapter 7** aimed at investigating managerial aspects of hygiene control. The results on compliance of food handlers with procedures to control the evisceration process were described. The evisceration process affected *Campylobacter*, ESBL/AmpC producing *E. coli* and generic *E. coli* concentrations differently between slaughterhouses (Chapters 3 and 5). It was expected that procedures and compliance with them could contribute to these differences. Thus, the procedures were analysed and the compliance of food handlers with the criteria to control the evisceration process was observed in the two slaughterhouses. In addition the effect of the evisceration process on the occurrence of carcasses with visible faecal contamination was measured. Visibly contaminated and clean carcasses were sampled to investigate the concentration of *E. coli*. Further the food handlers' characteristics that could contribute to non-compliance with set procedures were investigated.

In **Chapter 8** the cumulative findings of the thesis were discussed and suggestions were made concerning how this information can be applied by various stakeholders in the broiler meat chain to improve future control of public health hazards during broiler processing.

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Chapter 2

Propidium monoazide does not fully inhibit the detection of dead *Campylobacter* on broiler chicken carcasses by qPCR

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Abstract

A real time quantitative PCR combined with propidium monoazide (PMA) treatment of samples was implemented to quantify live *C. jejuni, C. coli* and *C. lari* on broiler chicken carcasses at selected processing steps in the slaughterhouse. The samples were enumerated by culture for comparison. The *Campylobacter* counts determined with the PMA-qPCR and the culture method were not concordant. We conclude that the qPCR combined with PMA treatment of the samples did not fully reduce the signal from dead cells.

Keywords: *Campylobacter*, qPCR, PMA, Broiler

1. Introduction

Consumption of broiler meat products contaminated with *Campylobacter* is estimated to cause 20 to 30% of nine million cases of human campylobacteriosis in the European Union each year (European Food Safety Authority, 2011; European Food Safety Authority, 2012). Most of the human infections are associated with thermotolerant *Campylobacter* such as *C. jejuni*, *C. coli* and *C. lari*. The species *C. jejuni* is by far the most important source, causing 80% of human cases of campylobacteriosis annually (European Food Safety Authority, 2011). Reducing *Campylobacter* contamination along the broiler chicken supply chain could decrease human infections. The most effective intervention would be strategies at farms; however those cannot be achieved in the short term (Havelaar et al., 2007; European Food Safety Authority, 2011). Therefore alternative intervention strategies should be explored in the entire broiler supply chain (Havelaar et al., 2007).

During slaughtering operations *Campylobacter* contamination on broiler carcasses fluctuate (Rosenquist et al., 2006). Interventions at the steps that contribute the most to *Campylobacter* increase on the carcasses could significantly improve safety of broiler meat (Rosenquist et al., 2003; Berrang et al., 2004). Thus, quantitative data on *Campylobacter* contamination on broiler carcasses during processing are needed, not only for evaluation of effectiveness of intervention strategies, but also for risk assessment studies.

The detection of *Campylobacter* at slaughtering operations is currently based on time and labour consuming culture methods. Molecular methods e.g. real time PCR are a rapid and high throughput alternative to traditional culture techniques. A disadvantage of real time PCR is that it does not offer any improved sensitivity over culture methods for detection and quantification of *Campylobacter* in biological samples. Another disadvantage is that dead cells may also be detected, providing unreliable information on public health risks. Live cells with intact membranes can be differentiated from dead cells by their ability to exclude DNA binding photoreactive dyes, e.g. ethidium monoazide (EMA) (Nogva et al., 2003) and propidium monoazide (PMA) (Nocker et al., 2006). Those dyes easily penetrate dead or membrane-compromised cells (Nocker et al., 2006). Inside cells dyes intercalate into double-stranded nucleic acids from cells with compromised membranes, and upon light exposure, cross-linking of the DNA occurs and its PCR amplification is inhibited (Nocker et al., 2006; Nocker et al., 2007; Nocker et al., 2009).

Real time PCR protocols combined with PMA treatment (PMA-qPCR) have been implemented to quantify live bacteria, e.g. *Escherichia coli* O157:H7, *Listeria monocytogenes*, *Listeria innocua*, *Salmonella* and *Campylobacter* (Elizaquível et al., 2011; Nocker et al., 2009; Pan and Breidt, 2007; Løvdal et al., 2011; Josefsen et al., 2010; Banihashemi et al., 2012).

The PMA-qPCR developed by Josefsen et al. (2010) was tailored to quantify live *C. jejuni, C. coli* and *C. lari* in chicken rinse samples obtained immediately post slaughter, thus of interest in our study. The protocol is based on the amplification and detection of a 287-bp sequence of the 16S rRNA gene of *C. jejuni*, *C. coli*, and *C. lari*. The protocol incorporates an internal amplification control (IAC) that allows monitoring of false negatives that can be caused by PCR inhibitors. In the real time PCR assay the IAC co-amplifies with the *Campylobacter* target, but both are detected with a specific probe labelled with different dyes (Josefsen et al., 2004; 2010).

The aim of this study was to evaluate the usefulness of the PMA-qPCR protocol to quantify live *C. jejuni*, *C. coli*, and *C. lari* on broiler chicken carcasses after selected processing steps in the slaughterhouse and compare PMA-qPCR results with culture. Firstly the PMA treatment was optimized and validated under laboratory conditions, including the effect of sample storage conditions.

2. Materials and Methods

2.1 PMA treatment

The treatment of samples with propidium monoazide (PMA) (Biotum Inc., Hayward, CA) was implemented following the manufacturer's guidelines regarding handling of the samples during the treatment and as described by Josefsen et al. (2010) with modifications of exposure of samples to 1000 W halogen lamp at a distance of 40 cm. The effect of two variables was assessed to differentiate between live and dead cells in a culture of *C. jejuni* NCTC 12665: (i) the concentration of PMA and (ii) the time of light exposure. In the first experiment 3 concentrations of *C. jejuni* NCTC 12665 (5.3, 3.6 and 2.8 log CFU/ml) were divided in 7 aliquots that were heated at 95°C for 5 min for killing the bacteria. One aliquot of each concentration was used as non-PMA treated control. Six remaining aliquots were treated in different combinations of PMA concentrations (20 or 50 μM) and light exposure-time (one, two or three minutes) (Figure 1). In the second experiment 5 concentrations of the *C. jejuni* NCTC 12665 (4.7, 4.1, 3.4, 2.7 and 2.1 log CFU/ml) were each divided in 7 aliquots from which 4 were heated at 95°C for 5 min for killing the bacteria and 3 aliquots were unheated live bacteria (Figure 1). One heated and one unheated aliquot of each concentration were used as non PMA treated controls. The remaining aliquots were treated with 20 μM PMA and exposed to light (one, two or three minutes) to determine the effect of signal reduction from dead cells and impact of the PMA on live cells. In both experiments confirmation of the absence of live cells in the heated samples was done by culture. All samples were subjected to DNA extraction as described under section 2.4, prior to being used as a template for the qPCR.

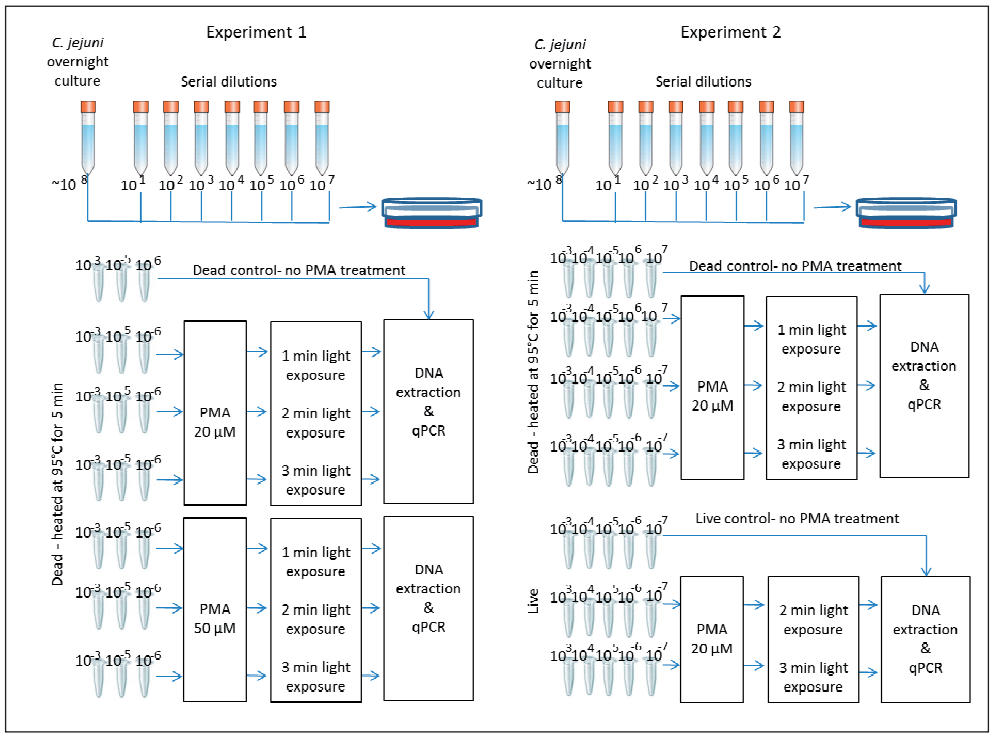

Figure 1
Summary of experimental work set up described under point 2.1. Selected dilutions were used in the experiments. Extrapolation of counts in the dilutions resulted in the following concentrations: experiment 1 (5.3, 3.6 and 2.8 log CFU/ml), experiment 2 (4.7, 4.1, 3.4, 2.7 and 2.1 log CFU/ml). Aliquots of the selected dilutions were treated in different combinations of PMA concentration and light exposure time. Controls containing live or dead bacteria were included.

2.2 Analysis of inoculated samples

The PMA-qPCR protocol (Josefsen et al. 2010) for quantification of live *C. jejuni, C. coli* and *C. lari* in the broiler chicken carcass rinse samples was initially applied to inoculated rinse samples from broiler chicken parts purchased at a retail store. In the same experiment the impact of one day storage and storage temperature (controlled and abused) of the rinse samples on concentration of *C. jejuni* was investigated. Five chicken parts (drumstick with thigh) from different batches were rinsed vigorously by hand for 60 seconds in 125 ml peptone saline each. The rinses were inoculated with *C. jejuni* NCTC 12665 that was subcultured overnight in brain heart infusion broth to final concentrations in the samples of 3 and 6 log CFU/ml of the rinse. The inoculated samples and controls were stored for 24h under controlled temperature (4°C) and abused temperature that mimicked a failure of the cooling system (2 hours at 4°C, then exposed to room temperature for 2.5 hours and stored again at 4°C). Before and after storage the samples were subjected to *Campylobacter* culture on mCCDA (bioTrading,

Mijdrecht, The Netherlands) for enumeration according to ISO 10272-2. In parallel the samples were subjected to PMA treatment as described by Josefsen et al. (2010), with modification of the light source and distance. After the PMA treatment the DNA was extracted and the qPCR was performed.

2.3 Analysis of naturally contaminated samples

The PMA-qPCR protocol was used to quantify live *C. jejuni, C. coli* and *C. lari* in rinse samples from whole broiler chicken carcasses, collected after the following processing steps: bleeding, scalding, defeathering, evisceration and chilling, during nine visits in two slaughterhouses. In total 242 samples were collected. The rinses were prepared according to ISO 6887-2 (Anonymous, 2000). In short: whole broiler chicken carcasses were removed from the line after the indicated steps, the cloacae of the birds were plugged with a fibre tampon in order to avoid faecal and intestinal leakage during sample preparation, the birds were placed in sterile plastic bags (Hevel, Zaandam, The Netherlands), and rinsed vigorously by hand for 60 s in 500 ml of peptone saline. The rinse samples of approximately 100 ml were subjected to laboratory analysis the next day, except for three visits where samples were analysed on the same day. Samples subjected to laboratory analysis at the day following their collection were stored overnight at 3°C +/- 2°C. The samples were cultured on Campy Food Agar (bioMérieux SA, Marcy l'Étoile, France) and enumerated according to ISO 10272-2 (Anonymous, 2006). *Campylobacter* presumptive colonies were confirmed by microscopic observation after Gram staining.

In parallel, aliquots of the rinse samples collected in the slaughterhouses, without and with PMA treatment were subjected to DNA extraction. The PMA treatment was performed for three min at 40 cm distance from the light source, as determined in 2.1 to be the optimal treatment conditions. The real time qPCR protocol with and without the PMA treatment was applied as described below.

2.4 DNA extraction and qPCR detection

DNA from the rinse samples was extracted with the High pure PCR template preparation kit (Roche Diagnostics GmbH, Mannheim, Germany). The real time PCR protocol of Josefsen et al. (2010) was adapted for detection on the Light Cycler 480 (Roche Diagnostics GmbH, Mannheim, Germany). The PCR mixture consisted of: 10 µl Light Cycler 480 Master hybridisation probe (Roche Diagnostics GmbH, Mannheim, Germany), both primers at a concentration of 625 nM each, 50 nM of the *Campylobacter* target probe and 75 nM internal amplification control probe, 5 µl of extracted template DNA and molecular grade water to obtain a reaction volume of 20 µl. The cycling conditions consisted of an initial denaturation at 95°C for 10 min, followed by 45 cycles of 95°C for 10 s, 60°C for 20 s and 72°C for 20 s. The fluorescence measurements were performed by the Light Cycler 480. The limits of detection as well

as the linearity of the qPCR assay were calibrated using increasing amounts (1 fg to 100 ng) of purified genomic DNA from *C. jejuni* NCTC 12665. The lower limit of DNA detection was reached with 1 fg of genomic DNA, which is nearly equivalent to the genome of 1 to 2 *C. jejuni* bacterial cells (1 fg/cell). *C. jejuni* was quantified using a standard curve that was based on genomic DNA and their bacterial cell equivalent. For determination of the total amount of *C. jejuni* cells in 1 ml of the rinse samples these values were multiplied with 200 and transformed to logarithmic scale.

2.5 Statistical analysis

Statistical analysis was performed using the R software (2.15.2, 2012, R Development Core Team). Analysis of variance was used to determine if there was a difference between *Campylobacter* concentrations obtained by culture and PMA-qPCR methods in the inoculated chicken rinse samples. Orthogonal regression was used to determine the relationship between the results obtained by culture, PMA-qPCR and qPCR in rinse samples collected in the slaughterhouses. Orthogonal regression was used as in this regression the errors are measured perpendicularly to the regression line, without treating one variable as exact. A bootstrap percentile algorithm was used to estimate the 95% confidence intervals of the slope and intercept. Estimation of the amount of dead cells in the samples collected in the slaughterhouses was done in two ways: (1) subtracting culture results from qPCR results, (2) subtracting PMA-qPCR results from qPCR results.

3. Results

3.1 PMA treatment

A PMA concentration of 20 µM versus 50 µM and three minutes of light exposure were the most effective in reducing the signal from dead cells (results not shown). Samples with a concentration higher than 4.1 $\log_{10}$ CFU/ml remained still detectable (Figure 2) however the signal was reduced as reflected by an increased Cp value. The Cp values from live cells treated with 20 µM of PMA and exposed to the light source for one, two and three minutes were similar to the control (Figure 2).

3.2 Campylobacter detection in the inoculated rinse samples

Analysis of variance of the *Campylobacter* concentration obtained by culture and PMA-qPCR methods in the inoculated rinse samples showed no statistical differences (p>0.05) across inoculation categories (Figure 3). Based on the culture data the storage of the rinse samples under studied conditions did not impact *Campylobacter* counts (data not shown).

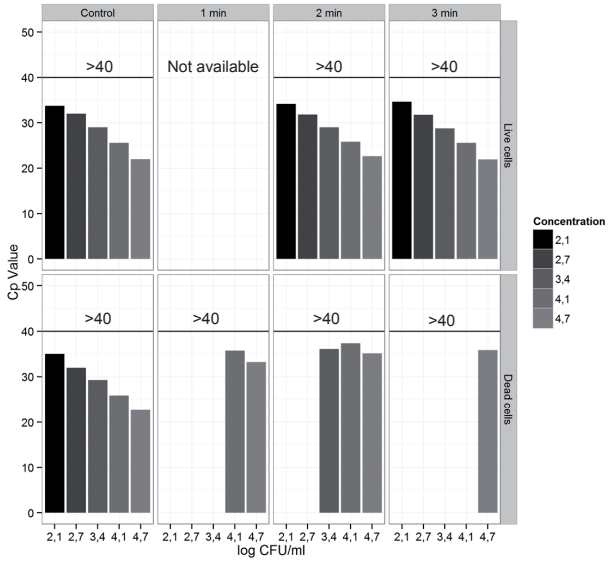

Figure 2
Effect of 20 µM PMA concentration and different light exposure time on the signal reduction from live and dead cells of *C. jejuni* NCTC 12665 cultures. No bar means that samples were not detected as they were above the detection level of 36 to 40 Cp value.

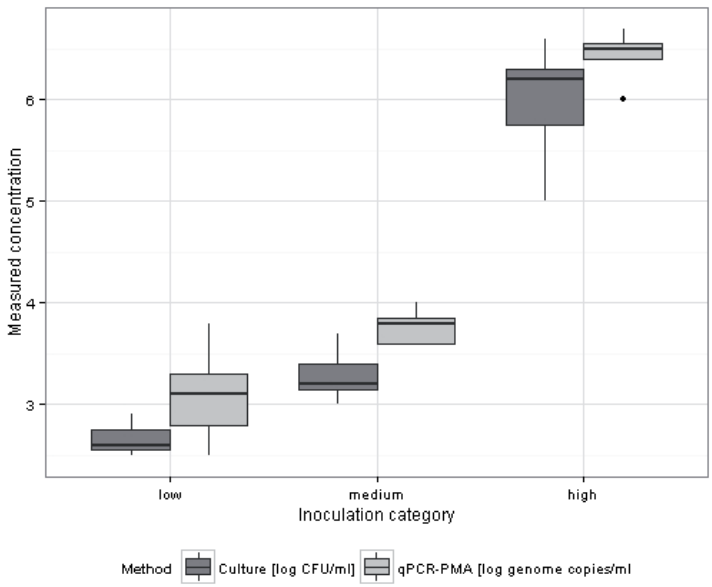

Figure 3
C. jejuni NCTC 12665 concentration in inoculated rinse samples and controls (low – not inoculated controls, in two cases naturally contaminated, medium –inoculated to level of 3 log CFU/ml, high- inoculated to level of 6 log CFU/ml). The length of the box indicates the interquartile range of a sample; the whiskers a sample maximum and minimum and the dots outliers.

3.3 Campylobacter detection in naturally contaminated rinse samples

Campylobacter concentrations in the whole broiler chicken carcass rinse samples collected at selected steps in the slaughterhouses determined by culturing and qPCR with and without PMA are presented in Figure 4. *Campylobacter* numbers obtained by PMA-qPCR were higher than by culture, but lower than obtained by qPCR (Figure 4). The results from orthogonal regression of the *Campylobacter* concentration obtained by culturing, qPCR with and without PMA are shown in Table 1 and Figure 5. Regression analysis of 1) culture and PMA-qPCR data and 2) culture and qPCR data resulted in similar equations. Estimation of the number of dead cells demonstrated that in 197 out of 217 analysed samples, the difference between *Campylobacter* concentrations obtained by subtracting culture results from qPCR results was larger (up to 2 log) than by subtracting PMA-qPCR from qPCR (up to 1 log, Figure 6).

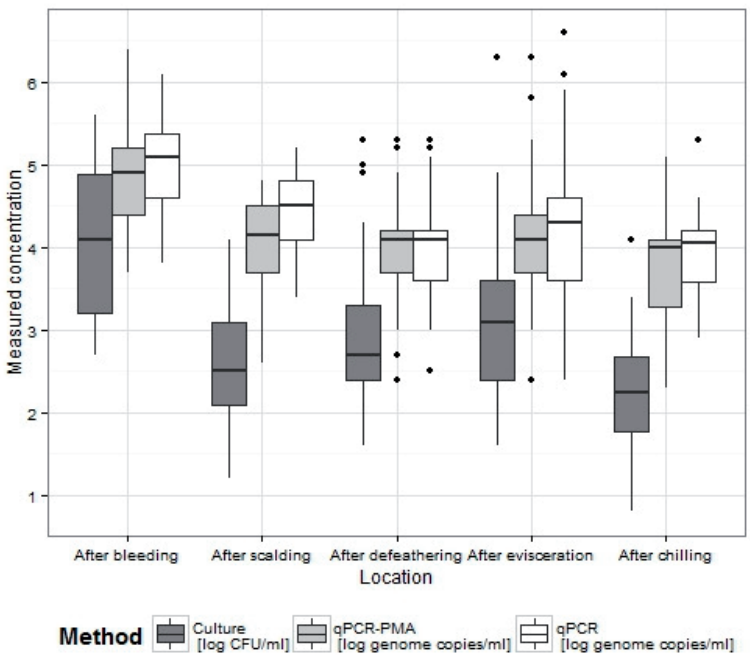

Figure 4

Campylobacter quantification in whole broiler chicken carcass rinse at several processing steps in the slaughterhouse obtained by culturing, PMA-qPCR and qPCR. The length of the box indicates the interquartile range of a sample; the whiskers a sample maximum and minimum and the dots outliers.

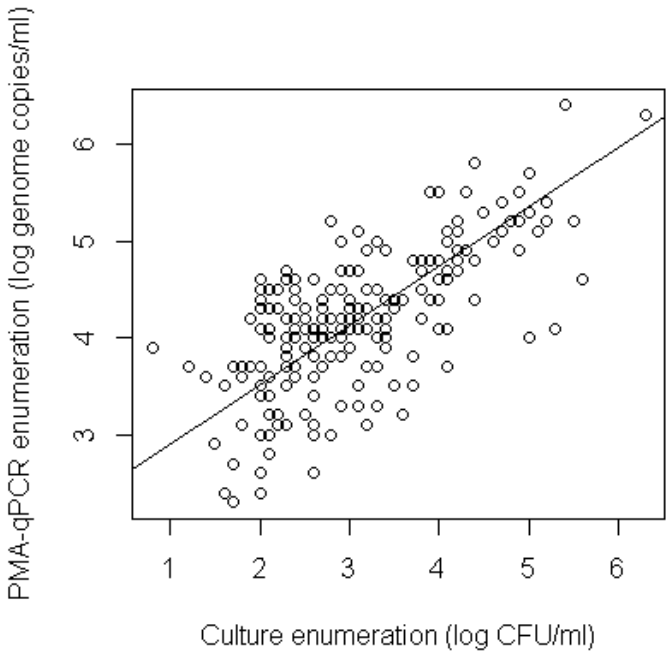

Figure 5
Relationship between *Campylobacter* count obtained by culture enumeration and PMA-qPCR in whole broiler chicken carcass rinse at several processing steps in the slaughterhouse. The sampling location and the sampling occasion are not taken in consideration.

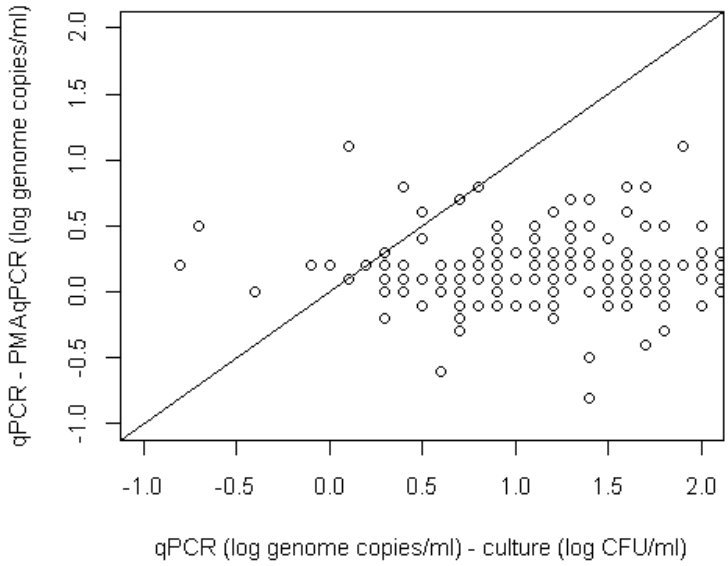

Figure 6
Estimation of the amount of dead bacteria in the analyzed samples by calculating the differences in *Campylobacter* concentrations obtained by qPCR and PMA-qPCR (y-axis), and by qPCR and the culture method (x-axis).

Table 1
Regression analysis results of the data obtained by culture and PMA-qPCR (1) and culture and qPCR (2). Detected samples were used in the regression analysis.

Sampling point	No Samples					(1) Culture (x) PMAqPCR (y)				(2) Culture (x) qPCR (y)			
	Total collected	Detected			Regression analysis	y= intercept + slope* x				y= intercept + slope* x			
		Culture	PMA qPCR	qPCR	Analysed (1)/ Analysed (2)	Slope (95% CI)		Intercept (95% CI)		Slope (95% CI)		Intercept (95% CI)	
1 after bleeding	46	46	46	46	46/46	0.39	(0.156, 0.620)	3.26	(2.317, 4.346)	0.37	(0.188, 0.521)	3.50	(2.844, 4.236)
2 after scalding	49	47	46	49	44/47	0.22	(-0.464, 0.997)	3.47	(1.340, 5.025)	0.16	(-0.240, 0.447)	3.98	(3.220, 5.029)
3 after defeathering	49	47	49	49	47/47	0.64	(0.390, 0.853)	2.10	(1.468, 2.786)	0.60	(0.380, 0.797)	2.27	(1.730, 2.863)
4 after evisceration	49	48	49	45	48/44	0.67	(0.458, 0.876)	2.03	(1.363, 2.658)	0.81	(0.588, 1.063)	1.67	(0.748, 2.458)
5 after chilling	49	46	40	44	37/41	1.01	(0.366, 2.032)	1.34	(-1.255, 2.879)	0.67	(0.246, 1.130)	2.35	(1.204, 3.275)
All locations	242	234	230	233	222/225	0.61	(0.521, 0.709)	2.29	(1.957, 2.607)	0.59	(0.502, 0.679)	2.54	(2.246, 2.809)

4. Discussion

In the present study usefulness of the PMA-qPCR to enumerate live *C. jeuni, C. coli* and *C. lari* on broiler chicken carcasses after crucial processing steps in the slaughterhouses was evaluated.

Optimization of the PMA treatment conditions on heat inactivated *C. jejuni* NCTC 12665 from pure culture, confirmed the effect of the 20 μM PMA concentration on signal reduction from dead cells. Cawthorn and Witthuhn (2008) reported however this concentration as ineffective in complete inhibition of dead *Enterobacter sakazakii.* Banihashemi et al. (2012) found 15 μM PMA to be optimal for *C. jejuni*. An effective PMA concentration is specific for the studied microorganism as reported by many authors (Yáñez et al., 2012; Løvdal et al., 2011; Pan and Breidt, 2007; Elizaquível et al., 2012). Extended light exposure time confirmed stronger signal reduction from dead cells as was also described by Nocker et al. (2006) and Yáñez et al. (2012). The light exposure time for optimal PMA efficiency was reported to depend on the intensity of the light source in excitation wavelength range of the dye (around 464 nm) which can vary between different light sources (Yáñez et al., 2012). The efficiency of PMA to reduce signal from dead cells was concluded to depend on PMA concentration, light source, studied organism, the structure of the outer membrane (Løvdal et al., 2011) and PCR amplicon length (Banihashemi et al., 2012).

In this study there was no impact of PMA on signal reduction from live *C. jejuni* NCTC 12665 cells, similar as observed by Nocker et al. (2006) and Elizaquível et al. (2012).

In the experiment with the inoculated rinse samples no differences in *Campylobacter* concentration obtained by culture and PMA-qPCR method was observed. This can be explained by the fact that there were no dead bacteria in the samples as the storage of the samples did not diminish the live *Campylobacte*r concentration as expected (results not shown).

With respect to samples collected in the broiler chicken slaughterhouses some effect of PMA treatment on signal reduction from dead *Campylobacter* cells was observed, as PMA-qPCR results are lower than qPCR (Figure 4). However the *Campylobacter* counts determined with the PMA-qPCR and the culture method were not concordant, opposite to the study on inoculated samples. The only difference in *Campylobacter* culturing method used in the study with samples collected at the slaughterhouses was using Campy Food Agar (CFA) instead of mCCDA agar plates. We found it easier to distinguish typical and atypical colonies on the transparent background of the CFA in comparison to the mCCDA. Culturing results obtained on both media were reported to be comparable (Habib et al., 2011; own results not presented). Thus, different media cannot explain the weak relation of culture and PMA-qPCR results in the naturally

contaminated samples versus the strong relation in the inoculated samples. An uncertain factor that was not considered in this study might be a shift of the obtained PMA–qPCR and qPCR numbers on logarithmic scale with 0.3 or 0.6 as a result of two to four copies of the 16S rRNA gene, present in *C. jejuni*, which is the target of the qPCR primers.

Regression analysis of data from naturally contaminated samples resulted in similar regression equation in case 1) culture and PMA-qPCR and 2) culture and qPCR, suggesting a limited effect of the PMA. In the study with naturally contaminated samples, dead bacteria were found (Figure 6) this is opposite to the inoculated samples. The range of dead cells calculated by subtracting qPCR and PMA-qPCR was narrower than in case of subtracting qPCR and culture data. The broader range of live cells indicates more discriminatory power of culture versus PMA-qPCR method in identifying live cells. Insufficient inhibition by PMA of signals from dead cells in naturally contaminated samples is the most likely explanation. Overestimation of *Campylobacter* counts by PMA-qPCR differed between steps, being higher after bleeding, scalding and chilling and lower after defeathering and evisceration which can be explained by the characteristic of these processing steps. Broiler chicken entering the processing might carry dead *Campylobacter* on the surface and feathers, what could explain the overestimation of PMA-qPCR results after bleeding. Inactivation of heat and drying sensitive *Campylobacter* during scalding and chilling is a result of immersing the carcasses in the scalding water tank at temperature 51°C to 58°C and air/air-spray chilling. During defeathering and evisceration leakage of faecal and intestinal origin might occur, providing more live *Campylobacter* cells on the surface, thus the lack of inhibition of dead cells by PMA is less readily observed.

According to Josefsen et al. (2010) using rinse samples from naturally contaminated chickens after chilling, culture and PMA-qPCR results were highly correlated, r^2=0.84 and also without PMA treatment the correlation was r^2=0.88, opposite to our results. The good fit of the regression obtained by Josefsen et al. (2010) could be a result of small variation introduced in the sampling of the carcasses originating from only one flock. This is in contrast to our experiments where variation is introduced due to differences in sampling day and slaughterhouse.

Lack of concordance of the results obtained by the culture and PMA-qPCR methods in our study can most probably be explained by insufficient inhibition of high numbers of dead *Campylobacter* cells in the rinse samples collected in the slaughterhouses. The impact of the amount of dead bacteria in a sample on the quantification of live bacteria by PMA-qPCR has been reported by others (Yáñez et al., 2012; Løvdal et al., 2011; Wagner et al., 2008; Pan and Breidt, 2007). It was stated that a high amount of membrane-compromised cells in the sample might impact the capacity of the dye to modify the entire DNA in the region targeted by the primers (Yáñez et al., 2012). A high initial cell density through a high concentration of dead bacteria in the samples

might inhibit the PMA crosslinking during the light activation and might result in detection of dead cells (Løvdal et al., 2011). In our study at the entrance to the processing operations, after the bleeding step, the *Campylobacter* concentration in the rinse samples in 25 out of 46 cases was higher than 4 log CFU/ml above which the PMA was found ineffective (our results section 3.1; Yáñez et al., 2012 and Elizaquível et al., 2012). Initial concentration could have impacted limited effect of the PMA treatment in this study.

5. Conclusions

In the presented study the usefulness of the PMA-qPCR protocol to detect the live *C. jejuni, C. coli* and *C. lari* in rinse samples from the whole broiler chicken carcasses, collected at crucial processing steps in the slaughterhouse was studied. The effect of the PMA treatment on the signal reduction from dead cells was observed. The results obtained by the PMA-qPCR and culture from the rinse samples collected in the slaughterhouses were not concordant, demonstrating an insufficient PMA effect on signal reduction from dead cells. The causes of restricted PMA capacity in penetrating the membrane-compromised cells in samples concentrated above 10^4 CFU remain a challenge to be addressed in further research.

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Chapter 3

A comparison of fluctuations of *Campylobacter* and *Escherichia coli* concentrations on broiler chicken carcasses during processing in two slaughterhouses

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Abstract

The causes of differences in *Campylobacter* and *Escherichia coli* concentrations on broiler chicken carcasses after chilling between slaughterhouses are not fully identified. Therefore, it is a challenge for slaughterhouses to comply with Process Hygiene Criteria for broiler meat. The aim of the study was to identify which processing steps contribute to increases or decreases in *Campylobacter* and *E. coli* concentrations within and between two slaughterhouses. Identifying the processing steps with variable performance could explain the differences in bacterial concentrations after chilling between slaughterhouses. Thermotolerant *Campylobacter* and *E. coli* concentrations on carcasses during broiler processing were measured during the summer period in 21 trials after bleeding, scalding, defeathering, evisceration and chilling. In two slaughterhouses with comparable *Campylobacter* and *E. coli* concentrations in the incoming batches (after bleeding), the mean $\log_{10}$ concentrations are found to be significantly different after chilling. *Campylobacter* concentrations decreased by 1.40 $\log_{10}$ in Slaughterhouse 1 and by 1.86 $\log_{10}$ in Slaughterhouse 2, whereas *E. coli* decreased by 2.19 $\log_{10}$ in Slaughterhouse 1 and by 2.84 $\log_{10}$ in Slaughterhouse 2. Higher concentrations of *Campylobacter* and *E. coli* on carcasses after chilling were observed in Slaughterhouse 1 in which an increase in concentrations was observed after evisceration. The effect of processing on *Campylobacter* and *E. coli* concentrations in Slaughterhouse 1 did not differ between batches. In Slaughterhouse 2, the effect of processing on the concentrations of both bacteria varied over batches. Changes in *E. coli* concentration levels during processing were similar to *Campylobacter* except for defeathering. *E. coli* concentration significantly decreased after defeathering in both slaughterhouses, whereas *Campylobacter* increased in Slaughterhouse 2 and in Slaughterhouse 1 no significant changes were observed. The patterns of increases and decreases in bacterial concentrations during processing are specific for each slaughterhouse. Inhomogeneous patterns potentially explain the differences in concentrations after chilling between slaughterhouses. Critical processing steps should be validated in each slaughterhouse by longitudinal studies and potentially based on *E. coli*. *E. coli* has a potential to be used as an indicator of processing hygiene, because the impact of most of the studied processing steps was similar as for *Campylobacter*.

Keywords: Poultry, Slaughter Hygiene, Process Hygiene Criteria

1. Introduction

Campylobacter has remained the major gastrointestinal bacterial pathogen in humans in the European Union (EU) since 2005 (European Food Safety Authority, 2014). The annual number of campylobacteriosis cases was estimated at 9 million in the European Union (Havelaar et al., 2009). Broiler meat is recognised as a major source of human infections. An estimated 20-30% of cases of campylobacteriosis in EU may be attributed to the handling, preparation and consumption of broiler meat (European Food Safety Authority, 2010a). According to risk assessment studies, the most effective reduction of human infections in the short term could be achieved by reducing *Campylobacter* numbers in contaminated slaughtered batches (Nauta et al., 2009). Compliance of batches sold as fresh meat with a threshold value of 1000 or 500 CFU/g of neck and breast skin would reduce the health risk by more than 50% or even 90% (European Food Safety Authority, 2011). These risk assessment results drive the initiative to establish Process Hygiene Criteria (PHC) for broiler meat to stimulate further control measures aiming at reducing carcass contamination (European Food Safety Authority, 2012a). Moreover, the PHC could be used as a tool to classify slaughterhouses according to their capability to prevent or reduce hazards and as a tool to monitor risk and verify hygiene management in slaughterhouses (European Food Safety Authority, 2012a). Compliance to the PHC is, however, a challenge for the industry. In 20% of the tested batches in the European Union, the *Campylobacter* concentrations in neck and breast skin after chilling exceeded 1000 CFU/g (European Food Safety Authority, 2010b). In The Netherlands, 30% of all produced batches would not meet the threshold of none of 5 samples per batch exceeding 1000 *Campylobacter* CFU/g of breast skin after chilling (Anonymous, 2011; Swart et al., 2013). Differences in *Campylobacter* concentrations after chilling between slaughterhouses were identified in the baseline surveys (Anonymous, 2011; European Food Safety Authority, 2010b). However, the causes of the differences were not fully identified. *Campylobacter* concentrations change along the processing line with typically a decrease after scalding and chilling, an increase after defeathering and an increase or no change after evisceration (Berrang and Dickens, 2000; Izat et al., 1988; Klein et al., 2007; Oosterom et al., 1983; Rosenquist et al., 2006; Seliwiorstow et al., 2012; Tchórzewska et al., 2013). It has not been investigated whether these changes in concentrations are maintained at similar levels between batches and between slaughterhouses. Identifying the processing steps with variable performance within and between slaughterhouses could explain the differences in *Campylobacter* concentrations after chilling and thus improve the ability of the slaughterhouses to comply with potential Process Hygiene Criteria.

Setting a PHC based on *Escherichia coli* instead of on pathogenic bacteria has been proposed (European Food Safety Authority, 2012a; European Food Safety Authority,

2012b), because indicator microorganisms are recognised to reflect better the process hygiene than pathogenic microorganisms. The advantage of using *E. coli* to monitor processing performance is also related to easier, lower-cost, omnipresent enumeration techniques and frequent occurrence of *E. coli* on the carcasses that is not impacted by seasonality as in case of *Campylobacter* (European Food Safety Authority, 2012a; Habib et al., 2012). Furthermore, targets based on *E. coli* concentration levels on carcasses after chilling proved to be a useful tool to reduce *Campylobacter* levels on postchill carcasses (Habib et al., 2012).

The aim of the study was to identify which processing steps contribute to increases or decreases in *Campylobacter* and *E. coli* concentrations in two slaughterhouses. Moreover the purpose was to identify whether the impact of the processing steps on bacterial contamination levels varied within and between slaughterhouses and whether the impact was similar for both *Campylobacter* and *E. coli*.

2. Materials and Methods

2.1 Slaughterhouses

The samples were taken in two commercial broiler chicken slaughterhouses. Slaughterhouse 1 is located in Germany and daily processes 130 000 broilers, whereas Slaughterhouse 2 is situated in The Netherlands and daily processes 240 000 broilers. The slaughterhouses were selected because of similarities in the processing equipment applied. During the study, the stunning, scalding and defeathering equipment was modernized in Slaughterhouse 1 prior to trials in 2013. The processing parameters remained the same.

2.2 Collection and preparation of samples

Thermotolerant *Campylobacter* and *E. coli* concentrations during broiler processing were measured in 21 trials. Eleven trials were performed in Slaughterhouse 1 (trials' ID: A, B, C, I, K, L, N, O, Q, R, U). Ten trials were performed in Slaughterhouse 2 (trials' ID: D, E, F, G, H, J, M, P, S, T). The trials were performed between June-October 2012 (trials A-M) and June-October 2013 (trials N-U). This sampling period was chosen in order to increase the probability of *Campylobacter* positive flocks (European Food Safety Authority, 2010c). Each trial was performed on a different day and included one batch, defined as a group of chickens raised together in one shed (European Food Safety Authority, 2011).

The *Campylobacter* status of the batch was ascertained by the slaughterhouses. In Slaughterhouse 1, bootswabs (in 2012 and 2013) and cloacal swabs (in 2012) at farms were taken 2-3 days prior to sampling in the slaughterhouse. The bootswabs were enriched in Campy Food broth (bioMérieux SA, Marcy l'Étoile, France), the cloacal

swabs in Preston broth (prepared according to manufacturer guidelines - Oxoid). From the enrichment broth 1 ml was taken for further analysis and the positivity was checked by PCR with a detection limit of 100 CFU/ml. In Slaughterhouse 2, faecal droppings were collected at farms one week prior to sampling in the slaughterhouse. The faecal droppings were streaked on mCCDA, incubated and confirmed according to a Dutch national method (Anonymous, 2010). The limit of detection was 100 CFU/g.

During the trials, the first samples were collected after at least 1000 carcasses of the investigated batch had passed through the line, in order to avoid potential cross-contamination from the previously slaughtered batch. Samples were collected after the following processing steps: 1) just after bleeding, 2) just after scalding, 3) just after defeathering, 4) after evisceration and evisceration spraying cabinet but before inside and outside washing and 5) just after chilling. These steps were chosen, because the most dynamic changes in bacterial contamination levels were reported after these steps (Rosenquist et al., 2006).

The sampling plan to collect quantitative data is presented in Table 1. Whole carcass rinse was performed as described previously (Pacholewicz et al., 2013). The carcasses were removed from the line after selected processing steps. The cloacae of the non-eviscerated carcasses were plugged with a fibre tampon to prevent faecal and intestinal leakage while rinsing. Prevention of leakage of faecal material as a result of plugging was compared to results from rinsing the carcasses that were plugged and sealed (results not shown). Plugging and sealing of the vent were previously reported to prevent the faecal leakage (Berrang et al., 2001). After placing the carcasses into sterile plastic bags (Hevel, Zaandam, The Netherlands), 500 ml of peptone saline was added and the carcasses were shaken by hands for 60 s (Anonymous, 2000; Nauta et al., 2007; Reich et al., 2008). The same volume of the rinse was used for carcasses with feathers, defeathered or eviscerated carcasses to overcome differences in bacterial recovery, because removal rate was reported to differ with different volumes of rinse fluid (Williams et al., 2010).

In addition positivity of caeca from carcasses sampled after evisceration was checked in trials J-U. Caecal material was plated on Campy Food Agar and in case of no growth, enrichment was done according to the Dutch national method (Anonymous, 2010).

Breast skin samples after chilling were additionally collected during trials in 2013. The purpose of collecting the breast skin samples was to compare results from rinse and breast skin. The skin samples were collected as previously described (Anonymous, 2011). In short: 25g (+/- 5g) of skin from breast corpus was cut and placed in a stomacher bag.

Samples collected after different processing steps do not correspond to the same carcass, except for the caecal samples. These samples were collected from the same eviscerated carcasses that were sampled by the whole carcass rinse method.

Table 1
Sampling plan presents processing steps after which the samples were collected, type of collected samples, number of samples collected in that particular trial (A-U) and enumeration threshold.

Processing step	Sample type	Unit	Enumeration threshold CFU/ml or CFU/g *Campylobacter*/ *E. coli*	Trials 2012											Trials 2013											Sum
				A	B	C	D	E	F	G	H	I	J	K	L	M	N	O	P	Q	R	S	T	U	
				Slaughterhouse 1 or Slaughterhouse 2																						
				1	1	1	2	2	2	2	2	1	2	1	1	2	1	1	2	1	1	2	2	1	
After bleeding	Whole Carcass Rinse	CFU/ml	10/10	3	3	3	0	4	4	4	8	8	8	8	8	8	8	8	8	8	8	8	8	8	133
After scalding	Whole Carcass Rinse	CFU/ml	1/10	3	3	3	3	4	4	4	8	8	8	8	8	8	8	8	8	8	8	8	8	8	136
After defeathering	Whole Carcass Rinse	CFU/ml	1/10	3	3	3	3	4	4	4	8	8	8	8	8	8	8	8	8	8	8	8	8	8	136
After evisceration	Whole Carcass Rinse	CFU/ml	1/10	3	3	3	3	4	4	4	8	8	8	8	8	8	8	8	8	8	8	8	8	8	136
After chilling	Whole Carcass Rinse	CFU/ml	1/1	3	3	3	3	4	4	4	8	8	8	8	8	8	8	8	8	8	8	8	8	8	136
	Breast skin sample	CFU/g	10/10	0	0	0	0	0	0	0	0	0	0	0	0	0	8	8	8	8	8	8	8	8	64
Total analysed samples				15	15	15	12	20	20	20	40	40	40	40	40	40	48	48	48	48	48	48	48	48	741
Number of samples below *Campylobacter* enumeration threshold				8	0	0	0	0	0	1	1	19	0	1	0	0	0	7	0	1	0	7	1	0	
Number of samples below *E. coli* enumeration threshold				0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	2	0	0	

The number of samples collected per trial for each sampling location was different (Table 1). In trials A-D – 3 samples were collected, in trials E-G – 4 and in trials H-U – 8. The difference in the number of samples between the trials was related to the fact that samples collected during trials A-G were not only analysed by the culture method but also by the PMA-qPCR method (Pacholewicz et al., 2013). The purpose was to validate the PMA-qPCR method for quantifying viable *Campylobacter* cells, as an alternative for the culture method. It was not feasible to analyse more samples per location with both methods. As the PMA-qPCR method was not validated, the culture method only was used in trials H-U and more samples could be accommodated.

All collected samples were stored at 3°C (+/- 2°C), as specified in ISO 10272-2 (Anonymous, 2006), until laboratory analysis. In the case of 15 trials (A, B, D-G, I, J, L, N, O, Q, R, T, U) the samples were stored overnight. Due to the slaughter time of the batches and travelling time from the slaughterhouses to the laboratory, it was not feasible to perform the analytical work on the same day. Overnight storage of the rinse samples did not affect the number of recovered *Campylobacter* (results not shown). In the case of the remaining 6 trials (C, H, K, M, P, S) the analytical work was done on the day on which the samples were collected.

2.3 Analytical methods

The serial dilutions of whole carcass rinse samples were done in Butterfield's Buffer (3M The Netherlands, Zoeterwoude, product number BPPFV9BF) or peptone saline. No differences in counts were observed while using both media (results not shown). The breast skin samples were homogenised with 9 volumes of peptone saline. Serial dilutions of the homogenate were done in Butterfield's Buffer. *Campylobacter* enumeration was done on Campy Food Agar (bioMérieux SA, Marcy l'Étoile, France, product number 43471), according to ISO 10272-2 (Anonymous, 2006). Five *Campylobacter* presumptive colonies per sample were confirmed by microscopic observation after Gram staining. The *Campylobacter* isolates were not identified at species level. The enumeration threshold for the rinse samples was 10 CFU/ml for samples after bleeding, because higher concentrations were expected in the samples after bleeding than after the following steps or 1 CFU/ml for samples after scalding, defeathering, evisceration and chilling. The enumeration threshold for breast skin samples was 10 CFU/g.

E. coli enumeration was done on Petri films (3M™ Petrifilm™ from 3M, The Netherlands, Zoeterwoude, products numbers for *E. coli* 64140). Blue colonies with associated gas bubbles were counted with the 3M™ Petrifilm™ Plate Reader (Model 6499, 3M, Germany) after 24 hour incubation at 37°C. The *E. coli* enumeration threshold for the rinse samples was 10 CFU/ml (samples after bleeding, scalding, defeathering, evisceration) and 1 CFU/ml (samples after chilling). The enumeration threshold for breast skin samples was 10 CFU/g.

2.4 Statistical analysis

Several analyses were performed in order to identify, on a per slaughterhouse basis (1), whether trends (i.e. the whole pattern of increases and decreases over the processing steps) were different between the trials, and (2) which processing steps significantly increased or decreased the mean numbers of organisms on the carcasses. Furthermore, for each of these questions, it was analysed (3) whether the effect differed between the slaughterhouses. The analysis was performed separately for *Campylobacter* and *E. coli*.

The *Campylobacter* and *E. coli* concentrations (per ml of rinse sample and per g of breast skin sample) were transformed to the $\log_{10}$ scale. The normality and homoscedasticity of the residuals were checked by diagnostic plots. Counts below the enumeration threshold were replaced by half the threshold according to Rosenquist et al. (2006). Sensitivity analysis was carried out to investigate the different replacements of the enumeration threshold. There was no effect of various replacements of the enumeration threshold on the results. Trials with many samples below enumeration threshold (around 50%) or negative results from caeca were not included in the analysis. For statistical analysis we used trials that had consistent types of samples collected.

The models used to analyse the data did not accommodate zeros; therefore, 100% of prevalence in a positive batch was assumed. Data analysis was performed in R software, package lme4 (3.0.3, 2014, R Development Core Team).

2.4.1 Models developed for the identification of critical processing steps in a slaughterhouse

Two linear mixed effect models were prepared to identify whether the impact of the processing steps varied between trials (Figure 1). The first model had processing step as an explanatory factor and trial as a random effect (model 1). In model 1, the intercept (β_0 = mean concentration level after bleeding) varied over trials (b_0 = random effect over trials with mean 0) and the effect of the processing step (slope) was the same for each trial (β indicates a fixed effect), the residual error (ε) varied over carcasses. The "Concentration" is the mean concentration ($\log_{10}$ CFU/ml) in samples collected after a particular step ("Scalding", "Defeathering", "Evisceration", "Chilling", dummy variables with 1 for the respective process step and 0 otherwise).

(1)

$$\text{Concentration} = b_0 + \beta_0 + \beta_1 \text{Scalding} + \beta_2 \text{Defeathering} + \beta_3 \text{Evisceration} + \beta_4 \text{Chilling} + \varepsilon$$

In the second model, not only the intercept (initial concentration level after bleeding) varied over trials, but also the impact of the processing step (slope). The random effects *bi* were added to each processing step.

(2)

$$\text{Concentration} = b_0 + \beta_0 + (b_1 + \beta_1)\,\text{Scalding} + (b_2 + \beta_2)\,\text{Defeathering} + (b_3 + \beta_3)\,\text{Evisceration} + (b_4 + \beta_4)\,\text{Chilling} + \varepsilon$$

Since model 1 and model 2 are nested models, the comparison was done using a likelihood ratio test. Based on the selected model (model 1 or model 2), an increase or decrease in bacterial concentration after particular processing steps (effect of a processing step) was computed at each slaughterhouse. The effects of the processing steps were calculated as e.g. the predicted concentration after defeathering minus the predicted concentration after scalding.

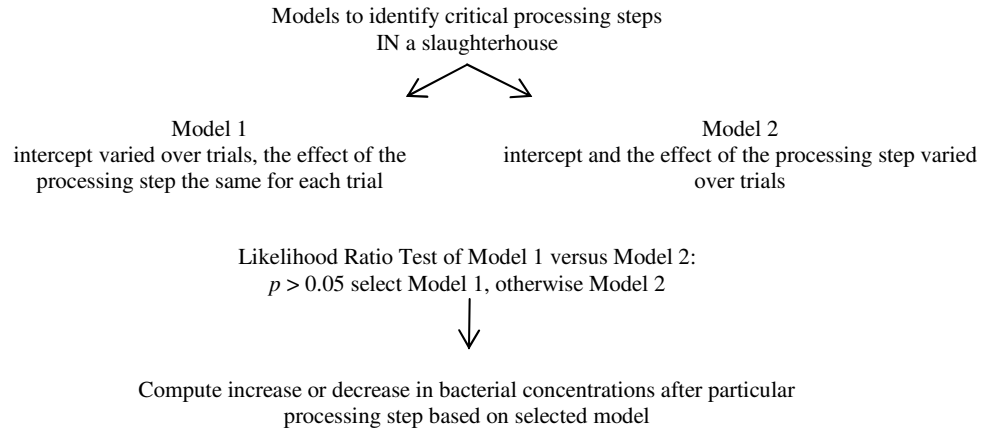

Figure 1
Overview of selection of a model to identify the critical processing steps in a slaughterhouse.

2.4.2 Models developed for the identification of critical processing steps between slaughterhouses

In the following analyses it was computed whether, and if so, how the impact of processing steps differed between slaughterhouses (Figure 2). Model 1 was run fitting the data from both slaughterhouses together. It was analysed whether the impact of the processing steps was the same at both slaughterhouses.

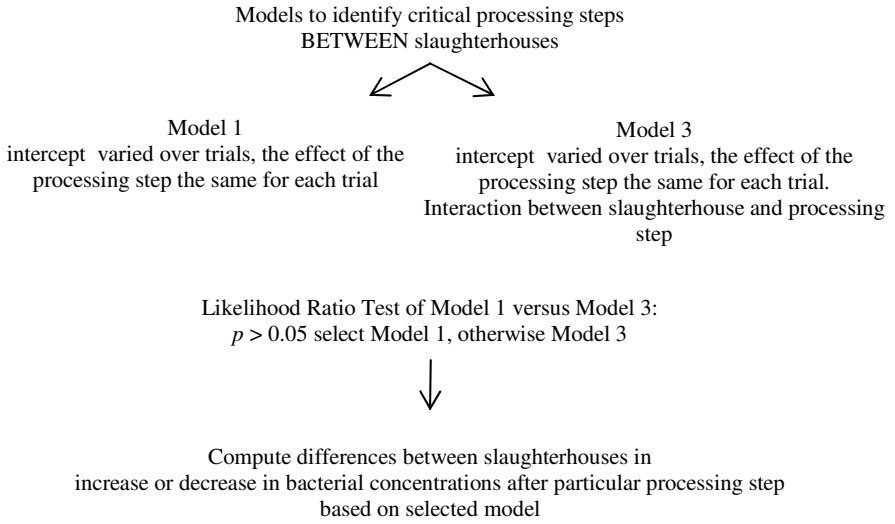

Figure 2
Overview of selection of a model to identify the critical processing steps between the slaughterhouses.

Another model was prepared (model 3) to address the question whether the patterns of the impact of the subsequent processing steps differ between the slaughterhouses. An interaction formula between slaughterhouses and processing step was added to the model 3.

(3)

$$\begin{aligned}\text{Concentration} = {} & b_0 + \beta_0 + \beta_1 \text{Scalding} + \beta_2 \text{Defeathering} + \beta_3 \text{Evisceration} \\ & + \beta_4 \text{Chilling} + \beta_5 \text{Slaughterhouse} + \beta_6 \text{Scalding} * \text{Slaughterhouse} \\ & + \beta_7 \text{Defeathering} * \text{Slaughterhouse} + \beta_8 \text{Evisceration} * \text{Slaughterhouse} \\ & + \beta_9 \text{Chilling} * \text{Slaughterhouse} + \varepsilon\end{aligned}$$

Models 1 and 3 were nested and, thus, compared by the likelihood ratio test. The significance of any coefficients β_6 to β_9 indicated a difference between the slaughterhouses for the respective processing step. The differences in the effect at processing step were calculated with Slaughterhouse 1 as the reference, as e.g. predicted concentration after defeathering minus the predicted concentration after scalding in Slaughterhouse 2 subtracted from the predicted concentration after defeathering minus the predicted concentration after scalding in Slaughterhouse 1.

Table 2

Comparison of models used to identify critical processing steps within and between slaughterhouses. *P* values determine selection of the models, if *p* value < 0.05 Model 2 was selected to identify critical processing steps within a slaughterhouse or Model 3 was selected to identify critical processing steps between slaughterhouses.

	Campylobacter						*E. coli*					
	Slaughterhouse 1		Slaughterhouse 2		Both slaughterhouses		Slaughterhouse 1		Slaughterhouse 2		Both slaughterhouses	
	Model 1	Model 2	Model 1	Model 2	Model 1	Model 3	Model 1	Model 2	Model 1	Model 2	Model 1	Model 3
	Run 1	Run 1	Run 2	Run 2	Run 5	Run 1	Run 3	Run 3	Run 4	Run 4	Run 6	Run 2
df	7	21	7	21	7	12	7	21	7	21	7	12
AIC	650.6693	662.7846	777.0657	777.1634	1442.054	1426.995	577.3792	593.1349	621.8779	613.3394	1246.27	1209.512
logLik	-318.3346	-310.3923	-381.5329	-367.5817	-714.027	-701.4973	-281.6896	-275.5674	-303.939	-285.6697	-616.1486	-592.7561
chi-square	13.791		26.251		33.491		11.344		35.851		58.903	
p value	0.465		0.024		3e-06		0.659		0.001		2e-11	

2.4.3 Additional analysis

Two additional analyses were performed, 1) an ANOVA to analyse whether concentrations after chilling at each slaughterhouse differed between batches for both *Campylobacter* and *E. coli*; and 2) a linear regression model (model 4) to analyse the effect of sample type (rinse and skin), organism (*Campylobacter* and *E. coli*), slaughterhouse (Slaughterhouse 1 or Slaughterhouse 2) and interaction of organism and slaughterhouse on concentrations after chilling (Concentration).

(4)

$$\text{Concentration} = \beta_0 + \beta_1\,\text{Type} + \beta_2\,\text{Organism} + \beta_3\text{Slaughterhouse} + \beta_4\,\text{Organism} * \text{Slaughterhouse} + \varepsilon$$

3. Results

3.1 Critical processing steps within slaughterhouses

Different models fitted the data best at different slaughterhouses. Model 1 fitted the data collected in Slaughterhouse 1 best for *Campylobacter* (p=0.465) and for *E. coli* (p=0.659) (Table 2). Thus, in Slaughterhouse 1, the *Campylobacter* concentration levels as shown in Figure 3 and *E. coli* concentration levels as shown in Figure 4 varied with respect to the initial external contamination of the batches (concentration in rinse from carcasses collected after bleeding). The impact of the processing steps did not vary over batches in Slaughterhouse 1 (Figures 3 and 4). *Campylobacter* concentration on broilers after bleeding in Slaughterhouse 1 varied widely from 2.3 to 6.4 $\log_{10}$ CFU/ml (Figure 3) whereas *E. coli* concentrations varied from 3.1 to 5.7 $\log_{10}$ CFU/ml (Figure 4).

In Slaughterhouse 2, model 2 fitted the data best for *Campylobacter* (p=0.024) and for *E. coli* (p=0.001) (Table 2). Thus, in Slaughterhouse 2, *Campylobacter* concentration levels as shown in Figure 5 and *E. coli* concentration levels as shown in Figure 6 varied with respect to the initial external contamination. In addition, as shown in Figures 5 and 6, the impact of processing steps varied between batches. *Campylobacter* concentration on broilers after bleeding in Slaughterhouse 2 varied widely from 1.6 to 6.1 $\log_{10}$ CFU/ml (Figure 5) whereas *E. coli* concentrations varied from 3.6 to 6.4 $\log_{10}$ CFU/ml (Figure 6). Based on the selected models (Model 1 in Slaughterhouse 1 and Model 2 in Slaughterhouse 2) the changes in the *Campylobacter* and *E. coli* concentrations in each slaughterhouse were computed as the average increase or decrease per processing step (Table 3). In both slaughterhouses, the concentrations decreased significantly after scalding for *Campylobacter* by 1.17 $\log_{10}$ in Slaughterhouse 1 and by 1.58 $\log_{10}$ in Slaughterhouse 2 and for *E. coli* by 0.64 $\log_{10}$ in Slaughterhouse 1 and by 1.29 $\log_{10}$ in Slaughterhouse 2 (Table 3). Defeathering contributed to a significant increase in the *Campylobacter* concentration by 0.41 $\log_{10}$ in Slaughterhouse 2 (p=0.01). No significant differences were found in Slaughterhouse 1 after defeathering in comparison to the

concentrations after scalding (p=0.92). A significant decrease in *E. coli* concentrations by 1.26 $\log_{10}$ in Slaughterhouse 1 and by 0.44 $\log_{10}$ in Slaughterhouse 2 after defeathering was found (Table 3). Evisceration in Slaughterhouse 1 caused a significant increase in *Campylobacter* (0.75 $\log_{10}$) and in *E. coli* (0.46 $\log_{10}$) concentrations. In Slaughterhouse 2, the *Campylobacter* and *E. coli* concentration levels after evisceration were not significantly different from the levels after defeathering (Table 3). Significantly lower concentration levels in *Campylobacter* and *E. coli* were found after chilling in comparison to concentrations on carcasses collected after evisceration in both slaughterhouses (Table 3). The concentrations on carcasses after chilling, as compared by ANOVA, differed significantly (p<0.001) between batches in each slaughterhouse.

Average *Campylobacter* concentration levels were significantly reduced through the processing by 1.40 $\log_{10}$ in Slaughterhouse 1, and also significantly reduced by 1.86 $\log_{10}$ CFU/ml in Slaughterhouse 2 (Table 3, Figures 3 and 5). With respect to *E. coli*, the concentrations after chilling were significantly lower by 2.19 $\log_{10}$ in Slaughterhouse 1 and also significantly lower by 2.84 $\log_{10}$ in Slaughterhouse 2 (Table 3, Figures 4 and 6).

Table 3
Increases and decreases in *Campylobacter* and *E. coli* concentrations after selected processing steps. *P* values < 0.05 indicate significant increase or decrease in concentrations.

	Processing step	*Campylobacter*		*E. coli*	
Slaughterhouse		$\log_{10}$	*p* value	$\log_{10}$	*p* value
Slaughterhouse 1	scalding - bleeding	-1.17	<0.01*	-0.64	<0.01*
	defeathering - scalding	0.01	0.92	-1.26	<0.01*
	evisceration - defeathering	0.75	<0.01*	0.46	<0.01*
	chilling - evisceration	-1.00	<0.01*	-0.74	<0.01*
	Total decrease: chilling - bleeding	-1.40	<0.01*	-2.19	<0.01*
Slaughterhouse 2	scalding - bleeding	-1.58	<0.01*	-1.29	<0.01*
	defeathering - scalding	0.41	0.01*	-0.44	0.01*
	evisceration - defeathering	-0.03	0.86	-0.05	0.72
	chilling - evisceration	-0.65	<0.01*	-1.06	<0.01*
	Total decrease: chilling - bleeding	-1.86	<0.01*	-2.84	<0.01*

Significant *p* values are marked with asterisks.

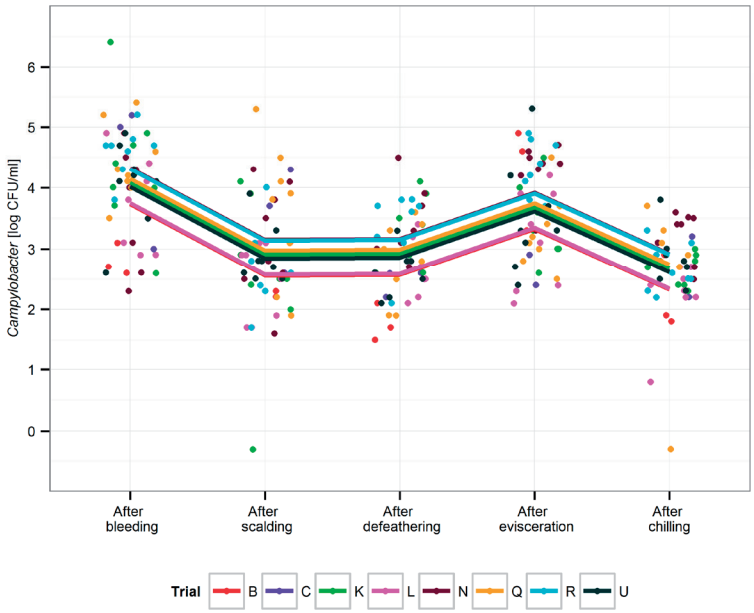

Figure 3
Campylobacter concentrations in whole broiler carcass rinse samples (log CFU/ml) after selected processing steps in Slaughterhouse 1. The lines indicate the concentrations per sampled batch (trial), based on the selected model (Table 2); the points indicate the concentrations in the individual samples.

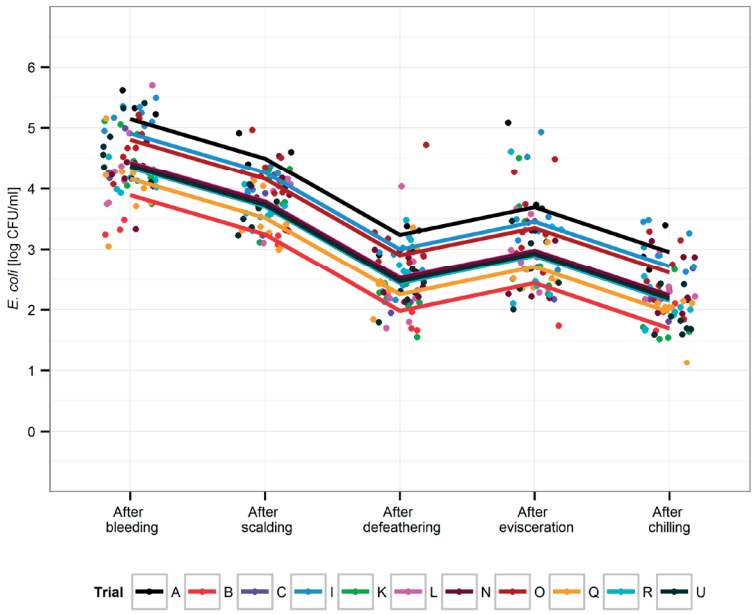

Figure 4
E. coli concentrations in whole broiler carcass rinse samples (log CFU/ml) after selected processing steps in Slaughterhouse 1. The lines indicate the concentrations per sampled batch (trial), based on the selected model (Table 2); the points indicate the concentrations in the individual samples.

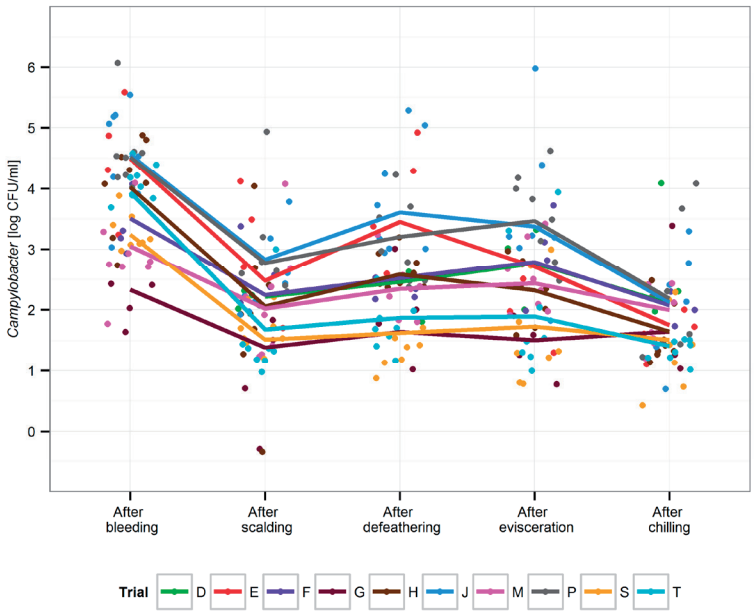

Figure 5
Campylobacter concentrations in whole broiler carcass rinse samples (log CFU/ml) after selected processing steps in Slaughterhouse 2. The lines indicate the concentrations per sampled batch (trial), based on the selected model (Table 2); the points indicate the concentrations in the individual samples.

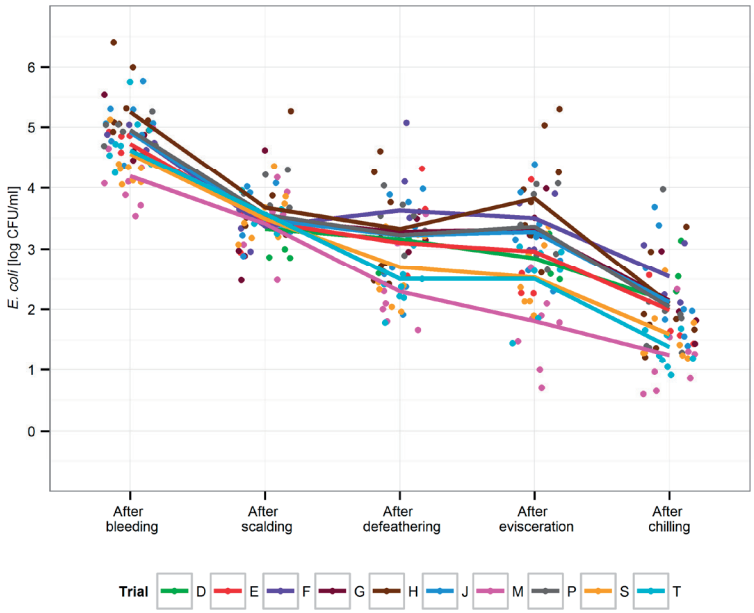

Figure 6
E. coli concentrations in whole broiler carcass rinse samples (log CFU/ml) after selected processing steps in Slaughterhouse 2. The lines indicate the concentrations per sampled batch (trial), based on the selected model (Table 2); the points indicate the concentrations in the individual samples.

3.2 Difference in the effect of processing steps between slaughterhouses

The impact of slaughterhouse on bacterial concentrations at the processing steps was best described by model 3 for *Campylobacter* (p=3e-06) (Table 2) and for *E. coli* (p=2e-11) (Table 2). No significant differences were observed between the slaughterhouses with respect to the *Campylobacter* and *E. coli* mean initial contamination level (after bleeding) (Table 4). The average *Campylobacter* concentration after bleeding in Slaughterhouse 1 was 4.08 $\log_{10}$ CFU/ml, and 3.80 $\log_{10}$ CFU/ml in Slaughterhouse 2. The average *E. coli* concentration after bleeding in Slaughterhouse 1 was 4.48 $\log_{10}$ CFU/ml, and 4.77 $\log_{10}$ CFU/ml in Slaughterhouse 2.

With respect to *Campylobacter* concentrations, significant differences in the impact of the processing steps between slaughterhouses were observed after scalding and evisceration. Reduction after scalding was 0.51 $\log_{10}$ higher in Slaughterhouse 2 than in Slaughterhouse 1. Evisceration in Slaughterhouse 1 contributed to an increase in concentrations that was 0.78 $\log_{10}$ higher than in Slaughterhouse 2 (Table 4). Increase in *Campylobacter* concentrations after defeathering was higher by 0.39 $\log_{10}$ in Slaughterhouse 2 than in Slaughterhouse 1; however, the difference was barely significant (p=0.05).

Table 4

Outcome of Model 3: comparison of the increases and decreases in *Campylobacter* and *E. coli* concentration levels at selected processing steps between Slaughterhouses 1 and 2. *P* values < 0.05 indicate significant difference in the impact of the processing step between slaughterhouses.

Processing step	*Campylobacter*		*E. coli*	
	Differences Slaughterhouse 1-Slaughterhouse 2 [$\log_{10}$]	p value	Differences Slaughterhouse 1-Slaughterhouse 2 [$\log_{10}$]	p value
bleeding	0.23	0.36	-0.32	0.08
scalding - bleeding	0.51	0.01*	0.62	<0.01*
defeathering - scalding	-0.39	0.05	-0.74	<0.01*
evisceration - defeathering	0.78	<0.01*	0.48	<0.01*
chilling - evisceration	-0.32	0.11	0.36	0.01*
bleeding - chilling	0.58	<0.01*	0.72	<0.01*

Significant p values are marked with asterisks.

With respect to *E. coli* concentrations, significant differences in the impact of the processing steps between slaughterhouses were observed after scalding, defeathering, evisceration and chilling. Reduction after scalding was 0.62 $\log_{10}$ higher in Slaughterhouse 2 than in Slaughterhouse 1 (Table 4). Reduction after defeathering in *E. coli* concentrations in Slaughterhouse 1 was 0.74 $\log_{10}$ higher than in Slaughterhouse 2. Evisceration in Slaughterhouse 1 contributed to an increase in concentration that was

0.48 $\log_{10}$ higher than in Slaughterhouse 2 (Table 4). Decrease in *E. coli* concentration after chilling was higher in Slaughterhouse 2 by 0.36 $\log_{10}$ than in Slaughterhouse 1 (Table 4). Overall reduction through the processing was significantly higher by 0.58 $\log_{10}$ for *Campylobacter* and by 0.72 $\log_{10}$ for *E. coli* in Slaughterhouse 2 than in Slaughterhouse 1.

Based on the linear regression model (model 4), sample type (rinse and skin samples) did not have a significant effect (p=0.44) on *Campylobacter* and *E. coli* concentrations after chilling (Figure 7). Slaughterhouse had significant effect on concentrations after chilling (p<2e-16). The concentrations were significantly lower in Slaughterhouse 2 by 1.41 $\log_{10}$. Organism had significant effect on concentrations after chilling. *E. coli* concentrations were significantly lower by 0.85 $\log_{10}$ (p=2.01e-13) comparing to the baseline (C*ampylobacter* in rinse samples in Slaughterhouse 1). The interaction between slaughterhouse and organism was significant (p=2.23e-06), with *E. coli* in Slaughterhouse 2 lower by 0.8 $\log_{10}$ as compared to the baseline.

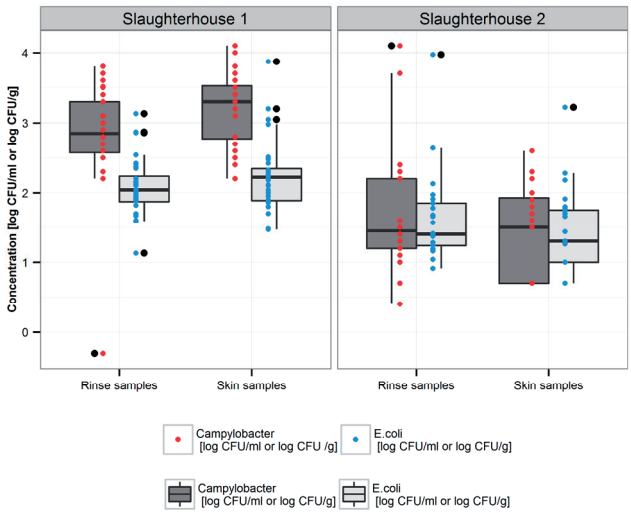

Figure 7
Campylobacter and *E. coli* concentrations in whole carcass rinse (log CFU/ml) and breast skin (log CFU/g) samples collected in trials (N, Q, R, U in Slaughterhouse 1 and P, S, T in Slaughterhouse 2). The line inside each box indicates the median, the upper whiskers indicate 75th percentiles and the lower whiskers indicate 25th percentiles. The black dots indicate the outliers.

4. Discussion

4.1 Impact of the processing steps on bacterial concentrations after chilling

Data collected in this study confirmed that certain slaughterhouses produce carcasses with lower bacterial concentrations after chilling, as previously reported (Anonymous, 2011; European Food Safety Authority, 2010c). This study revealed

that, even if the *Campylobacter* and *E. coli* concentrations in the incoming batches were similar in the studied slaughterhouses, the concentrations after chilling were significantly lower in Slaughterhouse 2 (Figure 7). The concentration levels in the incoming batches (after bleeding) were highly variable in each slaughterhouse as reported by other studies (Berrang and Dickens, 2000; Oosterom et al., 1983; Seliwiorstow et al., 2012). Our study identified that the pattern of increases and decreases in bacterial concentrations along the processing steps is specific for each slaughterhouse. The pattern was similar in Slaughterhouse 1 for all tested batches. This regularity can be potentially explained by the processing parameters applied consistently for the processed batches even after equipment modernisation. The regular peak after evisceration suggests insufficient control of this step what may have caused higher concentrations after chilling in comparison to Slaughterhouse 2. There the impact of processing steps varied between batches and *Campylobacter* concentration increased after defeathering. Irregularity in the pattern in Slaughterhouse 2 suggests that, potentially, the control of the processes was not always done in the same way. In-depth analysis of batch-related characteristics, batch handling operations, processing parameters and hygienic conditions in each slaughterhouse is needed to explain the variations in the observed patterns and its impact on contamination of the carcasses after chilling.

Different patterns of increases and decreases in *Campylobacter* concentration along the processing steps were included also by different risk assessment models (Nauta et al., 2009); with a slight increase (Nauta et al., 2005) or a considerable decrease (Hartnett et al., 2001) after defeathering and small (Nauta et al., 2005) or large (Hartnett et al., 2001) increases after evisceration. Despite the different dynamics during processing predicted by various models, similar *Campylobacter* concentration levels after chilling were predicted (Nauta et al., 2009). As shown in the current study however, these levels differed both between slaughterhouses and between batches. Therefore such variability between batches and slaughterhouses can further inform risk assessment models.

Defeathering and evisceration cause an increase in bacterial concentration on carcasses. However based on data collected in our study and on the risk assessment models (Nauta et al., 2009), an increase was not always observed after these steps. The differences in the impact of defeathering and evisceration on *Campylobacter* concentrations estimated by various models were a consequence of the assumptions made on the faecal contamination during those processing steps (Nauta et al., 2009). It suggests that differences observed in our study in the impact of these processing steps between slaughterhouses could potentially be explained by the degree of control of faecal contamination. Such contamination is the major cause of increase in *Campylobacter* concentration after defeathering and evisceration (Berrang et al., 2001; Berrang et al., 2004; Musgrove et al., 1997; Rosenquist et al., 2006). However, if the concentration on the carcasses entering these processing steps is high, the additional contamination

from leaking faeces may not be observed on the log scale. Furthermore, the bacterial concentrations after defeathering and evisceration are not only impacted by faecal leakage and concentration on incoming carcasses, but also by washing. To explain the causes of the differences in the impact of defeathering and evisceration on *Campylobacter* and *E. coli* concentrations, a detailed analysis of processing parameters and factors impacting the extent to which the faecal and caecal material contaminate the carcasses is needed.

Our results also confirmed the decrease in *Campylobacter* and *E. coli* concentrations after scalding and chilling as previously reported in a review (Guerin et al. 2010). Other authors observed higher reduction in concentrations after scalding than is the case in our study (Berrang and Dickens, 2000). The concentration of *Campylobacter* decreased after chilling in the analysed slaughterhouses in agreement with other studies (Huezo et al., 2007; Rosenquist et al., 2006). Drying explains the decrease in *Campylobacter* concentration after chilling (Alter and Scherer, 2006; Murphy et al., 2006; Oosterom et al., 1983). *Enterobacteriaceae* were reported to be less sensitive to drying than *Campylobacter* (Oosterom et al., 1983). The observed decrease in *Campylobacter* and *E. coli* concentrations after chilling in comparison to concentration after evisceration can additionally be explained by washing prior chilling.

4.2 Campylobacter and E. coli along the processing line

Changes in the concentration levels during processing were similar for both *Campylobacter* and *E. coli* except for defeathering (Table 3). In addition, models that fitted *Campylobacter* data did also fit *E. coli* data in each slaughterhouse. *E. coli* concentrations significantly decreased after defeathering in Slaughterhouse 1 by 1.26 $\log_{10}$ and in Slaughterhouse 2 by 0.44 $\log_{10}$, whereas *Campylobacter* increased in Slaughterhouse 2 by 0.41 $\log_{10}$ and in Slaughterhouse 1 no significant changes were observed. Decrease after defeathering in *Enterobacteriaceae* (Göksoy et al., 2004; Oosterom et al., 1983) and in *Coliforms* (Göksoy et al., 2004) was previously reported. The opposite impact of defeathering on *Campylobacter* and *E. coli* can be related to potential differences in their ability to attach to the skin. Differences within even one bacterial species in the attachment to inert surfaces were previously reported (Hue et al., 2011; Sulaeman et al., 2010). Good correlation of *Campylobacter* and *E. coli* during processing was reported (Duffy et al., 2014); however, the defeathering step was not investigated. On the contrary other studies reported weak correlation (Berrang and Dickens, 2000; Williams and Ebel, 2014). Based on orthogonal regression applied for our data, the *Campylobacter* and *E. coli* concentrations were not correlated (results not shown). Although there is a lack of correlation, the direction of the changes in concentration (either increase or decrease) is similar for *Campylobacter* and for *E. coli* at most of the tested processing steps, except for defeathering (Table 3). *E. coli* has thus the potential to be used as an indicator of the hygienic status of the processing and to be used to identify the critical processing steps; however, more understanding is needed of the opposite impact of defeathering on *Campylobacter*.

4.3 Sampling methods

As the sample type had neither effect on *Campylobacter* nor on *E. coli* concentrations (Figure 7), both methods (whole carcass rinse and breast skin collected after chilling) can be used to evaluate the hygienic status during processing.

Batches sampled during trials A, I, and O although determined as positive on farms, resulted in many samples below the detection limit (50% in trials A and I) or negative caeca results in the slaughterhouse (trial O). These trials were not informative for the model; hence, they were not included in the analysis. Positive results on the farms versus negative results in the caeca collected in the slaughterhouse can be explained by potential early stage of colonisation in these batches. At the early colonisation stage not all broilers are colonised and the level is low. In future studies, it is advisable to use highly contaminated flocks in order to determine the impact of the processing steps on *Campylobacter* concentrations. Highly contaminated batches were also reported to result in the major risk for consumers (Nauta et al., 2009).

5. Conclusions

Differences in *Campylobacter* and *E. coli* concentrations on carcasses after chilling between slaughterhouses are potentially caused by specific increases and decreases in concentrations during processing. Our results confirmed that defeathering and evisceration are the most critical steps during processing, leading to increase in *Campylobacter* concentrations on carcasses. There are however slaughterhouses that are able to control increases in concentrations after these steps and to maintain the impact of all processing steps at similar levels for each slaughtered batch. Critical processing steps should be validated in each slaughterhouse individually by longitudinal studies and potentially based on *E. coli*. *E. coli* has a potential to be used as an indicator of the steps, because the impact of most studied processing steps on *E. coli* and *Campylobacter* concentrations was similar, except for defeathering.

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Chapter 4

Explanatory variables associated with *Campylobacter* and *Escherichia coli* concentrations on broiler chicken carcasses during processing in two slaughterhouses

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Abstract

This study aimed at identifying explanatory variables that were associated with *Campylobacter* and *Escherichia coli* concentrations throughout processing in two commercial broiler slaughterhouses. Quantative data on *Campylobacter* and *E. coli* along the processing line were collected. Moreover, information on batch characteristics, slaughterhouse practices, process performance and environmental variables was collected through questionnaires, observations and measurements, resulting in data on nineteen putative explanatory variables. Analysis was conducted separately in each slaughterhouse to identify which variables were related to *Campylobacter* and *E. coli* change in concentrations during the processing steps: scalding, defeathering, evisceration and chilling. Determined associations with explanatory variables were different between studied slaughterhouses. In the first slaughterhouse there was only one significant association: poorer uniformity with less decrease in *E. coli* concentrations after defeathering. In the second slaughterhouse significant associations were determined with variables including age, uniformity, average weight of carcasses, *Campylobacter* concentrations in excreta and caeca, *E. coli* concentrations in excreta. Bacterial concentrations in excreta and caeca were found to be the most prominent variables, because they were associated with concentration on carcasses at various processing steps. While slaughterhouses produce specific assortments and have different batch characteristics and processing parameters, the effect of the significant variables was not always the same for each slaughterhouse. Therefore slaughterhouses need to determine individually relevant measures for hygiene control and process management.

Keywords: Poultry, Processing hygiene, Risk factors

1. Introduction

Campylobacteriosis is a zoonosis caused by thermotolerant *Campylobacter* spp. The most frequent symptom in patients is self-limiting gastroenteritis, but severe complications such as reactive arthritis and neurological disorders such as Guillain–Barré syndrome may also occur and even result in death (European Food Safety Authority, 2015). Campylobacteriosis is the most frequently reported zoonosis in the European Union, with 214,779 campylobacteriosis cases reported in 2013 (European Food Safety Authority, 2015). The main source of infection in humans is the poultry reservoir (European Food Safety Authority, 2011). The control of *Campylobacter* throughout the poultry supply chain, including at slaughter, is crucial to reduce the number of human campylobacteriosis cases. It is expected that reducing levels on carcasses at slaughter will reduce the number of campylobacteriosis cases in humans (European Food Safety Authority, 2011; Swart et al., 2013).

The levels of *Campylobacter* on carcasses after chilling differ depending on the batch of origin and the slaughterhouse where the batches were processed (Anonymous, 2011; European Food Safety Authority, 2010; Pacholewicz et al., 2015; Zweifel et al., 2015). In order to determine potential causes of the differences the changes in *Campylobacter* concentration through broiler processing steps were investigated (Pacholewicz et al., 2015). Processing steps such as defeathering and evisceration were identified as crucial for control, because they may lead to an increase in bacterial concentrations on carcasses. The effect of processing steps including defeathering, evisceration, scalding and chilling on increase or decrease in bacterial concentrations, differed between studied slaughterhouses. The causes of different impact of processing steps between the slaughterhouses have not yet been fully identified or quantified.

Currently, factors that influence *Campylobacter* colonisation in batches on farms have been identified (Bouwknegt et al., 2004; Näther et al., 2009). At slaughter, factors influencing *Campylobacter* prevalence after chilling have been identified (Hue et al., 2010; Malher et al., 2011). Little is known as yet of factors that influence changes (increases or decreases) in *Campylobacter* concentrations on carcasses throughout the entire broiler processing. Identifying such factors would enable implementation of control measures to reduce the *Campylobacter* concentration on carcasses at the end of slaughter and thus the number of human infections. In addition, determining whether similar factors influence *E. coli* and *Campylobacter* concentration on carcasses would support the control of hygienic performance during processing based on *E. coli*, as proposed by the BIOHAZ panel of EFSA (European Food Safety Authority, 2012). Thus, the objective of this study was to identify variables associated with *Campylobacter* and *E. coli* concentrations on carcasses during processing in two slaughterhouses mentioned earlier.

2. Materials and Methods

2.1 Experimental design

The investigations were conducted in two commercial broiler chicken slaughterhouses, characterised in Table 1. The study took place between June and October in 2012 and 2013. Batches were considered as the analytical unit, a batch being a group of chickens raised in one house (European Food Safety Authority, 2011). In total, 21 batches (Table 1), positive for *Campylobacter* on–farm, were sampled (Pacholewicz et al., 2015).

Table 1
Characteristics of slaughterhouses.

	Slaughterhouse 1	Slaughterhouse 2
Number of lines	1	2
Line speed	8,500 birds per hour	7,200 birds per hour
Number of shifts	2	2
Capacity (number of broilers slaughtered per day)	130,000 broilers	240,000 broilers
Production fresh/frozen	95 % fresh; 5 % frozen	70 % fresh; 30 % frozen
Unloading	Tilting system	Belt system
Live hanging	Carousel system	Carousel system
Bleeding time	180 s	185 s
Stunning	Water bath high frequency	Water bath high frequency
Killing	Left side and frontal cut	Frontal cut
Immersion scalding Temperature: Time:	Fresh product (epidermis on) 51.5 - 52.8 °C 3 min	Fresh product (epidermis on) 52.8 – 53.5 °C 2.5 min Frozen product (epidermis off) 54 -57.2 °C 2.5 min
Defeathering Time: Water consumption:	 37 s 0.5 l per carcass	 46 s 0.8 l per carcass
Evisceration (Key machines)	Vent cutter, opener, eviscerator, spraying nozzles	Vent cutter, opener, eviscerator, spraying cabinet
Chilling	Immersion tanks Air chilling: 105 min	Immersion tanks Air chilling (carcasses with epidermis): 80 min Air + spray chilling (carcasses without epidermis): 80 min
Batches ID	A, B, C, I, K, L, N, O, Q, R, U	D, E, F, G, H, J, M, P, S, T

2.2 Collection of samples

Whole carcass rinse samples were collected after five processing steps: bleeding, scalding, defeathering, evisceration and chilling. In total 680 rinse samples were collected (Pacholewicz et al., 2015). These data provided insight in the changes in concentrations throughout processing.

Additional samples were collected including excreta from carcasses after scalding. For this the abdomen of the carcasses was pressed to obtain at least 1 g of sample. In total 136 excreta samples were collected. These samples provided data on bacterial concentration in material that may leak during defeathering. To confirm positivity and investigate concentrations of *Campylobacter* in the sampled batches, eight eviscerated packages were collected at the veterinary inspection station in 12 batches (J-U). This resulted in collection of 96 caecal samples.

In batches A-G 24 additional carcasses after scalding were collected (3 in batchess A-D and 4 in batches E-G) and defeathered manually. From these, whole carcass rinse samples were prepared, and removed feathers were weighted. The rinse samples were used to compare the bacterial concentrations between manually and mechanically defeathered carcasses.

Samples from the defeathering environment were collected during batches A-G. Forty water samples (approximately 100 ml) and 40 feathers samples (approximately 25 g) were collected from the first and last defeathering machine.

2.3 Sample preparation and enumeration

Whole carcass rinse samples were prepared as described previously (Pacholewicz et al., 2015). The eviscerated packages were transported to the laboratory, where at least 1 g of caeca content per carcass was collected. Both caeca samples and excreta samples were homogenised and serial 1:10 dilutions were made in Butterfield's Buffer (3M The Netherlands, Zoeterwoude, product number BPPFV9BF). *Campylobacter* was enumerated using CampyFood Agar (bioMérieux SA, Marcy l'Étoile, France, product number 43471), following ISO 10272-2 (Anonymous, 2006). *E. coli* was enumerated using Petri films (3M™ Petrifilm™ from 3M, 148 The Netherlands, Zoeterwoude, product number 6414).

2.4 Explanatory variables

The nineteen explanatory variables, collected for each batch, relate to batch characteristics, slaughterhouse practices, process performance and environment (Table 2). Batch related variables were collected at farms and slaughterhouses via a questionnaire administered to farmers and quality managers. Variables related to slaughterhouse practices and environment were obtained based on measurements conducted during the sampling of each batch. Process performance variables were collected through

observation of both defeathering and evisceration processes. Defeathering performance was assessed by the number of remaining feathers on parts of the carcass as wings, hocks, and abdomen and breakages (of wings and hocks) on the twenty carcasses observed in each batch. Remaining feathers on the twenty carcasses were evaluated by scoring each assessed part of the carcass (scores 1 to 4). Score "1" was assigned when all feathers were removed, score "2" when the number of remaining feathers was below 5 on assessed part, score "3" when the number was between 5 and 10, and score "4" when the number was above 10. The scores from all parts were summed per carcass and averaged per sampled twenty carcasses in each batch to obtain an overall batch score. Higher average score indicated insufficient defeathering and potentially inadequate settings of the equipment. To evaluate breakages on wings and hocks, parts of the carcasses (wings and feet) were assigned the scores 0 (not broken) and 1 (broken). For each batch, scores from twenty tested carcasses were averaged to obtain an overall score for breakages after defeathering. Higher average score indicated more frequent breakage potentially caused by a too tight setting of defeathering equipment.

Evisceration performance was assessed by counting carcasses with visible faecal contamination after evisceration during one minute of processing. The results were presented as a percentage of carcasses with visible faecal contamination and indicated a potentially poor setting of the evisceration equipment.

Uniformity of a batch was calculated based on the coefficient of variation which is the ratio of the standard deviation and the average of the weight after chilling (Bilgili, 2004). This coefficient was presented as percentage.

Table 2
Descriptive statistics of the explanatory variables of the tested batches.

Explanatory variables	Slaughterhouse 1				Slaughterhouse 2			
	Mean	SD	Min	Max	Mean	SD	Min	Max
Batch characteristics								
Age [days]	31	2	28	35	41	5	33	48
Feed withdrawal time [hours]	13	4	9	22	10	2	7	12
Distance from farm to slaughterhouse [km]	109	58	24	200	78	52	11	173
Size of the batch [number of broilers in a batch]	15394	10563	2500	41040	20070	10322	7316	35300
Uniformity coefficient of variation [%]	13	1	11	16	15	3	10	19
Average weight of carcasses [g]	1190	146	1048	1533	1764	316	1238	2211
Campylobacter in excreta [log CFU/g]	5.2	2.1	1.5	7.2	6.1	1.1	4.3	7.7
E. coli in excreta [log CFU/g]	5.3	0.9	4.3	6.9	5.9	0.6	5.0	6.8
Campylobacter in caeca [log CFU/g]	7.8	1.5	4.7	9.1	7.4	0.7	6.7	8.4
E. coli in caeca [log CFU/g]	5.9	1.2	3.9	7.9	6.3	1.1	4.5	7.3
Slaughterhouse practices								
Slaughter time [hours*]	11 h 55 min	4 h 55 min	4 h	18 h 25 min	11 h 7 min	5 h 49 min	4 h 30 min	18 h 55 min
Line speed [broilers per hour]	8247	206	8040	8640	7358	127	7200	7560
Scalding temperature [°C]	51.7	0.6	51.2	52.8	54.8	1.8	52.8	57.2
Environmental variables								
Outside temperature [°C]	20.0	8.2	10.8	35.0	14.5	7.0	6.1	25.4
Temperature in the scalding and plucking area [°C]	25.5	3.5	21.9	31.7	23.1	2.5	20.0	27.3
Temperature in the evisceration area [°C]	18.4	3.1	13.0	21.9	21.3	3.1	18.6	26.7
Process performance								
Feathers score after defeathering	1.7	0.3	1.2	2.1	1.4	0.3	1.1	1.8
Breakages score after defeathering	0.2	0.2	0.0	0.4	0.2	0.1	0.1	0.3
Carcasses with visible faecal contamination after eviscerator [%]	3.0	3.5	0.0	11.0	2.7	4.8	0.0	15.0

*number of hours after midnight

2.5. Statistical analysis

Analyses were performed using the R software (3.2.0, R Development Core Team).

2.5.1 Explanatory variables selection

Firstly variables were selected as potentially affecting *Campylobacter* and *E. coli* concentrations for a particular processing step based on expert opinion. Pearson's correlation coefficient (r) of the bacterial counts and explanatory variables was computed for these variables (Table A1-Appendix A) and variables with |r|>0.7 were considered to be statistically associated. For each set of the associated variables one was choosen for further analysis and the rest was disregarded. Calculation of the correlation was a preliminary step to exclude the variables that could confound the model. Correlation does not necessarily indicate a technological causal relation between the variables, it was performed to inform the following data analysis. The selected variables were analysed based on the model below to identify a significant association between explanatory variables and the impact of the processing step on both *Campylobacter* and *E. coli* concentrations (Table 3). The Bonferroni-Holm correction (Holm, 1979) was applied to address the multiple comparsions of selected variables in the model. Two significance levels were considered, p=0.05 and a less strict level of p=0.1. The following equation describes the bacterial concentrations on carcasses during studied processing steps including selected variables.

$$Concentration = b_0 + \beta_0 + \beta_1\, Explanatory\ variable + \beta_2\ Processed$$

$$+\ \beta_3\, Explanatory\ variable * Processed + \varepsilon$$

In the model "concentration" is the *Campylobacter* or *E. coli* concentration in the whole carcass rinse sample. The intercept $b_0 + \beta_0$ (bacterial concentrations during the processing steps at zero value of the explanatory variable) varied between batches. The "explanatory variable" is a variable from the list as described in Table 3. "Processed" is a variable indicating whether the concentrations were measured before (processed=0), or after (processed=1) a particular step. A significant interaction of "Processed" and "Explanatory variable" indicates that the explanatory variable affected the change in concentration during processing. This form of analysis allowed using each data point, even though the samples before and after processing steps were not paired. The analyses were computed separately for *Campylobacter* and *E. coli,* in each slaughterhouse.

2.5.2 Evaluation of the defeathering process

Comparison of the contamination levels in the rinse samples obtained from mechanically and manually defeathered carcasses for both *Campylobacter* and *E. coli* was done by the two sample Wilcoxon test.

In order to investigate differences in the impact of defeathering between slaughterhouses and bacterial species, the concentrations in samples collected before (in rinse after scalding), during (in excreta, water and feathers) and after defeathering (rinse after defeathering) were compared for both *Campylobacter* and *E. coli*. The concentrations in the samples were converted for a carcass as an analytical unit and presented as $\log_{10}$ CFU/carcass. Concentrations in rinse were calculated per 500 ml of peptone saline as used to rinse each individual carcass. Concentrations in 1 g of excreta were converted to a total weight of excreta collected from each carcass. Concentrations in 1 g of feathers were converted to the weight of feathers obtained from each carcass. Concentrations in 1 ml of water were converted to 500 ml in Slaughterhouse 1 and 800 ml in Slaughterhouse 2. These are the volumes, reported by each slaughterhouse (Table 1). The bacterial concentrations in these samples (before, during and after defeathering) were compared by ANOVA and the *post-hoc* Tukey HSD test.

3. Results

3.1 Variables associated with changes in Campylobacter and E. coli concentration

The correlated variables in one slaughterhouse were not always correlated in the second slaughterhouse (Appendix A, Table A1). Out of nineteen explanatory variables selected for the study, three variables were significantly associated with changes in *Campylobacter* concentrations during the tested processing steps in Slaughterhouse 2, whereas none in Slaughterhouse 1. There was one variable associated with *E. coli* concentrations in Slaughterhouse 1, whereas four in Slaughterhouse 2 (Table 3).

In Slaughterhouse 1, poorer uniformity of batch was associated with less decrease in *E. coli* concentrations during defeathering by 0.14 log per each percent point. On the opposite, in Slaughterhouse 2 the poorer uniformity was associated with more decrease in *E. coli* concentrations during defeathering by 0.15 log per each percent point.

Age was associated with a smaller decrease in *E. coli* concentrations during scalding in Slaughterhouse 2 by 0.07 log per each day of age. Age was poorly related to the concentrations of both *Campylobacter* and *E. coli* in excreta and in caeca (Figure 1). Within the measured range, each log increase in *Campylobacter* concentration in excreta in Slaughterhouse 2 corresponded with more decrease during chilling by 0.35 log. In caeca, each log increase in *Campylobacter* concentrations was associated with higher increase during defeathering by 0.67 log. Each *E. coli* log increase in the excreta in Slaughterhouse 2 was associated with less decrease during defeathering by 0.61 log.

The weight of the carcasses in Slaughterhouse 2 was significantly associated with an increase in *Campylobacter* concentrations on carcasses after evisceration, by 0.002 log per gram.

Defeathering efficiency, indicated by the number of remaining feathers (presented as a score) was associated with higher decrease in *E. coli* concentrations during defeathering in Slaughterhouse 2.

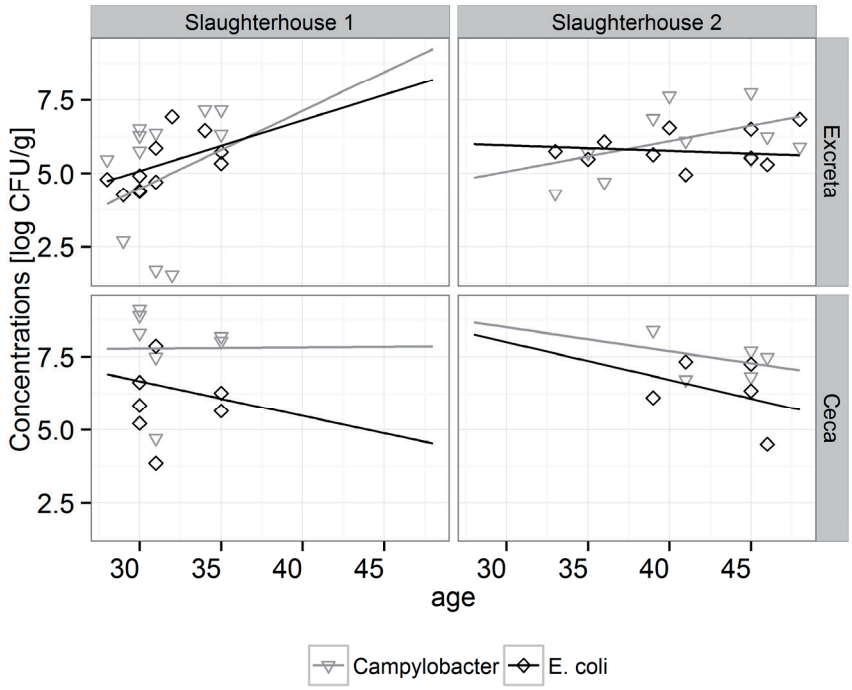

Figure 1
Poor relation between age [in days] of the sampled batches and concentrations of both *Campylobacter* and *E. coli* [log CFU/carcass] in excreta (collected after scalding) and caeca samples in Slaughterhouse 1 and 2.

Table 3

Explanatory variables associated with *Campylobacter* and *E. coli* concentrations on carcasses at several processing steps in two studied slaughterhouses. Numbers in the cells indicate the estimate β_3 in the model as described in 2.5.1. The estimates in bold indicate the explanatory variable that significantly contributed to change in concentration during processing (below p value = 0.05 indicated by **, and below a p value = 0.1 indicated by *). Empty cells indicate that particular variable was either not assigned by experts for analysis at particular processing step or was correlated with other variable as specified in Table A1.

Explanatory variables		Slaughterhouse 1								Slaughterhouse 2							
		Campylobacter				*E. coli*				*Campylobacter*				*E. coli*			
		Scalding	Defeath-ering	Eviscera-tion	Chilling	Scalding	Defeath-ering	Eviscera-tion	Chilling	Scalding	Defeath-ering	Eviscera-tion	Chilling	Scalding	Defeath-ering	Eviscer-ation	Chilling
Batch character-istics	Age [days]	-0.11	0.05	-0.08	0.1	-0.06	-0.03	0.03	-0.05	0.03			-0.01	**0.07****			0.06
	Feed withdrawal time [hours]	-0.02		-0.02	0.05	-0.03		-0.02	-0.03								
	Distance from farm to slaughterhouse [km]			-0.003				-0.002									
	Size of the batch [number of broilers in a batch]	0.00001	-0.00002	-0.00001	0.00001	0.00001	0.000002	-0.00001	-0.000004	-0.00002	-0.00001	0.00002	-0.00002	-0.000004	-0.00001	0.00001	-0.00002
	Uniformity coefficient of variation [%]	0.25	-0.17	0.01		0.02	**0.14***	-0.15	0.02	-0.06	-0.05	-0.002		0.08	**-0.15****	0.01	-0.003
	Average weight of carcasses [g]			-0.001				0.001			-0.001	**0.002****			-0.001	-0.0004	
	Campylobacter in excreta [log CFU/g]	-0.55	0.66	-0.53						0.07	-0.1	0.26	**-0.35****				
	E. coli in excreta [log CFU/g]					-0.19	-0.07	0.16	-0.005					-0.25	**0.61****	0.13	-0.09
	Campylobacter in caeca [log CFU/g]				0.22						**0.67***		-0.65				
	E. coli in caeca [log CFU/g]					0.03	-0.09	0.08	0.01					-0.14	0.09	0.25	-0.28
Slaugh-terhouse practices	Slaughter time [hours]	0.06	-0.04		-0.01	-0.01	-0.004	0.01	0.03			-0.03				-0.01	0.03
	Line speed [broilers per hour]		0.001				-0.0005			-0.001	-0.001	0.0005	-0.001	0.001	-0.001	0.001	-0.001
	Scalding temperature [°C]	-0.72	0.71	-0.45	0.06	-0.08	-0.41	0.5	-0.23	0.02	0.17			-0.13	0.15		
Environ-mental variables	Outside temperature [°C]																
	Temperature in the scalding and plucking area [°C]	0.09	-0.08	0.03		0.04	0.04	-0.07		-0.09	0.08	-0.01			0.06		
	Temperature in the evisceration area [°C]																
Process per-formance	Feathers score after defeathering		-0.34				0.11								**-1.3****		
	Breakages score after defeathering										-1.28						
	Carcasses with visible faecal contamination after eviscerator [%]			-0.02				0.005				-0.02				0.01	

3.2 Defeathering in two slaughterhouses

Campylobacter concentrations in whole chicken rinse samples did not differ between manually and mechanically defeathered carcasses in Slaughterhouse 1 (p = 0.23) and in Slaughterhouse 2 (p = 0.84) (Figure 2). Concentrations of *E. coli* did not differ significantly in Slaughterhouse 2 (p = 0.58). In Slaughterhouse 1 however, *E. coli* concentrations in mechanically defeathered carcasses were significantly lower (p = 0.002) by 1.4 log.

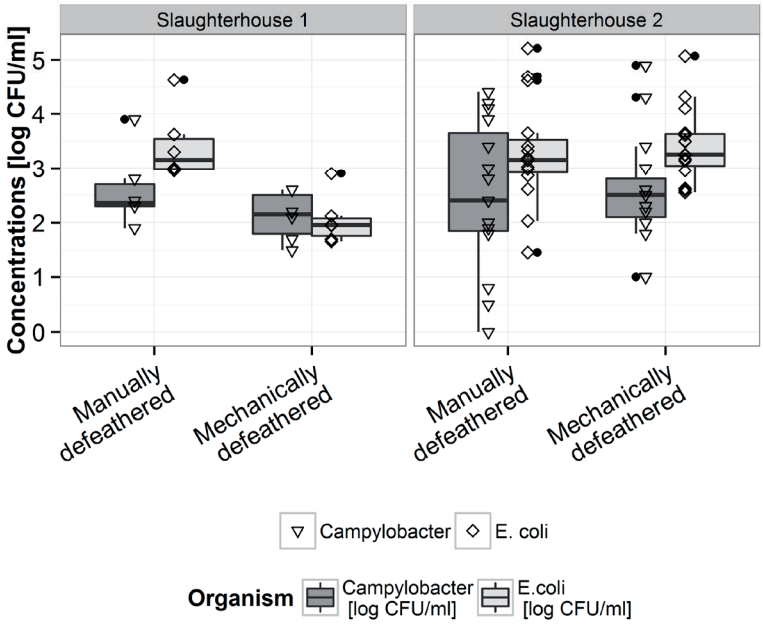

Figure 2
Comparison of *Campylobacter* and *E. coli* concentrations in rinse samples [log CFU/ml] from mechanically and manually defeathered carcasses in each slaughterhouse. The length of the box indicates the interquartile range of a sample; the whiskers a sample maximum and minimum and the black dots outliers. The bold line in the box indicates a median. The triangles indicate the *Campylobacter* concentrations in a particular sample and rhombusses indicate *E. coli*.

The *Campylobacter* and *E. coli* concentrations on carcasses before, during, and after defeathering are presented in Figure 3. It shows that when higher concentrations of bacteria were found on the excreta than in rinse samples before defeathering, then the concentrations in the rinse after defeathering generally increased and vice versa (Table 4). In addition, both *Campylobacter* and *E. coli* were removed during defeathering with feathers and water at high concentrations (Figure 3).

Table 4
Comparison of *Campylobacter* and *E. coli* concentrations between rinse samples collected before and after defeathering and between rinse samples after scalding and excreta leaking during defeathering [log CFU/carcass]. Results are based on ANOVA and post hoc Tukey HSD test. Results are based on batches B, C, D, E, F, G.

Slaughterhouse	Organism	Concentration in excreta - concentration after scalding [log CFU/carcass]		Concentration after defeathering - concentration after scalding [log CFU/carcass]	
		difference	*p* value	difference	*p* value
Slaughterhouse 1	*Campylobacter*	1.38	<0.001	0.01	0.99
	E. coli	-0.81	<0.001	-1.26	<0.001
Slaughterhouse 2	*Campylobacter*	1.51	<0.001	0.41	0.2
	E. coli	-0.1	0.96	-0.52	0.002

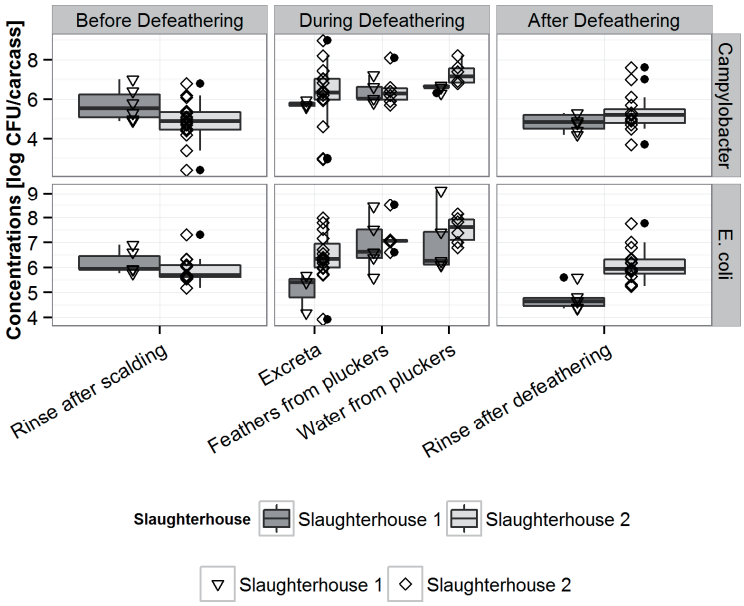

Figure 3
Campylobacter and *E. coli* concentrations before, during and after defeathering [log CFU/carcass]. Results on bacterial concentrations in the samples are based only on batches B, C, D, E, F, G. The triangles indicate the *Campylobacter* and *E.coli* concentrations in a particular sample in Slaughterhouse 1 and rhombus in Slaughterhouse 2.

4. Discussion

Trends in *Campylobacter* and *E. coli* concentrations along the processing line differed between slaughterhouses and were either regular (in Slaughterhouse 1) or variable (in Slaughterhouse 2) between tested batches (Pacholewicz et al., 2015). This difference can be potentially explained by the differences in the batch characteristics and

processing parameters applied in each slaughterhouse (Tables 1 and 2). Also variables associated with changes in both *Campylobacter* and *E. coli* concentrations on carcasses through the broiler processing differed between the slaughterhouses. The determined differences demonstrate that risks differ between slaughterhouses. Although the hygiene control should be based in each slaughterhouse on HACCP principles, the magnitude of the critical control points might differ between slaughterhouses. Control measures should be thus determined individually by each slaughterhouse. Effectiveness of the measures could be measured by the process hygiene criteria based on *E. coli* which application was adviced by European Food Safety Authority (2012).

In our study most of variables associated with *Campylobacter* concentrations were different from variables associated with *E. coli* concentrations on carcasses (Table 3). However for both bacteria the associations were determined between their concentrations on carcasses and in the excreta or caeca at various processing steps. These findings are in line with previously reported associations between *Campylobacter* concentrations in caeca and on the carcasses after defeathering and evisceration (Reich et al., 2008; Rosenquist et al., 2006). Based on our finding the reduction during chilling was higher in case of batches having higher *Campylobacter* concentration in the excreta. The reason of this association is unclear. Other studies contradict our findings. According to Reich et al. (2008) higher concentration in caeca was associated with higher concentrations on carcasses after chilling. A recent study on air chilling in a laboratory scale settings demonstrated that *Campylobacter* reduction was not observed on carcasses carrying concentration above 3 log CFU/g of tested chicken carcass parts (Rivoal et al., 2015).

Presence of carcasses with visible faecal contamination during broiler processing e.g. after evisceration (Table 2) is not uncommon (Cibin et al., 2014; Rosenquist et al., 2006). Percentage of carcasses with visible faecal contamination (Table 2) can be explained a.o. by explanatory variables (this manuscript) and by food handlers' compliance with process control procedures. The last factor was studied and described in our following manuscript (Pacholewicz et al., 2016). Leakage of faecal and caecal material during processing is recognised as an important source of cross contamination of the carcasses (Berrang et al., 2001; Cason et al., 2004; Musgrove et al., 1997). Our results show that when the concentration of bacteria in the excreta was higher than on exterior, there was a greater increase in bacterial concentrations after defeathering (Figure 3, Table 4). Differences in bacterial concentrations on the exterior and in the excreta of carcasses might explain the different impacts of defeathering on *Campylobacter* and *E. coli* concentrations observed in our previous study (Pacholewicz et al., 2015). Potentially the bacteria present on the carcasses after scalding are removed with water and feathers during defeathering, and replaced by bacteria from leaking excreta. Our experiment on manual defeathering confirms that this could be the case. While eliminating the faecal leakage during manual defeathering, the bacterial concentration

on carcasses was not reduced (Figure 2), what points to the role of concentrations of the exterior of carcasses. This concentration did not decrease, potentially because while defeathering manually the carcasses were not sprayed what is the case during mechanical defeathering. Spraying water as well as feathers dropping from the defeathering machines carried high concentrations of bacteria (Figure 3) what confirms removal of bacteria during defeathering. Interpetation of bacterial concentrations in the inputs and outputs to the defeathering step needs however a mechanistic model.

Poor uniformity of the batch influences the leakage during evisceration, because the equipment cannot be adjusted to individual size of each carcass within a batch (Malher et al., 2011; Rosenquist et al., 2006). Based on results from our study, the uniformity was associated with changes in *E. coli* concentrations on carcasses also during defeathering. The fact that a poorer uniformity had different effect in the studied slaughterhouses suggests the potential impact of equipment setting. Each slaughterhouse might set equipment differently while processing heterogenous batches. These settings potentially affect the faecal leakage which contributes to increase in bacterial concentrations on carcasses during defeathering. A better uniformity is expected to lead to better equipment performance, in every slaughterhouse.

In addition the association between *E. coli* concentrations during defeathering in Slaughterhouse 2 and the remaining feathers points as well to the role of equipment settings. Less tight settings of defeathering machines impact the effectiveness of feathers' removal, and might contribute to less pressure on the abdominal area of the carcasses, and thus less faecal leakage. As a result increase in bacterial concentrations on carcasses during defeathering might be lower than in case of thight setting leading to complete removal of feathers.

In conclusion, this study demonstrates that the bacterial concentrations in intestines of the carcasses are the prominent explanatory variables of carcass contamination because they were associated with *Campylobacter* and *E. coli* concentrations at various processing steps. Our findings confirm that control of bacterial concentrations both at farm and during processing are needed to reduce carcass contamination post-chilling. Control during processing should be focused on reducing faecal leakage that leads to cross contamination throughout processing, e.g. defeathering. The determined associations provide however an indication of areas of potential control which should be confirmed by additional studies. As each slaughterhouse is unique, each should identify individually bottlenecks in process control and implement relevant measures to improve processing hygiene.

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Appendix A

Table A1

Results on Pearson's correlation (r) of the explanatory variables. Variables with correlation coefficient are considered statistically associated and are marked in grey. Consistent results between slaughterhouses (with the same directioin of the correlation coefficient) are marked in bold. Results presented horizontaly come from Slaughterhouse 1, whereas vertically from Slaughterhouse 2.

Explanatory variables		Age [days]	Feed withdrawal time [hours]	Distance from farm to slaughter-house [km]	Size of the batch [number of broilers in a batch]	Uniformity coefficient of variation [%]	Average weight of carcasses [g]	*Campylobacter* in excreta [log CFU/g]	*E. coli* in excreta [log CFU/g]	*Campylobacter* in caeca [log CFU/g]	*E. coli* in caeca [log CFU/g]	Slaughter time [hours]	Line speed [broilers per hour]	Scalding temperature [°C]	Outside temperature [°C]	Temperature in the scalding and plucking area [°C]	Temperature in the evisceration area [°C]	Feathers score after defeathering	Breakages score after defeath-ering	Carcasses with visible faecal con-tamination after eviscerator [%]
		Slaughterhouse 1																		
Age [days]	Slaughterhouse 2	1.00	0.54	**-0.09**	**-0.04**	-0.23	**0.65**	**0.24**	**0.53**	**-0.03**	0.03	**-0.64**	**0.58**	0.30	**-0.71**	**-0.53**	**-0.88**	-0.39	0.70	**-0.07**
Feed withdrawal time [hours]		-0.08	1.00	-0.39	**-0.07**	**-0.03**	-0.18	**0.41**	-0.31	**0.37**	**-0.64**	-0.12	0.88	-0.19	-0.71	**0.01**	-0.70	**-0.71**	0.74	**0.37**
Distance from farm to slaughterhouse [km]		**-0.52**	0.61	1.00	**-0.19**	0.49	0.03	**-0.29**	0.32	-0.63	**-0.02**	**0.01**	**-0.75**	-0.36	**0.03**	-0.05	**0.24**	0.64	**-0.37**	**0.03**
Size of the batch [number of broilers in a batch]		**-0.06**	**-0.18**	**-0.16**	1.00	**-0.25**	**0.27**	**0.39**	-0.13	0.41	-0.28	**-0.16**	**-0.12**	0.07	0.68	0.60	**0.14**	**-0.29**	-0.11	**-0.47**
Uniformity coefficient of variation [%]		0.24	**-0.71**	-0.20	**-0.24**	1.00	-0.28	**-0.41**	0.02	**-0.75**	**0.52**	0.04	-0.14	-0.67	0.11	**0.40**	0.42	**0.09**	0.53	**-0.17**
Average weight of carcasses [g]		**0.95**	0.05	-0.47	**0.17**	0.02	1.00	**0.13**	**0.51**	**-0.21**	0.24	**-0.72**	**0.09**	0.67	**-0.41**	**-0.39**	**-0.50**	**0.23**	**-0.07**	**-0.45**
Campylobacter in excreta [log CFU/g]		**0.48**	**0.29**	**-0.13**	**0.09**	**-0.41**	**0.49**	1.00	-0.53	**0.91**	-0.61	-0.37	**0.44**	-0.02	0.06	**0.18**	0.15	**-0.19**	**-0.20**	**0.35**
E. coli in excreta [log CFU/g]		**0.09**	0.29	-0.33	0.09	-0.69	**0.22**	0.30	1.00	-0.69	**0.46**	-0.23	-0.17	0.20	-0.54	**-0.49**	-0.65	0.03	**0.38**	**-0.48**
Campylobacter in caeca [log CFU/g]		**-0.36**	**0.86**	0.69	-0.43	**-0.96**	**-0.12**	**0.67**	0.48	1.00	**-0.44**	**0.30**	**0.30**	**0.10**	**0.33**	**0.10**	**0.34**	**-0.51**	**-0.88**	**0.45**
E. coli in caeca [log CFU/g]		-0.34	**-0.58**	**-0.52**	0.32	**0.16**	-0.46	0.29	**0.28**	**-0.24**	1.00	-0.003	**0.19**	0.04	-0.17	-0.09	-0.19	0.43	-0.45	-0.17
Slaughter time [hours]		**-0.44**	0.62	**0.46**	**-0.31**	-0.42	**-0.46**	0.15	0.02	**0.50**	0.11	1.00	**-0.35**	-0.26	**0.47**	**0.05**	0.28	0.07	**-0.19**	**0.44**
Line speed [broilers per hour]		**0.41**	-0.40	**-0.47**	**-0.22**	0.09	**0.28**	**0.31**	0.24	**0.18**	**0.22**	**-0.45**	1.00	0.07	**-0.49**	**-0.12**	-0.48	**-0.74**	**0.69**	**0.20**
Scalding temperature [°C]		-0.52	0.77	0.75	-0.20	-0.47	-0.52	0.16	-0.07	**0.62**	-0.14	0.97	-0.45	1.00	-0.47	-0.66	-0.39	0.22	**-0.30**	-0.12
Outside temperature [°C]		**-0.94**	0.06	**0.42**	-0.51	-0.06	**-1.00**	-0.54	0.41	**0.13**	0.85	**0.65**	**-0.24**	0.54	1.00	**0.89**	**0.67**	-0.04	-0.83	-0.26
Temperature in the scalding and plucking area [°C]		**-0.35**	**0.10**	0.35	-0.41	**0.09**	**-0.53**	**0.17**	**-0.05**	**0.15**	0.84	**0.58**	**-0.29**	0.46	**0.91**	1.00	0.55	**-0.28**	0.09	-0.36
Temperature in the evisceration area [°C]		**-0.74**	0.13	**0.12**	**0.42**	-0.72	**-0.52**	-0.52	0.90	**0.87**	-0.02	-0.004	0.13	0.05	**0.65**	-0.21	1.00	0.27	-0.92	**0.11**
Feathers score after defeathering		0.79	**-0.42**	-0.30	**-0.17**	**0.70**	**0.75**	**-0.003**	-0.50	**-0.72**	-0.18	-0.58	**-0.22**	-0.59	-0.70	**-0.08**	-0.80	1.00	**-0.99**	0.02
Breakages score after defeathering		-0.22	-0.39	**-0.68**	0.30	-0.28	**-0.21**	**-0.002**	**0.80**	**-0.06**	0.64	**-0.28**	**0.67**	**-0.38**	0.49	-0.03	0.68	**-0.41**	1.00	**-0.23**
Carcasses with visible faecal contamination after eviscerator [%]		**-0.13**	**0.69**	**0.68**	**-0.27**	**-0.35**	**-0.07**	**0.22**	**-0.10**	**0.84**	-0.34	**0.50**	**0.08**	0.67	0.04	0.12	**0.15**	-0.50	**-0.42**	1.00

Appendix B

Table B1

Estimates of explanatory variables for which association with *Campylobacter* or *E. coli* concentrations on carcasses during particular processing step was determined.

Slaughterhouse	Organism	Processing step	Explanatory variable	Estimate	Estimate value	Std. Error	DF	t value	*p* value	Corresponding figure
Slaughterhouse 1	*E. coli*	Defeathering	Uniformity [%]	β0	3.80	0.90	133	4.24	<0.001	B1
				β1	0.00	0.07	9	0.04	0.97	
				β2	-3.14	0.73	133	-4.29	<0.001	
				β3	0.14	0.06	133	2.58	0.01	
Slaughterhouse 2	*E. coli*	Scalding	Age [days]	β0	7.40	0.78	111	9.54	<0.001	B2
				β1	-0.07	0.02	8	-3.46	0.01	
				β2	-4.06	0.76	111	-5.33	<0.001	
				β3	0.07	0.02	111	3.71	<0.001	
Slaughterhouse 2	*E. coli*	Defeathering	Uniformity [%]	β0	3.39	0.50	114	6.80	<0.001	B3
				β1	0.01	0.03	8	0.22	0.83	
				β2	1.78	0.63	114	2.85	0.01	
				β3	-0.15	0.04	114	-3.73	<0.001	
Slaughterhouse 2	*E. coli*	Defeathering	*E. coli* in excreta [log CFU/g]	β0	3.59	0.83	114	4.33	<0.001	B4
				β1	-0.02	0.14	8	-0.11	0.91	
				β2	-4.05	1.10	114	-3.68	<0.001	
				β3	0.61	0.19	114	3.22	0.002	
Slaughterhouse 2	*E. coli*	Defeathering	Feathers score	β0	3.61	0.51	95	7.09	<0.001	B5
				β1	-0.03	0.35	5	-0.07	0.94	
				β2	1.29	0.60	95	2.16	0.03	
				β3	-1.34	0.41	95	-3.29	0.001	
Slaughterhouse 2	*Campylobacter*	Defeathering	*Campylobacter* in caeca [log CFU/g]	β0	-3.01	1.44	73	-2.09	0.04	B6
				β1	0.69	0.19	3	3.59	0.04	
				β2	-4.60	1.87	73	-2.46	0.02	
				β3	0.67	0.25	73	2.65	0.01	
Slaughterhouse 2	*Campylobacter*	Evisceration	Average weight of carcasses [g]	β0	3.54	1.36	114	2.60	0.01	B7
				β1	-0.00	0.00	8	-0.75	0.48	
				β2	-2.95	0.89	114	-3.31	0.001	
				β3	0.00	0.00	114	3.31	<0.001	
Slaughterhouse 2	*Campylobacter*	Chilling	*Campylobacter* in excreta [log CFU/g]	β0	-0.74	0.76	114	-0.97	0.33	B8
				β1	0.53	0.12	8	4.32	0.003	
				β2	1.49	0.81	114	1.83	0.07	
				β3	-0.35	0.13	114	-2.70	0.01	

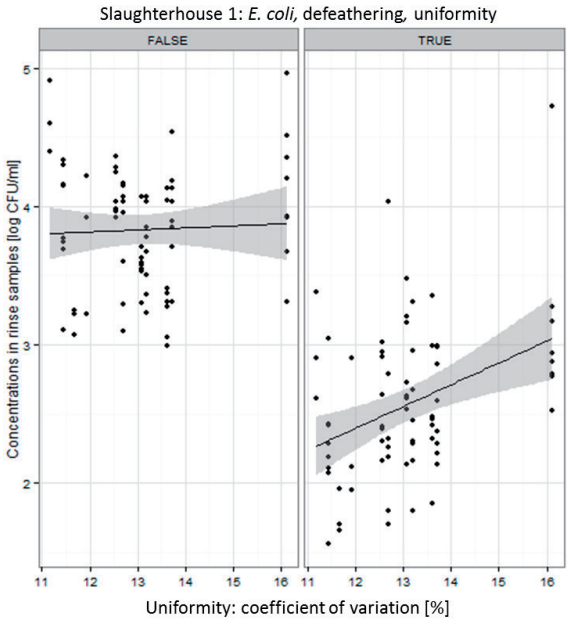

Figure B1
Association between change in *E. coli* concentrations on carcasses during defeathering [log CFU/ml] and uniformity (coeficient of variation [%]) in Slaughterhouse 1.

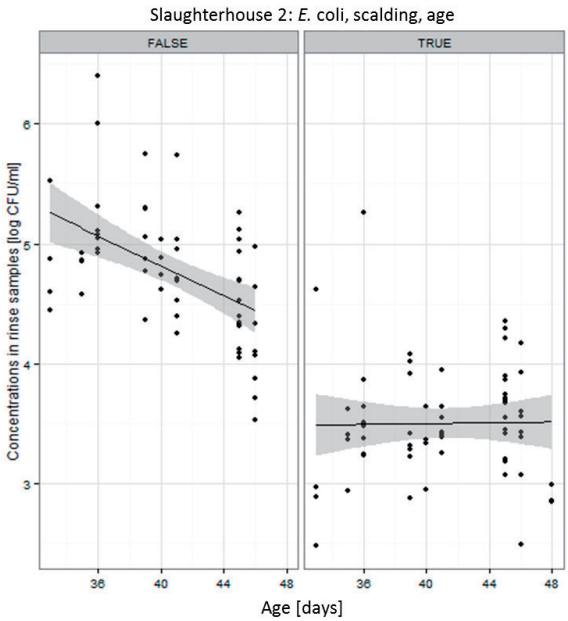

Figure B2
Association between change in *E. coli* concentrations on carcasses during scalding [log CFU/ml] and age of batches [days] in Slaughterhouse 2.

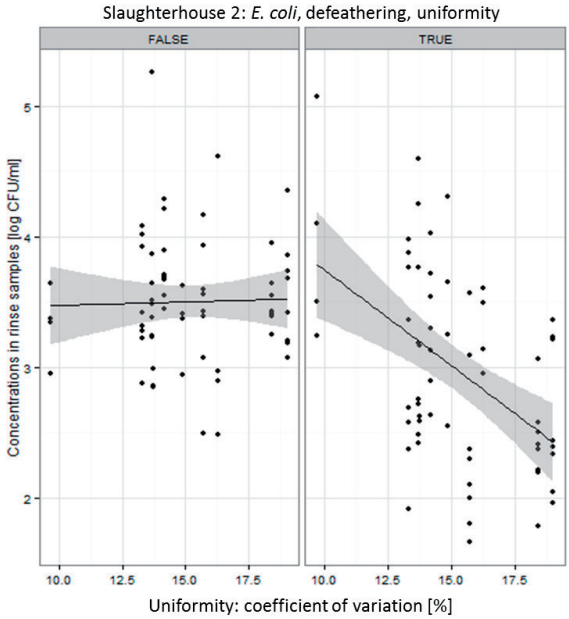

Figure B3
Association between change in *E. coli* concentrations on carcasses during defeathering [log CFU/ml] and uniformity (coeficient of variation [%]) in Slaughterhouse 2.

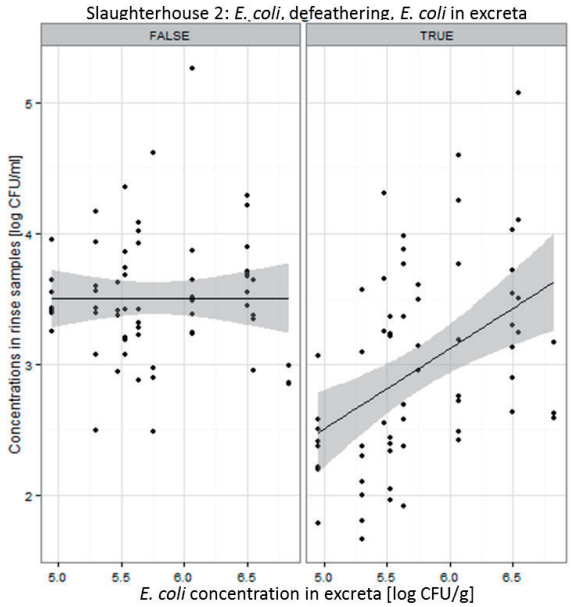

Figure B4
Association between change in *E. coli* concentrations on carcasses during defeathering [log CFU/ml] and *E. coli* concentration in excreta [log CFU/g] Slaughterhouse 2.

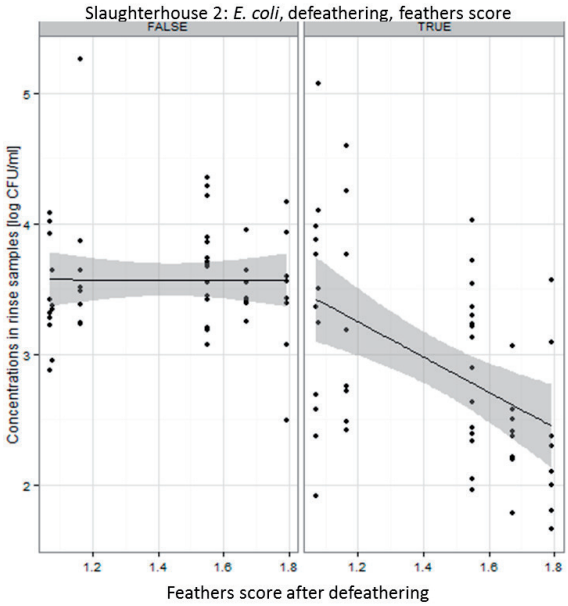

Figure B5
Association between change in *E. coli* concentrations on carcasses during defeathering [log CFU/ml] and feathers score in Slaughterhouse 2.

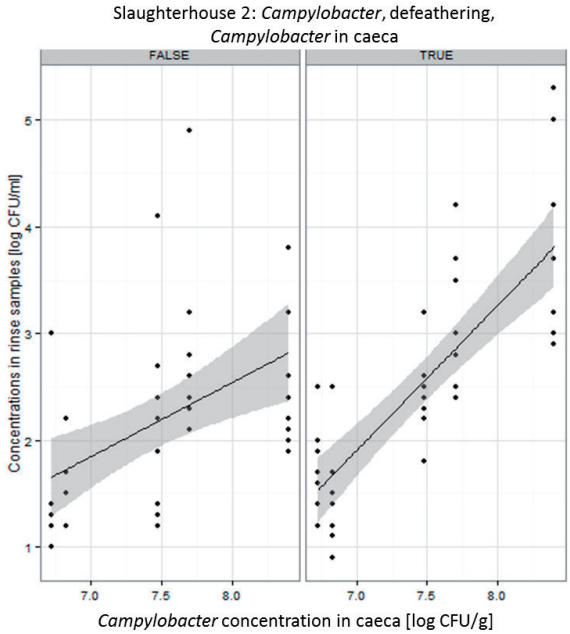

Figure B6
Association between change in *Campylobacter* concentrations on carcasses during defeathering [log CFU/ml] and *Campylobacter* concentration in caeca [log CFU/g] in Slaughterhouse 2.

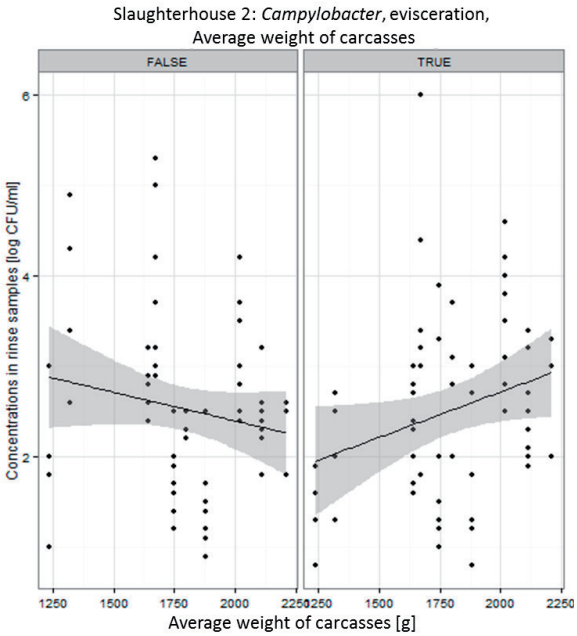

Figure B7
Association between change in *Campylobacter* concentrations on carcasses during evisceration [log CFU/ml] and average weight of carcasses [g] in Slaughterhouse 2.

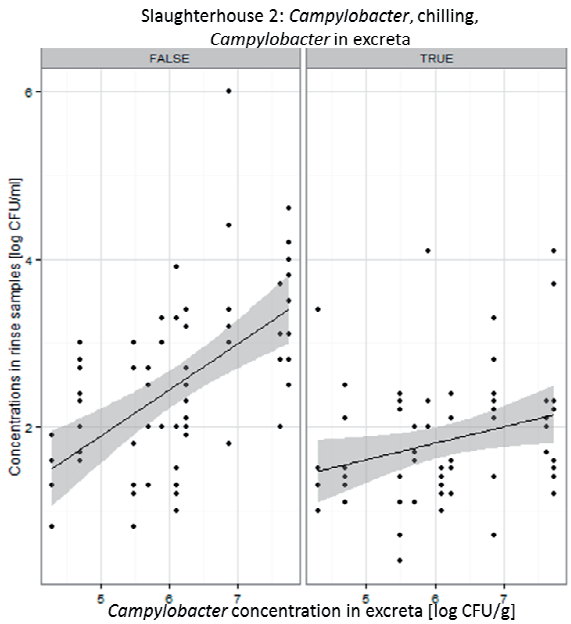

Figure B8
Association between change in *Campylobacter* concentrations on carcasses during chilling [log CFU/ml] and *Campylobacter* concentration in excreta [log CFU/g] in Slaughterhouse 2.

Chapter 5

Reduction of extended-spectrum-β-lactamase- and AmpC-β-lactamase-producing *Escherichia coli* through processing in two broiler chicken slaughterhouses

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Abstract

Whilst broilers are recognized as a reservoir of extended-spectrum-β-lactamase (ESBL)- and AmpC-β-lactamase (AmpC)- producing *Escherichia coli*, there is currently limited knowledge on the effect of slaughtering on its concentrations on poultry meat. The aim of this study was to establish the concentration of ESBL/AmpC producing *E. coli* on broiler chicken carcasses through processing. In addition the changes in ESBL/AmpC producing *E. coli* concentrations were compared with generic *E. coli* and *Campylobacter*. In two slaughterhouses, the surface of whole carcasses was sampled after 5 processing steps: bleeding, scalding, defeathering, evisceration and chilling. In total, 17 batches were sampled in two different slaughterhouses during the summers of 2012 and 2013. ESBL/AmpC producing *E. coli* were enumerated on MacConkey agar with 1 mg/L cefotaxime, and the ESBL/AmpC phenotypes and genotypes were characterised. The ESBL/AmpC producing *E. coli* concentrations varied significantly between the incoming batches in both slaughterhouses. The concentrations on broiler chicken carcasses were significantly reduced during processing. In Slaughterhouse 1, all subsequent processing steps reduced the concentrations except evisceration which led to a slight increase that was statistically not significant. The changes in concentration between processing steps were relatively similar for all sampled batches in this slaughterhouse. In contrast, changes varied between batches in Slaughterhouse 2, and the overall reduction through processing was higher in Slaughterhouse 2. Changes in ESBL/AmpC producing *E. coli* along the processing line were similar to changes in generic *E. coli* in both slaughterhouses. The effect of defeathering differed between ESBL/AmpC producing *E. coli* and *Campylobacter*. ESBL/AmpC producing *E. coli* decreased after defeathering, whereas *Campylobacter* concentrations increased. The genotypes of ESBL/AmpC producing *E. coli* ($bla_{\text{CTX-M-1}}$, $bla_{\text{SHV-12}}$, $bla_{\text{CMY-2}}$, $bla_{\text{TEM-52c}}$, $bla_{\text{TEM-52cvar}}$) from both slaughterhouses match typical poultry genotypes. Their distribution differed between batches and changed throughout processing for some batches. The concentration levels found after chilling were between 10^2 and 10^5 CFU/carcass. To conclude, changes in ESBL/AmpC producing *E. coli* concentrations on broiler chicken carcasses during processing are influenced by batch and slaughterhouse, pointing to the role of both primary production and process control for reducing ESBL/AmpC producing *E. coli* levels in final products. Due to similar changes upon processing, *E. coli* can be used as a process indicator of ESBL/AmpC producing *E. coli*, because the processing steps had similar impact on both organisms. Cross contamination may potentially explain shifts in genotypes within some batches through the processing.

Keywords: Poultry, Slaughter Hygiene, Antibiotic resistance

1. Introduction

Extended-spectrum-β-lactamase- and AmpC-β-lactamase (ESBL/AmpC) producing *Escherichia coli* have been classified as a hazard for public health of medium to high relevance on poultry meat (European Food Safety Authority, 2012). Since 2000, ESBL/AmpC-producing *E. coli* have been increasingly identified in patients suffering from infections (for instance: the urinary tract, soft tissue and intra-abdominal area) (Livermore et al., 2007; Pitout and Laupland, 2008; Rodriguez-Bano et al., 2004; Woodford et al., 2004). The presence of ESBL/AmpC-producing *E. coli* in humans hinders the effectiveness of the 2nd-, 3rd- and 4th- generation cephalosporins often applied in initial treatments of the infections (Marchaim et al., 2010; Rodriguez-Bano et al., 2004; Rodriguez-Bano et al., 2010).

The main reservoirs and transmission routes of ESBL/AmpC producing *E. coli* have not yet been fully identified (European Food Safety Authority, 2011a). Potential routes include food and food producing animals because, in Europe, the presence of ESBL/AmpC producing *E. coli* in animals (i.e. broilers, poultry, swine) and food of animal origin has been increasingly reported in the last decade (Blanc et al., 2006; Dierikx et al., 2010; Escudero et al., 2010; Rodríguez et al., 2009). Among all food producing animals the prevalence of ESBL/AmpC producing *E. coli* is the highest in broiler chickens (Anonymous, 2014). The phenotypes, ESBL/AmpC genotypes and plasmids (mobile genetic elements) found in chickens can be similar to those found in humans (Been et al., 2014; Leverstein-van Hall et al., 2011; Overdevest et al., 2011). The plasmids were recently suggested to spread the resistant genes for cephalosporin in animals and humans and the potential transmission of the ESBL/AmpC producing *E. coli* through the food chain was suggested to be minor (Been et al., 2014). Nevertheless control measures of ESBL/AmpC producing *E. coli* across the poultry chain, including the slaughtering process are needed.

The control of public health hazards, including ESBL/AmpC producing *E. coli*, *Campylobacter*, and *Salmonella* during poultry slaughter was proposed to be included in a future poultry meat inspection system (European Food Safety Authority, 2012). In this system, slaughterhouses would be categorised according to hygiene standards in order to facilitate risk management by directing high risk flocks to slaughterhouses with higher standards. The hygiene standards would be based on generic *E. coli,* since high levels of *E. coli* indicate faecal contamination (European Food Safety Authority, 2012; Ghafir et al., 2008; Habib et al., 2012). The targets of *E. coli* would be specified by Process Hygiene Criteria (PHC) (European Food Safety Authority, 2012). However, the relation of PHC based on *E. coli* with public health hazards has not yet been fully investigated.

The effect of processing steps on selected organisms such as *Campylobacter* has been studied by several authors (Berrang and Dickens, 2000; Pacholewicz et al., 2015; Rosenquist et al., 2006; Seliwiorstow et al., 2015), as has the relation of *Campylobacter* to *E. coli* contamination (Duffy et al., 2014; Habib et al., 2012). With respect to ESBL/AmpC producing *E. coli*, the prevalence and genotypes of ESBL/AmpC producing *E. coli* in broilers (Dierikx et al., 2013) and on broiler meat in retail (Leverstein-van Hall et al., 2011; Stuart et al., 2012) have been studied. Furthermore, quantitative data and genotype characterisation in caeca and on carcasses at the end of processing have been reported (Reich et al., 2013). However, there is currently a lack of quantitative data on ESBL/AmpC producing *E. coli* and its relation to generic *E. coli* and *Campylobacter* throughout poultry processing. Such data is needed to validate a future meat inspection system based on PHC, and to identify effective interventions for both ESBL/AmpC producing *E. coli* and *Campylobacter*.

In this study, we determined the concentrations of ESBL/AmpC producing *E. coli* on broiler chicken carcasses during processing in two slaughterhouses. Our aim was to investigate how ESBL/AmpC producing *E. coli* concentrations on carcasses change during processing between slaughterhouses. We compared the pattern to generic *E. coli* to determine whether *E. coli* can be used as a surrogate for ESBL/AmpC producing *E. coli* during processing. A comparison with *Campylobacter* was made to investigate whether interventions in processing hygiene will have similar effect for both ESBL/AmpC producing *E. coli* and *Campylobacter*. Finally, we identified the ESBL/AmpC producing *E. coli* genotypes and their changes along the processing line, in order to identify whether the genotypes at slaughterhouse match the typical poultry genotypes ($bla_{\text{CTX-M-1}}$, $bla_{\text{SHV-12}}$, $bla_{\text{CMY-2}}$, $bla_{\text{TEM-52c}}$, $bla_{\text{TEM-52cvar}}$) (Anonymous, 2014).

2. Materials and methods

2.1 Slaughterhouses

Sampling of the carcasses along the processing was conducted in two broiler chicken slaughterhouses, located in Germany (Slaughterhouse 1, slaughtering daily 130 000 broilers) and in the Netherlands (Slaughterhouse 2, slaughtering daily 240 000 broilers).

2.2 Experimental design

Sampling was performed during the summer months (between June and October) in 2012 and 2013, because it was conducted parallel to a sampling campaign for *Campylobacter* (Pacholewicz et al., 2015). During the campaign the samples were collected only from *Campylobacter* positive batches. A batch is defined as consisting of broilers from one shed (European Food Safety Authority, 2011b). In total 17 batches

were sampled each on a separate day. Eight batches were sampled in Slaughterhouse 1 (I, K, L, N, O, Q, R, U) and nine in Slaughterhouse 2 (E, F, G, H, J, M, P, S, T).

Per slaughterhouse, carcasses were collected after five processing steps: bleeding, scalding, defeathering, evisceration, and chilling. Per processing step, four carcasses were sampled in the first three batches (E, F, G), whereas the number of replicate carcasses was extended to eight in all later batches (H-U). The total number of the collected samples was 620.

The carcasses were sampled by the whole carcass rinse method which means that they were rinsed in 500 ml of peptone saline for one minute (Pacholewicz et al., 2015). Approximately 100 ml per rinse sample was subjected to laboratory analysis. The samples were (ten-fold) serially diluted in Butterfield's Buffer (3M The Netherlands, Zoeterwoude, product number BPPFV9BF).

2.3 Isolation and enumeration

For the enumeration and isolation of presumptive ESBL/AmpC producing *E. coli*, decimal dilutions of the rinse samples were plated onto MacConkey (MCA) agar supplemented with 1mg/L cefotaxime (MCA+). From the undiluted rinse samples, 1 ml was distributed over 4 plates, whilst for the decimal dilutions (10^{-1} to 10^{-2}); one plate was inoculated per dilution with 0.1 ml. Incubation was for 24 h at 37°C. From each sample, only colonies with an *E. coli* phenotype (pink to red colonies) were counted and recorded as presumptive ESBL/AmpC producing *E. coli*. From each sample, one presumptive ESBL/AmpC producing *E. coli* was stored at -80 °C in 1 ml peptone with 30% glycerol for further analysis. Four to eight isolates were stored per location per batch depending on the number of replicates taken, resulting in a total number of 500 isolates that were collected and analysed for ESBL/AmpC production.

Enumeration of generic *E. coli* and *Campylobacter* was done based on the same samples as enumeration of ESBL/AmpC producing *E. coli* and was performed as described previously (Pacholewicz et al., 2015). *E. coli* was enumerated on Petri Films (3M™ Petrifilm™, 3M, The Netherlands, product number 64140), incubated for 24 hours at 37ºC and afterwards blue colonies with gas bubbles were counted by the Petri Film Reader (3M, Germany, number 6499). *Campylobacter* enumeration was done on Campy Food Agar (bioMérieux SA, Marcy l'Étoile, France, product number 43471), following ISO 10272-2 (Anonymous, 2006b).

2.4 Identification and phenotypic characterisation

E. coli identity was confirmed by indole testing and for the majority of the samples by oxidase testing (except for batches I, L). In addition, strains included for analysis of ESBL/AmpC genes (described in 2.5) were also confirmed for *E. coli* by MALDI-TOF (MALDI Biotyper, Bruker, Germany).

The production of ESBL and/or AmpC was determined phenotypically by a combination disk test, including cefotaxime (BD, MD 21152 USA, reference 231607), cefotaxime combined with clavulanic acid (BD, MD 21152 USA, reference 231752), ceftazidime (BD, MD 21152 USA, reference 231633), ceftazidime combined with clavulanic acid (BD, MD 21152 USA, reference 231754) and cefoxitin (BD, MD 21152 USA, reference 231591). Disk zones for cefotaxime and ceftazidime with and without clavulanic acid were interpreted according to CLSI (2012). Susceptibility to cefoxitin (inhibition zone ≤17 mm) was used to screen for possible AmpC producers (Dierikx et al., 2012).

2.5 ESBL/AmpC genotype identification

ESBL/AmpC genotype identification was performed for 170 isolates recovered from samples collected after bleeding and after chilling solely. Genomic DNA was extracted using the DNeasy Blood and Tissue kit (QIAGEN, Hilden, Germany). The DNA was used to screen for the ESBL/AmpC gene families (bla_{SHV}, bla_{TEM}, bla_{CTX-M}, bla_{DHA}, bla_{ACC}, bla_{MOX}, bla_{FOX}, bla_{CMY} and $bla_{ACT/MIR}$) by the Check-MDR CT-101 array platform (Check-Points, Wageningen, The Netherlands). ESBL/AmpC genes presence was further confirmed by PCR and sequencing analysis (Dierikx et al., 2012).

2.6 Statistical analysis

ESBL/AmpC producing *E. coli* concentrations obtained from the whole carcass rinse samples are presented as $\log_{10}$ CFU/ml. The minimum number of colonies per plate to be counted was set to 1, in contrast to an ISO standard (Anonymous, 2006a), in order to be able to calculate concentrations also for the processing steps with the lowest ESBL/AmpC producing *E. coli* levels. The number of presumptive ESBL/AmpC producing *E. coli* (pink to red colony on MCA+) was corrected for each batch with the percentage of confirmed *E. coli* isolates (positive ESBL/AmpC combination disk test). The term "ESBL/AmpC producing *E. coli*" was used for the strains screened on selective agar with 1 mg/L cefotaxime and confirmed ESBL or AmpC phenotype.

For samples with number of presumptive ESBL/AmpC producing *E. coli* below the enumeration threshold (below 1 CFU), the values were replaced by half of the threshold (Rosenquist et al., 2006).

The changes in ESBL/AmpC producing *E. coli* concentrations during processing were analysed for each slaughterhouse separately based on linear mixed effect models (Pacholewicz et al., 2015). Three models were used to identify whether the impact of processing steps varied between batches (Model 1 and 2) and slaughterhouses (Model 3). Model 1 had a random intercept for each batch, whereas Model 2 had a random intercept and random slope for each batch. Model 3 had an additional interaction term between the slaughterhouse and the processing step. Data analysis was performed in the R software, package lme4 (3.1.2, R Development Core Team).

The proportion of ESBL/AmpC producing *E. coli* in generic *E. coli* was calculated based on data presented in this study and by Pacholewicz et al. (2015).

Genotype dissimilarity at the beginning (after bleeding) and at the end of the processing (after chilling) were analysed by Bray-Curtis dissimilarity analysis for each batch.

3. Results

3.1 Impact of processing steps on bacterial concentration on carcasses

Overall, ESBL/AmpC producing *E. coli* could be enumerated in 81.6% of the collected samples. Quantitative data were used to establish the pattern of changes in ESBL/AmpC producing *E. coli* through the processing steps. To this end, samples below the enumeration threshold (18.4%) were replaced by various values (Appendix Figure A1). Although different replacements of the threshold changed the outcome of single trials (Figures S1 in supplemental materials), the overall patterns of changes in ESBL/AmpC producing *E. coli* through the processing steps did not vary.

Specific patterns were obtained for each slaughterhouse (Figure 1). In Slaughterhouse 1, the initial concentration (on carcasses after bleeding) varied significantly between incoming batches (from 1.70 to 4.86 log CFU/ml) and the changes in ESBL/AmpC producing *E. coli* concentrations after particular processing steps were relatively similar for all slaughtered batches. This was confirmed by the superiority of the simpler model (fixed slopes, Model 1, i.e. processing steps similarly affect all slaughtered batches) above the more complex model (random slope, Model 2, i.e. effect of processing steps differs between slaughtered batches), as judged by likelihood-ratio tests (Appendix Table A1). In Slaughterhouse 2, the initial concentration also varied significantly between incoming batches (1.70 to 5.51 $\log_{10}$ CFU/ ml). Here, the changes in ESBL/AmpC producing *E. coli* concentrations after particular processing steps varied between the batches. This was confirmed by the need to include a random intercept and random slope into the model (Table A1).

In general, processing reduced the ESBL/AmpC producing *E. coli* concentration on carcasses. The overall reduction (between bleeding and chilling) was 2.16 $\log_{10}$ CFU/ml in Slaughterhouse 1 and 2.64 $\log_{10}$ CFU/ml in Slaughterhouse 2 (Table 1). Scalding and chilling led to a reduction in concentrations in both slaughterhouses. In Slaughterhouse 1, the highest reduction was observed after scalding (on average 0.97 $\log_{10}$, $p<0.01$) (Table 1; Figure 2), followed by defeathering (0.74 $\log_{10}$, $p<0.01$) and chilling (0.55 $\log_{10}$, $p<0.01$). In Slaughterhouse 2, the ESBL/AmpC producing *E. coli* concentration on carcasses decreased after each analysed processing step. The highest reductions in ESBL/AmpC producing *E. coli* concentrations were observed after scalding (by 1.44 $\log_{10}$, $p<0.01$ followed by chilling (by 1.09 $\log_{10}$, $p<0.01$) (Table 1, Figure 2).

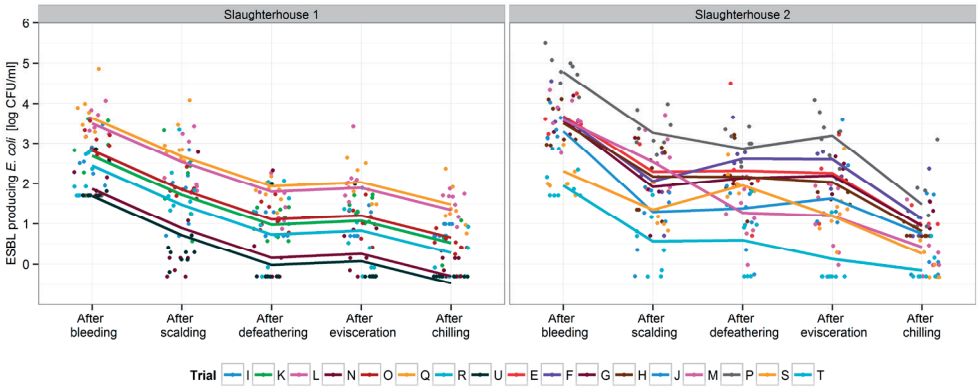

Figure 1
ESBL/AmpC producing *E. coli* concentrations in whole broiler carcass rinse samples (log_{10} CFU/ml) after selected processing steps in Slaughterhouse 1 and in Slaughterhouse 2. The lines indicate the concentrations per sampled batch (trial), based on the selected statistical model; the points indicate the concentrations in the individual samples, based on the selected statistical model.

Table 1
Effect of different processing step on ESBL/AmpC producing *E. coli* [log_{10}].

	Slaughterhouse 1		Slaughterhouse 2	
Processing step	log_{10}	*p* value	log_{10}	*p* value
scalding - bleeding	-0.97	<0.01*	-1.44	<0.01*
defeathering - scalding	-0.74	<0.01*	-0.02	0.94
evisceration - defeathering	0.1	0.35	-0.1	0.57
chilling - evisceration	-0.55	<0.01*	-1.09	<0.01*
Total decrease chilling - bleeding	-2.16	<0.01*	-2.64	<0.01*

* *p* value <0.05 indicates a significant increase or decrease in concentration.

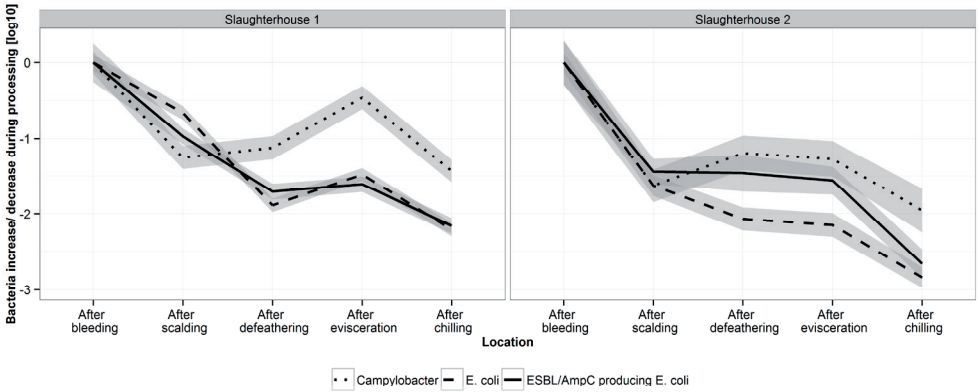

Figure 2
Average change in ESBL/AmpC producing *E. coli*, generic *E. coli* and *Campylobacter* concentrations after processing steps (concentrations after bleeding were artificially set to zero). Lines show an average effect of a processing step and shadow a standard error of an effect.

When comparing the data from both slaughterhouses, the concentrations after bleeding were relatively comparable (Table 2). The effect of some processing steps on ESBL/AmpC producing *E. coli* concentrations differed between slaughterhouses as confirmed by a significant interaction between slaughterhouses and processing steps (Model 3, Table S1). Defeathering was significantly more effective in reducing the ESBL/AmpC producing *E. coli* (by 0.71 $\log_{10}$, $p<0.01$) in Slaughterhouse 1 than in Slaughterhouse 2 (Table 2). The small but not significant difference between slaughterhouses was observed in reductions after scalding that were higher by 0.23 $\log_{10}$ in Slaughterhouse 2. The effect of evisceration on ESBL/AmpC producing *E. coli* concentration on carcasses did not differ between slaughterhouses ($p=0.48$, Table 2). Reduction in concentrations after chilling was higher in Slaughterhouse 2 (by 0.49 $\log_{10}$, $p=0.04$, Table 2) than in Slaughterhouse 1. The total reduction through the processing was slightly but insignificant higher by 0.13 $\log_{10}$ in Slaughterhouse 2 than in Slaughterhouse 1 ($p=0.61$) (Table 2).

Table 2
Comparing effects of a processing step on ESBL/AmpC producing *E. coli* [$\log_{10}$] between slaughterhouses.

	ESBL/AmpC *E. coli*	
Processing step	Differences Slaughterhouse 1- Slaughterhouse 2 [$\log_{10}$]	*p* value
bleeding	-0.28	0.31
scalding - bleeding	0.23	0.21
defeathering - scalding	-0.71	<0.01*
evisceration - defeathering	0.12	0.48
chilling - evisceration	0.49	0.04*
Total reduction: bleeding - chilling	0.13	0.61

* *p* value <0.05 indicates a significant difference in the effect.

3.2 Concentration on carcasses after chilling

The ESBL/AmpC producing *E. coli* concentrations on the carcasses after chilling varied between 2.4 and 5.1 $\log_{10}$ CFU/carcass (i.e. in 500 ml of peptone saline used to rinse one carcass) in Slaughterhouse 1 and between 2.4 and 5.8 $\log_{10}$ CFU/carcass in Slaughterhouse 2 (Figure 3).

3.3 Relation with E. coli and Campylobacter

The average proportion of ESBL/AmpC producing *E. coli* in the generic *E. coli* including all processing steps per batch varied between 0.1% to nearly 100% (Figure 4). It was higher in batches slaughtered in Slaughterhouse 2.

The tested processing steps had similar impacts on the concentrations of ESBL/AmpC producing *E. coli* and generic *E. coli* (Figure 2). Although the overall impact of

processing was the same for ESBL/AmpC producing *E. coli* and generic *E. coli*, slight differences were observed. The increase in *E. coli* bacteria after evisceration and the decrease after defeathering were greater for generic *E. coli* but smaller and not significant for ESBL/AmpC producing *E. coli*. The differences between the effects of slaughter steps for both *E. coli* and ESBL/AmpC producing *E. coli* were between 0.18 and 0.49 (Slaughterhouse 1) and between 0.04 and 0.47 (Slaughterhouse 2). When comparing both to *Campylobacter,* it appeared that defeathering decreased concentrations of both ESBL/AmpC producing *E. coli* and generic *E. coli*, whereas *Campylobacter* concentrations increased in Slaughterhouse 2 and did not change in Slaughterhouse 1 (Figure 2).

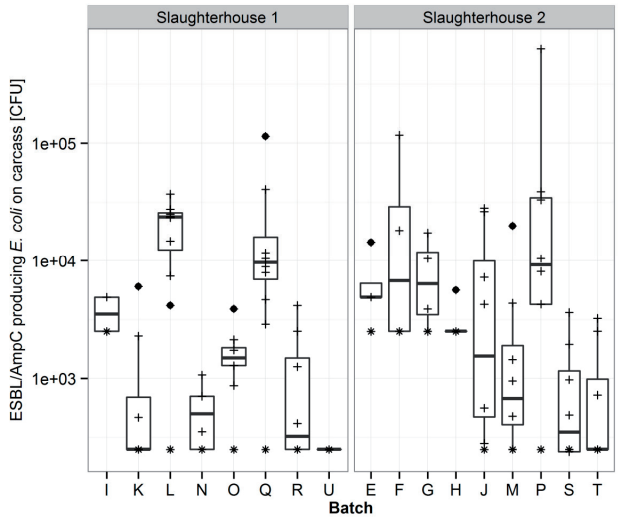

Figure 3
ESBL/AmpC producing *E. coli* concentrations on carcasses after chilling (CFU/carcass) in Slaughterhouse 1 and 2. Crosses show concentration on a particular carcass, big dots indicate outliers, asterisks indicate an enumeration threshold, lines in boxes a median, upper whiskers 75th percentiles and lower whiskers 25th percentiles.

3.4 ESBL/AmpC phenotypes and genotypes

In most of the sampled batches (E, F, G, H, I, L, M, O, P, Q, T, U), the ESBL phenotype was predominant (Table 3). Three batches (I, J, N) had about equal proportions of ESBL and AmpC, and in three batches (K, R, S) AmpC was the dominant phenotype.

Isolates from samples collected after bleeding and chilling harboured the genotypes $bla_{\text{CTX-M-1}}$, $bla_{\text{SHV-12}}$, $bla_{\text{CMY-2}}$, $bla_{\text{TEM-52c}}$ and $bla_{\text{TEM-52cvar}}$. Often, several genotypes were detected in one batch, and the frequency of a given genotype varied within and between batches (Table 3). The similarity between genotypes, identified in isolates from samples collected after bleeding and after chilling within a batch, was higher in batches slaughtered at the beginning of the production day (Figure 5). Higher Bray-Curtis dissimilarity score indicates more differences between the genotype patterns detected in the samples collected after bleeding and after chilling within a batch.

Table 3
Identified phenotypes and genotypes of ESBL/AmpC producing *E. coli* in two slaughterhouses.

Slaughterhouse	Batch	Isolates from samples collected after selected processing steps and subscribed to genotype analysis		Batch origin	Slaughter time	Phenotype [%] of all isolates from all processing steps		
		Bleeding	Chilling			ESBL	AmpC	Unclassified
Slaughterhouse 1	I	CMY-2 (1) SHV-12 (3) TEM-52cVar (1)	NA	GER	16:20	50.0	37.5	12.5
	K	CMY-2 (8)	CMY-2 (5) SHV-12 (1)	GER	4:10	3.33	90.0	6.67
	L	CMY-2 (2) SHV-12 (6)	CMY-2 (6) SHV-12 (1)	GER	18:25	64.71	27.45	7.84
	N	CMY-2 (2)	CMY-2 (1) SHV-12 (1) TEM-52cVar (1)	GER	12:15	46.88	53.13	0
	O	SHV-12 (7)	SHV-12 (4) TEM-52c (2)	GER	10:15	83.96	4.72	11.32
	Q	TEM-52cVar (3) TEM-52c (4)	SHV-12 (1) TEM-52cVar (6)	GER	14:00	71.52	2.53	25.95
	R	SHV-12 (3) CMY-2 (1)	SHV-12 (1) CTX-M-1 (1) TEM-52c (1)	GER	8:00	26.67	61.67	11.67
	U	CMY-2 (1) SHV-12/CMY-2 (1) SHV-12 (2)	NA	NL	11:30	69.23	15.38	15.39
Slaughterhouse 2	E	SHV-12 (2) TEM-52c (1) CMY-2 (1)	CMY-2 (2) CTX-M-14 (1)	NL	15:55	60.87	30.43	8.70
	F	CMY-2 (3)	CTX-M-1 (1) CMY-2 (1)	NL	18:55	59.1	31.82	9.1
	G	SHV-12 (1) TEM-52c (2)	SHV-12 (1) TEM-52c (2)	GER	14:15	87.5	12.5	0
	H	SHV-12/CMY-2 (3) TEM-52cVar (1) TEM-52c (1) CTX-M-1 (3)	CTX-M-1 (1)	NL	5:10	74.36	25.64	0
	J	CTX-M-1 (3) SHV-12 (1) CMY-2 (1)	CTX-M-1 (3) SHV-12 (1) CMY-2 (4)	GER	17:00	51.35	29.73	18.92
	M	SHV-12 (6) TEM-52c (1)	SHV-12 (1) TEM-52c (4)	GER	5:00	100	0	0
	P	CTX-M-1 (6) SHV-12 (1)	CTX-M-1 (8)	GER	4:30	100	0	0
	S	CMY-2 (7)	CMY-2 (7)	NL	4:30	5.56	86.11	8.33
	T	CMY-2 (3) TEM-52c (1)	CTX-M-1 (1) SHV-12 (1) TEM-52c (1)	NL	11:00	62.5	25.0	12.5

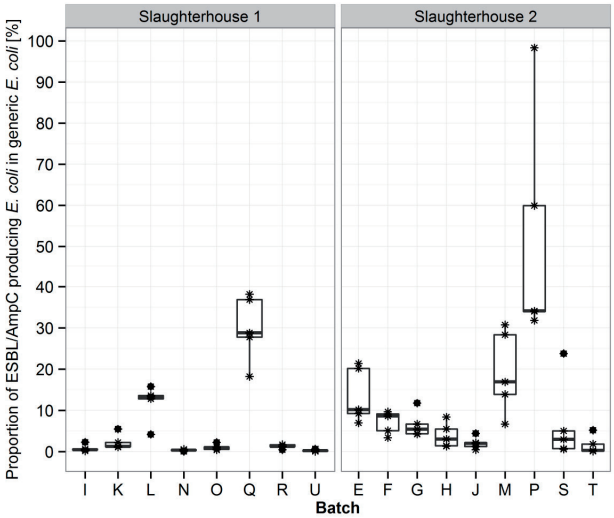

Figure 4
Proportion of ESBL/AmpC producing *E. coli* in generic *E. coli* [%] in Slaughterhouse 1 and 2. Asterisks present the proportion of ESBL/AmpC producing *E. coli* in generic *E. coli* per location in particular trials. The big dots indicate outliers.

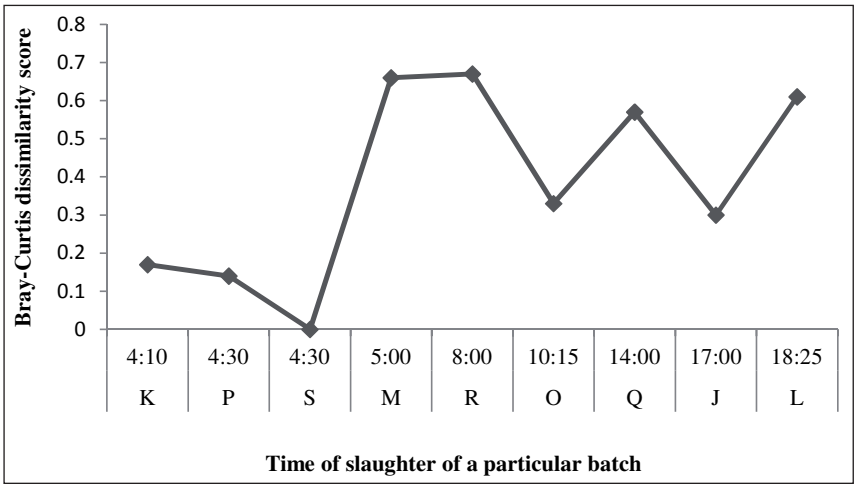

Figure 5
Bray-Curtis dissimilarity score between genotypes detected in the isolated from samples after bleeding and after chilling in relation to time of slaughter of a particular batch.

4. Discussion

In order to address public health hazards of inter alia ESBL/AmpC producing *E. coli*, suggestions have been made for future poultry meat inspection. These include the use of process hygiene criteria for the characterisation of slaughterhouse performance based on *E. coli* as a surrogate for, amongst other ESBL/AmpC producing *E. coli* (European Food Safety Authority, 2012). However, to our knowledge, quantitative data on ESBL/AmpC producing *E. coli* concentrations through the broiler processing have not been previously described.

This study reports on changes in concentrations through carcass processing in two slaughterhouses. For all tested microorganisms, high variability in concentration levels per batch was observed. Higher proportions of ESBL/AmpC producing *E. coli* were observed in batches slaughtered in Slaughterhouse 2 (Figure 4). There the variability in ESBL/AmpC producing *E. coli* concentrations in the incoming batches corresponds with the variation in the proportion of ESBL/AmpC producing *E. coli* over generic *E. coli* between batches (0.1-100%). The higher proportions of ESBL/AmpC producing *E. coli* in Slaughterhouse 2 seems unrelated with the country of origin of the batch, because both slaughterhouses processed Dutch and German batches (Table 3). The variability in ESBL/AmpC producing *E. coli* concentration levels in the incoming batches confirms that identifying high risk flocks at farm level and directing those to slaughterhouses with higher hygiene standards, as proposed by European Food Standard Authority (2012) could be a useful measure to reduce exposure of consumers to these resistant bacteria. This batch effect points to the role of primary production and/or transport. Thus, one possible intervention strategy consists of controlling ESBL/AmpC producing *E. coli* either at farm level and transport or upon slaughterhouse entrance. A slaughterhouse specific pattern was found for the influence of processing steps on tested microorganisms. The ESBL/AmpC producing *E. coli* pattern differed in a similar manner to that observed for generic *E. coli* and *Campylobacter* (Figure 2) and processing steps showed a regular impact on the concentrations in Slaughterhouse 1, whilst Slaughterhouse 2 had a more variable performance (Pacholewicz et al., 2015). Most of the processing steps affected the concentrations of the three tested organisms in a similar way. More similarities were observed between ESBL/AmpC producing *E. coli* and generic *E. coli* than with *Campylobacter* (Figure 2). The maximum difference between the estimates between ESBL/AmpC producing *E. coli* and *E. coli*, i.a the effect of a certain step on *E. coli* counts as compared to the effect on ESBL counts, was log 0.5. This confirms appropriateness of *E. coli* as a surrogate for ESBL/AmpC producing *E. coli* during processing. Differences between *Campylobacter* and both ESBL/AmpC producing *E. coli* and generic *E. coli* were particularly observed for the impact of defeathering in both slaughterhouses. That is in agreement with reported lack of concordance of the impact of defeathering on

Campylobacter and *Enterobacteriaceae* (Oosterom et al., 1983) and total viable counts (TVC) (Zweifel et al., 2015). Possibly, the relative role of faecal leakage (Berrang et al., 2001; Berrang et al., 2004; Musgrove et al., 1997) as compared to contamination of the exterior of the carcasses during defeathering differs between *Campylobacter* and other organisms. *Campylobacter* is sensitive to air drying and thus present at lower external concentrations already at the beginning of the processing (Berrang et al., 2000).

Despite this difference, all of the tested bacteria provided a concordant indication of the efficiency of processing steps between slaughterhouses. Scalding, evisceration and chilling were more effective in Slaughterhouse 2, whereas defeathering was more effective in Slaughterhouse 1. In addition, processing steps had a regular impact on the concentrations in Slaughterhouse 1, whilst Slaughterhouse 2 had a more variable performance. The slaughterhouse differences could be potentially explained by processing parameters as scalding temperature and time applied (Zweifel et al., 2015), impact of initial contamination (after bleeding) on defeathering (Seliwiorstow et al., 2015) and control of faecal leakage during evisceration (Rosenquist et al., 2006). Moreover, the similarity of slaughterhouse outcomes for all tested organisms suggests that a PHC based on *E. coli* can be used to differentiate the hygienic status of slaughterhouses and their ability to reduce hazards such as *Campylobacter* and ESBL/AmpC producing *E. coli*. Similar differences in the reduction of foodborne pathogens between slaughterhouses have been reported previously (Pacholewicz et al., 2015; Paulin, 2011; Rosenquist et al., 2006; Zweifel et al., 2015) suggesting that improvements in processing hygiene are attainable and that interventions implemented at slaughter level can have a similar effect on all tested organisms. Next to control at the primary production stage, control of slaughter processing thus forms a second possible intervention, in a similar way as proposed for *Campylobacter* (European Food Safety Authority, 2011b) and for ESBL/AmpC producing *E. coli* (Depoorter et al., 2012). To conclude, a reduction of carcass contamination during slaughter represents a strategy targeting multiple public health hazards.

The identified genotypes of ESBL/AmpC producing *E. coli* from both slaughterhouses ($bla_{\text{CTX-M-1}}$, $bla_{\text{SHV-12}}$, $bla_{\text{CMY-2}}$, $bla_{\text{TEM-52c}}$, $bla_{\text{TEM-52cvar}}$) correspond with genotypes common for poultry in The Netherlands (Anonymous, 2014; Dierikx et al., 2013) and in Germany (Laube et al., 2013; Reich et al., 2013). Differences in the genotypes, which we observed between beginning (samples after bleeding) and end (samples after chilling) of processing, can possibly be explained by cross contamination between processing batches as reported for *Salmonella* (Rasschaert et al., 2007). Carcasses could have been contaminated with genotypes that originated from previous batches. This explanation is supported by the fact that isolates from samples after chilling were more dissimilar than those collected after bleeding in most of the batches slaughtered later in the day (Figure 5). However, this conclusion is limited by relative small number

of isolates genotyped at these steps. Another potential explanation of the differences in genotypes can be related to sampling different carcasses after each processing step. Different individuals might carry different genotypes at different concentrations. Despite a significant reduction of ESBL/AmpC producing *E. coli* through processing (Table 1), ESBL/AmpC producing *E. coli* were detectable on carcasses after chilling at concentrations between 2 and 5 $\log_{10}$ CFU/carcass. Narrower range in the concentration levels (3.7-4.2 $\log_{10}$ CFU/carcass) was reported by Reich et al. (2013). Human exposure to ESBL/AmpC producing *E. coli* through cross contaminating during preparation of a meal with broiler meat in kitchen appears likely, as confirmed in a Belgian model for human exposure through chicken meat consumption (Depoorter et al., 2012).

In summary, the concentration of ESBL/AmpC producing *E. coli* on carcasses after chilling is impacted both by concentrations in incoming batches and by reductions in a slaughterhouse. Whilst ESBL/AmpC producing *E. coli* has high prevalence in incoming batches, processing reduces concentration on carcasses. This reduction was more successful in one of two studied slaughterhouses, suggesting that improvements are possible.

Acknowledgements

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Appendix

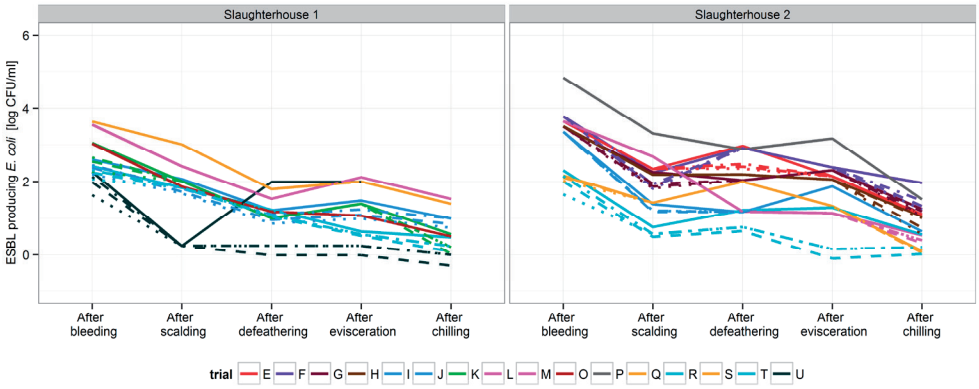

Figure A1
Role of different substitutions for values below enumeration threshold in Slaughterhouse 1 and 2. Lines represents: (—) omitted values below the threshold; (- - -) values set to 0.5 of the threshold; (⋯) values set to square root of the threshold; (-.-.-) values set to the threshold.

Table A1
Selection of the mixed effect models to the quantitative data on ESBL/AmpC producing *E. coli.*

	ESBL/AmpC producing *E. coli*					
	Slaughterhouse 1		Slaughterhouse 2		Both slaughterhouses	
	Model 1	Model 2	Model 1	Model 2	Model 1	Model 3
df	7	21	7	21	7	12
AIC	615.687	623.715	692.202	677.051	534.546	525.927
logLik	-300.844	-290.858	-339.101	-317.526	-260.273	-250.963
chi-square	18.872		41.205		27.001	
p value	0.166		0.00017		0.00006	

Chapter 6

Brushing of broiler carcasses at the beginning of processing reduces bacterial concentrations

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Submitted

Abstract

The aim of the study was to investigate the effect of brushing prior to scalding on reducing the *E. coli* and *Enterobacteriaceae* concentrations on carcasses. Four visits were arranged to a commercial slaughterhouse in which carcasses were cleaned in a separate line. In the first three visits, ten batches were sampled to compare the *E. coli* and *Enterobacteriaceae* concentrations on carcasses before and after a stand-alone brushing unit. Per batch, eight carcasses before and eight after brushing were sampled by the whole carcass rinse method. Furthermore dry matter content and pH were determined in these samples, as these parameters influence directly (pH) or indirectly (dry matter) the scalding lethality. During the fourth visit the effect of brushing on the treated area of the carcasses was examined by sampling the skin with feathers from the breast and vent areas. In addition, bacterial concentrations were examined on feathers from breast, vent, wings, legs and from the remaining surface of the carcass and on feet and heads. Results revealed significant reduction in *E. coli* and *Enterobacteriaceae* concentrations on the brushed carcasses. The concentrations on whole carcasses were reduced on average by 0.3 log for both *E. coli* and *Enterobacteriaceae.* On the breast skin *E. coli* was reduced by 0.7 log and *Enterobacteriaceae* by 0.8 log, whereas on the vent skin *E. coli* was reduced by 1.2 log and *Enterobacteriaceae* by 0.9 log. Rinse samples from treated carcasses had significantly less dry matter on average by 2.5 g (p<0.001) and significantly higher pH by 0.08 units (p<0.001). High concentrations of both *E. coli* and *Enterobacteriaceae* were present on feathers from non-brushed areas and on heads and feet. This study confirms that brushing reduces bacterial concentrations on carcasses, which could be increased by enlarging the brushed surface of the carcass. Further in-line investigations are needed to observe the effect of brushing on bacterial concentrations in scalding water, and on carcasses after scalding and at the end of processing.

Keywords: Hygiene, Poultry, Slaughterhouse, Intervention

1. Introduction

High numbers of campylobacteriosis cases in humans originating from broiler meat consumption urge implementing intervention strategies during broiler processing, aiming at reducing the concentration of *Campylobacter* on carcasses after slaughtering (European Food Safety Authority, 2011). *Campylobacter* and other bacteria such as e.g. *E. coli* are present in high concentrations in the colon and caeca of broilers and on their exteriors including feathers and skin (Berrang et al., 2000; Kotula and Pandya, 1995). Concentrations vary significantly between incoming batches (Pacholewicz et al., 2015; Seliwiorstow et al., 2015), potentially explaining the observed variations between slaughterhouses in *Campylobacter* and *E. coli* concentrations on chilled carcasses (European Food Safety Authority, 2010; Habib et al., 2012). Currently, the concentration in the incoming batches cannot be controlled by farmers or slaughterhouses, because specific measures are not available. Various interventions have been studied so far to reduce the *Campylobacter* load on carcasses after chilling. Widely applicable and effective measures are still lacking, because they are either not acceptable for consumers (e.g. chemical decontamination or irradiation (MacRitchie et al., 2014)), or have low effectiveness (e.g. logistic slaughter (Havelaar et al., 2007)). In addition many of the tested interventions were applied at the end of the processing line (European Food Safety Authority, 2011), where their effect can be inhibited because bacteria become firmly attached to the skin within several seconds (Notermans and Kampelmacher, 1975). This urges exploring interventions at the beginning of processing in order to reduce the bacterial concentrations on carcasses entering the processing line and thus on the final product. Little is known about the impact of interventions applied before scalding on bacterial concentrations on carcasses after scalding and at the end of processing. Scalding directly follows bleeding and with its temperature and washing effects contributes to significant bacterial reductions (Berrang and Dickens, 2000; Pacholewicz et al., 2015; Zweifel et al., 2015). This reduction can be affected by various factors. One of them is faecal material entering the scald tank together with broiler carcasses, which contributes to an increase in the dry matter and a decrease in the pH value of the scalding water (Humphrey, 1981). The pH decreases because the ammonium urate present in the chicken faeces dissociates to uric acid and ammonium hydroxide (Humphrey, 1981). Maintenance of pH of scalding water at the level of 9 by adding sodium hydroxide was reported to significantly reduce heat resistance of *Campylobacter jejuni* in scalding water and marginally reduce the *Campylobacter jejuni* prevalence on carcasses after scalding (Humphrey and Lanning, 1987). Also adding acetic acid to scalding water increased the death rate of *S.* Newport, *S.* Typhimurium and *Campylobacter jejuni* in the water (Okrend et al. 1986). Thus, when the pH in the scalding water is away from the optimum for *Campylobacter* and *Salmonella* heat resistance, the death rate of these bacteria in the

water increases. Next to the pH value also dry matter, proteins and temperature of the scalding water were reported to affect the death rate of *Salmonella* Typhimurium in scalding water (Humphrey, 1981; Humphrey et al., 1981).

It is expected that by reducing the faecal contamination on carcasses entering the scalding tank, not only the bacterial concentration on carcasses could be reduced, but also the reduction in pH level of scalding water could be prevented leading to an increase in bacterial reduction during scalding.

Recently a prototype intervention was developed by Meyn Food Processing Technology B.V. Oostzaan, The Netherlands (European Patent Application EP 2 974 601 A1) aiming at mechanical cleaning of broiler chicken carcasses before scalding by a set of brushes. The goal of this study was to investigate the effect of brushing on bacterial concentrations using *Escherichia coli* and *Enterobacteriaceae* as indicator organisms. Measuring concentrations of these organisms on carcasses was proposed to indicate the efficiency of control measures in slaughterhouses (European Food Safety Authority, 2012a; European Food Safety Authority, 2012b).

The specific objectives were to A) investigate whether a reduction in bacterial concentrations through brushing of the carcasses is realized, and if so, whether the reduction occurs on whole carcasses or on the treated areas only; B) to investigate the effect of brushing on the dry matter content and pH and C) to investigate the bacterial concentrations on various parts of the carcasses before their entrance to scalding.

2. Materials and Methods

2.1 Intervention equipment

A prototype intervention apparatus was designed to brush the breast and vent area of the carcasses before scalding (Figure 1). Its purpose was to remove solids such as faecal material, especially faecal clumps, and thus reducing solid matter and bacterial concentrations on carcasses before scalding.

The intervention was a standalone unit with three brushes that were installed in a stainless steel frame and connected with water and electrical supply. The first brush was designed to brush the vent area of the carcass, whereas the second was aiming at the breast and the third at the neck. The brushes had nylon bristles which were shaped as a loop for better removal of faecal contamination, especially clumps. The length of the bristle of the breast and neck brush was 23 cm and of the vent brushes 27 cm. The bristles had a diameter of 1 mm. Bristles were fixed on the shaft of each brush in 12 rows, each row had 7 bristles, resulting in 84 bristles per brush. The length of each brush was 70 cm and the diameter with bristles was 30 cm for the breast brush and 26 cm for the vent brush. Under the vent brush a conveyor belt was present to tilt and position the carcasses in order to expose the vent area for brushing. The position of the first and

second brush in the frame was horizontal and the third brush was set in an angle in order to brush the neck area of the carcass.

Carcasses were removed from the processing line just after bleeding and placed in the shackles of the brushing unit. First, the carcasses entered the vent brush and were subsequently directed by the shackle guide to the breast brush and then to the neck brush. The vent brush rotated in a downward direction whereas the breast and neck brushes rotated in an upward direction. All brushes rotated with a speed of 2240 rpm (37 rps). Water sprayers supplied water to clean the brushes. Each brush had 5 water nozzles providing a flat spraying pattern to enable spraying the whole brushes. The line speed in the brushing unit was 77 carcasses per minute. Water consumption to spray the brushes was 15 l/min, corresponding with 190 ml/carcass. The water temperature was 16°C.

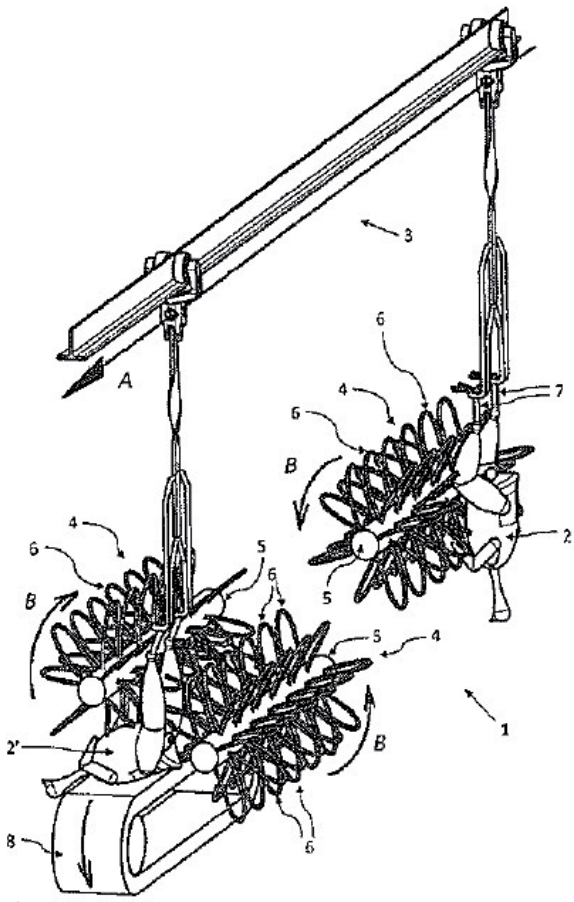

Figure 1
Intervention equipment to brush the exterior of carcasses before scalding. Source: European Patent Application EP 2 974 601 A1.

2.2 Experimental design

Two experiments were performed. The first aimed at evaluating the effect of brushing on the *E. coli* and *Enterobacteriaceae* concentration on carcasses measured by the whole carcass rinse method. The second aimed at investigating the concentrations of bacteria before and after brushing only on the treated areas of carcasses (breast and vent skins with feathers). In addition, in the second experiment, the distribution of *E. coli* and *Enterobacteriaceae* concentrations on feathers from various areas of the carcasses and on feet and heads was investigated.

The samples were collected in a Dutch slaughterhouse between October 2014 and February 2015. In the first experiment, 10 batches were sampled during three sampling visits. Four batches were sampled during the first visit, three during the second visit and three during the last visit. In each batch 16 carcasses were sampled. First 8 reference carcasses were collected after bleeding before the brushing. Afterwards 10 carcasses were removed from the commercial line and hung in the brush unit, treated and 4 carcasses out of the ten were randomly collected and used to prepare rinse samples. This procedure was repeated once and resulted in sampling of 8 treated carcasses per batch.

The whole carcass rinse method was applied according to Pacholewicz et al. (2015) with modification. Prior to rinsing, in addition to cloaca plugging, the head was cut and the neck was closed with a tie wrap, and the feet were cut. Rinse samples were cooled in water with ice and stored at 3 °C (+/–2 °C) until laboratory analysis for a maximum of 20 h.

For the second experiment, samples were collected from various parts of the carcasses after bleeding. Samples were collected in groups including 1) skin with feathers from breast area before brushing, 2) skin with feathers from breast area after brushing, 3) skin with feathers from vent area before brushing, 4) skin with feathers from vent area after brushing, 5) feathers from the breast area after bleeding before brushing, 6) feathers from the vent area after bleeding before brushing, 7) feathers from wings after bleeding before brushing, 8) feathers from legs after bleeding before brushing, 9) feathers from all remaining areas of the carcass, after bleeding before brushing. Additionally, rinse samples were taken from 10) the collected heads after bleeding before brushing, and 11) the collected feet after bleeding before brushing. The feathers were plucked manually and weighed. In total there were three samples collected in each group 1-11, each sample was made out of eight carcasses coming from one batch. A batch was defined as a group of chickens from one house (European Food Safety Authority, 2011). Samples of skin and feathers were rinsed with peptone saline in a ratio 1:10. Pooled heads and feet samples were rinsed in one liter of peptone saline. All samples were rinsed manually for 60 s. The rinse fluid was collected, cooled and stored at 3°C (+/–2 °C) until laboratory analysis for a maximum of 20 h.

2.3 E. coli and Enterobacteriaceae enumeration

Laboratory analysis was performed on the day following the collection of the samples. The concentration of both *E. coli* and *Enterobacteriaceae* was examined using Petri Films (Petrifilm™ 3M, Zoeterwoude, The Netherlands, products numbers 6414). Serial dilutions were prepared in Butterfield's Buffer (3M, Zoeterwoude, The Netherlands, product number BPPFV9BF). After incubation for 24h at 37°C, colonies were counted by Petrifilm™ Plate Reader (Model 6499, 3M, Neuss, Germany). For *E. coli* blue colonies with gas bubbles were counted, whereas for *Enterobacteriaceae* red colonies with yellow zones and red colonies with gas bubbles with or without yellow zones were counted. The detection limit in the first experiment was 10 CFU/ml for *E. coli* and 100 CFU/ml for *Enterobacteriaceae*, whereas in the second experiment it was 1000 CFU/ml for both organisms. Results from the whole carcass rinse samples were converted to log CFU/carcass, whereas from feathers, skin, feet and heads as log CFU/sample by scaling to the volume of the original rinse sample.

2.4 pH and dry matter content

pH and dry matter content were measured in rinse samples collected in the first experiment. The pH was measured by pH-Tester (HI 98103, HANNA instruments Deutschland GmbH, Kehl am Rhein, Germany) on the day following collection of the samples. Dry matter was measured by freeze drying of 20 ml of the rinse sample, collected in a 50 ml plastic tube. The samples were frozen at -80 °C overnight. Afterwards the samples were dried for 48-72 h in the freeze dry system Freezone 2.5 (Labconco, Beun De Ronde, Abcoude, The Netherlands). After drying, the samples were weighed on a XS105 DualRange scale (Mettler Toledo, Tiel, The Netherlands). The measurement precision was 0.1mg. Results were expressed as the dry matter content in g/carcass.

2.5 Statistical analysis

The lognormal distribution of *E. coli* and *Enterobacteriaceae* concentrations obtained in the first experiment was checked by diagnostic qq plots. In the second experiment too few samples were collected to conduct statistical analysis.

Data analysis was performed in the R statistical software (3.2.0, 2015, R Development Core Team). The effect of brushing in the first experiment on *E. coli*, *Enterobacteriaceae*, dry matter and pH was analysed by a linear mixed effect models (Pacholewicz et al., 2015). The models were used to analyse whether the effect of brushing on bacterial concentrations, pH and dry matter was regular or variable between the tested batches. The first model (1), as described below, was used to analyse the data on dry matter content and pH. In the model "Value" is the mean pH or dry matter in the samples collected from reference or treated carcasses. Term "group" indicating reference or treated carcasses, was an explanatory factor and batch was modelled as a random

effect of the intercept. The intercept (b_0+β_0) varied between batches, and the effect of brushing (slope) was either regular for each batch (β_1 is a fixed effect, b_1=0) or varied between batches (b_1>0, random effect). The second model (2) was used to analyse data on *E. coli* and *Enterobacteriaceae* concentrations. In the model "Value" is the mean of *E. coli* concentrations or *Enterobacteriaceae* concentrations collected from reference or treated carcasses. The intercept and slope are as in model one. In addition the second model has terms "organism" and interaction of "organism and group", in order to analyse whether the effect of brushing was the same for both *E. coli* and *Enterobacteriaceae.* Significance of the interaction term estimate (β_3) indicates differences in the effect of brushing for both organisms. In both models the residual error (ε) varied over carcasses. The models used are given by,

1)

$$Value = b_0 + \beta_0 + (\beta_1 + b_1)group + \varepsilon,$$

2)

$$Value = b_0 + \beta_0 + (\beta_1 + b_1)group + \beta_2\, organism + \beta_3\, group * organism + \varepsilon.$$

3. Results

3.1 Reduction of E. coli and Enterobacteriaceae on brushed carcasses

Concentrations of both *E. coli* and *Enterobacteriaceae* in the whole carcass rinse samples before and after brushing are presented in Figure 2. According to the model, the bacterial concentrations on carcasses before brushing varied significantly between batches as indicated by the random intercept in the model. Furthermore, the model indicated that the effect of brushing was not statistically different between tested batches, because the slope was fixed (b_1=0). The effect of interaction of group and organism shown by estimate (β_3) was not significant (p=0.84), which confirmed that the effect of brushing did not differ between the *E. coli* and *Enterobacteriaceae*. The concentrations on brushed carcasses were reduced on average by 0.3 log for both *E. coli* and *Enterobacteriaceae* in each tested batch.

Figure 3 shows *E. coli* and *Enterobacteriaceae* concentrations in samples of skin with feathers collected from the breast and vent areas of the reference and treated carcasses during the second experiment. The average *E. coli* concentration in the skin samples from breast before brushing was 7.1 log CFU/sample and after the brushing it was 6.4 log CFU/sample. The average *E. coli* concentration in vent skin samples before brushing was 6.7 log CFU/sample and after brushing 5.6 log CFU/sample. The average *Enterobacteriaceae* concentration in the skin samples from breast before brushing was 7.3 log CFU/sample and after brushing 6.5 log CFU/sample. The average *Enterobacteriaceae*

concentration in vent skin samples before brushing was 7.0 log CFU/sample and after brushing 6.1 log CFU/sample. This suggests a higher reduction in concentration on the treated area than observed in the whole carcass rinse samples, however the data set was not sufficient to confirm it by data modelling.

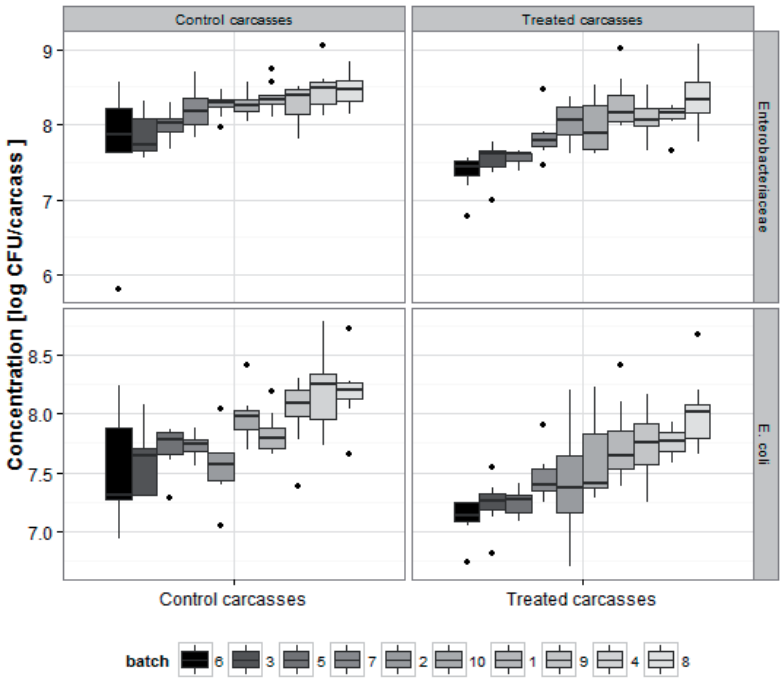

Figure 2
Concentrations of *E. coli* and *Enterobacteriaceae* (log CFU/carcass) in whole carcass rinse samples obtained from reference and treated carcasses in ten batches (sorted by increasing median). The length of the boxes indicates the inter-quartile range of concentrations; the whiskers maximum and minimum concentrations, the dots indicate the outliers and the lines indicate the median.

3.2 Reduction of dry matter

Figure 4 presents the weight of dry matter recovered from whole carcass rinses obtained from reference and treated carcasses. The model with random intercept and random slope ($b_1 > 0$) fitted the data, indicating differences in dry matter between batches before brushing, and differences in removal of faecal material between batches. According to the model the average content of dry matter in the rinse samples obtained from carcasses before brushing was 4.5 g (standard deviation 1.6 g), which decreased significantly ($p<0.001$) after brushing by 2.5 g (standard deviation 1.3g).

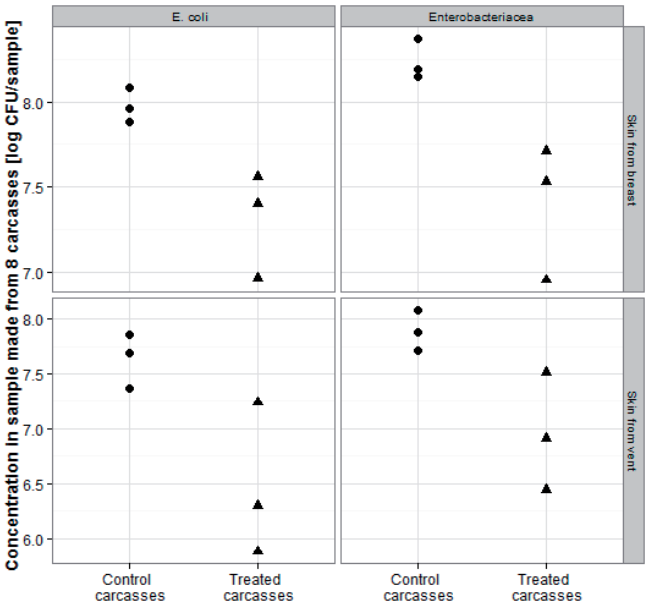

Figure 3
Effect of the brushing on treated areas of the broiler chicken carcasses. The dots indicate the *E. coli* and *Enterobacteriaceae* concentrations (log CFU/carcass) on skins with feathers from breast and vent before brushing (control samples), whereas the triangles after brushing (treated samples).

3.3 pH

Figure 5 presents the results on pH of the rinse samples collected from reference and treated carcasses by brushes. Here the model with fixed slope ($b_1 = 0$) and random intercept fitted the data best. It confirmed that pH varied between batches. According to the model the average pH value in the rinse samples obtained from the carcasses before brushing was 6.19 and in the samples obtained after brushing the pH value was significantly ($p<0.001$) higher by 0.08 on average. This result confirmed that brushing prevented reduction of, and even increased, the pH value in the rinse sample.

3.4 Distribution of the bacteria on carcasses

Results on *E. coli* and *Enterobacteriaceae* concentrations on feathers obtained from various parts of the carcasses after bleeding and weight of the feathers are presented in Table 1. The weight of the feathers from breast and vent areas was lowest comparing to the weight of feathers from other parts of a carcass. But, breast, wings and the remaining area were highest contaminated, in absolute numbers. Concerning concentrations, vent and breast scored highest (Table 1). Table 1 shows also *E. coli* and *Enterobacteriaceae* concentrations on feet and heads from carcasses after bleeding. The average *E. coli* concentration from three samples was 5.9 log CFU/feet and 6.9 CFU/head, whereas *Enterobacteriaceae* concentrations were 7.7 log CFU/feet and 6.9 log CFU/head.

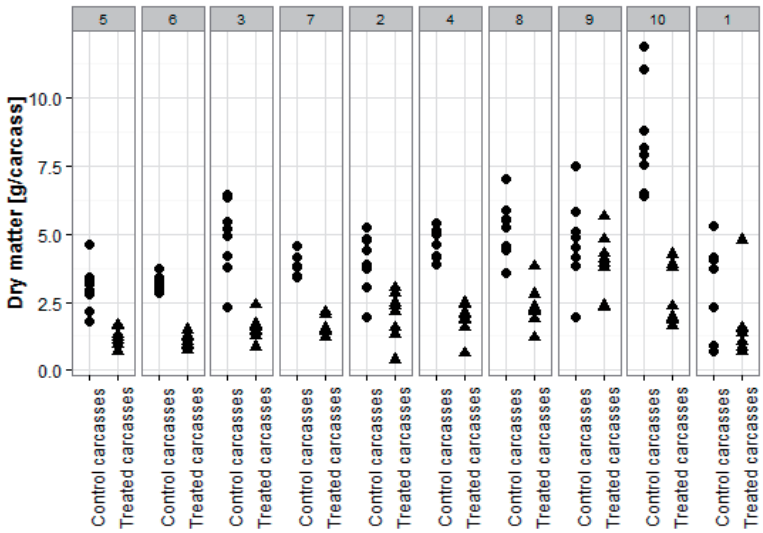

Figure 4
Effect of brushing on the dry matter content in the whole carcass rinse samples of reference and treated carcasses.
The dots present the weight (g/carcass) of dry matter recovered from whole carcass rinse samples from control carcasses, whereas triangles from treated in particular batches (1-10). The dry matter content is sorted on the figure by increasing median in the tested batches.

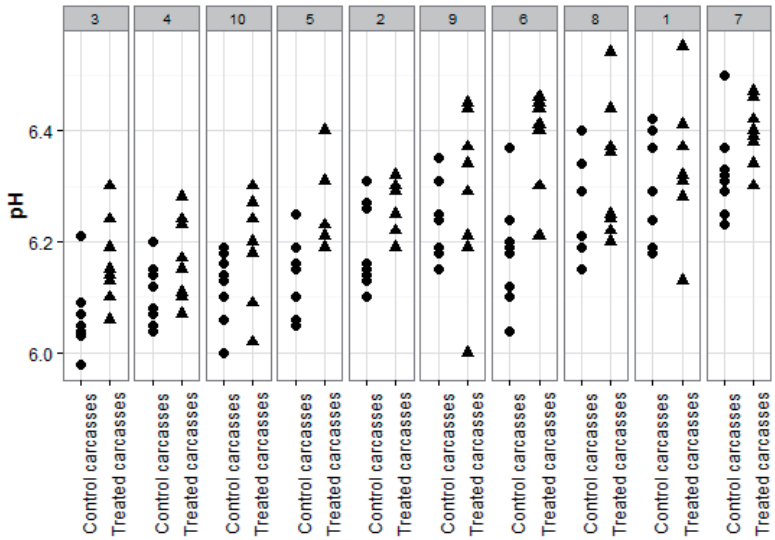

Figure 5
Effect of brushing on change of pH level in the whole carcass rinse samples collected before and after brushing.
The dots present pH in each of the whole carcass rinse sample from control carcasses, whereas triangles from treated in particular batches (1-10). The pH value is sorted on the figure by increasing median in the tested batches.

Table 1
Average concentrations of *E. coli* and *Enterobacteriaceae* [log CFU/sample and log CFU/g] on exterior of the carcasses and weight of particular samples (g/sample). Each sample was made from eight carcasses.

	Feathers					Feet	Head
	Breast	Legs	Remaining area	Vent	Wings		
Average weight [g/sample]	11.6	50.1	156.6	11.3	174.2	619.5	528.8
Average *Enterobacteriaceae* [log CFU/sample]	7.3	6.6	7.2	6.8	7.2	7.7	6.9
Enterobacteriaceae [log CFU/g]	6.3	4.9	5.0	5.7	4.9		
Average *E. coli* [log CFU/sample]	6.8	6.5	6.9	6.5	7.0	5.9	6.4
E. coli [log CFU/g]	5.8	4.7	4.7	5.4	4.7		

4. Discussion

Slaughterhouses have limited influence on the faecal contamination and thus *Campylobacter* concentrations on broilers entering the slaughterhouse, but interventions are needed to reduce bacterial concentrations as early in processing as possible. An intervention to brush the exterior of the carcasses before scalding was tested and was found to significantly reduce both *E. coli* and *Enterobacteriaceae* concentrations on the treated carcasses. These bacteria are an indicator for the potential effect of brushes on *Campylobacter*, as it originates from faecal material as well. Similar impact of processing steps on both *E. coli* and *Campylobacter* concentrations on carcasses was found (Pacholewicz et al., 2015).

Reduction in bacterial concentrations caused by prescald brushes was also evaluated by other authors (Berrang and Bailey, 2009). Although these authors brushed the whole surface of the carcass, in contrast to selected areas in our study, they observed an insignificant reduction in *Campylobacter* concentrations by 0.46 log and in *E. coli* concentrations by 0.19 log. The differences in reduction observed in our study and by Berrang and Bailey (2009) may be related to different types of brushes (loop brush in our study versus conventional brush, confirmed by communication with the authors). Data on other parameters applied in that study as e.g. water consumption is not available, making it difficult to further compare the results with our findings. Although the prescald conventional brush tested by Berrang and Bailey (2009) caused insignificant reductions in bacterial concentrations, its combination with other interventions, according to the authors contributed to significant reductions in concentrations at the end of processing. These findings confirm that application of brushes is a potentially effective hurdle to reduce bacterial concentrations on carcasses. The impact of brushes tested in our study on bacterial reductions is expected to increase by brushing a bigger area of the carcass. Other parts of carcasses than breast and vent carry also high concentrations of bacteria

as was demonstrated in our study (Table 1). In addition, a high load of bacteria is present on feet and heads of the carcasses (Table 1). This is in agreement with another study (Kotula and Pandya, 1995) and suggests that cleaning of these areas is of great importance.

The brushes tested in our study removed the faecal clumps effectively, in contrast to brushes tested by Shackelford et al. (1992). In that study, the effectiveness of brushes on removal of solids from carcasses before scalding depended on the consistency of the faecal material adhering to the feathers. The differences in the effect of clumps removal in our study, compared to the study by Shackelford et al. (1992) may also be explained by the type of brushes used. The loop brush is more effective in removing adhered faecal material to feathers than brushes with straight bristles. Moreover, in our study, the carcasses were tilted during vent brushing, thereby exposing the vent area and facilitating brushing. Other brushing parameters applied by Shackelford et al. (1992) differed slightly from those used in our study e.g. 140 ml water used to spray the brush, compared to 190 ml in our study.

Brushing significantly reduced the dry mater content in rinse samples from treated carcasses (Figure 4). Reduction of dry matter differed between the tested batches, in agreement with Shackelford et al. (1992) who reported between batches variations in the total solids removed from carcasses. These authors, despite limitations on effective removal of clumps, reported reduction of solids in the scalding water as a result of application of brushes.

Our results have shown that the pH in rinse samples from treated carcasses was higher compared to reference samples. The difference in pH was relatively small (0.08 units) which might not have a biological relevance, but this result confirms that brushing of the carcasses contribute not only to a decrease in solids but also prevents the decrease in pH of scalding water. This will potentially increase the lethality of this processing step when inline brushes are applied. As reported previously (Okrend et al. 1986; Humphrey and Lanning, 1987) the pH in the scalding water at value away from the optimum for *Campylobacter* and *Salmonella* heat resistance influences their specific D_T in the water. The effect of brushing on bacterial concentrations in scalding water and on carcasses after scalding will be evaluated in further studies. In addition, there is a need to investigate the effect of the brushes tested in our study on *Campylobacter* reduction on carcasses. In the study by Berrang and Bailey (2009) the reduction in *Campylobacter* concentrations after brushing was slightly higher than *E. coli*.

In summary, the tested brushes are a potentially effective approach to reduce solids and bacterial concentrations on carcasses before scalding. It is likely that the reduction can be increased by brushing larger surface areas of carcasses than tested in this study. Further studies should investigate the impact of brushing on bacterial concentrations in scalding water and on carcasses after scalding and at the end of processing.

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Chapter 7

Influence of food handlers' compliance with procedures of poultry carcasses contamination: a case study concerning evisceration in broiler slaughterhouses

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Abstract

Campylobacter remains the most commonly reported zoonotic agent worldwide. Reducing the concentration of *Campylobacter* on chicken meat is seen as the most efficient strategy to diminish the number of human campylobacteriosis cases. Analysis of risk factors related to characteristics of broiler batches and processing conditions could, however, not fully explain differences in impact of processing on contamination levels between slaughterhouses. Our study aimed at investigating whether compliance of food handlers with procedures on setting and controlling evisceration process parameters could explain differences in microbial concentrations on carcasses between slaughterhouses. The study was conducted in two commercial broiler chicken slaughterhouses. Analysis of documentation provided insight in the adequacy of procedures, and observational studies revealed insight in compliance with procedures by using a set of criteria for evisceration control. The frequency of carcasses with visible faecal contamination was counted and *E. coli* concentrations on carcasses classified based on visible contamination was analysed. *E. coli* was found to be a valid indicator for *Campylobacter* during evisceration. Food handlers' knowledge, attitude and practices related to evisceration control tasks were analysed based on a validated questionnaire. Documentation analysis revealed obvious differences in the procedures between slaughterhouses. The observation study revealed that in the slaughterhouse with advanced procedures, the food handlers more often complied with these procedures and a lower frequency of carcasses with visible faecal contamination was observed. Carcasses contaminated with visible faecal spots, even at a low level, carried significantly higher concentrations of *E. coli* than visibly clean carcasses. Food handlers in both slaughterhouses revealed a good knowledge level. The attitude of food handlers differed between slaughterhouses. In one slaughterhouse, where food handlers complied more frequently with procedures their attitude was at a good level, and practices at good and moderate levels. In the other slaughterhouse the attitude of food handlers was at moderate level and practices at moderate and poor levels. In conclusion, the results from our case study suggest that management factors like availability of adequate monitoring procedures and food handlers' compliance with these procedures may influence the bacterial concentrations on carcasses. Our study suggests that managerial improvements, in the area of supervising and motivating food handlers are an important control point. To validate the observed relation between compliance with procedures and contamination of carcasses, an intervention study is needed.

Keywords: *Campylobacter*, Processing hygiene, Food handler, Compliance with procedures

1. Introduction

Campylobacter remains the most commonly reported zoonotic agent worldwide. A high fraction of campylobacteriosis cases in humans is accounted to the poultry reservoir and 20 to 30% of the cases to the handling, preparation and consumption of broiler meat (European Food Safety Authority, 2010). Risk assessment studies indicate that compliance of broiler meat batches with a *Campylobacter* microbiological criterion is the most efficient strategy to diminish human infection (European Food Safety Authority, 2011). Setting a hygiene target based on *E. coli* concentrations of carcasses after chilling was proposed to be useful as an indirect sanitary tool for reducing the level of *Campylobacter* contamination of post-chilled broiler carcasses (European Food Safety Authority, 2012a; European Food Safety Authority, 2012b). *Campylobacter* as well as *E. coli* concentrations on broiler chicken carcasses after chilling vary between slaughterhouses (Anonymous, 2011; Habib et al., 2012; Pacholewicz et al., 2015; Seliwiorstow et al., 2015). Identifying the causes of variation in the bacterial concentration between slaughterhouses could support the development of strategies to reduce the bacterial concentrations on chicken meat and thus the number of campylobacteriosis cases in humans.

The impact of processing steps on *Campylobacter* and *E. coli* contamination levels was reported to vary between two slaughterhouses (Pacholewicz et al., 2015). These slaughterhouses have similar equipment and operational food safety management systems based on HACCP principles and prerequisite requirements, and comparable contamination levels of *Campylobacter* and *E. coli* in the incoming batches. The effect of processes such as evisceration on bacterial concentration on carcasses has frequently been reported to differ between slaughterhouses, causing either an increase or no change in concentrations (Pacholewicz et al., 2015; Rosenquist et al., 2006; Seliwiorstow et al., 2015). Various explanatory variables related to processing parameters and the characteristics of incoming batches were analysed to explain the variable impact of processing steps between slaughterhouses on *Campylobacter* and *E. coli* contamination levels. Nevertheless, the analysis did not explain fully the observed differences between the slaughterhouses (Pacholewicz et al., in preparation).

Other variables than those related to broiler chicken batches and processing parameters may further explain observed differences in bacterial concentrations on broiler meat between slaughterhouses. Luning and Marcelis (2006) hypothesized that food quality is not only affected by the behaviour of the food systems (i.e. the properties of the product and processes), but could also be affected by the decision making behaviour of people operating the food production system within a certain company context. Moreover it was observed that food handlers did not always follow prescribed hygiene practices (Baş et al., 2006; Jianu and Chiş, 2012; Walker et al., 2003). The impact of variable compliance on product safety parameters was demonstrated (Sanny et al., 2012;

2013). Compliance with adequate procedures is necessary to produce products with an acceptable bacterial contamination level (Luning et al., 2008).

Despite the high automation level in poultry processing (Barbut, 2014), certain activities still need to be executed by food handlers, e.g. adjusting the equipment to the size of the carcasses and taking corrective actions in case processes do not perform properly. These settings can have an essential impact on hygiene, e.g. at the evisceration process. Proper adjustment of equipment prevents the leakage of faecal contamination and thus prevents an increase in bacterial concentration on carcasses. Presence of visibly contaminated carcasses after evisceration was previously reported (Burfoot and Allen, 2013; Cason et al., 2004; Cibin et al., 2014; Smith et al., 2007). To our knowledge, the compliance of food handlers with hygiene and food safety procedures in broiler chicken slaughterhouses and its impact on microbiological concentration has not yet been studied.

This study aimed to investigate whether compliance of food handlers with procedures on setting and controlling evisceration could explain differences in the impact of the evisceration process on *E. coli* concentrations between slaughterhouses. To reach this goal, the structure of existing procedures in the slaughterhouses was analysed against Good Manufacturing Practices. Furthermore, a set of criteria for optimal control of evisceration was developed and it was observed whether the available procedures and food handlers complied with these criteria. The frequency of carcasses with visible faecal contamination after evisceration was calculated and the *E. coli* concentration on the contaminated carcasses was analysed. In addition, the level of knowledge, attitude and self-reported practices were investigated among the food handlers to understand a relationship with their compliance with the criteria.

2. Materials and Methods

2.1 Slaughterhouses

The study was performed in two commercial broiler slaughterhouses in which the evisceration process had different effects on bacterial concentrations as described previously (Pacholewicz et al., 2015). In Slaughterhouse A both *Campylobacter* and *E. coli* concentrations increased after the evisceration process, whereas concentrations did not increase in Slaughterhouse B.

2.2 Development of the assessment criteria

A set of assessment criteria for evisceration process control was developed in order to conduct both a documentation analysis and observational study of food handlers. Food handlers included operators, responsible for setting and controlling the equipment and post mortem inspectors. The criteria included activities that the food handlers should carry out in order to control the evisceration process and were based on a literature survey

and preliminary observations as recommended by Martin et al. (1993). Moreover, quality managers were interviewed and the available procedures were analysed. This resulted in fifteen assessment criteria: ten criteria dedicated to operators and the other five dedicated to post mortem inspectors (Table 1). During observations, three scores were used to rate the actions performed by the food handlers: good, sufficient and poor compliance. These scores were prepared based on the notational coding method (Clayton and Griffith, 2004). A criterion was scored as good compliance when the food handlers completed the task in a consistent way within the specified time interval and took sufficient time to perform observations and activities. A sufficient score indicated that food handlers performed the activities as specified by the criteria incompletely, e.g. only a hasty evaluation, or performed actions inconsistently. A poor score was given when the food handler did not perform the tasks or was not present at the production site.

Table 1
Checklist with assessment criteria to observe food handlers involved in the evisceration process. The table includes a comparison of the procedures available in two slaughterhouses with the assessment criteria.

Assessment criteria	Degree of Compliance [filled during observations] Good/Sufficient/ Poor	Does the procedure comply with the assessment criterion? Slaughterhouse	
		A	B
1. Equipment Setting			
Vent cutter			
1.1. Control performance of vent cutter by observing carcasses		yes	yes
1.2. Observe and adjust height		no	yes
1.3. Observe and adjust shackle guide		no	yes
1.4. Observe and adjust water nozzles		no	yes
Opener			
1.5. Control performance of opener by observing carcass		yes	yes
1.6. Observe and adjust height		no	yes
1.7. Observe and adjust shackle guide		no	yes
Eviscerator			
1.8. Control performance of eviscerator by observing carcasses		yes	yes
1.9. Observe and adjust height		no	yes
1.10. Observe and adjust shackle guide		no	yes
2. Visible Faecal Contamination Inspection			
2.1. Remove carcasses with high visible faecal contamination from the line		no	yes
2.2. Remove part of carcasses with low visible faecal contamination by trimming or cutting		no	yes
2.3. Sterilize/clean knife before and after each trimming or cutting		no	yes
2.4. Remove part of remaining viscera in carcasses which were not properly eviscerated		no	yes
2.5. Record number of bile contamination, visible faecal contamination and rejected carcasses per each batch		yes	yes
Percentage of compliance [yes score]		27%	100%

2.3 Analysis of existing procedures

We analysed the available procedures on the evisceration process against the assessment criteria as specified in Table 1. Compliance of procedures with the criteria was expressed as a percentage of the assessment criteria present in the existing procedures in each slaughterhouse. The structure of existing procedures was analysed according to requirements specified by Good Manufacturing Practices (Table 2).

Table 2
Assessment of the procedures on evisceration provided by two slaughterhouses according to the requirements on the structure of procedures.

Requirements to procedures and instructions		Slaughterhouse A	Slaughterhouse B
1	Are the procedures present	yes	yes
2	Information about who wrote the procedures	no	no
3	Procedure number	no	yes
4	Authorization	no	no
5	Effective date	no	yes
6	Purpose: Clear purpose of the procedure, why it is it is written and why it is performed	no	yes
7	Scope of the procedures: when (frequency) the procedures needs to be performed and where the procedure applies	yes	yes
8	Responsibility who performs the procedure who is responsible to see it is performed correctly	no	yes
9	Materials and equipment: what is needed to perform the test	no	yes
10	How: clear and concise description how to perform the procedure	no	yes
11	Reporting: Where results should be recorded	yes	yes
12	Specify corrective action	no	yes
	Criteria that were met [%]	25%	83%

2.4 Observation of compliance of food handlers with assessment criteria

This observational study was designed based on previous observational studies of food handlers (Clayton and Griffith, 2004; Fischer et al., 2007; Green et al., 2006; Redmond and Griffith, 2003). Both slaughterhouses were visited between April and July 2015 to observe the activities of the food handlers involved in the evisceration process. The observations were performed on three separate days in each slaughterhouse. A batch was used as an observational unit and defined as a group of chickens raised together in one shed (European Food Safety Authority, 2011). In total, twenty-six batches were observed, namely 14 in Slaughterhouse A and 12 in Slaughterhouse B. Compliance of operators was observed during the first 15 minutes of processing each new batch in the evisceration area. The compliance of post mortem inspectors was observed during the following nine minutes. Food handlers were not informed about the specific objectives of the study in order to assure reliability of the observation. The presence of observers may affect the behaviour of the person being observed, known as reactivity or the Hawthorne effect (Clayton and Griffith, 2004; Redmond and Griffith, 2003). In order

to prevent the reactivity influencing the outcome of observations, the results obtained from the first one to two observed batches were discarded. This approach enabled the food handlers to adjust to the presence of observers in the slaughterhouses. Moreover, the two researchers involved in the observational study were wearing protective clothing typical for the employees in the slaughterhouses to limit the effect of reactivity (Clayton and Griffith, 2004; Green et al., 2006).

2.5 Frequency of carcasses with visible faecal contamination

After completing the observations of food handlers, we observed the presence of visible faecal contamination on carcasses in the 26 batches studied and classified the carcasses as without visible faecal contamination, with low and high level of visible faecal contamination as shown in Figure 1. A low level of visible contamination indicated a single spot of faecal material, whereas substantial leakage of the material on carcasses was classified as high level.

The number of carcasses with visible faecal contamination was counted at four locations including the key evisceration machines: 1) after the vent cutter, 2) after the opener, 3) after the eviscerator, and 4) after post mortem inspection. At each location, contaminated carcasses were counted three times for a three minute interval, adding up to nine minutes of counting per batch. Further a frequency of carcasses with visible faecal contamination was calculated based on line speed in each slaughterhouse.

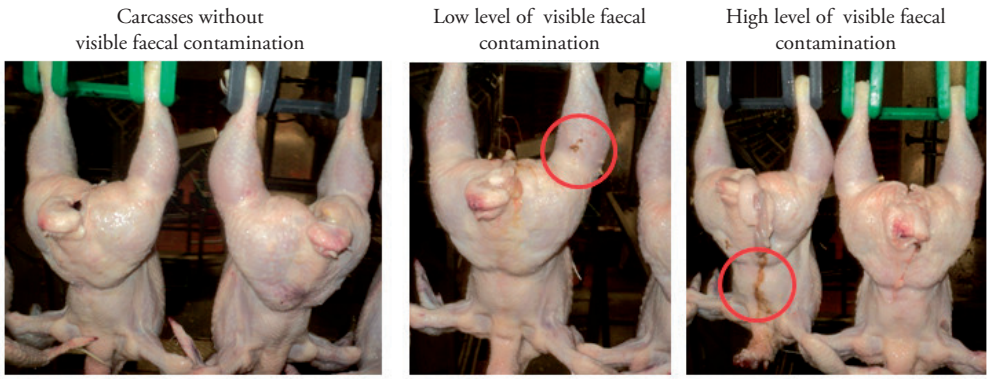

Figure 1
Visual aids to judging the level of visible faecal contamination on carcasses.

2.6 Microbiological sampling

After the observations of food handlers' practices and observations of the carcasses with and without visible faecal contamination, we collected samples to investigate whether carcasses with low and high level of visible faecal contamination had different concentrations of *E. coli* than visibly clean carcasses. In total 165 carcasses were collected, from five batches sampled in Slaughterhouse A and six in Slaughterhouse B.

Per batch, 15 carcasses were sampled including five carcasses visibly clean, five with low and five with high contamination level. The samples were taken using the whole carcass rinse method and analysed for *E. coli* concentration as described in our previous study (Pacholewicz et al., 2015). The obtained results were transformed to the logarithmic scale. The limit of detection was 100 CFU/ml of rinse sample. In samples below the detection limit, the results were expressed as a half of the detection limit (Rosenquist et al., 2006).

2.7 Questionnaires on knowledge, attitude and practices

In July 2015, the slaughterhouses were visited again in order to identify possible reasons for inadequate compliance with procedures using questionnaires. One questionnaire was dedicated to the operators and the second to post mortem inspectors. Both questionnaires were divided into four parts covering food handlers' socio-demographic characteristics, knowledge, attitude and self-reported practices. The questionnaire was developed according to the guidelines provided by Tan et al. (2013).

Each questionnaire contained 35 questions. The socio-demographic part included gender, age, education level, training followed and years of employment in the studied slaughterhouse. The knowledge part contained ten statements for which the food handlers could choose answers 'yes', 'no', and 'do not know'. The attitude part contained ten statements for which the food handlers were asked to specify the level of agreement as 'strongly agree', 'agree', 'uncertain', 'disagree' and 'strongly disagree'. The section on self-reported practices contained ten statements for which the food handlers were asked to rate their practices based on the five point scale: 'never', 'rarely', 'sometimes', 'often', and 'always'. This questionnaires contained negative statements, providing incorrect information without using negative words.

The questionnaires were translated into Dutch and German. Firstly, recruited native speakers with a background in veterinary medicine and animal sciences translated the questionnaires from English. Afterwards, two other native speakers with similar expertise as the first pair translated the questionnaires back to the English language in order to assure the equivalent interpretation of the questions (Young et al., 2010). Following both translations, we analysed the differences and if needed we implemented modifications. Experts in the field including veterinarians from Utrecht University verified the questionnaires for adequacy of their content.

Twelve food handlers in Slaughterhouse A participated in the study, including six operators and six post mortem inspectors. In Slaughterhouse B four operators and ten post mortem inspectors participated.

The answers given by food handlers in the questionnaires were scored. In the knowledge part the correct answer was scored with 4 points, whereas incorrect or 'do not know' with 0 points. In the attitude and practice parts a scale was used as follows,

4 for 'strongly agree' or 'always', 3 for 'agree' or 'often', 2 for 'uncertain' or 'sometimes', 1 for 'disagree' or 'rarely' and 0 for 'strongly disagree' or 'never'. For a negative statement the points were given in a reverse order. The maximum number of points that a food handler could gain in each part was forty. In each part an arbitrary scale was used to interpret the overall scores for knowledge, attitude and practice. If 80% and more questions were answered correctly by food handlers a score 'good' was assigned, between 50-79% 'moderate score', and below 50% 'poor'.

2.8 Pilot test

A pilot test was organized to check the reliability of the questionnaires. Thirty independent respondents were recruited by Wageningen University, including students with a background in veterinary medicine, animal science, food technology, food safety, and/or food quality management. Homogeneity of answers given by the responders was checked by computing Cronbach's alpha coefficient (Tan et al., 2013). The coefficient based on all questions in the pilot was 0.8, which is above 0.7 and indicates reliability of words, phrases, subjects and point of view.

2.9 Statistical analysis

Statistical analysis of the concentration of *E. coli* on carcasses with visible faecal contamination classified as low or high level and on clean carcasses was performed using a mixed effects model. Comparisons were made between groups (clean, low, and high) including a fixed effect of the slaughterhouse and random effect of batch.

The frequency of carcasses with visible faecal contamination was presented as a percentage. The frequency of fulfilling the actions specified in the assessment criteria on good, sufficient and poor level was calculated separately for each slaughterhouse and each group of food handlers.

The percentage of food handlers in each socio-demographic group was calculated for each slaughterhouse and the percentage of correct answers on knowledge, attitude and practice parts was calculated for each slaughterhouse (Tan et al., 2013).

Furthermore, a coherence test (Ferreira, 2015; Rosenbaum, 2002) was performed for each slaughterhouse separately in order to test the presence of association between number of carcasses with visible faecal contamination and the compliance of operators with criteria to control the evisceration process at locations as vent cutter, opener, eviscerator and post mortem inspection. Based on a list of 15 criteria (Table 1) an overall compliance score was computed stratified by batch (b), location (m), and slaughterhouse (s). Although not all criteria apply for each location where the compliance was observed, an overall score for batch, location and slaughterhouse was computed by giving a score 0 when the criterion was not applicable, score 1 when performed poor, score 2 when performed sufficient and score 3 when performed good. These numbers were added up

to arrive at the overall scores b, m, and s, denoted by $T_{b,m,s}$. Furthermore, the percentage of carcasses that were not scored as clean per batch, location and slaughterhouse, was calculated and denoted by $r_{b,m,s}$. However the food handler at location m (i.e. $T_{b,m,s}$) does not influence the level $r_{b,m,s}$ directly, but rather has influence over the increase in percentage of visibly contaminated carcasses with faeces, hence it is more applicable to study the difference $d_{b,m,s} = r_{b,m,s} - r_{b,m-1,s}$. This implies that the compliance scores for the first location (vent cuter) cannot be used in the analysis. The null-hypothesis was formulated as: for fixed slaughterhouse (s) and location (m), the compliance score ($T_{b,m,s}$) is uncorrelated to $r_{b,m,s}$. Correlation was measured using Pearson's product moment correlation coefficient. The basic idea behind a coherent test is that under the null hypothesis, a permutation of b (say $\pi(b)$) should not influence the correlation between T and R. Hence we may compare the data statistic

$$s_{m,s} = \sum_{b=1}^{B} R_{b,m,s} T_{b,m,s}$$

to the distribution of

$$S_{m,s} = \sum_{b=1}^{B} R_{b,m,s} T_{\pi(b),m,s}$$

arising from many permutations $\pi(b)$ of b. Several terms of the Pearson correlation coefficient are unused, as they have no bearing on the result. Similar to the determination of a p-value in classical hypothesis testing, we may determine the fraction of values of S to the left of s, and if this value is small, the null-hypothesis is likely to be false. This procedure tests associations (Ferreira, 2015; Rosenbaum, 2002) and was computed for data from all locations pooled together. Validity of the model was checked and revealed that it performed as intended.

3. Results

3.1 Adequacy of procedures

In Slaughterhouse A, the existing procedures complied with 27% of the assessment criteria, whereas in Slaughterhouse B the procedures fully corresponded with the criteria (Table 1). Moreover, the structure of the procedures in Slaughterhouse A met 25% of the requirements, whereas this was 83% in Slaughterhouse B (Table 2).

3.2 Compliance of operators with assessment criteria to control evisceration process

Figure 2 presents the number of batches for which the food handlers complied with the assessment criteria as specified in Table 1. It shows that food handlers from Slaughterhouse A complied less frequently with the criteria than food handlers from Slaughterhouse B.

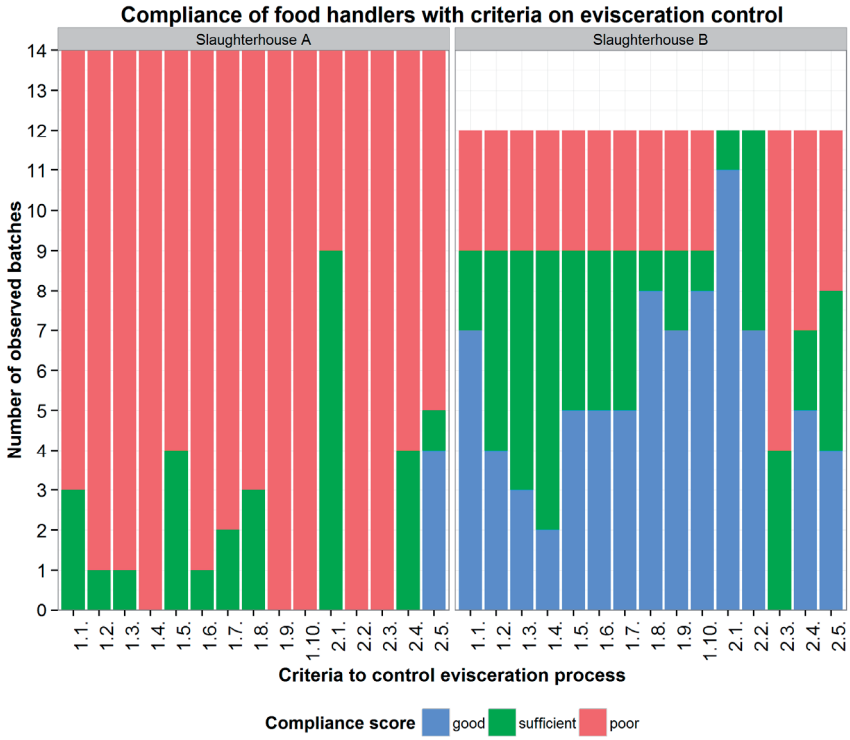

Figure 2
Compliance of food handlers with criteria on evisceration control in two slaughterhouses on: good (blue), sufficient (green) and poor (red) levels.

3.3 Frequency of carcasses with visible faecal contamination

Figure 3 presents the number of carcasses with visible faecal contamination assessed at four locations in the slaughter line. It shows that at most of the tested locations the number of carcasses with visible contamination was higher in Slaughterhouse A than in Slaughterhouse B. The results from the coherent test revealed that there was an association between compliance with control criteria and frequency of carcasses with visible faecal contamination based on combined results from all locations in Slaughterhouse A but not in Slaughterhouse B.

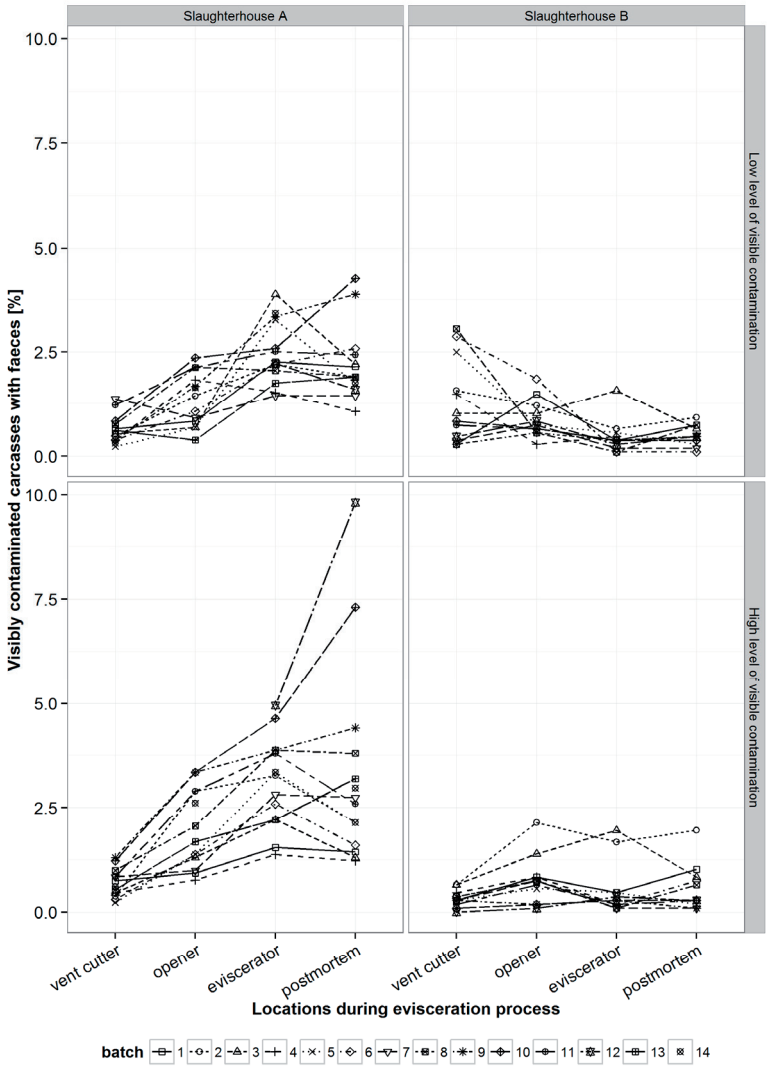

Figure 3
Frequency of carcasses with visible faecal contamination at low and high levels measured at four locations in two slaughterhouses.

3.4 E. coli concentration on carcasses visibly contaminated with faeces

Figure 4 shows *E. coli* concentrations on carcasses with different levels of faecal contamination in the two slaughterhouses. The concentrations on carcasses with low visible contamination were higher than on visibly clean carcasses and differed on average by 0.4 log. On the highly contaminated carcasses, the concentrations were on average 1.5 log higher than on the visibly clean carcasses. In Slaughterhouse A the concentrations on carcasses in all sampled groups were higher by 0.6 log than on

carcasses in Slaughterhouse B. The *E. coli* concentrations in all groups were significantly different between batches from which they originated as confirmed by the significance of the intercept in the mixed effect model used for the analysis. These results are based on a model with random intercept on batch and fixed slope on the slaughterhouse and the level of carcass contamination. This model fitted data better comparing to model with an interaction between slaughterhouse and level of carcass contamination.

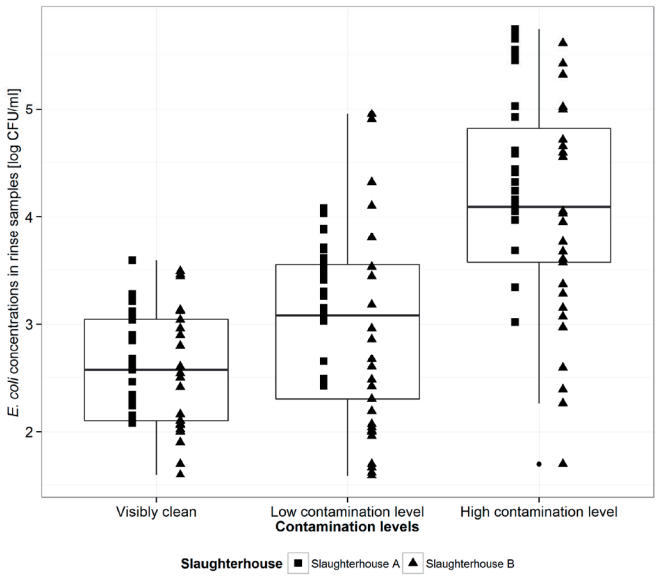

Figure 4
E. coli concentrations (log CFU/ml) on carcasses with different levels of faecal contamination. Samples were obtained from two slaughterhouses. The squares indicate concentrations in the samples obtained in Slaughterhouse A, whereas triangles in B. The line inside each box indicates the median, the upper whiskers indicate 75th percentiles and the lower whiskers indicate 25th percentiles.

3.5 Food handlers' characteristics

Table 3 shows the overview of food handlers' socio-demographic characteristics in the two slaughterhouses. In Slaughterhouse A, most food handlers (9 out of 12) were male and 6 out of 12 food handlers were between 41 and 50 years old. The majority of food handlers (9 out of 12) had secondary school education and 2 food handlers followed training given by the slaughterhouse. One third of the food handlers (4 out of 12) worked there for 15-20 years and one quarter for more than 20 years. In Slaughterhouse B, 13 out of 14 food handlers were male. Five out of the 14 food handlers were between 41 and 50 years old and 5 out of 14 were between 31-40 years old. Almost all food handlers (13 out of 14) had followed training given within the slaughterhouse. One third of the food handlers (5 out of 14), worked there more than twenty years and 4 out of 14 for 10-15 years.

Table 3

Socio-demographic characteristics of food handlers in two slaughterhouses. In total 26 food handlers participated in the study.

Demographic characteristics	Category	Slaughterhouse A		Slaughterhouse B	
		n=	[%]	n=	[%]
Gender	Male	9	75	13	93
	Female	3	25	1	7
Age (years)	Under 21	0	0	0	0
	21-30	0	0	1	7
	31-40	2	17	5	36
	41-50	6	50	5	36
	51-60	3	25	3	21
	Over 60	1	8	0	0
Education	Primary school	0	0	0	0
	Secondary school	9	75	13	93
	High school	1	8	1	7
	University	2	17	0	0
Training	Yes	2	17	13	93
	No	10	83	1	7
Duration working in a slaughterhouse (years)	Under 1	1	8	0	0
	1-5	1	8	2	14
	5-10	1	8	1	7
	10-15	2	17	4	29
	15-20	4	33	2	14
	Over 20	3	25	5	36

Tables 4 to 6 present the outcome of the questionnaires. Overall six answers were excluded, because the food handlers either did not give any answer or gave two contradictory answers. Overall, the knowledge level of food handlers on evisceration process control was good (80% and above) (Table 4). In Slaughterhouse A the level was 87% for operators and 98% for post mortem inspectors, whereas in Slaughterhouse B it was 80% for operators and 91% for post mortem inspectors. The attitude of food handlers in Slaughterhouse A was scored 'moderate' for both operators (77%) and post mortem inspectors (67%), whereas in Slaughterhouse B it was scored as 'good' for both operators (86%) and post mortem inspectors (86%). The score on self-reported practices was 'poor' in Slaughterhouse A for operators (47%) and 'moderate' for post mortem inspectors (70%), whereas in Slaughterhouse B it was scored as 'moderate' for operators (75%) and 'good' for post mortem inspectors (88%).

Table 4

Knowledge of the food handlers on the assessment criteria to set and control the evisceration process obtained in two slaughterhouses. An asterisk next to the number of question indicates negatively coded statements, an hashtag indicates statements in which some answers were excluded.

			Slaughterhouse A							Slaughterhouse B						
	Knowledge of food handlers responsible for controlling and setting of the evisceration equipment	Correct answer	Yes		No		Do Not Know		Score	Yes		No		Do Not Know		Score
			n=	[%]	n=	[%]	n=	[%]	[%]	n=	[%]	n=	[%]	n=	[%]	[%]
1	Bacterial contamination can occur during processing in a slaughterhouse	yes	5	83	0	0	1	17	83	4	100	0	0	0	0	100
2	Proper setting of evisceration equipment may prevent damage of intestine	yes	6	100	0	0	0	0	100	3	75	1	25	0	0	75
3	Change of evisceration equipment setting may be needed after change of each batch	yes	6	100	0	0	0	0	100	3	75	1	25	0	0	75
4*	Proper adjustment of evisceration equipment settings may cause rupture of intestine and increase bacterial contamination	no	1	17	3	50	2	33	50	1	25	3	75	0	0	75
5	Setting of the height of vent cutter depends on the size of carcasses	yes	6	100	0	0	0	0	100	2	50	2	50	0	0	50
6*	Proper adjustment of spraying nozzles in the vent cutter may increase faecal contamination	no	6	100	0	0	0	0	100	1	25	3	75	0	0	75
7	Setting of shackle infeed guide in the opener depends on the size of carcasses	yes	6	100	0	0	0	0	100	4	100	0	0	0	0	100
8*	Correct position of carcasses in the opener may cause rupture of intestine	no	1	17	5	83	0	0	83	1	25	3	75	0	0	75
9*	Controlling the setting of eviscerator may cause bacterial contamination	no	1	17	4	67	1	17	67	0	0	3	75	1	25	75
10	Adjusting shackle infeed guide in the eviscerator can reduce visual faecal contamination on carcasses	yes	5	83	0	0	1	17	83	4	100	0	0	0	0	100
	Average score of correctly answered knowledge questions								87							80
	Knowledge of food handlers responsible for post mortem inspection															
1	Bacterial contamination on carcasses can be caused by presence of faecal material on carcasses	yes	6	100	0	0	0	0	100	10	100	0	0	0	0	100
2	Rupture of intestine may cause visual faecal contamination on carcasses	yes	6	100	0	0	0	0	100	10	100	0	0	0	0	100
3	Correct Post Mortem Inspection after evisceration may reduce the presence of visual faecal contamination on carcasses	yes	6	100	0	0	0	0	100	6	60	3	30	1	10	60
4*	Carcasses with diseases and deficiencies may continue to chilling step	no	6	100	0	0	0	0	100	0	0	10	100	0	0	100
5#	Faecal contamination can be visible as dirty marks or spots on carcasses	yes	6	100	0	0	0	0	100	8	80	0	0	0	0	80
6*	Carcasses with bile contamination may continue after post mortem inspection to the following step	no	6	100	0	0	0	0	100	0	0	10	100	0	0	100
7#	Visual faecal contamination can be eliminated by trimming, cutting, extra washing and removing contaminated carcasses	yes	6	100	0	0	0	0	100	7	70	2	20	0	0	70
8*	Cleaning or sterilizing knife after trimming and cutting can be ignored during inspection	no	6	100	0	0	0	0	100	0	0	10	100	0	0	100
9*	Remained viscera or part of viscera may stay in carcasses after post mortem inspection	no	1	17	5	83	0	0	83	0	0	10	100	0	0	100
10	Number of rejected carcasses per each batch and the cause of rejection can be reported to manager	yes	6	100	0	0	0	0	100	0	0	10	100	0	0	100
	Average score of correctly answered knowledge questions								98							91

Table 5

Attitude of the food handlers on the assessment criteria to set and control the evisceration process obtained in two slaughterhouses. An asterisk next to the number of question indicates negative coded statements, hashtag indicates statements in which some answers were excluded.

			Slaughterhouse A											Slaughterhouse B									
	Attitude of food handlers responsible for controlling and setting of the evisceration equipment	Answer with max. score	Strongly Agree		Agree		Uncertain		Disagree		Strongly disagree		Score	Strongly Agree		Agree		Uncertain		Disagree		Strongly disagree		Score
			n=	[%]	n=	[%]	n=	[%]	n=	[%]	n=	[%]	[%]	n=	[%]	n=	[%]	n=	[%]	n=	[%]	n=	[%]	[%]
1	Preventing bacterial contamination during slaughtering is part of my responsibility	strongly agree	4	67	2	33	0	0	0	0	0	0	92	2	50	1	25	0	0	1	25	0	0	75
2*	Learning how to prevent bacterial contamination is crucial only for manager	strongly disagree	0	0	0	0	1	17	1	17	4	67	88	0	0	0	0	1	25	1	25	2	50	81
3	Setting of the evisceration equipment is part of my responsibility	strongly agree	2	33	1	17	0	0	0	0	3	50	46	3	75	1	25	0	0	0	0	0	0	94
4*	Adjusting the setting of the evisceration equipment is less essential to avoid rupture of intestine and visual faecal contamination	strongly disagree	2	33	0	0	2	33	2	33	0	0	42	0	0	0	0	0	0	2	50	2	50	88
5	Adjusting the setting of the evisceration equipment is needed while changing of each batch	strongly agree	3	50	3	50	0	0	0	0	0	0	88	3	75	1	25	0	0	0	0	0	0	94
6*#	Monitoring if the evisceration equipment runs well during processing is only manager's responsibility	strongly disagree	0	0	0	0	0	0	3	50	3	50	88	0	0	0	0	1	25	0	0	2	50	63
7*	Adjusting the height of vent cutter is necessary only at the beginning of day	strongly disagree	0	0	1	17	0	0	1	17	4	67	83	0	0	0	0	0	0	1	25	1	25	94
8*	Adjusting the height of opener is only important to do at the beginning of day	strongly disagree	0	0	1	17	0	0	2	33	3	50	79	0	0	0	0	0	0	1	25	3	75	94
9*	Checking the setting of eviscerator is only manager's responsibility	strongly disagree	0	0	0	0	0	0	3	50	3	50	88	0	0	0	0	1	25	1	25	2	50	81
10*	Controlling and monitoring the setting of eviscerator is done once per day	strongly disagree	0	0	1	17	0	0	2	33	3	50	79	0	0	0	0	0	0	1	25	3	75	94
	Average score of correctly answered attitude questions												77											86
	Attitude of food handlers responsible for post mortem inspection																							
1	Preventing bacterial contamination during slaughtering is part of my responsibility	strongly agree	0	0	3	50	0	0	0	0	3	50	88	6	60	4	40	0	0	0	0	0	0	90
2	Learning how to prevent bacterial contamination is crucial only for manager	strongly disagree	1	17	1	17	1	17	1	17	2	33	58	0	0	0	0	0	0	1	10	9	90	98
3	Inspection on visual faecal contamination on carcasses is part of my task	strongly agree	4	67	2	33	0	0	0	0	0	0	92	7	70	3	30	0	0	0	0	0	0	93
4*	Visible faeces on carcasses are acceptable	strongly disagree	0	0	1	17	1	17	2	33	1	17	54	0	0	0	0	1	10	1	10	8	80	93
5	Carcasses with diseases and deficiencies are allowed to continue to chilling step	strongly disagree	0	0	0	0	0	0	0	0	6	100	100	1	10	0	0	0	0	1	10	8	80	88
6	The acceptable number of carcasses with visual faecal contamination is 1%	strongly agree	0	0	0	0	3	50	2	33	1	17	33	1	10	2	20	2	20	1	10	4	40	38
7*	Broiler carcasses with visual faecal contamination was continued to next step	strongly disagree	0	0	2	33	0	0	2	33	1	17	50	0	0	0	0	0	0	2	20	8	80	95
8	Removing visual faecal contamination from carcasses is important to be done during inspection	strongly agree	2	33	3	50	0	0	1	17	0	0	75	6	60	4	40	0	0	0	0	0	0	90
9	Removing remained viscera or part of viscera from carcasses is manager's responsibility	strongly disagree	2	33	1	17	0	0	1	17	2	33	50	0	0	0	0	0	0	4	40	6	60	90
10	Recording and reporting number of visual faecal contamination per each batch to manager is part of my task	strongly agree	1	17	4	67	0	0	1	17	0	0	71	6	60	3	30	0	0	1	10	0	0	85
	Average score of correctly answered attitude questions												67											86

Table 6
Practices of food handlers on setting and controlling the evisceration process obtained in two slaughterhouses.

	Practices of food handlers responsible for controlling and setting of the evisceration equipment	Answer with maximum score	Slaughterhouse A											Slaughterhouse B										
			Never		Rarely		Sometime		Often		Always		Score	Never		Rarely		Sometime		Often		Always		Score
			n=	[%]	n=	[%]	n=	[%]	n=	[%]	n=	[%]	[%]	n=	[%]	n=	[%]	n=	[%]	n=	[%]	n=	[%]	[%]
1	Do you adjust the height of vent cutter after change of each batch?	Always	1	17	0	0	3	50	2	33	0	0	50	0	0	0	0	1	25	1	25	2	50	81
2	Do you change the vent cutter setting if carcasses are too small?	Always	0	0	0	0	0	0	4	67	2	33	83	2	50	0	0	0	0	1	25	1	25	44
3	Do you count number of missed carcasses after vent cutter?	Always	5	83	0	0	0	0	0	0	1	17	17	0	0	0	0	1	25	2	50	1	25	75
4	Do you adjust the shackle infeed guide of opener after change of each batch?	Always	1	17	1	17	2	33	2	33	0	0	46	0	0	0	0	2	50	1	25	1	25	69
5	Do you change the height of opener after change of each batch?	Always	1	17	0	0	2	33	2	33	1	17	58	0	0	0	0	0	0	3	75	1	25	81
6	Do you count number of carcasses with damaged intestine after opener?	Always	3	50	1	17	0	0	1	17	1	17	33	0	0	0	0	1	25	1	25	2	50	81
7	Do you adjust the shackle guide of eviscerator after change of each batch?	Always	1	17	0	0	4	67	1	17	0	0	46	0	0	0	0	1	25	2	50	1	25	75
8	Do you adjust the the height of eviscerator after change of each batch?	Always	1	17	1	17	3	50	1	17	0	0	42	0	0	0	0	0	0	2	50	2	50	88
9	Do you check feed withdrawal time of the carcasses per batch?	Always	2	33	1	17	0	0	2	33	1	17	46	0	0	0	0	1	25	0	0	3	75	88
10	Do you count the number of carcasses with visual faecal contamination after eviscerator?	Always	3	50	0	0	0	0	1	17	2	33	46	0	0	1	25	0	0	2	50	1	25	69
	Average score of correctly answered knowledge questions												47											75
	Practices of food handlers responsible for post mortem inspection																							
1	Do you inspect faecal contamination inside of carcasses?	Always	0	0	0	0	3	50	3	50	0	0	63	0	0	0	0	0	0	5	50	5	50	88
2	Do you check the presence of faecal spots outside of carcasses?	Always	0	0	0	0	0	0	2	33	4	67	92	0	0	0	0	1	10	3	30	6	60	88
3	Do you check the presence of diseases and deficiencies of carcasses?	Always	0	0	0	0	0	0	0	0	6	100	100	0	0	0	0	0	0	1	10	9	90	98
4	Do you check the presence of bile contamination on carcasses?	Always	0	0	0	0	0	0	2	33	4	67	92	0	0	0	0	0	0	3	30	7	70	93
5	Do you allow carcasses with faecal spots to continue to next steps?	Never	1	17	0	0	2	33	1	17	1	17	38	6	60	3	30	1	10	0	0	0	0	88
6	Do you remove carcasses with visual faecal contamination from conveyor?	Always	0	0	0	0	4	67	0	0	1	17	50	0	0	0	0	2	20	2	20	6	60	85
7	Do you remove visual faecal contamination by trimming, cutting, extra rinsing?	Always	2	33	1	17	2	33	1	17	0	0	33	0	0	1	10	2	20	3	30	4	40	75
8	Do you sterilize or clean your knife before and after trimming and cutting?	Always	0	0	2	33	0	0	1	17	2	33	54	0	0	0	0	1	10	3	30	6	60	88
9	Do you record number of visual faecal contamination on carcasses per each batch?	Always	0	0	0	0	0	0	0	0	6	100	100	0	0	0	0	0	0	2	20	8	80	95
10	Do you report rejected carcasses to manager?	Always	1	17	0	0	0	0	0	0	5	83	83	1	10	0	0	0	0	2	20	7	70	85
	Average score of correctly answered knowledge questions												70											88

4. Discussion

This observational study aimed at investigating whether compliance of food handlers' with criteria on controlling and setting the evisceration process may contribute to differences in the impact of evisceration between slaughterhouses determined previously (Pacholewicz et al., 2015). The current study showed that in Slaughterhouse A carcasses with visible faecal contamination were observed more frequently than in Slaughterhouse B (Figure 3). Also in Slaughterhouse A, non-compliance with the criteria on evisceration control was more frequent (Figure 2). Knowledge of food handlers in both slaughterhouses was scored on a good level. The attitude was scored as moderate among both groups of food handlers in Slaughterhouse A, whereas good in Slaughterhouse B. The self-reported practices fulfilled by food handlers were scored 'poor' for operators in Slaughterhouse A and 'moderate' for the post mortem inspectors. In Slaughterhouse B the practices were scores as moderate for both groups of food handlers.

Differences in the control of the evisceration step between the studied slaughterhouses observed in the current study may potentially explain the differences in the occurrence of visibly contaminated carcasses (Figure 3). Our results demonstrate that the visibly contaminated carcasses after evisceration have higher *E. coli* concentrations than visibly clean carcasses (Figure 4). This is in agreement with previous studies (Burfoot and Allen, 2013; Cibin et al., 2014). In the present study, carcasses with even small spots of faecal and caecal content after evisceration carried on average a significantly higher load of *E. coli*. Similar findings were reported with respect to *Campylobacter* (Berrang et al., 2004). Visibly clean carcasses in the current study carried *E. coli* in concentrations between 1.6 and 3.6 log CFU/ml (Figure 4). In Slaughterhouse A the concentrations on visibly clean carcasses were higher than in Slaughterhouse B, which is in agreement with previous findings (Pacholewicz et al., 2015) where in one slaughterhouse carcasses carried higher *E. coli* concentration after evisceration than in another. The variation observed in *E. coli* concentration on carcasses within a category of visibly contaminated carcasses (Figure 4) suggests that even if carcasses fall in one category based on visual assessment, they may carry variable concentration of *E. coli.*

Our study shows the importance of procedures that should document all activities that food handlers need to do in order to assure that they know their tasks and responsibilities to prevent deviations (Yiannas, 2008). The role of procedures is to support food handlers in taking appropriate and consistent decisions to meet food safety goals (Luning and Marcelis, 2007).

The compliance of food handlers with the criteria on evisceration control differed between slaughterhouses e.g. setting of the evisceration equipment and removing carcasses or their parts with visible faecal contamination in Slaughterhouse A was hardly ever carried out, whereas in Slaughterhouse B it was frequently carried out

(Figure 2). Data analysis in our study revealed an association between the presence of visibly contaminated carcasses and the compliance of food handlers with the criteria on evisceration control only in Slaughterhouse A. Lack of association between compliance with evisceration controls and contamination of carcasses in Slaughterhouse B suggests that the contamination may occur also due to other factors as e.g. uniformity of batch because the evisceration equipment cannot be adjusted to an individual carcass.

In both slaughterhouses the scores of knowledge level were good (>80%). However in Slaughterhouse A operators reported fulfilling 47% control tasks during the evisceration process and post mortem inspectors 70% of the tasks, whereas in Slaughterhouse B operators reported 75% and post mortem inspectors 85% (Table 6). This was confirmed qualitatively by actual observations, showing that food handlers in Slaughterhouse A complied with fewer tasks than in Slaughterhouse B (Figure 2). Likewise in a study of Clayton et al. (2002) food handlers had knowledge about their tasks, but they have not always implemented it into practice. Lack of implementation of knowledge in actual practices of food handlers in various food premises was widely reported (Abdul-Mutalib et al., 2012; Angelillo et al., 2000; Ansari-Lari et al., 2010; Baş et al., 2006; Clayton et al., 2002; Tokuç et al., 2009). Adoption of knowledge in practice is a challenge, even after providing training, as demonstrated by Sanny et al. (2013). In our study, only 2 out of 12 food handlers in Slaughterhouse A reported that they had training provided by the slaughterhouse, whereas the majority of food handlers (13 out of 14) from Slaughterhouse B reported this. Despite lack of training, the knowledge of food handlers in Slaughterhouse A was even scored as good likewise in Slaughterhouse B. In principle, theoretical training can improve the knowledge level of food handlers, but it can only have a limited effect on food handlers' attitude and practices (da Cunha et al., 2014). Various researchers suggested that training can influence practices of food handlers only if it employs adequate strategies to change attitude and motivation (da Cunha et al., 2014; Rennie, 1994; Tokuç et al., 2009). Ko (2013) reported that the attitudes of food handlers mediate the relationship between their knowledge and practices. More attention should be thus given to enhancing the attitude of food handlers. Additional measures beyond training were suggested to potentially influence the practices of food handlers, such as e.g. routine inspection (Bolton et al., 2008), strict monitoring of compliance with procedures (Sanny et al., 2013) or incentives for food handlers to reward practical implementation of knowledge (Mitchell et al., 2007).

Another explanation of the discrepancy between knowledge and practices can be seen in an "optimistic bias effect" (da Cunha et al., 2014; Wilcock et al., 2004) meaning that people perceive that the risk of a negative event is lower for them than for other people.

Furthermore, it was reported that the practices of food handlers can change only if the food safety culture changes and if the organization provides the necessary resources

(Clayton et al., 2002). Food safety culture was thus proposed to be considered as an "emerging risk factor" contributing to an increase in the likelihood of food poisoning (Griffith et al., 2010). Recently, food safety culture has been defined as an interaction between two routes, the human route and the techno-managerial route (De Boeck et al., 2015). The human route was defined as a food safety climate perceived by employees and managers in a company. The techno-managerial route was defined as the context in which the company operates. The components of the food safety climate (human route) include leadership, communication, commitment, environment, and risk perceptions (Griffith et al., 2010). These components should be further compared between broiler slaughterhouses to determine additional factors influencing practices of food handlers and thus bacterial concentrations on broiler meat.

The methods developed for the purpose of our case study could be applied across the poultry sector to recognise in depth the role of food handlers and organisation in *Campylobacter* control. Also these methods can be used to revise procedures available in the slaughterhouses. Similarly, the assessment criteria could be developed for other steps during poultry processing such as defeathering. In addition, implementation of the questionnaires in many slaughterhouses would enable managers to recognise how compliance could be improved. The questionnaires could be extended not only to food handlers but also to the management level. This could provide a picture of food safety culture in organisations that might influence the safety of broiler meat.

In conclusion, this case study demonstrates that adequate procedures to control the evisceration process and compliance of food handlers with these procedures may contribute to the reduction of the number of carcasses with visible faecal contamination, and to the prevention of an increase in bacterial concentrations on carcasses after this processing step. These findings suggest that managerial aspects of the organisation might influence food safety. However in order to demonstrate the influence of the compliance with procedures on contamination of poultry carcasses an intervention study needs to be performed.

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Chapter 8

General discussion

Broiler meat can be a source of biological hazards for consumers (European Food Safety Authority, 2012). One of these hazards, thermophilic *Campylobacter* spp., causes this decade's most commonly reported zoonosis in Europe: campylobacteriosis (European Food Safety Authority and European Centre for Disease Prevention and Control, 2015). Risk assessment studies have indicated that one way to diminish human cases in the short term is to reduce the number of thermophilic *Campylobacter* bacteria on broiler meat (Havelaar et al., 2007). Strategies to reduce *Campylobacter* concentrations on carcasses during broiler processing might also reduce other bacteria, such as e.g. *Salmonella*, ESBL/AmpC producing *E. coli* and generic *E. coli*, although quantitative data testing this hypothesis are missing. Importantly, *Campylobacter* concentrations on broiler carcasses at the end of processing differ among slaughterhouses and the causes of these differences have not so far been identified (European Food Safety Authority, 2010). Identification of such causes could support development of control measures in broiler slaughterhouses to diminish *Campylobacter* concentrations on carcasses.

In response, this thesis provides quantitative data on the effect of selected processing steps on *Campylobacter,* generic *E. coli* and also, for the first time, on ESBL/AmpC producing *E. coli,* giving insight into their changes in concentration on carcasses at various steps in broiler processing (Chapters 3 and 5). It also describes investigations of technological factors and, for the first time, managerial factors influencing these changes (Chapters 4 and 7). Further, it provides the results of preliminary tests of a novel technological intervention (Chapter 6) and gives suggestions for managerial interventions (Chapter 7). In order to further evaluate and implement the interventions proposed in this thesis, it would be desirable to have a rapid, high-throughput method for enumeration of *Campylobacter* concentrations on the carcasses. However, a published PMA-qPCR-method could not be recommended for this purpose (Chapter 2) as it was not sufficiently able to differentiate between live and dead *Campylobacter* cells.

The information presented in this thesis could support various stakeholders during the implementation, in the near future, of *Campylobacter* Process Hygiene Criteria (PHC) in broiler slaughterhouses: implementation of a PHC for *Campylobacter* in poultry was discussed by the European Commission with Member States for the first time in October 2015 (personal communication Mr. A. Ottevanger, Ministerie van Volksgezondheid, Welzijn en Sport). The current proposal includes implementation of a three class PHC, limiting *Campylobacter* to 1000 CFU/g of breast skin, as in the voluntary program implemented in the Netherlands since 1 March 2014. In this approach, the tolerated limit is that out of 5 collected samples, 3 can carry *Campylobacter* at a level between 1000 and 10000 CFU/g and no sample can exceed 10000 CFU/g (Anonymous, 2015). According to the risk assessment performed by the National Institute for Public Health and the Environment (RIVM), implementation of a two class criterion, in which out of 5 collected samples none exceeds the limit of 1000 CFU/g,

should lead to reduction of human campylobacteriosis cases by 70% (Swart et al., 2013). The three class criterion is likely to result in a lower preventable fraction of campylobacteriosis (personal communication Dr. A.N. Swart, RIVM).

Slaughterhouses

Compliance with the PHC might be a challenge for slaughterhouses, especially in countries where the *Campylobacter* prevalence in slaughtered flocks is high. In these countries a tendency for high concentrations on carcasses was also reported (European Food Safety Authority, 2010). The compliance can be also challenged by the seasonality. Data from one of the Dutch reports (Anonymous, 2015) suggests that between April and December 2014 the number of samples exceeding the limit was higher between May and October and lower in April, November and December.

The results presented in this thesis (Chapter 3) confirm that *Campylobacter* concentrations on broiler carcasses after chilling differed between tested batches in both slaughterhouses and between slaughterhouses in line with the previous findings (Anonymous, 2011; European Food Safety Authority, 2010). In addition to most previous studies, this research investigated the explanatory variables potentially contributing to the differences.

Campylobacter concentrations after chilling can be influenced by both batch (i.e. the concentrations on exterior of the carcasses after bleeding and concentration in their faeces and caeca) and slaughterhouse operations (Chapters 3, 4 and 5). The concentrations after bleeding differed between the tested batches in both slaughterhouses (Chapter 3), in agreement with another recent study (Seliwiorstow et al., 2015a). These concentrations can be influenced not only by hygiene control and other factors on farms, but also during transport. Transportation was demonstrated to increase the *Campylobacter* concentrations on the exterior of the carcasses (Seliwiorstow et al., 2013).

Further, this thesis revealed that despite similar *Campylobacter* concentrations on carcasses after bleeding in the two slaughterhouses, the overall reduction in concentrations on carcasses was different (Chapter 3). This implies that differences in the performance of processing steps contribute to variations in bacterial concentrations between the slaughterhouses at the end of slaughtering, as suggested by others (European Food Safety Authority, 2010; Zweifel et al., 2015). While in Slaughterhouse 1 the changes (increases or decreases) in *Campylobacter* concentrations through processing were consistent between the tested batches, they varied in Slaughterhouse 2 (Chapter 3, Figure 1). In both slaughterhouses, scalding and chilling contributed to reductions in *Campylobacter* concentrations. However, the magnitude of this reduction after scalding differed between the slaughterhouses. Further, while defeathering caused significant increase in *Campylobacter* concentrations in Slaughterhouse 2, in Slaughterhouse 1 it was

the evisceration process that caused significant increase in concentrations. The changes in *Campylobacter* concentrations through the processing correspond with previous studies (Guerin 2010; Rosenquist 2006) i.e. decrease during scalding, increase during defeathering, variable effect of evisceration, decrease during chilling. However this study showed that magnitude of increases and decreases differs between slaughterhouses. Thus the results obtained in this thesis confirm that the pattern of contamination is slaughterhouse specific (Chapter 3). However within each slaughterhouse, the increases and decreases in *Campylobacter* were similar to changes in both generic *E. coli* and ESBL/AmpC producing *E. coli*, except for during defeathering. During defeathering both *E. coli* and ESBL/AmpC producing *E. coli* concentrations on carcasses decreased (Chapters 3 and 5, Figure 1).

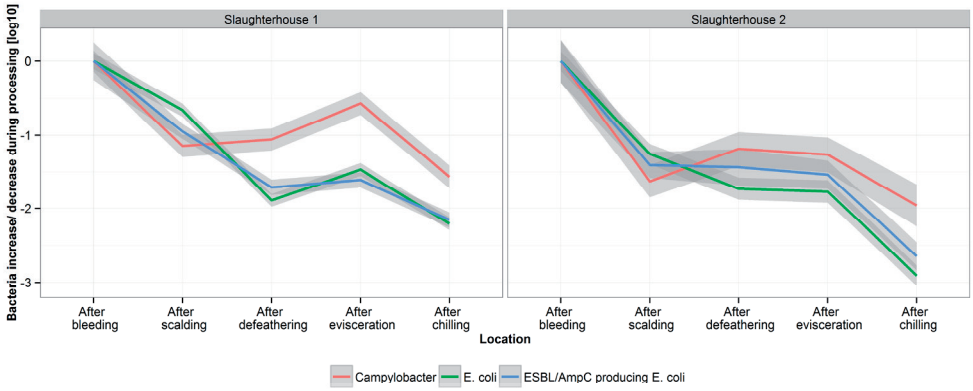

Figure 1
Average change in *Campylobacter,* ESBL/AmpC producing *E. coli* and generic *E. coli* concentrations after processing steps. Lines show the average effect, whereas the shaded area indicates one standard error of an effect.

The potential causes of the observed differences in the impact of the processing steps on bacterial concentrations were analysed considering the technological aspects including batch characteristic and processing parameters (Chapter 4). Also managerial aspects were considered including adequacy of procedures and compliance of food handlers with them (Chapter 7).

Higher *Campylobacter* reduction after scalding in Slaughterhouse 2 can be explained by the higher scalding temperature applied in that slaughterhouse in comparison to the other. Although the higher temperature effectively reduces the bacterial concentration, it affects adversely the quality of the carcasses i.e. sensory defects to the skin (Lehner et al., 2014). Higher scalding temperatures in the EU are usually applied to batches that will be frozen after processing.

With respect to defeathering a statistical association was found between the *Campylobacter* and *E. coli* concentrations on carcasses after defeathering and their concentrations in excreta (for *E. coli*) and caeca (for *Campylobacter*). Interestingly, these

associations were present in Slaughterhouse 2 where *Campylobacter* concentrations increased significantly during defeathering and the *E. coli* decrease was smaller than in the Slaughterhouse 1. This result suggests that although the leakage of the intestinal material is a well-recognised source of contamination during defeathering (Berrang et al., 2001; Musgrove et al., 1997) it might have been more pronounced in one slaughterhouse than in the other.

Another potential variable explaining the different effect of defeathering between slaughterhouses could be the uniformity of the processed batches (Chapter 4). A statistical association between uniformity and the *E. coli* concentrations on carcasses after defeathering was seen in both slaughterhouses. However, in Slaughterhouse 1, a greater decrease in *E. coli* was observed in more uniform batches, but the opposite was seen in Slaughterhouse 2. This difference might suggest a potential role of the settings of the defeathering equipment by food handlers while processing poorly uniform batches. A better uniformity of the batch is expected to make settings easier.

Food handlers' practices varied between slaughterhouses, as demonstrated for evisceration (Chapter 7), potentially causing differences in the effect of this step. It was observed that the procedures to control this process differed between slaughterhouses. One slaughterhouse had less detailed procedures, with the food handlers barely monitoring the evisceration process and seldom adjusting the equipment settings between batches. This slaughterhouse also had a higher frequency of carcasses with visible faecal contamination. Occurrence of such contamination is common in slaughterhouses (Rosenquist et al., 2006), but may vary between slaughterhouses as demonstrated in Chapter 7 and elsewhere (Seliwiorstow et al., 2015b). As visibly contaminated carcasses carried higher *E. coli* concentration (Chapter 7; Burfoot and Allen, 2013; Cibin et al., 2014), it can be concluded that compliance with procedures on setting and controlling the evisceration may influence bacterial concentrations on carcasses.

Additional potential explanations of the difference in the effect of evisceration between slaughterhouses could be the different spraying system following the eviscerator or differences in the weight of the carcasses in the eviscerated batches (Chapter 4). Weight of the carcasses in the slaughter batches was associated with changes in *Campylobacter* concentrations on the carcasses during evisceration (Slaughterhouse 2). This may suggest that in batches with heavier carcasses, an increase in *Campylobacter* concentrations on carcasses during evisceration is more likely. The evisceration process is an example of a process in which it became clear that the bacterial contamination on the carcasses may be influenced by interactions between product, equipment and food handlers.

The last studied processing step, namely chilling, consistently contributed to decreases in the *Campylobacter* concentrations on carcasses (Chapter 4). Although there were no statistical differences in the effect of chilling on *Campylobacter* reductions between the slaughterhouses (Chapter 3) in general, some parameters might still influence

the reduction. These include temperature, time, air velocity and initial concentrations (Rivoal et al., 2015).

Regardless of the complexity of the problem of *Campylobacter* contamination on carcasses during processing, slaughterhouses must strive to stay below the limit specified by the PHC. In one of the tested slaughterhouse, 20 out of 32 collected breast skin samples exceeded the limit of 1000 CFU/g (Chapter 3), whereas in the other, all of tested 24 skin samples were below the 1000 CFU/g (Slaughterhouse 2). Each slaughterhouse will need to address different issues to comply with the PHC. For example, one of the tested slaughterhouses needs to eliminate the cause of the higher concentrations, the other must address the cause of variation in the effect of the processing steps between the batches. Each slaughterhouse needs to identify its individual pattern of changes in bacterial concentrations on carcasses throughout processing in order to apply suitable control measures. For this, quantitative data needs to be obtained from the processing line. Ideally, collecting data on *Campylobacter* through the processing would be the best way to gain insight in the effect of processing on concentrations. However as rapid quantification methods are currently unavailable (Chapter 2), such data has to be gathered by a complex and time consuming traditional culture method. Alternatively, monitoring of the changes in the bacterial levels along the processing line could be based on *E. coli* concentrations, because its changes along the processing line share sufficient similarities with *Campylobacter* and ESBL/AmpC producing *E. coli* within a slaughterhouse (Figure 1). Monitoring based on *E. coli* is less expensive and easier to perform than *Campylobacter* quantification by culture (Altekruse et al., 2009; European Food Safety Authority, 2012; Habib et al., 2012). Monitoring based on *E. coli* can be less variable than monitoring *Campylobacter* because *Campylobacter* concentrations between the batches vary more than *E. coli*. In addition, hygiene monitoring based on *E. coli* can be done for all batches, even those that are *Campylobacter* negative. This collection of quantitative data along the processing line should not be limited to single occasions, but should be designed to recognise variation between batches. Although this data collection would require effort and resources, it would provide the slaughterhouse management valuable insights in both the variability in *Campylobacter* contamination at the beginning of the processing and the variability in the effect of processing steps on concentrations. Such insights could then be used to implement site-specific interventions that are likely to reduce multiple hazards on broiler chicken carcasses (Chapters 3 and 5, Figure 1).

The slaughterhouses may invest in technological or managerial interventions. Probably, an intervention combining both approaches would have the greatest benefit.

One example of the technological intervention could be a novel equipment to brush the exterior of the carcasses before scalding (Chapter 6). Such brushing reduces the *E. coli* and *Enterobacteriaceae* concentrations on the surface of the carcasses. Although the overall reduction on the whole carcass was low (0.3 log) it was significant

(p <0.05), opposite to previous study where insignificant reduction was reported (Berrang and Bailey, 2009). However in that study a conventional brush was used. In the current study, higher reductions (up to 1 log) were observed on the brushed area of vent and breast, what provided an insight to the manufacturer for further optimisation of the brushes. The brushes can be seen as an effective hurdle reducing bacterial concentration on carcasses before scalding. Combined with other interventions, they could lead to biologically relevant reductions (Berrang and Bailey, 2009). Reducing contamination at the beginning of processing is important, because it could prevent contamination at the following processing steps.

In addition, the brushes significantly reduced the dry matter on carcasses entering the scalding tank (Chapter 3). The dry matter, especially faecal material and dissociation of its constituent i.e. ammonium urate contributes to a decrease in the pH value of the scalding water (Humphrey, 1981). The pH of scalding water influences heat resistance of *Campylobacter* and *Salmonella* (Humphrey and Lanning, 1987; Okrend et al., 1986). Thus the effect of brushing on pH level of scalding water and bacterial concentrations in the water and on carcasses after the scalding should be addressed in future studies on brushes applied in a commercial processing line.

Another example of a technological intervention could be modernisation of the equipment. As observed, one of the tested slaughterhouses used a different way of spraying the carcasses after the evisceration machine than the standard cabinet provided by the manufacturer. This difference could potentially explain the occurrence of higher number of carcasses with visible faecal contamination and higher bacterial concentrations. The contamination was lower in the other slaughterhouse having such standard cabinet (Chapters 4 and 7). However parameters as water consumption used to spray the carcasses were not compared.

From a managerial perspective, the controlling and setting of the processing equipment may be an important intervention preventing bacterial contamination on carcasses (Chapter 7). For example, monitoring the settings of the evisceration equipment contributed to improvement in *Campylobacter* control during this process in some slaughterhouses in New Zealand (Biggs, 2015). The control of the evisceration equipment was not always performed in the slaughterhouses, as demonstrated in Chapter 7. The reasons for non-compliance were investigated through research into food handlers' knowledge, attitude and practices. Overall, it was observed that food handlers, despite having knowledge of evisceration process control, do not always implement this. Similar discrepancies between knowledge and practice have been commonly reported in other food businesses (Angelillo et al., 2000; Baş et al., 2006; Tokuç et al., 2009). In addition, the study described in Chapter 7 demonstrated that the level of discrepancy differed between the two studied slaughterhouses, as did the attitude of food handlers. The attitudes of food handlers seem to mediate the relationship between knowledge and practices (Ko, 2013).

As results described in Chapter 7 suggest, managerial interventions are needed to support the food handlers in fulfilling their tasks. These could include not only providing the food handlers with adequate procedures and other resources including education/training, time and infrastructure (De Boeck et al., 2015; Yiannas, 2008), but also implementing interventions that will encourage compliance. Theoretical training does not contribute to the implementation of knowledge in practice (da Cunha et al., 2014). Training may impact practice only if it aims at changing attitudes and motivation among the food handlers (da Cunha et al., 2014; Rennie, 1994; Tokuç et al., 2009). Some authors have suggested that compliance can be achieved by the stricter monitoring or inspection of food handlers (Bolton et al., 2008; Sanny et al., 2012; 2013). Potentially, incentives to reward food handlers for adequate behaviour (Mitchell et al., 2007) could be more effective as demonstrated e.g. in Sweden, where producers are rewarded for providing batches free from *Campylobacter* (Hansson et al., 2015). Further interventions could be related to organisational factors (work pressure, managerial commitment, policy and norms) that could influence the practices of the food handlers (Mitchell et al., 2007). Involvement of management is thus needed to create a food safety climate (De Boeck et al., 2015) in which desired practices to improve compliance of food handler's with procedures are performed.

Equipment manufacturers

Technological innovations are also needed to support the slaughterhouses in achieving the limit specified by the *Campylobacter* PHC. So far, the automation of broiler processing has reduced contamination of broiler carcasses with biological hazards, mostly because it limits human handling (Tsola et al., 2008). As the findings described in Chapter 3 demonstrate, mechanical defeathering can be as hygienic as removing the feathers manually: *Campylobacter* concentrations on carcasses defeathered mechanically were similar to those on the manually defeathered carcasses in both slaughterhouses. *E. coli* concentrations were similar on carcasses defeathered manually and mechanically in one slaughterhouse, whereas in the other slaughterhouse the concentration on mechanically defeathered carcasses was even lower than on manually defeathered carcasses. Another example of improvement through automation is given in the literature: the automation of the evisceration process and the hygienic design of the equipment used (including the continuous washing of the spoons used to remove viscera) contributed to better hygiene standards than did the manual performance of this process. With line speeds currently up to 13,500 birds per hour, hygienic standards such as washing hands after touching each carcasses would be impossible to meet (Barbut, 2014; Tsola et al., 2008). At the same time, hygienic standards are also challenged by automation, mostly related to the fact that currently available equipment is unable to address weight variation

of the broiler carcasses within and between batches. This can and does sometimes lead to viscera rupture and the concomitant faecal contamination of the carcasses (Rosenquist et al., 2006). Poor within-batch uniformity in carcass weight thus contributes to increase in prevalence in *Campylobacter* contamination during evisceration (Malher et al., 2011). For the same reason, variations in weight between batches are also a challenge. As demonstrated in Chapter 4, batches with heavier carcasses were associated with an increase in *Campylobacter* concentrations during evisceration. Uniformity of the batch was confirmed as an important factor also for the defeathering process (Chapter 4).

A statistical association between the presence of feathers on carcasses after defeathering and a more effective decrease in *E. coli* concentration during defeathering was observed in one slaughterhouse, suggesting that equipment settings may impact bacterial contamination. A less tight setting in the defeathering equipment may result in incomplete defeathering, but the pressure on the carcass abdomen is also lower, leading to less faecal leakage and thus less contamination.

The contributions made to contamination by equipment settings and their control by food handlers were explored in the study described in Chapter 7 in the context of evisceration. This revealed that human factors related to automated processes may influence the presence of faecal contamination and thus contribute to an increase in *E. coli* contamination. Even where the evisceration equipment was designed according to hygienic practices and was similar in two studied slaughterhouses, if it is not set and controlled between the batches by the food handlers its functioning can lead to contamination of carcasses with faeces and thus bacteria (Chapter 7). This conclusion may apply not only to evisceration equipment but in general to all processing equipment.

The effect of the equipment setting on processing hygiene and safety could be addressed during the training provided by manufacturers to the slaughterhouses. As demonstrated, knowledge gained during training decreases over time, and thus training should be repeated periodically (McIntyre et al., 2013). The slaughterhouses need to be aware that training is needed and that food handlers need to be motivated to implement the gained knowledge into practice.

The variation in weight of the carcasses within and between batches seems to influence the performance of the equipment, especially if the equipment is not adjusted by the food handlers during processing. Reduction or elimination of human handling during poultry processing could support the slaughterhouses in improving processing hygiene, and this might be a more realistic goal, given that changes in the behaviour of food handlers is difficult to achieve (Sanny et al., 2013). Potentially an option to deal with weight heterogeneity during broiler processing is to pre-sort a batch at early processing steps into certain weight groups to adjust equipment more precisely as e.g. in the fish deboning industry (Barbut, 2014). Other potential solutions include automating the adjustment of the equipment settings.

Technological improvements during defeathering are also needed, as this step can contribute to an increase in *Campylobacter* concentrations (Chapter 3). A statistical association between *Campylobacter* concentration in caeca and *Campylobacter* contamination on carcasses after defeathering was seen (Chapter 4), consistent with Reich et al. (2008). This implies that leakage of intestinal material during defeathering contributes to contamination of carcasses, as has been reported elsewhere (Berrang et al., 2001; Musgrove et al., 1997) and underlines the need for the development of interventions to prevent such leakage.

Cleaning the exterior of the carcasses in the batches prior to scalding by a novel brushing system was tested and described in Chapter 6. The small scale brushing system reduced the concentration of *E. coli* and *Enterobacteriaceae* significantly. While the reduction on the whole carcasses was low (0.3 log), a higher reduction (up to 1 log) was observed on the breast and vent area of the carcasses that were directly brushed. Bacteria are distributed over the entire carcass and extending the brushed surface might contribute to an increase in the bacterial reductions. Brushing the exterior of the carcasses prior to scalding is expected to contribute to a decrease in the bacteria in the scalding water and on the carcasses after scalding. However with the small scale brushing system this effect could not be tested. To demonstrate the effect tests are needed with the brushes installed in the processing line.

Government

Setting a PHC will stimulate improvements in broiler processing hygiene, by communicating the level that should be achieved to control *Campylobacter,* and to monitor the ability of slaughterhouses to reduce the *Campylobacter* on the carcasses measured after chilling (European Food Safety Authority, 2012). Indeed, these outcomes were observed in New Zealand, where implementation of microbiological criteria (there called the *Campylobacter* Performance Target (CPT)), has contributed to a reduction in broiler carcasses carrying high *Campylobacter* concentrations after chilling and to a reduction in human campylobacteriosis cases (Lee et al., 2014). The CPT was implemented in 2007, when the reported incidence of campylobacteriosis cases was 160 cases per 100 000 persons. By 2012 the number of cases had dropped to 80 per 100 000 persons. Based on source attribution studies, it is clear that campylobacteriosis cases attributed to poultry dropped by 74% (Sears et al., 2011). The Government of New Zealand has not set mandatory interventions to reduce the *Campylobacter* concentrations on carcasses. Each slaughterhouse had to decide which control measure to apply. The results presented in this thesis suggest that this is a good approach also for the European slaughterhouses, given the observed differences between slaughterhouses.

Implementation of the *Campylobacter* PHC across Europe can be seen as a first step towards improvement in poultry meat inspection, as described in an opinion of the EFSA BIOHAZ panel (European Food Safety Authority, 2012). In this opinion, a proposal was described to replace the current post mortem inspection based on visual evaluation with a PHC for the main biological hazards. A PHC can be more informative, given that visual assessment is unable to detect the major biological hazards to public health that are related to poultry meat consumption (European Food Safety Authority, 2012).

The findings presented in Chapter 7 can contribute to a discussion of the future of poultry meat inspection. They confirm that carcasses with visible faecal contamination after evisceration carry higher concentration of *E. coli* than visibly clean carcasses. On average, even a small spot of faecal material contributes to a significantly higher concentration of *E. coli* on that carcass (by 0.4 log). Although this difference was statistically significant (p=0.001) is may not be biologically relevant. In addition, a large variation was observed in the *E. coli* concentration in visibly contaminated carcasses with a single faecal spot. This suggests that bacterial concentrations on visibly dirty carcasses depend on the concentration in faeces and/or caeca. Thus it is not always the single visible spot of faeces that leads to an increase in bacterial concentration. Further, *E. coli* concentrations can differ significantly between carcasses without visible faecal contamination (Chapter 7). This confirms limitations of visual inspection on judging the level of biological hazards on carcasses and is in agreement with Cibin et al. (2014), who reported that inspectors have a low probability of classifying carcasses carrying high *E. coli* and *Enterobacteriaceae* concentrations based on visual evaluation. These results support the implementation of Process Hygiene Criteria as more appropriate measure of hygiene during the broiler processing.

The EFSA BIOHAZ panel (European Food Safety Authority, 2012) also proposed implementation of the PHC based on ESBL/AmpC producing *E. coli*. Pending more information on the relative role of poultry meat source attribution on ESBL/AmpC producing *E. coli* in humans, the quantitative data along the processing line, described for the first time in this thesis (Chapter 5), would contribute to setting such a target and its potential integration with criteria for other hazards. The data can also support the risk assessment on human exposure to 3rd generation cephalosporin resistant *E. coli,* so far based on prevalence data only (Depoorter et al., 2012). The exposure was reported to be influenced by both the proportion of 3rd generation cephalosporin resistant *E. coli* in the incoming batches and by the level of *E. coli* on carcasses. Data reported in this thesis (Chapter 5) confirm as well that the control of ESBL/AmpC producing *E. coli* both in the primary production and also at slaughter is important to reduce the level on the final product reaching the consumers. These levels varied from 2-5 log CFU/carcass (Chapter 5). A recent study in Germany reported that the ESBL

producing *E. coli* concentration in the final product was between 1-3.1 log CFU/g of neck skin (Reich et al., 2016). Although these results cannot be directly compared due to the differences in sample type, they indicate variation in concentrations between collected samples.

Not only did the ESBL/AmpC producing *E. coli* concentrations differ between batches, but also the proportion of the ESBL/AmpC producing among the generic *E. coli*, which is in agreement with the recent report by Reich et al. (2016). The observed difference in proportion points to the role of primary production and both horizontal and vertical spread of ESBL/AmpC producing *E. coli* isolates (Dierikx et al., 2013). As reported by Persoons et al. (2011) factors influencing the occurrence and levels of antimicrobial resistance include, in addition to antibiotic use, management factors (e.g. poor hygienic condition) and the hatchery from which the chickens originate.

In addition, the EFSA proposed an implementation of PHC based on *E. coli* to monitor the efficiency of slaughterhouses, as a way to reduce the bacterial concentrations on carcasses (European Food Safety Authority, 2012). However given the upcoming PHC based on *Campylobacter,* the need for PHC based on *E. coli* could be reconsidered. It would enable hygiene control in *Campylobacter* negative flocks. Further, if slaughterhouses implement measures to reduce *Campylobacter* below the required limit, this will most probably improve their general hygiene practices and reduce the level of generic *E. coli* as well ESBL/AmpC producing *E. coli*.

Further research

The research described in this thesis has identified several areas for additional investigation that could further support implementation of a *Campylobacter* PHC.

As the work presented in this thesis shows, there is a need for quantitative data on biological hazards during broiler processing. Such data can provide valuable insights into changes in bacterial concentrations through processing, which can help slaughterhouses to select control measures. This will require the development of novel rapid quantification methods, or optimisation of the existing e.g. PMA-qPCR (Josefsen et al., 2010). Such optimisations could potentially rely on dilution of the samples to overcome any limitation on the effect of PMA in highly concentrated samples (Duarte et al., 2015). Alternatively, they could rely on centrifuging and resuspending the samples before PMA treatment, given that such a procedure has been reported to reduce the signal from dead cells (it reduces the organic matter, improving the discrimination between live and dead cells) (Krüger et al., 2014). A third possibility, based on the study of *Campylobacter* quantification by ethidium monoazide (EMA)-qPCR in water samples, considers avoidance of the turbidity of the samples, which can inhibit the photoreactive dye (Seinige et al., 2014). Finally, including a control to monitor the signal reduction from dead cells, as proposed by Krüger et al.

(2014), could demonstrate whether the difference is due to the presence of viable but non-culturable (VBNC) cells (Duarte et al., 2015).

Collecting additional quantitative data will also support the further analysis of risk factors. For example, because the data available here covered only 10 batches per slaughterhouse and was insufficient for a multivariate analysis, the risk analysis described in Chapter 4 was based only on univariate analysis. Additional data need to be collected during intervention studies to confirm the statistical associations found between explanatory variables and bacterial concentrations at the different processing steps, e.g. an association between defeathering efficiency and *E. coli* concentration might, with additional data, point with more certainty toward the role of equipment settings. Results obtained in this thesis provided an interesting insight into changes in bacterial concentrations during defeathering. For example, defeathering had a different effect on concentrations of *Campylobacter* than on both generic *E. coli* and ESBL/AmpC producing *E. coli* (Chapters 3, 4 and 5). Identifying the cause or causes of these differences would assist in the development of measures to control *Campylobacter* concentration during this step (Chapter 3).

As reported in Chapter 4, bacterial concentrations on the exterior and in any excreta that may leak during defeathering can potentially explain the increases or decreases in concentrations after this step. The contribution of each source and their interactions need to be further investigated. Additional quantitative data on *Campylobacter* and *E. coli* concentrations, not only on carcasses before and after defeathering but also in inputs (leaking excreta) and outputs from the process (removed feathers and draining water), could be collected and a mechanistic model of contamination during this step could be used as described previously (Nauta et al., 2005). Collection of this data could be performed under controlled conditions e.g. in a research slaughterhouses. There variation in processing could be implemented and its effect on the concentrations in inputs and outputs to the defeathering step could be measured. Such data could help to determine the most important sources of contamination during defeathering and support thus development of an intervention.

Quantitative results presented in this thesis provide a valuable data source for risk assessment models. Review of various risk assessment models on *Campylobacter* in broiler meat (Nauta et al., 2009) revealed importance of variability in concentrations within and between the batches and variability in the effect of the processing steps on concentrations. Those are important parameters for the microbiological criteria (Nauta et al., 2012). This thesis presents quantitative data that can further inform risk assessment models about the ratio of between and within batch variability. Additional analysis of these data, comparing within and between batch variations, is needed.

This thesis suggested that human-related factors might influence bacterial contamination on broiler chicken carcasses. The results described in Chapter 7 pointed

to the role of management of the organisation in improving processing hygiene. Additional research is needed to identify any factors that might influence food handlers' practices. This could be achieved by studying the food safety climate, namely leadership, communication, commitment, resources and risk awareness of food safety and hygiene (De Boeck et al., 2015; Griffith et al., 2010). A recently developed questionnaire on food safety climate components could be a useful tool in such studies (De Boeck et al., 2015). Its application in broiler slaughterhouses and the linking of its outcome with quantitative data as generated in this thesis could complement the identification of managerial risk factors that might impact *Campylobacter* contamination and other public health hazards during processing.

Conclusions

The research described in this thesis confirms the complexity of controlling hygiene during broiler processing. Bacterial levels after processing are affected by the initial bacterial contamination, which varies between incoming batches. Moreover they are affected by changes in the concentration of bacteria during processing that are specific for each slaughterhouse (Chapters 3 and 5). In addition, as this thesis reveals for the first time, processing hygiene can be affected by the practices of food handlers, indicating that factors affecting their compliance to process control should be further investigated and controlled (Chapter 7).

Regardless of all these variations, European slaughterhouses may have to comply with a process hygiene criterion (PHC) for *Campylobacter* in the near future. They will thus need to develop abilities to overcome these variations and effectively reduce *Campylobacter* concentrations on carcasses to an acceptable limit. To achieve this limit, they will need to implement improvements using technological solutions that provide reliable uniformity in meeting the PHC. Improvements could target e.g. cleaning the exterior of carcasses at the early processing steps and minimise contamination during subsequent processing steps, especially during defeathering and evisceration. The need for such technologies to address the PHC may trigger equipment manufacturers to seek further innovation and supporting research. Slaughterhouses may also implement managerial interventions, such as revisions of procedures and measures to ensure the compliance of food handlers. Most promising is a combination of both technological and managerial approaches, which might produce the best outcome in terms of improvement in broiler processing hygiene. Different interventions are probably relevant for different slaughterhouses because contamination patterns differ between slaughterhouses (Chapters 3, 4, 5 and 7). To determine which risk factors and what measures are the most relevant, slaughterhouses will need to monitor bacterial contamination throughout their processing line, an activity for which quantitative data are needed. This monitoring could be based on generic *E. coli* because similarities in the

effect of processing steps have been identified between this organism and ESBL/AmpC producing *E. coli* and *Campylobacter* at most of the tested processing steps. Once the risk factors are determined, and interventions are in place, those might effectively reduce multiple hazards on broiler carcasses.

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Summary

Poultry can be a source of biological hazards for consumers including *Salmonella*, *Campylobacter* and ESBL/AmpC producing *E. coli*. Campylobacteriosis, for example, has been the most frequently reported zoonosis in European Union since 2005. To diminish the number of campylobacteriosis cases in short term, risk assessment studies estimate that reducing the number of *Campylobacter* on chicken meat is needed. This has driven an initiative to implement Process Hygiene Criterion (PHC), a limit of *Campylobacter* for broiler meat that could stimulate implementation of control measures to reduce contamination. The number of *Campylobacter* varies between the batches and slaughterhouses, for reasons that are not yet clear. Identifying the causes would enable developing interventions to control *Campylobacter* contamination. Potentially, such interventions could be effective for other hazards as ESBL/AmpC producing *E. coli*, and this effectiveness could be measured by generic *E. coli*. However concentrations of *Campylobacter*, ESBL/AmpC producing *E. coli* and generic *E. coli* along the processing were not yet known.

The aim of this thesis was to study changes in the contamination of broiler chicken carcasses with *Campylobacter*, ESBL/AmpC producing *E. coli* and generic *E. coli* through the broiler processing line, and to identify and quantify factors related to processing technology and management that might influence these changes (Chapter 1).

Currently, quantification of *Campylobacter* is based on traditional culture method that is complex and time consuming. In Chapter 2 a study on implementation of a rapid method (PMA-qPCR) for quantification of live *Campylobacter* cells was described. Compared with the culture method the results obtained by PMA-qPCR insufficiently differentiated between live and dead *Campylobacter* cells. Therefore this method was not recommended.

Further, quantitative data on *Campylobacter* and *E. coli* concentrations on carcasses throughout five processing steps were collected (Chapter 3). Changes in *Campylobacter* concentration during processing within and between slaughterhouses were analysed as was their correspondence with quantitative data on *E. coli.* For that mixed effect models were used. The concentrations of both organisms at the beginning of processing varied significantly between batches, which pointed to the role of primary production in the contamination. Although concentrations differed between the batches, they did not differ between slaughterhouses. Interestingly the concentrations at the end of processing (after chilling) did differ, confirming that reduction is more efficient in some slaughterhouses. While in one slaughterhouse the processing had similar effect between slaughtered batches, it was variable in the other. Processing steps as scalding and chilling reduced both *Campylobacter* and *E. coli* concentrations on carcasses; however the magnitude of the reduction differed between slaughterhouses. Further, defeathering increased *Campylobacter* concentrations but it was significant only in one slaughterhouse and *E. coli* concentrations decreased during this step in both slaughterhouses. Evisceration

increased both *Campylobacter* and *E. coli* concentrations in one slaughterhouse, but not in another. These results confirmed that each slaughterhouse have its own pattern of contamination, suggesting that these differences might influence the variation in the *Campylobacter* and *E. coli* concentrations after chilling.

Potential causes of the observed differences were studied (Chapter 4). The presence of statistcal associations between explanatory variables and increases and decreases in *Campylobacter* and *E. coli* concentrations during tested processing steps was modelled. The variables were analysed separately in each slaughterhouse and for each organism. These differences confirmed that each slaughterhouse needs to identify individually bottlenecks in process control and to implement measures to improve processing hygiene. Bacterial concentrations in excreta and caeca were the most prominent variables, because they were associated with concentration on carcasses at various processing steps.

Further it was investigated for the first time whether the concentrations of ESBL/AmpC producing *E. coli* change through the processing in a way similar to those of *Campylobacter* and *E. coli* (Chapter 5). Results revealed that the ESBL/AmpC producing *E. coli* concentrations on broiler chicken carcasses during processing are influenced by batch and slaughterhouse. This points to the role of both primary production and process control in reducing ESBL/AmpC producing *E. coli* levels in final products, similarly as for *Campylobacter* and *E. coli* (Chapter 3). The similar changes seen at various processing steps indicate that *E. coli* can be used as a process indicator of ESBL/AmpC producing *E. coli*. With respect to *Campylobacter*, defeathering had different effect on ESBL/AmpC producing *E. coli*. Nevertheless, all of the tested bacteria provided a concordant indication of the efficiency of processing steps between slaughterhouses. This suggests that effective interventions reducing *Campylobacter* concentrations could target multiple public health hazards. In addition, the genotypes of ESBL/AmpC producing *E. coli* ($bla_{\text{CTX-M-1}}$, $bla_{\text{SHV-12}}$, $bla_{\text{CMY-2}}$, $bla_{\text{TEM-52c}}$, $bla_{\text{TEM-52cvar}}$) from both slaughterhouses matched typical poultry genotypes.

Further the effect of technological intervention i.e. novel brushes on *E. coli* and *Enterobacteriaceae* reduction before scalding was tested (Chapter 6). For this, the prototype brushes were installed next to commercial processing line. Results revealed that on the whole carcass the effect of brushes was small but significant. Higher reduction was observed on the brushed areas, suggesting that improvements could be implemented. As bacteria are distributed on the entire carcasses, extending the brushed surface could further increase bacterial reductions by brushing. In addition, the brushes removed from carcasses significant amounts of dry matter, including faecal material. This had an effect of pH of rinse samples obtained from brushed carcasses. Based on these findings, it is expected that brushing will impact the bacterial reduction during scalding, because these parameters directly (pH) or indirectly (dry matter) influence scalding lethality. This hypothesis needs to be confirmed, however, through additional trials with the brushes operating in the processing line.

In addition, the need for managerial interventions was presented, based on investigation of the compliance of food handlers with procedures to control the evisceration process (Chapter 7). Evisceration affected *Campylobacter*, ESBL/AmpC producing *E. coli* and generic *E. coli* concentrations differently between slaughterhouses (Chapters 3 and 5). In these slaughterhouses, the procedures were analysed and the compliance of food handlers with the criteria to control the evisceration process was observed. The results revealed that one slaughterhouse had less detailed procedures, with the food handlers barely monitoring the evisceration process and seldom adjusting equipment settings between batches. This slaughterhouse also had a higher frequency of carcasses with visible faecal contamination in comparison to the other. It was confirmed that the compliance with procedures may be associated with presence of visible contaminated carcasses with faeces. As those carcasses carried higher *E. coli* concentrations, it can be concluded that compliance influences food safety. To demonstrate the influence of the compliance with procedures on contamination of poultry carcasses, an intervention study needs to be performed. Further, the causes of non-compliance were studied by research into the food handlers' knowledge, attitude and practices. This revealed that although the food handlers had knowledge on process control they did not always implement this into practice. Attitude, recognised to influence relationship between knowledge and practices, varied between slaughterhouses and was less positive in slaughterhouse with frequent non-compliance. Managerial interventions might thus focus on assurance of food handlers' compliance with process controls by providing adequate procedures, training focused on the motivation of food handlers or their supervision or potentially compliance incentives. To achieve this, involvement of management of the slaughterhouse is needed.

Finally, the findings of this thesis were discussed (Chapter 8) within the perspective of upcoming *Campylobacter* Process Hygiene Criterion. To achieve the limit specified by PHC, the slaughterhouses will need to intervene. A combination of both technological and managerial interventions might result in the best improvement in broiler processing hygiene. The need for such interventions may trigger slaughterhouses and equipment manufacturers to seek solutions and the research supporting this. Firstly, however, slaughterhouses need to monitor individually how processing influence bacterial contamination, because such patterns are slaughterhouse specific. This monitoring could be based on *E. coli*, because sufficient similarities have been identified between this organism and ESBL/AmpC producing *E. coli* and *Campylobacter* at most of the tested processing steps. Once the risks are determined, and interventions are in place, those might effectively reduce not only *Campylobacter* but multiple hazards on broiler carcasses.

Samenvatting

Pluimvee kan voor consumenten een bron zijn voor biologische gevaren waaronder *Salmonella*, *Campylobacter* en ESBL/AmpC producerende *Escherichia coli*. Campylobacteriosis is bijvoorbeeld de meest frequent gerapporteerde zoonose binen de Europese Unie sinds 2005. Om het aantal gevallen van Campylobacteriosis te verminderen, hebben risico assessment studies uitgewezen, zal vermindering van het aantal *Campylobacter* bacteriën op kippenvlees een goede methode zijn. Dit uitgangspunt is de drijvende kracht achter de Process Hygiene Criterion (PHC) een limiet niveau voor het aantal *Campylobacter* op kippenvlees. De PHC zal het implementeren van controle maatregelen in de keten stimuleren. Het aantal *Campylobacter* bacteriën op kippenkarkassen varieert per aangeleverde koppel en per slachthuis. De redenen van deze variatie zijn niet duidelijk. Duidelijkheid over deze redenen zal het mogelijk maken interventie maatregelen te ontwikkelen om *Campylobacter* contaminatie te beheersen. Mogelijk zullen interventie maatregelen tegen *Campylobacter* ook werken tegen andere gevaren zoals ESBL/AmpC producerende *E. coli*. De effectiviteit van de maatregen zou door het bepalen van de generieke *E. coli* niveaus gemonitord kunnen worden.

Het doel van het onderzoek, beschreven in dit proefschrift, is om de veranderingen in de contaminatie van kippen karkassen met *Campylobacter*, ESBL/AmpC producerende *E. coli* en generieke *E. coli* tijdens het slachten te bepalen en daarvan uit factoren (gerelateerd aan slacht technologie en management) te identificeren en kwantificeren die deze veranderingen beïnvloeden (hoofdstuk 1).

Momenteel worden *Campylobacter* bacteriën via de traditionele kweek methoden geïdentificeerd en gekwantificeerd. In hoofdstuk 2 is een onderzoek beschreven over het gebruik van een snelle methode (PMA-qPCR) voor de kwantificatie van levende *Campylobacter* bacteriën. Ook zijn beide methoden (traditioneel en PCR) vergeleken waaruit bleek dat de PMA-qPCR onvoldoende onderscheid maakte tussen levende en dode *Campylobacter* bacteriën. Daarom is deze methode dan ook niet gebruikt.

In hoofdstuk 3 is onderzoek beschreven om kwantitatieve data voor *Campylobacter* en *E. coli* te verzamelen gedurende 5 stappen in het slachten van pluimvee. Veranderingen in de *Campylobacter* concentratie op kippen tijdens het slachtproces werden geanalyseerd en vergeleken met de kwantitatieve data van *E. coli.* Hiervoor werd een mixed effect model gebruikt. De concentraties van beide organismen op kippen karkassen varieerde significant aan het begin van het slachtproces tussen verschillende koppels, wat wijst op invloed van de primaire productie kolom. Hoewel de concentraties varieerden tussen de aangevoerde koppels varieerden ze niet tussen de slachthuizen. Echter de concentratie varieerden wel aan het eind van de slachtlijn (koeling) wat bewees dat reductie van de concentratie slachthuis specifiek is. Het ene slachthuis kan dat dus beter dan de andere. Ook deze vermindering bleek in het ene slachthuis telkens van hetzelfde niveau te zijn terwijl het in het andere slachthuis varieerden. Slacht stappen zoals broeien en koelen reduceerde *Campylobacter* en *E. coli* concentraties op karkassen in

dezelfde richting echter de grootte van het effect verschilde tussen de slachthuizen. Het ontvederen zorgde voor een significante toename in het aantal *Campylobacter* bacteriën op karkassen in één slachthuis. *E. coli* hoeveelheden verminderden in beide slachthuizen bij deze stap. Evisceratie liet zowel de concentratie van *Campylobacter* als *E. coli* in één slachthuis toenemen terwijl dit niet gebeurde in het andere slachthuis. De beschreven resultaten laten zien dat elk slachthuis zijn eigen specifieke patroon van beïnvloeding van contaminatie heeft wat mogelijk de variatie in contaminatie na het koelen voor *Campylobacter* en *E. coli* mede kan verklaren.

Onderzoek naar mogelijke verklaringen van deze variaties is beschreven in hoofdstuk 4. Statistisch significante associaties tussen verklarende variabelen en toe en afnames van *Campylobacter* en *E. coli* concentraties tijdens het slachten werden gemodelleerd. De variabelen werden onafhankelijk per slachthuis en per organisme geanalyseerd. De resultaten laten zien dat elk slachthuis specifieke problemen heeft en dat slachthuis eigen maatregelen zouden moeten worden ingevoerd om slacht hygiëne te bevorderen. Bacteriële concentratie in de excreta en het caecum waren de meest veel belovende variabelen omdat zij gerelateerd zijn aan de concentraties van de bacteriën in diverse stappen van het slachtproces.

Bovendien wordt in deze thesis onderzoek beschreven waarin voor de eerste maal is gekeken of de concentratie van ESBL/AmpC producerende *E. coli* op kippenkarkassen tijdens het slachtproces op een zelfde manier veranderen als *Campylobacter* en generieke *E. coli* (hoofdstuk 5). De beschreven resultaten laten zien dat de concentratie van ESBL/AmpC producerende *E. coli* op kippenkarkassen tijdens het slachten worden beïnvloed door de koppel en het slachthuis. Dit laat zien dat zowel de primaire productie kolom als de slachthuisketen een rol spelen in het reduceren van de concentratie van ESBL/AmpC producerende *E. coli* op het eindproduct. Dit is gelijk aan hoe het verloopt voor *Campylobacter* en *E. coli* (hoofdstuk 3). Generieke *E. coli* kunnen dus als een indicator organisme voor ESBL/AmpC producerende *E. coli* worden gebruikt. Ontvederen lijkt voor de *Campylobacter* concentratie fluctuaties een ander effect te hebben dan voor ESBL/AmpC producerende *E. coli.* Desalniettemin lieten alle geteste bacteriële species een vergelijkbaar patroon zien van beïnvloeding tijdens het slachten. Hier uit kan geconcludeerd worden dat interventies om *Campylobacter* concentraties te verminderen ook een andere public health gevaren zou verminderen. Daarnaast bleek in dit onderzoek dat de gevonden genotypes van de ESBL/AmpC producerende *E. coli* ($bla_{\text{CTX-M-1}}$, $bla_{\text{SHV-12}}$, $bla_{\text{CMY-2}}$, $bla_{\text{TEM-52c}}$, $bla_{\text{TEM-52cvar}}$) uit beide slachthuizen overeenkwamen met typische pluimvee genotypes.

In hoofdstuk 6 is beschreven wat het effect was van een technologische interventie, het gebruik van borstels voor het broeien, op *E. coli* en *Enterobacteriaceae* reductie op kippenkarkassen. Om dit te meten is een prototype borstel geplaatst naast een commerciële slachtlijn. De resultaten van de experimenten lieten zien dat het effect van

de borstels op de microbiële besmetting van het hele karkas beperkt maar significant was. Sterkere reductie werd gezien op de geborstelde oppervlakken wat een aanmoediging is om deze interventie verder te ontwikkelen. Dit zou kunnen door bijvoorbeeld het te borstelen oppervlak te vergroten. Bovendien verwijderden de borstels ook veel droge stof waaronder feces. Dit heeft waarschijnlijk een effect op de bacteriële reductie tijdens het broeien omdat de daardoor veranderde pH (direct) en de aanwezige droge stof (indirect) de afdoding beïnvloed. Deze hypothese moet bevestigd worden door het plaatsen van de borstels in de slachtlijn.

De noodzaak voor management gerelateerde interventies gebaseerd op compliance van slachthuis medewerkers voor maatregelen om de evisceratie van kippen te borgen, zijn beschreven in hoofdstuk 7. Evisceratie beïnvloedt de *Campylobacter*, ESBL/AmpC producing *E. coli* en generieke *E. coli* concentraties verschillend in de beide slachthuizen (hoofdstuk 3 en 5). In deze slachthuizen werd de compliance tot beheerstaken in de evisceratie door slachthuis medewerkers beoordeeld en geanalyseerd. De resultaten toonden aan dat in één slachthuis de procedures slecht en beperkt gedetailleerd beschreven waren en dat de slachthuis medewerkers maar beperkt toezicht hielden op het evisceratie proces. Bovendien pasten zij nauwelijks de apparatuur aan tijdens het slachten van verschillende koppels. In dit slachthuis werd een hoger aantal fecaal bezoedelde karkassen gevonden dan in het andere slachthuis. Aangetoond kon worden dat compliance tot procedures effect heeft op aantal fecaal bezoedelde karkassen. Op deze karkassen werden ook *E. coli* bacteriën gevonden zodat geconcludeerd kan worden dat compliance tot procedures effect heeft op voedsel veiligheid. Om dit effect daadwerkelijk aan te tonen zou een interventie studie uitgevoerd moeten worden.

De redenen van non compliance zijn verder bestudeerd door te onderzoeken wat de relatie hiervan was met kennis, attitude en uitvoering. Wat bleek was dat hoewel de slachthuis medewerkers voldoende kennis hadden over het proces zij deze kennis onvoldoende gebruikten tijdens de werkzaamheden. De attitude, de schakel tussen kennis en uitvoering, varieerde tussen de beide slachthuizen en was lager bij het minder goed functionerende slachthuis. Management interventies moeten zich dus focussen op het verbeteren van de compliance van medewerkers door deze medewerkers heldere procedures te verstrekken en het verhogen van de motivatie van medewerkers om zich aan deze procedures te houden. Voor deze motivatie verhoging is inzet van het gehele management noodzakelijk.

Uiteindelijk zijn de resultaten beschreven in dit proefschrift bediscussieerd in hoofdstuk 8. De resultaten en conclusies werden in perspectief geplaatst met de geplande *Campylobacter* Process Hygiene Criterion (PHC). Om aan deze eisen van de PHC te voldoen moeten slachthuizen inzetten op gecombineerde technologische en management gerelateerde interventies. De noodzaak voor zulke interventies kan de slachthuizen en de producenten van slachtlijnen uitdagen om te komen met verbeteringen in slachthuis

procedures. Voor dit alles moeten slachthuizen echter eerst individueel hun processen controleren en doormeten. Om zo te laten zien hoe hun procedures invloed hebben op bacteriële contaminatie van hun product. Voor deze controle en monitoring kan *E. coli* worden gebruikt vanwege de overeenkomsten in contaminatie patronen met ESBL/AmpC producerende *E. coli* en *Campylobacter* bij de verschillende proces stappen. Interventies die worden ingezet zullen waarschijnlijk niet alleen effectief *Campylobacter* contaminatie op kippen karkassen verminderen maar gelijk ook andere microbiële hazards bestrijden.

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Curriculum Vitae

Ewa Pacholewicz was born on 23 October 1983 in Pleszew, Poland. After completing secondary school in Kalisz in 2002, she started to study Food Technology and Human Nutrition in the department of Food Science and Nutrition at the Poznań University of Life Sciences. During her studies in 2005-2006 she participated in a student exchange arranged with the CAH Vilentum University of Applied Sciences in Dronten, the Netherlands, as part of the Erasmus programme. There she obtained her BSc in International Food Chain Management. During this programme she followed an internship at the Meyn Food Processing Technology B.V., Oostzaan. Afterwards, as part of the Leonardo da Vinci scholarship programme, she followed an internship at the Central Veterinary Institute in Lelystad. Coming back to Poland she finalised her MSc and graduated in 2007. After graduation she started to teach at the Poznań University of Life Sciences. In the meantime she established cooperation with Technologie-Transfer-Zentrum (ttz), Department of Food Technology and Bioprocess Engineering in Bremerhaven, Germany where she worked as a Project Manager from 2008-2010. While there, she became involved in European Projects from the 6th and 7th Framework Programme (e.g.: CHILL-ON, NAFISPACK) and prepared the successful grant application of the IQ-Freshlabel project. In 2010 she started to work at Meyn Food Processing Technology B.V. Supported by the company, she initiated cooperation with Utrecht University, the Institute for Risk Assessment Sciences (IRAS) and a project on hygiene during poultry processing. She was seconded by Meyn Food Processing Technology B.V. to work on this project at IRAS between 2011 and 2016. The results presented in this thesis were obtained in the context of this project.

Contribution to scientific conferences

E. Pacholewicz, A. Liakopoulos, A.N. Swart, B.G.M. Gortemaker, C. Dierikx, A.H. Havelaar und H. Schmitt. Reduzierung von ESBL- und/oder AmpC-bildenden *Escherichia coli* in der Geflügelfleischgewinnung. Oral presentation at the 16. Fachtagung für Fleisch- und Geflügelfleischhygiene, Berlin, Germany, 1-2 March 2016.

E. Pacholewicz, S.A.S. Barus, A.N. Swart, A.H. Havelaar, L.J.A. Lipman, P.A. Luning. Effect of food handlers' compliance with procedures on poultry contamination: a case study in broiler chicken slaughterhouses. Poster presentation at the Veterinary Science Day, Bunnik, 19 November 2015.

E. Pacholewicz, H. Schmitt, A. Liakopoulos, A.N. Swart, M. Schipper, B.G.M. Gortemaker, C. Dierikx, L.J.A. Lipman, J.A. Wagenaar. Similar control points for *Campylobacter* and ESBL/AmpC producing *E. coli* during broiler processing. Oral presentation at the18th International Workshop on *Campylobacter*, *Helicobacter* and Related Organisms (CHRO), Rotorua, New Zealand, 1-5 November 2015.

E. Pacholewicz, A. Liakopoulos, A.N. Swart, B.G.M. Gortemaker, C. Dierikx, A.H. Havelaar, H. Schmitt. Quantification of extended-spectrum-β-lactamase- and AmpC-β-lactamase-producing *Escherichia coli* through processing in two broiler chicken slaughterhouses. Oral presentation at the XXII European Symposium on the Quality of Poultry Meat, Nantes, France, 10-13 May 2015; and at the GKZ-symposium, Utrecht, The Netherlands, 13 May 2015

E. Pacholewicz, A.N. Swart, M. Schipper, B.G.M. Gortemaker, J.A. Wagenaar, A.H. Havelaar, L.J.A. Lipman. Trends in *Campylobacter* and *E. coli* contamination on broiler chicken carcasses during slaughtering. Oral presentation at the Workshop on *Campylobacter*, *Arcobacter* & Related Organisms (CARO), Berlin, Germany, 20-21 November 2014.

E. Pacholewicz, A.N. Swart, L.J.A. Lipman, A.H. Havelaar. Identifying factors affecting safety of broiler chicken carcasses during slaughtering. Poster presentation at the 55. Arbeitstagung des Arbeitsgebietes Lebensmittelhygiene der DVG, Garmisch – Partenkirchen, Germany, 23-26 September 2014; and at the XIVth European Poultry Conference, Stavanger, Norway, 23–27 June 2014.

E. Pacholewicz, A.N. Swart, B.G.M. Gortemaker, W.J.C. Heemskerk, J.A. Wagenaar, A.H. Havelaar and L.J.A. Lipman. Hygienic processing performance with respect to *Campylobacter* along the processing line within and between broiler processing plants. Oral presentation during the XXI European Symposium on the Quality of Poultry Meat, Bergamo, Italy, 15-19 September 2013; and poster presented at the 17th International Workshop on *Campylobacter*, *Helicobacter* and Related Organisms, Aberdeen, Scotland, UK, 15 - 19 September 2013.

E. Pacholewicz, W.J.C. Heemskerk. Zur Bedeutung der Nüchterungszeit von Geflügel für die Schlachthygiene. Oral presentation at the 54. Arbeitstagung des Arbeitsgebietes Lebensmittelhygiene der DVG, Garmisch – Partenkirchen, Germany, 24-27 September 2013**.**

E. Pacholewicz, M.M. Throns, I. Habib, A.H. Havelaar, L.J.A. Lipman. Survival of *Campylobacter* spp. in chicken carcass rinse during sample storage. Poster presentation at the XXIV World's Poultry Congress, 5-9 August 2012, Salvador, Bahia, Brazil.

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The general aim of this thesis was to study changes in the contamination of broiler chicken carcasses with *Campylobacter*, ESBL/AmpC producing *E. coli* and generic *E. coli* through the broiler processing line, and to identify and quantify factors related to processing technology and management that might influence these changes. The purpose was to obtain background knowledge necessary for developing interventions that will reduce the bacterial contamination on carcasses during and after processing. The information gathered in this thesis can be applied by various stakeholders in the broiler meat chain to improve future control of public health hazards during broiler processing.

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