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Cellulitis in broiler chickens

Studies of the pathogenesis, a diagnostic tool and risk factors

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Cellulitis in broiler chickens – studies of the pathogenesis, a diagnostic tool and risk factors

Abstract

Cellulitis in broiler chickens is a subcutaneous inflammation that is caused by the bacterium *Escherichia coli*. It is one of the main causes of condemnation of broiler carcasses at slaughter in Sweden and elsewhere. The aim of this thesis was to gain more knowledge of the pathogenesis and risk factors of this disease. Samples from cellulitis lesions and organs (n=327) were collected from carcasses at slaughter. By using whole genome sequencing, clonal spread of *E. coli* strains within some flocks and systemic spread in individual chickens with cellulitis were demonstrated. In two experimental studies, in which *E. coli* was inoculated subcutaneously to induce cellulitis, clinical signs, immune parameters in blood and the use of infrared imaging as a diagnostic tool were evaluated. Chickens showed clinical signs of varying severity depending on *E. coli* isolate. Analyses of blood showed that some chickens were bacteraemic post-inoculation. Signs of activated monocytes and increased *in vitro* adherence to *E. coli* by heterophils, monocytes and thrombocytes were observed. Infrared thermography did not reliably identify cellulitis. Mixed effect modelling of production data was used to identify risk factors for condemnation due to dermatitis/cellulitis. Identified risk factors were *e.g.* old parent flocks, late slaughter and slaughterhouse identity. *Post-mortem* examinations of 491 chickens culled or found dead near slaughter on 10 farms, showed that cellulitis occurred in approximately 9% of the chickens.

In conclusion, *E. coli* associated with cellulitis can spread systemically at time of slaughter and inoculation of *E. coli* can cause clinical signs and changes to immune parameters in blood. The occurrence of cellulitis may be underestimated, and a multifactorial approach is probably needed to reduce it.

Keywords: broiler chickens, cellulitis, *Escherichia coli*, cgMLST, systemic spread, experimental infection model, infrared thermography, immune response, risk factors

Cellulit hos slaktkycklingar – studier kring patogenesen, ett diagnostiskt verktyg och riskfaktorer

Abstract

Cellulit hos slaktkyckling är en inflammation i underhuden som orsakas av bakterien *Escherichia coli*. Sjukdomen är en av de främsta orsakerna till kassation av kycklingar vid köttinspektionen vid slakt i Sverige och i andra länder. Syftet med avhandlingen var att öka kunskapen om patogenes och riskfaktorer för cellulit. Prover från cellulit och organ (n=327) samlades in från kycklingar vid slakt. En klonal spridning av *E. coli*-stammar inom vissa flockar samt systemisk spridning hos kycklingar med cellulit, kunde påvisas genom helgenomsekvensering. Kliniska tecken, immunparametrar i blod och användning av infraröd termografi som en möjlig diagnostisk metod utvärderades i två experimentella studier, där *E. coli* inokulerades subkutant för att framkalla cellulit. Kycklingarna visade en varierande grad av kliniska tecken beroende på inokulerat *E. coli*-isolat. Blodanalyserna visade att vissa kycklingar hade bakteriemi efter inokulering. Monocyter visade tecken på aktivering, och i *in vitro*-test observerades en ökad adherens av heterofiler, monocyter och trombocyter till *E. coli*. Infraröd termografi kunde inte påvisa cellulit på ett tillförlitligt sätt. Effektmodellering av produktionsdata användes för att identifiera riskfaktorer för kassation på grund av dermatit/cellulit. Riskfaktorer som identifierades var bland annat föräldraflockarnas ålder, ålder på kycklingflocken vid slakt och slakteri. Obduktion av 491 kycklingar som avlivats eller hittats döda på 10 gårdar i nära anslutning till slakt, visade att cellulit förekom hos ungefär 9%.

Sammanfattningsvis kan *E. coli* som är förknippad med cellulit, spridas systemiskt vid slakt och inokulering av *E. coli* kan orsaka kliniska tecken och förändringar av immunparametrar i blod. Förekomsten av cellulit kan vara underskattad och en kombinerad strategi behövs sannolikt för att minska den.

Nyckelord: slaktkyckling, cellulit, underhudsinflammation, *Escherichia coli*, cgMLST, systemisk spridning, experimentell infektionsmodell, infraröd termografi, immunsvar, riskfaktorer.

Dedication

To my family.

Contents

List of publications

This thesis is based on the work contained in the following papers, referred to by Roman numerals in the text:

I. Jonare, L., Östlund, E., Söderlund, R., Hansson, I., Aspan, A. & Jansson, D. S. 2023. Core genome multilocus sequence typing (cgMLST) confirms systemic spread of avian pathogenic *Escherichia coli* (APEC) in broilers with cellulitis. Veterinary Microbiology, 282:109755. https://doi.org/10.1016/j.vetmic.2023.109755

II. Jonare, L., Östlund, E., Jacobson, M., Wall, H. & Jansson, D. S. 2024. Infrared thermography as a diagnostic tool for coliform cellulitis in broilers. Submitted.

III. Jonare, L., Wattrang, E., Östlund, E., Wall, H., Jacobson, M. & Jansson, D. S. 2024. Subcutaneous inoculation of *Escherichia coli* in broiler chickens causes cellulitis and elicits innate and specific immune responses. Submitted.

IV. Jonare, L., Egenvall, A. & Jansson, D.S. Risk factors and late grow out occurrence of coliform cellulitis in broiler chickens. Manuscript.

Paper I is reproduced with the permission of the publishers.

The contribution of Liv Jonare to the papers included in this thesis was as follows:

I. Participated in the planning and organisation of the study. Executed the study together with one co-author. Performed all laboratory work, data processing and analysis, except the whole genome sequencing and genomic analyses. Drafted the manuscript, revised it based on feedback from co-authors and corresponded with the journal.

II. Participated in the planning and organisation of the study. Executed the study together with one co-author. Performed all laboratory work, data processing and analysis, except the whole genome sequencing and genomic analyses. Drafted the manuscript, revised it based on feedback from co-authors and corresponded with the journal.

III. Took part in the planning and organisation of the study. Executed the study together with mainly two co-authors. Performed parts of the laboratory work, data processing and analyses. Drafted the manuscript, revised it based on feedback from co-authors and corresponded with the journal.

IV. Took part in the planning and organisation of the study. Executed the practical part of the study together with one co-author. Performed data processing and analysis with support from co-authors. Drafted the manuscript and revised it based on feedback from co-authors.

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Abbreviations

APEC	Avian Pathogenic *E. coli*
cgMLST	Core genome multilocus sequencing
CFU	Colony forming units
CI	Confidence interval
EM mean	Estimated marginal mean
Ex-PEC	Extraintestinal Pathogenic *E. coli*
FAdV	Fowl adenovirus
GPs	Grand parents
IRT	Infrared thermography
MST	Minimum spanning tree
PCR	Polymerase chain reaction
PRRs	Pattern recognition receptors
PFGE	Pulse-field gel electrophoresis
PI	Post infection
PM	*Post-mortem*
PRRs	Pattern recognition receptors
SVA	Swedish Veterinary Institute
TLRs	Toll-like receptors
WGS	Whole genome sequencing

1. Background

The broiler chicken is by far the most numerous farm animal in Sweden. Approximately 112 million broiler chickens were slaughtered in 2022, producing 176 000 tonnes of meat (Lannhard Öberg 2023). The production has increased from 44 million slaughtered chickens and 74 000 tonnes of meat in 1995 (The Swedish Board of Agriculture 2024). Worldwide, the chicken meat production was 124 million tonnes in 2022, with USA and Brazil as top producers, with a production of 20 and 15 million tonnes of meat, respectively (FAO 2024).

1.1 Swedish broiler production

Sweden has approximately 120 large-scale commercial broiler farms, most of them situated in the south of the country (The Swedish Poultry Meat Association 2024). Each farm produces on average seven batches per year that consist of on average 85,000 broilers chickens per batch. The production of chickens runs through the so-called breeding pyramid, with a small number of elite breeding birds at the top that multiply in numbers through every generation down to the meat-producing chickens (Figure 1). In each generation, a strict crossbreeding with pure lines is applied to achieve the combination of traits considered as optimal in the broiler chick generation. In Sweden, fast-growing broiler genotypes dominate, and grandparent birds (GPs) are imported as day-old chicks from Scotland. The GPs lay eggs that will become the parent stock, which in turn will generate broilers (Berndtson *et al.* 2019).

Chickens are incubated and hatched at commercial hatcheries and are placed as day-old chicks on farms. Hatching on farm instead of hatcheries is sometimes carried out but is not common practice. The chickens are raised

on litter with *ad libitum* access to feed and water, in broiler house compartments of between 300 and 3,000 m^2 in size, with a regulated stocking density of up to 36 kg/m^2 (The Swedish Board of Agriculture 2019). The chickens are slaughtered when they have reached a body weight of 2,000–2,300 g, which is achieved approximately at 35 days of age. Some farms practice thinning, *i.e.* slaughter of a part of the flock, when the chickens have reached a body weight of 1,600 g, which is generally seven days before the main slaughter.

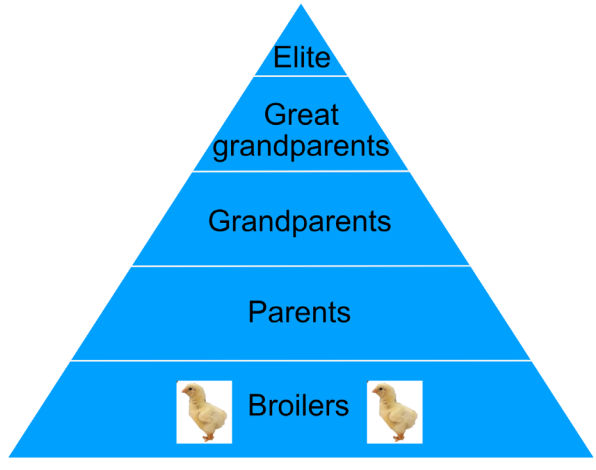

Figure 1. The broiler breeding pyramid. Grandparents are imported to Sweden, producing parents, which in turn produce the broiler chickens raised for meat consumption. High up in the pyramid are great grandparents and elite animals and between each step, the number of animals are multiplied.

The majority of chicken farms participate in health control programs that set common standards for biosecurity and management routines (Berndtson *et al.* 2019). However, despite the high biosecurity, disease caused by bacterial infections do occur.

1.2 Cellulitis

1.2.1 Occurrence

Cellulitis is one of the most common causes for condemnation or downgrading of broiler carcasses at slaughter in Sweden. In 2022, the condemnation percentage for cellulitis/dermatitis (which are reported as an entity) at commercial slaughterhouses in Sweden was 0.65% (Majewski *et al.* 2024). Other recent examples of countries that have reported a condemnation rate for cellulitis include Finland (0.28% in 2019, Törmä *et al.* (2022)), Germany (0.38% in 2017–2018, Schulze Bernd *et al.* (2020)), Denmark (0.1% in 2018, Poulsen *et al.* (2018)) and United Kingdom (0.33% in 2022, Majewski *et al.* (2024)). It should be noted that comparisons of condemnation data across countries are challenging, because most countries use different national or regional code systems (Majewski *et al.* 2024). Cellulitis was first described in 1984, and thereafter the condemnation rate due to the disease increased considerably, *e.g.* from 0.001% in 1986 to 0.69% in 1996 in the Quebec province in Canada (Randall *et al.* 1984; Kumor *et al.* 1998).

1.2.2 Pathogenesis

Cellulitis is an inflammation caused by bacterial infection of the subcutaneous tissue, usually also affecting the skin (Nolan *et al.* 2020). The main causative agent is *Escherichia coli*, although other bacteria also have been associated with cellulitis, such as *Proteus* spp. and *Streptococcus dysgalactiae* (Randall *et al.* 1984; Messier *et al.* 1993; Sanches *et al.* 2020). In coliform cellulitis, skin scratches inflicted by other chickens in the flock are believed to be an important part of the pathogenesis as a possible portal of entry for *E. coli* to the subcutaneous tissue, although this has not been fully investigated (Messier *et al.* 1993; Peighambari *et al.* 1995a; Alfifi *et al.* 2022; Kromann *et al.* 2023).

The characteristic gross finding of the disease is a subcutaneous distinct, yellow to brown inflammatory, sheet-like exudate called plaque (Figure 2) (Nolan *et al.* 2020). The plaque is more or less loosely attached to the underlying tissue, where congestion, discoloration and haemorrhage can be present. The skin overlying the subcutaneous plaque often appears thickened and discoloured in yellow or green. The lesion is most commonly located on one side of the abdomen or thigh, but can affect other parts of the body, and

the infection can spread locally to other areas. Lesion size normally varies from 1 to 10 cm (Elfadil *et al.* 1996b). Histologically, heterophils and lymphocytes have been observed in congested tissue containing fibrinonecrotic foci, and in more mature lesions, connective tissue surrounds the inflammatory plaque (Elfadil *et al.* 1996b; Fallavena *et al.* 2000). Secondary systemic spread of the bacteria has been suggested, resulting in gross lesions such as pericarditis, perihepatitis and coelomitis/peritonitis (Poulsen *et al.* 2018; Silva *et al.* 2021).

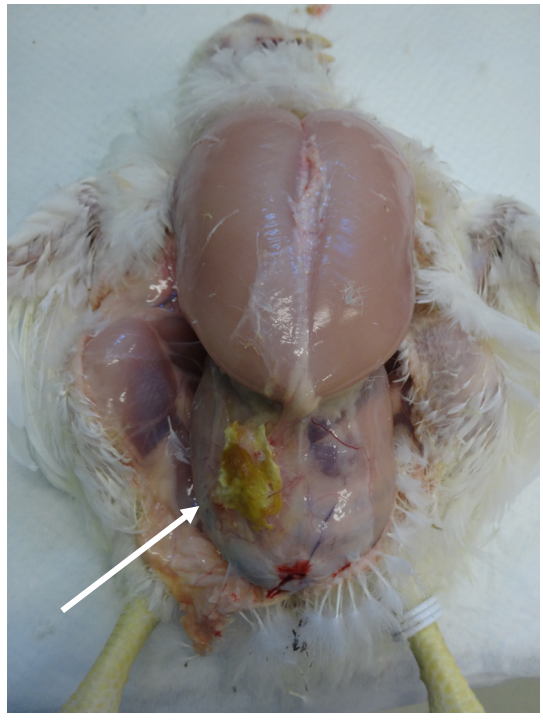

Figure 2. Broiler chicken with cellulitis. The arrow points at the characteristic plaque, the inflammatory exudate that forms due to the *E. coli* infection. Photo: Liv Jonare

There are still gaps in the understanding of the pathogenesis of cellulitis, and it is not known when, during grow-out in a commercial large-scale chicken setting, that cellulitis develops. The late grow-out period has been suggested since lesions develop fast, and gross findings indicative of acute lesions have most commonly been observed at slaughter (Norton *et al.* 1997; Poulsen *et al.* 2018; Nolan *et al.* 2020; Jonare *et al.* 2023). Further, chickens

experimentally infected before 16 days of age often develop a systemic *E. coli* infection in addition to cellulitis, compared to the local cellulitis lesion that is most commonly identified at processing (Johnson *et al.* 2001; Jonare *et al.* 2023). The morbidity and mortality due to cellulitis during grow-out on the farm have not been reported, although chickens with cellulitis have been found in flocks with increased mortality due to colibacillosis at the end of the production period (Poulsen *et al.* 2018; Davam *et al.* 2024).

1.2.3 Clinical signs

According to the early literature, chickens with cellulitis show few or no clinical signs (Randall *et al.* 1984; Glunder 1990). However, there are no published observational studies of clinical signs in broiler chicken from commercial farms and therefore, the possible impact on bird welfare is not known. Reports of cellulitis in experimental models with subcutaneous inoculation of *E. coli*, describe no clinical signs (Peighambari *et al.* 1995a; Gomis *et al.* 1997b; Norton *et al.* 1997). However, experimental studies of other types of coliform infections in poultry, have reported clinical signs of increasing severity that correlated with the extent of gross lesions found during *post-mortem* (PM) examinations (Pors *et al.* 2014; Kumari *et al.* 2020).

1.2.4 Diagnostics

There are no diagnostic tools available for cellulitis in live birds on farms. As the bird's plumage covers the skin on the abdomen and there are no specific clinical sign associated with the disease, it is difficult to identify birds with lesions in a commercial setting. Infrared thermography (IRT) has been suggested as a diagnostic tool for cellulitis (Tessier *et al.* 2003), but there are currently no published reports on the use of this technique to diagnose coliform cellulitis in broiler chickens. The technique registers the infrared radiation emitted from an object and transforms it into images that display surface temperatures (Yahav 2012). As inflammation produces heat, possible increased skin temperature at the location for cellulitis might thus be detected (Cavaillon 2021).

In research of broiler health, IRT has been used to study *e.g.* femoral and tibial head necrosis (Weimer *et al.* 2019), footpad dermatitis (Jacob *et al.* 2016) and metabolic heat loss (Ferreira *et al.* 2011; Alvarez *et al.* 2022). There are also studies that have investigated the correlation of the surface

temperature of the broiler chicken with the core temperature (Giloh *et al.* 2012; Aardsma *et al.* 2018), as well as the normal variation in broiler surface temperature (Tessier *et al.* 2003; Cangar *et al.* 2008; Naas *et al.* 2010; Bloch *et al.* 2020).

Tessier *et al.* (2003) used IRT to assess the surface temperature on the lateral abdomen in 18–35 days-old broiler chickens and the variation of temperature depending on location (right vs. left abdomen), time of day, and the effect of handling. The study showed that the right abdominal side was warmer than the left side and that the skin temperature has a circadian 24 hour cycle. Handling of the chickens did not appear to affect skin temperature (Tessier *et al.* 2003).

In another study, the surface temperature of the comb, head and eye decreased as a possible effect of vasoconstriction, when 72-week-old broiler parents were handled, while the core temperature increased (Edgar *et al.* 2013). When using IRT, Naas *et al.* (2010) showed that the broiler's surface temperature decreased with a denser feather cover. However, the feathered areas showed a significant increase in temperature in higher ambient temperature, although the actual temperature on the feathered areas was lower than the temperature of featherless areas.

1.2.5 Risk factors

Cellulitis appears to be a multifactorial disease, with risk factors distributed across the broiler production chain. The origin of the chickens (hatchery source) has been reported to influence the occurrence of cellulitis and the age of the parent flocks may also influence the outcome (Buzdugan *et al.* 2020; Schulze Bernd *et al.* 2020; Junghans *et al.* 2022). A common cause of mortality in parent hens as they gain age is coliform salpingitis, and it has been shown that these hens can transmit *E. coli* vertically to their progeny with ensuing transmission between chicks (Poulsen *et al.* 2017; Naundrup Thøfner *et al.* 2019). The route of transmission from hen to chick has not been fully investigated, but may involve transmission via the eggshell or in the hen's oviduct or both. Schrader *et al.* (2004) reported a difference in cellulitis condemnation rates between broiler chicken farms, suggesting farm management to be an important risk factor. Large chicken farms and houses with a large capacity (large flock sizes) have been associated with a higher condemnation rate from cellulitis (Elfadil *et al.* 1996a; Schulze Bernd *et al.* 2022). Small broiler houses may speculatively facilitate daily inspection of

the chickens, making it easier to notice diseased birds and remove dead birds at an early stage (Schulze Bernd *et al.* 2022). A significant association between condemnation due to cellulitis and the stocking density (kg/m^2) has been found (Forseth *et al.* 2023), while others found no such significant association (Elfadil *et al.* 1996a; Schulze Bernd *et al.* 2022). The effect of daily inspection of the chickens was investigated by Schulze Bernd *et al.* (2020), who concluded that fewer inspection visits in the broiler house correlated with a lower occurrence of cellulitis at meat inspection. The number of persons inspecting the flocks had no such influence. Inspection was proposed to excite the flock and increase the risk for chickens to scratch one another, which in turn can make it possible for *E. coli* to enter the subcutaneous tissue (Schulze Bernd *et al.* 2020). Other factors in the broiler houses that have been observed to have an effect on cellulitis occurrence include type and moisture level of the litter, lighting conditions, total downtime between flocks, distance between feeding trays and use of trays under the water nipples (Elfadil *et al.* 1996a; Schrader *et al.* 2004; Xavier *et al.* 2010; Schulze Bernd *et al.* 2020). Increased age of the chicken flock at slaughter have been associated with increased condemnation due to dermatitis/cellulitis (Forseth *et al.* 2023). Further, other morbidities that lead to condemnation of chicken carcasses at meat inspection, such as the ascites syndrome and polyserositis, and pododermatitis, which is recorded at slaughter, have been positively correlated with cellulitis (Elfadil *et al.* 1996b; Schulze Bernd *et al.* 2020; Forseth *et al.* 2023). Differences in the condemnation rate of cellulitis between slaughterhouses in the same country and during the same time-period have been observed, as well as differences within a slaughterhouse, depending on day or night shifts (St-Hilaire & Sears 2003; Gustafson 2019; Buzdugan *et al.* 2020). Together, these studies suggests that slaughterhouses and meat inspectors evaluate or record cellulitis inconsistently at meat inspection, and that there might be a need for a more uniform assessment.

1.3 Escherichia coli

The bacterium *E. coli* belongs to the order *Enterobacterales*, family *Enterobacteriaceae* and genus *Escherichia* (Janda & Abbott 2021). It is a Gram-negative, rod-shaped and motile bacterium with peritrichous flagella. When cultured on blood agar, colonies are 2–3 mm in size, opaque and grey

to white, and some isolates show a clear haemolytic zone (Figure 3). *Escherichia coli* is found in the normal intestinal flora in warm-blooded animals, including humans.

Figure 3. Growth of *E. coli* on blood agar that displays the typical morphology of 2 mm grey colonies. Photo: Liv Jonare

1.3.1 *Escherichia coli* diversity in poultry

Although most *E. coli* strains are considered commensals, some possess virulence factors that enable them to cause disease, either as a primary pathogen or as an opportunist. Avian pathogenic *E. coli* (APEC) belongs to the group of extraintestinal pathogenic *E. coli* (ExPEC) and is associated with extraintestinal disease in poultry, *i.e.* colibacillosis (Nolan *et al.* 2020). Colibacillosis in poultry is the most common bacterial infection occurring in poultry, regardless of species and age category and exists in several forms, such as cellulitis, air sacculitis, salpingitis, and colisepticemia. Avian pathogenic *E. coli* is a heterogeneous group and there is no clear definition of the pathotype with regards to virulence factors (Ewers *et al.* 2004; Mehat *et al.* 2021). However, some combinations of virulence genes that may distinguish APEC from avian faecal commensal *E. coli* have been suggested (Ewers *et al.* 2004; Johnson *et al.* 2008).

1.3.2 Characteristics of *E. coli* isolates associated with cellulitis

Serotypes and multilocus sequence types

Strains of *E. coli* can be classified into serotypes, which is based on the presence of cell surface antigens (*e.g.* somatic, O and flagellar, H), or into multilocus sequence type (ST), based on the allelic profile in a set of housekeeping genes. The APEC serotypes associated with cellulitis are the same as those found in other types of colibacillosis in poultry (Nolan *et al.* 2020). The most common serotypes of isolates from cellulitis lesions are O78 and O2, although studies also have reported others, including O25, O83 and O115 (Messier *et al.* 1993; Peighambari *et al.* 1995b; Ngeleka *et al.* 1996; Jeffrey *et al.* 2002). Sequence types of APEC that are associated with cellulitis include STs 23, 95, 101, 117 and 428 (Poulsen *et al.* 2018; Mehat *et al.* 2021; Alfifi *et al.* 2022; Jonare *et al.* 2023). Of these, STs 23, 101 and 117 have been reported to cause increased occurrence of colibacillosis including cellulitis in Nordic countries during 2017–2020 (Poulsen *et al.* 2018; Alfifi *et al.* 2022; Kromann *et al.* 2023).

Virulence factors

Avian pathogenic *E. coli* isolated from cellulitis lesions have been shown to carry virulence genes associated with adhesion, iron acquisition and serum resistance (Barbieri *et al.* 2013; Poulsen *et al.* 2018; Alfifi *et al.* 2022). Pathogenicity has been associated with the virulence genes *kpsMTII* (capsule antigen), *gimB* and *ibeA* (invasins) (Barbieri *et al.* 2013). Further, the cytotoxic *E. coli* vacuolating factor (ECVF) has been suggested to be an important factor in the inflammatory response caused by APEC in cellulitis, as it presumably induces microscopic lesions (Quel *et al.* 2013).

In general, cellulitis-derived *E. coli* isolates have been shown to harbour the same characteristics and virulence genes as *E. coli* isolated from other types of colibacillosis in poultry (Ngeleka *et al.* 1996; Jeffrey *et al.* 2002). de Brito *et al.* (2003) showed that virulence factors were more frequently detected among *E. coli* isolated from cellulitis and that these isolates had a higher capability to cause cellulitis, than randomly selected faecal isolates of *E. coli*. The differences in the cellulitis reproducing ability between *E. coli* isolated from cellulitis and isolates from other types of colibacillosis and random isolated from faeces, have been demonstrated in earlier studies, but genomic characterization was not presented for these isolates (Peighambari *et al.* 1995a; Johnson *et al.* 2001).

Genetic relationships

Singer *et al.* (1999), used DNA fingerprinting by pulse-field gel electrophoresis (PFGE) and showed that birds with cellulitis from an individual flock or farm can harbour isolates that are genetically closely related. Further, other results based on PFGE indicate that closely related isolates can persist in the broiler house and cause cellulitis in the consecutive flock (Singer *et al.* 2000). When investigating isolates within and between farms, genetically diverse as well as closely related isolates have been reported (Elfadil *et al.* 1996a; Singer *et al.* 1999; de Brito *et al.* 2003; Poulsen *et al.* 2018). These studies suggests that cellulitis can be caused by a range of different *E. coli* isolates and that cellulitis-associated isolates can persist on a farm, as well as most likely be transferred within the broiler production chain, however, how this occurs is not known.

1.4 Chicken immune response

1.4.1 Immune response to bacterial infections

The innate immune response includes a wide variety of non-specific mechanisms that are activated immediately upon encounter of bacterial infection, such as physical barriers and cellular recognition and elimination of pathogens. Important physical barriers include the skin and surface epithelium (Alber *et al.* 2019; Kaspers *et al.* 2021). Substances like mucus and antimicrobial molecules further reinforce these barriers. Trauma to the physical barriers or invasion of bacteria lead to activation of innate immune cells (Alber *et al.* 2021). These cells, *e.g.* heterophils (orthologue to mammalian neutrophils), monocytes and thrombocytes, have cell surface or soluble pattern recognition receptors (PRRs), *e.g.* toll-like receptors (TLRs) that detect pathogens and host-derived molecules. Pathogens are recognised by binding of PRRs to structures common for groups of pathogens, so called pathogen associated molecular patterns (PAMP). In *E. coli*, the antigenic O segment of lipopolysaccharides, which is a key component of the cell wall, is an important PAMP, but there are also others, like the flagellar antigen H (Alber *et al.* 2019). Recognition activates immune cells by intra-cellular signalling pathways. Once activated, heterophils and macrophages may *e.g.* pursue phagocytic activity at the site of infection and produce cytokines that

activate and modulate the immune response (Juul-Madsen *et al.* 2014; Alber *et al.* 2021).

Heterophils are the dominating phagocytosing innate immune cells and are considered important in the defence against *E. coli* (Genovese *et al.* 2013; Alber *et al.* 2021). They have cell surface receptors that facilitate phagocytosis and these receptors depend on either the opsonisation of bacteria by complement proteins and antibodies or are non-opsonisation dependent, such as the mannose receptor. After recognising the bacterium, it is engulfed by the cell in a membrane bound vesicle (phagosome) that merges with cytoplasmic granules, consisting of anti-microbial substances, which kills the pathogen. (Genovese *et al.* 2013).

Knowledge about the adaptive response to *E. coli* in chickens is relatively limited. Studies have demonstrated that antibodies can opsonize *E. coli*, potentially enhancing its clearance. Further, after vaccination against *E. coli*, T-cell responses have been demonstrated (Alber *et al.* 2021).

1.4.2 Cellulitis

There is a lack of knowledge regarding the immune response during the development of cellulitis in broiler chickens. The role of the first line defence was evaluated in a study where broiler and leghorn chickens of the same age were subcutaneously inoculated with an APEC strain aiming to induce cellulitis (Olkowski *et al.* 2005). Broilers chickens developed larger cellulitis lesions and recruited fewer heterophils and macrophages with lower phagocytic activity to the lesions compared to leghorn chickens. It was concluded that broilers were more susceptible to cellulitis due to deficiencies in the first line defence (Olkowski *et al.* 2005). Further, an adaptation of the immune response in line with the expected difference in lifespan in broiler and layer-type chickens has been suggested, based on humoral and cellular response after immunization with 2,4,6-Trinitrophenol (Koenen *et al.* 2002). Broiler chickens generated a short humoral response with primarily IgM antibodies, while layer chickens of the same age generated a more long-term humoral response consisting of both IgM and IgY antibodies and a strong T-cell response. The findings in these studies were suggested to be associated with selective breeding towards better meat-yielding traits, such as feed conversion ratio, where other physiological functions might have been altered (Koenen *et al.* 2002; Olkowski *et al.* 2005).

Aims of the thesis

The overall aim of this PhD project was to gain knowledge about the pathogenesis and the underlying risk factors for cellulitis in broiler chickens. Increased knowledge can provide a better and more reliable basis for implementation of preventive measures. The specific objectives of the studies reported in papers I–IV were to:

- Investigate if systemic spread of *E. coli* can occur in broiler chickens with cellulitis at the time of slaughter.
- Analyse and describe genomic characteristics of *E. coli* isolated from cellulitis lesions.
- Analyse immune parameters in chicken blood during the development of cellulitis.
- Study clinical signs during development of cellulitis.
- Assess if infrared thermography may be used as an *in vivo* diagnostic tool for cellulitis in chickens.
- Analyse broiler chicken management and production data to identify possible risk factors for cellulitis.
- Describe condemnation percentages due to cellulitis in Sweden in relation to various broiler production parameters in 2021-2022.

2. Comments on materials and methods

Detailed materials and methods for each study are described in papers I–IV. This section provides some additional comments.

2.1 Paper I

2.1.1 Bacterial sampling and culture

Bacterial sampling from the subcutaneous tissue (with or without a lesion), the pericardium, and the spleen parenchyma were systematically performed on 101 chicken carcasses that were condemned due to dermatitis/cellulitis at a Swedish large-scale broiler chicken slaughterhouse. Several precautions were taken to minimize the risk for bacterial contamination during sampling:

- Upon collection from the slaughter line, the carcasses were individually placed in plastic bags, transported to a nearby building and sampled within 60 minutes.
- The sampling was done before the PM examination.
- Samples from the cellulitis lesions were taken as far away as possible from any meat inspection incision(s). The surface of the spleen was seared with a refillable kitchen torch before incision with a sterile scalpel.

Swabs were streaked on 5% bovine blood agar and Cysteine Lactose Electrolyte Deficient (CLED) agar [Swedish Veterinary Agency (SVA), Sweden]. The CLED agar plates were used to prevent swarming of *Proteus* spp. The growth on agar plates was classified as pure or mixed based on colony morphology. Presumed *E. coli* colonies were counted in case of sparse growth. At least one *E. coli* colony from each plate was confirmed

with matrix-assisted laser desorption ionization-time of flight mass spectrometry (MALDI-TOF MS) technique. MALDI-TOF MS is simple to use and considered as a reliable method to identify bacterial species (Torres-Sangiao *et al.* 2021).

2.1.2 *Post-mortem* examinations

After sampling, a PM examination was performed. A routine PM examination protocol from SVA (Sweden) was used, with focus on the cellulitis lesions and gross findings indicative of bacterial systemic spread, such as presence of exudate on the surface of organs, organ enlargement and suspected necroses. The definition of cellulitis used was according to Nolan *et al.* (2020):

> "Cellulitis lesions are primarily unilateral and located on the abdomen and thigh. Skin color varies from normal to yellow or red-brown, and the skin may be swollen at the site of inflammation. […] Beneath the skin, there is subcutaneous edema, exudate, and muscle hemorrhage. A fibrinous to caseous plaque between the muscle and the subcutis is the characteristic lesion."

2.1.3 Characterization of isolated *E. coli*

Escherichia coli isolates were selected for whole genome sequencing (WGS), resulting in 36 isolates from 26 carcasses. Due to the presence of mixed bacterial growth in some of the samples, the selection of *E. coli* isolates for WGS were based on growth results on the primary plate. Only samples with a pure growth of *E. coli* or with minor growth of other bacteria in addition to *E. coli*, were selected for WGS. This step was added to minimize the risk that the *E. coli* isolates used for WGS represented contaminating bacteria.

Additionally, ten other *E. coli* isolates were included, which originated from cultures of carcasses that had pure growth of *E. coli* in characteristic cellulitis lesions (Nolan *et al.* 2020), but with no or non-specific growth in samples from organs. The selection of these isolates was done randomly from two flocks with five isolates from each flock. The addition of these isolates made it possible to compare the genetic characteristics of *E. coli* isolated from chickens with and without putative systemic spread of the bacteria.

2.2 Paper II & III

2.2.1 Experimental infection studies

Overview

In the studies described in papers II and III, an experimental model was set up to induce cellulitis in chickens for the evaluation of clinical signs and gross findings, the immune response and infrared imaging as an diagnostic tool. In each study, 50 broiler chickens were included. In paper II, there were five groups with ten chickens each, where four groups were inoculated with either of four *E. coli* isolates. In paper III, there were three groups with 15 chickens in each group, where two groups where inoculated with one out of two *E. coli* isolates.

Experimental models

There are previously published papers that describe experimental models for cellulitis (Peighambari *et al.* 1995a; Gomis *et al.* 1997b; Norton *et al.* 1997). Two different models have been described to successfully induce cellulitis.

A. Scratching the skin with a needle and then rub a swab dipped in an overnight broth culture of *E. coli* on the scratched surface.
B. An injection into subcutaneous tissue of an overnight broth culture of *E. coli*.

In previous studies, the "scratch-and-swab" method (A) resulted in cellulitis in 42–100% of birds depending on the *E. coli* isolate (isolated from avian cases of cellulitis or air sacculitis), and the subcutaneous inoculation method (B) induced cellulitis in 54–100% of the birds depending on the dose of *E. coli* (Peighambari *et al.* 1995a; Gomis *et al.* 1997b; Norton *et al.* 1997). Broth culture swabbing on intact skin or on skin with plucked feathers did not induce cellulitis (Peighambari *et al.* 1995a). None of these studies reported changes in the general clinical condition of the inoculated chickens, despite that gross findings of systemic colibacillosis were observed in 8–77% of cases at PM examination (Peighambari *et al.* 1995a; Gomis *et al.* 1997b). Several later studies have used the subcutaneous inoculation model to induce cellulitis experimentally (Jeffrey *et al.* 1999; Johnson *et al.* 2001; Macklin *et al.* 2009; Allan *et al.* 2012). Based on these earlier papers, we chose the subcutaneous inoculation method for papers II and III, because of the slightly

higher likelihood of birds developing cellulitis. Moreover, we speculated that the "scratch-and-swab" method would possibly be associated with a higher risk of contamination with *E. coli* from the litter compared to subcutaneous inoculation.

Inoculation dose

In paper II, we aimed for an inoculation dose of 10^8 CFU of *E. coli*. Studies describing the subcutaneous inoculation method used inoculation doses of 10^6–10^9 CFU of *E. coli*, which caused cellulitis in 100% of the chickens (Gomis *et al.* 1997b; Norton *et al.* 1997). Later studies reported a cellulitis rate of 33–100% when using doses of 10^6–10^8 CFU of *E. coli* (Jeffrey *et al.* 1999; Johnson *et al.* 2001; Macklin *et al.* 2009; Allan *et al.* 2012). In paper III, the inoculation dose was lower (10^7 CFU), with the aim to reduce the number of chickens with systemic bacterial spread.

Selection of E. coli isolates for inoculation

In the earlier published papers, no data on genomic characteristics of inoculated *E. coli* have been provided (Peighambari *et al.* 1995a; Gomis *et al.* 1997b; Norton *et al.* 1997; Jeffrey *et al.* 1999; Johnson *et al.* 2001; Macklin *et al.* 2009; Allan *et al.* 2012). In paper II, we used four *E. coli* strains that had been isolated from cellulitis lesions in carcasses condemned due to cellulitis at a Swedish slaughterhouse. The isolates originated from birds from three different flocks, with cellulitis lesions of different sizes (small or large) and from birds with or without gross findings of systemic bacterial spread. With this selection, we aimed to increase the likelihood that at least one strain would induce cellulitis. For paper III, we chose the two strains from paper II that had induced local cellulitis lesions, but a lower proportion of chickens with systemic bacterial spread, as compared with the other strains.

2.2.2 Infrared imaging

Rationale for the evaluation of infrared imaging

As IRT is non-invasive and requires a minimum of handling of the birds, it could be advantageous to use as a diagnostic tool for chickens with cellulitis in research studies. As described in the introduction of this thesis, IRT has been successfully used in several applications in poultry research. Further, Tessier *et al.* (2003) showed that a featherless area on the broiler abdomen

had small within and between bird variations and suggested that the technique might be suitable for detecting cellulitis. In addition, feathered areas appear to increase in temperature along with featherless areas if the environmental temperature is increased, although on a relatively lower scale (Naas *et al.* 2010). Hence, we considered IRT to be promising for evaluation of possible increase in surface temperature due to inflammation in the abdominal skin on both feathered and non-feathered areas.

Overview

In the study described in paper II, IRT imaging was evaluated as a diagnostic tool for cellulitis. After experimental inoculation with *E. coli*, images were obtained from all birds with an infrared FLIR E8 camera (Teledyne FLIR LLC, Wilsonville, OR, USA, Figure 4). According to recommendations for conducting measurements similar to those in our study, the camera had a resolution of 320*240 pixels, its thermal sensitivity was 0.05°C and the spectral range was 7.5–13 μm (Yahav 2012). The birds were placed on a table, and a right and left lateral and caudolateral view were obtained from approximately 0.6 m. The procedure was repeated at 9, 19, 31, 44 and 92 hours post inoculation (PI).

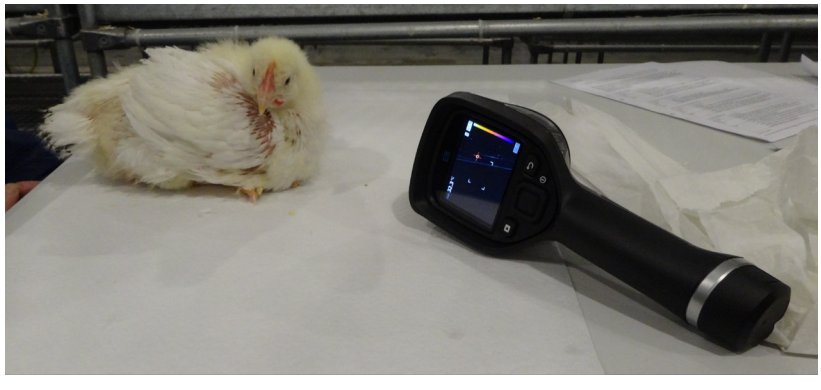

Figure 4. The handheld FLIR E8 infrared camera. Photo: Liv Jonare

2.2.3 Clinical assessment

As requested from the Ethics committee, the clinical condition of the birds was assessed using an assessment protocol developed by Linköping University (unpublished). The protocol includes assessment of each individual bird with focus on its general appearance, posture, gait, and the

body condition, plumage condition, eyes and skin. Additional monitoring parameters were added to our study protocol, such as a general group/flock activity assessment, that was carried out upon entry to the animal facility, *i.e.* vocalization, locomotion, feeding, drinking and resting.

2.2.4 Bacterial and tissue sampling

In paper II, all cellulitis lesions were systematically sampled for bacterial culture and in a subset of the chickens with gross findings of systemic bacterial spread, organs were also sampled. To complement the dataset, organs from the remaining chickens with gross findings of systemic spread were sampled at a later time-point from thawed carcasses. The number of samples was increased for a more complete assessment of the outcome of the experimental infection. In paper III, all chickens were sampled in the cellulitis lesion, pericardium and spleen parenchyma prior to the PM examination.

2.2.5 Blood analyses

In paper III, blood samples were drawn from the jugular vein on day -3 or -4 relative to day of inoculation (day 0), on days 1–4, and 7 PI. Blood for serology was collected *post-mortem* from the femoral vein.

Leucocyte counts

Total and differential leukocyte counts may be used to assess many aspects of the immune system. In this study, we wanted to evaluate the leucocyte cell counts before and after inoculation to detect a possible immune response to *E. coli*. Conventional haemocytometers cannot reliably quantify chicken blood cells because avian erythrocytes and thrombocytes are nucleated, and thrombocytes have similar size as the lymphocytes (Scanes 2015). Flow cytometry uses fluorescence and light scattering to differentiate cell populations, and combined with immunofluorescence labelling for cell-specific markers, the methodology can be used for identification and enumeration of chicken leukocytes in whole blood samples. Compared to cell identification under a microscope, flow cytometry is much faster (>1000 cells per second) and less subjective, which enables a robust dataset for analysis.

In the study described in paper III, several populations of chicken blood cells were analysed by immunofluorescence labelling and flow cytometry:

heterophils, monocytes, thrombocytes, total lymphocytes and some lymphocyte subpopulations including B-cells. The T-cell subpopulations are shown in Table 1.

Table 1. Subpopulations of T-cells identified by immunofluorescence labelling and flow cytometric analysis in paper III

TCR & co-receptors	Celltype
CD4+CD8-	"Classic" T-helper cell[1]
CD4+CD8α+	T-helper cell with possible memory function[1]
CD4-CD8αβ+	Cytotoxic T lymphocytes (CTL)[2]
CD4-CD8αα+	Several cell types, incl. natural killer (NK) cells[3]
TCRγ/δ+CD8-	T-cells expressing the γ/δ TCR
TCRγ/δ+CD8αα+	
TCRγ/δ+CD8αβ+	

[1]Luhtala (1998), [2]Smith and Göbel (2014), [3]Straub *et al.* (2013).

In addition, CD25 cell surface expression on the T-cell subpopulations (Table 1) was analysed as a marker of possible activation (Hála *et al.* 1986).

Detection and quantification of antibodies to E. coli

To quantify levels of *E. coli*-specific IgY in serum we used an in-house ELISA protocol (SVA). The method was slightly modified from Wattrang *et al.* (2020) and is briefly described in paper III. Sonicated preparations of each of the two inoculated *E. coli* strains were used as coating antigens. Before analysing the study samples, an alternative coating with heat-treated whole-cell bacteria was evaluated. A whole-cell bacterial solution presumably displays a higher proportion of cell-surface antigens, which should theoretically enhance the detection of *E. coli* strain-specific antibodies. However, when the ELISA was run on a subset of study samples, the results from the whole-cell bacterial coating were similar to the coating with sonicated bacteria. This result indicate that the whole-cell bacteria may have been lysed by *e.g.* the high pH in the coating buffer. An alternative would have been to use purified *E. coli* surface antigens (*e.g.* O antigens) from the inoculation strains as coating. However, there was not enough time to produce these in the lab and they were not found to be commercially available. This led to the decision to use sonicated bacteria, which were presumed to be more stable in solution over time than whole-cell bacteria.

Functional test for phagocytic cells: adhesion

Phagocytic activity of blood cells can be an indication of the immunocompetence of chickens. As the phagocytosis is initiated by attachment of the blood cells to bacteria, adhesion can reflect the phagocytic activity. We wanted to assess the *in vitro* adhesion of heterophils, monocytes and thrombocytes to the inoculated *E. coli* strains and evaluate the extent of adhesion before and after inoculation.

To be able to track the bacteria in the assay, the *E. coli* strains were fluorescence-labelled according to a previously published protocol (Atwal *et al.* 2016). A red CellTrace™ Far Red Cell dye was used, which enters the cell and remains in the cytoplasm by covalent binding to amines. This type of labelling was suggested to be suitable for studies of bacterial attachment (Atwal *et al.* 2016). Samples of whole-blood from the chickens were incubated with each of the labelled *E. coli* strains separately, and then analysed by flow cytometry, using an adjusted protocol based on an assay for phagocytosis in chicken (Naghizadeh *et al.* 2019). The flow cytometry assay allowed identification of the phagocytic activity of selected cell types identified by immunofluorescence labelling, heterophils, monocytes and thrombocytes. By multi-colour fluorescence detection, labelled *E. coli* that was adhered to the different cell populations were detected. Using whole blood in the assay has been suggested to resemble conditions *in vivo*, as compared to using preparations of isolated cells (Naghizadeh *et al.* 2019).

2.3 Study IV

2.3.1 Database extract

In the study described in paper IV, an extract from the database *Tuppen* was used to evaluate factors that could potentially affect the condemnation percentages for dermatitis/cellulitis at the slaughterhouse. The Swedish Poultry Meat Association maintains *Tuppen*, to which different companies throughout the broiler production chain, *e.g.* hatcheries, rearing farms and slaughterhouses, contribute data. The database is designed for use as a follow-up tool to monitor and assess production results, *i.e.* not primarily for research purposes. The provided dataset from *Tuppen* covered the years 2021 and 2022, but data from January to mid-May 2021 were excluded because

some farms reared Cobb chickens during that period. We aimed to include only one genotype (Ross 308) in the analysis.

2.3.2 *Post-mortem* examination of dead and culled chickens

Dead or culled chickens from broiler farms were examined PM to evaluate if cellulitis could cause mortality or disease during late grow-out. Birds from farms (n=10) with low or high mean condemnation percentage of dermatitis/cellulitis (<0.5% and >1.3%, respectively) during the year 2022 were included. The farmers were asked to collect 50 dead or culled chickens within 24 hours of farm visits, which were planned to occur within a few days prior to slaughter. The chickens were preferably from one broiler house compartment, but chickens from additional compartments were accepted in order to achieve a sufficient number of birds. The PM examination was simplified to allow the procedure to be completed during one day per flock and the primary focus was cellulitis.

2.4 Statistical analyses

Detailed statistical methods can be found in paper I–IV, below are some further considerations and comments.

2.4.1 Number of chickens in papers II and III

According to the guidelines of the 3Rs (replace, reduce, refine) (National Center for 3Rs 2024), calculations were made to determine the number of birds required to address the respective research question in papers II and III.

Study II: Based on the variation of earlier measurements (Tessier *et al.* 2003; Naas *et al.* 2010; Giloh *et al.* 2012) and a presumed difference of 1.0°C before and after infection, it was decided to use a group size of ten chickens. The sample size was calculated with 80% power and significance level of 5% using R software (R Core Team 2013).

Study III: According to Wattrang *et al.* (2022), five chickens in each sample group were necessary to observe significant effects on selected blood parameters in a systemic bacterial infection. The calculations were based on 80% power and a significance level of 5%. The subcutaneous *E. coli* inoculation was expected to produce a local infection that likely would affect the immune parameters to a lesser extent than a systemic infection.

Consequently, it was decided that each sampling group would consist of at least seven chickens.

2.4.2 Further details of the modelling in paper III

A mixed effect model was used to determine differences in mean body and skin temperature within the groups between the IRT measurement occasions (at 9, 19, 31, 44 and 92 hours PI). One model was run for each experimental chicken group and body area of interest. In the model, the temperature (outcome variable) was set as a function of the measurement occasion (independent variable/fixed effect). Each chicken received a unique number, which was set as a random effect, *i.e.* the model had a random intercept. A continuous autoregressive correlation was used for each individual and measurement occasion PI, as the time elapsing between the measurements differed.

2.4.3 Summary of the modelling in paper IV

A mixed effect model was used for the analyses in paper IV. Chicken flock was the unit of observation and a flock was defined as a group of broilers placed in the same compartment (broiler house section) on the same date of placement. The outcome was the condemnation percentage due to dermatitis/cellulitis, which was log-transformed to achieve normal residual distribution. The results of the model were back-transformed. A mixed effect model was chosen as it includes the variance on data depending on random effects on the included main effects (fixed effects). The random effects in our model were compartments nested within farms. Based on p values <0.2 from the initial univariable analysis, all fixed effects were brought together. This model was then reduced to only include significant fixed effects ($p<0.05$). Interactions considered to have a possible effect on condemnation due to dermatitis/cellulitis, were added to the reduced main effect model. This model was then further reduced to achieve significant fixed effects and interactions. Estimated marginal (EM) means and EM trends were used for comparisons between groups.

3. Results and discussion

The most important results from the studies described in paper I–IV are summarised and discussed in this section.

3.1 Post-mortem findings

3.1.1 Paper I

In addition to cellulitis, several other skin conditions were observed among the 101 broiler carcasses that had been condemned for dermatitis/cellulitis at the slaughterhouse (Figure 5).

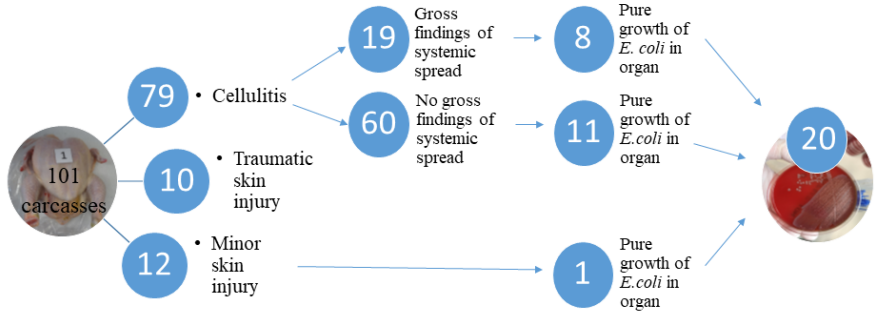

Figure 5. Summary of gross and bacteriological findings in the 101 PM examined broiler carcasses condemned at meat inspection due to dermatitis/cellulitis.

Cellulitis was diagnosed in 79 out of the 101 examined carcasses. It was expected that not all carcasses would be diagnosed with cellulitis, as there is

a joint condemnation code for dermatitis and cellulitis in Sweden (Swedish Food Agency 2021).

Cellulitis is most often described as a localized lesion, predominantly found on the abdomen (Messier *et al.* 1993; Peighambari *et al.* 1995b; Elfadil *et al.* 1996b; Fallavena *et al.* 2000; Gomis *et al.* 2000). In our study (paper I), the majority (57%) of the carcasses condemned with cellulitis had a lesion that involved two or more areas of the body, while 43% had a local lesion on the abdomen, thigh or proximal trunk. The higher proportion of carcasses that had a lesion involving two or more areas of the body, as compared to a local lesion, could be explained by the definition used, *e.g.* as we included subcutaneous oedema at the periphery of the lesion.

There were other types of skin lesions present in 96% of the carcasses with cellulitis, such as single or multiple linear abrasions (presumably skin scratches), and small multifocal and superficial circular spots. These were not always located in close association with the cellulitis lesion. As skin lesions, such as scratches, are presumed to be the portal of entry for *E. coli* in the pathogenesis of cellulitis, skin lesions were expected. However, carcasses condemned due to cellulitis with no skin scratches have also been observed (Alfifi *et al.* 2022). In that study, other factors, such as irritated skin in combination with *e.g.* stress, were suggested to be predisposing for cellulitis.

Nineteen (24%) of the carcasses diagnosed with cellulitis (paper I) had gross findings indicative of systemic spread (Figure 5), which was an expected result based on previous reports. In earlier studies, approximately 30% of chickens with cellulitis have been reported with gross findings indicative of systemic bacterial spread, such as perihepatitis or pericarditis, which is higher than in our study (Gomis *et al.* 1997a; Onderka *et al.* 1997; Gomis *et al.* 2001). A variety of flock-related factors, husbandry and bacterial characteristics are some of the factors that could influence the likelihood of systemic disease. There can also be a difference in the definition of cellulitis and assessment between studies. In Sweden, all inflammatory conditions involving the skin and the subcutaneous tissues are recorded under the same condemnation code at the slaughterhouse at meat inspection, which in this study was illustrated by a broader spectrum of gross findings associated with the skin than the general definition of cellulitis (Nolan *et al.* 2020). For example, three of the ten carcasses diagnosed with a traumatic skin lacerations on the craniolateral breast area had a small

underlying subcutaneous plaque-like formation close to the lesion (Figure 5). These carcasses were not added to the cellulitis group as the lesion, judging from its location and appearance, most likely had a different cause than the typical cellulitis lesion. Situations like this emphasizes the need for a clear definition of lesions.

3.1.2 Papers II and III

For the studies described in paper II and III, days PI for the PM examination and the number of chickens with gross findings of cellulitis and systemic bacterial spread are summarized in Table 2.

Table 2. Summary of the *E. coli* inoculated groups in the two experimental inoculation models and key findings

Paper	Group	*E. coli* strain	Dose (CFU)	Early euth.[1]	Euth. days PI[2]	Cellulitis	Systemic spread
II	1	ECA9	$1.4*10^8$	8/10	3	7/10	10/10
	2	ECA18	$1.8*10^8$	2/9	4	9/9	6/9
	3	ECB11	$1.3*10^8$	1/9	5	9/9	4/9
	4	ECCA689	$1.7*10^8$	6/10	6	9/10	8/10
III	1 (A)	ECA18	$1.1*10^7$	1/15	12–14	7/15	0/15
	2 (B)	ECB11	$1.8*10^7$	2/15	12–14	13/15	0/15

[1]Early euthanasia and mortality

[2]Number of days PI when the groups were euthanized and examined *post-mortem*

In paper II, the gross findings of cellulitis were in accordance with the general definition, *i.e*. a subcutaneous, yellow or brown-red fibrinous plaque that varied from 1–10 cm (Figure 6a). In paper III, the inoculation dose was lower and presumably less virulent strains were used, and chickens were PM examined later than in paper II (Table 2). The majority of these chickens had a lesion with plaque-like material partially or completely enclosed either in subcutaneous tissue or within the abdominal wall (Figure 6b).

Microscopy was not included in this study, which could have provided a broader understanding of the developed lesions in terms of development. In an earlier study by Peighambari *et al.* (1995a), in which broiler chickens were examined PM ten days PI, microscopy showed that connective tissue had developed around the lesions.

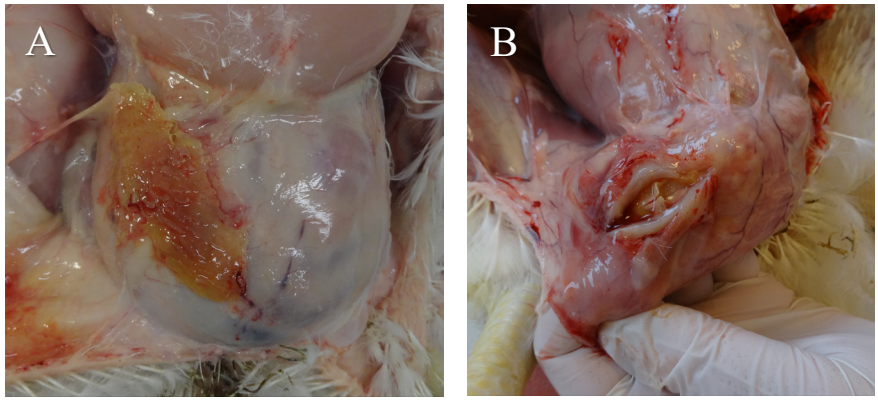

Figure 6. Gross findings of cellulitis in broiler chickens inoculated subcutaneously with *E. coli* described in papers II and III (A and B, respectively). Photos: Liv Jonare

Based on these results, it may be suggested for our study that the demarcation consisted of fibrous tissue, although this cannot be confirmed. In addition, other early studies have reported histological findings in carcasses with cellulitis condemned at slaughter, including fibrous thickening of the dermis and encasement of the plaque by connective tissue (Elfadil *et al.* 1996b; Onderka *et al.* 1997; Fallavena *et al.* 2000).

In general, the cellulitis lesions found in broilers at slaughter are most commonly described as having similar appearance to the gross lesions found in paper II and not demarcated as in paper III (Gomis *et al.* 1997a; Poulsen *et al.* 2018; Nolan *et al.* 2020). This suggests that cellulitis in commercial settings develop in the late grow-out phase, during the days prior to slaughter.

In paper II, the majority of chickens had gross findings of systemic bacterial spread (Table 2). Such findings have been reported from other cellulitis experimental studies and is a regular finding in chickens with cellulitis at slaughter (Peighambari *et al.* 1995a; Gomis *et al.* 1997a; Gomis *et al.* 1997b; Onderka *et al.* 1997; Silva *et al.* 2021; Alfifi *et al.* 2022). In paper II, the chickens were inoculated with bacteria that were not washed before dilution (see paper II and III for preparation of inocula). The washing in paper III might speculatively have eliminated endotoxins released by dead or dying bacteria in the *E. coli* culture, and thereby contributed to a milder inflammatory response upon inoculation. Endotoxins (lipopolysaccharides) from *E. coli* have been used in an experimental study in broilers to evaluate the inflammatory response (De Boever *et al.* 2009). In paper III, the *E. coli*

strains that had caused milder clinical signs, were selected for inoculation and with the lower dose (Table 2), there were no gross findings of bacterial infection other than at the site of inoculation. Hence, the success rate of cellulitis development in papers II and III was presumably influenced by several factors, including the preparation of the inoculum, the dose, and the *E. coli* isolate, with the latter two suggested as contributors previously (Jeffrey *et al.* 1999; Johnson *et al.* 2001).

In both paper II and III, gizzard erosions were observed at the PM examinations in the majority of chickens, in most cases the lesions were superficial and small to moderate in size. These types of lesions can be associated with various aetiologies including fowl adenovirus (FAdV), and serotype 1 which has previously been detected in flock outbreaks in Sweden (Lindgren *et al.* 2022). Tissue samples from the gizzard and caecal tonsils from a total of eight birds were analysed by routine PCR methodology at SVA (Meulemans *et al.* 2001). The samples from gizzards and caecal tonsils were negative for FAdV and the cause of the gizzard erosions was not determined.

3.1.3 Paper IV

The number of chickens that were examined PM in the study described in paper IV are specified in Table 3.

Table 3. Summary of the number of chickens that were dead and culled in the late rearing stage and examined PM after collection from farms. The number of birds that were diagnosed with cellulitis is specified for each category of farm (High and Low: mean percentage >1.3% and <0.5%, respectively, of condemnation due to dermatitis/cellulitis in 2022) and the percentage is calculated from the total number

	High	Low	Total
Dead	143	62	205
Cellulitis	*3*	*5*	*8 (1.6%)*
Culled	160	126	286
Cellulitis	*24*	*11*	*35 (7.1%)*
Total	303	188	491
Cellulitis	*27 (8.9%)*	*16 (8.5%)*	*43 (8.8%)*

These results showed that cellulitis occurred and that it was involved in almost one out of ten lost chickens during the late rearing period. For more definite numbers and variations between flocks and farms, further research

involving a larger number of chickens is needed. However, it can be concluded from our study that cellulitis most likely contributes to mortality and culling from disease on farms, which suggests that the occurrence of cellulitis based on meat inspection results most likely underestimates the true occurrence. Most of the cellulitis cases were observed in the culled chickens, indicating that at least some birds showed clinical signs. This observation contradicts the current opinion that broiler chickens with cellulitis show no or very mild clinical signs (Randall *et al.* 1984; Nolan *et al.* 2020). Seventeen of the 43 birds (40%) with cellulitis had gross findings of systemic bacterial spread, which might have been the main reason for culling. It appeared that there was no difference in cellulitis occurrence between farms with high or low condemnation percentage due to dermatitis/cellulitis, but more data is needed to confirm this.

3.2 Clinical assessment and mortality

3.2.1 Papers II and III

The chickens' clinical condition after the subcutaneous *E. coli* inoculation varied between studies and experimental groups (paper II and III). Inoculated chickens in paper II showed more pronounced clinical signs and were euthanized early to a higher extent, as compared with the chickens in paper III (Table 2). Two chickens in paper II (groups 1 and 4, respectively) and one in paper III (group B) died PI. In paper II, the clinical signs led to the decision to euthanize all chickens in group 1 three days PI and the remaining groups on the following days (Table 2). This outcome was unexpected, as previously published cellulitis experimental trials had not reported such effects (Gomis *et al.* 1997b; Norton *et al.* 1997; Jeffrey *et al.* 1999). However, the majority of birds in paper II were diagnosed with systemic spread of the inoculated *E. coli*. Systemic colibacillosis causes clinical signs in affected birds, which has been reported in coliform experimental trials in poultry using other routes of inoculation (Pors *et al.* 2014; Kumari *et al.* 2020; Nolan *et al.* 2020).

Further, the daily weight gain of the chickens in the inoculated groups in paper II and III was reduced PI (Figure 7). A lower bodyweight in *E. coli* inoculated birds was observed by Gomis *et al.* (1997b).

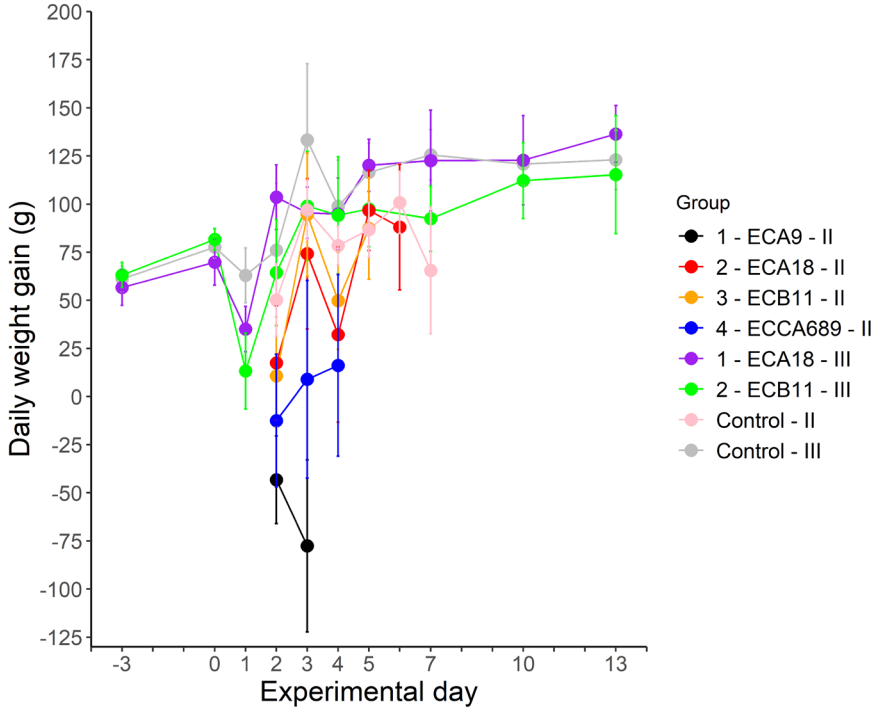

Figure 7. Mean daily body weight gain ± CI of the *E. coli* inoculated groups and controls in the cellulitis experimental model, described in paper II and III, respectively. Each group is named "group number - inoculated *E. coli* strain - number of paper (II or III)".

Thus, the assessment of the general condition and body weight gain were essential elements when evaluating how the chickens were affected by experimental *E. coli* inoculation. Further, the general assessment of the chickens from a distance when entering the animal house was a very important part in assessing the condition of the birds. At the individual examination, most of the birds appeared normal although the general condition sometimes appeared affected when observed in their pen. To observe the chickens from a distance has been described as a part of good stockmanship for Ross 308 broilers on commercial farms (Aviagen 2018).

3.3 Bacteriology and whole genome sequencing

3.3.1 Paper I

Bacteriology

In total, 327 samples from subcutaneous location and organs were cultured, of which 259 originated from birds diagnosed with cellulitis. Results are summarized in Table 4.

Table 4. Summary of culture results from 79 broiler carcasses condemned due to dermatitis/cellulitis at slaughter, and diagnosed with cellulitis at *PM* examination

Origin of sample	No growth	*E. coli*[1]	*E. coli* in mixed growth[2]	Mixed growth[3]	Other species[1]
Subcutis (*n*=79)	1	54	22	2	-
Pericardium (*n*=79)	29	8	14	21	7
Spleen (*n*=79)	31	11	9	13	15
Other organs (*n*=22)	3	4	3	12	-

[1]Pure or predominant growth in a mixed culture

[2]Some *E. coli* colonies in a mixed culture

[3]≥ 2 colonies with different morphology, no visible *E. coli* colonies

The majority of the plates showed pure growth of *E. coli* or growth of *E. coli* admixed with some other bacteria. This result indicate that *E. coli* was the predominant bacterial species in the cellulitis lesions, which is in agreement with previous publications (Randall *et al.* 1984; Nolan *et al.* 2020). Nineteen of the birds diagnosed with cellulitis also showed pure growth of *E. coli* in one or more of the sampled organs. Of these, eight birds had gross findings of systemic spread, while 11 of the birds had no visible lesions. As discussed in paper I, our findings indicate possible systemic spread of *E. coli* from the cellulitis lesion, as previously suggested (Gomis *et al.* 2001; Poulsen *et al.* 2018; Silva *et al.* 2021). Assessment of such finding has an economical importance, as it should be considered at meat inspection. Our results suggest

that it is difficult to reliably eliminate carcasses with cellulitis and systemic bacterial spread from the food chain. Therefore, it seems reasonable to recommend condemnation of carcasses with cellulitis regardless of the size of lesions, until further information is available. To test the sampling techniques, we performed a pilot study using approved broiler carcasses collected from the slaughter line after meat inspection (unpublished data). Sampling was performed immediately after gathering or four hours later, when carcasses had been subjected to storage at room temperature. In this pilot study, the majority of the samples showed no bacterial growth, only a few samples showed mild to profuse mixed flora after four hours, suggesting that the sampling technique was adequate and that carcasses should be sampled as soon as possible after collection. Therefore, it came as a surprise that approximately 30% of the samples from the subcutaneous tissue of birds diagnosed with cellulitis, showed non-specific growth, presumably from bacterial contamination, in addition to *E. coli*. As discussed in paper I, the mixed cultures were presumably a result of contamination of carcasses during processing (*e.g.* during the scalding and/or defeathering processes, at meat inspection when incisions are often made into the skin overlying suspected lesion), or during sampling.

The sampling was performed and blood agar were streaked in a non-laboratory facility at the premises of the slaughterhouse. It has previously been shown that various species of contaminating bacteria can be present on processed chicken carcasses (Moazzami *et al.* 2021; Alfifi *et al.* 2022). Hence, it may have been justified to eliminate all plates with a small number of *E. coli* colonies from WGS to avoid the risk of contaminating bacteria. However, this would have excluded birds with minor specific growth, which can indicate bacteraemia, early systemic spread or that the infection was being cleared (Gomis *et al.* 1997a; Onderka *et al.* 1997; Gomis *et al.* 2001). The majority of the sequenced isolates from organs in paper I, originated from samples with pure growth of >10 *E. coli* colonies (12/19 isolates, 63%). It might however be questioned, if the *E. coli* isolates from samples with pure growth of <10 *E. coli* colonies originated from the actual organ, or from contamination flora.

To conclude, our results indicate that it can be challenging to obtain non-contaminated samples for bacteriology from broilers with cellulitis at slaughter.

Whole genome sequencing

Forty-six isolates from 36 carcasses were WGS (paper I). The minimum spanning tree (MST) based on the core genome multilocus sequencing (cgMLST) data from the *E. coli* isolates can be found in paper I, Figure 1. The MST showed that the majority of isolates originating from the same carcass were genetically identical or differed in only one allele. Further, most of the isolates from the same flock clustered together and had the same ST. These results confirm a clonal spread of *E. coli* associated with cellulitis.

The virulence genes that were most commonly present among the sequenced isolates (*iss*, *TerC*, *hlyF*, *iroN*, *OmpT*, *chuA*, *fyuA*, *irp2*, *sitA* and *TraT*) were related to adhesion, iron acquisition and serum resistance, which is similar to earlier studies (Barbieri *et al.* 2013; Poulsen *et al.* 2018; Alfifi *et al.* 2022). The presence of *ibeA* was confirmed in a majority of the isolates, while *kpsM* was present only in a subset. These genes have earlier been suggested to contribute to the pathogenicity of cellulitis-derived *E. coli* isolates (Barbieri *et al.* 2013). There were several STs represented among the analysed isolates, including 23, 95, 117, 131 and 428. These STs have previously been identified in APEC isolated from colibacillosis in poultry (Mehat *et al.* 2021; Kravik *et al.* 2023).

3.3.2 Papers II and III

Whole genome sequencing

The serotypes and STs of the *E. coli* strains used for inoculation in papers II and III are summarised in Table 5.

Table 5. Summary of serotype and sequence type of *E. coli* strains used for inoculation in paper II and III

Paper	*E. coli* strain	Serotype	Sequence type
II	ECA9	O78:H4	117
II, III	ECA18	O24:H4	117
II, III	ECB11	O153:H9	38
II	ECCA689	O25:H4[1]	131[2]

[1]The O group shows 95% resemblance to O25.

[2]The sequence type showed close resemblance to ST131, but differed by a single base in the *PurA* loci.

All strains used for inoculation harboured virulence genes that are common to APEC, where *chuA*, *hlyF*, *iss*, *ompT*, *sitA*, *terC*, *traT* were present in all four strains, and *etsC*, *hra*, *iutA*, *iucC* in three. The former genes were also identified among the isolates from the chickens in paper I and includes adhesion, iron acquisition and serum resistance genes. The same STs as the inoculated strains were present among the slaughterhouse isolates in paper I, with the exception of ST38 (Table 5). In general, the finding of various combinations of virulence factors in our studies and those by others, suggests that the development of cellulitis is not solely associated with a specific APEC pathotype, but more likely to APEC in combination with other factors, such as bird management and general condition (Jeffrey *et al.* 2002; Schulze Bernd *et al.* 2020).

It has previously been reported that APEC with ST117 caused outbreaks of colibacillosis, including cellulitis, in Denmark, Norway and Finland (Ronco *et al.* 2017; Poulsen *et al.* 2018; Alfifi *et al.* 2022; Kravik *et al.* 2023). It was suggested that ST117 had been vertically transmitted through the broiler breeding pyramid (Figure 1), and that the potential source was broiler parents imported from Sweden, possibly even transmitted from breeding stock higher up in the breeding pyramid (Ronco *et al.* 2017). Sequence type 117 has not been reported in Sweden earlier, but was found in one flock in paper I, with serogroup O161:H4, and among two of the inoculation strains in paper II and III, ECA9 and ECA18, with serotypes O78:H4 and O24:H4, respectively (Table 5). Sequence type 117 in combination with these serotypes can thus be confirmed to have been present in Sweden in 2020 and 2021, but there is no information from earlier years.

Bacteriology

There was a higher number of positive *E. coli* cultures in paper II, than in paper III; a summary is available in Table 6.

In paper II, groups 1 and 4 had a higher occurrence of *E. coli* positive cultures from organs in chickens (10/10 and 7/8, respectively), compared with the other groups (group 2: 3/6 and group 3: 3/4). This indicate that the infection was more severe in groups 1 and 4 and that the birds were not able to clear the infection, which agrees with the extent of clinical signs and gross findings. The milder outcome in paper III and the fact that there was a longer timespan before euthanasia, which might have provided time for the birds to clear the bacteria, are reflected by the number of birds with positive *E. coli* culture from the subcutaneous tissue (Table 6).

Table 6. Summary of the re-isolation of *E. coli* in samples from the inoculated chickens diagnosed with cellulitis at the PM examination in papers II and III

	Re-isolated *E. coli from*	
Paper	**Subcutaneous tissue**	**Organs**
II	33/34[1]	19/34
III	4/20	0/20

[1]Culture result from one chicken was missing

Whole genome sequencing of the re-isolated *E. coli* isolates (subcutaneous tissue *n*=4 and organ *n*=6 in paper II; *n*=2 in paper III) confirmed that they were almost identical to the inocula. A maximum of three single nucleotide polymorphisms (SNPs) were detected in four of the WGS isolates. It is likely that these mutations had occurred during laboratory cultivations or in the live birds.

3.4 Infrared imaging and skin conditions

In the study described in paper II, surface temperatures of chickens inoculated subcutaneously with *E. coli* were evaluated to assess the potential of IRT as a diagnostic tool for cellulitis.

3.4.1 Maximum temperatures and skin reactions

The maximum temperature detected by the infrared camera on the ventral and lateral side of the body of the chickens, showed significantly higher temperatures at 9 hours PI in the inoculated groups, compared to the control group (Figure 8). The difference between the control and the inoculated groups was approximately 1.5 °C. The temperature decreased, but groups 1, 2 and 4 had a significantly higher maximum lateral temperature at 19 hours PI as compared to the control group (Figure 8B). At 44 hours PI, the temperature did not differ between groups.

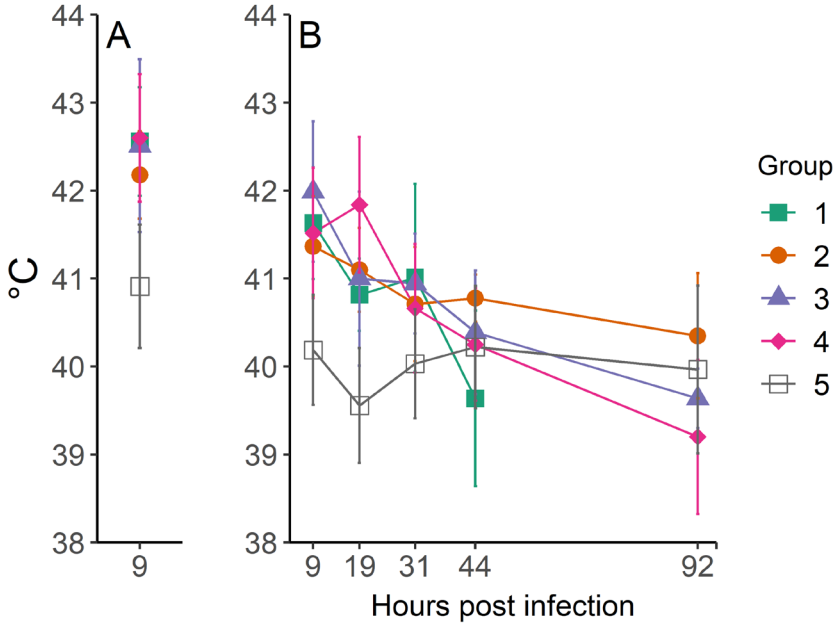

Figure 8. Maximum temperature ± SD detected on the ventral (A) and the lateral (B) view of the chickens in the inoculated groups (1–4) and the control group (5).

The increase of the maximum body temperature suggests a transient pyrexia, which has been observed previously after inoculation of poultry with bacteria or lipopolysaccharide (D'Alecy & Kluger 1975; Johnson *et al.* 1993; Gray *et al.* 2019). However, even though pyrexia is plausible in response to cellulitis, an experimental infection does not necessarily follow the course of a natural disease. Another possibility is that the increase in temperature could have been caused by stress due to the handling of the chickens during the IRT imaging procedure. A rise in the surface temperature of the head has been seen during handling/restraint of broiler chickens, which was suggested as a response to stress (Moe *et al.* 2017). However, this does not explain the differences in temperatures between the *E. coli* inoculated chickens and the control chickens.

Concurrent measurement of the core temperature by data loggers was discussed but not included in the study, due to time restraints and lack of loggers of appropriate size. Retrospectively, a validated method to estimate

the core body temperature would have been advantageous to compare with the IRT data.

There were no consistent patterns in the surface temperature changes at the site of *E. coli* inoculation (abdomen's right side). At 31 hours PI, there was a significant increase of the surface temperature of chickens in group 1, compared with chickens in all other groups. In groups 2–4, the left side of the abdomen had significantly higher temperatures at 19 hours PI, compared to chickens in the control group, which was also observed at 31 hours PI in group 1, compared to group 3 and the controls. Mild or moderate swelling and erythema were the most common findings at the site of inoculation, which was present in 35 of the 38 *E. coli* inoculated birds (Figure 9). There were no such skin reactions in the control group.

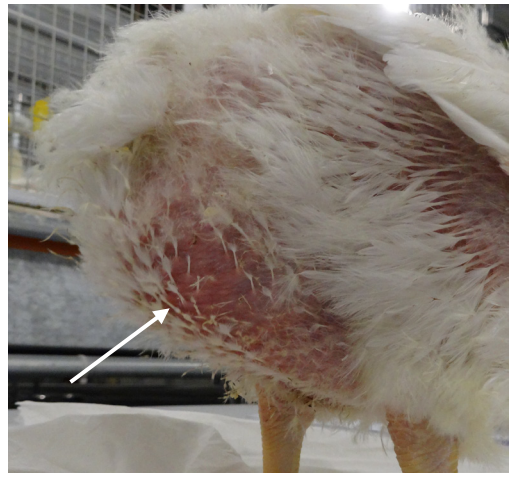

Figure 9. Chicken with erythema at the location for the subcutaneous inoculation of *E. coli*. Photo: Liv Jonare

From these results, we conclude that IRT is not a useful tool in detecting cellulitis. Possibly, the mild nature of the skin lesions was a limitation. An earlier studies has suggested that broilers might have a less developed cellular immune response compared to layer-type genotypes, which might affect the inflammatory reaction in tissue (Koenen *et al.* 2002; Olkowski *et al.* 2005). Further, it has been suggested that the handling of broilers can cause a peripheral vasoconstriction that lowers the skin temperature detected by the infrared camera (Edgar *et al.* 2013; Moe *et al.* 2017). However, when abdominal skin temperature was evaluated with IRT in healthy broilers, it

did not appear to be affected by the handling (Tessier *et al.* 2003). Nonetheless, getting the chickens used to handling is an effective way to reduce the impact of handling on the surface temperature.

Compared to a previous study where skin temperatures were measured in healthy broilers, there was more variation of the temperature in our study, which may have reduced the ability to detect smaller differences in temperature change or certain patterns (Tessier *et al.* 2003). Small differences in the position of the infrared camera and the chicken's body posture may have contributed to the variation in temperature, by creating different conditions for the image analysis.

3.5 Blood analysis

3.5.1 Blood culture

In the study described in paper III, cultures were set up on blood drawn from the chickens before and after the inoculation of *E. coli* strain ECA18 in group A, and ECB11 in group B. All cultures were negative before inoculation, but 1–3 days PI, five chickens inoculated with strain ECB11 were culture-positive with 10^2–10^4 CFU *E.coli*/mL blood, despite the absence of clinical signs. In an earlier study, up to 10^4 CFU *E.coli*/mL blood was observed at six hours and one day following inoculation of 10^7 CFU of *E. coli* subcutaneously (Gomis *et al.* 1997b). All inoculated broilers in the sampled groups were bacteriaemic at some point in time up to seven days PI. The majority of the birds had lesions consistent with systemic bacterial spread observed at PM examination (Gomis *et al.* 1997b). This was not the case in paper III, although the inoculated *E. coli* dose was the same, indicating that Gomis *et al.* (1997b) used a more pathogenic *E. coli* strain. In addition, other host-associated factors may have contributed.

3.5.2 Blood leukocyte counts

The numbers of heterophils analysed in paper III varied more in *E. coli* inoculated groups PI, compared to the control group, although no significant alterations were observed. The numbers of circulating monocytes and thrombocytes did not differ between infected and uninfected chickens. Heterophils and monocytes are important cells in the innate immune response and are recruited via the circulation to the site of infection following

a bacterial infection (Wigley 2013). Considering the mild clinical outcome PI with localized gross findings, low/no alterations in blood leukocytes numbers was an expected finding. However, some of the bacteraemic chickens showed up to a 3-fold increase in heterophils on days 4 and 7. Hence, bacteraemia induced heterophilia as expected.

The numbers of lymphocytes in blood showed a decrease in chickens in group B on day 1 PI (non-significant). The B-cells and the subpopulations of T-cells tended to be decreased in group B on day 1 PI, with a significant decrease in the number of TCRγδ+CD8αβ+ cells, compared to the control birds. The decreased levels of these celltypes on day 1 PI may be an effect of migration of lymphocytes to the subcutaneous tissue. Peighambari *et al.* (1995a) observed increased numbers of lymphocytes in subcutaneous tissue on the abdomen in chickens with experimentally induced cellulitis, compared with normal skin at the same location. The decreased numbers of lymphocytes in our study could also be due to bacterial toxins. However, the virulence genes that was exclusively detected in the ECB11 strain (used for inoculation in group B) have to our knowledge not been associated with toxins that specifically target lymphocytes. T-cells in the circulation seemed not to be activated, assessed as the expression of CD25, which seems consistent with the mild outcome of the inoculation.

3.5.3 Cell surface expression of MRC1L-B on monocytes

One of the most prominent changes in the evaluated immune parameters described in paper III, was the significant increase in the cell surface expression of the MRC1L-B receptor on monocytes on day 1 PI in both inoculated groups A and B, compared to the control group (Figure 10). In group A, the increase was transient and returned to pre-inoculation levels on day 2, while in group B it remained slightly elevated, but gradually returned to pre-infection levels on day 4 PI. A similar increase of MRC1L-B receptor expression has been observed after experimental inoculations in layer pullets with the Gram-positive bacterium *Erysipelothrix. rhusiopathiae* (Wattrang *et al.* 2020; Wattrang *et al.* 2022). These consistent observations may indicate that the increased expression of the MRC1L-B receptor on monocytes in the circulation might be a regular characteristic of the chicken immune response to bacterial infections.

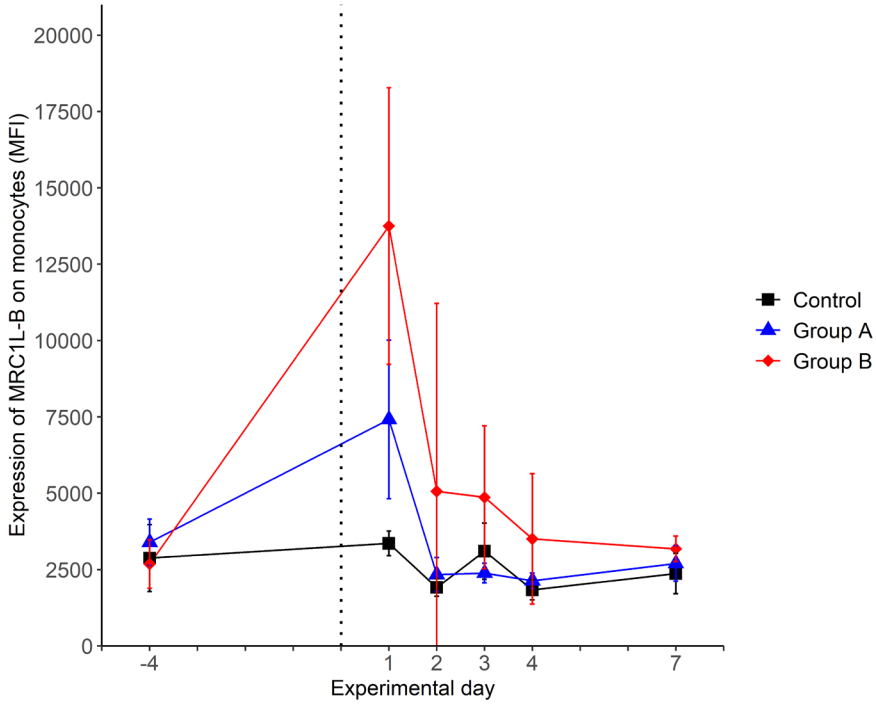

Figure 10. Expression of the MRC1L-B cell surface receptor on monocytes (mean values ± 95% CI) in the experimental *E. coli* inoculation model (paper III). The dotted line represents day of inoculation.

The function of the monocyte MRC1L-B receptor in bacterial infection in chickens is not completely known. The receptor is considered to be a homologue to the mammalian endocytic MRC-1 receptor, that can be expressed in both monocytes, dendritic cells and nonvascular endothelium, having functions involving facilitation of antigen presentation and regulation of glycoproteins released during inflammation (Martinez-Pomares 2012; Staines *et al.* 2014). In chickens, studies have observed a changed expression of the MRC1L-B receptor upon *in vitro* maturation of chicken monocytes, and as a response to *in-vitro* stimulation with innate defence peptides (Kraaij *et al.* 2017; Peng *et al.* 2020). The increased expression of the chicken MRC1L-B receptor on monocytes could be due to *e.g.* activation or maturation, or a reallocation of monocyte populations with a different

MRC1L-B expression, as proposed by Wattrang *et al.* (2020), which in turn may promote the phagocytic elimination of bacteria (Peng *et al.* 2020).

3.5.4 *E. coli* leukocyte adhesion assay and quantification of IgY antibodies to *E. coli*

The *in vitro* adhesion assay showed that approximately 5% or less of heterophils and monocytes adhered to strains ECB11 and ECA18 in blood collected before the *E. coli* inoculation. For thrombocytes, the adhesion was higher, approximately 25% to ECB11, while it was approximately 5% for ECA18. In blood collected 7 days PI, the adherence to strain ECB11 increased significantly in all cell types in group B, with a 4-fold increase in heterophils (Figure 11). The adhesion remained on low levels to both inoculated strains in group A and the control birds.

Before inoculation, all groups showed low levels of IgY titers to both strains ECB11 and ECA18, which remained low PI in the control birds. The mean IgY titers to the antigens from ECB11 was significantly increased for chickens in group B on days 7 and 14 PI, and in group A on day 14 PI. For the antigens from ECA18, the mean IgY levels were significantly increased on day 7 and 14 PI for both *E. coli* inoculated groups. However, the mean titer to ECA18 was >200 in only a few birds in group A and B at day 7 and 14 PI. For strain ECB11, approximately half of the birds in groups B had a titer >200 at day 7 and 14 PI, while this was true for only one bird in group A on day 14. There was a significant correlation between IgY titers to the ECA18 and ECB11 antigen within a chicken in all groups at each sampling occasion (Spearman's rank correlation coefficient, $r_s = 0.79$, $p<0.05$). Correlation analysis between the proportion of cells that adhered to the inoculated *E. coli* strains and the titer of antibodies to respective strains within chickens showed a positive correlation for group B ($r_s = 0.70$, $p<0.05$) for heterophils adhering to ECB11. No such correlation was found for monocytes or thrombocytes, nor to strain ECA18 for any of the cell types in group B. In group A, a weak to intermediate negative correlation was found between the antibody titer and monocytes adhering to both inoculated strains (ECB11: $r_s = -0.42$, $p<0.05$ and ECA18: $r_s = -0.46$, $p<0.05$), while no such correlations were identified for the other cell types and the *E. coli* strains.

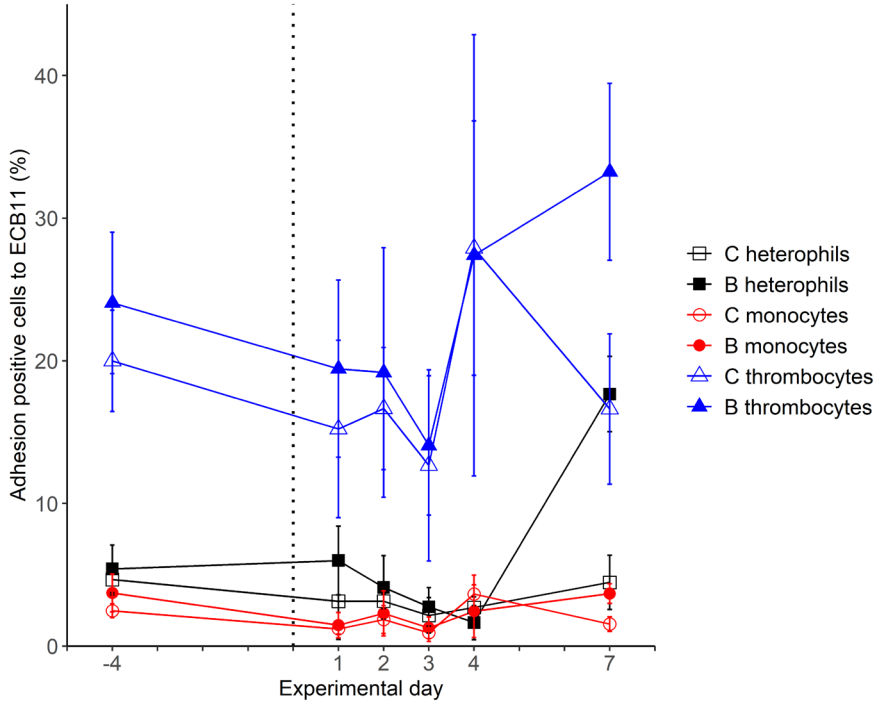

Figure 11. Adhesion positive heterophils, monocytes and thrombocytes (mean values ± 95% CI) in blood from chickens in Group B and control birds, respectively, in the experimental *E. coli* inoculation model. The dotted line represents day of inoculation, C = control birds, B = group B.

The adhesion assay results suggest opsonisation of the ECB11 strain in the B group by strain specific antibodies, which is supported by the increased titers of IgY to the ECB11 antigen. In an early *in-vitro* study by Stabler *et al.* (1994), the bacterium *Salmonella* Enteritidis was observed to be more efficiently phagocytosed by heterophils and monocytes after opsonisation by incubation in antibody-containing serum. Heterophils showed the most pronounced increase (depending on assay) (Stabler *et al.* 1994), which was also observed in our study.

IgY recognising both *E. coli* strains was detected in serum from inoculated chickens irrespective of inoculation strain. A strong correlation between increased antibody titers to both *E. coli* strains within birds was observed. Hence, results from our ELISA assay suggested that IgY cross-reacted between the two strains. On the contrary, results from the leukocyte

adhesion assay suggested opsonisation by strain-specific antibodies in the serum samples. Our inoculation strains were of different serotypes (Table 5), which means that antibodies specific to these surface antigens should be able to distinguish between these strains. Thus, our results show that mainly bacterial surface antigens were exposed in the bacterial preparations used in the leukocyte adhesion assay, which allowed antibody opsonisation in this assay to be strain-specific. In contrast, the sonicated whole-cell lysate used in the ELISA assay likely contained a large proportion of *E. coli* antigens common for both strains. Consequently, this assay was *E. coli*-specific but not strain-specific. Similar to our study, Li *et al.* (2017) inoculated broiler breeders with either one of two different *E. coli* strains. Using an ELISA assay with whole-cell sonicate, an increase in IgY antibody titers to both *E. coli* strains in both groups was demonstrated independent of the inoculated strain. A strain-specific ELISA assay would probably been achieved by coating with purified serotype-specific bacterial surface antigens.

To conclude, the immune response to the *E. coli* inoculation of chickens included immediate possible activation/redistribution of monocytes (increased expression of MRC1L-B) and later increase in levels of *E. coli*-specific antibodies that showed opsonic capacity in the *in vitro* assay of adherence of leucocytes to *E. coli*. Further, bacteraemia may cause heterophilia. It was shown that broiler chickens could clear a considerable dose (10^7 CFU) of subcutaneously inoculated *E. coli* although minor alterations in the blood leucocyte count, as assessed in this study. Evaluation of the cellular response by histology would have been valuable due to possible migration of immune cells to tissue.

3.6 Risk factors

3.6.1 Final dataset

After the cleaning of the dataset, it consisted of 4,697 broiler chicken flocks and 159,358,144 chickens reared at 90 farms in 446 compartments. The mean percentage of condemnation due to dermatitis cellulitis was 0.59% (SD 0.74) and the median 0.34% (Q1 0.16% and Q3 0.73%). Age of the parent flocks was divided into three categories: 23–28 weeks (providing chickens to 424 flocks, 9%), 29–33 weeks (846 flocks, 18%) and >33 weeks (3,427

flocks, 73%). There were four slaughterhouses included in the data, that processed between 586 (12%) and 1,904 (41%) flocks each.

3.6.2 Mixed effect model analysis

The final mixed effect model contained 13 fixed effects and three interactions: season, age of parents flock, number of birds/m2, size of broiler compartment, feed mill, feed factory, age of flock at slaughter, slaughterhouse, total condemnation percentage, condemnation percentages due to ascites and inflammation in joints/tendons, total footpad score and slaughter of a flock over >1 day. Included interactions were between: number of birds/m^2 and age of flock at slaughter; slaughterhouse and the total condemnation percentage; slaughterhouse and footpad score. Results are presented below as back-transformed estimated marginal means with standard errors.

In flocks originating from the oldest parent age category flocks (cat. 3: >33 weeks old), there was a higher condemnation percentage for dermatitis/cellulitis (0.29%±0.027) compared with younger flocks (cat. 1: 24–28 weeks old, 0.21%±0.021, and cat. 2: 29–33 weeks old, 0.26%±0.025, $p<0.0001$). A similar association has been shown in an earlier study that analysed several causes of condemnation in 55,918 broilers flocks in the United Kingdom (Buzdugan *et al.* 2020) and in a recent German study (Junghans *et al.* 2022). Chicks from old parents appears to have better early prospects, with higher body weight at hatch, compared to chicks from young parents (Jacobs *et al.* 2016). However, higher first week mortality has been observed in chicken flocks originating from old parents, as compared to young parents (Poulsen *et al.* 2017). The possible association between first week mortality, which is associated with *E. coli* infection, and condemnation at meat inspection due to dermatitis/cellulitis was not investigated in this study, as mortality data were not provided in the dataset.

The interaction between slaughterhouses (S) and total condemnation showed that S3 had a higher condemnation percentages due to dermatitis/cellulitis with increased total condemnation compared to S1, S2 and S4, except at 2.80–2.99% where there was difference only with S2 and S4 ($p<0.05$, Table 7). Slaughterhouse S1 and S4 did not differ, while S2 and S4 differed except for 1.00–1.19% in total condemnation ($p<0.0001$) and S1 and S2 differed except for 0–1.19% in total condemnation ($p<0.01$). EM means for three representative condemnation levels for each slaughterhouse

is available in Table 7. It has been suggested that the variation between slaughterhouses may depend on different assessment at meat inspection (St-Hilaire & Sears 2003; Buzdugan *et al.* 2020; Törmä *et al.* 2022). However, the immunosuppressive very virulent infectious bursal disease virus (vvIBDV) has been detected in Sweden in layers and organics broilers in 2017-2019 (Mató *et al.* 2020), and later in fast-growing broiler chickens in recent years (unpublished data). Theoretically, a vvIBDV followed by a secondary *E. coli* infection could be associated with a higher condemnation percentage due to colibacillosis including cellulitis in those geographic areas where the virus has been identified. Further studies are needed to investigate the causes for the differences in condemnation percentages due to cellulitis/dermatitis at different slaughterhouses.

Table 7. Back-transformed estimated marginal (EM) means of condemnation due to dermatitis/cellulitis stratified by slaughterhouse (S) 1–4 and total condemnation (three selected levels), from mixed models analysis of 4697 broiler flocks placed between 20 May 2021 and 28 November 2022 in Sweden. Different superscript letters indicate significant difference for each total condemnation percentage (p<0.05)

S	Total cond.	EM means (%)	SE
1		0.07[a]	0.015
2	0–0.99%	0.02[a]	0.010
3		0.39[b]	0.046
4		0.10[c]	0.010
1		0.24[a]	0.061
2	2.00–2.19%	0.09[b]	0.013
3		1.03[c]	1.03
4		0.27[a]	0.27
1		0.46[a]	0.10
2	>3%	0.17[b]	0.020
3		2.01[c]	0.233
4		0.40[a]	0.041

Although there was an interaction between the total footpad score and slaughterhouse, the condemnation due to dermatitis/cellulitis was similar for all the four slaughterhouses. Specifically, in flocks with high footpad scores (>40) there was a lower condemnation due to dermatitis/cellulitis, compared to low footpad scores (≤40). The finding was significant for S1, S2 and S4

(ratio of EM means for high/low categories: S1:0.37, p=0.0006; S2: 0.64, p=0.0010 and S4: 0.84, p=0.030). The footpad scores reflect the litter condition, where poor litter condition leads to higher footpad score (Ekstrand *et al.* 1997; de Jong *et al.* 2014). Even though the association between high footpad scores and low condemnation due to dermatitis/cellulitis has been observed earlier (Schulze Bernd *et al.* 2020), this result was somewhat surprising. Poor litter condition was expected to increase the condemnation due to dermatitis/cellulitis, as the chicken's skin on the abdomen would be exposed to chemical irritants and moisture while at rest/laying down. Schulze Bernd *et al.* (2020) suggested that this could be associated with the covering of the broilers' claws by moist litter, which would protect them from injuries from sharp claws, as scratches are believed to be an important portal of entry for *E. coli* to the subcutaneous tissue (Norton *et al.* 1997). Another hypothesis, suggested by several broiler farmers during the course of our studies, is that a dry environment in the broiler compartment would reduce the condition and integrity of the skin and make it less resilient to scratches.

In European countries, ascites and cellulitis are two of the most common causes for condemnation of chicken carcasses at meat inspection. In paper IV, the condemnation due to dermatitis/cellulitis increased from 0.17%±0.017 to 0.32%±0.031 when the condemnation level due to ascites decreased from the highest percentages (0.4% or higher) to the second lowest percentages (0.050–0.99%). One possible explanation would be that cellulitis and ascites may be present in the same chicken and is reflected in the condemnation percentages, as only one cause of condemnation cause is registered per bird in most countries, including in Sweden. Earlier, a positive association between these two reasons of condemnation has been observed (Tessier *et al.* 2001; Forseth *et al.* 2023).

There was an interaction between age of flock at slaughter and the stocking density at placement (birds/m^2), which is demonstrated in Figure 12. Condemnation due to dermatitis/cellulitis increased with stocking density for all age categories, where the largest effect was seen in age group 29–32 days, followed by age group 36–40 days. Higher age of the flock at slaughter and increased stocking density possibly lead to more cumulative interactions between birds, which in turn can increase the risk for skin scratches. Interestingly, palpation or massage of the skin overlying a cellulitis lesion promoted inflammation in an early study, compared to skin that was not subjected to palpation, which suggested that body contact

between birds may increase the occurrence of cellulitis (Glunder 1990). A significant association between increased stocking density and increased condemnation due to skin lesions, including cellulitis, was found in a recent epidemiological study analysing 1,843 chicken flocks (Forseth *et al.* 2023). Notably, this study also found that decreased condemnation due to cellulitis was associated with high flock age at slaughter (Forseth *et al.* 2023), which is contrary to our results. Other studies have investigated and not found significant associations between stocking density, age of slaughter and condemnation due to cellulitis (Elfadil *et al.* 1996a; Schrader *et al.* 2004; Schulze Bernd *et al.* 2020).

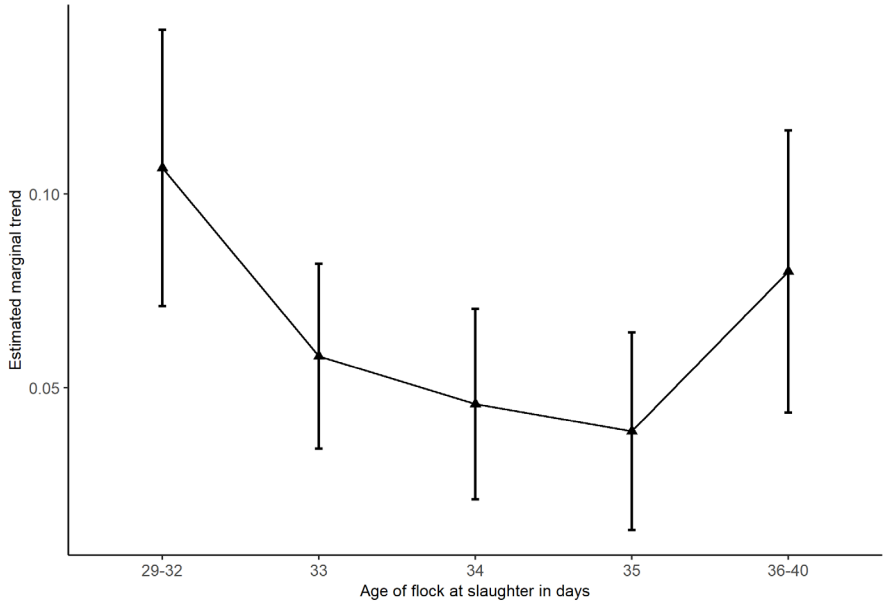

Figure 12. Estimated marginal trends (±SE) for the interaction between age of flock at slaughter and stocking density at placement (birds/m^2), modelled with data from 4,697 broiler flocks placed between 20 May 2021 and 28 November 2022 in Sweden. The positive trend indicates that condemnation due to dermatitis/cellulitis increases with higher stocking density, where the highest increase was in flock age group 29–32 days and the lowest at flock age 35 days. The interaction effect at each level was significant. EM trend is in log scale as the outcome variable was log-transformed.

Low condemnation rates due to dermatitis/cellulitis was associated with small broiler houses in a previous study, which suggested that daily

inspection of the chickens is easier to perform in small houses leading to identification of chickens with cellulitis prior to slaughter (Schulze Bernd *et al.* 2020). The findings in paper IV was consistent with that study, as small compartments (<750 m^2) had an EM mean of 0.17%±0.019, as compared with the other size categories, which varied between 0.26–0.27% (p<0.0001.

Flocks that were slaughtered on more than one day had a higher (p=0.007) condemnation percentage due to dermatitis/cellulitis (0.26% ± 0.026), compared with flocks that were slaughtered during one day (0.24% ± 0.022). No significant association to the condemnation due to dermatitis/cellulitis was however found for flocks practicing thinning with more than 4 days between the slaughter occasions.

3.6.3 Limitations of the model

There were potential limitations to paper IV, despite the use of a large dataset to address the complexity of the data and account for possible clustering. The database used is primarily a tool for monitoring of production results and not for research purpose. Several companies in the broiler production chain contribute data, *e.g.* hatchery and rearing farm, which could lead to varying data quality across parameters. There were some important variables that were either not available (mortality during rearing and number of slaughtered chickens) or considered unreliable (number of placed chickens), and hence not included in the model and could not be accounted for in the analysis. There is a risk for other unmeasured effects that affected the condemnation due to dermatitis/cellulitis. One example could be the time that farmers spend observing each flock. To reduce for such confounders we decided to remove the Cobb genotype from the analysis and only include the Ross 308 genotype. The EM means in our model differed quite considerably from the ordinary raw means. Estimated marginal means are group means that are adjusted for the influence of other factors in the model, as if the data would have been balanced in all aspects. Therefore, we needed a model strategy that could account for the structure of the dataset, *e.g.* differences between farms, to accurately compare how various variables affected the condemnation due to dermatitis/cellulitis.

4. Conclusions

- *Escherichia coli* may spread systemically in broilers with cellulitis.
- Avian pathogenic *E. coli* associated with cellulitis can cause clonal spread within a broiler chicken flock and closely related strains can be isolated from chickens with cellulitis from different farms.
- Experimentally induced coliform cellulitis in broiler chickens may cause clinical signs of varying severity, likely depending on inoculation dose, *E. coli* strain and preparation of inoculum.
- Evaluation of the general appearance and body weight gain are essential elements when chickens are assessed after an experimental *E. coli* inoculation.
- Immune responses to an *E. coli* inoculation in chickens may include possible activation/redistribution of monocytes and increased levels of *E. coli* specific antibodies, with putative opsonic capacity.
- Subcutaneous inoculation of *E. coli* can cause bacteraemia that may induce heterophilia.
- Infrared thermography is not suitable as a diagnostic tool for cellulitis.
- Risk factors for cellulitis are distributed across the broiler production chain and include *e.g*. old parent flocks and a significant interaction between slaughterhouse and total condemnation. A multifactorial approach is likely needed to decrease the occurrence of cellulitis.
- The occurrence of cellulitis is currently based on the condemnation percentage at meat inspection at slaughter. This is likely an underestimation of the true occurrence because cellulitis is also diagnosed among culled and dead chickens during the late stage of the rearing period.

5. Future perspective

The studies included in this thesis involved several aspects of cellulitis and raised questions that would be interesting for future research.

- Determine the reasons for the differences in condemnation rates due to dermatitis/cellulitis between slaughterhouses in Sweden.
- The meat inspection code for skin lesions used at slaughterhouses in Sweden includes a range of different skin pathologies and injuries. The proportions of chickens with cellulitis and other types of lesions need further investigation.
- The occurrence of cellulitis in broiler production in different countries should be determined. For this purpose, harmonized meat inspection codes and assessment routines are necessary prerequisites.
- Findings in studies included in the thesis suggest that some chickens with cellulitis may show clinical signs. How can this be studied on commercial farms? A diagnostic tool for use in live chickens is still needed.
- The role of skin lesions in the pathogenesis of cellulitis is still unclear and should be investigated. Prevention of skin scratches and other skin lesions in broilers may be another potentially important future field of research.
- The role of parent flocks with regards to chick quality, vertical transmission of *E. coli*, mortality (first week and total) and condemnation at meat inspection in broiler chickens should be considered as a highly prioritised field of research.
- Is there a link between first week and/or total mortality in broiler flocks and condemnation due to dermatitis/cellulitis at slaughter?

- Can vaccination of broiler chickens against APEC decrease the condemnation percentage from dermatitis/cellulitis?
- Based on result from paper IV, management-related factors such as compartment size, stocking density and broiler house conditions (temperature, relative humidity and litter quality) should be further studied and explored to reduce the occurrence of cellulitis in broiler chickens.

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Popular science summary

Cellulitis in broiler chickens is a disease caused by the bacterium *Escherichia coli*, leading to discoloured skin and inflammation under the skin, primarily on the abdomen and thighs. Affected chickens are condemned as food at slaughter, resulting in economic loss. The purpose of this thesis was to increase knowledge about how and when cellulitis develops, identify risk factors, and evaluate whether a thermal camera can be used to diagnose the disease. Increased knowledge can provide a basis for measures aimed at reducing the occurrence of cellulitis.

By bacterial sampling and analyses of DNA of *E. coli* found in chickens condemned due to cellulitis at a slaughterhouse, we found that *E. coli* had spread from the cellulitis lesions to other parts of the body. Two studies where cellulitis was induced by injection of *E. coli* bacteria under the skin showed that chickens could develop symptoms as the disease progressed, a fact that has not been determined yet on farms. Further, blood sampling in these studies showed that some blood cells increased in numbers and that the level of antibodies against *E. coli* increased. The thermal camera, which was hypothesised to detect heat in the skin caused by inflammation, was found unsuitable to diagnose cellulitis. Furthermore, data analysis from various parts of the chicken rearing process indicated that factors such as the age of the chickens' parents, the age of the chicken flock at slaughter, and the slaughterhouse to which the flock is transported to, can affect the proportion of chickens condemned at the slaughterhouse due to cellulitis. Cellulitis was also observed in chickens that had died or been culled due to disease near slaughter.

In summary, the studies included in this thesis show that cellulitis is a disease that is affected by many different factors and that measures at several

levels are likely needed to reduce the occurrence and the condemnation at slaughter due to cellulitis.

Populärvetenskaplig sammanfattning

Cellulit (underhudsinflammation) hos slaktkyckling är en sjukdom som orsakas av bakterien *Escherichia coli,* där kycklingen får missfärgad hud och en ansamling av inflammatoriskt sekret under huden, främst på buken och låren. Kycklingar som har sjukdomen kasseras vid slakt och blir därför inte till livsmedel, vilket orsakar ekonomiska förluster. Syftet med avhandlingen var att öka kunskapen om hur och när cellulit utvecklas, vilka riskfaktorer som finns och att utvärdera om värmekamera går att använda för att diagnostisera sjukdomen. Ökad kunskap kan ligga till grund för rekommendationer och åtgärder som syftar till att minska förekomsten av cellulit.

Genom att samla in bakterieprover och analysera DNA från *E. coli* från kycklingar som kasserats på grund av cellulit på slakteri, kunde vi visa att *E. coli* hade spritt sig från celluliten till andra delar av kroppen. Två studier där cellulit framkallades genom injektion av *E. coli*-bakterier under huden, visade att kycklingar kan få symptom under utvecklingen av sjukdomen, något som ännu inte fastställts på gård. Med blodprover konstaterades att vissa blodceller ökade i antal och att nivån av antikroppar mot *E. coli* ökade. Värmekameran, som antogs kunna detektera värme i huden orsakad av inflammationen, visade sig inte fungera på ett tillförlitligt sätt. Vidare så analyserades data från olika delar av kycklinguppfödningen, vilket tydde på att ålder på kycklingarnas föräldrar, hur gamla kycklingarna är när de slaktas och vilket slakteri de skickas till, kan påverka hur stor andelen kycklingar som kasseras på slakteriet pga. cellulit. Cellulit förekom hos kycklingar som antingen dött eller avlivats pga. sjukdom under dagarna före slakt, något som inte har rapporterats förut.

Sammanfattningsvis visade studierna som ingår i avhandlingen att cellulit är en sjukdom som påverkas av många olika faktorer och att det sannolikt behövs åtgärder på flera plan för att minska förekomsten och kassationen.

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I

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Core genome multilocus sequence typing (cgMLST) confirms systemic spread of avian pathogenic *Escherichia coli* (APEC) in broilers with cellulitis

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ABSTRACT

Broiler cellulitis has emerged as an important cause of economic losses for farmers and slaughter plants from carcass condemnation at processing. Avian pathogenic *Escherichia coli* (APEC) has been identified as the main causative agent. The aim was to characterize *E. coli* isolated from cellulitis and organs in broilers at slaughter by whole genome sequencing analysis to study if systemic spread could be confirmed. Isolates were collected post-mortem from 101 carcasses condemned due to dermatitis/cellulitis from five commercial farms and six flocks. Forty-six isolates were characterised to determine serotypes, sequence types and virulence-associated genes. Analysis by cgMLST was performed to study the genetic similarity between isolates from the same broiler, among birds from the same flock and between flocks. *Escherichia coli* was isolated from 90% of birds from subcutaneous samples. In 20 broilers, *E. coli* was isolated from organs in pure culture or mixed with sparse growth of other bacteria. In eight of these, there were post-mortem findings suggestive of systemic bacterial spread. The majority of the isolates from the same bird and flock belonged to the same serotype and sequence type and were genetically indistinguishable, but differed when compared between flocks. Common APEC virulence genes, i.e. *chuA*, *fyuA*, *hlyF*, *iroN*, *irp2*, *iss*, *ompT*, *sitA*, *TerC*, *TraT*, were present in > 87% of the isolates. We conclude that evidence of systemic spread of *E. coli* from cellulitis was present in some birds at time of slaughter but cannot be reliably detected at meat inspection.

1. Introduction

Cellulitis in broiler chickens was first described in 1984 (Randall et al., 1984) and has become economically important as it leads to complete condemnation or down-grading of affected carcasses at slaughter plants. The definition of cellulitis in broilers includes a subcutaneous serosanguineous to caseated fibrinous plaque covered by discoloured and thickened skin, often located on the abdomen (Randall et al., 1984; Nolan et al., 2020). Avian pathogenic *Escherichia coli* (APEC) is believed to be the main causative agent. As of today, there is no common *E. coli* serotype or virulence gene profile defined in association with cellulitis in broilers. The pathogenesis is believed to involve bacterial infection of the subcutaneous tissue introduced through skin scratches or other skin lesions often caused by other broilers (Messier et al., 1993; Peighambari et al., 1995). The portal of entry and timing of events are however difficult to study as chickens with cellulitis display few clinical signs during grow-out and are therefore first identified at meat inspection. The disease development has been associated with managemental risk factors such as large flock size and certain types of litter (Elfadil et al., 1996; Schulze Bernd et al., 2020).

In some cases, concurrent systemic manifestations of *E. coli* infection can be observed, such as pericarditis, airsacculitis and perihepatitis suggesting systemic bacterial spread from the cellulitis lesion as the primary site (Gomis et al., 1997). In a study by Gomis et al. (1997), *E. coli* that belonged to the same O serogroup was isolated from the cellulitis lesion and organs from the same broiler, suggesting systemic spread of bacteria. In addition, *E. coli* was isolated from organs even in the absence of gross findings (Gomis et al., 1997). More recently, Poulsen et al. (2018) found the same pulsed-field gel electrophoresis (PFGE) profile in *E. coli* isolates from cellulitis and spleen from the same

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broiler. Further, Silva et al. (2021) demonstrated that *E. coli* isolates from cellulitis and liver lesions from the same broilers possessed the virulence-associated genes *iss* and *iutA* as determined by PCR. However, more definite proof of systemic spread to organs is needed. This can be done through whole genome sequencing (WGS) and core genome multilocus sequence typing (cgMLST) of isolates from the same carcass. A similar approach based on core genome single nucleotide polymorphism analysis of *E. coli* isolates collected from broiler flocks with increased first week mortality was recently published (Kravik et al., 2022). The analysis of isolates from multiple sample sites of the same bird showed no sequence diversity, suggesting identical isolates in all organs. The question whether systemic spread is present at slaughter in carcasses with cellulitis is important in terms of production economics. Partial instead of complete condemnation of carcasses with cellulitis could possibly mitigate economic losses if applied when signs of systemic manifestations of *E. coli* infection are absent. This aspect has not yet been thoroughly studied with WGS sequencing, however several authors have suggested that full condemnation of birds with cellulitis should be implemented at slaughter (Poulsen et al., 2018; Silva et al., 2021).

Another reason to study *E. coli* from broilers with cellulitis is the possible influence on food safety. Concerns regarding genetic similarity among *E. coli* isolated from cellulitis in broiler chickens and human extraintestinal pathogenic *E. coli* (ExPEC) were raised early (Kumor et al., 1998). It has been shown that APEC strains can share certain traits with uropathogenic *E. coli* (UPEC) and neonatal meningitis *E. coli* (NMEC) based on phylogenetic cluster analysis, presence of virulence genes and plasmid replicon types (Jorgensen et al., 2019). Johnson et al. (2008) reported a common phylogenetic cluster for APEC, NMEC and UPEC isolates, which could represent isolates having zoonotic potential, although most of the characterized APEC, NMEC and UPEC clustered separately in subgroups and could be considered as separate subpathotypes.

The aim of the present study was to characterise *E. coli* isolates from broiler chickens condemned due to cellulitis and to investigate if systemic spread could be confirmed from the cellulitis lesion as the assumed primary portal of entry for *E. coli*. To this end, *E. coli* isolates were collected from broiler chickens at a slaughter plant and isolates were studied by WGS and cgMLST analysis.

2. Materials and methods

2.1. Study population

The study population consisted of Ross 308 broiler chickens (target body weight 2200 g) from large conventional farms that were approved by an official veterinarian at ante-mortem inspection at a Swedish slaughter plant. Flocks included in the study were those that were available for sampling on six weekdays during five consecutive weeks in May and June 2021.

In total, 101 broilers slaughtered at 35–38 days of age were examined post-mortem ($N = 6$ flocks, $N = 5$ farms). The collection of carcasses was performed by slaughter plant staff based on the condemnation code for dermatitis/cellulitis (Swedish Food Agency, 2021). One flock was included in the study per day and was represented by 12–20 carcasses each. The carcasses were retrieved at the first meat inspection position after passing through the scalding tank, defeathering and removal of head and feet, but before evisceration. During the first three study days, carcasses with gross evidence of a subcutaneous plaque were specifically targeted for sampling. Carcasses were collected sequentially from the slaughter line in batches of up to six birds. They were individually placed in sealed plastic bags, transferred to a separate building, sampled and examined by two poultry veterinarians within approximately 60 min.

2.2. Sampling and post-mortem examination

Sampling for bacterial culture was performed before post-mortem examination. All sampling was performed with aseptic techniques using clean instruments and Amies swabs (Copan, USA), with minimal handling of tissues and organs to avoid cross-contamination. First, the site of cellulitis was sampled as far away as possible from meat inspection incisions, if present. Then, after incision of the body cavity with sterile instruments, the pericardium and spleen were sampled from all carcasses. The pericardium was swabbed and the spleen was incised with a sterile scalpel after surface searing before sampling of the parenchyma. In addition, a maximum of two additional organs or tissues per carcass were sampled if gross signs of bacterial infection were observed. Following sampling, post-mortem examinations were performed according to a routine but simplified avian necropsy protocol (National Veterinary Institute SVA, Sweden). Information on sex and body condition (thin, normal, obese) and gross findings were documented. The skin and subcutaneous tissues were examined for all types of abnormalities such as discoloration, thickening, congestion, oedema, presence of exudate, scratches and other skin defects. Organs and tissues were examined with special attention to signs of bacterial infection such as enlargement, presence of exudate and parenchymatous lesions. The femoral head and the proximal tibiotarsus were incised and assessed for osteomyelitis, and tendon sheaths, hock, knee and hip joints were opened and examined for signs of infection.

2.3. Bacterial culture and MALDI-TOF MS

The swabs were streaked immediately after sampling on 5% bovine blood agar and Cysteine Lactose Electrolyte Deficient (CLED) agar (SVA, Sweden). CLED agar plates were used to prevent swarming of *Proteus* spp. The agar plates were placed in bags on ice packs and transported to the laboratory in the evening on the same day and incubated at 37 °C for 24 h. One presumptive *E. coli* colony (i.e. 2–3 mm in diameter, opaque, grey on blood agar or yellow on CLED) from each tissue or organ was randomly selected for confirmation by matrix-assisted laser desorption ionization-time of flight mass spectrometry (MALDI-TOF MS), using a Maldi Biotyper Microflex LT System (Bruker Daltonik GmbH) with the MBT BDAL 8468 MSP library. Upon species confirmation, the same colony was re-cultured on 5% bovine blood agar at 37 °C for 24 h, followed by storage in 1.5 ml Brain Heart Infusion broth supplemented with 15% glycerol (SVA, Sweden) at − 80 °C pending further analyses.

2.4. Whole-genome sequencing and analyses

Forty-six *E. coli* isolates from 26 carcasses were selected for whole genome sequencing (WGS) analysis independently of gross findings. *Escherichia coli* isolates ($n = 36$) from subcutaneous tissue i.e. cellulitis and one to two organs from the same broilers were included if isolated in pure culture of with minimal growth of other bacteria upon primary culture. Isolates that met these criteria originated from four out of the six flocks (flock 1, 3, 4 and 5). In addition, to increase the number of characterized isolates, ten isolates with pure *E. coli* growth from cellulitis on primary plates were analysed from two randomly selected study flocks ($n = 10$ birds, flocks 3 and 5). Cultures from organ swabs from these ten birds showed no growth or sparse growth in mixed culture of no specific pathogenic bacteria. The isolates were named according to their flock of origin, carcass number and origin of sample, e.g. *3:5:Sc* (flock number 3: carcass number 5: subcutis). For DNA preparation, the selected isolates were retrieved from the frozen stock, passaged twice on 5% bovine blood agar, slurried in 500 µL nuclease-free water (Sigma-Aldrich, Germany) and lysed by heating at 100 °C for 5 min. DNA was extracted with the IndiMag Pathogen kit (Indical) on a TANBead Maelstrom-9600 system (TANBead). Library preparation was performed using Nextera chemistry and sequencing was done producing 2 × 150 bp reads on an Illumina NovaSeq instrument at SciLifeLab Clinical

Genomics, Solna, Sweden. Reads were checked for quality with FastQC (Andrews, 2010), trimmed with Trimmomatic (Bolger et al., 2014) and assembled with Unicycler (Wick et al., 2017). Serotype was determined with SerotypeFinder (Joensen et al., 2015) and multi-locus sequence typing (MLST) was done with mlst (Seemann, 2015) using the Achtman 7-locus MLST scheme available in the PubMLST database (Jolley et al., 2018). Assemblies were screened for virulence factors with VirulenceFinder (Camacho et al., 2009; Joensen et al., 2014; Tetzschner et al., 2020). The Enterobase cgMLST V1 scheme was used for the cgMLST analysis (Zhou et al., 2020) and clustering and visualization was done with Ridom SeqSphere+ (Junemann et al., 2013). The Enterobase database was also searched for isolates of human origin within the same HC50 HierCC V1 clusters as the isolates from the broiler chickens. A subset of the isolates of human origin were screened for virulence genes. Program versions and parameters are listed in Supplementary material T1. All raw sequencing data have been submitted to the European Nucleotide Archive and Enterobase and are available under accession number PRJEB52616.

2.5. Statistical analyses

All data were entered into Microsoft Excel 2016 (Microsoft Corporation, USA) where descriptive statistics (percentage, number of events and mean value) were calculated. Minor gross findings at post-mortem examination that could not be linked to a specific diagnosis were removed from the dataset.

3. Results

3.1. Slaughter data and post-mortem findings

The mean body weight of the sampled broiler flocks ranged from 2214 to 2312 g and the condemnation rate due to dermatitis/cellulitis ranged from 0.18% to 0.53%.

Among the 101 examined carcasses, 52% were males and 48% females. The mean carcass weight without head and feet was 1969 ± 274 g (out of 98 records). The majority of the carcasses were in normal body condition (89%) while 11% were thin (out of 94 records). A majority (n = 73, 94%) had an incision at the location of the skin lesion made at meat inspection (of 78 records).

A variety of different skin conditions were present in all 101 broilers. Gross findings in agreement with the definition of cellulitis (see introduction) or presence of a subcutaneous exudate considered as an early stage of plaque formation were observed in 79 out of 101 carcasses (78%). Of these, a majority had a subcutaneous plaque, intradermal green or yellow discoloration, subcutaneous oedema, subcutaneous congestion and inflammatory exudate/fibrin. In 35% of the carcasses with cellulitis, a localized lesion was observed on the abdomen, in the vent region or in dorsal or lateral position to the tail. Fewer carcasses (8%) showed a local lesion on the thigh or proximal trunk. In another 57%, the cellulitis lesion involved several areas of the body. The size of the primary cellulitis lesion ranged from 10 to 80 mm (recorded in 67 carcasses). Ninety-six percent of the carcasses with cellulitis displayed other localised skin lesions. These included single to multiple linear abrasions or lacerations up to 80 mm long in 84%, of which the majority were superficial and located on the abdomen (presumed scratches from claws). There were also single to multifocal dark discoloured 1–2 mm superficial circular erosions located on the abdomen in 68% of the carcasses and other skin defects in 33%. Twenty-two carcasses (22%) had gross findings not in agreement with the definition of cellulitis. Ten of these carcasses had a 5–40 mm lesion overlying the craniolateral breast muscle extending through the dermis with haemorrhage and inflammatory exudate on the muscle fascia. The other 12 carcasses had minor skin lesions on the abdomen, thigh and/or in lateral position to the tail. Other gross findings are summarised in Table 1. There was no sign of breast blisters or burns in any of the 101 carcasses. Signs of suspected systemic bacterial infection were observed in 19% (n = 19) of the examined carcasses and only in broilers diagnosed with cellulitis. The most common gross findings indicative of systemic spread were splenomegaly and perihepatitis, followed by air sacculitis, pericarditis, hepatomegaly, other liver pathology and coelomitis.

Table 1
Summary of gross findings in addition to cellulitis and skin lesions at post-mortem examination of 101 broiler chickens from six flocks condemned on account of dermatitis/cellulitis at meat inspection at a Swedish slaughter plant.

Number of carcasses / Finding	Cellulitis n = 79	Other skin lesions n = 22
Splenomegaly	22	2
Hepatomegaly	18	-
Perihepatitis	18	-
Miliary white spots in liver	12	-
Airsacculitis	11	-
Pericarditis and/or epicarditis	10	-
Coelomitis	8	-
Salpingitis	2	-
Focal serositis spleen	1	-
Other		
Ascites syndrome	5	2
Retained yolk sac	3	-
Dehydration	1	-
Right Müllerian duct cyst	-	1

3.2. Bacteriology

A total of 327 samples were cultured and *E. coli* was confirmed by MALDI-TOF MS in 44% of the samples and in 90% of the carcasses (Table 2). As seen in Table 2 there was variable growth in samples from subcutaneous tissue and organs. In samples originating from organs, pure growth of *E. coli* was found in 11%. In some of these, there was sparse growth of *E. coli* (1–10 colonies). Table 3 summarises culture results based on carcasses with and without gross findings suggestive of systemic bacterial infection.

Twenty out of the 101 carcasses had one or more sampled organ with growth of *E. coli* in pure culture or with sparse growth of other bacteria, of which 19 were diagnosed with cellulitis. Further, eight of these 20 carcasses (40%) had gross findings suggestive of systemic bacterial infection.

Table 2
Bacterial growth from cellulitis and organs samples (N = 327) obtained at post-mortem examination of broiler carcasses condemned because of dermatitis/cellulitis (N = 101). Numbers in brackets refer to bacterial growth from cellulitis and organ samples from the subgroup of carcasses with gross findings indicative of cellulitis (n = 79).

Number of sampled birds / Origin of sample	No growth	*E. coli*[1]	*E. coli* in mixed culture	Other species[1]	Mixed growth[2]
Subcutis n = 101	2 (1)	54 (54)	37 (22)	- (-)	8 (2)
Pericardium n = 101	42 (29)	9 (8)	15 (14)	7 (7)	28 (21)
Spleen n = 101	47 (31)	11 (11)	10 (9)	18 (15)	15 (13)
Other organ 1[3] n = 21	3 (3)	3 (3)	4 (3)	- (-)	11 (10)
Other organ 2[3] n = 3	- (-)	1 (1)	- (-)	- (-)	2 (2)

[1]Pure growth or growth in mixed culture with sparse growth of other bacteria
[2] ≥ 2 colonies with different morphology
[3]any 4th and 5th sample taken per carcass in one of the following location: liver, joint, air sac, tendon sheet, skeleton, coelomic cavity, pancreas or oviduct; liver being the most common sampled organ (n = 12).

Table 3
Distribution of bacterial growth or no growth, acquired at post-mortem examination from cellulitis and organs in broiler carcasses with gross findings indicating systemic spread of *E. coli* (S+, $n = 19$) and no systemic spread (S-, $n = 82$).

Origin of sample	Group	No growth	*E. coli*[1]	*E. coli* in mixed culture	Other species[1]	Mixed growth[2]
Subcutis	S+	-	13	5	-	1
$n = 101$	S-	2	41	32	-	7
Pericardium	S+	3	3	4	1	8
$n = 101$	S-	39	6	11	6	20
Spleen	S+	7	5	2	3	2
$n = 101$	S-	40	6	8	15	13
Other organ	S+	2	2	2	-	6
1[3]	S-	1	1	2	-	5
$n = 21$						
Other organ	S+	-	1	-	-	2
2[3]	S-	-	-	-	-	-
$n = 3$						

[1]Pure growth or growth in mixed culture with sparse growth of other bacteria
[2] ≥ 2 colonies with different morphology
[3] any 4th and 5th sample taken per carcass in one of the following location: liver, joint, airsac, tendon sheet, skeleton, coelomic cavity, pancreas or oviduct; liver being the most common sampled organ (n = 12).

3.3. Whole-genome sequencing and bioinformatics

Among the 46 sequenced isolates, 34 originated from organs and cellulitis lesions from 15 individual birds. Another ten isolates represented broilers with cellulitis without coliform growth in organs (single samples from cellulitis) and two isolates came from a carcass with a lesion on the craniolateral breast muscle (not diagnosed with cellulitis) (Table 4).

There were 11 serotype/sequence type (ST) combinations represented among the sequenced isolates (Table 4; Supplementary material T2). The serotype-sequence types differed between flocks, except for O2/O50:H5-ST95 and O120:H4-ST428 that was present in both flocks 4 and 5. One to five serotype-sequence type combinations were represented within flocks, while the majority of isolates from the same bird had the same serotype-sequence type.

All 46 isolates harboured the virulence genes *iss* (increased serum survival) and *TerC* (tellurium ion resistance protein), while 45 isolates (98%) harboured genes *hlyF* (hemolysin F), *iroN* (enterobactin siderophore receptor protein) and *ompT* (outer membrane protease) (Supplementary information T2). Another five genes were present in 40–43 isolates (87–94%) (*chuA*: outer membrane hemin receptor; *fyuA*: siderophore receptor; *irp2*: high molecular weight protein 2 non-ribosomal peptide synthetase; *sitA*: iron transport protein and *traT*: outer membrane protein complement resistance).

The minimum spanning tree (MST) showed that the majority of isolates from the same flock clustered with up to two allele differences between isolates and had the same sequence type (Table 5, Fig. 1). Isolates originating from the same carcass but from different locations showed an identical allelic profile or differed in one allele, with the exception of the two carcasses 3:13 and 4:1, in which the isolates from cellulitis and organ differed in the majority of alleles and had different serotypes and sequence types (Table 4; Fig. 1). In addition, the isolates from carcass 4:1 (isolates 4:1:Sc and 4:1:Sp) fell into two different clusters of isolates from flock 4. In two cases, isolates from different flocks were more similar than isolates from the same flock. Four isolates from flocks 4 and 5 of sequence type 428 were located in a shared cluster, with up to four allele differences between isolates and one isolate from flock 5 (5:9 Sc) was located 27 alleles from isolates from flock 4.

In Enterobase, 26 *E. coli* isolates of human origin were identified within 50 allelic differences from the current study isolates forming five clusters (Fig. S1). One to five isolates of human origin were present in four of the five clusters harbouring two to six genetically identical isolates from the current study in each cluster. The fifth cluster harboured 18 isolates of human origin and one isolate from the current study. Out of the 26 isolates from humans, 11 were isolated from urine, one from liver and blood respectively, and 13 lacked this information. When comparing the presence of virulence genes in one to two of the isolates of human origin within each cluster with the current study isolates in the same cluster, 14–22 shared virulence genes were identified in both groups. The most commonly occurring genes were *lpfA* (long polar fimbriae), *ompT* (outer membrane protease) and *terC* (tellurium ion resistance protein) that were present in all analysed isolates of human origin and isolates from the current study in the five clusters. The genes *cvaC* (microcin C), *fyuA* (siderphore receptor), *gad* (glutamate decarboxylase), *hlyF* (hemolysin F), *iroN* (enterobactine siderophore receptor protein), *irp2* (iron regulatory protein 2), *iss* (increased serum survival), *mchF* (ABC transporter protein) and *sitA* (iron transport protein)

Table 4
Identification, serotype and sequence type for *E.coli* isolates ($n = 46$) acquired at post-mortem examination from subcutaneous tissue and organs in broilers condemned for dermatitis/cellulitis at a slaughter plant. The isolates were named according to flock, carcass number and location of sampling (Sc=subcutaneous tissue, Pc=pericardium, Sp=spleen, Li=liver, LA=liver and air sac). Isolates marked with * belonged to the carcass group not diagnosed with cellulitis.

Flock: Carcass no: Location	Serotype	Sequence type
1:6:Sc	O103:H21	101
1:6:Pc	O103:H21	101
3:3:Sc	O25:H4	131
3:5:Sc	O25:H4	131
3:5:Pc	O25:H4	131
3:6:Sc	O25:H4	131
3:6:Sp	O25:H4	131
3:7:Sc	O25:H4	131
3:7:Pc	O25:H4	131
3:7:Sp	O25:H4	131
3:8:Sc	O25:H4	131
3:11:Sc	O17/O44/O77:H18	69
3:12:Sc	O25:H4	131
3:13:Sc*	O132:H28	13199
3:13:Sp*	O23:H6	69
3:15:Sc	O25:H4	131
3:18:Sc	O78:H4	23
3:18:Sp	O78:H4	23
3:18:Li	O78:H4	23
4:1:Sc	O161:H4	117
4:1:Sp	O2/O50:H5	95
4:2:Sc	O161:H4	117
4:2:Pc	O161:H4	117
4:7:Sc	O120:H4	428
4:7:Pc	O120:H4	428
4:9:Sc	O161:H4	117
4:9:Sp	O161:H4	117
4:9:LA	O161:H4	117
4:12:Sc	O2/O50:H5	95
4:12:Pc	O2/O50:H5	95
5:1:Sc	O2/O50:H1	135
5:2:Sc	O2/O50:H1	135
5:2:Pc	O2/O50:H1	135
5:2:Sp	O2/O50:H1	135
5:3:Sc	O2/O50:H1	135
5:5:Sc	O2/O50:H1	135
5:5:Sp	O2/O50:H1	135
5:6:Sc	O2/O50:H1	135
5:7:Sc	O2/O50:H1	135
5:7:Pc	O2/O50:H1	135
5:9:Sc	O2/O50:H5	95
5:11:Sc	O2/O50:H1	135
5:13:Sc	O120:H4	428
5:13:Pc	O120:H4	428
5:19:Sc	O11:H25	457
5:19:Sp	O11:H25	457

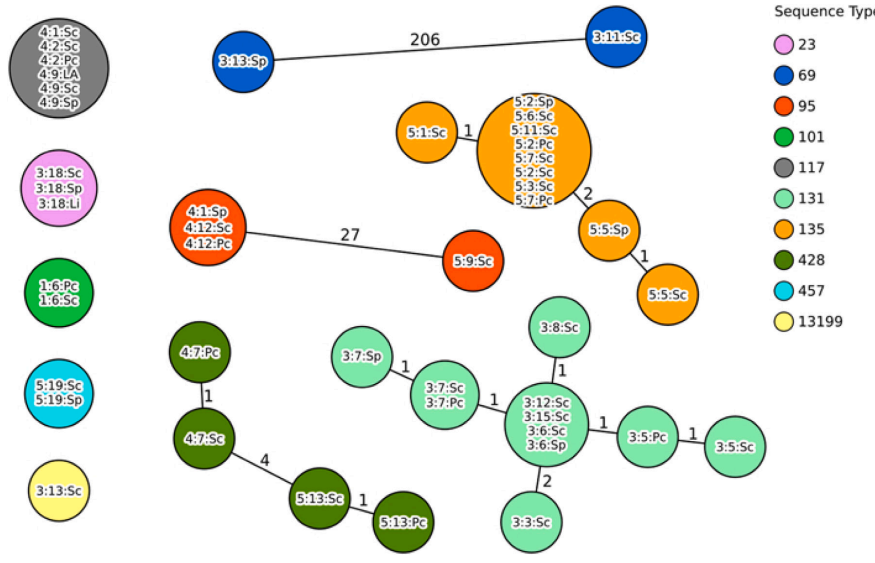

Fig. 1. Minimum spanning tree based on the cgMLST data from *E. coli* isolates (n = 46) from carcasses condemned for cellulitis at a Swedish slaughter plant. Colours represent different sequence types. Each circle represents an allele profile with lines that connects closely related isolates forming clusters. The number on the line denotes how many alleles that differed between the isolates. No line implies that the isolates differs in > 1870 alleles.

occurred in both isolates of human origin and current study isolates in four out of five clusters. The gene *lpfA* (long polar fimbriae) was present only in the 14 isolates from the current study that clustered with those of humans origin, as well as in a single cellulitis isolate (3:11:Sc) that differed by 206 alleles from the isolates from the current study that clustered with isolates of human origin.

4. Discussion

In the present study, *E. coli* in pure culture was isolated from organs both with and without gross findings of suspected bacterial infection beyond the assumed primary site of cellulitis in broiler chickens. The isolates originated from six different healthy flocks as suggested by ante-mortem inspection and mean body weight. The condemnation rate of the flocks due to dermatitis/cellulitis was similar to figures reported in previous studies in Europe (Poulsen et al., 2018; Schulze Bernd et al., 2020). The majority of *E. coli* isolates from cellulitis and organs in the same carcass were genetically indistinguishable. Our results thus confirm earlier studies (Gomis et al., 2001; Poulsen et al., 2018; Silva et al., 2021) that *E. coli* may have spread systemically in broiler carcasses with cellulitis sampled at slaughter. The present study also suggests that gross findings of suspected systemic bacterial infection is not a reliable tool for identification of this subset of birds at meat inspection.

As cellulitis is difficult to diagnose in live broilers on farms, the present study and most earlier studies have relied on sampling at slaughter plants. The commercial slaughter of broilers results in various species of bacteria being present in the slaughter plant environment and on the broiler skin (Moazzami et al., 2021). A likely cause is that water is used during the slaughter process, e.g. during scalding and defeathering, which could introduce contaminating bacteria on and under the skin and possibly within the body cavity. Moreover, many carcasses are incised at meat inspection to confirm cellulitis, which could introduce contaminants to the subcutaneous sampling location. Therefore, it was an expected finding that some samples in the present study showed growth of bacteria in mixed culture, with or without *E. coli* (Table 2). This phenomenon occurred at a similar rate on all six sampling days despite an aseptic sampling technique, sampling in a separate building within a short time of death and prior to post-mortem examination. In accordance with our results, Messier et al. (1993) reported growth of bacteria in mixed culture in samples from cellulitis when sampling was performed at a slaughter plant. Another study described that *E. coli* was isolated from cellulitis lesions and organs but no results were included regarding the level of mixed bacterial growth (Onderka et al., 1997). Other researchers have reported only pure cultures (Gomis et al., 1997; Poulsen et al., 2018). The present study clearly illustrates the difficulties in obtaining pure primary cultures from broilers at slaughter plants, despite a careful sampling strategy designed to obtain representative and good quality samples.

Several previous studies have suggested that *E. coli* can spread systemically from the primary cellulitis lesion to organs (Gomis et al., 2001; Poulsen et al., 2018; Silva et al., 2021). In contrast to these earlier studies, which were based on serotyping, virulence gene detection by PCR and fingerprinting (PFGE), our study is the first to report evidence of systemic spread based on cgMLST. Core genome-MLST analysis is a genome-wide genotyping method representing the core genome and it thus provides a solid and reliable way to identify identical or closely related *E. coli* isolates. Ideally, signs of systemic spread should be detected at the slaughter plant during meat inspection. However, in the present study we confirmed findings of other researchers, that *E. coli* can be isolated from organs in carcasses even when no gross findings of systemic spread were observed (Gomis et al., 1997; Onderka et al., 1997). Interestingly, in the present study only a few *E. coli* colonies were cultured from some organ samples. This could be a sign of early or low-grade systemic infection, possibly bacteraemia or sample contamination. In contrast, there were also carcasses with gross findings of systemic infection from which cultures revealed no bacterial growth. This could indicate bacterial clearance as discussed in a previous paper by Gomis et al. (2001). Thus, it is most likely difficult to reliably identify birds with systemic spread at slaughter, especially taking in consideration the short time available to inspect each carcass and the mild nature

of gross lesions sometimes present in early infection stage. It should be mentioned that although it is assumed in the current study that cellulitis is the primary lesion from where the systemic spread occurred, the sequence of events in coliform cellulitis have not been fully studied and determined.

Moreover, the cgMLST analysis in our study confirmed that most *E. coli* isolates from the same flock of origin belonged to the same genetic cluster, while isolates from different flocks belonged to separate clusters. This suggests that each flock has its own predominant cellulitis-associated *E. coli* populations. This has previously been suggested by Singer et al. (1999) based on PFGE analysis. In addition, in our study, one cluster consisted of isolates with close genetic similarity, which originated from two different farms. This indicates that in addition to endemic *E. coli* populations, the same genotypes associated with cellulitis can be found on several farms, which also is in accordance with previous results by de Brito et al. (2003) using extragenic palindromic PCR (REP-PCR) fingerprint technique. Together, these results point to the possibility of bacterial transmission of APEC within the broiler industry network.

Avian pathogenic *E. coli* is a heterogeneous group of bacteria that is not well defined when it comes to the presence of virulence genes (Mehat et al., 2021). While cellulitis-associated *E. coli*, including the isolates in the present study, fits in the general APEC definition, there is no shared set of virulence genes defining this group of bacteria (Jeffrey et al., 2002; de Brito et al., 2003; Barbieri et al., 2013; Poulsen et al., 2018). The genes *kpsMTII*, *ibeA* and *gimB* have been suggested as clinically important virulence genes in association with cellulitis (Barbieri et al., 2013). In our study, the *ibeA* gene was identified in a majority of the sequenced isolates. On the other hand, the *kpsM* gene was present only in a subset of isolates. In another study, the cytotoxin *E. coli* vacuolating Factor (ECVF) was shown to induce microscopic skin lesions in an animal experimental setting (Quel et al., 2013). The author suggested that ECVF contributes to the pathogenesis of cellulitis. As no ECVF sequence yet has been published, further analysis of our study isolates was not possible.

Our study showed that cellulitis could be associated with multiple serotypes and sequence types, virtually all of which have previously been associated with the broader APEC pathotype. Our findings included STs 23, 95, 117, and 428, which commonly cause systemic disease in poultry (Mehat et al., 2021). Our results strengthen the proposed pathogenesis that cellulitis development is dependent on both an infecting strain specialised in causing extraintestinal infection and the condition of the host together with management factors (Jeffrey et al., 2002; Schulze Bernd et al., 2020). Poulsen et al. (2018) identified a predominant *E. coli* sequence type (ST117) and PFGE profile in broilers with cellulitis in Denmark. They studied *E. coli* isolates from 34-days-old broilers with cellulitis and carcasses condemned at slaughter due to cellulitis. A subset of eight study isolates with ST117 were subjected to WGS and six of the isolates differed by only 7–36 SNPs. In another study by Ronco et al. (2017), closely related isolates of sequence type ST117 were reported as a predominant pathogen in several Nordic countries. Ronco et al. (2017) studied 114 isolates from broilers and broiler parents, and ST117 was identified in a clade consisting of 62 isolates. Moreover, 47 of these isolates belonged to serotype O78:H4. In both studies, it was suggested that the O78:H4-ST117 could have originated from a common breeding stock source in Sweden (Ronco et al., 2017; Poulsen et al., 2018). In our study, there were only six ST117 isolates, all originating from the same flock and cluster, but with a different serotype (O161:H4) than the one presented by Ronco et al. (2017). Serotype O78:H4 was however present in three isolates from another flock but in combination with ST23. It should be added that there is no study published on APEC isolates from Sweden confirming the presence of O78:H4-ST117 during the same time period as in previous reports (Ronco et al., 2017; Poulsen et al., 2018). Thus, our study could not confirm the presence of O78:H4-ST117 as the predominant *E. coli* lineage in Sweden in 2021. Although speculative, there could have been another unknown common source, or other strains could have since replaced this particular serotype-sequence type in Sweden.

In our study, a similar set of virulence genes were detected among the genetically related isolates of human origin and the study isolates. There was limited information on the source and disease conditions of the isolates of human origin, but at least 11 of 29 isolates were collected from urine indicating that the isolates belong to the UPEC group. These results support previous research that APEC and human pathogenic *E. coli* are similar in terms of both phylogeny and virulence factors, although it is not clear if APEC cause infections in humans (Manges, 2016; Jorgensen et al., 2019). There is no routine genomic characterization of ExPEC isolates collected in the Swedish healthcare system, and therefore no direct comparison on the national level was possible in the present study.

5. Conclusion

Based on genomic comparison we conclude that *E. coli* isolated from cellulitis in broiler chickens frequently have characteristics in agreement with other APEC and can spread systemically. Identifying broilers with systemic spread of *E. coli* based on gross findings is however not a reliable method that can be used at slaughter plants, as *E. coli* isolates that are genetically indistinguishable from the cellulitis isolates can be found in organs without gross findings.

We also conclude that sampling for bacterial isolation from broiler carcasses in the slaughter plant is challenging, due to the bacterial flora in the slaughter process environment as well as on the carcasses.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.vetmic.2023.109755.

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Cellulitis in broiler chickens is a subcutaneous inflammation caused by the bacterium *Escherichia coli* and is one of the main causes of condemnation of chicken carcasses at slaughter. This thesis aimed to gain more knowledge of the pathogenesis and risk factors. The results showed that cellulitis-associated *E. coli* could spread systemically, cause clinical signs and affect immune parameters in blood. Risk factors for cellulitis were identified within the broiler chicken production chain.

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